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INFECTIOUS DISEASES

NATIONAL VACCINE PROGRAM OFFICE

WORLD HEALTH ORGANIZATION

EVOLVING SCIENTIFIC AND REGULATORY PERSPECTIVES
ON CELL SUBSTRATES FOR VACCINE DEVELOPMENT

WORKSHOP

TUESDAY,
SEPTEMBER 7, 1999

The workshop took place in the Plaza Ballroom,
DoubleTree Hotel, 1750 Rockville Pike, Rockville, MD
20852 at 7:45 p.m., William Egan, Ph.D., Chair,
presiding.

PRESENT:

WILLIAM EGAN, Ph.D., Chair
ELWYN GRIFFITHS, Ph.D., D.Sc., Co-Chair
REGINA RABINOVICH, M.D., Co-Chair
LEONARD HAYFLICK, Ph.D., Speaker

ANDREW LEWIS, Jr., M.D., Speaker
PHILIP KRAUSE, M.D., Speaker
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(7:54 p.m.)

DR. RABINOVICH: Good evening. I would like to ask those who would like to participate in this evening's session to please have a seat so we can get started. My name is Regina Rabinovich. I am here from the Institute of Allergy and Infectious Diseases from the NIH in the U.S., and on behalf of my co-sponsoring agencies and colleagues, the Center for Biologics Evaluation and Research, the International Association for Biologicals, the National Vaccine Program Office, which includes Marty Myers, who is supposed to be a co-chair for this session and is currently either grounded or wandering around Newark, New Jersey, and the World Health Organization, I welcome you to the meeting on Evolving Scientific and Regulatory Perspectives on Cell Substrates for Vaccine Development.

I have a couple of housekeeping comments.

I won't bother to tell you where the bathrooms are. I am sure you can find those. There are two documents that I think would be very useful, if you haven't noticed them already, because they are key to the workshop. The first is the document prepared by our colleagues from the Food and Drug Administration,
which is under your tab entitled, Draft CBER document.

Because I think that the creation and comment upon this is central to the scope of this meeting, as will be elucidated further by Dr. William Egan.

Because the sponsoring groups as well as the FDA particularly are so interested in the concepts within the document and the discussion that will take place, this meeting is being taped. And for that reason, we must ask that speakers as well as those that are asking questions or getting answers speak into the microphones, and that we will work with the moderators to make sure that everyone that wants to ask a question, regardless of where they are in the room, have a microphone available to them because it is key that it be part of the written record.

The steering committee would like to thank a couple of individuals that have worked very hard to make the meeting possible. Two of our colleagues at FDA, Dr. Keith Peden and Dr. Phil Krause, and Dr. John Daugherty at NIAID.

In thinking about eight months ago about this workshop, the scope and what it should cover, we never in our wildest dreams considered that in September of 1999 we would have gone through a summer where there would be no question about how to spell
thimerosal, and that early information coming out of
sort of a group analysis of all clinical trial data
for the rotavirus vaccine would give us the first
inkling to the potential connection to
intussusception. Indeed, the issues regarding this
topic, vaccine safety, which never makes sense to
anyone, what we are talking about in terms of vaccine
safety, are elucidated by the kinds of thoughts and
strategies that will evolve from this meeting. And if
ever there was a connection between basic research and
what we do with human subjects as an outgrowth of your
deliberations, I think this is it.

So on behalf of my colleagues at the
National Institute of Allergy and Infectious Diseases,
I thank you for your participation. I would like to
go ahead and introduce Dr. Elwyn Griffiths from the
World Health Organization, who is my co-chair in my
role as Dr. Marty Myers, and will introduce the first
speaker. Thank you.

DR. GRIFFITHS: Can you hear? Oh, yes,
okay. (Speaking in Welsh.) It is the only
opportunity I have ever had to introduce a meeting
such as this in Welsh. I know there is one colleague
of mine, Merfyn Williams, who used to be at FDA, and
he is in the audience. So at least he understood that.
It is really an honor and a pleasure for me to co-chair this session, the first session of this important meeting with Regina here. Let me also say that the World Health Organization is very pleased indeed to be a co-sponsor of this workshop. Because I think it considers the subject to be vitally important from a global perspective, and we take that view of course, for future development and production of vaccines in general.

I see this evening's activities really as a sort of an hors d'oeuvre, I guess you might say, or an appetizer to the entrees that come during the next two days or so. So it is an overview of where we are going and where we have been. I think perhaps we should, because of the time scale -- I think we should move forward with the program, taking particular pity on any colleagues who have just flown in from Europe, because they would be suffering from some jet lag or biological clock problem at the moment. So I think we should move forward fairly quickly.

So it is a pleasure then really to call upon Dr. Bill Egan, who is now the Acting Director of the Office of Vaccines at the Center for Biologics Research and Evaluation, CBER, who will actually give us a review of the works or purpose and the goals of
this. So he will set the scene for where we are going
to go during the next few days. So, Bill?

DR. EGAN: Thank you, Elwyn. Can I have
the first slide, please? I too would like to thank
everybody who has been able to come to the meeting and
everybody who helped in the organization of the
meeting and all of the participants in the meeting.
This is a workshop and everyone will be working
together to try and address some very interesting,
some practical, and some very difficult questions.

Every now and then I think we need to
remind ourselves that viruses can propagate only in
live cells, and this of course holds true for whole
viral vaccines. They can only be produced in cells.

The choice of suitable cell substrates for the
manufacture of viral vaccines has over the years
engendered considerable discussion. The primary focus
in these discussions on cell substrates have been
safety, in particular the potential safety concerns
from residual cellular DNA and from adventitious viral
agents.

As history has shown, the need for concern
about cell substrate issues was real. We have only to
think back to the finding of SV40 in polio virus
vaccines to realize the extent of the risk that any
cell substrate may pose, and there is still great need
for concern.

Early discussions on cell substrates
allowed only for the use of "normal" cells, that is,
cell populations that were derived from normal tissues
and that had undergone no subculture. And right now,
I am referring primarily to the 1954 decision of the
Armed Forces Epidemiological Board.

I can't read this -- it is hard to read
from this perspective back here. The use of only
normal cells has prevailed for many years. For
example, in 1971, the regulations of the Public Health
Service for the production of OPV stated that only
primary monkey kidney tissue cultures may be used in
the manufacture of polio virus vaccines. There were
similar regulations in place for the production of
inactivated polio virus vaccine, live attenuated and
inactivated measles vaccines, mumps vaccine, rubella
vaccine, and the adenovirus vaccine.

This was taken from the regulations in
1971, and it was in 1972 that the Federal Register
notice was placed that contemplated the use of other
cell lines other than these primary animal tissue
cells for use in viral vaccines.

Now our thinking and our practices with
regard to cell substrates has evolved over time. At present, for example, in addition to primary animal cell culture, diploid cell lines, both of human and animal origin, are in routine use, as, for example, the mumps, rubella, IPV vaccines. And in addition to being manufactured in the human diploid cell, live IPV is also produced in viro cells, a continuous cell line.

We now wish to consider additional cell substrates or additional cell substrate uses. In particular, we would like to consider the use of continuous cell lines for live attenuated viral vaccines, and the use of other neoplastic cell lines for both live attenuated and inactivated viral vaccines. Indeed, the question that the Armed Forces Epidemiology Board met to consider 45 years ago, namely the use of tumor cells, specifically HeLa cells, as a cell substrate is a question that we are again raising.

The goals of the workshop are simple. They are two. The first is to identify the various concerns that are associated with using neoplastic cell lines, to catalog them as completely as possible. The second is to determine approaches and to identify approaches to determining the level of the risk that
are associated with these concerns. In the decisions
that we will make with regard to the use of neoplastic
cells as potential substrates for viral vaccines, we
need to be guided by data; either data that already
exists in the literature or the data that we need to
generate. We should not be guided by intuition or by
opinion. If I may quote Dr. Hilleman from a
discussion about the use of tumor cell lines from a
meeting that was held in 1968, he said, "If the debate
has taken on an ecclesiastical or philosophical
overtone, it should not be unexpected, since it is an
area in which the definitive information for making a
judgment is clearly missing. In fact, I feel that the
greatest problem of all may lie in ascertaining
whether there is a problem in the first place."

The workshop in which we will be
participating over the next four days is designed to
see how far we have progressed and what we still need
to do. Thank you.

DR. GRIFFITHS: Thank you very much, Bill.
I think we have been given the task, then, for the
next few days, that is, to identify these concerns and
issues associated specifically with neoplastic cells
for production of vaccines. We are focusing on
vaccines. And to identify some approaches in
determining this risk associated with their use and
really how to move forward on these issues. I think
those are going to be our main concerns over the next
days.

So we now move on to the second talk and
we are really looking at historical perspective to a
certain extent here, the evolution of cell substrates
used for the production of human biologicals. Because
as Bill already mentioned, they have been in use for
a long time and there has been a tremendous amount of
discussion over the years. And who better to give us
this talk and to take us through from the beginning of
the evolution of these cell substrates than someone
who has been involved right from the very beginning,
and the name is synonymous really with diploid cells,
Dr. Hayflick. So would he come forward and give his
talk on the evolution of cell substrates for the
production of human biologicals. Dr. Hayflick?

DR. HAYFLICK: Although the first cell

culture is generally attributable to Ross Harrison in
1907, it was not until 1949 that contrary to the then
existing dogma, which held that the polio virus could
replicate only in neural cells, that Enders, Weller
and Robbins showed that the virus could replicate in
cultured human fibroblasts. This set the stage in the
late 1950's for the development by Salk and his colleagues of an inactivated polio vaccine prepared in primary monkey kidney cells.

I should define primary cells, because neophytes are using it to mean something entirely different and are oblivious to the fact that its original meaning still has a legal FDA definition. A primary culture is simply a population of cells derived from impact tissue that has not undergone any subcultivations. After a few years of use of primary monkey kidney cells, it became apparent that these cells contained many unwanted viruses, some of which were lethal to humans. At about this time in 1961, we described the development of normal human diploid cell strains that were free of contaminating viruses, and we suggested that they may be useful for the preparation of human virus vaccines. It took about 10 years before our suggestion became generally accepted in the scientific community.

Normal human diploid cell strains have several interesting properties. If I can have the first slide? The properties that we described are listed here. Very briefly, point number one, if derived from human embryos, they undergo about 50 population doublings. Contrary to the belief that
this is a serious limitation, the limit is actually
about 50 million metric tons after 50 doublings.

Human diploid cells undergo a number of population
doublings inversely proportional to donor age. This
suggested to us that the finite replicative capacity
of cultured normal cells is an expression of aging at
the cell level. This notion received considerable
experimental support in subsequent years and is now
the basis for the field of cell senescence or
cytogerontology.

We also found, of course, that the cells
derived from this tissue had the diploid karyotype and
are incapable of replication in suspension culture.
The next point, number four, human cell strains will
not produce tumors when inoculated into the hamster
cheek pouch or even when we directly introduced them
into terminal human cancer patients, something that
was relatively easy to do in the 1960's.

Human diploid cell strains can be
cryogenically preserved. When, for example, WI38,
which was the first highly characterized human diploid
cell strain which we developed in 1962, is preserved
at a particular doubling level and then reconstituted,
the number of doublings remaining is equivalent to 50
minus the number of doublings spent prior to
preservation. The cells have an extraordinary memory
and remember at what doubling level they are
preserved, even after 37 years of storage in liquid
nitrogen. WI38 has been cryogenically stored longer
than any other normal human or animal cell population.

We also reported that human diploid cell strains had
the broadest virus spectrum of any cell population
known, and even heretofore unknown common cold viruses
were isolated with these cells at that time.

As a result of this characterization, we
suggested that cell populations in culture could be
classified into three groups: primary cultures, which
I have already described: cell strains, which are
populations that have a finite capacity to replicate
and don't produce tumors when inoculated into
experimental animals, have the karyology of the tissue
of origin and are anchorage-dependent; and the third
category, cell lines, which are populations consisting
of immortal cells that may produce tumors when
inoculated into lab animals, don't have the karyology
of the tissue of origin, and are usually anchorage
independent.

Our description of these three fundamental
cell classes in which we define normal cell strains to
be mortal overturned a dogma that existed from the
turn of the century. The dogma was that all cells
placed into culture have the capacity to replicate
indefinitely. And if they do die, you simply do not
have the proper culture conditions. We upset this 60-
year-old dogma by proving that normal cells do have a
finite capacity for replication and showed further
that normal cells have an intracellular counting
mechanism and that the replicometer is located in the
nucleus. Without our having proven that there exists
mortal normal cells, then the field of cancer research
rooted in the concept of immortalization of normal
cells clearly could not have developed.

Our efforts to define these classes of
cells were not universally accepted, and
terminological chaos has reigned for the last 40
years. A good recent example can be found in the very
first paragraph of the major document that you all
received in the package of materials for this
workshop. It is titled, "A Defined Risks Approach to
the Regulatory Assessment of the Use of Neoplastic
Cells as Substrates for Viral Vaccine Manufacture."
Reference is made to a 1954 U.S. Armed Forces
Epidemiological Board recommendation that "normal
cells, rather than cell lines established from human
tumors be used for the development of adenovirus
vaccines." By normal cells, this Board meant immortal cell lines as I have just defined them but derived from normal tissue. The cell lines referred to by the Board are not normal by any criterion. In fact, the normal human diploid number was not known in 1954, and no normal cell had yet to be described.

This confusion led Maurice Hilleman to prepare a live adenovirus vaccine in the Henley intestine cell line, which was thought to have been derived from normal human tissue, but was in fact a grossly abnormal cell line later identified as HeLa. The six human recipients of this vaccine fortunately did not suffer any ill effects after 25 years of follow-up. It was not until 1961 that we showed how to make normal human diploid cell strains that normal cells became available.

The advantages of these cells are shown on the next slide. In 1961, we suggested that these normal diploid cells have definite advantages over primary cell populations. I won't go into detail on each of these items, but simply give you some details on two of them, the first and the last actually. In respect to latent viruses, primary adult monkey kidney cells, as we all know now, harbor quite a number of serologically distinct latent viruses, several of
which were demonstrably lethal for humans. The B
virus and the Marburg agent both were lethal for 23
people after accidental percutaneous inoculation of
handlers of monkeys and their primary kidney cultures.
SV40, as was mentioned earlier, known to produce
tumors in rodents and with the unknown potential to do
so with humans was also an early contaminant of
primary monkey kidney cells. SV40 was also capable of
transforming normal human cell strains into cancer
cell lines in vitro. SV40 was in fact inadvertently
administered to several million recipients of early
lots of inactivated and attenuated polio virus
vaccines. Latent viruses have never been found in
WI38. To this day, no evidence exists for an
endogenous human oncogenic agent, latent virus,
transforming principle, slow virus or endogenous
intact retro in any human diploid cell derived human
virus vaccine.
I won't discuss cost of cell procurement,
available cells, cell storage. These are all fairly
obvious to most of us by now. But the last point I
think is worth emphasizing, and that is that the
essential point that we tried to make was that a
diploid cell strain was more attractive than a primary
cell population in its ability to be thoroughly tested
before use. In a word, standardization. Thus, the
concept of cell standardization was first introduced.
Once standardization is achieved, cryogenic storage
permits the acceptable strain to be held indefinitely
for subsequent use. It is not possible, practical or
economic for primary cells to be similarly handled.

The argument holds equally well now for
cell lines that all of you now take as perfectly
obvious. But it was not so obvious from 1962 to 1972.
In the late 1950's and early 1960's, cell lines were
generally believed to be a forbidden candidate for the
production of human biologicals because they shared
properties with cancer cells. Our suggestion that
human diploid cells could provide a safe and superior
substrate for the production of human virus vaccines
was met with considerable resistance for the next
decade. The major reasons for this resistance were
the belief that hypothetical cancer viruses might be
present and that spontaneous transformation might
occur resulting in the presence of human cancer cells.
Latent viruses were such a problem with primary monkey
kidney cells that a worldwide moratorium on the
licensing of all polio virus vaccines was called in
1967 because of death and illnesses that occurred in
monkey kidney workers and vaccine manufacturing
facilities in Germany and in Yugoslavia. The responsible virus, of course, was the Marburg agent. The arguments against the use of human diploid cells for the preparation of human virus vaccines were used as the basis for the philosophy of the Division of Biologics standards, the precursor of what is today CBER, that "the devil you know is better than the devil you don't know."

In 1961, together with Dr. Stanley Plotkin, we prepared the first human biological produced in a human diploid cell. It was an oral polio vaccine which we showed to be both safe and efficacious. By 1963, 7,000 people received a similar vaccine produced in WI38 with no safety problems. Many of you use the concept of the master working cell bank in the production of biologicals, but few know of its origins. In 1963, I suggested the concept of the two-tiered system of master and working cell banks for WI38, although these terms were not used. The terms I used were master and working cell seeds. I based this reasoning and the terms on the identical way in which virus seeds were then utilized. The first publication of these procedures was made at the landmark Opatia, Yugoslavia conference in 1963.

By the 1980's, when heteroploid and mortal
cell lines like CHO lines were used for the
manufacture of human biologicals, the procedures for
characterizing and handling WI38 were lifted over for
use with cell lines with very few changes even to this
day. In the mid-1960's, several WI38 users expressed
difficulties growing the cells that were traceable to
variations in the medium reagents used by various
laboratories. As a consequence of this, we developed
a practical standardized cell culture powdered media
in kilogram lots, that unlike wet media was easily
transported worldwide and used by many labs
simultaneously and for which only two variables
existed, water and serum. As a result of our
development of powdered media, the WI38 growth problem
was quickly resolved and the use of powdered media has
now become commonplace throughout the world.
In the years following our development of
WI38 in 1962, as a matter of national pride other
countries produced their own strains. The Medical
Research Council in the UK produced MRC5. In 1970,
the Japanese made TIG1 and the Chinese made 2BS, et

cetera. Today, most of the world's virus vaccines are
made on WI38 or similar strains. It has been used to
provide more individual doses of a biological than any
other cell substrate ever used. This slide following
gives you a summary of or a rough idea of the extent
of the use of these populations.

I am frankly astonished to observe that
after 10 years of debate on the risks of moving from
frequently contaminated primary monkey kidney cells to
virus free normal human cells that the time required
to make the final leap to abnormal cell lines for
producing human biologicals occurred in the 1980's in
a matter of weeks. Today, the molecular mechanism for
our finding that normal cells have a replicometer has
been substantially described as a result of a
fascinating story that time constraints prevent me
from describing. The explanation is briefly described
on the next slide and consists, as most of you here
know by now I am sure, of the shortening of telomeres
in normal cells each time DNA replicates. And for
those cells that are immortal, they escape the
inevitability of telomere shortening by switching on
an enzyme called telomerase that adds on the
nucleotides at each round of DNA replication, thus
maintaining the telomere length constant.

The remaining question, as I just
described, in respect to telomere shortening was the
discovery of this enzyme called telomerase, an enzyme
that contains an RNA template and a catalytic moiety,
among other components. This is the only enzyme that contains a reverse transcriptase and is part of normal cell biology. 90 percent of cancer cells express the enzyme. Only normal stem cell populations do so.

As for the use of abnormal and mortal cell lines for the production of human vaccines, that has already been done. The Vero cell line derived from the kidney of an African green monkey kidney has been licensed in France, Belgium, the United States and Thailand for the production of both live and attenuated polio and rabies vaccines. There are now more than 20 million vaccinees. The Chinese have made a Japanese encephalitis vaccine in the abnormal cell line, BHK21. Recombinant vaccines have been licensed for hepatitis B grown in a CHO cell line, and phase III trials are currently underway for an HIV vaccine in these same cells.

There are other immortal cell lines that bear consideration as substrates for the preparation of human virus vaccines. About two years ago, I initiated a culture of fibroblasts from a skin biopsy that I took from my knee. This culture was the first to be immortalized by transfection with human telomerase reverse transcriptase. Three other human cell strains were similarly treated and these results
were published in Science in January of 1998. These immortalized normal cell populations have now undergone well over 400 population doublings. This opens up enormous opportunities, not only to immortalize WI38, but many other normal cell types for applications like -- for exploitation actually as vaccine substrates among many other potential uses. Another category of immortal cell lines that merits consideration are those transformed by agents other than viruses. SUSM1 is a cell line derived from normal human lung that we transformed with a chemical carcinogen. KMSD6 is also an immortal cell line derived from normal human fibroblasts and transformed by exposure to cobalt 60 gamma radiation. I might add that contrary to the belief of some, early passage WI38 does exist in ample quantities, and anyone wishing to obtain these cells should feel free to contact me.

Thus, there exists several methods for transforming well-characterized normal human and animal cell populations to immortal cell lines using at least three proven methods. What should not be overlooked, however, in our zeal to condemn any immortal abnormal cell line from consideration as a human virus vaccine substrate is the fact that
downstream processing has reached such a level of efficiency that the preparation of a human virus vaccine containing only the purified moieties capable of eliciting an immune response is possible now. Affinity technology in particular is clearly capable of meeting this exacting goal. Nevertheless, as we all know, economic considerations will surely guide most of these decisions. Thank you very much.

DR. GRIFFITHS: Thank you very much indeed. We do have time for one or two questions if people want to start the ball rolling and ask Dr. Hayflick any questions. I don't see any immediate -- oh okay. Sorry, I can't see your name from here. Can you introduce yourself? Is there a microphone?

PARTICIPANT: I am Jim Cook from Chicago.

What all types of animals have WI38's been inoculated into for tumorigenicity testing besides the hamster cheek pouch?

DR. HAYFLICK: Oh my.

PARTICIPANT: I mean, have they been put into newts. Have they been put into other kinds of immunocompromised animals?

DR. HAYFLICK: All immune compromised lab animals -- mice, rats --

PARTICIPANT: What about things like the
odd observation that Balb C3T3's when hooked onto plastic disks will make tumors in newts? Has anything unusual like that been done with WI38's?

DR. HAYFLICK: Yes. And to the best of my knowledge, there has been no tumor formation. There have been occasional reports of nodules where the cells will replicate to a point where they reach a macroscopic size. But usually what happens is that they fail to grow beyond that state.

DR. GRIFFITHS: Johannes?

PARTICIPANT: Johannes Loewer. You mentioned that the human diploid cell lines have a very broad host range for many different viruses. Is it known whether neoplastic cells have even a broader host range or is it more or less similar to diploid cells?

DR. HAYFLICK: You are talking about cell -- immortal cell lines?

PARTICIPANT: Yes, immortalized cell lines or neoplastic cell lines.

DR. HAYFLICK: From human material?

PARTICIPANT: Yes.

DR. HAYFLICK: To the best of my knowledge, the human diploid cell strains have a far broader virus range than the immortal cell
populations. Most or many of the rhinoviruses were
first discovered on WI38 and later on MRC5. But the
fact that they could ultimately be adapted to growth
in immortal cell populations was also revealed.
However, I think it is still fair to say that normal
human diploid cells are the most sensitive cell
population to most of the viruses that are now well
calibrated.

DR. GRIFFITHS: Any other points that we
should take? Because we are right on time, so we are
really doing quite well this evening. If not, then
thank you very much indeed.

DR. HAYFLICK: Sure.

DR. RABINOVICH: Our next speaker is Dr.
Elwyn Griffiths from the World Health Organization,
who has also worked in this field for -- I won't even
venture to guess how many years. He is going to talk
about major issues associated with cell substrates.

DR. GRIFFITHS: Thank you very much. I
must say I was very impressed when I got this badge
actually from the organizers. I mean, I don't know if
they get a prize for the number of flags that is on
it. It is very impressive. I mean, I was only asked
to do this particular talk and stand in just a couple
of weeks ago. I think it should have been Dr.
Petricciani who would have done this and done it far better than I am sure. But in standing in, perhaps I should have stood in for the patron committee or something just to have another badge on here.

Anyway, for this evening to a certain extent this talk really is something of a trailer to the more detailed discussion we are going to have over the next few days, and really an extension of the points already touched upon by Dr. Hayflick.

The way I thought I would like to do it is to take a different stance in the beginning. Before dealing with the major issues which really revolve around these different cell substrates, to start with a reminder. And I think I would like this reminder to be borne in mind throughout the meeting that what we are dealing with has great benefits for mankind. I mean, the benefits of the vaccines produced on cell substrates. There has been enormous success in cell substrates for producing vaccines. These particular vaccines have had a tremendous influence on human health.

I would just want to pick up on polio, for example, because that is something which the WHO clearly has a -- and everybody else, I guess -- has a major interest in. The last case of polio in the
whole of the Americas, that is PAHO, was in 1991. In
the Western Pacific region, it was in March of 1997.
The world is moving towards global eradication of
polio in roughly about -- it is expected in 2000. I
am not sure if 2000 is realistic -- 2000, 2002 or 2003
or so. You can see the difference from -- the top one
is 1988 really to 1998. There are patches left in
1998, and this is gradually getting smaller and
smaller.

So essentially, global eradication of
polio, which will have enormous benefits for mankind,
has been achieved primarily through live polio
vaccines. These vaccines are produced on a number of
cell lines -- primary monkey kidney cells, diploid
cells, and also in vero cells. I think it is true to
say on a global perspective that the majority of the
producers are producing on primary monkey kidney
cells.

Now having said that, I just wanted to
have that borne in mind. When we deal with the
issues, we really do have some major benefits from
these products. So it really is how much of a
risk/benefit equation which we have to deal with here
and not forget the actual benefits by focusing too
much on the issues. That isn't to say the issue is
not important.

The major issues associated with the use of cell substrates really relate to safety. I mean, that is our main problem here. This is something which has been really extensively debated over the last 40 years or so, with the acceptance of cells for production really being a hot topic as they come up from time to time -- primary cells, diploid cells and continuous cell lines, as Dr. Hayflick already mentioned. The perceived risks being attributed to contaminants coming from the cells. The main ones of interest really being viral contamination, DNA, and transforming proteins.

The whole of these issues have been discussed -- I wouldn't say ad nauseam, but they have been extensively discussed over -- what is it, from 1967 -- over 30 years or so. And what is interesting is when you look back over this, this is a selection of meetings. I am sure there are many, many more meetings. You see, I am only just taking this from 1967. It is the same players essentially or the same cast as we have for this meeting. We have the FDA. The IABS features strongly in this, the WHO, the NIH. So they are all there really right from the very beginning.
The main point here is that some of the
issues -- I mean, each meeting will have a special hot
topic, if you like. In 1967, it was diploid cells.
Then in 1978, that meeting was organized essentially
to see whether we could move forward with the use of
continuous cell lines, primarily because namalva cells
have already been produced by the Wellcome Institute
for producing interferon. And I suppose the 1978 Lake
Placid conference gave us a cautious yes to the use of
continuous cell lines for the production of
biologicals. And I am broadening this now not just
for vaccines, but for the production of biologicals.

But there was still a lot of "yes, but" at
that meeting. I wasn't there, and I am sure that John
and other people who would have been there would say
the same thing. It seems that it was, yes, we can go
forward, but, but. And certainly not in the USA, I
guess. That was the sort of general feeling at that
time. It was really the meeting in 1986, the WHO
meeting, a study group in Geneva, which really allowed
the field to move forward in so far as use of
continuous cell lines were concerned for the
production of biologicals.

At that time, there was an agreement from
this particular study group that continuous cell lines
could be used for the production of biologicals

provided that the level of residual DNA or residual
host cell DNA was kept below 100 picograms of DNA per
dose. That is the origin of the magic figure which has
been used for the last 10 years or so. And this
really allowed -- this decision really allowed the
field to move forward in the expansion of the
biotechnology industry to produce recombinant DNA
biologicals in continuous cell lines. This has been
the situation for the last 10 years or so or more now.

At the last meeting of the WHO, which was
1996, a WHO and NIABS consultation at Merieux

Fondation in Annecy, that is the time where the field
was reviewed, if you like. Where are we after 10
years or so of using this figure of 100 picograms?

Incidentally, that figure of 100 picograms was used as
the guidance for all the national regulatory
authorities. The FDA went a little bit further and
went down to 50 at one time, less than 50 picograms.

Industry was pushing toward the limited detection.
And it is interesting to see the European
Pharmacopoeia has a limit for recombinant hepatitis B
vaccine produced in continuous cell lines of 20
picograms per dose, which is very low and very
stringent. This meeting in 1996 was to review that,
and things have changed a little since then and I will
come to that later on.

The present meeting, of course, really
focuses on cell substrate for vaccine production, not
for biologicals in general. I think there is this
distinction which I would like to make later on. We
are now moving to potential use of overtly neoplastic
cells for vaccine production, and I think this is a
very timely meeting.

The sort of landmarks -- and I am really
going over a little bit the ground which has already
been covered by Dr. Hayflick. These were sort of, I
think, the sort of landmarks as you might say. The
gradual overcoming of the hurdles -- the use of
primary cells for vaccine production in polio really
exemplifies that in the 1950's. In the 1970's,
diploid cells were accepted for vaccine production,
but not without problems as we have already heard.
Then in the 1980's, after the WHO meeting, continuous
cell lines then became acceptable for vaccine
production -- namalva cells for interferon, vero cells
and so on -- hybridomas for monoclonal antibodies
before then of course, and recombinant DNA
derivatives.

Now the progress during this period of the
1960's to the 1990's has been really on a number of fronts. I think it is fair to say -- I can say this because the WHO isn't really a regulatory agency. But I think one can now say that regulatory decisions are made more on a scientific basis and not on opinions as such. And the progress that has been made in scientific knowledge really over this period is enormous. We have to remember that. I just put a few up here to flag them -- new diagnostic procedures, let's say, such as PCR; better understanding of molecular mechanisms of pathogenicity; and new concepts, the concept of validation has been introduced.

So we are now in a position -- it is perhaps unfair to say that the regulatory decisions can be made more on a scientific basis. We have got more scientific basis to make the decisions. I think that is perhaps the main point to make there. We can make decisions, better informed decisions, and I think this is an important part of the gradual move to acceptance of different cell substrates.

Part of this process as well has been co-defining, if you look -- perhaps co-define is too strict. Having regulations and guidelines and recommendations for production and quality control of
these products using cell substrates has also been a major development since that time, both on a national basis, on a regional basis, such as in the European Union and the European Commission, and the WHO, of course, on a global perspective. And I think what we are doing is really learning how to manage the risks to take advantage of the benefits. And I think that is the important -- what we want to do to take the field forward.

Very briefly then I will go through just to remind ourselves and really to reiterate what Dr. Hayflick has said. The primary cells -- I mean, I will just mention the disadvantages here in the sense that many people tend to use them because they tend to be easy to prepare and so on. But they really do have this major disadvantage of microbial contamination and especially of viruses, of course.

Can we go to diploid, the next one, and just go through these very quickly. Again, and it has already been eluded to, the great advantage of using diploid cells is that they can be well characterized and standardized. And so production then becomes based on well characterized cell banks, the master cell bank and a working cell bank. And this is true also for the continuous cell line.
But they all have their advantages and disadvantages. I just want to focus on the advantages. An infinite life span for the continuous cell lines, so you can keep them going forever I guess really. And again, production on well characterized and standardized cells for an indefinite time basis really.

Now when you look at the disadvantages amongst these cell lines, the primary cells had the problem with the contamination. But the main disadvantage of the continuous cell line is that many do express endogenous viruses and there has always been this concern over tumorigenic potential, should we say, associated with cellular DNA.

The main three risks then with these different cell lines for producing biologicals are contaminating viruses, and we must include here, the TSEs, the transmissible spongiform encephalitis agents, whatever that may be in the end, residual host cell DNA, and growth-promoting proteins. And as I said, the trick really is how to manage these. And by developing procedures, we can actually move forward onto guidelines and so on.

In fact, there are three principles essentially really to production of biologicals. And
these are really the basis for moving forward on all these cell lines. Control of the starting material, such as the baseline data, characterization of the host cell, and so on, if you are able to do that depending on what sort of cell line you're using.

Then you control the manufacturing process itself, this is for adventitious agents and so on, and also control of the final product. And that includes tests performed during development. Because not all tests will be carried out on a routine lot-to-lot basis. There will be some testing on development. And then there is a subset of those tests, which will be carried out on a routine basis. These are the lot-to-lot releases.

Now historically, assuring the quality and safety of what you might call traditional biologicals has been what I call disaster-led. And you can make a nice list of all the problems we have had over the years and you find that control procedures are really established or altered following some major mishap, let's say. For example, Creutzfeldt Jakob disease from the bottom there in recipients of pituitary-derived growth hormone. I mean, that meant that we weren't now going to use the pituitary-derived growth hormone and move over to recombinant. So it is these
sorts of issues which really trigger the traditional.

But if you look at the situation which I call applied to biotechnology products -- I mean novel biotechnology products. I mean, it is all biotechnology I guess really, but I am thinking of recombinant DNA, monoclonal antibodies, cell cultures and so on. The approach has been somewhat different. Guidelines have been established early-on in the development of the new field to try and pre-empt any disaster. I mean, the idea here is that we think of what the problems might be and try to make sure that we don't land ourselves in these sort of situations.

And developing guidelines and points to consider, whatever you might want to call them -- and the different countries and different agencies call them different things -- I think these have been instrumental in establishing safety and quality of these newer type of biologicals, the biotech products if you like. Because they are by far the best characterized and best purified biological medicines in clinical use.

You see during this period in the 1970's and 1980's, guidelines on production and control or you might call them points to consider have been derived by many agencies. The main ones being the CBER
FDA points to consider, Japan has guidelines, the European CPMP biotech working group, those are the European Commission guidelines, and WHO. Now what is interesting is that the majority of these guidelines do have the benefit of mutual consultation during their development. If you look at them in some detail, you find that essentially they all say the same thing, but they are actually viewing it from a slightly different perspective with slightly different words. The ICH guidelines, for example, on cell substrates, that actually deals with more of the characterization of the cell lines and so on. So we find that these different type of guidelines do all have the same thrust, but they take it slightly in different perspectives. But they all say essentially the same thing.

Now as far as WHO is concerned, the first requirements for cell cultures for production of biologicals was the 1959 requirements for primary monkey kidney cell production of polio vaccine. Now all the other major agencies -- FDA and in Europe and the individual national states -- would also have their requirements as well. But WHO is the embodiment of a global situation. And using the primary cells, monkey kidney cells, for polio vaccine really did lead
to some confidence in developing other primary cell
lines for producing a range of vaccines. And these
are still in use. We mustn't forget that. The
primary cell is still a major source -- for example,
the chick embryo cells for measles vaccine and mumps
vaccine as well. So the development of these at this
point was a sort of major move forward.

Now I am not going to try and sort of
compare and contrast the different guidelines, because
I don't think that is particularly helpful. But what
I will try and do is just to scan briefly through the
guidelines and points to consider which have been
developed in relation to the cell cultures. These,
again, reflect considerable discussion. I mean, I have
just put the WHO ones. The first one there was the one
for oral OPV, and these are updating. As time goes on,
there is always a need for updating because some
technology changes and things move forward.

I have already mentioned the meeting of
the study group in 1986. It was published in 1987,
which allowed the field to move forward, and that was
the acceptability of cell substrate for production of
biologics. And then in 1987 as well, there was the
continuous cell line for the production of
biologics. The FDA, the ICH, the CPMP also have
their guidance documents as well specifically on cell
substrates.

Now I just want to say something about the
situation of 1998 as far as the WHO cell substrate
document is concerned. This document actually
reflects considerable international consultation. I
think this is probably the latest one or major
international one. It was published last year, but the
actual meeting -- there was a meeting in 1996 with
WHO, IBS, and we had the benefit of input from the
ICH, which went into great detail about how we should
control the quality of the biologicals produced on
primary, diploid and continuous cell lines. It covers
the whole three for production of viral vaccines and
the biologicals. It is not restricted to viral
vaccines. And it is interesting that this document is
very clear in that it encourages the move away from
primary cells to cell substrates which can be
generated from well characterized cell lines. As we
have already mentioned -- Dr. Hayflick mentioned this
idea of the well characterized cell line goes back a
long time, probably to the meeting -- I think there
was a meeting in Zagreb where this was developed of
having the master cell bank and so on.

Now in this particular document, there has
been a major change in certain parts of the way we handle the control of some of these cell lines. There is, for example, for well characterized diploid cells, a relaxation of the karyology and tumorigenicity testing and so on. But I just want to touch on one particular point which is important for the next part of the issues. And that is related to the DNA and the viruses. The main risks -- in this document, we still see that the main risks still are for the continuous cell lines residual DNA and maybe growth promoting proteins, and I will come to that in a moment, and for all cell lines, contaminating viruses.

Now as far as the DNA is concerned, there has been a major departure in the previous recommendation regarding residual DNA. And this is really due to a reevaluation of the situation and new data over this decade from 1986. But also new technologies have appeared on the scene -- gene therapy and DNA vaccines, which actually are involving injection or administration of in the case of DNA vaccines quite large quantities of DNA. So one has to be consistent here. Either DNA is really a big problem or it isn’t. Or is it the type of DNA? And this is the issue.

If you look at the 1986 summary, I guess
really, the cause of concern for that time was transmission of oncogenes. And the 1986 consultation provided this way forward by saying that if you can get your DNA down to less than 100 picograms per dose, then there is no realistic risk, and this is what everybody went forward on. The perceived problem was really not DNA post-op, but the specific sequences of DNA which may be encoding oncogenes and the possible insertion of endogenous or host cell protooncogenes or inactivation of suppressor genes by the DNA. The DNA itself was largely ignored, I think, and that is were the move forward is on DNA vaccines. We can go forward there because although there is a risk, the acceptance of this minimum risk is something which has been actively discussed and debated for the last two or three years.

So the figure now is that we now think -- by we, the current opinion. I think this is everybody. As I say, everybody has partaken into these general discussions here. They are based on current state of knowledge. The suggestion is that DNA from continuous cell lines can be considered -- the DNA itself now -- as really a cellular contaminant rather than itself as a significant risk factor requiring removal to extremely low levels which needed
validation and testing of each lot and so on. And the relaxation now is that we think that a figure of 10 nanograms of residual DNA from a continuous cell line will apply. And this applies primarily, of course, to purified recombinant DNA biologicals.

This is something which I want to touch on here. What we are talking about is removal of DNA to reasonably low levels. But the guidelines do make the point that residual DNA from continuous cell line may pose a higher risk if it might include infectious retroviral or provirion sequences, and then we may well still have to go down to lower levels. Now this is something I am sure that is going to come up in this meeting. What is the actual level which is applicable to vaccines. Can you actually go to that lower level.

I don't want to say much about the growth of multi-proteins. These are sort of more or less dismissed, I suppose, in the WHO guidelines. Growth factors that are produced by cells, of course, the risk is very limited. Yes, they do affect cell growth. It is usually transient and reversible. They don't replicate and many are inactivated in vivo. So the general thrust of the argument there was that they are not such a problem as we originally thought.
Now that might be different today, but the viruses remain the major and real concern for all cell types. The test for viral contamination really is a major part of cell bank characterization. In the guidelines, in the WHO ones, it covers the primary cells here. Now, here we are taking cells directly from animals. Sometimes in the wild animals for polio -- from monkeys for polio. And quite clearly now, we have to move into a situation if you are going to use animals that they must be healthy animals subject to veterinary and laboratory monitoring, very closed colonies wherever possible, not from the wild if possible. And animals, if they do come in from the wild, then they really have to be quarantined and appropriately monitored in the time before you actually take the cells for preparation of vaccines. And serological screening of donor animals for relevant pathogens.

Now you might say what is a relevant pathogen. Well that really is changing as time goes on, of course. The problem with doing this for a primary cell is that you have to do it for every vaccine batch. I mean, it is not the case of doing it once. You need to test the cells vigorously. It is an awful lot of work. And of course you can only test
for a certain number of known viruses. You can have a sort of catch-all, but there are mishaps. As we know, SV40 was missed, and this is an issue that comes back to haunt us as time goes on.

With a cell bank, we can actually do this testing, extensive testing for exogenous and endogenous agents, once only. You can do it and then you can make sure that this is the master cell and then you move on to the working cell bank. The further testing really is on the working cell bank or production cells really to detecting common adventitious agents, and that could be sort of a catch-all. It is not such a major issue.

It is interesting that the working group, the WHO study group, in 1986 valued the well characterized cell banks so much that it recommended the establishment of a well characterized cell line that would be available to national control authorities and manufacturers of biologicals globally.

And, in fact, the WHO master cell bank for vero cells was established following the recommendation by this group. It comes from African green monkeys and it was selected -- the vero cell was apparently selected because of the immediate prospect for improving the quality and quantity of several vaccines. Now it has
been used but perhaps not used as widely as was thought at the time.

Now the strategy for preventing viral contamination then is basically what all the documents go through. Tests on starting materials -- not just the cells. We have to remember that some of the agents will come in from the reagents -- the serum and so on. We have to be very aware of excipients as well.

Evaluation of production processes. That is the ability to remove or inactivate potential viral contaminants. That is your validation essentially.

This is the concept where validation has come in. And then tests on final product or appropriate intermediates. And essentially what people think is that it is not a single approach. One approach isn't sufficient. It is the combination of approaches which gives you this degree of security.

Now if you look at the WHO guideline, and I think this is true of most of the points to consider in the guidelines, they are really focusing on biotech products, that is, highly purified recombinant DNA products essentially. Now if you look down at the bottom, the purified biotechnology product -- you can have a very robust virus clearance system that you can
check for validation of virus clearance downstream and
so on. So even if you are -- I mean, I don't think
there are any biotech products produced or recombinant
DNA products produced in primary cells, but even if
you were doing it that way, you could be sure that --
you could actually be sure that everything was going
to be cleared up by the system. When you come to virus
vaccines, you have much less purification, and the
possibility of virus/virus interaction during
production needs to be borne in mind, and this is an
issue which will come up later in the meeting.

So virus vaccines, though, are a different
ballpark really from the general biologicals, which
everybody -- you know, most of these guidelines are
focusing on clearance of DNA and clearance of viruses,
looking at the characterization of cells. When you
move to virus vaccines, there is less you can do.

Now we mustn't think, of course, that we
know everything at this stage. I mentioned that we
have had enormous progress in the last 30 years or so.
We must be prepared for surprises. Two recent events
I think have raised awareness of the challenge there
is in dealing with viral contamination of cell lines
and vaccines which are used in their preparations and
the consequences. One was the detection of very low
levels of reverse transcriptase in chicken cell-

derived vaccines using this newly developed assay --
we call it PERT assay. This is from -- like measles
vaccine is an example and mumps is another example.
All the vaccines produced in eggs will contain very,
very low levels of RTAs. And this, of course, when it
first was discovered raised a lot of alarm bells.
People were thinking, yes, there must be retroviruses
in here and so on. And this really led to quite a lot
of activity trying to show where this activity was
coming from. Is it real retrovirus or what do we have
there? What is the problem? Or do we just have some
non-specific activity which really looks like reverse
transcriptase? And much work -- and WHO was very much
interested in this, of course, because if national
control authorities of one country ban a vaccine
because it is considered to be potentially unsafe,
this has ripples throughout the whole of the vaccine
community. I mean, what is not safe for one set of
kids in one country must be unsafe for other kids of
course.

So there was a lot of work to try and make
sure that this activity -- where did it come from and
so on. And manufacturers and the national control
authorities were very active here. It has now been
shown to be particle associated, but extensive studies have shown no transmission or productive infection, and that of course is good news. But as we will hear later on in the meeting, what about the potential for interaction between these particles and some of the viral vaccines -- the actual virus which is used for producing the vaccine during growth. We need to be sure -- I am thinking of pseudotypes and so on here.

This is an issue which we need to address in this meeting.

The other surprise was the detection of SV40 genome in rare human tumors. This is something which has come back to haunt us after 30 years or so. I am sure you all know that SV40 was a contaminant of some of the early batches of primary rhesus monkey kidney cells used to produce polio vaccines. This is no surprise. During the 1950's, these were actually used in a large number of people -- in the millions. There was follow-up with that to see whether they actually caused any problems, and nothing much materialized. And then suddenly about three or four years ago, the SV40 sequences were picked up in various rare human tumors. That raised the issue of was the vaccine -- was the polio vaccine made in primary kidney cells actually still transmitting SV40
or SV40 sequences. Because right in the beginning when SV40 was discovered, measures were introduced very quickly by national regulatory authorities to exclude SV40 from polio vaccines. For example, I am thinking here of the WHO. The 1959 requirements had to be modified and they were updated and the regulations in all the major national authorities were also changed to exclude SV40. And for more than 30 years then, polio vaccine made in primary monkey kidney cells have been shown to be free of SV40. Now by shown to be free, it was shown to be free by technology of the 1960's, I guess. I mean, it is old technology. And the question which everybody was rather anxious about was were we actually missing something here? And then the application of new highly sensitive PCR techniques for detecting SV40 genome was then introduced to see whether batches of vaccines were actually carrying any SV40 sequences. This is work carried out by CBER and also by NIBSC, and we are looking back on historical samples as being quite clear that the methods introduced in the 1960's were effectively excluding SV40.

Now if I can sort of come back to where I started from in the beginning in relation to polio, the polio eradication is going to be with us in about
two or three years, and this is really an end-game and
how do we move forward here. Once polio has been
eradicated, transmission of wild polio would be
stopped. And the global certification of polio
eradication will come in around 2005, I guess. But
there will be continued use of the OPV expected for
maybe 5 or 10 years. It is unclear. The end-game here
is unclear really and how we move forward. But WHO is
looking towards cessation of immunization with OPV
around 2005 or 2010. USA will be moving to IPV in
2000 -- in January of the year 2000. So the question
is do we need to do anything about the vaccine, the
OPV. And we need now, because -- I think now as we
move to eradication, I think we do need to be
absolutely sure of the vaccine and introduce an
additional level of security. This is provided by
ensuring that SV40 sequences are absent from polio
virus seed. Because during the actual survey of the
vaccines, one seed I think from a manufacture was
found to be positive. The seed was positive. There
was no actual live SV40 there. There were some SV40
sequences there. It is considered that it is much
better to have the seed free of SV40. And we are now
actually updating the original requirements -- this
will come up this year -- making sure that all seed
will be tested for absence of SV40 sequences. The primary monkey kidney cells for production will now have to come, I think, from closed, intensely monitored colonies of animals and not from the wild. As time goes on, of course, new viruses are discovered and new problems arise. The foamy virus has been identified as one that we should be really sure is absent from these vaccines. So updating is something which we are moving forward towards in this end-game for on the polio. What is the next step then? What are the challenges for the future? The use of neoplastic cells or novel cell lines for vaccine production I think is really a challenge. And I think one could say as with any new technology, new sets of safety issues are generated for consideration. Not just by regulatory agents but by industry and regulatory agencies together. I mean, everybody is on a learning curve here. It is not one or the other dictating. Everybody is together here. We need to consider again, I think, some of the issues of residual DNA. Is it oncogenic? What is the issue there? Is there an infectious DNA in relation to what sorts of cell line you've got and what is in the cell? It is really timely to review and assess the risks in light of a
much better understanding of the molecular mechanisms of geo-originicity and of viral/viral interaction. As I mentioned, I think we will touch on that during the meeting.

What I think we have to do is to come to some realistic and scientifically sound decisions concerning the use of these substrates. Now, a point which I from sort of a WHO and a global perspective think is very important -- I think science and commerce in biologicals -- I mean, vaccines are made in one country and travel the world. They are not necessarily just used in one country. It really is an international situation. And so too are the public health questions which the use of these products raise. So if there is an issue in relation to a problem in one country, it is a global issue. And the international dimension of the discussion of these issues is vital. And I am glad that we do have an international group here, and I think it is important that WHO is part of this and we are very pleased to be co-sponsoring this meeting. Thank you very much.

DR. RABINOVICH: Are there any questions specifically for Dr. Griffiths? Dr. Rubin?

PARTICIPANT: I was not quite clear on what you were saying about the presence of
retroviruses in chick embryo cell cultures. Whether
you thought that in fact it was present or not. And
the reason I am asking is we had a lot of experience
with chicken leukosis viruses in chick embryo cells
beginning back in 1960. And the thing about them is
they are not easy to detect because they don't produce
any pathogenic effect and they have to be detected in
indirect means. The indirect means in those days when
they were first found by us, at least, was
interference with RAS sarcoma virus preventing. And
then much later on came -- at least 10 years later
came reverse transcriptase. The thing about these
leukemia viruses or leukosis viruses, these
retroviruses, other than the really pathogenic ones,
is that they are highly species specific for one
thing. So chicken viruses in general, with some
exceptions, will not infect other species. So if we go
up the scale, I think, to the mouse leukemia viruses,
I think it is generally true of them also that they
are not cytopathic in culture. They can't be detected.
And probably they are of no concern to other species
unless they are very closely related. The concern I
would have of vaccines made in higher species, monkeys
or humans, is that probably there are -- or not
probably, there may be some stealth viruses like these
that don't produce any obvious effect and that we
don't even know about their presence to even detect
them in animals. Let's say the chicken virus requires
a flock of chickens that is not infected with them
that has to be infected at a very early age and you
have to practically wait for half a lifetime of the
animal to produce any statistical effect among them.
So I wonder if there is any concern about these points
with the retroviruses.

DR. GRIFFITHS: Can I just clarify the
point about the ALV, the avian leukosis virus.
PARTICIPANT: Yes.

DR. GRIFFITHS: The vaccine production
stipulates that the eggs are free from the -- the
chickens are free from the avian leukosis virus.

PARTICIPANT: Yes.

DR. GRIFFITHS: So the reverse
transcriptase activity wasn't -- it wasn't expected.
There are very low levels of RTAs. I mean, it has
been tested by the ordinary methodologies -- the sort
of standard methods. It was only when you went down
to these very low levels. And the origin is now known.

Where it comes from is endogenous viruses, retros.
And they do form particles, but as I said they are
non-infectious particles. But on a global basis,
yellow fever for example -- the yellow fever vaccine

-- this is some sort of a risk/benefit which the WHO
has to sort of wrestle with which a national authority
has the benefit of not necessarily -- like the USA,
for example, doesn't have to. It can make a decision
right away. But, for example, WHO requirements --
they will be changing, but they have allowed
production in ALV positive eggs because you cannot get
ALV-free flocks in many -- in places where you are
actually making yellow fever vaccine, and it was
considered that the lack of yellow fever vaccine was
much more of a risk than the presence of the ALV.

Nevertheless, Western Europe and USA making yellow
fever vaccine is all in ALV-free -- from eggs from
ALV-free chickens. And that will now be introduced
into WHO requirements as well, I think, in the future
because these countries which were producing in ALV-
positives can now actually get the eggs free.

PARTICIPANT: Okay.

DR. RABINOVICH: One last question?

PARTICIPANT: I was really going to
comment on actually the last comment from Dr. Rubin.

And reiterate your point that the sequence that appear
to be responsible for the RTA activity appear to be
the AEV gene and not the ALV gene.
DR. GRIFFITHS: That is right. Yes.

PARTICIPANT: But actually both the AEV and ALV genomes can actually undergo recombination. One of the subgroups of ALV is actually based on the AEV envelope gene sequence. But I really wanted to challenge the assumption that we should not be concerned about the species specificity of the retroviruses. In fact, you can induce tumors quite readily in certain species quite diverse from any species with RAS sarcoma virus that contains the ALV envelope gene in subgroups like Subgroup D. So it is, I don't think, a correct assumption to state that viruses like the avian retroviruses are not of concern. They could be.

DR. GRIFFITHS: I think David's point -- did you introduce yourself David? That is David Onions for the record.

DR. RABINOVICH: Thank you.

DR. GRIFFITHS: But I think the point made is very important that although we assume that some of these things are innocuous, I think it is much better if you can get rid of them to have them out of the way, and I think this is the point really. Because there are a lot of interactions going on and there is a lot of new information coming through, which I am
sure will be discussed over the next few days.

DR. RABINOVICH: Thank you, Dr. Griffiths.

I would like to introduce our next speaker, who every single presenter and panel chair at this conference knows well because he has been contacted individually by him. It is Dr. Andrew Lewis from the Center of Biologics and Review at the FDA.

DR. LEWIS: Thank you, Regina. I think pestering is a better word perhaps.

DR. RABINOVICH: Persistence, leadership.

DR. LEWIS: We have had a lot of conversations with everybody. As you've heard from Dr. Hayflick and Dr. Griffiths, until the end of the 1980's, the use of all types of neoplastic cells for the production of biologicals was controversial. With the development of the defined 100 picogram limits of residual DNA, the development of the concept of viral clearance, and the World Health Organization's acceptance in 1987 of interferons and monoclonal antibodies that were produced in tumor cells, the issues regarding the use of neoplastic cells for the production of purified biological products were resolved. However, the use of neoplastic cells as substrates for live virus vaccines continue to be controversial.
A number of factors are motivating the need to reconsider using neoplastic cells for vaccine development, and I have listed these factors on the next slide. These factors include the development of the whole virus or traditional vaccines to HIV, bioengineering approaches to viral attenuation to vaccine development, the rapid development of vaccines to emerging viruses such as the H5N1 and the H9N2 influenza viruses, significant progress in understanding carcinogenesis and in detecting adventitious agents, and finally the very successful experience with the highly purified biologicals that were derived from neoplastic cells.

Now conflicting with the need to use neoplastic cells as vaccine substrates are the regulatory concerns over the possible risks associated with vaccine manufacturers in these cell types. I think it is no secret that issues regarding vaccine safety receive a very high level of public attention. It is also no secret that public confidence in vaccine safety is a critical component for the success of immunization programs that are important to the public health.

The scientific and regulatory challenge is to develop the concepts and the technologies that can
be used to assess the risks perceived to be associated
with neoplastic cell substrates in a manner which
sustains public confidence in vaccine safety. To
achieve this goal, we at CBER believe that it is
essential that an approach be developed which can be
used to objectively and critically assess using state
of the art technology those issues associated with the
use of neoplastic cells as substrates for vaccine
manufacture.

The purpose of my talk tonight is to
outline an approach that CBER is proposing to
establish an objective and systematic means of
managing the regulatory concerns that are associated
with the development of vaccines in neoplastic cells.
To begin the discussion of this approach, I would like
to define exactly what we mean by the term neoplastic
cells.

For my talk, the term neoplastic cells
refers to immortalized cells that are derived from
either tumors or from transformation of instant tissue
culture, and these cells can either be tumorigenic or
non-tumorigenic when injected into animals.

The approach that CBER has followed to
consider neoplastic cells as possible vaccine
substrates is based on the approach worked out in the
1980's to evaluate the use of continuous cell lines to manufacture biologicals. Now as shown in the next slide, this approach consisted of five components. These components included identifying the issues, developing theoretical and experimental models to evaluate each issue, validating the models for issue-associated risk evaluation, developing criteria to evaluate the levels of risk, and finally discussing these issues and the approaches in public meetings, which we are doing over the next several days.

The process of developing the CBER approach began with organizing and presenting a discussion of the use of neoplastic cells as vaccine substrates before CBER's Vaccines and Related Biological Products Advisory Committee in November of 1998. Following this meeting, CBER drafted a proposal which evolved from the Advisory Committee presentations. This proposal represents the first attempt in CBER to formally address the regulatory issues associated with the use of neoplastic cells as substrates for vaccine development.

Perhaps I should also point out that the draft proposal which has been distributed in your meeting package, as Dr. Rabinovich mentioned in the introductory remarks, is not -- and I will emphasis
the word not -- an official, approved FDA document

offering guidance on the use of neoplastic cell substrates. The issues and concerns that were presented to the Advisory Committee were developed in greater detail in this CBER draft proposal, and these issues and concerns were use to develop the agenda for this meeting. And I am going to review them for you in the next series of slides.

Now in this series of slides, I have attempted to organize the issues in a common format. The title of the slide represents the issue of concern. The first bullet represents the cell substrates that are involved. The second bullet illustrates the issues that are responsible for the concern. And the third bullet is a very brief summary of data that documents why the concern or why the issue is in fact a concern.

Now concern 1 represents tumor cell contamination. With regard to the presence of tumor cells as contaminants, at first glance this concern appears to be trivial, as viable cell substrates are almost always or always removed from viral vaccines. However, if cells from human tumors are used as a manufacturing substrate, the issue becomes a bit more complex. As noted in bullet 3, the inoculation of
human tumor cells into humans has in fact produced
tumor allografts on some occasions. Thus, for
regulatory purposes, not only will it be necessary to
remove or eliminate the cell substrate, it will
probably be necessary to document by validated
procedures the efficiency with which the manufacturing
process removes tumor cells from the final product.
The second concern is a concern with
adventitious agent contamination. All cell
substrates, as Dr. Griffiths has pointed out, are
subject to contamination with adventitious agents.
Such agents might infect vaccine recipients, and the
ample history of substrate contamination with viruses
and with other agents has provided reason enough for
this concern. Without saying much more about this,
Dr. Phil Minor is going to review this topic in
considerable detail during the Friday meeting.

Concern 3 is the concern with cell
substrate DNA contamination. Most vaccines consisting
of whole varions contain some level of residual DNA
from the cell substrate in which the vaccine was
manufactured. There has been considerable discussion
over the years as to the possibility that residual DNA
could be the source of activated oncogenes or other
manifestations of a neoplastic process and might
result in the transfer of neoplastic activity to vaccine recipients. Even though some live virus vaccines contain microgram quantities of DNA from their diploid cell substrates, there is no evidence that any adverse event has resulted in an exposure to residual cell DNA. While diploid cell DNA is thought to be free of all the accoutrements of neoplasia, immortalized cells and tumor cells contain various combinations of genetic alterations that contribute to their neoplastic state. For this reason, we believe it is necessary to revisit the possible risk associated with the residual DNA from neoplastic cell substrates.

Now in addition, tumor cells can carry part of or the entire genome of oncogenic viruses. Furthermore, the need to produce or develop retrovirus vectors and retrovirus vaccines means, as Dr. Griffiths has pointed out, that retrovirus proviruses can also be present in any substrate DNA that remains in retrovirus vaccines that are manufactured in neoplastic cells. These possibilities generate the concerns produced by the presence of residual cell DNA in viral vaccines manufactured in neoplastic cells and are the reasons why this topic is covered in the session on Thursday.
Concern 4 represents cell protein contamination. Tumor cells produce a variety of cytokines and lymphokines and other biologically active proteins. Some of these proteins have the capacity to produce local and systemic reactions. In addition, random selection of cells from the human population as well as alterations that occur with some frequency in the genomes of neoplastic cells might result in the presence of abnormal PRP proteins, and whether such proteins might be able to produce spongiform encephalopathy so far as I am aware is not really known, but the theoretical possibility that such events can occur means that we should be addressing these concerns and discussing them at this meeting.

Concern 5 is the concern with viral/viral and viral/cellular interactions. In cells infected with more than one virus, a variety of interactions are known to occur between the replicating viral genomes. These interactions include genetic recombination, genome reassortments, pseudotyping or sequestering of the genomes of one of the viruses within the capsid of another virus, and finally the parasitism of the genome of large viruses by the entire genomes of the smaller viruses. If vaccines
are manufactured in cells containing adventitious agents, each of these processes could be associated with the transfer of non-viral components to vaccine recipients or their non-vaccine viral components. Evaluating the possibility that these events could occur and developing methods to search for novel virus derivatives and establishing baselines or threshold levels considered to be necessary to document the absence of such novel agents will occupy two sessions of the workshop.

The final and sixth concern is the concern with genomic instability. In contrast to diploid cells, immortalized cells and especially tumor cells evolve by changing the configuration and expression of their genomes. The question, and therefore the concern, raised by this characteristic of neoplastic cells is whether such changes could over time mobilize occult viruses or proviruses or result in the production of biologically active proteins resulting in adverse events. Although we are not aware of any evidence that such events have occurred, the ability of tumor cells to evolve over time is well established and we suggest that the consequences of neoplastic evolution for the safety of vaccines manufactured in neoplastic cells needs to be considered.
Now once the issues and concerns associated with the possible use of neoplastic cells as vaccine substrates were identified, it was necessary to consider an approach that would allow the possible risk posed by these issues to be assessed.

As issues of risk assessment are best evaluated using quantitative data and as current technology provides the opportunity to measure most biological events, we are attempting to develop a systematic, quantitative approach to assessing the concerns associated with neoplastic cell substrates using what we have designated as a defined risk approach algorithm. This algorithm is presented in the next slide.

The basic features of this algorithm include assessing the levels of risk posed by each issue quantitatively, establishing the probability of a worst case scenario for each issue, using the data to evaluate the risk individually and cumulatively, and then using the data to assess the relative risk of the product. In the next talk, Dr. Phil Krause is going to discuss risk factors and the use of experimental data and the application of this algorithm to specific concerns.

I would like to end my talk with sort of a short story. In June of 1998, I was visiting with
two friends from medical school. Both of these
friends are practicing physicians and have been in the
business of delivering health care for many years.
During one of the many conversations about
professional issues that always come up when we get
together, I asked my friends if they would consider
giving vaccines produced in cancer cells to their
family and their patients. There was a slight pause
in the conversation and then one of them answered with
a seven-word remark that was both an expression of
confidence as well as a reminder of responsibility.
When this remark is committed to paper, it also turns
out that it can be somewhat poetic as well as
prophetic. And since it has been following me around
for the past 14 months, I thought it would be
appropriate to share it with you as my last slide.

In response to the question about using
vaccines made in cancer cells, my friend simply
replied, "If the FDA says they are okay." That
question is being addressed at this meeting. The
question that is actually being addressed at this
meeting is how to find out if they are okay.

Beginning with the controversy over the distribution
of non-defective adeno SV40 hybrids that were
developed in my laboratory in the late 1960's, with
the Oscillimar (Phonetic) Conference on recombinant DNA in 1975, and with xenoscience transplantation issues since I have been with CBER, I have observed that when technological advances make it necessary to address precedent setting issues, it is essential to first bring together knowledgeable scientists and interested individuals from other disciplines to discuss the issues and the data that are available for informed decision making. Following these initial instructions, consultations, collaborations and cooperation among the interested parties, we have usually been able to resolve many or most of these issues.

Technological advances are once again asking us to address a precedent setting issue in the field of vaccinology. With this meeting on cell substrates, we are in the process of reviewing and evaluating the data that are available for informed decision making. And I would like to conclude by saying that those of us at CBER who have been working on cell substrate issues believe that through consultation, collaboration and cooperation with our colleagues in academia, industry and the regulatory community that it should be possible to develop an effective approach to evaluate and manage the use of
neoplastic cell substrates for vaccine manufacture.

Thank you.

DR. RABINOVC: Are there any questions specifically for Dr. Lewis? Please identify yourself.

PARTICIPANT: Dr. Tevethia from Penn State. In the study that you cited where the allografts were accepted in the patients, you didn't mention, did they progress to the tumor stage or did they regress or disappear?

DR. LEWIS: I am sorry?

PARTICIPANT: Did they progress?

DR. LEWIS: Yes, they did. These studies -- if you look at that literature, Tev, it goes back to the late 1940's and early 1950's most of it, and the cell line that was particularly problematic was called hep-3. And most of these studies were done in patients with terminal cancer. And so it is very hard to assess the implications of that for a more modern problem. But preceding that information, there were several reported cases in the literature, and also cited by Dr. Luci Gross in his book "Oncogenic Viruses". So reviews -- this is where most of his information came from. But there were several cases in the literature in which surgeons had actually grafted themselves with a tumor cell from patients who
they were operating on in the 1920's and 1930's. So it is an unusual phenomenon, but it is not unheard of. And I think when we are talking about vaccines that are going to go into possibly millions of people, we have to consider the breadth of the experience that the population would be subjected to.

PARTICIPANT: Dr. Fried from ICRF. How many cells were injected to get these nodules?

DR. LEWIS: Mike, a lot of those things were done not with cells but with explants. That was before the days when trypsin was used to break up cells. So they would just take a little piece of the tumor with a trocar and stick it in. The tumors were generally several mm, but it was not -- that is just a rough estimate because this is a fragment. Of course with the surgical -- when the surgeons inoculated themselves, there is no way to know.

DR. HAYFLICK: Actually, many of those studies were done by Chester Savin.

DR. LEWIS: Yes, and Alice Moore.

DR. HAYFLICK: And Alice Moore. And they were done with prisoner volunteers at the state penitentiary in Ohio. So they were not terminal cancer patients.

DR. LEWIS: Well, yes. I went back and
looked at that data, Dr. Hayflick, and the prisoners uniformly rejected the tumors.

DR. HAYFLICK: Correct.

DR. LEWIS: Yes.

DR. HAYFLICK: Correct. And I think that is important to know.

DR. LEWIS: Yes.

DR. HAYFLICK: However, when we inoculated HeLa into terminal human cancer patients, the HeLa cells did indeed grow to the point that they had to be extirpated.

DR. LEWIS: Yes.

DR. HAYFLICK: I also should point out that hep-3 later turned out to be HeLa.

DR. RABINOVICH: Thank you very much, Dr. Lewis. Last but not least, Dr. Phil Krause has the unenviable position of being between you and bed before tomorrow morning. So I welcome him and his presentation of setting the stage for conceptual and experimental approaches to address product safety issues raised by novel cell substrates. And he becomes our guinea pig for testing of the computer system.

While he is doing that, let me remind all speakers that the manuscripts are to go to our
colleagues at IQ Solutions, and that there is, I believe, still space at the late breaker session for tomorrow evening which was thought up by Dr. Lewis, but we welcome comments, arguments and particularly data that people may want to share at the late breaker session.

DR. KRAUSE: What I want to talk to you about tonight is the defined risks approach that Andy just alluded to. And the idea is that we would like to develop a quantitative approach that would enable us to use laboratory data to assess risks associated with cell substrates. The change in precedent against the use of neoplastic cells as vaccine substrates needs to be based on objective scientific data that can be used to evaluate levels of risk.

This approach is an example of science-based regulation in which FDA attempts to gather the relevant scientific data before making a decision. And as Andy pointed out or as Dr. Lewis pointed out, this could be implemented by assessing the level of risk posed by each issue quantitatively, establishing the probability of a worst case scenario for each issue, using data to evaluate the risks individually and cumulatively, and using the data to assess the relative risk of the product. So I am going to go
through these four points in this talk.

The first of which is to quantitatively assess the level of risk posed by each issue. I am also going to go through a simple example of how the defined risks approach can be applied to an issue. In order to quantitatively assess the level of risk posed by each issue, it is necessary to first assess what are the issues associated with the plausible risk to vaccine recipients, and Andy Lewis went through some of these, evaluate the need for and availability of quantitative data to assess the risks in a regulatory setting, to evaluate factors that could increase or decrease this possible risk, and to consider development of assays that could be used in evaluating specific products and the general issue.

As it happens, these are four of the major points which are going to come up in a recurrent way in the panel discussions later on during this meeting. So much of the discussion in this meeting is going to focus on this first part of the defined risks approach algorithm.

Now I am going to briefly discuss how this algorithm could be applied to the issue of infectious residual DNA in biological products. And I don't mean to prejudice the discussions that will occur later in
the meeting, but I think it would be useful to go through these steps as we have considered them so far for this issue to illustrate the kind of analysis that we think will be valuable for all of the issues to be discussed at the meeting.

You have already heard some of this from Dr. Hayflick and from Dr. Griffiths, but according to the recently revised WHO requirements for the use of animal cells as in vitro substrates for the production of biologics, the amount of cellular DNA in biological products should be limited to 10 nanograms per dose. This is an increase from a previously established limit of 100 picograms per dose. This limit is meant to apply to continuous cell lines but not to products given orally or to products derived from microbial, diploid or primary cell culture systems. The 10 nanogram figure was derived by considering data and theoretical calculations regarding the tumorigenicity of injected DNA.

While CBER is attentive to WHO guidelines, CBER evaluates products on a case-by-case basis in determining appropriate limits for cell substrate DNA. Moreover, it should be noted that for live viral vaccines and other less purified products, it may not be possible to limit the total DNA to 10 nanograms.
Thus the question is raised, what data would be required to provide assurances that this or any specific limit would be appropriate for vaccines produced in novel cell substrates, including neoplastic cells?

The first question is whether there is a plausible risk or not. Viral genomic DNA is infectious when injected into animals. Moreover, tumor cells and primary cells may contain virus genomes and may harbor latent viruses. And DNA from cells used to produce retroviral or DNA-viral vaccines may contain viral genomes as a by-product of vaccine production. Thus, based on the data, it would appear that under some circumstances there could be an infectious risk from residual DNA.

Several factors may influence an assessment of the tumorigenicity or infectivity risk associated with residual DNA. These include the total quantity of DNA in the vaccine, the number of doses to be given, the size of the DNA, sequence-related properties of the DNA, for example whether it encodes a virus, the number of copies of potentially infectious sequences per cell, and the state of the DNA, which I take to mean such factors as whether it is chromatin-associated, whether it is integrated into
a cellular genome, whether it is linearized or circular, et cetera. For many of these kinds of considerations raised on the slide, although it is likely that they have an effect on the ultimate infectivity or tumorigenicity of the DNA, these studies have not in general been performed in a quantitative fashion that would enable us to apply a quantitative risk assessment model.

This slide presents the quantitative data that we are aware of regarding the potential infectivity of cloned or purified viral genomic DNA. Information on tumorigenicity is also presented based on the assumption that viral genomic DNA is at least as infectious as it is tumorigenic. In these experiments, cloned or purified genomic DNA was injected directly into various animal models. On the right-hand side of the table, I have calculated the theoretical risk associated with a product that contains one microgram per dose of cellular DNA that contains a single genome per cell. This calculation accounts for the dilution of the viral genome and the cellular genome, and assumes that the viral genomic DNA is as infectious or tumorigenic when incorporated in cell substrate DNA as it is when it is linearized and injected directly.
The estimated risk of infection associated with the theoretical product would thus range from as high as 1 in 8,000 for polyoma virus DNA to 1 in tens of millions for other DNAs. An improved understanding of the relative infectivity of different types of DNAs might assist in developing tests to ensure that unacceptable quantities of infectious DNAs are not in biological products produced in novel cell substrates.

Another key point for discussion at the meeting will be the development of appropriate assays or methods to better evaluate these risks. It should be pointed out that direct assessment of the tumorigenicity and infectivity of residual DNA associated with a particular product may be impractical. This is because in order to achieve meaningful safety margins, very large quantities of DNA would need to be purified and tested in multiple animal models for infectivity and tumorigenicity. The example on this slide, for example, if a dose of a product contained one microgram of residual cellular DNA, assessment of a million doses in a single type of assay would require testing of an entire gram of DNA.

This is why I believe it is important to take a more general approach and understand the potential underlying infectivity and tumorigenicity of different
types of DNA.

Based on the data that I have presented, one might conclude that additional work could be done to achieve a more complete understanding of this issue. This might include studies of how to detect latent genomes, including for unknown viruses, how to quantitatively assess the effect of various factors on DNA infectivity, and improved assessment of DNA infectivity for different types of viruses.

Now it may be helpful to think about this issue in the context of a hypothetical example. Suppose an unpurified, live, attenuated viral vaccine is grown in a cell line that contains 50 copies per cell of a latent virus. This is the approximate number of copies of HPV DNA that are in HeLa cells.

If there were 10 nanograms of residual DNA per dose, which is the current WHO recommendation, and if two doses were recommended per child, as for instance is the case with MMR vaccine, and the infectivity of viral DNA in the vaccine were comparable to that of a purified polyoma virus DNA, we can calculate the theoretical infectivity risk from the DNA in this product, which I proceeded to do on this slide. Multiplying these numbers together with the infectivity of polyoma virus DNA yields a theoretical
infectivity risk of 1.2 infections per 10,000 children. For a vaccine that is universally administered to the U.S. birth cohort of 4 million children, this would represent about 500 infections per year, clearly an unacceptable rate. Of course, this assumes that the DNA is as infectious as polyoma virus DNA. It assumes that this DNA is as infectious as residual DNA in a vaccine as it is when it is cloned and purified. And it doesn't account for the potential effect of DNA fractionation or other measures that might reduce its infectivity. Based on the limited data I showed you, retrovirus DNA may be about 1000 to 10,000-fold less infectious than polyoma virus DNA. Thus, this calculation is very dependent on a clear understanding of the infectivity of the viral DNA and a clear understanding of what type of viral genomes a neoplastic cell could harbor.

The example I just went through could be thought of as a worst case scenario. And in fact, the second element of the defined risk approach algorithm is to establish the probability of a worst case scenario for each issue. Thinking of these kinds of issues in the context of their worst case is justified by the need for public confidence in vaccines and their safety, which requires that we take a very
conservative approach. This includes identification and discussion of all of the issues, determining which can be dismissed, which are relevant, and which are amenable to a resolution with additional data.

The third element of the defined risk approach algorithm is to use data to evaluate the risks individually and cumulatively. In this meeting, we will be individually considering a broad variety of potential risks posed by neoplastic cell substrates. Each of the meeting sessions is devoted to discussion of these potential risks. The meeting starts tomorrow morning with the consideration of mechanisms of oncogenicity because this is a final common pathway for many of the potential concerns with neoplastic cells. We then consider virus/virus and virus/cell interactions, residual DNA, and adventitious agents. We then turn to a discussion of how cell substrates might be designed or chosen to minimize these types of concerns.

In lieu of a poster session, there will be a session on miscellaneous topics on Thursday evening to permit participants to present additional relevant data. The final panel discussion on Friday afternoon will then summarize the results of the meeting. It is our hope that a comprehensive discussion of these
individual potential risks will help CBER also
consider them cumulatively.

The fourth element of the defined risks
approach algorithm is to use the data to assess the
relative risk of the product. Potential risks must be
placed in the context of the potential benefits of the
product. This is something which may need to be
considered by CBER on a product-by-product basis.

These risks also must be placed in the context of
risks already found acceptable and unacceptable by
society, and this can be a changing target. For
example, society has been willing to accept the one in
a few million or so risks of a vaccine associated with
paralytic polio for many years, but more recently it
appears less willing to do so.

We also hope the discussion will include
some consideration of the relative risks of using
different types of cell substrates, including the role
of species in cell type, whether it be primary,
diploid or neoplastic, and for neoplastic cells, what
the role of the transforming event and the known
history of the cell plays in the safety assessment.

I would like to close by putting this
discussion into the context of the real world. CBER
currently has applications to study the use of various
types of neoplastic cells in vaccine development.

Some of these products could be amenable to production in other types of cells, but others might not. By the time CBER gets an application, the manufacturer usually has already made a commitment to one cell substrate or another. It is our hope that this meeting will both raise awareness of these issues among manufacturers in the academic community and will provide CBER with a sound scientific foundation to answer these critical regulatory questions, which include what types of data regarding these cells are necessary to ensure that vaccines, including unpurified ones that are produced in them, are safe. And are there some vaccine cell substrate combinations for which current technology simply precludes an adequate safety assessment. I thank you very much and good night.

DR. RABINOVICH: I think we should entertain some questions. I think your approach will be provocative and will raise points for discussion throughout the meeting. Are there any questions at this time? Dr. Rubin?

PARTICIPANT: Well, I just thought this was an opportunity to raise a question about DNA that you, yourself, raised in a recent paper, of which you
were a co-author and which we were just talking about
and which I haven't seen proposed for discussion at
any point, which is that DNA are double-stranded RNA
of as small as 25 base pairs when incorporated into
the DNA of the cell and actually not incorporated even
in the cytoplasm, I take it, of the cell can cause the
production of protein molecules -- what are they MLH-1
and 2 or something like that -- that are supposed to
be very active in autoimmune reactions. And I wonder
how much you have thought about that and how much do
you think that is relevant to the considerations that
we have here. It is a very surprising development to
me that at least as little as 25 base pairs can be
inserted and produce a fairly large biological effect.

DR. KRAUSE: Yes. Of course, that was a
study in which these small molecules were transfected
into cells and the cells started to display some of
the characteristics of antigen presenting cells. And
then the question then is, is that something which
could subsequently be related to autoimmune responses
or things like that. I think those are legitimate
kinds of questions. I know that the world has a
fairly long experience with unpurified viral vaccines
which do contain eukaryotic DNA as residual DNA in
quantities greater than 10 nanograms. In particular
I am referring to WI38 or MRC5 DNA, which appear to be safe from that perspective. I was on the committee that licensed the -- or actually I chaired the committee that licensed the chickenpox vaccine, and that was actually an issue that we considered at that time as well, and actually looked among recipients of the vaccine for evidence of an autoimmune response associated with the DNA that was included in that vaccine. Actually, we didn't look, we asked the company to look and they did not find one. Of course, in the context of DNA vaccines, we are talking about injecting even larger quantities of DNA into people which then -- and one might imagine that this response is at least related to some of the responses associated with the DNA vaccine.

I think in the context of the meeting, we had envisioned the discussion covering mostly issues that are specific to neoplastic cells, and at least in our initial thinking about the issue didn't think that this was an issue, given the small sizes of the molecules that were involved, that would be likely to be more of a problem with neoplastic than with other cells. So we decided not to emphasize it at this time, in part because the plate is already very full. But you raise a very interesting point.
DR. RABINOVICh: Please identify yourself.

PARTICIPANT: Fried from ICRF London. Do you think it is fair in your polyoma example to use linear DNA which can recircularize and then go into the mouse and replicate and amplify and compare it to integrated, where it can't come out and amplify?

DR. KRAUSE: You know, I guess the question isn't necessarily is it fair, but the question is what data does one need to know or to have in order to be certain that a given level of residual DNA from a certain cell type is safe. Now the experiment that I -- the trouble is that not a lot of information is available on this. The only study that I am aware of where these data come from is that which was published by Marc Israel and Mel Martin's lab some 20 or more years ago I think. And what they did is they looked at simply circularized DNA, and that was more infectious than linearized DNA. And then depending on where you linearized the DNA, you then became more likely to either get infection or tumor. But I think these kinds of experiments could certainly legitimately be done in other ways, whether linearizing with an enzyme which leaves overhangs to yield a different result than linearizing with an enzyme which leaves blunt ends and thereby potentially
precluding recircularization. But I think --

PARTICIPANT: It doesn't preclude it. I mean, it will recircularize even when you cut --

DR. KRAUSE: Okay, reduce the likelihood.

PARTICIPANT: I mean, you could put it in a plasmid, so you have a small piece of DNA and it is integrated in that, and then you can compare that to say cellular DNA, where the virus can't get out and replicate itself.

DR. KRAUSE: You are raising a very legitimate point. To my way of thinking, these are the kinds of questions which need to be answered. The question is do they need to be answered on an intuitive basis or do they need to be answered on an experimental basis.

DR. RABINOVICH: Thank you. I think at this point, for those of us who have to coerce our children to go to school an hour earlier than usual, we will go ahead and close the session tonight and expect everyone to be here awake and coherent at 8:00 in the morning to start. Thank you very much.

(Whereupon, at 9:53 p.m., the session was concluded.)
U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE

FOOD AND DRUG ADMINISTRATION
CENTER FOR BIOLOGICS EVALUATION AND RESEARCH
INTERNATIONAL ASSOCIATION FOR BIOLOGICALS
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES
NATIONAL VACCINE PROGRAM OFFICE
WORLD HEALTH ORGANIZATION

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EVOLVING SCIENTIFIC AND REGULATORY PERSPECTIVES ON CELL SUBSTRATES FOR VACCINE DEVELOPMENT
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WORKSHOP
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WEDNESDAY, SEPTEMBER 8, 1999
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The workshop was held in the Plaza Ballroom, Doubletree Hotel, 1750 Rockville Pike, Rockville, Maryland 20852, at 8:30 a.m., Harry Rubin, DVM, and Martin Myers, M.D., Co-Chairs, presiding.

PRESENT:

HARRY RUBIN, DVM Co-Chair
MARTIN MYERS, M.D., PhD Co-Chair
NAOMI ROSENBERG, PhD Session Chair
HENRY PITOT, M.D., PhD Speaker
ALEX VAN DER EB, PhD Speaker
JAMES McDOUGALL, PhD Speaker
PRESENT: (continued)

STEPHEN BAYLIN, M.D. Speaker
WALTER DOERFLER, M.D. Speaker
JAMES COOK, M.D. Speaker
SATVIR TEVETHIA, PhD Speaker
FRANK SISTARE, PhD Speaker
MICHAEL FRIED, PhD Speaker
SANDRA RUSCETTI, PhD Session Chair

CLIVE PATIENCE, PhD Session Chair
LEONARD EVANS, PhD Session Chair
DAMIAN PURCELL, PhD Session Chair
PAUL JOLICOEUR, M.D., PhD Session Chair

ALSO PRESENT:

DAVID ONIONS, PhD
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CO-CHAIRMAN MYERS: On behalf of the National Vaccine Program, welcome. Our first speaker this morning will be Henry Pitot of the University of Wisconsin, and he's going to start talking about animals of neoplastic development.

DR. PITOT: Hopefully, this thing works.

Does it? Both Harry and I chose to speak down here. I guess the other speakers can decide whether or not they can see the slides from up here or down there.

I think my function this morning was basically to try to cover in about 15 or 18 minutes most of the more commonly used animal models for cancer development, and probably without further ado, I'll just start with the first slide, which is, I think, familiar to most of you.

It's probably the most widely used animal model for neoplastic development. It's, obviously, used by the regulatory agencies, and some people have considered it what might be considered the gold standard of carcinogenesis.

Unfortunately, it has a lot of drawbacks, as those of us that work in experimental systems know. But looking at the literature, in fact, probably most
of the data on whether or not a chemical is
carcinogenic really comes from this sort of data that
you see here, which really is based on studies that
went back many years, actually to the 1930s where
animals were given a carcinogen for an extended period
of time until tumors developed.

In this particular system there's
actually, as you can see, a fairly standardized type
of thing which allows one over a two-year period to
determine the development of neoplasm.

Now nice as it might look, there are a
number of problems, and I don't have the time this
morning to go into all of the difficulties. But one
of the more interesting facets that's come out of this
whole series is this table which I borrowed from Dr.
Ames' publication several years ago, which basically
demonstrates, looking at a series of some 380
different chemicals that were tested, the relationship
between whether or not they were carcinogenic or
whether or not they were mutagenic.

I think you can look at that table and
immediately see that perhaps one of both the advantage
of this chronic bioassay and the disadvantage is that
there are a variety of chemicals which, obviously, are
mutagenic and carcinogenic, which one might expect,
but there are also a large number that are perhaps not
mutagenic but still are carcinogenic.

So this type of system has led us to the
finding that we can do certain things with it, but
clearly we cannot begin to study mechanisms and
looking at dissecting the whole process of
carcinogenesis.

So as a result of that, of course, one
goes back a number of years, actually to the 1940s
when some of the earlier studies on what might be
called, what we call today at least, multi-stage
carcinogenesis were carried out.

This slide is just a classic experimental
slide which shows the studies that were done
originally by Barren, Blum and Schubik and others in
the 1940s which basically was done on the back of a
mouse, painting with a chemical, then administering a
material which at that time was an irritant called
croton oil, but today we call a promoting agent,
eventually ending up, if the format of the system is
appropriate, with neoplasm.

Now what I mean by appropriate is that
there is first an initiating agent, this material that
is given on the back of the skin, followed by a
promoting agent. If you reverse the process, it
doesn't seem to work, at least in most instances.

If you, for example, try just the promoting agent, nothing happens; and the interesting experiment that was done by Bautwell some 20 years later was that, if you change the format of the administration of the promoting agent, then you do not get neoplasm.

So here was a system which allowed for carcinogenesis to occur. The endpoint of this particular system is seen in this slide, which is the typical papilloma of the mouse. It is not a malignant neoplasm. It is benign, but it is useful in this particular system, which really tried to understand what might be called the latent period of carcinogenesis.

So this system was used, actually, for about 30 years, during which time the chronic bioassay developed. This system never became really important with respect to regulatory agencies or anything of that sort, but it was used primarily in academic circles in trying to understand the whole process of carcinogenesis.

It was in the 1970s that Dr. Carl Pareno at the Argon laboratory actually first sort of broke the ice by demonstrating that other tissues besides
mouse skin showed this exact same phenomenon -- that

is, of a stage phenomena in the development of

neoplasia.

Dr. Pareno's system, which is sort of

shown very briefly here, was to administer a
carcinogen -- in this case, this is actually a
modification of his which is done in our laboratory --
with a mitotic stimulus followed by the administration

of some promoting agent.

This material, this promoting agent, which
did the same thing as the irritant in the skin system,
now had become sort of a much more well recognized of

what we call today promoting agent.

Over the years since Pareno's
demonstration, there's been sort of a parallel
development of both the mouse skin system and the rat
liver system, which is what Pareno studied, in
addition to -- and I won't have time to name them all
here -- probably a dozen other different tissue

specific multi-stage models of carcinogenesis.

So it's not just unique as it was a few
decades ago just to the mouse skin. It clearly goes

well beyond that.

So I'll spend just a few minutes talking

about the liver system, simply because that's what's
done in our laboratory. I think potentially it can be
utilized perhaps to go on to other model systems as
well.

One of the big advantages in the liver
system is that we think, and there's certainly
reasonable evidence to argue, that one may identify
initiated cells. That is the very first step in the
development of neoplasia. This is just a histologic
section which shows in the middle of this section a
single cell which now expresses a gene, the piriform
of glutathionase transferase, which normal hepatocytes
do not express.

We have found this to be a very useful
what is called a marker for identifying initiated
cells and, as you'll see, these cells then under the
influence of a promoting agent go on to clonally
develop into small colonies in the organ -- this is
all done in vivo -- expressing the same particular
genetic component.

Now this -- It turns out not only is the
eexpression of this particular gene in these cells
abnormal, but a whole series of others which have been
studied over the years by many, many different
laboratories, either as markers or trying to
understand what actually is going on during this
process of promotion.

As a result of that, looking to mining years of work and many, many experiments, one can come up with a series of conclusions for the effect of promoting agents.

Now I'm sure in the discussion we can spend an awful lot of time on various other aspects, but I'm going to home just in on these two characteristics which I feel are probably the most critical aspects of these agents which cause a selective enhancement of cell replication basically of initiated cells. That is, they cause the replication of these cells different from the normal cells. They will also cause normal cells to replicate, but the initiated cells they cause much more effectively and selectively. That was shown several years ago by Farber and others.

The other aspect that they also do has been shown recently by Dr. Schulte Herman in Vienna. They selectively inhibit the programmed cell death of cells. So these two selective actions, actually, I would propose -- and we can discuss it -- actually can explain virtually all of the actions of promoting agents.

Now, of course, one easily says what is
the molecular mechanism of this. I think at the
moment we really don't know. We certainly have a lot
of ideas about what's going on, but there's still a
lot of work to be done.

You can also see in this system, perhaps

unlike the mouse skin system, that it is possible to
quantitate the development of neoplasia. So if one,
for example, administers a -- initiates cells by just

a single dose, a very small dose of a carcinogen, you
can see you increase the number of these putatively
initiated cells by three orders of magnitude, which
perhaps is not unexpected.

Also notice that there are a certain

number which occur spontaneously. Then if you promote
and cause these to develop into these small colonies,

only about one percent of these develop into that; and
although I don't have the quantitation on there, one
can also go on into neoplasm and, in fact, of this
perhaps one-tenth of one percent of these cells
develop into neoplasm.

So it allows one in a model system to
quantitate the various stages and really get an idea

of how effective the various stages are.

Now to do this, there have been model
systems developed -- this is one of them -- where you
can actually dissect each of the three stages. One

can initiate with this very small dose of a
carcinogenic agent, an initiating agent, which by
itself will do nothing.

One can then promote, as shown with the

blue line here, with a promoting agent. Then
somewhere down the line after these focal lesions,
these small lesions, have occurred, one can administer

another agent which Bernie Weinstein coined the term
progressor agents, which actually then causes during
this period the development of malignant neoplasms.

So during this period from the
administration of the initiating agent to this point,
basically you have these focal lesions. The cells are
different phenotypically. They are undoubtedly
different genetically, at least from point mutations
and other things, but they are not different
cytogenetically. They have perfectly normal
cytogenetics.

It is in the stage of progression that the
cytogenetics of cells become abnormal, as we all know.
Not only do they become abnormal, but they
continuously get worse. That is, they continually
evolve in what is called evolving karyotypic
abnormalities.
From this, both this model and others,
again one can determine certain characteristics. I've just listed some of the consequences of this major factor of the stage of progression. Four of them here, gene amplification, gene deletion, rearrangement, and a very effective way, certainly cells that are in the stage of progression are much more effective at accepting external genetic information by transection mechanisms than are normal diploid cells.

Consequences of -- You might say the functional consequences of this particular phenomenon can be seen in this slide, just some of them. There are probably a number of others. I've listed a few of these here, and probably for the discussion of this particular symposium, one of the most important ones is this particular part right here.

It is well known that cells in the stage of progression lose the expression of the MHC determinant which, of course, makes them a very difficult target for the immune system, because there will be no interaction between the MHC system and the T-cell receptors.

I'm sure that will be discussed in many other components here, but it does allow one by using
this multi-stage phenomenon, then to dissect this
compartment into its various units and actually try to
determine changes which occur at each of the different
stages.

Finally, and I've taken this from an
article by Ray Tennant from the NTP, the more modern
models of carcinogenesis are transgenic models, either
transgenesis itself by adding genes in the standard
transgenic mechanism or by gene targeting of
knockouts.

What I've listed here are three --
actually, two of the more common ones, the upper two
here, the T-53 knockout animal, both the homozygote
and the heterozygote, and the so called TGAC animal in
which the viral Harvey ras gene has been associated
with either a Zeta hemoglobin promoter or also, more
recently, with a keratin promoter, such that one can
actually do many of the skin tumor painting without
worrying about initiation, because basically one
already has initiated cell populations. So promoting
agents actually under this system become complete
carcinogens, in quotes at least.

Just briefly, looking at the upper one,
the P53 deletion mutation, this is taken from an
article that was recently published. This just shows
the tumors that develop in these animals, and you'll notice that -- these are mice -- that the normal animals spontaneously, at least out to this number of weeks, develop very few spontaneous neoplasms. But as; you might expect, in both the heterozygotes and the homozygote P53 animals, the spontaneous development of neoplasia is really extremely high.

This creates a problem perhaps when one actually is trying to determine mechanisms, because it is rather difficult to add to the system something to perturbate it, while at the same time the system is spontaneously developing a very large number of neoplasms.

On the other hand, if you look at the neoplasms that are produced in such a system, you can see that there seems to be a somewhat, at least quantitatively, different spectrum. Perhaps in the homozygotes deletion animal, lymphomas are the predominant component, whereas in the heterozygotes, one can see that the lymphomas still are a significant component of it, but both the soft tissue sarcomas and osteosarcomas, which are really a very small part here -- these soft tissue tumors become very prominent.

Now there are -- This is not the least in this long list of modern models. This just shows you
Some of you in the back probably can't read this, and it's really not that critical. It's not complete by any means. There are a whole series of transgenic models which are now developing. The difficulty is, if, of course, one wants to study the development of a specific neoplasm, it may be extremely useless. On the other hand, because the animal is programmed for certain things, it becomes perhaps difficult to study the earlier stages in the development of neoplasia and, rather, what one will be looking at is the later stages themselves.

So just to sort of sum up, what I've shown you are three basic models: This chronic bioassay which is perhaps, from the regulatory standpoint, the gold standard, but from the investigatory standpoint it has many problems; the multi-stage model which allows one to look and dissect at the various stages of neoplasia some of its mechanisms and its characteristics; and finally, the newer transgenic and knockout models which, certainly in specific areas, can answer specific questions, but probably they have to be geared and tooled to do this, and it seems that they will probably be most significant in answering questions in this final stage of neoplasia -- that is,
So I'll stop there, and hope I kept on time.

(APPLAUSE)

CO-CHAIRMAN MYERS: We have time for a few questions. If you would come to the microphone and, as we are transcribing the meeting, if people could identify themselves, both by name and institution.

DR. LEOWER: Johannes Loewer. Has ever DNA of neoplastic or normal cells been tested as initiator or promoter in these -- If so, what was the outcome?

DR. PITOT: I'm not sure of all the experiments. I know it was tested once in mouse skin, but probably not appropriately. It didn't work. But I guess that, certainly, if one can use in -- as we'll turn in the next discussion, in cell culture, one can clearly by transection of DNA get a situation where one can get -- I'm not quite sure what the stage one is dealing with, but certainly a transformation.

DR. FRIED: I'm Mike Fried, ICRF, London. You said in the beginning that certain carcinogens were not mutagenic. What is the mechanism, how they work, if it's not a genetic one?

DR. PITOT: Well, we can spend another
three hours on that one. I'll give you my opinion, and I'm sure it can be discussed otherwise.

I showed you that in the putatively initiated cells in the liver, there are a number of spontaneous lesions. We well know that spontaneous carcinogenesis is very characteristic of all mammals probably, more so of some than others.

So one could make the argument that the so-called non-genotoxic carcinogens which are not mutagenic in the chronic bioassay or in the Ames system and other systems are, in fact, promoting agents. What they are doing is they are causing the development of spontaneously initiated lesions through promotion and then spontaneously into progression.

That's a fairly simplistic answer, but I think it follows the facts.

CO-CHAIR RUBIN: I want to call attention to some recent work by Zarbell and Tilley with -- I think it was NMU carcinogenesis in the mammary gland of rats where they found that this so-called mutagenic agent, in fact, was causing mammary cancer, mammary epithelial cancer. But it turned out that all the tumors had a Harvey ras mutation in them, and that Harvey ras mutation preexisted in the mammary epithelium, and the agent -- you could call it then a
promoter, but in effect what it did was select with a clonal expansion of those clones in the mammary gland.

DR. DOERFLER: My name is Walter Doerfler from Cologne in Germany.

Isn't it a problem perhaps with the transgenic model as people are now finding applying the DNA array technology that, when one does a knockout, perhaps quite a number of functions are altered, not at all exclusively the functions that you are knocking out, but other functions.

So the interpretation must become extremely complex.

DR. PITOT: I certainly agree. I think that the use of the gene targeted and transgenic animals may be very useful, but I think it's new enough that we really don't know all of the problems that may occur.

AUDIENCE PARTICIPANT: In your multi-stage approach, how specific to the species that you are testing, the fact is, particularly related to the MHC expression, it is clearly published -- the regulation of the expression differs from species to species.

So, therefore, what could be changing expression in your rat model may not be applied to humans. You know very well that expression of MHC
genes is all over the place. It's up-regulation, down-regulation.

So how can you apply this overall and generalize your results?

DR. PITOT: I think that you see the sort of thing that you're discussing in the human also in the rodent, not only in the rat but the mouse, and the same sort of system. So that you will find neoplasms that will up-regulate the MHC components, others that will down-regulate them.

Unfortunately, that it will happen in the same neoplasm, that some cells will up and some will down. So, therefore, you're going to be faced with a problem, I think, from the vaccine question actually of trying to get cells to up-regulate, and there are mechanisms to do this, so that some of the vaccines may well be able to work.

I think it is applicable to all different systems, and certainly the human is perhaps the best example.

CO-CHAIRMAN MYERS: Thank you very much. Our next paper will be presented by our Co-Chair, Dr. Harry Rubin, Professor of Molecular and Cell Biology at Berkeley, and he's going to talk about multi-step carcinogenesis.
CO-CHAIRMAN RUBIN: As most of you know, our speaker was supposed to be Larry Loeb. He was supposed to be the first speaker. Larry Loeb suffered a back accident trying to climb into a cave, which he shouldn't have done, with his grandson.

We tried to find at a late date some alternate speakers. It was too late to get them to come, and so as the designated chairman I volunteered to speak. That will explain the rather primitive state of some of my displays, which were -- Some of them were made at four o'clock this morning.

So I would like to talk about --

basically, about the transformation of cells in culture, both the transformation to the neoplastic state of primary cells, if you like, of normal cells obtained from the animal and in some cases even from humans, and then into permanent cell, the highest.

The first observation that was made in a semi-systematic way about the transformation of normal mouse cells into sarcoma cells beginning with fiber blast was made, of course, by Wilton Earle of the National Cancer Institute, beginning in about 1943, and working together with Sanford they investigated this problem for many years.

Of course, as many of you know, at first
what they were working with was carcinogen induction
of transformation in mouse cells, but they found that
their controls were transforming at the same rates as
the carcinogen treated cells were, and that started
the whole study of spontaneous transformation of cells
in culture.

Probably the earliest really systematic
investigation of the transformation of mouse cells in
culture, although that's not what it was called in the
particular paper, was by Todaro & Green in 1963,
Journal of Cell Biology, and a similar systematic
investigation was carried out by Paul Kraemar and
associates in New Mexico with Chinese hamster cells.

What I've listed here are the basic -- not
necessarily the sequence of changes, but an indication
of a variety of changes that occur during spontaneous
transformation of rodent cells in culture. So let me
just run down them.

You take the mouse fibroblast, in the case
of Todaro & Green, and while they multiply fairly well
when they're first put in cell culture, the rate of
multiplication decreases with every passage. They go
through a crisis. If you're transferring a small
number of cells -- in that case like three times 10^5 --
you may, in fact, lose the culture, in a sense like
you lose human cells eventually.

If you have enough cells at each transfer, every third day you go through a crisis period, which means it looks like the cell culture is dying out, but then there begin to appear variance in the culture, and gradually you get an increase in cloning efficiency. You get an increase in the rate of multiplication back to the original rate and perhaps, in some cases, an increased rate.

Then you find that the growth -- the cells start to multiply to a higher saturation density. Roughly about the same time, they will grow in low serum concentrations or lower serum concentrations than they would grow in before.

Then later on at a later step the cells will grow in suspension in soft agar or methyl cellulose. This is very often associated with an ability to produce tumors in the animal, but not always. It is not an invariant accompaniment of ability to produce tumors.

Anywhere along these lines -- and we'll go into this in a few minutes -- you begin to get transformed colony morphology and transformed foci in the culture. Some of these things such as the transformed foci or production of tumors are spoken
about in a qualitative way when, in fact, they're quantitative, progressive increases in the neoplastic transformation of the cells.

You see that, I think -- In particular, this was shown by the group that worked with Chinese hamster cells at Los Alamos, Kraemer & Kramm, etcetera. They found that their hamster cells went through many of these stages, but as they were going along, they were continually testing them for ability to produce tumors in the isologous animals, in the Chinese hamsters.

What they found, that even though all these changes were occurring -- there were tissue culture representations of changes in the behavior of the cells after they had gone through their crisis also -- they would not produce tumors, and that was the case even where every cell in the culture had shown chromosome aberrations. After the 20th passage in culture, every cell in the culture was chromosomally abnormal.

They were still not producing tumors when injected back into Chinese hamsters, but about the 40th or 50th passage they could produce tumors, but they could produce tumors only under certain conditions.
The particular conditions were (a) to use 10 million cells -- a lot of cells -- and instead of inoculating the cells directly subcutaneously into the hamster, they were inoculated into gelatin sponges that had been implanted subcutaneously. Under those conditions, they produced tumors of the gelatin sponge, which up to that time didn't worry the hamster too much, but in fact they spread into the rest of the hamster after that.

Then with further passages when chromosome aberrations really became quite severe in all of the cells, the tumors could be produced by direct subcutaneous inoculation, and even later tumor production would occur with smaller numbers of inoculated cells.

Now the cells would also change -- this was shown first, I think, in the mouse -- would change after they produced the tumor in the mouse, if you take the tumor out of the mouse, and the tumor required the inoculation, let's say, of a million cells. Once you got the tumor, you could produce a tumor with 1,000 cells.

So that there was continuous progression.

What you see here is really a complexity of progression, that each one of these stages that we're
talking about is, in fact, an indication of another
change in the population of cells.

Could I have the next transparency? Well, this I did draw at about 4:30 this morning, and they're from memory. So if someone has a better memory than I do, please inform me.

This is taken from Todaro & Green's paper in 1963, which is real classic. What's shown in these top two graphs here is a plot on the horizontal axis of the number of passages of the cells in culture, and the increase in the cell population every three days, n-zero being the number inoculated. Sometimes they used n-1, that if they counted cells at one day and then they counted them in three days when they passage them.

At first, you could start out with a population that would increase about eightfold in those three days, doubling every day, but with each successive passage the cells would multiply slower.

You saw a lot of pathology in the culture.

Then at about the tenth passage it looked like the whole culture is going to die out. This is in passaging three times 10^5 cells, which at least at that time was thought to be a low inoculant of cells.

Then with further passages variance appeared. That
could be distinguished morphologically from the original cells.

They continued to grow faster and faster, and they reached a plateau, which was a relatively low plateau. These cells would not produce tumors in immunocompromised mice, for example, which is usually said to be the case, but in fact if you wait three, four, five months, very often a tumor will appear.

Again, when you get that tumor and reinoculated it into mice, it will produce a tumor very fast. So all that time these cells have been incubating in an occult stage in the animal, and finally a variant appears there, just like it appeared in cell culture.

Now if one increases the concentration of cells at every passage -- and this is a 3T12 passage which means 12 times 105 or 1.2 times 106 cells were passaged at each passage; three simply means they were passaged every third day -- then you get a decline in the rate of multiplication of the cells, but it's a much shallower decline, for one thing. Although there's something that resembles a crisis, it does not appear to be a very serious crisis.

Then you start to get the upswing in the rate of multiplication of the cells, and they end up
multiplying faster than these -- maybe not much faster -- but what they do do is multiply to a much higher saturation density.

These cells, when inoculated into mice, will now produce a tumor fairly quickly. The only difference here is that these cells have been passaged at four times higher concentration than the 3T3 cells. So there's something about the higher population density of the cells that furthers the transformation.

In fact, a few years later in 1968-1969 Aaronson & Todaro found that if you took cells directly from the mouse, start passaging them in culture, and inoculating them back into mice every few passages, that the higher the density of the cells that you made your passages at, the quicker the tumor would start to show up in the mouse. So there was something either selective or inductive about high population density in inducing tumors. It is obviously the selective aspect that you select for cells and that you grow at high density.

Now I want to say a word about human cells. This is a paper by Smith & Hayflick in 1974, and Hayflick can correct me on this. I'm sure I've got some of it wrong. Again, it's from memory. Basically, this was an experiment with
clones of human fibroblasts. I think it was the WY38 line. So what you're looking at here is the proportion of cells that go through a certain number of divisions in culture.

So I think it's widely thought that human cells will go through 50 divisions and sort of fall off the end of the cliff. That's based on work with large populations of cells, but when you study clones and their capacity to continue multiplication in culture, the capacity drops off in some clones right away, and right at about roughly the tenth passage or so -- I can't really give you an exact figure -- about half the cells have lost the ability to multiply.

So it looks like the loss of capacity to multiply is a stochastic event. It's a random loss of capacity to multiply. What you appear to end up with is the last surviving clone. That's the clone that goes 50 plus or minus ten divisions in culture.

That result was basically confirmed by Peter Rabinovitch of Seattle in 1983. He used a new technique for labeling the DNA of the cells, gromodeoxyuridine label. He worked with whole populations of cells, and he found that the percentage of human cells that were cycling decreased linearly with the number of divisions that the cells went
through in culture.

Now if you look at published literature also, it looks as though human cells will not undergo spontaneous transformation. There are many ways of eliciting long term growth in culture, but there's been some unpublished work, the senior author of which was a cytogeneticist, Tamara Ignatova, was working in Marguerite Vogt's lab at the Salk Institute a few years ago -- unpublished work using Li-Fraumeni cells, which are unstable -- relatively unstable human fibroblasts.

Again, what she found was that, if you kept the cells in a condition of confluence, which as we all know results in contact inhibition of the cells -- but I think it's not often appreciated, there's also a good deal of cell death that occurs at confluence -- then after a month or two of leaving the cells intact in a confluent layer, you start to see large scale chromosome rearrangements and indefinite growth.

So these cells apparently have undergone some kind of genetic changes involving chromosomal changes that are fairly easy to see that results in indefinite growth. That is, in effect, spontaneous transformation. Unfortunately, as far as I know, this
work has never been published, and I think there's
some disagreement among the group that's done it about
aspects of it should ultimately get published.

Do I have another? That's it? Well, I
want to turn to some slides now. I want to deal now
for a few minutes with a cell line, but what I'm going
to say about this cell line, I think, can be
generalized to cell lines in general.

So what I want to talk about is the NIH
3T3 cell line, which is famed in modern molecular
genetics as the first line in which there was
demonstrated a transformation of an animal cell line
by DNA extracted from a human tumor, the EJ bladder
carcinoma.

A problem with that original finding --
there are many problems with the original finding
which three laboratories reported back in 1981 -- was
the fact that again, if you take the original line of
NIH 3T3 cells and you let them sit at confluence for
a couple of weeks, that you start to see spontaneous
transformation.

In fact, probably the outstanding
characteristic of Jane Hill, Todaro and Aaronson's
original line of NIH 3T3 cells is the ease of
spontaneous transformation. All you have to do is
leave the cells for two weeks at confluence and

transfer them once, and foci start to show up

throughout the culture. I'll give you some examples

of that.

So again there's something about

surprisingly enough from conventional genetic thinking

where one thinks that at the highest rate of mutation

or genetic change is during cell division when DNA is

being replicated. Actually, it appears that the

highest rate of chromosomal change of mutational

change -- we're not sure which, or maybe all of them

together -- occurs when the cells are inhibited at

confluence.

You might ask why. Well, there's

something else which I already inferred, that there is

a considerable amount of cell death when these cells

or many cells are left at confluence for an extended

period of time. So there's considerable damage done

to the culture.

So let me show you a few slides of the

kind of observations that one makes. Let's go back to

the first one. Okay.

What you see here is a culture of NIH 3T3

cells which ordinarily would look like this, a mono-

layer of cells, nontransformed cells, but when left
for a couple of weeks or if left for a couple of weeks
and transferred, you get a high density population.
This is a transformed focus where the cells are no
longer in any regular arrangement. They're criss-
crossing. You can't identify individual cells,
because they're piled so thickly.

This is an unstained slide, which was one
of the first observations we made back in about 1988-
1987, which surprised us that the cells which people
were talking about as being used as targets for
transformation by the Harvey ras oncogene would
transform by themselves.

Now another feature is, when one looks at
independent transformations -- and now we're getting
to another point in transformation. So you start out
with a single culture, and you start splitting it, and
you let each one of them go to confluence various
numbers of times. You get independent
transformations.

If you look at the foci in each one of
these dishes -- these are the cuts from individual
dishes -- in a rough sense, the foci, which are these
thick aggregations of cells on a background of mainly
monolayers of cells -- each of the groups of foci look
different from one another.
So you can't simply talk about transformation of cells by producing foci. These --

If you collect the cells from any of these foci and inoculate them into mice, they'll produce tumors, and the tumors in the mice will all look like sarcomas.

But in culture where you can find a distinction in the appearance of the cells, each one of the independent transformations is different from every other one,

which means that the genetic changes that are occurring in these cells are different in each one of these cases.

So there are genetic changes, chromosomal rearrangements, deletions, etcetera, that are going on in these cultures that cause that kind of variation, which is reminiscent of what pathologists were telling us way back in the Thirties and Forties, that no two tumors look exactly the same.

Well, okay, now I want to show you want will happen with the NIH 3T3 cells if you culture them in a certain way. So what you see here are a series of cells that have been through what we call a primary assay, which means two weeks at confluence, and every two weeks they are transferred to become confluent again, and this is a tertiary assay, and this is after eight such sequences.
You start out with a culture that has no foci. By the third round of confluence, even when you place only 1,000 cells on a background of nontransformed cells, you see these thick foci. By the eighth passage at confluence, even 200 cells are producing about 100 foci. So that's a high rate of transformation.

Here is a culture derived from this same initial culture, but transferred originally about 100 times at low density. You go through the same operations, and you see much less evidence of transformation.

So there's continuous selection and change going on in these established cultures, and one cannot rely on them by saying, well, they're going to remain the same as long as we continue passaging them. It's very dependent, just like the original transfer of cells directly from the mouse -- dependent on population density. Even the established lines depend on population density.

If you look at another criterion of what was happening to these cells, in these cultures if you took the undiluted cell populations, you got a very rapid rise in saturation density, which remained roughly constant after that; whereas, in this case
even by the seventh round of confluence, there was only a minimal increase in saturation density. Another important aspect of these observations, contrary again to what you might expect -- You might think the cells which are becoming transformed would certainly grow more at high density. If you would grow them at low density, would they grow any faster? Quite the opposite is true. Actually, at low density these cells grow slower and slower, which is another indication that what confluence -- extended confluence is doing to these cells is damaging them, and apparently damaging their DNA. The problem is that occasionally in some of the cells, that particular type of damage results in transformation. Now if you take the original line of NIH 3T3 cells and clone it out, what you see here is three different clones. We've done this with very large numbers of clones, but these are illustrative of the heterogeneity you get within any single population. So this clone 1A actually started to show even in the first round of confluence very tiny dense foci. By the second round, it was showing these huge foci. Then we had to dilute it out, and it continued
to produce large foci.

This clone of cells transformed more gradually, and here you can see progressive transformation within a clone. At first, light foci, broad foci are produced. Then denser foci begin to show up, and finally you get foci with this clone that are just about the same roughly as this clone.

You take clone 4B here, and in spite of many rounds of confluence, here you seem to get one light focus, but in the sister dish which was being transferred and not being fixed, apparently were no foci. Even after five rounds of confluence, there's only a minimal amount of transformation.

So what this means is every time you are working with a mixed population or an uncloned population, it's always a heterogeneous combination of clones that behave differently from one another in transformation.

Okay. Now I want to talk about one other point. I tried to emphasize the point that an important aspect in transformation, both of primary fibroblast or at least explants directly from the animal, and in established cell lines is high density, is contact inhibition of the cells.
been clearly established in bacteria, which is always our great sounding board for genetic change -- Warner Arbor who won a Nobel prize working with bacteria that led to very important discoveries found that, if he left a culture, several cultures of bacteria sitting around for 17 years so they hadn't grown at all during that time except the first day that they were sitting there, that there were enormous chromosomal rearrangements that occurred even in a single chromosome of bacterium.

So cells that are being starved, in effect, are great candidates for genetic change, both in bacteria and apparently in animal cells.

Now what I'm going to illustrate in this last pair of slides is something that came as a great surprise to me. Having worked with cells for about 50 years now, I thought it was pretty hard to surprise me, but these cells are pretty clever.

It was this. We were working with this subline of NIH 3T3 cells which was very difficult to transform by keeping them at confluence, and we decided, well, we ought to look at what happens with clones of those cells to see how heterogeneous they are.

What we found was quite a shock to us.
Here we have two clones -- they're representative clones, clone 1A of this resistant line and clone 2E. Then the parental population, which we're calling large A prime here. Again, we go through one round of confluence, and then this is the fourth round of confluence and the sixth round of confluence. The shock was that, while the parental culture -- which is, after all, made up of all of these clones -- was exhibiting very little, if any, transformation or the barest minimum, the clones were showing a lot of transformation.

So what does this mean? I won't go into this point here. So we quantitated that on the next slide. This is the last slide. And we made a scale of transformation, which is shown here. So none of them transformed to the extent of producing these really thick foci, but they would produce distinguishable foci, and they would produce foci even on a background of nontransformed cells. Just look at this first panel here. We don't have to go into the second one. It's sort of a cumulative observation of relatively heavily transformed cells -- that is, this type here -- or an accumulation of those plus more moderately transformed
ones -- these here -- or more moderately and lightly transformed ones.

So the combination of all of them after five rounds of confluence had involved about 80 percent of the clones that we had. At that time, shown on the scale at the top, the parental culture had shown no transformation at all, in spite of the fact that it was made up of thousands of those clones.

So could I have the lights, please? So the right panel is a repetition of the same experiment. What that says to us, that when you get a heterogeneous mixture of clones from the parental population, there is some kind of mutual protection against transformation.

We later learned that back in 1981 George Post had found something very similar to this with melanoma cells in the mouse, that parental populations retained their consistency of behavior; whereas, the clones obtained from those populations underwent a great deal of variation.

What we have to think about in that case is what that means for progression in the animals, because the general model that Henry Pitot was presenting to you, and general model that Wallace Clarke, the great melanoma specialist, has emphasized,
that in many systems in humans, including the formation of melanomas, I think, of liver cancers, of colorectal cancers, of skin cancers, all seem to go through a sequence, let's say, -- colorectal is probably the best known these days -- of the formation of foci of cells developing into polyps, into adenomas, all of which are of monoclonal origin.

It's from those monoclonal, benign tumors, whether they are moles or adenomas or warts or, in the case of the liver cancer models in animals, these altered hepatic foci, that the next step is most likely to occur.

So that's a new parameter that we have to deal with where we get an association between the changes in the cell culture reflecting, we think, the effect of clonal expansion in the organism. Thank you.

(APPLAUSE.)

DR. ONIONS: Harry, I think you've proved that you don't need much warning to --

CO-CHAIRMAN RUBIN: Oh, I didn't go over. That's a first.

DR. ONIONS: Is there time for one or two questions?

DR. COFFIN: John Coffin, Tufts. What is
known, if anything, about the molecular changes that
are associated with these different colony
morphologies, for example?

CO-CHAIRMAN RUBIN: If you're asking me
what have we done, nothing.

DR. COFFIN: Well, what is known
elsewhere?

CO-CHAIRMAN RUBIN: Well, let me tell you
something negative. This is work Stuart Ansen did
with the NIH 3T3 cells before we ever got them.
So he encountered this spontaneous
transformation as well. So he tested the spontaneous
transformation by extracting DNA and doing the
classical transfection experiment into nontransformed
NIH 3T3 cells.

He found essentially none of them were --
the DNA of none of them was able to transform more
rapidly than as spontaneous transformation went on.
So there's no indicating, at least, that the Harvey
ras gene had mutated there.

What we know from classical genetics of
cells in culture, actually, was done with other cell
lines that was independent of transformation is that
when you get chromosome rearrangements or deletions,
like the deletions that you get in loss of
heterozygosity where you lose real chunks of the genome, is you end up with cells that will grow slower than the original cell.

If you get point mutations, it's very rare that that slows down the rate of multiplication, at least in the thymidine kinase gene, which has been looked at.

So we think that the correlation that we see of reduced growth rate at low density of the transformed cells parallels the classical genetic findings, and it's likely that what we're seeing is a lot of chromosome rearrangements and deletions in the cell, but we don't have any proof of it.

DR. COFFIN: If I may raise another question.

CO-CHAIRMAN MYERS: Make it short.

DR. COFFIN: Yes. Can you separate the slow growth property from the transformation property by forcing rapid passages to transform cells, for example?

CO-CHAIRMAN RUBIN: Yes. Well, you always then select for faster growers, and it varies with whatever population you use. In one population you can select from the faster growers, and they turned out to be less transformed, and in another population
you don't. But you can do it.

Not all cells that grow slower are transformed. That seems to be a more general finding that involves the whole population. It's only a subset of those, presumably particular chromosomal rearrangements, deletions, etcetera, that result in transformation. But a large proportion of those changes result in a slowing down of the growth of the cell at low density. Okay? Thank you.

CO-CHAIRMAN MYERS: Thank you. Our next paper is the transformation by DNA viral oncogenes by Dr. Alex van der Eb from Leiden University.

DR. VAN DER EB: So as you have heard last night and you will hear in the coming thoughts during this meeting, diploid human cells have a finite life span in vitro. They divide a certain number of times and then they stop dividing.

This property limits to some extent their usefulness for the production of viral vaccines or production of viral factors for gene therapy.

Now cell lines, continuous cell lines, are immortal and are, therefore, more suitable in certain aspects. However, there may be certain risks associated with the use of continuous cell lines. Therefore, it would be helpful if we would be able to
immortalize diploid human cells without transforming them.

So far the only means of immortalizing diploid human cells is by -- reproducibly immortalizing is by transforming them with a DNA virus. Whereas spontaneous transformation or spontaneous cancer is a multi-step process which requires a large number of different gene mutations or changes or alterations in genes that accumulate over the years, transformation by DNA virus apparently seems to be a one-step event.

This is due to the fact that the viral transforming genes are -- or gene is a multifunctional protein that alters simultaneously a number of different regulatory pathways in the cells.

I would like to discuss briefly transformation and immortalization by DNA tumor viruses, and particularly focus on SV40, an adenol virus, and also say a few words about HPV, but that will be more extensively dealt with by Dr. McDougall later during this meeting.

If I can have the first slide, please.

This slide shows the SV40 large T antigen, which is the major transforming gene of the SV40 virus, and it also shows the three main transforming
domains in this gene.

On the righthand side you see the P53 binding sites which overlaps with ATPase binding site, which is very important for transformation by SV40. A second site is more to the left, and that coincides with CR1, although CR2, the conserved regions 1 and 2, also is important. That is a site that's responsible for binding of the RB protein and the RB family proteins as well as the coactivator B300 or CBP.

In addition -- So this is the second important part for transformation, and in addition a third part is the N terminus which is the DNAJ-like protein -- like domain which resembles the DNAJ proteins of E. coli that have an important role in molecular chaperons for -- in conjunction with HSB proteins.

How the DNAJ and terminus of SV40 contribute to transformation is still unclear, but it is clear that P53 binding and inactivation of the RB proteins or P300 really very importantly disrupts the main growth control pathways in the cell.

Adenovirus transforms basically in a rather similar way, and the next slide shows the adenovirus E1A gene, which is the major transforming gene of adenovirus. As you can see here, there are
two again conserved regions here. One is CR2 which are important for transformation.

The third region, CR3, is not important for transformation, but another region, the N terminus which is not conserved among adenoviruses, is on the other hand again essential for transformation.

So the N terminus, CR1 and CR2 are important for transformation of primary cells, as well as association to cellular proteins. The N terminus and CR1 are responsible for binding to P300 and CBP as well as the P400 coactivator which is similar to -- more or less similar to P300, but as CR2 and CR1 are important for binding to the retinoblastoma protein and the related pocket proteins, so again basically in a rather similar way compared to the SV40.

Now the adenovirus E1A gene confers such a strong growth promoting effect on cells that these cells apparently -- certainly, if one has primary cells in which E1A is expressed to a reasonable extent, that these cells respond by activating their P53, and this will lead to either growth arrest or apoptosis.

So to counteract this growth arrest or apoptosis, the E1B region is necessary. So you see here the E1A region with the two proteins -- with the
two RNAs of proteins that I just showed you with CR1 and CR2, as well as the N terminus which is located here. But the E1B region calls for two proteins, a large protein and a small protein, and these are essential for neutralizing the apoptotic and cell cycle arrest activity of the E1A region.

So E1A and E1B are needed in order to transform cells, because of the effect of E1A on P53. Therefore, it is almost impossible to transform -- obtain E1A transformed cells alone, and very few cells -- and those are rodent cells that are obtained that are transformed by E1A alone -- express E1A to very low levels.

HPV, the human papilloma viruses, basically transform again in a similar way. Next slide, please.

The two transforming genes or papilloma viruses are E6 and E7. E6 targets P53 and causes its degradation, but E7 again targets the other growth regulatory proteins, PRB, the RB protein and its family members as well as probably P300, but that we'll hear later in more detail. So they resemble again the adenoviruses which have basically the same properties.

Now not all these cells react in the same
way to these transforming genes or viruses. Next slide, please. Here I show you SV40, how SV40 transforms cells, human fibroblasts, diploid fibroblasts or epithelial cells and keratinocytes. Fibroblasts are very efficiently transformed by SV40. Immortalization, however, is very rare, at least 10^-7 events per senescent cell, and there is a pronounced extended life span.

Epithelial cells, on the other hand -- not on the other hand, but epithelial cells more or less at the same weight are responding more or less at the same rate as SV40. There is morphological transformation. There is also rare immortalization. It's not completely clear how much their extended life span is, but I believe that there is an extended life span again in these epithelial cells.

The situation is different for adenovirus E1. Forget E1B. This is an old slide where I thought that I had E1A transformed human cells, but this is not the case.

Fibroblasts or epithelial cells appear to be surprisingly resistant to transformation by adenovirus. Frank Rijm in 1974 has done many attempts to transform human embryonic retinal cells with DNA of adenovirus, and he was initially completely
unsuccessful until he found one clone, and this clone
gave rise to the well known 293 cell, and that is
really the only clone that he has seen transfected
with adenovirus.

Fibroblasts similarly do not show
transformation, and I mean now transformation
according to the focus assay. You can introduce by
transfection the adenovirus E1 genes into human cells
where they are expressed, but we have the impression
that expression is lost after a while. There is no
morphological alteration.

These transformed cells -- and I'm talking
now about 293 cells -- They immortalize probably after
short crisis periods. Efficiency of immortalization
is not so clear, because there's only, as far as I
know, only one example. It's not so clear whether
there is an extended life span.

So we then switch to embryonic retinal
cells, because we had heard from work of Phil Denimore
and others that neural cells, cells of neural origin,
could be transformed more easily also in the rate
system, the rodent system; and we switched to
embryonic -- primary embryonic retinal cells, and they
can be transformed quite efficiently by adenovirus E1,
although still at relatively low numbers compared to
transformation of rat kidney cells, for example, but it is reproducible.

Also there is a high frequency apparently of immortalization, and there is no apparent crisis, which is rather surprising.

I will not say much about HPV E6 and E7. There is little interaction with fibroblast. There may be reported very rare immortalizations by E6, E7, but epithelial cells at keratinocytes are immortalized more frequently, and apparently there is no distinct crisis.

So transformation of human cells by SV40 has been studied most extensively. So I will briefly turn now -- go back now to SV40.

If diploids and diploid fibroblasts -- If diploid fibroblasts are transformed by SV40, then a number of changes occur, and these are depicted here in this slide.

The normal diploic fibroblasts grow to a certain saturation density during a number of population doublings, and then they stop dividing, and an irreversible arrest, growth arrest, which is called senescence, and it has been mentioned before.

No immortalization will ever occur, as far as I know, from these senescent cells. They do not
die, but they sit there just sometimes for many
months.

If you transform the culture at this stage
for a -- with SV40, then the saturation density
increases, and the transformed cells seem to ignore
this senescence arrest phase and just go on for a
number of -- for a large number of passages sometimes,
which causes an extended life span. However, in the
dead cell -- the whole protein will die and enter
into so called crisis where the cells -- the
transformed cells really die, unlike the situation in
senescence.

Only in a very few cases, very rare cases,
an immortalization will occur after a shorter or
longer period, and this will etherize to a cell which
is the same as the SV40 transformed cell before the
crisis, but now they are immortal and can grow
indefinitely.

So what is the basis of the appearance of
the senescence and the crisis? That has to do with
the telomeres. In contrast to the germ cells which
have telomeres active in these cells, the normal
somatic cells are telomeres repressed and have no
telomeres activity.

So during population doublings, t he
telomeres are lost, become smaller and smaller until a certain stage is reached, which may be about two-thirds of the length of the telomeres, approximately two-thirds, but certainly not all telomeres have been used up, and at that stage the cells start senescence.

This senescence is called M1 or mortality phase 1. If you, in contrast, inform with SV40, again the M1 senescence is ignored, and the cells continue dividing, and also the telomeres become shorter and shorter until they are almost completely disappeared. That coincides with the crisis which is also called mortality stage 2. So the M2. If there is no immortalization, the cells -- all cells will die here. Immortalized cells have now in most cases active telomerase.

So this is then the extended life span between senescence and crisis, and it's also this period which is characterized by the onset of chromosomal abnormalities. So there are -- and that effect already was also seen in the normal cells. When they approach crisis, there is chromosomal rearrangements. There are dicentrics formed, and so on.

That is probably due to the fact that during senescence the P53 pathway, P53-P21 pathway, is
activated for some reason. So what happens during

senescence is that at a certain critical length of the
telomeres -- we don't know exactly what the signal is
-- will cause activation of preexisting P53. There is
probably not more transcription of the P53 gene, and

this P53 causes accumulation of active P53 which
causes activation of transcription of the P21 gene,
the WAF-1 gene, which in turn is an inhibitor of
cyclin/CDK and inhibits the cell cycle. So this
triggers apparently a certain minimum length of
telomeres.

    Now catalytic subunits of the human
telomerase has been isolated. The question can be
raised would it be possible just to introduce
telomerase in cells and immortalize them in diploid
cells and immortalize them in that way?

    Indeed, there has been reports that this
is indeed the case. Dr. Hayflick has already
mentioned an example yesterday. Also Dr. McDougall
will probably talk about this later, but as Botnar and
coworkers have also found, that fibroblasts as well as
retinal epithelial cells become apparently immortal
when the human telomerase catalytic subunit is
introduced into the cells.

    Rather, Piono et al. showed that mammary
epithelial cells as well as keratinocytes do not immortalize just with only introduction of the telomerase, and this works that immortalization is obtained when, in addition, the E7 gene is added to the cells. That, I think, will be discussed later in much more detail by Dr. McDougall.

So this means that, if you inactivate the RB, then that would lead to immortalization. This may be due to the fact that there is apparently a third type of senescence, and that third type of senescence is called N0 and occurs usually before M1.

It is known that, unlike fibroblasts that can be found a long time, 50 to 60, 70 or sometimes 80 population doublings, depending on the cell strain and also depending on the lab, I have the impression, that many other cells when taking a tissue culture form human tissues will not grow very long, and after ten or maybe 20 passages they just stop dividing. This is long before the telomeres have reached the size, the minimum size which corresponds to the senescence, M1 senescence.

Now Weinberg has suggested that this M0 state is due to a kind of physiological stress and is caused somehow by suboptimal growth conditions that occur in vitro. So introduction of telomerase in
cells that still have to undergo an M0 may not work,
because M0 is cause for physiological stress and has
nothing to do with the size of the telomeres. On the
other hand, cells that have no M0 but only an M1,
there it may work, but this is all theory, and we'll
see if it is really true.

So the last slide then summarizes here for
the two senescent stages that occurred in certain
cells, including the mammary epithelial cells but
probably many more cells, and that is that M0 occurs
after -- in the case of mammary epithelial cells,
after about 20 population doublings, and may be
activated by physiological stress.

It's controlled by the RB P60 pathway, and
can be bypassed by HPV. It must be E7. This must be
E7, right? It's wrong. Jim, it's wrong. It is E7.
I'm sorry.

This was made just before I left, and
there are more mistakes here, as you can see. The
computer did a trick and did not exactly what it was
supposed to do.

Anyway, it's bypassed by HPV E7. One, on

the other hand, may be triggered by certain extent of
telomere shortening, and which is actually the mitotic
clock, and is controlled by the P50-CP21 pathway, and
that is bypassed by HPV E6.

M2 is called crisis leading to cell death and is probably caused by extensive telomere shortening. It has nothing to do with all these things.

So I think I'll stop here. Thank you.

(APLAUSE.)

CO-CHAIRMAN RUBIN: We can have a few questions now.

CO-CHAIRMAN MYERS: Could we have the lights, please.

CO-CHAIRMAN RUBIN: Lights, please.

Could we have the lights? Yes, thank you.

DR. HAYFLICK: Hayflick, UCSF. Unless I misunderstood your introductory remarks, there are indeed two other ways in which one can transform normal human fibroblasts to an immortal cell population. Namely, we have done this with exposure to cobalt 60 radiation, producing a cell line called SUS M1.

It's also been done with chemical carcinogens, producing a cell line called KMS T6, in addition to H terp which you've mentioned. I also should mention that, contrary to popular belief, there are several publications in the open scientific
literature reporting spontaneous transformation of normal human cells.

For those of you who would like those references, please contact me, and I'll be happy to supply you with them. Thanks.

DR. VAN DER EB: Thank you. Yes, you can immortalize -- transform or immortalize human cells also by radiation or chemical carcinogens. I think this is often quite difficult. You have to expose the cells many times, as far as I know at least, to the chemicals or to the radiation, and it is a lot more easy just to take SV 40 large-T for fibroblast. But you are completely right, yes.

DR. HAYFLICK: Well, the reason I emphasize that --

CO-CHAIRMAN RUBIN: Can you get to the microphone?

DR. HAYFLICK: The reason I thought it was worth emphasizing is that the thrust of this meeting is the development or the consideration of immortal cell populations other than those that might be currently used for vaccine production.

Two of the obvious choices, if one wants to avoid the overt introduction of viruses or their fragments into cells, is by the use of radiation or
chemical carcinogens.

DR. VAN DER EB: I know from cells that are spontaneously immortalized at least and also clearly transformed that they have lost quite a lot of things like P53 and also the RB pathway. So what you do then is basically the same as what occurs or happens in spontaneous transformation, I think.

CO-CHAIRMAN RUBIN: One more question.

DR. BERKOWER: Ira Berkower from the FDA. One concept that we've been struggling with is the notion of cells that are more malignant and less malignant, more transforming and less transforming, with the idea that if vaccines could be made in less transformed cells, that will be safer. Does that make any sense in terms of these molecular mechanisms that you're studying, that there would be a more transformed phenotype?

DR. VAN DER EB: What is a more transformed phenotype?

DR. BERKOWER: Or a more malignable phenotype?

DR. VAN DER EB: This is difficult, I think. If I see it now -- If I look at the literature, then I have the impression that if you take immortalization as the important final step that
you want to reach, that then HPV genes may be more suitable.

I have the impression that they are -- The cells look less transformed at least than in the case of SV 40, for example, but also in the case of adeno. But there's very little I can say to that, I think. It's so difficult. What is immortal? Which cell is more transformed than the other?

You would have to compare things like tumorigenicity. I should say that cells transformed by viruses are often not yet immortalized, I showed you, but also if they are immoral, they are not yet immediately tumorigenic.

So maybe tumorigenicity would be more important than just looking at the cells, how they grow.

CO-CHAIRMAN RUBIN: Okay. We'll have to move on to the next speaker, who is Dr. James McDougall from the Fred Hutchinson Cancer Research Center at Seattle, Washington.

DR. MCDougALL: Well, as Alex has already pointed out, the two things that I would rather talk about are human papilloma virus and telomerase immortalization. But when Andy called me -- Andy Lewis called me -- he said that somebody else had
dropped out of talking about hit and run, and so I got
lumbered with the job.

It's interesting. It's interesting, but it's controversial, without doubt. I'll just put my
first slide on. In the olden days, and there are a
few of us that were there in the olden days, one of
the most interesting studies that was going on in
terms of looking at how viruses might transform cells,
and particularly how they might contribute to human
cancer, was the belief that herpes viruses might very
well be responsible for, for example, cervical cancer.

There were good reasons for believing this. For example, serology gave very good evidence
that women with cervical cancer had high levels of antibodies for herpes simplex, and it became clear
that this was herpes simplex Type 2.

So as time went on, it looked reasonable to try and sort out whether or not this virus had
fragments, had subgenomic fragments that could
actually transform cells. That was one of the studies we concentrated on in the early 1980s.
The studies were carried out mostly in rat
cells or hamster cells, and lo and behold, we were
very pleased to find that we could actually transform these cells and, if we grew these cells up from an
initial transformation by exposing them to herpes virus DNA and to fragments of herpes virus DNA -- and I'll show you the specific fragments in a minute -- that those cells would then produce tumors in their rat or mouse host.

So this looked very good, and we felt we were on the track of what might be the key genes that were involved in the development of an important human tumor, in this case cervical carcinoma.

So over the years there were a lot of examples of how herpes viruses might perhaps have a hit and run type of effect rather than a similar situation that we see with the adeno viruses and SV 40 and human papilloma viruses where it's clear that very specific fragments persist in the cells that are either immortalized or transformed by those.

I just put up a series of papers that all refer to the hit and run phenomenon related to herpes viruses.

So if we look at the herpes virus genome -- and here is the genome along the top here, and what I really want to bring your attention to are these fragments here that map in this region of the genome.

We initially show that this one called morphological transforming region 1 would carry out
that transformation of both rat and mouse cells at a reasonably efficiently level, almost at the same level as SV 40 would transform these cells.

Subsequently, there were other studies which identified two other regions which also were capable of transforming, in this case, rat cells -- in both of these cases, rat cells.

Now that was somewhat surprising in that there would be more than one region of this genome that would actually transform or immortalize cells but, of course, this is a very large genome anyway.

So that that might not be too unreasonable.

So we continued with a large series of experiments to try and track down exactly what the region of the genome might be that was producing this transformation effect. Unfortunately, we were able to make smaller and smaller fragments of this MTR1 region until we got down to a region of that DNA which, in fact, was too small to have its own open reading frame, and immediately one has got to start worrying. If there's not an open reading frame, what in fact, is affecting the cell? Is there a protein being produced? In this case, clearly not. So we began to worry what the mechanism might be in this situation.
So one of the mechanisms we thought about was that there might be just random insertion of sequences into the host cell genome. So we looked at these MTR1 fragments, and particularly the MTR1 fragment of herpes simplex virus type 2, and by sequencing that we were able to show there was a structure in there very similar to a bacterial insertion sequence in its structure, and felt that it was highly likely that this was perhaps integrating and then excising randomly from cells, and this might in fact have a mutagenic effect upon the cell.

Now interestingly, we were able to take HSV1 and take a similar -- the identical region from the genome of HSV1 and look at the sequence and structure of that.

What we found was that that structure, although for half of this loop, is pretty well identical. The other loop did not pair sufficiently to make an insertion sequence-like structure. That region of HSV1 will not transform cells, whereas this one will transform cells at a reasonable efficiency.

So this seemed to be a reasonable suggestion, and perhaps we should look at, in that case, mutagenesis by this fragment, the MTR1 fragment.

Now while we were doing these experiments,
we also realized that there was another herpes virus
that we should look at, and that was the human
cytomegalovirus. The genome is shown up here.

Again, the region that we were
particularly interested in was this early region, the
immediate early region of the genome, which is
transcribed very early after infection by the virus.
So one of the experiments we did was to try and work
out whether we could actually, first of all, transform
cells with this region, and the answer was yes, and
this was repeated in two or three different labs.

Then we wanted to find out, if we used
very sensitive PCR experiments, could we in fact
identify regions of that CMV immediate early region
present in cells that were transformed by CMV. By
going through two rounds of PCR to pick this up, we
were able to in fact identify a fragment from CMV that
persisted in some of the cell lines, but really in a
very low percentage.

Most of the cell lines, just like the
herpes cell lines, had lost the DNA that we
transfected into them.

Again, when we looked at the transforming
region of CMV and made lots of deletion mutants of
that region -- so we started off with a region here
which was quite a reasonable sized piece of DNA, 2.9
kilobases, and just made deletions of this all the way
down.

Again, to our surprise, we could get right
down to here, and we were still capable of
transforming cells with that region. So again, this
suggested to us that maybe we were looking at the same
situation with CMV that we had seen with herpes
simplex type 1.

In fact, in that very small region of CMV
DNA, again you find this same structure present, and
in most cases that structure was lost from the cells,
that it had been put in by transfection, and we
actually carried out not only transfection with DNA
but made retroviral constructs and put that region in,
could again show that we could transform cells with
that region, but that it was lost from the cells on
passage and in tumors produced by those cells.

So the DNA clearly had been present there,
but had been lost fairly on in the process, despite
the fact that these cells are capable of making
tumors.

Obviously, one of the ways to look at this
is that maybe these viral DNAs are acting in some way
as mutagens, and there was certainly a history of the
fact that herpes virus infection would produce damage
to chromosomes.

I must qualify this, of course, by saying
that in general herpes virus is lytic to cells, and so
you don't expect cells to survive. However, in most
herpes virus populations there's a lot of defective
virus present, which might very well allow some cells
to survive in an infection.

We could also show experiments done in Zur
Hausen's lab in Germany that you could find mutations
in host cell genes after transfection of herpes virus
DNA-N. So clearly the virus is acting as a mutant,
and this is just a demonstration of some thioguanine
resistance with a number of these herpes virus
transforming regions, showing that there is in fact a
mutation frequency that is measurable in these
experiments.

So this immediate early region in CMV
became a region of interest to us, but we really
stopped those experiments because they had been
started out of an interest in cervical carcinoma, as
I initially said. We stopped those, because human
papilloma viruses raised their beautiful heads, and
obviously we now know that this is the prime
initiating cause of anogenital carcinomas.
So we really stopped looking at this, but about ten years later it gave us a lot of pleasure when Tom Shent suddenly discovered the idea of hit and run, and reported in the literature that they've used immediate early genes of CMV and had essentially come to the same answer, that these regions would produce increased foci, increased transformation of cells, but in fact the DNA was then lost from the cells, and so this again looked like a hit and run phenomenon.

I'll just go past this one for the moment. And the type of experiment that came from Tom Shent's lab, which were published in 1997 in PNAS, was that we heard from Alex van der Eb already about the effects of adenovirus E1A, and what they showed was that if you combined E1A with the CMV early regions, E1 or preferable the E1 and E2, you could get a much higher number of foci produced in those transfected cells than if you had just E1A alone, and they showed this in a number of different experiments.

So clearly, the CMV region, which was not then retained in these cells, was nevertheless contributing to the transformation of the cells, the same sort of picture that we've seen earlier. The other picture that they also found was that there was an increased mutation frequency,
looking at APRT in this case and HPRT in this case, that again if you put these immediate early genes in -- and you can do this on their own or in combination with E1A again -- you could demonstrate that there clearly was a mutation frequency resulting from the transfection of this DNA into, in this case, rat cells or mouse cells.

So, clearly, there's no question that these small fragments of viral DNA that can transform cells most likely act as mutagens and are lost from the cells upon passage of these cells, but the cells retain their transformed phenotype. That's probably the most important thing to remember out of this.

So in conclusion for this talk, I'd just like to make these points: That it's clear that subgenomic fragments of herpes simplex virus and cytomegalovirus can transform cells in vitro. Now these experiment are not conducted in human cells, but in rodent cells, as I've already said. It's clear from a number of experiments that have been carried out now in certainly more than one or two labs that that virus DNA, which is capable of transformation, may not persist over the long term in the transformed cells.

Although you can detect early on -- If you
put a complete open reading frame in, you can detect
the expression of viral proteins. You only see these
early, and they do not persist, again suggesting that
there is not only a loss of that sequence but perhaps
a loss of sequence over time out of these transformed
cells.

Lastly, it's clear that these viral
transforming regions can indeed be mutagenic, shown in
a number of experiments both by our labs and by Tom
Shent's labs. So that still leaves the possibility
that herpes viruses can be responsible for some -- the
development of some tumors, and there are still
experiments being looked at and some that have been
published showing that, even in cells that are
immortalized by human papilloma virus, the presence of
some of these morphological transforming regions
results in a more rapid conversion of those cells to
tumorigenicity.

So hit and run may seem an old phenomenon,
but it's not dead. Thank you.

(APPLAUSE.)

CO-CHAIRMAN RUBIN: Questions?

DR. KUNG: Hsing-Jien Kung from UC-Davis.

I'm just wondering, do you have some
hypothesis whether this is due to insertion of
mutagenesis or due to the fact it knocked out P53,
make the genome just generally unstable; therefore, it
takes its own course.

DR. McDUGALL: Well, I don't think P53 is
not found in these cells. So I still believe the

hypothesis, that what you're seeing is integration of
this and then a random excision of it is probably the
reason, and that the integration is random, the

excision is random, and probably the way to get at
this, if we wanted to go back to these experiments, is
really to do much more detailed sequencing of these
regions and look at the flanking areas on any DNA that

might still be there.

It is very clear that this DNA is
generally lost from these cells.

DR. KUNG: But you do have some residual,
right, in some of the recent experiments?

DR. McDUGALL: Some of the experiments
with CNV there is some material that remains there,

and we could go back to that and ask those questions,
yes.

DR. FRIED: Did you --

CO-CHAIRMAN RUBIN: Identify yourself.

DR. FRIED: Fried from ICRF. Do you take
the region of that secondary structure that you
pointed to, and did that stop the mutagenesis?

DR. McDougall: No. What we did was just
to use the HSV1 region as a control. So we didn't use
APH -- We didn't do anything to the HSV2 or CMV
regions. We just used the HSV1, which is, as you see,
a tail. It does not produce a nice insertion
sequence. We used that as our control.

DR. Fried: And in Shent's experiments,

were there any specificity to the type of mutations
that were formed, based changes or --

DR. McDougall: I confess, I don't
remember the answer.

Co-Chairman Rubin: We go to the back now.
Someone has been waiting there.

DR. Krause: Phil Krause, FDA. To your
knowledge, has anybody ever taken MQR regions and
injected them into nude mice or something like that
and looked for an in vivo tumorigenicity endpoint?

DR. McDougall: Not to my knowledge, no.

Dr. Coffin: John Coffin. Stem loop
structures such as you drew must, of course, be very
common, you know, kind of DNAs, and actually -- I
mean, is this effect specific for herpes virus DNAs or
have they been studied so much more than almost any
other DNA of similar kinds or complexities, if you
take bacteria for HDNA or --

DR. McDougall: Well, in the bacterial systems, right. I don't know of many studies in this sort of viral system, and I don't know of any that have been done very recently either.

DR. Coffin: So, in fact, it could be that all kinds of DNAs may carry regions --

DR. McDougall: I wouldn't be at all surprised.

DR. Coffin: And it's just the herpes viruses that have been looked at.

DR. McDougall: It just happens that that was a good candidate.

CO-CHAIRMAN Rubin: One more question, and we'll have to move on.

DR. Broker: Jim, has anyone taken synthetic stem loop structures or is it specific to the particular sequences that these various herpes viruses that are produced?

DR. McDougall: Well, in a way, that's the same as John's question. I think the answer is no. At least, I don't know of any experiments like that.

As I say, we got hopelessly diverted by papilloma virus.

CO-CHAIRMAN Rubin: Could you please give
us your name?

DR. BROKER: Oh, Tom Broker, UAB. A good choice, Jim.

CO-CHAIRMAN RUBIN: We'll have to go to the next speaker. Thank you very much.

The next speaker is Stephen Baylin, Johns Hopkins University School of Medicine, who will talk about DNA methylation and epigenetic mechanisms of carcinogenesis.

DR. BAYLIN: Thank you very much. I'd like to thank the organizers for inviting me. As I hear some of the talks going before, with respect to the area our work is in and our group works on, I think of another kind of change other than the classic genetic changes in DNA that we've been hearing about, which is also heritable and involves DNA probably through chromatin structures which are variable.

In terms of contribution to oncogenesis right now, I think one of the interesting aspects of that which has emerged is that process as it regards epigenetic gene silencing during all phases of tumor progression. If I can have the first slide, please.

Now for those of you who don't work in this particular area of epigenetics, let me just quickly remind you that in the eukaryotic genome and
certainly in humans and rodents, essentially the only
place that we methylate our DNA is at the base
cytosine and only at those cytosines that occur 5
prime to a guanosine.

We talk about the CPG dinucleotide as the
substrate for this event. Now it's an event which is
catalyzed by what up until recently was thought to
just be one mammalian enzyme, certainly DNA methyl
transferase.

There are at least two others now that
have activity, and this is going to probably emerge as
a very important part of the story in tumorigenesis,
the interplay and the roles of these different DNA
methyl transferases. But they each catalyze a
reaction in which acidinosine methionine is used as
the methyl donor group, and the methyl group is placed
on the 5 position of the ring of cytosine.

The other important aspect of this for
tumorigenesis which is reduced to real simplicity here
in terms of what's being understood about methylation
and its role in the genome is that in the eukaryotic
genome the CPG dinucleotide has been drastically
reduced over evolution to a small fraction of its
predicted frequency.

So most of the genome has a reduced CPG
content. However, in most of the genome there is a high percentage of those CPGs that actually get methylated in rodents and humans. So we have heavy methylation, as shown by the yellow dots, in most of the genome.

This methylation may play various roles, one of which may be to participate in chromatin that is transcriptionally repressive for repeat elements in areas of unwanted transcription in the bulk of the genome.

Now the exception to that type of distribution occurs in and around about half of the genes in our genome where the CPG dinucleotide frequency maintained at its predictive level, and these are the so called by Adrian Byrd and others CPG islands.

For most genes -- say, for inactive genes on the X chromosome of the female and selected silenced alleles of imprinted genes, these CPG islands are maintained free of methylation, and this is thought to be a permissive state. It doesn't matter whether the gene is actively transcribed or not, but it's a permissive state for active transcription of the gene.

What's being more and more frequently
recognized in the cancer cell genome is an increasing
list of genes in which this balance is disrupted, such
that in the face of loss of methylation from large
regions of the genome other than the promoter regions,
CPG islands around an increasing list of genes
actually have become methylated.

Whether it's the cause or the result is
still being worked out. This correlates with a
chromatin organization around that gene which is
unfavorable for transcription of the gene.

Now the impact for this on carcinogenesis
and tumorigenesis, I think, can be looked at from the
types of genes where this has now been defined. For
those genes which are known tumor suppressor genes by
virtue of the fact that when they're mutated in the
germ line of families, those families have inherited
forms of cancer.

About half of those genes, as shown in the
yellow, in somatic forms of cancer, noninherited
cancer, have had some frequency now shown and well
worked out of this promoter region CPG
hypermethylation.

I would point to you that one of the most
frequent times when this is seen is in the cyclin DRB
pathway for the P16 gene, and now in a number of
experimental systems, including mammary epithelial
cells, loss of M0 that we just heard about, at least
experimentally and probably in several natural tumors,
may be in association with hypermethylation of this
cyclin dependent kinase inhibitor gene P16.

So it can be a very early event in
carcinogenesis and a very important one, as shown by
just that one gene involvement. Next slide, please.

Other genes other than the classic tumor
suppressor genes are emerging, and these are just a
few, and they play a role in various kinds of
processes, some for enzymes that guard against DNA
damage such as methyl transferase, 06NGMT GSP pi.

Recently, for the P53-like gene where
mutations of -- or P73 where mutations have not been
found, very frequent to see hypermethylation of this
gene in lymphomas. So an increasing list of genes
which can play a potentially important read in
oncogenesis.

Just to remind you, that in many of the
instances where this has been defined, or in several,
that gene might not have been recognized through
classic mutational changes in the coding region for a
role in tumors, because several of these genes such as
P73, in some tumors even P16 like colon cancer, don't
seem to be altered through mutational change but have a high frequency of this promoter change. Next slide, please.

Just to remind you again how frequent this is, -- this is not to meant to be read -- this is a survey of major human tumors where Jim Harmon in our laboratory and others have been working out these hypermethylation events, and just to show you that virtually every kind of human neoplasm has multiple genes, not every tumor in every patient but, for example, in breast cancer, if you take the list of genes here shown to be hypermethylated and examine a series of breast cancers, you will find that every one of those breast cancers has one or more of these genes hypermethylated. This now includes in about a quarter of them actually in the nonfamilial type of breast cancer the BRCA1 gene. Next slide, please.

Now in terms of what this might mean for therapy of cancers, the difference, of course, here from coding region mutations is that theoretically, if there are no base changes that inactivate the gene on a permanent basis, this is a potentially reversible situation, although indeed, if you look at it in culture and you look at it over time, for the most part it is a heritable event, the maintenance of this
kind of gene silencer. But it certainly has raised the
question can reactivation of these hypermethylated
genes serve as some sort of therapeutic target for
cancer.

This is not a new concept. There has been
a drug around for a long time -- next slide, please --
Peter Jones and others introduced years ago when they
found that drugs and congeners of 5 azacytidine can
cause demethylation within the genome and reactive
genes. So this has been the drug that people have
concentrated on.

It's only more recently that, I think,
people are thinking about it in terms of specific gene
reactivation for events in cancer, like John talked
about. Various other series of molecules, antisense
again, so called DNA methyl transferase 1 and other
molecules directed at the major mammalian DNA methyl
transferase are being tried.

It must be remembered that these other DNA
methyl transferases that have recently been described
are probably going to also play important roles in
their separate genes with separate sequences, and so
that's going to have to be taken into account. Next
slide.

In our laboratory, it just shows you that
most of the genes where you see the CPG island methylation in culture, you can achieve at least partial reactivation of any or all of these genes through administration of the drug 5 deoxy azacytidine. Next slide.

And you can bring back functional events in the cells by reactivating these genes. The classic example has been the loss of the mismatch repair gene hMLH1, which is probably the leading cause for the microsatellite instability phenotype in colon cancer. Most of those colon cancers have this epigenetic change at the promoter of this gene.

If you throw different mismatches at cultured cancer cells -- This is a Hela cell in Tom Kunkle's lab. Actually, this is the 100 percent for each of the three different bases in a Hela cell which has effective repair.

Here's a hypermethylated colon cancer cell before azacytidine and, if you give 5 azacytidine to that cell, you can restore considerable mismatch function -- repair function to the cell by reactivating the mLH1 gene. Next slide.

In terms of manipulating the genome, I think what's become exciting in this area is to consider holistically the role of DNA methylation in
chromatin which is transcriptionally repressive, and
this has been an exciting arena in the past year or
two.

Adrian Byrd's laboratory and Wolff's
laboratory at the National Institutes of Health have
made a big contribution to this recently when they
showed that proteins such as mECP2 actually can bind
preferentially to methylated cytosines in the genome
and recruit proteins in a transcriptionally repressive
complex which include a major player, histone de-
acetylases, various histone de-acetylases.

So what this does is suggest -- and
there's been long an argument about the chicken or egg
participation of methylation in the science -- that
methylation can potentially through recruitment of
proteins such as mECP2 actually see the repressive
complexes around these methylated sites in the
promoters of these genes. Next slide, please.

There are drugs that inhibit histone de-
acetylase and will reactivate many genes. This slide
just shows you that in our hands for the
hypermethylated genes in cancer cells and now in
multiple others, that if you block histone de-
acetylase alone with drugs like trichostatin at any
doses -- and here is mLH1 in a colon cancer cell which
is hypermethylated, not expressed by rtPCR -- you can't reactivate, or we can't, using trichostatin alone. However -- next slide -- if one combines a low amount of a drug like 5 deoxy azacytidine to achieve just a little bit of demethylation -- and here's the hMLH1 again.

So here's the azacytidine alone, and now you give trichostatin. Within six hours of doing that, you can effectively reactivate the gene. We've seen at least four or five genes where this is the case.

So this is suggesting to us the following type of scenario for work in a working construct, I think, that could prove fertile in this arena for understanding. Just let me show you that this type of activation also brings the protein back to the cells.

This is mLH1. Here's an unmethylated colon cancer cell line, the green staining. Here is a line that's methylated, and this is trichostatin alone. Aza will bring back a few cells, and then the combination brings back in this short study a fraction of three or fourfold greater number of cells. Next slide.

In thinking about this, again in cartoon form is a working hypothesis, the following type of
scenario is one that is appealing to us. As many people working in transcription machinery are showing, around the promoter of a gene generally there are a series of activating complexes, shown in the green here, and repressive complexes around the chromatin of this promoter that are simultaneously bound to the gene.

The activator complexes, importantly, include coactivators, some of which, like CBP, pcaf, include acetylase activity. The repressor complexes often include corepressors like syn 3 which are tethered to hdac, and the balance of these two, determined by incoming cell signals, would then determine the level of expression of that gene. Next slide.

What I think changes when you have the hypermethylation introduced into that promoter is that you change the nature of this repressive complex around the methylated CPG, shown here in the black, so that perhaps at least two things happen. One is that you tightly tether a complex that, through syn3 and probably other transcriptional repressive complexes which will behave in this way, to be shown, you tether hdac and deacetylase inhibition to that promoter region.
Our studies with the TSA would also suggest it's possible that you block or have less favorable access to a number of these coactivator complexes which have acetylase activity, and that leads to a balance of the acetylase activity and methylation around the gene that results in a rather tight transcriptional repression.

What we may be doing when we begin to relieve this methylation with the drug in a little way is to allow return perhaps of these acetylases and coactivator complexes, and now the cell cares if you block the deacetylase, and it contributes to gene reactivation. Next slide.

This needs to be worked out by showing who the players and the chromatin setup around these promoters really are at these genes in cells where they are methylated versus nonmethylated, which many groups obviously are now working on.

So it has raised a possibility, which actually is getting some trials now in places like our institution and others, that by administration of several days of low dose 5 azacytidine to patients which may avoid some of the toxicity that's been seen with these kinds of compounds, much of which may not be due to actually effects on methylation but actually
binding of the drug to the DNA, that if you could do
this in combination with drugs like phenylbutarate and
others that are coming that inhibit histone
deacetylase activity, one might get a favorable ratio
for reactivating some of these key genes like P16 and
others, which might have therapeutic benefit. Next
slide, please. That may be my last.

I think that is my last slide. Thank you
very much.

(APPLAUSE.)

DR. DOERFLER: I would like to comment on
the promoter models. Now to my experience, the
situation is slightly more complicated than you have
depicted in some of these slides.

For instance, the finding that some of the
promoters are rich in CPT dinucleotides would be
completely unmethylated, I don't think, is so clearly
true. We have looked at many promoters, some of which
are as you showed and are active or inactive. Others
are methylated in spurious nucleotides. However, you
find them only if you apply the genomic sequencing
technique which detects all methyls in a promoter.

Lastly, when one talks about the
mechanism, of course, the one you proposed is very
plausible. On the other hand, I think there are
examples where a methide group inhibits the finding of
a transcription factor.

So there are several ways of how this
scheme might work.

DR. BAYLIN: Well, I think Dr. Doerfler's
comments -- I hope you could hear them -- are exactly
right in the time frame. So let me address the last
one first.

There are certainly genes which do not
have CPG islands, most of them, in which occasionally
a methylation event right in and around a
transcription factor recognition site is important to
access of that transcription factor to the gene, and
can play a role in expression. No question about
that.

Probably even within CPG islands, although
density seems to be the most important determinant,
there are also regions within the island of
methylation that are important for where it occurs,
but density is extremely important.

I think he's again right that, if you do
genomic sequencing around these promoters -- every
dogma changes a bit -- do you find no methylation in
virtually all of these CPG islands? And no, you find
methylation. But what is often very much protective
is a region right in and around the transcription
start site.

Often the regions of methylation that you
do find in normal cells in these islands is distal to
that region right around the transcription start site,
for the genes that we've sequenced at least, and this
is true for Ecad here, and it's true for mLH1. It may
be that that's the regions in which their methylation
is seeded into these areas during tumor progression
that causes the increased density and begins to more
and more affect the expression of that gene. But
you're right, it is more complex than the dogma would
have indicated.

CO-CHAIRMAN RUBIN: Thank you. I think
we'll have to move to the next speaker, because
otherwise we're not going to have a break.

The next speaker is Dr. Walter Doerfler of
the University of Cologne, who will speak on a new
concept in viral oncogenesis.

DR. DOERFLER: I'd also like to thank the
organizers for inviting me to this very interesting
and constructive meeting. Could I have the first
slide, please.

For actually not 50 years ago, but for
more than 30 years we have used the adenovirus model
to look at problems of viral oncogenesis. Here's a
model of one of the adenoviruses. We have been mainly
using adenovirus type 12, and we've been following the
model established in 1962 by Trenton Yabe and Taylor
who showed that, when adenovirus type 12 in small
doses is injected into newborn hamsters, actually
about 70 percent of the animals that survive the
injection develop tumors within 30 to 50 days.

That model we have been following most of
our studies. The interesting pursuit mainly is that
of viral DNA integration and its possible
consequences, and that's what I'd like to discuss with
you today.

So here is my model and some of the
concepts which at least for me are new and which we --
I can't say we have proven these concepts, but we'd
like to pursue them in future research.

So definitely the adenovirus is
integrating its DNA, and tumor cells are transformed
cells. There's no free viral DNA. The site of
insertion is nonspecific. It's different in each
tumor, and there are multiple copies inserted.

The inserted DNA, much like any foreign
DNA -- if you use lambda DNA, if you use plant cells
and put transgenes in, the foreign DNA becomes de novo
methylated, an observation we made way back in '78.

Recently, we have been able to show and represent some of the data today that as a consequence probably of this insertion, changes of DNA methylation occur at places distant, remote, from the site of insertion, although the immediate site of insertion can also be affected.

Now changes in methylation, as the previous speaker just discussed with you, can lead to changes in transcription, and we'd like to pursue the idea that these changes might play a role in viral oncogenesis.

Now from a totally different point of view -- and that project we've been pursuing only for 12 years; I may not have time now to present all the data on that. So I've asked for some minutes on Thursday night. A much more frequent occurrence, a much more frequent encounter of an organism, this foreign DNA, is not necessarily the viral infection but it may be uptake of foreign DNA in the foods supply, and we've been studying that to some extent, and I may have a chance to present the data.

Now here is again the concept. Viral DNA or other foreign DNA inserts into the host genome. This DNA is de novo methylated. We'd like to think of
this de novo methylation as an ancient host defense mechanism which defends against the foreign genes. As a consequence of this insertion, changes in the patterns of DNA methylation occur at remote sites in cellular DNA. Perhaps, depending on the site of foreign DNA insertion, this may also lead -- it's not been shown too stringently, at least not in our lab -- in the instances we have looked at, there's only indications, but there is evidence from other laboratories that alterations in methylation accompanied by alterations in chromatin structure, and these numerous changes can lead to alterations in transcription. I will show you some evidence for that.

Now let's look at the data. When we infect cells, hela cells, with adenovirus Type 12 or when we -- Maybe we could dim the lights a little bit -- or when we look at transformed cells -- this is a transformed cell line, transformed by adenovirus Type 12, and a stretched chromosome preparation -- chromosomes have been subjected to low centrifugal force, and you can see the multiple copies of integrated viral DNA, or you could see them if we could dim the lights a little bit perhaps. Thank you very much.
A very similar picture emerges when we look at the chromosomes in infected cells. For a long time we're trying to show that also infected cells, productively infected cells at least early after infection do show some association, actually massive association, of viral DNA with the host chromosome and the pictures between infected cell upon stretching the chromosome and the transformed cells that we have definitely shown of this DNA integrated by cloning and sequencing junction fragments do look very similar.

So we've pursued the possibility that at least transiently some of the DNA becomes also inserted and productively infect the cells. As long as we have the lights down, this is a blow-up of the transformed cell.

You might imagine that there's multiple copies aligned in a string, pearl-like fashion, in the stretched DNA, in this case hamster DNA.

Now just a brief summary of some of the observations, summarizing many years of work. At the chromosomal and nucleotide levels there is no specificity of insertion. There may be rare exceptions. The integration may occur preferentially at transcriptionally or otherwise may be replication active sites.
The consequences are, as I mentioned before, de novo methylation of the integrated foreign DNA, and secondly, changes in patterns of methylation in cellular DNA segments. The integration is in general stable, but we have found revertants which have lost all or most of the viral genome, and are still oncogenic. Actually, we reported that first in 1982 in a paper with Ingrid Kuhlman, and in this case hamster tumors were induced by injecting adenovirus Type 12.

The integrated state was demonstrated at that time by Southern blotting and also by cloning junction sites. Upon cultivation of these cells, occasional loss of the viral DNA was observed, initially by Southern blotting in the old days, and these Southern blot negative cells, clonal lines, were still oncogenic just as much as the original cells from the tumor.

Now when we used more recently, with Anna Pfeffer, PCR analysis, we found very similar to what Jim McDougall was just telling us, tiny bits of the adenovirus Type 12 DNA in several of these lines, none of the lefthand end but only snippets from the righthand end.

Each clonal line seemed to have a
different persistent pattern of these small fragments.

When these cells were injected again, they were as oncogenic as the cells that carried the viral DNA. So we had a discussion before on the hit and run mechanism. It's a possibility, but of course, there are other ways of interpreting these data, but it's certainly interesting in this context.

I will skip this slide, not to spend too much time, and return to the cell line T637, hamster cell line, and adenovirus Type 12 transformed. It carries about 15 to 20 copies of viral DNA. As you see on this fish preparation, there's a green spot that contains the adeno sequences. The red regions are representing endogenous retroviral genomes. That's about 900 copies per cell, often located on the short arms of the chromosome.

Now in the following slide I will use this cell line and others to look at changes in methylation patterns in these regions, which are abundant, and changes might be readily apparent.

In fact, when we do such a comparison, work done by Hilda Heller in our laboratory, and compare patterns of methylation in the IAP region of the BHK hamster cell -- that's the parent line for this transformed cell line that carries the adeno 12 -
- we see both for the hepa-2 and for the hhl cleavage, both sensitive to methylation in CCCG or GCGC sequences, a very striking increase in DNA methylation as compared to the parent line. This increase persists when the TR3 line is investigated, which has lost all of the adeno 12 copies. This is both based on Southern blotting, but there may be still snippets left. We haven't analyzed that extensively yet. But it's important, I think, to our way of arguing that these changes in methylation persist.

Hilda Heller has looked at another of other cellular regions, for instance, like the part of the major histocompatibility complex and others in these tumor cell lines and in other cell lines, and has found changes in some, not in others. So certainly, in these transformed tumor cells there are extensive changes in DNA methylation patterns. Now this may not be so surprising, because we are talking about transformed cells with a transformed phenotype. That most likely plays a role in the eliciting of changes in DNA methylation.

We have considered other possibilities, in addition, like the function of early viral gene products which are expressed, but when we infect the
same cells, in the case of adeno 12 which also
expresses early viral gene function under these
conditions, we do not see these changes. But we have
not pursued this possibility, although it's not
definitely ruled out.

The third possibility we have investigated
more extensively, namely, the notion that just the
integration of foreign DNA nonviral and nonviral
transforming could perhaps also elicit similar
changes.

So Hilda Heller and Kristina Kaemmer made
a number of bhk hamster cell lines that were
transgenic, not for adeno DNA but for bacteriophage
lambda DNA. In these panels you see -- You may not
see it well in the back, but up front you can clearly
see that these cell lines are all lambda DNA
transgenic, as determined by FISH analysis. This is
a control, and again the lambda DNA is located in
different positions and different clones, becomes de
novo methylated, just like adeno DNA behaves like any
foreign DNA in a cell line like bhk21 cells.

Now we've now looked at these cells for
changes in DNA methylation, and we've concentrated
again on a subsegment of the IAP 1 region, which is
shown here in full detail. You see all the CG
dinucleotides. These are the lollipops here.

The HP sides are just these two sides. So you see my concern when conclusions are drawn about DNA methylation using just restrictases, because with upper you just catch two out of 35, and with HP you just catch two out of 35, and with Hh, the dots, you catch maybe six out of 35, just a subpopulation of nucleotides -- dinucleotides that can be subject to changes in DNA methylation.

So we first looked again at Hh and HP, cleaved DNA from these lambda transgenics. Could I have the next slide, please? Thank you.

You see it mainly for the Hh. For the HP it's less striking, although some changes are observed, but for the Hh it's very noticeable that even in sub-clones, different clones of lambda transgenic cell lines show these changes.

I should mention that one of these cell lines has also now been subjected by Knut Mueller to an analysis on changes in transcription, by doing a differential hybridization of C-DNAs, and Knut can find quite considerable changes in the transcription level of these -- not only the total level but of cloned segments out of these cell lines.

Now to convince ourselves of these
changes, and particularly the reviewers who were
doubtful, we used the bisulfide treatment of the DNA.
In brief, the bisulfide converts a cytosine to a
uracil, but a methyl C is resistant to this
conversion.

So then after complete conversion of the
dNA, we PCR out the segment we are interested in -- in
our case, the IEP1 region -- clone individual PCR
products, and sequence up to several hundred of these
clones.

Whenever we find a C in a known sequence,
it's a 5 methyl C after the treatment, and we see a U
or a T, it's a C.

I just give you a quick summary of the
data. What you see here are the positions 1 to 34 in
the segment I showed you on the map out of IAP. Each
of these numbers represents one CG dinucleotide, and
in hundreds of clones we plot here the percentage of
clones which are methyl C in a particular location.

So let's first look at the bhk 21 cell
line, which is sort of the base. This has a certain
pattern of methylation, as I showed you before in the
Southern blot. Of course, we didn't see any -- All
this 34, you just saw the HP and Hh side.

Now when we look at P637 cell line where
we find a blatant, a very obvious increase in DNA methylation by HP and Hh blotting -- cleavage, you find the same effect when we look at all the CGs and the percentage of those CGs that were methylated.

Now the lambda lines are somewhere in between. I've shown two examples here, and there are changes in some positions, not in others. When we look at clonal sublines of CPHK21 cell line, we do not see these increases. The possibility existed with respect to methylation IAP in the bhk population per se, but by this and another method we have applied, we did not see that. So we don't pursue that any further.

We conclude that after insertion of lambda DNA in these transgenic cell lines, we do see quite distinct changes in levels of methylation, and in one cell line investigated so far we also see changes in transcription, as done by differential hybridization and CDNAs.

Now how might this change come about? Of course, I don't know. So I show you a cartoon, and one possibility we consider is that, due to the insertion of foreign DNA as an alteration or you might call it a destabilization of DNA surrounding here, and this may be -- since chromosomes have a unique
neighborhood in a nucleus, this may be somehow transmitted to other parts of the genome, and the parts affected naturally depend on where the foreign DNA inserts.

This adeno is a recent immigrant of 30 years ago. The IAPs have been there for 5 million years, and the ancient genes like MHC can also be affected. So this is a way of thinking about it. Of course, no proof whatsoever, but I think the observation is quite clear, and we are now preparing other approaches to make -- obtain additional evidence.

Particularly, we are thinking about using perhaps the primary human cell lines, because then we could have much better information about the genes in which the DNA methylation patterns are changing.

Now lastly, and this I will just summarize very quickly, the more frequent encounter of an organism with DNA, at least to my judgment, is the daily uptake of food. So we asked the question whether DNA that is orally administered to a mouse -- we used M13 DNA or a green fluorescent protein cloned DNA or, more lately, we used leaf from soybeans and watched for one of the genes out of the light side, the ribulose biphosphate carboxylase, for its
persistence in the gut and places beyond.

I will not have time to do just to the data, which were mainly obtained by Reiner Schubert and Gerte Holbeck. The concept behind this, perhaps at first a strange experiment, is its foreign DNA integrated into an established genome consistently or very frequently becomes de novo methylated, and I consider this a defense mechanism.

If you postulate a defense mechanism, the next question is, well, where is the attack. The attack very frequently, naturally, is in the gastrointestinal tract with a huge surface and a tremendous immune system.

So we have asked the question, can intestinal depotea take up the DNA. They seem to be able. We find, first of all, the DNA persisting at certain times after feeding to ease the control of unfed animals, TE, EDTA buffer and then there is observation of M13 DNA one hours to eight hours after feeding, not later on, in the cecum or in the small intestine, the large intestine. At about ten-to-hundredfold lower level we also find persistence in the blood of fragmented forms of DNA. The largest fragments here are about 1700 out of 7,250 of the M13 DNA, and about 800 in the blood. So it's not the
total DNA that's persisting.

When we go to sections -- again, you will have difficulty seeing that in the back. If you want to see my printouts, I can show them to you. We have persistence in gut epithelia, in pia patches or in liver cells that are controls, and Reiner Schubert looked at hundreds of sections through the gut and through the liver and the spleen, and in some of these section he found -- by applying the FISH, he found DNA persisting over in the nuclei of these cells.

Now FISH is nice, and PCR is nicer, but you always have nightmares, not only when you are in jet lag. You wake up four o'clock in the morning thinking maybe this is the wrong interpretation.

So what Reiner Schubert and Doris Renz did was to reclone the DNA from the spleen of animals that have been fed for a week daily, and in 18 hours after the last feeding spleen DNA was prepared and clones were -- DNA was cloned into lambda-2 vector, and lambda plaques were investigated.

Amongst several hundred million of plaques, about five to six were found positive for M13 and for the mouse DNA. Then what you see here are the coordinates of the M13 phage DNA. I told you, it's one 7,250 nucleotide long molecule, and these are the
coordinates that we find persisting in individual

clones.

Two clones were of particular interest, because they had, as the nearest neighbor to the M13 DNA, DNA with 70 and 80 percent homology to known mouse genes. So we feel that perhaps the M13 DNA in rare instances has become integrated into mouse DNA in the neighborhood perhaps of pseudogenes.

It did not escape our attention that in some clones we found 100 percent homology to known E. coli genes. So if you have some fantasies, you can say, well, perhaps there's a steady stream of foreign DNA from the gut to the spleen, and then the uptake and perhaps also some of it integrated in rare instances in individual cells.

I will not go into details now on feeding experiments where we have also looked at pregnant animals and transferred to the next generation, which can occur, and also found DNA in some cells in the fetus and in the newborn, perhaps also in an integrated state, but I will not have time to discuss that.

Let me just say in closing, when Gerte Holbeck has investigated animals which have been fed soybean -- this is not naked DNA like we did in these
series of experiments which are a little bit way out, but we figured first let's try an extreme case. If that DNA persists, then perhaps we can also expect natural feeding experiments to give results.

Now when we look at the, as I said before, ribulose biphosphate carboxylase gene after feeding leafs of soybeans, and we can also find this DNA to persist actually in practically unfragmented form in the gut. Of course, we don't know is it still in a cell or has it been liberated. But in rare instances now in three cases, we could also see by PCR some of the DNA which has no homology to mouse, because mouse doesn't have such a gene, has no life cycle in spleen and in liver.

So perhaps even by that route, the natural route, if you wish some of the foreign DNA, masses of which we ingest daily can persist in the gut. I have no doubt about that, and can enter probably via columnar epithelia in the gut, pia patches, thoracic duct, white blood cells, spleen and places beyond.

Whether it has any effect, any long range effects on the cells in the way I have been speculating in my first slide, of course, remains to be determined, and this will be a much harder task than looking for a tiny needle in a haystack, like in
So in summary, we'll continue to pursue this concept that the insertion of foreign DNA may have much further effects, much more far reaching effects on an established mammalian genome than just a local perturbation, but this local perturbation may be transmitted to other parts of the genome and change transcriptional and certainly methylation patterns.

Thank you very much for interest.

(APPLAUSE.)

CO-CHAIRMAN RUBIN: Any questions?

DR. COFFIN: You seem to reject -- Oh, John Coffin, sir. You seem to reject hypotheses that relate to selection, say from a variety of expression of methylation in preexisting cell clones. But at the same time you suggest that methylation is -- methylase is a guardian of the genome against integration expression of foreign genes. If that's the case, could not your data also be explained, at least so far as you presented it, by the idea that preexisting cells with lower amounts of methylation, the rare preexisting cells are much more sensitive to transformation events and, therefore, selected by the transformation experiments that you showed?
DR. DOERFLER: Thank you very much for this question. Of course, we are still considering this possibility, and it was one of the possibilities mentioned on my slide under number 4.

Now I have two lines of thought which are at least unsupported -- can't rule it out. One, when we look at many clones, subclones, of the bhk cell line that we use sort of as a total which could be heterogeneous, and look at -- I think Ralf Remos has looked at 70 or 80 different clones, again by HP and Hh cleavage. Doesn't see any differences.

Now that may not be good enough. So we --

In the CGG genomic sequencing analysis in the region that I discussed, IAP-1, Ralf also did the same type of genomic sequencing analysis, and again he found no striking differences -- I mean, there's always some variation, of course -- to the basic total cell line.

So although when you have looked at 70 clones or at five or ten clones, subclones, this is not very much, when you have to look at millions, it's difficult to rule it out completely. But certainly, the data -- and we're continuing this sort of analysis -- do not support this notion. It's difficult to rule it out completely.

CO-CHAIRMAN RUBIN: Are there anymore
questions or is everybody so eager to get to the
break? Okay, thank you very much, Dr. Doerfler.

We're going to take a break now, and we'd
like to be back by eleven o'clock to start again, for
sure.

(Whereupon, the foregoing matter went off
the record at 10:33 a.m. and went back on the record
at 11:01 a.m.)

CO-CHAIRMAN MYERS: If everybody could
take their seats, we'll reconvene. Probably the most
difficult thing that has to occur in a meeting is
ending the breaks.

We're going to continue our morning
session. Our next speaker is Dr. James Cook, who is
Professor of Medicine at the University of Illinois,
who is going to talk about the role of nonspecific
NK/macrophage cell host responses in assessing
tumorigenicity.

DR. COOK: Well, Dr. Lewis asked me to
talk about the terminus of tumorigenicity from the
perspective of the recipient of the challenged host,
what kinds of things determine the ability of cells to
form tumors, what the limitations are of the
tumorigenicity assays, and how the immune response may
affect our perception of tumorigenicity, depending on
what's used as the animal.

What I'll do is give an overview of the model system we've been studying. The focus in these studies have been on DNA virus oncogenes. Dr. van der Eb gave a nice summary this morning of how SV40-T and E1A of adenovirus immortalize and transform cells, and we're going to be looking at cells that are expressing E1A without worrying so much about whether they're immortalized, because most of these will be super transfected cells that are artificially created to express the adenovirus E1A oncogene, and to ask what effect that has on the tumorigenicity of the cells in various hosts.

I'm going to focus on so called innate immune responses or natural killer cells, activated macrophages to a lesser extent, and Dr. Tevethia will talk more about specific immune responses in a following talk.

The correlations that we'll discuss are between oncogene induced susceptibility of neoplastic cells to killer cell injury. So this is sort of counterintuitive here in an oncogene that's supposed to be causing a cell to become immortalized and become virulent is actually inducing the susceptibility of the cell to immortalize it to destruction, and then
the tumorigenicity in various animals with a focus on
the value of immunodeficient animals in assessing
tumorigenicity, and then later on probably in the
discussion session we'll talk about the
generalizations of some of these data to broader terms
for human cell evaluations.

The points that I'll make are that viral
oncogenes in certain contexts can sensitize neoplastic
cells to the destruction by killer cells, can thus
actually reduce or eliminate tumorigenicity if the
host is immunocompetent, and that the results of
tumorigenicity assays are highly dependent on the
immunocompetence of the host.

So inducing a tumor is not the same. I
think we all know that, but tumorigenicity is a very
relative phenomenon and can't be perceived as an
absolute, and the level of tumorigenicity among
different types of neoplastic cells can be widely
variable. So we can't assume that a single dose of a
cell can always tell you the same thing.

This all started with studies that we were
doing looking at an explanation for the reason that
adenovirus 2 or 5 transformed hamster cells were
unable to form tumors in immunocompetent hamster,
although they could form tumors in very young, two- to
three-day-old hamsters.

Through a long series of experiments, the upshot of that is the expression of this E1A immortalizing gene or oncogene of human adenovirus serotypes 2 and 5 appears to sensitize cells to host defenses that are absent in the immuno-immature animal or in animals that lack sufficient NK activity, and this is just a representation of the generality of this kind of observation.

If E1A is transfected and caused to be expressed in early passage or low passage NIH 3T3 cells in a rat cell line called RN 12 that actually is pre-immortalized with rats in a bhk 21 subclone or in a human fibrosarcoma cell, in every circumstance when these cells are tested for susceptibility to killing by the relevant natural killer cell population, they're much more sensitive.

So it's not just the adenovirus transformed cell. It appears to be perhaps many, if not almost any, type of cell in which E1A can be expressed in reasonable concentrations.

Now just to tell you a summary of a lot of work about E1A, as I've just shown, E1A expression in cells can sensitize them to natural killers from many species of origin. It also sensitizes to a wide
variety of injuries. There's nothing unique about
this NK cell injury. In other words, it's not a
specific killer cell receptor interaction with the
cell surface. It's something to do with injury per se
of certain types, and those are injuries that tend to
stimulate apoptosis or proapoptotic injuries.

From an immune perspective, natural killer
cells, cytotoxic lymphocytes, activated macrophages or
the dominant cytokine that macrophages produce -- that
is a cytotoxic molecule tumor necrosis factor alpha --
all can elicit this kind of apoptotic response in
cells if they express E1A, and these are cells that
are inherently resistant to these types of injuries.

It's not unique to immune injuries, and
the chemical injuries can do the same thing. A
topicide, atopoisummarys 2 inhibitor, beauvericin,
a calcium regulator, hydrogen peroxide and hygromycin
or protein synthesis inhibitor all can trigger the
same kind of response in E1A expressing cells.

So there's something inherently weak about
E1A expressing cells in an Achilles heel kind of
fashion that makes them sensitive to undergoing
apoptosis when injured by exogenous agents.

Now the immune mediated apoptosis per se
is somewhat unusual from other types of E1A
sensitization in that it is P53 independent. So it works equally well in cells that can't express P53, and it's also resistant to blockade by the E1B 19kD molecule, one of the small E1B proteins that Dr. van der Eb told you about that normally blocks E1A induction of apoptosis during the immoralization phenomenon.

Now what I'll do is focus more on the animal model side of the story to show you something about how tumorigenicity of cells that have this phenotype are being sensitized to immune mediated injury can vary, depending on the type of host in which it's tested.

So in this model bhk21 hamster cells were subcloned and then transfected in stably expressed E1A or E1A + E1B. It turns that E1B expression is irrelevant for this model. So I won't talk about it further.

We tested the E1A expression for its ability to induce sensitivity to killing, as I've shown you, and then asked whether tumorigenicity in different types of animal hosts, depending on their cellular immune responses, varied.

For example, in the bhk model we used adult hamsters that have, in fact, NK cell and NT cell
responses both, nude mice which are unusual in this

respect in that they have defective T-cell responses
which we all know about, but they also have something
wrong with their NK cells when it comes to recognizing
ElA oncoprotein expressing hamster or rat cells.

So the NK cells are defective in this
regard, whereas they will kill other types of NK
susceptible targets, and then nude rates which are

more like we think of as an aphonc animal. That is,
they have defective NK cell responses but have
perfectly healthy active NK cell responses -- I'm
sorry, they have defective T-cell responses but active

NK cell responses when it comes to killing ElA
expressing cells.

So the question was: If we have ElA
expressed in these bhk cells, we use these animals as
the recipients, what happens in vitro and what happens
in the in vivo tumor induction model?

So here are the in vitro data, using

hamster spleen natural killer cells as the source. As
I've shown you before, they can kill the bhk
expressing cells perfectly well, as long as they

express ElA.

The nude mouse cells don't do very well.

They have this defect for killing hamster cells
expressing E1A, in contrast to the nude rats which kill them quite well unless the nude rats are previously depleted of NK cells with an antibody such as anti-AZ allow GM1. So there is an NK recognition of E1A expressing bhk cells in these different types of animals.

Now this is a busier slide looking at the results of tumor induction studies in these different animals, and what we're talking about are the NK resistant bhk 21 parental cells or the E1A expressing clone that was derived from that, that are inherently NK susceptible or resistant, and what happens when different animals are challenged, considering their NK phenotype when it comes to killing the E1A positive cell.

So hamster that have competent natural killer cells, that can kill the E1A expressing cells develop tumors with about 1,000 cells, and this has been true for over 30 years of testing of bhk 21 cells, if you look back in the old literature. It takes about 1,000 cells to form tumors that are 50 percent endpoint in an adult hamster with bhk 21.

If you ask what happens when those NK and T-cell competent animals are challenged with the E1A expressing cells, you virtually can't get tumors with
10 million cells. So there is at least four orders of magnitude change in the ability of these cells to induce tumors in immunocompetent adult hamsters when the cells express E1A and become NK susceptible.

If we ask what happens in the nude mice that lack both T-cell and NK cell defenses against the E1A expressing bhk cells, there's not a huge change, although there is some.

They go from again about 1,000 cells making a tumor in nude mice, a little less, to about 10 to 80 times more cells being able to make tumors in nude mice, but certainly not the magnitude of increase of resistance of the animals against E1A expressing cells, as was seen in the normal hamster.

In fact, nude mice have activated macrophages which can kill E1A expressing bhk 21 cells, and so it may be that there are other defenses other than their Nk cells that can explain this smaller change in increased resistance to tumor development.

Then there are the nude rats that are highly NK competent against E1A expressing bhk cells.

It takes about 30,000 cells to make a tumor in a nude rate, and 10 million cells is insufficient to make any tumors in these animals.
So again, there's a several orders of magnitude change in the resistance of nude rates against the E1A sensitized bhk 21 cells unless the nude rates are NK depleted in advance, in which case - Now this was just a single challenge with 10 million cells, but in which case the animals become more susceptible to tumor development.

So it appears that E1A can reduce the tumorigenicity of these cells if the animals have a competent NK cell response and, obviously, there are other things going on in these animals as well that might lead to changes in tumor susceptibility.

Now if we look at a completely different animal model with the same basic scenario -- that is, transfecting and expressing E1A and asking what happens to the susceptibility of the tumor cell in immunocompetent and immunodeficient animals.

In this case, these are studies done with Jack Rudis in which the methylcholanthrene cell line MCA-102 was studied. We tested them for susceptibility to killing, and also tested them across the range of animals, adult mice, nude mice, and in this case CD3 epsilon transgenic mice that are defective for both T and NK cell responses.

The one difference here is that nude mouse
NK cells are competent to kill these E1A expressing mouse cells. So we have a range of activities of intact NK and T, defective T but intact NK, and then defective both in these animals that basically lack the function of either of these types of killer cells.

This just shows you that the nude mouse in a black 6 background can in fact kill E1A expressing methylcholanthrene sarcoma cells in contrast to the E1A negative cells.

These are the survival curves of these three different kinds of animals challenged with the E1A expressing cells. So this is the adult C57 black 6 mouse that has both the ability to generate a T-cell response and a killer NK cell response.

This is tumorigenicity induced by the methylcholanthrene cells. So you can think of this as a survival curve over the 12 week time of tumor challenge, and this is the survival curve of the E1A expressing cells.

So there's basically very little detectable tumor induction even at 10 million cells in these animals. So I think of this difference in the 50 percent endpoints for tumor production after three months as reflecting the cumulative antineoplastic activities of these mice against the E1A expressing
sarcoma cells, realizing that some of those are immune
mediated and some of those are likely to be other.

If you do the same experiment in nude mice
and follow them over time, you get a similar type of
curve, although you'll notice that the tumor inducing
capacity in these animals is lower.

It only takes about 100 cells to reach an
endpoint in the nude mice, whereas it takes a little
over 1,000 cells to reach an endpoint in black 6 mice.
So they are inherently more susceptible to the
methylcholanthrene cells, in the first place.

There still is some resistance that's
present in the nude mice against the E1A expressing
cells, but the amplitude of this difference at the end
of the assay is smaller.

Then if you look at the animals that lack
any detectable T-cell or NK cell defenses because of
the CD3 epsilon transgenic status, basically what you
see is that the survival curves are not much
different, and the simplest way that I think of these
is that these mice don't really have any defenses, at
least from the immune response point of view, against
E1A expressing methylcholanthrene sarcoma cells.

So it probably suggests that these
defenses are pretty important. If it were other
nonimmune defenses, you would think of these mice as being able to mount them. Here the difference in tumorigenicity is not significant.

So what I'd like to conclude is that E1A can sensitize neoplastic cells to killer cells and markedly reduce the tumorigenicity of those neoplastic cells, again considering the fact that you test this in some kind of immunocompetent animal.

The lack of tumor formation by one cell dose in one host at one time point may not indicate nontumorigenic phenotype. So what I would do is go back to the previous slide and say that, if we picked a dose in here and said that we're just going to challenge with a single dose of maybe a million cells -- So here's a million cells at a given point in time -- if you looked at any point in time across this spectrum in the black 6 mice, you would find no tumor formation by the E1A+ methylcholanthrene sarcoma cells.

If you looked at the nude mice, it would kind of depend on when in the follow-up period you picked. If you looked at two weeks, you should see no tumor formation. If you looked at four weeks, you would see tumor formation, and in the CD3 epsilon animals you would see tumor formation at two weeks.
So it depends on the animal. It depends on the cell dose, and it depends on the time at which you look at these kinds of things as what result you might find.

So I think we can't just assume something is not tumorigenic based on a single dose, single time, single host assay, and that the tumorigenicity likely reflects a collection of neoplastic cell traits, not just a single trait in terms of susceptibility to one thing or another.

In the context of the cumulative effects of the host defense, probably there are things such as natural killer cells, activated macrophages, host growth factors and a variety of other things that determine the outcomes of these experiments.

So I think it's important not to think too simplistically about tumor development in animals, irrespective of the type of neoplastic cell that's being tested.

Thank you.

(APPLAUSE.)

DR. RUSSO: Carlo Russo from Merck.

You didn't mention anything about kera -- the inhibitory -- that expressed by NK cells and what role this receptor may have in modulating the NK
activity, in view of the changes in MHC expression by
the tumor.

DR. COOK: So the question is: Could oncogenes alter class I expression on the cell
surface of target cells?

It's known that high levels of class I expression and inhibitor expression on target cells
can actually lead to repressed NK cell activity, in contrast to CTL responses where high levels of class
I expression are necessary. So actually, these are probably complementary host defenses.

What I can tell you is that class I expression level appears to be unrelated to this
sensitizing phenomenon in the E1A system. So you can have cells that have normal levels of class I compared
to normal fibroblasts for levels of class I that are very suppressed, and in both cases the cells are
highly susceptible to NK killing.

So class I as an inhibitor or a class I-like molecules as an inhibitor for NK killing appears
not to be a major phenomenon in this system.

DR. RUSSO: Do you think that in the experiment you should include the factors that may up-
regulate MH expression in order to rule out the possibility of inhibition of NK cells, such as gamma
DR. COOK: Well, you could treat -- Well, those experiments have been done. You can treat the cells with interferon gamma. Class 1 expression can go up. It doesn't seem to change the cells' resistance, as long as they express E1A.

Certainly, in other models that's not the case. Dr. van der Eb?

DR. VAN DER EB: van der Eb, Leiden.

If I understand it correctly, you talked about adeno 2 or 5 E1A expressing cells. Right?

DR. COOK: Right. Correct.

DR. VAN DER EB: Do you have any comparison with adeno 12 transformed cells which are oncogenic?

DR. COOK: We haven't done it in these exact models. We've looked at adeno 12 transformed cells from viral transformation events in hamster and in mouse. When you contrast adeno 2 or 5 transformed cells using virus to adeno 12 transformed cells, the adeno 12 transformed cells are much less NK susceptible, despite the fact -- and this kind of goes back to the last question -- despite the fact that they all express low levels of class 1.

So again, I think adenovirus violates
these models of natural killer cell recognition and killing in that adeno 2 and 5 appear to allow class 1 to be expressed most of the time, and yet sensitized to natural killing which normally requires low levels of class 1.

Adeno 12 represses class 1 and doesn't appear to sensitize to natural killing, despite the fact that the class 1 is reduced, and you would expect that to trigger a natural killer cell response.

CO-CHAIRMAN MYERS: One last question.

DR. JOLICOEUR: Jolicoeur from Montreal.

I was wondering if you have other examples of the oncogene or viral oncogene which behave like that?

DR. COOK: I'm sorry?

DR. JOLICOEUR: If you have other examples of an oncogene which will behave as E1A here.

DR. COOK: A nonviral oncogene?

DR. JOLICOEUR: Nonviral or a viral, an autoviral.

DR. COOK: Some other example? Well, in mouse systems SV40 T antigen can do something like this, or in rat where it can sensitize cells to killing by activated macrophages, and in some models natural killer cells.
There are examples of overexpressed MIC that can cause sensitivity to natural killer cell activity. So I don't think E1A is unique in this regard, although it appears to be unique in the fact that it can do it in multiple different species.

SV-40, for example, if you compare hamsters and mice, SV-40 transformed mouse cells or sensitive SV-40 transformed hamster cells aren't, even though they express T antigen at equally high levels.

So there may be some species specificity to other oncogenes' ability to convey these same kinds of traits to the targets.

CO-CHAIRMAN MYERS: Thank you very much.

Continuing on in the same theme, our next speaker is Dr. Tevethia from Penn State, who will talk about the role of CTL responses and their implications for tumorigenicity testing and the use of tumor cells as vaccine substrates.

DR. TEVETHIA: Thank you very much. If I could have the first slide.

Everybody had a favorite virus and a favorite oncogene. Mine is SV40, and the SV40 large T antigen is my favorite oncogene. This has already been introduced. So I can move on to the second slide.
The point here I want to make -- and I had to go back to some of the work we had done in the Sixties and Seventies to make this slide -- if you transform cells, either hamster cells or mouse cells, BALC mouse cells or C57 black 6 mouse cells, with the SV40 large T antigen, and try to transplant in nude mice, all of these are transplantable in nude mice. But if you try to transplant in the adult animals, in hamsters they are immediately transplantable. In BALC they are rarely transplantable, but only after prolonged custody in vitro and passaging through the immunosuppressed host.

In C57 black 6 mice, they are never transplantable. They are only transplantable if you force them to lose MHC Class 21 antigen.

There's obviously a very simple conclusion to the role of immune response and the transplantation of these in vitro transformed cells. Let me just give you a clue, that every time you transplant a cell line transformed by oncogene, there the oncogene acts as an antigen, exogenous antigen.

It is processed quite differently as compared to the transgenic mouse. If you express the same oncogene T antigen in transgenic mouse, we have 60 or 70 examples of this. I picked three of them,
expressed T antigen and its own promoter produces tumors in choroid plexus.

The point I'm trying to make, no matter which promoter you pick, you can induce neoplasia in almost any tissue you like, and these tumors will continue to grow progressively until they kill the host at different times after birth.

So the question really that I want to address is why is it hard to transplant the cell line without tumorigenic in nude mice into the adult immunocompetent mice, but the same tumors induced by the same oncogene in vivo as a transgene continue to grow progressively?

I can tell you, the difference is where this oncogene becomes as a self-antigen and that is processed quite differently and the way it interacts with the immune response is quite different.

Just to give you a just a little bit of introduction, and the only thing I want to point out, that the purified T antigen can induce the generation of cytotoxic T-cells and can induce in the participation of the tumor rejection.

This is just a cartoon slide, just to indicate how the antigen processing works. I'll go very, very briefly. When the antigen comes from
outside, broken down into peptides, peptides are
shunted to the tap into the endoplasmic reticulum
where it is assembled with the molecules, and to
microglobulin, is then transported back to the cell
substrates where it is recognized by the cytotoxic T-
cells.

Just a cartoon slide to show the 3-D
structure of the classical molecule with a peptide
bound in the groove. This is the immuno terminal.
This is the C terminal. What I'd like to point out,
there are two residues in the peptide. They are known
as the anchor residues. They are responsible for
binding to the MIC molecule in a particular way.
So these anchor residues will determine
which peptide will bind to which MIC classical
molecule.

In SV40 large T antigen, there are four
epitopes that we have defined, 206-215, 223-31, 404-
411, 489-497. They are restricted either by H2 DFP
molecule or H21 molecule. We have the cytotoxic T-
cell clone for these -- especially for these epitopes,
and these are the epitope designation 1, 2, 3, 4 and
five.
In order to work -- It is a complicated
slide. What I'd like to point out is we have cloned,
expressed these epitopes individually in the common vaccinia viruses in cooperation with John Yewdells and Jack Bennick, and either by themselves or behind the ES signal sequence of the glycoprotein E19.

That allows these F clones to be delivered directly into the endoplasmic reticulum much more efficiently.

Okay. So what I'd like to describe is the two-transgenic system that inactivates the immune response, cytotoxic T-cell immune response by different mechanisms.

One of them is a 501 transgenic mouse system. It's driven -- T antigen driven by the late alpha amylase promoter and appears in salivary glands and osteopaths. It produces osteosarcoma about one year of age, and metastasized to lung, liver, and T-cells are positively selected. Means that T-cells are present that are expressly for the large T antigen.

SV11 tumor system is driven by its own promoter. Tumors appear in choroid plexus. T antigen most importantly appears in the thymus, expressed in thymus, as a result of which there is a negative selection in the T-cells, and these mice essentially can be considered as naive, because they don't select the T-cells.
So let me just describe the SV11 system.

This is a choroid plexus of the 100 day old normal mice, and this is histochemistry for T antigen. Obviously, this is negative.

In the SV11 transgenic mice, by 40 days of age you barely see a few T antigen positive cells over here. By 60 days you see a focus formation and corresponding expression of the T antigen in this focus.

By 80 days tumor grows, and you can see, it contains -- all cells contains the large T antigen, and by 100 days they are described by Terri Wendyke.

There's a big explosion in the tumor. Means that it grows extremely large in a short period of time, and it will die at exactly 104 to 105 days after birth, a very predictable system.

Now this is a very, very complicated slide. The message I want to give is this, that if you immunize these mice, SV11s, either with a full T antigen vaccinia virus or with its epitopes, you can see they don't respond. That's all you need to look at.

The graph here shows they all respond to vaccinia virus vectors. That means they are not immunosuppressed to general immunosuppression. But
these are the normal mice, litter mates that don't have the transgene. They respond to all of these epitopes.

So what we have done is to -- carried out studies in which we could -- We have taken the transgenic mice. We irradiated them briefly with low dose of the radiation 450r. Then we reconstituted them with the normal T-cells from the normal animals.

Then we can immunize them with either the full NT antigen or with immunogenes or we can leave them alone. Then we look for the development of cytotoxic T-cells and look for the progression of the tumors.

Okay. If now you irradiate them, transfer the spleen cell, immunize them with a full NT antigen, you have the appearance of the cytotoxic T-cells only to epitope 4. This is the most dominant epitope at this particular point.

What is important is that, if you irradiate them first, as I said before, reconstitute them but don't immunize, they still develop high levels of cytotoxic T-cells to the dominant epitope 4.

Now this is important, because tumors now after radiation is starting to release the antigen, and this particular T antigen there is the one which has been
processed and inducing the development cytotoxic T-
cells.

By irradiation or reconstitution, you can start a program immune response against the tumor cells. This is the tumor looks like. I showed you what the tumor looks like at 40 days of age, very big tumor. In this case, the tumor remains a very small size, hardly recognizable, but still you will see the T antigen positive cells.

Now if you keep these animals long enough, until they die, and I'll show you when they die, that they will eventually die of the tumor, the tumor we grow. This is the life span of these animals. You can see the transgenic animals die, which are un-irradiated, by 107 days.

Irradiation plus the spleen cell from the transgenic mice again, then they don't live very much longer; but if you irradiate and transfer normal spleen cells and don't immunize, they live over 320 days. But they eventually develop tumors and die.

The second one that I want to discuss -- So the previous example indicates that the T-cells are completely deleted in the thymus.

The second example where the T-cells are positive selected -- this is driven by amylate late
promoter, and it has all of the epitopes 1, 2, 3, 4 and 5, and they come down with osteosarcoma before one year of age.

This is what it looks like, the osteosarcoma impinging on the spinal cord. This is the metastasizing to the lungs, and this is the metastasis to the liver. You can see that all of the cells in the tumor have the T antigen by histochemistry. So they have not lost the antigen.

What I have done is to just simply summarize the immune responses to it. What's interesting here is that, if you look at the four months of age, then all -- no matter how you immunize, they respond -- almost all -- they respond to almost all of the epitopes except 2 and 3, which is a lethal epitope. They respond to vaccinia virus.

If you test the very same animals at 12 months of age, then you can see they have lost the response to full NT antigen all to epitope 1, and also epitope 5. They do respond on occasions to epitope 4.

So we wanted to know what the mechanism may be, and to -- we want to differentiate between different possibilities, and one of the ones we zeroed in, that these T-cells which are already there are dying by clone deletion.
The way we have done that is to -- We have made the tetrameres. The tetrameres are made by folding the class 1 molecules with the peptide and the beta 2 microglobulin. Now these soluble class 1 molecules have a signal for the biotin -- the biotinylated, and then with strep evident, you can see they will make tetrameres in three of them and can bind to a single T-cell to different receptors.

You simply to FAS analysis. What one does is -- this is an example for it, and I'll just illustrate one of them. We have used one of our CTL clone to epitope 1, for example. They have the epitope 1 tetrameres, and with the antibodies, here are the CD 8. With the tetrameres, you can see all of the CTL clones, they line up in the corner over here.

It means that -- So you can count -- you can actually count the T-cells that are especially for a particular epitope, and you can see that the -- This is a different tetramer. It does not bind to the same CTL clone.

So we have used this approach to identify the mechanism in the 501 mice, why these T-cells are being deleted. If you look at the three-month-old mice 501, only 3.5 percent of the entire CDA positive population is directed to epitope 4.
You can see in the control mice over 14 to 16 percent of the T-cells here are directed to RC epitope 4, once you immunize the animals with epitope 4. So they are much reduced in number, to begin with. By the time 11 months comes around, you have only half a percent left of the epitope at specific T-cells.

Now if you ask the question, do these animals have a tumor or do not have a tumor, and we assay the animals to determine if they have tumor or not, once they have tumors they have lost all of the T-cells, and you can see, once you have lost it, you cannot even expend them in vitro for lytic activity.

What's interesting is that these are not anergic. Even the .5 percent of the T-cells can be expended with full function.

Okay. The last thing will take just a minute for me. This is a study, not done in my laboratory, done by the laboratory of Judy Trevethia, and what we were interested in this study was the fact that she made the transgenic mice, which is the black 6, that only have 1 through 128 -- actually 1 to 127 amino acids of T antigen. It had the RB binding site.

It had the intact J domain, and that's really about it.

You can see -- What I want to emphasize
from our point of view here, that all of the epitopes are outside the region of this transgene. That will be, in my opinion, might not be as good a news, because you cannot do anything immunologically, you know, to these animals.

So these animals can come down with a SNR tumor. These are the adeno carcinomas. They are driven by the elastase promoter, and you can see this is the adeno carcinoma of the pancreas. You can see a lot of duct formations. You can see this is a normal pancreas with islets intact, metastasizes to the liver.

This is again liver metastasis. What is interesting about this is that, if you take the newborn within four hours after birth and check the pancreas, you see a dysplastic pancreas at that particular point within four hours after birth. As the normal pancreas of the four-hour-old, you know, newborn will look like this.

So I guess I'll pass this one. So I think my message is the following. I think that transplantability may or may not give you the right answers, because there's a lot of variation, and beside the engine that's present in those tumor cells or transformed cells, then the exogenous engine
process differently as compared to the transgenic mice.

These are some of the people that did the work. These are the people we did work in collaboration, Bennick Yewdell, Barbara Knowles, Arnold Levine of Princeton. Thanks very much.

(APPLAUSE.)

CO-CHAIRMAN MYERS: We have time for one or two questions. Thank you.

Our last paper this morning is by Frank Sistare and was on transgenic animals that might be useful in identifying unsuspected oncogenic factors in tumor cell substrates, from CDER.

DR. SISTARE: I want to thank the organizers for this opportunity to speak. May I have the first slide?

What I want to do is, first of all, spend some time on how our center -- I'm from the Center for Drug Evaluation and Research -- got involved in sort of changing, as we heard this morning, the gold standard, standard two-year bioassay, for ascertaining the carcinogenic potential of pharmaceuticals.

This was an initiative that was an outgrowth of an ICH process that started a few years ago. A guidance was developed, again with the premise
that there was a general dissatisfaction with the

standard two-year bioassay, and are there alternatives

for assessing pharmaceutical carcinogenicity.

So a consortium was formed as an outgrowth

of that by the International Life Sciences Institute.

This consortium has over 50 labs worldwide. There are

28 pharmaceutical companies. There's members from the

United States government regulatory agencies, Japanese

and European regulatory agencies as well, that are

part of that.

Why we're focusing on alternatives -- Like

I said, there are often ambiguities and

dissatisfaction with the conventional "gold standard"

two-year rodent bioassay. Saccharin, as an example,
comes to mind as the strain or species specific event

which is deemed irrelevant to humans, and there are a

host of others.

The feeling is that transgenics will carry
target genes that are involved in human and rodent
cancers. During the prime course of these transgenic

assays, there are much lower backgrounds as opposed to

the backgrounds associated with some of the two-year

bioassays.

It's a six-month assay which is a
tremendous savings in time in terms of making a
decision on whether a pharmaceutical is going to be
developed or not, and there's tremendous cost savings
just in the conduct of the assay. In often cases, the
endpoints are very simple.
The ILSI initiative has named a number of
specific models. As you've heard this morning, there
are a host of transgenic models that could be
explored, but several were brought into the limelight.

There were the initiation promotion
models, which is not a transgenic model, which Dr.
Pitot reviewed for you this morning. There's a
newborn rodent assay, which again is not a transgenic
model, but I'm going to spend some time talking about
that, because that is one model system that the ICH
consortium -- I'm sorry, the ILSI consortium has
decided to focus on.
Then several transgenic rodent models,
though not named by ICH, are emerging from this ILSI
initiative: The two knockout models, the P53 knockout
mouse heterozygous, and the XPA homozygous knockout
mouse, the TJC mouse and the TJ RAS H2 mouse.
So what I want to do for you now is
describe these test models, give a brief overview of
some of the pragmatic protocol design issues that are
going to impact on whether or not some of the models
might be practical for assessment of vaccines.

I want to review for you some of the test compound results. Again, these are going to be based on pharmaceuticals, and then give you some mechanistic speculations that are based on some of the data that we're seeing now.

First of all, the neonatal mouse model: This model has been around for probably the longest time of the models that the ILSI consortium is being focused on. A number of compounds have been tested. The ILSI consortium has formed a standard protocol for assessing these. So some of these other compounds that have been tested have been used have been used in different schedules and different strains.

The protocol is -- One nice virtue of it is the protocol, there's only two dosing periods, one on day eight and one on day 15 of the mouse. One-third of the total dose of the compound is given initially and then two-thirds seven days later. The test agent can be given either IG or PO by gavage. The doses that are decided on are based on the 28 day dose range finding study, and you simply dose them for that period of time and feed them, take care of them for a year, and then one year in necropsy
and do some histopathology, and then assess the outcome.

The general concepts that are forming are that this model will pick up genotoxic carcinogens. Nongenotoxic or indirect active carcinogens appear negative and, specifically, the genotoxic carcinogens that seem to be picking up are mutagens.

There's some question about whether it can pick up deletionogens, and some of those questions are being addressed presently. Conceptually, the model is -- It's like an initiation or mutation just prior to periods of normal high DNA replication. So it's like an induced initiation of that early on, and then the normal promotion processes.

The P53 alluded to earlier -- mouse model, P53 tumor suppressive gene is the most frequently mutated gene in cancers. The human Li Fraumeni syndrome has a mutated P53. Those patients have greater cancer risk.

There's a conservation of P53 across species, and the function of the P53 gene product involved in cell cycle regulation, involved in trafficking toward apoptosis, and also involved in DNA repair, makes it an attractive product in terms of a target for mutation that might knock out a lot of
these functions.

The assay is a six-month assay with continuous dosing. There is -- It's a hemizygous model. So there's one functional allele in all cells. What's been found is that induced tumors, chemically induced tumors -- the tumors are associated with a loss of the wild type P53 allele.

One pragmatic concern is that one thing we're learning is that, when there are subcutaneous injections, when that's the route by which the drug may want to be delivered, tumors are sometimes associated with injection site. So the control animals will often show tumors at the injection site. So that can be a problem.

The TJC mouse model: The mouse zetaglobin promoter linked to v Harvey RAS gene, and assay 40 terminus. This is a micro injected mouse model. There were thought to be three to ten copies in the initial founder mice that were generated here, but it looks like these animals have wanted to go on an amplification of transgene, and in our own we've shown that there's about 40 copies presently now of this transgene.

The rationale for the use of v Harvey RAS is that human cancers are associated -- 25 percent of
human cancers are associated with a mutated RAS gene, and conceptually all cells have this mutation. All cells in this mouse were preinitiated, and what this model is waiting for is a strong promotion event. That becomes the event in the induction of tumors.

It's been found that tumors that result are associated with a loss of methylation, and all tumors that have been examined to date for expression do express the RAS -- mutated RAS gene. So it is expression dependent.

In terms of practical aspects of its utility, it is primarily a skin paint model. So the test compound is painted onto the skin. Approximately 200 microliters is applied to the skin. As a positive control for bolestra, PMA is applied three times a week.

Issues surround how the drug is going to be delivered. You have a very aqueous, soluble drug. That could be a problem. It has to be soluble in a solvent that can be delivered to the animal's skin and will evaporate and deliver the dose.

There is an alternative route that is being explored. Oral gavage with dimethylvinylchloride has been shown as a positive control to result in four stomach tumors. So again
it's almost like a local effect, and also some rare leukemic tumors. Leukemias have been seen with, for example, benzine administration.

Oddly enough, one example of systemic cyclosporin A given by gavage has resulted in topical skin tumors, but that's the only example that I know of where a systemic drug has resulted in a skin tumor.

The TgrasH2 transgenic mouse model: This model is reported to have five to six copies of the human normal ras gene with its own promoter and its own enhancer region. There are no mutations in the coding region of this transgene.

What's been found in transgenic mice is that the 221 protein seems to be up regulated. It's about two to threefold higher expression in cells, and that when tumors are looked at -- when one examines tumors from -- chemically induced tumors, that there are a lot of somatic point mutations that are found, found very frequently in these tumors. This is also a six-month assay.

Some of the data: This is some of the data that preceded the whole ILSI initiative, and this is the kind of thing we're looking for. Can these models pick up known human carcinogens? When you look at the ones that have been tested, yes, they all can
pick up the genotoxic in carcinogens.

We look, for example, at P53. It doesn't pick up cyclosporin A, Tcdd or diethylstilbestrol; whereas, Tgac and TgrasH2. So Tgac seems to pick up both genotoxic and nongenotoxic. So it picks up the promoters and the complete carcinogens; whereas, P53 is limited to the genotoxic.

TgrasH2 so far seems to be able to pick up both categories, although you'll see there are some other examples where they can't.

If you look only at compounds that are associated with rodent tumors, the same sort of pattern falls out. Tjc can pick up genotoxic and nongenotoxics. For the most part there are a few deficiencies here that you'll see. Ethyl acolate, for example, Tjc did not pick up.

P53 again, for the most part, picks up the genotoxic. Glysodol is an example where both P53 and Tjc did not result in a positive finding, but this was multi-site, two-species, both male and female carcinogen, conventionally treated bioassay. The reason for these negative findings are being explored presently in Dr. Tennant's lab.

Finally, you want models that do not react to everything that's given to them. So you want to
check the question of specificity. Do they not react
to noncarcinogens?

Again, for the most part, for Tgac except
for resorcinol here, and rotenone, the specificity is
good. P53 specificity is good, and rasH2 specificity
is good. But again, there is a lot more work being
done presently with these things.

The other question is not just things that
are four-cell negative, meaning negative in both male
and female rats and mice into your bioassay, or things
that are positive in male and female rats and mice to
your bioassay. What about those things that get us
into regulatory dilemmas where it's positive in the
mouse but it's not in the rat, or it's positive in the
males but not in the females, and what do you do with
that kind of data?

Some of the compounds that gave us these
sort of ambiguous findings where there were some mouse
only findings or male only findings in the mouse and
rat were put into Tgac and P53. For the most part,
these things are not viewed as like "overly"
sensitive.

There is some level of selectivity, in a
sense, that again these things were negative in the
rat and positive in the mouse, but they came out
negative in Tgac. It wasn't sure what was going to happen here.

In terms of our charge today is to ask questions about -- You know, when we're talking about vaccines, we ask questions about DNA -- when these transgenic models are exposed to DNA. I don't know the answer to that question, how these things will respond to exogenous DNA, but one abstract that I was able to locate, Gina Clarke at Genentech asked the question about growth factors, mouse abnormal growth factor or recombinant human IGF-1.

It was administered subcutaneously for six months to both P53 and Tgac, and it came out negative. Again, like I say, Tgac is primarily a skin model. This was a subcutaneous injection. This was an example where they did see some subcutaneous injection site tumors, but there were no increases in the ones with EGF or IGF-1.

The XPA deficient transgenic mouse has not received near as much testing as the other models, but the thinking here is that there is deficient repair capabilities, and it's expected that this model will be able to pick up genotoxic carcinogens.

For the most part, the few chemicals that it has been exposed to, it seems to be bearing out.
A question of whether it can respond to nongenotox is wide open, and what about genotoxes don't rely on repair. We don't know the answer to that yet. There are promising prospects sort of off in the wings, if you want to think of it that way.

There is a sort of Tgrash2 equivalent rat model. Again, if you want to ask this question about across species, there's another very interesting model, a K6 promoter, ontheondecarboxylase transgenic mouse made by Tom O'Brien. This mouse behaves like a pre-promoted model. Apparently, all the polyamines being synthesized by ODC have the skin that occurred to the sites as a state of promotion. What they're waiting for is an initiation of that. They've shown with some chemicals that a preinitiator will activate this model. Again, it's a skin paint model. So it's sort of like the slip side of the coin of the TgAC mouse. There are also some very creative kind of things. I just put an example from Dr. Inoue's group in Japan where they're taking Tgrash2 mouse and then giving it IP urethane, to accelerate the whole thing. So you've got an initiator here. Then you add your test compound, and they can get an answer in three weeks as opposed to waiting six months.
So there's some efforts like that to accelerate even further from the six month assay on forward.

So in conclusion, these short term models to define transgene carcinogens are promising. There are strengths and limitations that are being elucidated, and mechanistic understanding of these models is expanding.

The P53 and the neonatal mouse identifies genotoxic carcinogens but really not nongenotoxic carcinogens. TgAC identifies both. TgrasH2 identifies genotoxic and the nongenotoxic so far. Looks promising. There's further evaluation going on there, and with XPA we don't have a lot of test results yet to go with that.

I'll entertain any questions. Thanks.

(APLAUSE.)

CO-CHAIRMAN MYERS: -- cell substrates been tested in any of these models?

DR. SISTARE: I am unaware of anything like that having been done. It certainly is something that could be done, and the beauty of these things is it can be done pretty rapidly, because they are six-month assays. The cost is not prohibitive.

The animals are $150-$200 a piece. Some
of these transgenic animals, $150 a piece, but you
only need like 15 animals per group. So these things
are doable, but to my knowledge they haven't been done
yet.

DR. ONIONS: David Onions, Glasgow. I
enjoyed the talk very much. I just wanted to ask a
question in terms of how the endpoint is read. Mike
has very kindly allowed me to show some data later,
and one of the points that we find is that by looking
at the actual kinetics of the tumor development, that
actually gives you much finer data than looking at a
single time endpoint. But by looking at the kinetics,
you can actually discriminate between very low
carcinogenic events in multiple transgenic animals.
I was wondering how the readouts are done
in your particular case.

DR. SISTARE: In the TgAC, for example,
you can look at both the numbers of animals developing
papillomas and also the number of papillomas per
animal as a function of time, and that data is
analyzed in that way. But, you know, how we are
ultimately going to decide whether something develops
a tumor faster than another thing, is a more potent
carcinogen, that's something that has to be done
later.
With P53, you don't know until you dissect the animal at the end. So there's really not a time component that goes in there, but certainly, they will look at numbers of tumors per animal, number of sites, you know, is it a multi-site or is it a single site, that kind of thing? Is it only the males, only the females? So all these kind of things.

DR. ONIONS: I just have one very quick comment about the P53 heterozygotes. We've used a slight different line, which is the Edinburgh knockout mouse, but its function is the same.

If you use the heterozygote, what is quite surprising with some carcinogenic insults -- for instance, a retroviral insertion -- you would expect the normal allele to be the primary target of insertion in tumor development, and it's not. It actually is our other genes, but actually universally the heterozygote P53 normal allele is rearranged. So it's, I think, very important to be very careful about assuming that that is always the primary target and that you need to be very careful about that assumption.

DR. SISTARE: Yes. No, I agree with that.

There has been some data that's shown that there is some rearrangement in that locus, and also that, you
know, one has to keep in mind that the P53 is sort of

-- having only one allele, having sort of a gene
dosage effect there, that the loss of that function of
that protein is going to have these other cells in a
state of readiness for whatever. They're going to be

less prone to apoptosis -- less capable of apoptosing;
so other injuries, yes. I agree with that.

DR. LEWIS: Andrew Lewis, CBER. Has there

been any attempt to establish tissue culture cells
from these various models and to see whether you can
replicate the findings with genotoxic versus
nongenotoxic carcinogens in these tissue culture

systems?

DR. SISTARE: Well, there are P53
deficient cells, as we know, which kind of looks a lot

like the P53 model, Li Fraumeni cell lines, for
example. But from these particular cells, the TgAC,
for example -- The thing about TgAC is that it appears
that the target cell, at least for skin tumorigenesis,

is a follicular cell, a cell at the base of a hair
follicle. So it's not just any keratinocyte there.

There have been attempts, none of which I

know have been successful. Now there have been some
tumors -- tumor lines based on papillomas, but those
aren't as useful. Tgrash2, none to my knowledge.
DR. LEWIS: And how frequent are these tumors that appear at the wound site in these P53 mice?

DR. SISTARE: In P53s with the subcutaneous injection sites, it's not an insignificant frequency. It's something that we've got to reckon with. We've got to work out things like is it pH related, what is it, that kind of thing.

With TgAC, if there's a wound, a full length wound, you will get a papilloma every time there's a wound. So there are concerns about fighting, wounding, that kind of thing. You usually have to guard against those kinds of things.

CO-CHAIRMAN MYERS: After lunch Dr. Fried and the morning speakers are going to try and put this all -- lead us in a discussion to put this all into perspective, and we'll reconvene at 1:20 promptly.

There's one announcement.

DR. PEDEN: Yes. For the late breaking session on Thursday night, there's about an hour for a number of presentations. So if you want to present data that will appear in the publication that comes out of this meeting, come and see me, and we'll decide, if we have too many, which gets presented.

So I'm Keith Peden, and I'll be around
here. Thanks.

CO-CHAIRMAN MYERS: Thank you.

(Whereupon, the foregoing matter went off the record at 12:01 p.m.)
AFTERNOON SESSION

Time: 1:24 p.m.

DR. FRIED: Welcome to the first panel-audience discussion on mechanisms of neoplastic development and neoplastic cell tumorigenicity:

implications for cell substrate development.

What we're going to have first is two short talks which are relevant to the information we will discuss, and the first one will be given by David Onions, and it's the use of transgenics to detect rare oncogene events.

DR. ONIONS: Thank you very much, Mike.

It's kind of you.

What I'd like to just talk to you about is some work that I've been doing with my colleagues, Jim Neal and June Cameron at Glasgow and relates to using transgenic mice with transgenes targeted to the lymphoid compartment using the CD2 locus control region, and also controlling the functional expression of these genes using an estrogen receptor so you can control the actual target into the nucleus with tamoxifen. So you can either have an inactive or an active transgene.

Next slide, please. Now this is a survival curve showing 100 percent survival here and
survival in days along this axis here. What you're
looking at at the green line is the survival of
transgenic mice carrying the human CD2 myc transgene.

As you can see, what happens is there's a
background rate of about 13 percent of lymphomas in
these mice over a period of about a year, and that's
been replicated by other people.

On the second curve here is a quite
different mouse. This is the P53 knockout mouse. In
this case it's the homozygous knockout mouse. It's
the Edinburgh strain of mice, and again there is tumor
development, again predominantly lymphomas, over about
a year period. In fact, they're all dead by a year
from lymphoma.

So that's the starting point. But what
becomes interesting is when you make genetic crosses
of these mice. So if you look at this curve here,
this is a cross now between this mouse and this mouse,
and you see that there is a very rapid acceleration in
tumor development. This shows at least two genes are
collaborating in the development of lymphoma.

What's more interesting, I think, from the
point of view of this discussion here is that you can
even push this system further. Notice these mice are
all dead by 90 days. But if you superinfect these
mice with murine leukemia virus, you can actually get
insertional mutagenesis that results in further
acceleration of tumor development.

By cloning the sites where that virus is integrated, you can identify new genes, as we've done -- in this case, new progression genes. But the functionally important point I want to make here, I think, from the issues we've been discussing about the oncogenicity of residual DNA is that you can discriminate even in mice that have this very, very short life span by doing kinetic studies and looking at the pattern of tumor development over time.

You can actually pick up very rare oncogenic events or very weak oncogenic events that you would certainly miss, even if you put these insults into mice that carry the myc gene. So I think that's the first point I would like to make.

This might be a very useful model system, for instance, for looking at certain insertional vectors like AAV that have got the mutated -- or have got lacorep gene or certain retroviral vectors, as well as perhaps residual DNA.

Could I have the next slide, please. The second point I wanted to make is that not all oncogenes collaborate. This just shows genes that we
have identified as collaborating genes.

In this case this is a collaborating we identify with myc which we just published called CBFA1, and CBFA was identified by this retroviral tagging technique.

When we make transgenics with that and then superinfect murine leukemia virus, we can ask what genes are activated, and we find, not surprisingly, that myc is activated; because we know that myc interacts with CBFA1. But you can also find that other genes like pim and pal are activated, but they tend to be exclusive. So that we know now that pim and pal lethal form the same complementation group.

Could I have the next slide? So in fact, we can begin to bring a pattern of progression starting in this case with myc, and then we can put pim and pal which we now put in the same complementation group, and we've identified further progression genes.

So you can begin to build up a pattern of tumor -- of genes necessary for tumor progression.

The final point I wanted to make in relevance to the kind of use of these models in a functional sense to screen out of the carcinogens or
the oncogenic effect of residual DNA is this

conjecture slide. This is not data slide. This is a
c conjecture slide, and it's a simplification.
For instance, we heard from Dr. van der Eb
today about the complexity of transformation

associated with DNA viruses. They cram a lot of
functions into a small space. But predominantly E1A
has a myc-like function.

So that if you were to test the
oncogenicity of E1A regions in a myc transgenic model,
it's unlikely that you would detect an acceleration of
tumor development.

On the other hand, if you were to take
another mouse like the CBFA mouse, which we know
collaborates with myc, you would probably find that
this wouldn't collaborate, and you would detect
deceleration.

So my final point is that when picking
these models, it will have to be models plural,
because I think you need to use a range of different
mice with different oncogenes to cover the
complementation groups.

(APPLAUSE.)

DR. FRIED: Are there any questions or

comments?
DR. ONIONS: Yes, we do. I didn't actually show that one, but those were critical experiments for us. If you take --

DR. FRIED: Is your microphone on?

DR. ONIONS: Well, the question was, if you take the myc transgenic mice and superinfect those with murine leukemia virus, do you see acceleration.

The answer is, yes, you do. We published this in Oncogene a few years ago.

In fact, we used those mice to actually identify the new oncogenes like CBFA1 as a new oncogene.

AUDIENCE PARTICIPANT: I would like to add a note of complexity to this scheme. I would like to add that not only with a single oncogene you can detect a complementing -- depending on the strain of mouse, the MULV you are using, you will get a different complementation.

For example, with myc we have detected over 60 percent of notch activation, while you don't detect that in your system.

DR. ONIONS: That's right. That's correct, yes. That's absolutely true. I think that really just highlights one of the issues here, that
all animal models are extremely complex.

In instance, the LTRs and the retrovirus used will bias which particular genes are activated and so on. So that's absolutely correct. Again, I think, reinforces that, if you are to use these model systems, that you need to use a variety of model systems and not rely on one.

DR. FRIED: Does E1A and myc collaborate in vitro?

DR. ONIONS: Not as far as I'm aware. I think that goes back to early experiments of Helmut Lang who did these experiments and showed that predominantly E1A had a myc-like function. So that it didn't seem to collaborate.

Of course, if you put in E1B, then you've got other functions there.

DR. FRIED: Thank you very much. Is Jim Cook here? Okay, I think we'll go on. Jim was going to give us a short talk about the generalities about tumor genecosities, but I'm sure we could take that later if he shows up.

From the talks we have heard, we know that to generate a fully neoplastic or tumor state is not simple, and it requires multiple events and multiple stages. You know, we need sort of activation of
mitogenic signals. This is usually through oncogenes
and the oncogenes -- we might need more than one. We
might need cooperation.

We also probably need inactivation of
tumor suppressor genes, at least in the P53 and the RB
pathways. We have to overcome senescence. We have to
make sure, if the tumor is going to grow in the
animal, that the division is greater than the death
rate of the apoptosis.

This probably changes at the cell surface.

You need vascularization for the tumor and, of course,
we've heard a lot about overcoming immune
surveillance.

It's quite clear that the formation of
transformed cells or immortalized cells in vitro are
not necessarily the same as forming a tumor in vivo.

So the first panel-audience discussion
should probably highlight features that the regulatory
agencies should consider when assessing neoplastic
cell substrates for vaccine development.

I would ask the audience to actually
participate as much as possible, because I'm sure that
everybody here could answer all the questions that you
may have questions. So it will be a blend of both the
panel and the audience.
Also, if you want to ask people questions that you've thought of later after they gave their talk, this is the time. So I think one of the first questions we should ask is should we use neoplastic cells for vaccine substrates, and what are the positive features of neoplastic cells? Does anybody want to start on that?

I mean, we have heard that we can grow them to large numbers, and we can get their reproducibility, always having the same one, freezing them down. We certainly might need certain types of neoplastic cells for virus growth. For high titers we might need to get specific cell types. Also, more recently, growth of disabled virus has been done in sort of P93 cells, which Alex van der Eb highlighted in his talk, which actually contain some adenovirus genes, and they're sort of, I guess you would say, immortal but not tumor producing.

They don't produce tumors at all, Alex?

DR. VAN DER EB: They produce tumors now, I think, but initially -- That has been tested many years ago, I think. They were not tumorigenic in the nude mouse system, but they became it later.

DR. FRIED: But when you say they produced
tumors, was this on passage or do you need a lot of cells?

DR. VAN DER EB: I think it was on passage. This happened in 1974-75 or something like that, and so it's a long time ago, and there is not much written down, not by me.

DR. FRIED: Because that's mentioned in the document, the CBER document, but this type of cell is really used, and people are injecting things, because most of the adenovectors which people are using for gene therapy are grown in 293 or equivalent cells, because they complement the E1A gene and allow you to get disabled virus, which then is produced and will not replicate.

On the other hand, we know Onyx is using a virus for sort of gene therapy to selectively kill tumor cells which do not have P53, and they also grow their virus in those cells because they have E1B, and that's a complementation.

So could you think of any other cases where we would need neoplastic cells?

DR. PITOT: Well, being very naive in this, I just have one question. Since I think already on the market or at least in some trials a product of neoplastic cells that is basically a product of
myeloma cells have been used already in the treatment
of neoplasia. So it seems that question already, in
a sense, has been answered in that it's been done.

That's quite different from the sort of
things you've been talking about up here. But perhaps
if one is talking about a protein that's going to be
produced, I personally wouldn't see any problem with
the neoplastic.

So I think where it may get into a
difficulty is in the DNA vaccine production.

DR. FRIED: Right. Well, in the case of
the hybridomas you're purifying a protein. In the
case of growing the viruses that you want live, you're
actually just -- You don't purify it to the same
degree as you would purify the antibody molecules, and
then you would inject in virus; because you want it to
get into -- infect a lot of cells.

So it's slightly different, I think the
less critical.

DR. SHEETS: Might I respond to your
question about other reasons we might want to use
neoplastic cells?

My name is Becky Sheets, and I work in
vaccines at CBER.

A couple of other things that we have
considered in terms of why we are thinking about the
acceptability of these sorts of cells is (1) you
mentioned adenovirus as a recombinant vector, but also
traditional HIV vaccine -- traditional approaches to
HIV vaccines have not been tried in preventive
settings, and by traditional approach I mean live
attenuated and inactivated. Part of that is because
HIV only really grows well in human tumors, in T-cell
lines.

Another basis for using these sorts of
cell substrates for vaccine production is because they
can be grown in -- They can be adapted to growth in
serum free medium. Therefore, you're taking away the
risk of an animal derived substance that you would be
adding extraneously to the vaccine. So you're
removing some of the risk for BSE or for some of the
other bovine viruses.

So there are additional reasons for
considering neoplastic cells for vaccine production.

DR. FRIED: Right. Also, I guess, people
are going to use, say, adenovirus vectors to bring in
other antigens, maybe HIV-type antigens.

DR. SHEETS: We have been approached about
that. Some of the adenovirus vectors are replication
competent and can be grown in other things, but those
that are replication defective would need to be grown in a cell line such as 293. Correct.

DR. FRIED: I think that's a strong point.

Also, I guess certain viruses that one would want, higher titers might be achieved in neoplastic cells than some of the cells that they're growing; because you can get them from different tissues where might need tissue specificity for the virus to really grow well.

Okay. So that's some of the positive features. Then the question is what are some of the negative features for the substrate?

I guess the ones are that they contain activated oncogenes, and the question is whether these oncogenes could somehow make it over to transfer through the vaccination procedure.

Has anybody any feelings on that? That would be -- The other thing is, if there were cell contamination, would cells of a neoplastic cell be transferred, and could that cause a tumor? I guess one could very carefully get rid of most of the cells in the preparation, but how many cells do we need for tumors when we transfer it into the vaccination?

AUDIENCE PARTICIPANT: I have to express some bewilderment about some of these issues, simply
because, as I mentioned last night, the vero cell, which under many conditions is neoplastic, has been licensed for the production of IPV and OPV in the United States, Thailand, Belgium and France, and these issues apparently have been resolved, at least to the extent that they apply to vero cells.

Is there something that I'm missing that applies to cells other than vero that are immortal in neoplastic?

DR. FRIED: Well, I think in the document it was considered that -- Bob?

AUDIENCE PARTICIPANT: Just to clarify, in the United States at least vero cells have not been licensed for an unpurified vaccine, for instance like OPV, to my knowledge. IPV can be produced in vero cells.

Of course, vero cells being used for OPV may be a special case, because whatever you're giving is being presented orally rather than parentally. So I think that these still are issues, even with vero cells, that the world is struggling with.

AUDIENCE PARTICIPANT: Well, we have enough representatives here from FDA to tell us whether IPV and OPV are licensed in this country, produced in vero cells. Does anyone have a definitive
answer? OPV is not? Yours is definitive?

Okay. So IPV is, which implies strongly that vaccines that are administered orally and produced in continuously propagable abnormal neoplastic heteroploid cell populations should be acceptable.

AUDIENCE PARTICIPANT: I would like to add that OPV prepared in vero cells is also pre-trial.

AUDIENCE PARTICIPANT: I was going back to try and look at the basis of our understanding of whether DNA -- residual DNA is a risk. I thought there were some interesting talks today that perhaps highlighted some of the issues. One of the issues that wasn't stressed, though, was in fact the nature of the DNA that we see in Crucip and Ames, which are the primary danger, it seems to me, because blood products are purified. Then most of the DNA is pretty badly sheared, and it's sheared below a size that would be normally associated with a functional gene. Now that's an average size, and we have to be careful because there's clearly a distribution in size.

It seems to me that you're left with two primary worries -- well, three worries perhaps. One is of promoter insertion, because there's still some
of these size of around about a promoter.

The second level of concern would be, I think, the still intriguing data on the MTR-like sequences in herpes viruses and whether there are other insertion elements like that, and they truly operate.

I suppose the third concern I would have really relates to adventitious agents we don't know about, like say a good example would be bovine polyoma virus which we didn't really know a lot about until recently. So you have an unknown polyoma virus in a cell line, and as we know, as we've heard today, you can get a lot of transforming functions in a small domain.

So I think those are the three areas which seem to me to be perhaps residual concern in residual DNA.

My only comment would be that you can talk about it a lot, but the only way of actually testing that, apart from large scale epidemiological studies, are the kinds of models that, I think, that Frank Sistare and myself mentioned.

DR. FRIED: But, David, I mean, none of those are really neoplastic cell specific.

AUDIENCE PARTICIPANT: No, they're not.
DR. FRIED: They're the same for both. I mean it's just DNA. I was thinking more of, you know, if you start with a cell that has an activated oncogene or something, could that get over.

I think, since we need multiple genes for neoplasia, the chance of getting two or three cellular genes to meet and get in is very rare.

AUDIENCE PARTICIPANT: I think I was working around the conservative approach to exactly that same conclusion. In other words, I think you can enumerate a number of issues, but those seem to me so remote at the moment that I would not, without further experimentation -- I would go in your direction, yes.

DR. FRIED: Right. But I think one should consider the viruses. Now the viruses put -- They get a lot for their money, because they have either overlapping reading frames, so one piece of DNA, in the case of polyoma viruses, three genes and SV40 is two genes, or they have common things that are hooked up to each other, are very closely linked.

So we don't need different parts of the genome together. I think that's something one might think about.

DR. BERKOWER: I'm from CBER. Ira Berkower is my name.
I'd like to make two points and ask for comments on these points. The first one is: I see vaccines, particularly anything that is an assembled antigen, as being entirely different from other products that might -- biomedical products that might be made by recombinant DNA methods and expressed in even in the same cells.

For example, monoclonal antibodies made in myeloma cells -- myeloma cells are -- In hybridoma cells myeloma cells are highly malignant, but the antibodies can be highly purified. Steps like protein A affinity columns and various other chromatographic steps can highly purify away adventitious agents, harmful DNA sequences and so on, and really should be considered, in my view, completely different from vaccines.

Vaccine purification, particularly if it's a particulate type of antigen, would tend to select for things that are the size of viruses. They would tend not to be inactivated by any harsh chemicals or denaturing agents. They don't -- They may not have a specific affinity step.

So -- and even some of the purification steps might tend to concentrate viruses into your fractions rather than remove them.
So I think it's a mistake to reason from other products that might be made in tumor lines to vaccines. Vaccines, I think, are quite distinct in that regard. In many cases, they either can't be purified away from harmful agents or they can't be inactivated to the same extent as would ordinarily be done for other products produced in the same cells. So arguing that the same cells are used would not apply to vaccines. That's my first point.

DR. FRIED: Well, but on that point, the differences between neoplastic and immortalized cells -- I mean diploid cells -- there's not much difference there.

DR. BERKOWER: That's true, but I think one of the arguments that these things are safe is that they've been used to make other products. That argument, I think, just doesn't hold water.

DR. FRIED: And your second point?

DR. BERKOWER: The second point was:

Seems to me that, something I asked about before, that there ought to be a notion of sort of the malignant potential of some of these lines that are being considered, that some lines are what I would call highly malignant and probably shouldn't be used to produce vaccines, even though viruses can grow in
those cells.

The cell itself would intrinsically have a higher risk, whereas other cells would be much closer to normal somehow with a lower, what I would call, malignant potential, and might -- Perhaps we could identify these cells and, because there are continuous lines, characterize them as well as possible and actually show somehow that they were less malignant and, therefore, a more suitable substrate for producing vaccines, say by virus growing on those cells.

I could imagine, for example, classifying cells based on some kind of oncogene inventory, and I wonder if that would be possible.

DR. FRIED: Does the panel have any comments on that?

DR. COOK: I think that gets back to the same point Mike Fried just made, which is that until you have some way to assess the relevance of what highly malignant is and whether the things that convey those traits to the neoplastic cells that you're using for the vaccine substrate -- whether those genes could be reasonably expected to be transferred across in some kind of residual DNA.

It's sort of a nightmare and a worry, but
there's not much science to it. I mean, the question is how can you say that a cell that varies by as much as a millionfold compared to another cell is any greater risk, other than the fact that it feels bad?

If nothing is reasonably expected to be transferred and you have four or five oncogenes in that cell, what's the likelihood that those genes are going to be transferred across? I mean, think of a simple experiment like a cell transformed by a pair of oncogenes, that you're required to get that cell to be reasonably immortalized and transformed in a conventional in vitro assay.

Shear up that DNA and try to transfect it into another cell, and see if that DNA itself rather than the purified genes like we all do in plasma preparations could actually create a focus in anything you could imagine. I think the answer is probably not. It's unlikely that that would be an issue.

Now how you test that is another question, and even more fundamental to your issue is what's highly oncogenic mean? It's relative to how you assess the cells, how you quantitate those sorts of things, which cells you test, what kind of host you put them in, and you could probably define it to meet whatever criteria you wanted to.
I think it's a real dilemma and it probably requires some experiments to come up with how you're going to define these issues. What do the terms mean, and how are you going to use these to set the guidelines that people would have to do this awful job will do?

DR. BERKOWER: I mean, one example, which is not directly related -- It seemed obvious to me that, if you were going to use, for example, EBV transformed B cells as a substrate, at least pick the nonproducer lines. Okay? That's obvious -- to grow the virus in.

DR. COOK: I think that's a different issue, and that is you're at risk of transmitting an infectious agent that can amplify itself. That's different than saying you have a neoplastic cell free of all known adventitious agents that contains either a known or an unknown gene that regulates the behavior of that cell.

I think transmitting infectious agents should be at a different level of concern, because then if that could survive the purification process, that's a big problem.

DR. FRIED: I mean, if it's a really highly tumorigenic cell, and we're not quite sure what
that means, then I think one has to be careful in whether cells are contaminating the vaccine. If it's four or five or six oncogenes together that are making it so powerful, the chance of getting them all over in the vaccine is very remote, because you need six pieces.

AUDIENCE PARTICIPANT: I find myself in the reluctant position of trying to argue against what you just said, because I actually don't believe that oncogenic -- DNA from the lines we've been talking about is a risk. But I think I'm not sure that I agree with the reasoning that you've just adduced.

I do think there's a difference between cell lines. I think the very fact that we use vero cells usually at a passage level below the level at which they're tumorigenic in animal models is a testimony to that.

I think there clearly is a difference. We know that, as we pass these cells, that different genetic changes are occurring, and we now know that -- it's a kind of paradigm of tumor biology that multiple genetic changes are required.

So if you're going to pick a line, why don't you pick one earlier on where there's likely only to be one or two changes and not one where
there's seven?

I think your argument, Mike, was that it's so unlikely to transfer four oncogenes into a single cell, but surely all our transfection data in gene therapy suggests entirely the opposite.

If you make a cocktail of a cytokine gene and a couple of other genes and inject them in, you can find generally the cells that take that out have all of those genes in. It's rather like a cell transfection.

DR. FRIED: But you're putting in the purified genes, not the whole cell.

AUDIENCE PARTICIPANT: Oh, sure. I accept that. That's why I still think that this is a highly, highly improbable effect.

DR. FRIED: I was only commenting to him, saying that there may be multiple genes, you know. If there are four or five, it's going to be worse.

AUDIENCE PARTICIPANT: But this is -- I would like to say I very much agree with you, that the idea is to take a cell that is barely immortalized rather than the cell that is soundly full of all kinds of mutations and could potentially transfer more than one at a time, like you just said.

DR. FRIED: I don't think anybody is
disagreeing with you, that you take it earliest and

safest. I mean, the question is what are the risks as

you passage it later?

AUDIENCE PARTICIPANT: Well, okay. To sum

up my question then, would there be some convenient

quantitative way to assess, oh, this one is earlier,

that one is later?

DR. FRIED: Does the panel have any

comments? Anyone?

DR. COOK: So you're talking about two

different cell lines.

AUDIENCE PARTICIPANT: Yes.

DR. COOK: Trying to assess the relative--

AUDIENCE PARTICIPANT: Which one would be

better for vaccine --

DR. COOK: -- that cell line?

AUDIENCE PARTICIPANT: -- for use as a

cell substrate, as we're trying to decide today.

DR. COOK: Well, you can create artificial

models in which you can do that, like the animal

models I described this morning, and at some point I

guess I can present these data. But you could say,

okay, we're going to decide that host A is the host in

which this is going to be defined.

Somebody is going to have to make that
decision, because you can always come up with a weaker
host, what I call a walking test tube. You know,
you've got an animal, but it lacks any kind of defense
against a tumor, and you're going to be able to change
the tumorigenicity of that cell, depending on what you
test it in.
 So somebody is going to have to decide,
well, what is the animal in which we test this. The
data I showed this morning was that, if you have a
cell line that can be easily rejected by a normal
mouse or a nude mouse at a given concentration, it can
be highly tumorigenic in a CD3 epsilon transgenic
mouse.
 So you've got to define the animal in
which this is going to be done, and then you can say
how can we test the cells. Well, there are
quantitative ways to compare cell line A with cell
line B, once you settle on that in vivo model.
 Then you have to accept the fact that
you're making an arbitrary decision about, okay, if
this one has a millionfold greater ability to make
tumors at this inoculum over this period of time or
over a range of inocula, then does that meet the
definition of a worse cell line versus a better cell
line.
AUDIENCE PARTICIPANT: Yes, but I'd prefer a molecular diagnosis, if you see what I mean.

DR. COOK: A molecular probe that will tell you what's the worst cell?

AUDIENCE PARTICIPANT: No. Multiple probes to try and define, you know, which ones have accumulated more activated oncogenes and which ones are running with fewer or almost none.

AUDIENCE PARTICIPANT: To respond to this, I would suggest that there's at least the possibility that focus on the malignancy of the cell is the wrong problem to be concerned with, that what we're really interested in is the ability of the information in that cell to be transferred, not the state of the information in the cell.

For example, primary tissues from monkeys, for example, can have pathogenic organisms in there that have nothing to do with transformation and would not score in a test for neoplasia, but would be certainly something we would want to avoid.

I mean, the assumption in the question is that there's a correlation between the presence of information of a neoplastic nature and its ability to be transferred from one cell to another. And it doesn't seem to me that we have any evidence to
suggest that that correlation holds.

Now, certainly, if you have a scenario in which you have two cells that are completely identical in terms of their ability to donate information, you want the one with the less damage or accumulated changes in it. But that's an assumption that I don't think there's any basis to make.

In fact, I would question whether there's any reason to believe that there's any correlation at all between neoplastic potential and ability to donate information in a vaccine problem.

DR. FRIED: That was one of the topics I wanted to bring up, is whether the mechanism by which the cells become neoplastic could contribute to their relative risk as a vaccine substrate.

You know, certainly, if you knock out tumor suppressive genes, that's a loss of a gene. That might, you know, make the cell more tumorigenic, but you're not going to really transfer that unless you're transferring a dominant negative P53. If it's just mutated and gone, it's gone, and it's not a positive situation.

I don't know about methylation, how people feel, whether that is something that's going to change that. Walter?
DR. DOERFLER: Well, you know, with methylation you have to be very cautious in interpreting into biological problems, because we -- To be quite honest, I don't think we understand in full detail the implication.

So from my judgment at this point, I would say, well, methylation may be -- the pattern across a certain stretch of the genome may be just a model on which chromatin structure is built, and in some instances this relates to expression of genes, permanent silencing of genes, and then how does that relate to the tumorigenesis?

Well, one model I've got up in my mind can pursue, that by changing a pattern like Dr. Baylin, for instance, gave you several examples where in tumor cells the patterns are changed, and early on we saw viral transformed cells at a very different pattern into different segments.

So, certainly, things change, but to say it is an increase or a decrease, I wouldn't be able to do that; because we have looked at a fair number of human tumors in a number of mainly lymphomas and Hodgkin in Cologne, and looked at different -- used different probes.

For some of the probes there was an
increase; for other ones there was a decrease, and for third ones there was no change at all.

So if I should translate that into biologic significance, my feeling at this point is perhaps chromatin structures are changed segment-wise in different parts of the genome in a tumor cell as compared to normal cell. That's almost a sort of a given.

That's a blatant thing that chromatin structure changes, but I always thought changes in methylation patterns would be an interesting indicator for such change, and then we could, once we have the technology, try to delve deeper and see what do these changes mean in terms of altered chromatin structure and altered methylation pattern -- and altered transcription patterns.

So when we saw these changes in lambda DNA transgenic cells, of course, we got very excited that this might be a more general phenomenon somehow relating to tumorigenesis. I don't know how Dr. Baylin would feel about these proposals.

DR. BAYLIN: Well, again, if the question becomes more the one of transfer, which I think is the issue, it's hard to quite envision. The biggest danger -- If you're using any virus, I think, as a
vehicle for inserting or carrying something, the virus
itself is the biggest threat.

I think there's going to be a lot more
learned, and some of these viral proteins, in addition
to binding up and taking away RB function and P53
function, I think, can cause probably -- It's going to
emerge that they probably can cause methylation
changes in trans by binding up or changing parts of
what is the methylation machinery itself, the ability
to target the methyl transferases and all this.

This is very little studied, but I think
most viruses can set up methylation changes, and part
of it may be that. It's harder to envision with
antigens and proteins things where the background of
the cell itself will influence what you could
transfer. That would be a danger, I think. That
would be my view.

DR. DOERFLER: Perhaps I could still make
-- cite one example, because in general it's -- One
uses the sort of the correlation or inverse
correlation between gene activity and levels of
methylation very frequently and loosely.

So over the years I have learned the hard
way that one has to be sort of very cautious, because
it may be only certain segments of a promoter, and
unfortunately one cannot give a blueprint to say, well, it has to be 35 or 38 base pairs upstream from such and such a site. It doesn't seem to be the case. It's more complicated. Then an extreme case, which has taught me a lot, is again a virus, frog virus 3, which was initially worked on by Dawn Willis and Allan Graniff in Memphis.

This is a DNA virus which replicates both in fish cells, frogs probably as well, and also in mammalian cells like BHK. When you look at this DNA with any methylation sensitive restrictase, it seems to be totally methylated.

Several groups, including our own, have used genomic sequencing techniques to look at all the CGs, and again this is a completely methylated genome. And yet, a late expression is going on, and with genomes that are totally methylated in all CGs. So Mark Monitz in our lab a number of years ago did an experiment in which he used one of these late promoters which are active, fully methylated state in a viral infection, and hooked it up to an indicator gene like Luciferase and transfected it in the fully methylated stage. Yes, it was active.
However, when he just did a Hapa 2 methylation, a CCGT which -- there were about eight sides -- it dropped its activity. So here's an example which would support the notion that perhaps a certain pattern has to be present for an active or an inactive promoter, and this frog virus, for reasons I don't think we understand, prefers a completely methylated promoter to attract proteins possibly that facilitate transcription.

So one has to look in each promoter and each biologic system separately what this methylation means. So my suggestion is it's a modulator of DNA protein interactions and that we have to be working very hard to find out for each promoter what is the important pattern for the active or inactive state or for building a chromatin structure that is consistent with biologic activity.

DR. FRIED: Thank you. I want to get back to -- DNA would be the things that we're really afraid of, if there are some oncogenes in a cell. So I don't know how efficient DNA transfer is.

I mean, are there people who do DND vaccines? Could they say there's a sort of naked DNA go over or do you have to shield it somehow? What's the efficiency if you put it in the microgram, how
many cells get it? Does anybody know those answers or can say anything?

AUDIENCE PARTICIPANT: Ron Desrosiers might make a comment, because of the SIV experiments, which I think are probably the pertinent ones where you get an actual readout of virus production from putting in intact genomes.

We've done some of the experiments to him using SIV, and you need to put in very large amounts of DNA relatively still to get a functional expression of a replication combinant virus.

So you're still talking in tens of micrograms into an animal before you get out a functional virus. So it does imply that the uptake of DNA to result in a functional end expression is actually extraordinarily rare, and we're talking in micrograms of DNA.

I think the SIV experiments are the same. AUDIENCE PARTICIPANT: The question was what was the nature of the DNA. This is linearalized proviral -- intact proviral DNA going in. We have actually shown that if you actually take a retrovirus and you split it into gag and pol and env constructs, just as in a packaging line, put those in, you can also get expression in vivo and get particle
production.

DR. PURCELL: Yes. My name is Damian Purcell from Australia. I was just going to make a comment of some work that -- on SIV that I'll present on Thursday night regarding infection similar to Ruth Ruprecht and de Rossier's experiments with injected the naked DNA as plasmid, circular plasmid, in small numbers of animals.

We found that we can very efficiently initiate infection. When we put this DNA onto, for example, gold beads, we get down below ten nanograms.

DR. FRIED: You're putting it on beads?

If you just --

DR. PURCELL: On beads as, yes, gene gum, and we haven't titrated it, but putting it on the method of transfer, the naked DNA can under some circumstances such as gene gum inoculation be extremely efficient.

DR. FRIED: But would the naked DNA be efficient? I mean, if some was -- there was some DNA that got out of a cell, what's the chance of that getting over?

DR. PURCELL: Yes. We can't really comment on that, because we didn't titrate the DNA, but in our experiments with naked DNA injection, we
just followed a 300 microgram amount, which --

DR. FRIED: When you say it was efficient
with the beads, what kind of numbers are we talking
about?

DR. PURCELL: In terms of numbers of
animals?

DR. FRIED: No, in terms of amount of DNA
in successful infection, how could you dilute it down
to?

DR. PURCELL: We've only gone as low as
ten nanograms, but maybe one additional piece of
information is that included among the DNAs are DNAs
where we've made deletions in the promoter region of
SIV in the U3, so that the expected expression might
be lower than wild type and still, even with ten
nanograms delivered by gene gum, there's efficient
initiation of infection under those circumstances.

DR. FRIED: Thank you.

DR. PETRICCIANI: John Petricciani.

There's a large body of data actually in the
literature that goes back a couple of decades. One of
the experiments -- these are in vitro experiments on
DNA transfer. One of the experiments that was
mentioned earlier today was the transformation of 3T3
by human tumor cell DNA.
In that particular set of experiments, the amount of DNA required to get any positive readout was 20 micrograms, and it required also carrier DNA in addition to the human tumor cell DNA. The bottom line is that in the studies of which I'm aware in vitro, let alone in vivo, it's extraordinarily difficult to get a positive uptake in expression of DNA, whether it's cell transformation or other gene expression.

DR. FRIED: I assume that, if somebody aware of these things, they would be nucleaseing up their preparations before they -- I mean, they would be using -- trying to break down the DNA as small as possible.

AUDIENCE PARTICIPANT: Well, it's a question of inactivation, reduction through one or another mechanism. Right.

DR. SHIVER: John Shiver from Merck. Our experience is that plasmid DNA is not efficiently taken up by direct injection into most tissues into animals, whether they're non-even primates or mice, and that it generally requires some degree of amplification of whatever response you're looking for in order to detect it, such as an immune response to a transgene encoded by the plasmid or actually a virus virion developing capable of replication, as perhaps
in the SIV or FIV model.

Looking for direct gene expressions systemically is generally not successful. Also, the uptake of the DNA is highly dependent upon the state of the DNA. It really needs to be compacted supercoiled to really -- to work even as well as it does, generally, with naked DNA injection.

DR. FRIED: So your feeling, if there was oncogenes inside of a DNA and some of that was taken up by recipient cell, it would be very inefficient to get that expressed?

DR. SHIVER: Should be very inefficient, especially in the linearized fragments and form that you would expect that DNA to be in.

DR. DOERFLER: Just in response to this interesting comment, in addition to feeding DNA with all that we're injecting DNA, mainly the green fluorescent plasmid construct, fewest cytomegalovirus promoter or SV40 promoter, recently also the ras sarcoma virus promoter but we haven't done many experiments on that yet, and we've also mainly failed to find expression except locally in the muscle where we injected it into.

It could be traced by FISH and PCR in various organs for quite a long time, and although
these data are repeated only a number of times and not
for years, we've also seen it in testing the contents.

So this may be an interesting observation
for people around here. It might be excreted probably
via the bile route. I'm not sure how it works. But
definitely we can find some of this DNA in the
intestinal contents.

DR. SHIVER: We would agree with that
experience overall. The DNA that is residual and
remains tends to be extrachromosomally located -- you
know, with some persistence time, but generally gene
expression only near the site of injection.

DR. DOERFLER: Right. Right. So you do
find expression at site of injection? We do, too.

DR. FRIED: So, I mean, I think the
general feeling is, if there was DNA, cellular DNA
with cellular oncogenes, that would be very
inefficiently transferred, and I think anybody
preparing a vaccine would take great precautions in
trying to destroy the DNA or make it as small as
possible. Is that what the panel would think?

So the way the cell -- the neoplastic cell
was formed, we don't really expect most of these
properties, methylation or loss of tumor suppressor
genes or viral oncogenes to be transferred over in a
vaccine preparation.

Is there anybody who disagrees with that?

AUDIENCE PARTICIPANT: I don't disagree, but maybe I could ask you to clarify a bit the statement that you made a moment ago, that you'd like to see the DNA made as small as possible in order to avoid these kinds of problems. And of course, from a regulatory perspective, somebody comes in with a product and they're doing a particular purification scheme on it and it makes the DNA a certain size, and so we can always say can you make it smaller. But the question is how small then is small enough?

What if somebody comes in with a product, and they haven't made the DNA smaller? Does that mean we should be worried about it? Likewise then, the question of if you have one cell line which is more tumorigenic than another, again the question is, if we truly think that one which is less tumorigenic is safer, then that should be something that one can put some numbers on. Then one has to, obviously, come with some kind of a threshold of sort of trying to define this.

So I know it's thorny and difficult when trying to put numbers on this kind of thing, but I think the kind of information that the FDA needs to
make these decisions requires some more definition.

DR. FRIED: I think you probably have to spike your preparations with, you know, some really efficient either phage DNA or something or some of these really nice, you know, circular small polyoma SV40, which are probably efficient in assays, and then you know, at different stages of the preparation see how much and where it goes.

AUDIENCE PARTICIPANT: That certainly sounds like a reasonable suggestion, if in fact the recommendation then is that one has to presume that such DNA is there, in the first place, and that one does have to get it to a size that's small enough to prevent these kinds of outcomes.

DR. FRIED: I mean, I was going to get to it. But the only -- Is there a comment in the back? The only thing that I was not including is possibly gene amplification, which people don't look at.

I would certainly look at cells, because those are cells that usually amplify oncogenes. They also amplify more than one oncogene. More and more information is coming out that oncogenes get together.

There are large circles that we could see satellogically, but there's also generated small circles of DNA. So there you're sort of amplifying
up, and I certainly -- If one was using neoplastic
cells -- I was going to get to this later -- you would
certainly look for -- you know, cytologically, for
double minutes or HSRs, and I wouldn't stay away from
those, and I certainly would look for polydispersed
circles which have been reported in cells, small
circular DNA.

We don't know where that comes from, but
certainly, a lot of the gene amplifications has to do
with oncogenes and myc. I think all -- If anybody is
using cells, they should certainly do EM analysis for
circular DNA and for HSRs and double minutes.

DR. KAPPES: John Kappes from UAB. I'd
like to suggest an alternative mechanism for genetic
transfer, at least where the vaccine would be
retroviral derived or relying on retroviruses for
cancer vaccination, for example, if our hopes of gene
therapy ever come to fruition.

Data I will present tomorrow could suggest
transfer of that information via the messenger RNA.
That is, instead of relying on DNA transfer, with HIV
based vectors I have evidence that the poly-A signal
could help in some way I don't understand yet mediate
-- transfer that information.

That is, if the oncogene message was
packaged and that polyadenylation somehow modulated recombination with the transfer vector, and if that was integrated, that might be an alternative concern to take into account for transfer that type of genetic information.

DR. NICHOLS: Warren Nichols, Merck.

There’s certainly been a lot of data published over several years now about taking tumorigenic DNA from cells that are tumorigenic and injecting very large quantities into a variety of laboratory animals and newborn rats, newborn hamsters, nude mice, antithymocyte treated rats and mice, and when giving up to 100 micrograms of these, things like HeLa cells, CHO cells, the DNA from HeLa cells, CHO cells, human bladder tumor cells, cloned activated onc genes, there has been no evidence of any tumor formation in any of them.

Just as a comment on distribution, this isn’t a comment on distribution of cellular DNA, but in looking at a plasmid from DNA vaccines, when injecting 1011 plasmids into a muscle and then looking at various time periods after that, we can see a great many plasmids still in the muscle six weeks or 12 weeks later. But in the other tissues, in blood and brain and spleen and a variety of tissues, 13 that we
looked at, by six weeks there's never any present.

If we look just to try to see where it has been distributed, in day one and day two we can find it in most organs. By day seven, it spontaneously has all gone. When we use purification methods to separate plasmid from a genomic DNA even at two days or three days, it all disappears.

We use in looking at this PCR methods that are sensitive to .005 to .01 femptograms. It's about one plasmid in 150,000 nuclei per microgram of DNA. So that it certainly does get around, but it certainly does disappear quite quickly. None of it has ever been demonstrated to be integrated in that kind of system.

I do think that the injections with the DNA derived from tumor cells in huge quantities compared to what is present in any vaccine is very important.

DR. FRIED: Could you repeat the question?

AUDIENCE PARTICIPANT: Were those done in normal animals?

DR. FRIED: Were which done in normal animals?

AUDIENCE PARTICIPANT: Injected from the DNA.
DR. NICHOLS: The injection of the plasmid? The question is were the injections of DNA done in normal animals? The injections of plasmid into animals and then following and looking -- that was in normal animals.

The injection of cellular DNA that's in the literature is injecting into newborn hamsters, newborn rats, newborn nude mice and rats and mice, newborn rats and mice that had antithymocytes here.

AUDIENCE PARTICIPANT: Dr. Nichols, excuse me. Have you published that data?

DR. NICHOLS: Which data?

AUDIENCE PARTICIPANT: The ones you're just referring to?

DR. NICHOLS: The data about the plasmid injections, that is published; and there are two other papers that are in preparation right now, but the first one is published. The other data from the standpoint of the cellular DNA being injected, that's all from literature that exists now, not from us but from other -- many other people.

DR. FRIED: Thank you.

DR. MINOR: Philip Minor from NIBSC. Warren, how do you square your lack of detecting any integration with Dr. Doerfler's comments this morning
about integrating M13 after you feed it to mice? It does seem to me, there's a little bit of ambiguity here about what's really going on.

DR. NICHOLS: Well, let's say that there are always other systems you can look at, but up to now with that sensitivity we haven't seen anything that has remained after separation techniques.

We were talking about the possibility with Dr. Doerfler of having a ligase in the process being able to hook up things that make it look like integration that isn't really integration, and he has told us that there are very rare events, you know.

There are just two different observations, and it's hard to explain what --

DR. FRIED: But one is cellular DNA, and Walter, yours was just adenovirus. It wasn't -- I mean, Walter didn't do the comparable experiment.

DR. DOERFLER: Well, if I just can comment on that, the DNAs we have been using were mostly DFH M13 DNA double stranded form, either linear or circular. Most of the experiments were done with ECO 1 linearized DNA.

I don't think there is so much of a discrepancy between these observations, because we looked for -- In most experiments, we looked
relatively, you know, early after application. So the recloning was done over 18 hours after feeding, from the last feeding, from the spleen. So we can see it in the nucleus and perhaps chromosomally associated or even linked to cellular DNA.

This was a recloning experiment and, I think, for the definite this is M13 DNA. Whether or not it's linked to mouse DNA, well, it was suggested it could be. But, of course, there are other possibilities, how we might find such a linkage. That was the latest we could do. That was 18 hours after the last feeding. If we do it later, we have great difficulties finding it. So we considered that a rare event that only -- persistence only for a certain time, although experiments we didn't discuss in feeding pregnant animals, there may be a longer persistence in the offspring.

DR. MINOR: It wasn't the question of persistence so much as the question of actually any integration at all, you see. I think what Warren was implying to me was that there is no integration of the plasmid DNA that he injects into the mice; whereas, as I understood it from your feeding experiments, there was evidence of such expression.

DR. DOERFLER: Well, you know, we have two
lines of evidence. One is the recloning, and the
second is data I didn't show, chromosomal association
in embryonic cells after feeding pregnant animals in
rare cases. So one really has to look hard for it.

DR. FRIED: How long after did you look?

DR. DOERFLER: Well, in the directly fed
animals, it was -- The animals had received a dose of
50 micrograms day before one week, and then 18 hours
after the last feeding the animal was sacrificed,
spleen extubated, and the DNA was cut and cloned.
For the offspring of pregnant animals, we
looked in fetuses and in embryos.

DR. MINOR: I've got a related sort of
technical question, too, I think. As Warren said, I
mean, it depends very much on what system you're
looking at. I wondered, most of the carcinogenicities
or assays that we've been hearing about today are
really being chemical carcinogenicity type approaches.
I wondered how appropriate those would be
to something like a DNA where you're talking about a
particular gene going in. I mean, to what extent is
this system actually appropriate for what you're
looking for, if you're looking for DNA oncogenicity.

DR. FRIED: Well, that gets to the next
sort of topic. How do we test for oncogenicity.
Maybe Jim will want to give his talk now.

AUDIENCE PARTICIPANT: Can I ask a question about one of the earlier comments? Did you use any transgenic mice in that DNA study, because it's for the very reason that was just mentioned. If an activated oncogene was involved, it wouldn't cause, you know, global changes. It might cause changes that would just be measured in an animal that only needed a promoter or only needed a knocking out of -- that was heterozygous and only needed a knocking out of one copy of RB or something like that.

The other comment: I also see a paradox here in saying that there's no real risk of this DNA being taken up. When you consider that almost everyone of these vaccines is injected right into the tissue, which is the preferred site for DNA gene therapy, which is the preferred site for DNA vaccines, which is the best way to get expression of extracellular just naked DNA -- the best place you could go is right into those same muscles.

I'm a little surprised. I'm actually very surprised to hear that you think that just injecting DNA there won't be taken up. In fact, the fact is DNA at that site will be taken up. That's why Dr.
Doerfler's results are what they are, and they will be expressed, and they will persist for quite a long time, surprisingly long.

I don't know about you. I was very surprised to see how long DNA injected directly into a muscle will be expressed there. Whether that oncogene being expressed at that site will cause cancer in a mouse, whether it even could cause cancer in a mouse, is maybe the next question. But I think you couldn't do much more to get the DNA expressed than to inject it into a muscle the way it's being done.

I mean you could. You could damage the muscle. You could inject lidocaine and various other things, but it would be hard to maintain that -- I'd like to interpret your result, which is that if you don't add enough DNA, you don't get it; and if you add too much, kill the cells that would have responded.

AUDIENCE PARTICIPANT: Let me just say there's a big difference between being taken up and being integrated. The DNA is certainly taken up by the cells, and it is expressed by the cells.

What we're saying is there's no evidence that it's integrated into the genome of the recipient cells that can be determined in our studies up to this
DR. FRIED: So it's taken up, but it's not necessarily integrated. Is that -- So there's no long term -- The long term persistence is extrachromosomally?

AUDIENCE PARTICIPANT: Yes, that's correct. At least with the sensitivity that we can get of the .005 to .01 femtograms, it all is taken away by the purification procedures that we do, and nothing remains.

AUDIENCE PARTICIPANT: And also somewhat related to this, I heard you say that you hope the DNA is reduced to its smallest possible size. I believe it's true, the statement that not a single vaccine is treated with DNAase and that any considerations of reducing DNA to its smallest size have simply been missed by people who make vaccines.

DR. RUUD: Rupert Rudd from Solfi. We are using DNAs or our vaccine, and in general here I want to state that we are now looking at cells, viruses that are being expressed and can be transferred into vaccines.

I think it's also fair to say what you define as vaccine -- I mean, if your final product is like a monoclonal antibody, we have no problems here.
If you have a purified inactive phage, it's a purer product which is also a protein, but it is from virus that has been grown on certain cells. Then you may have a problem.

I think this disparity should be broadened and more defined in what we call vaccines and what kind of purpose it is, and list a series of the amounts that are necessary for that particular line.

DR. FRIED: Walter?

DR. DOERFLER: Just one other comment to the ongoing discussion on persistence and in what form. Now when we look by sectioning different organs and fish after feeding DNA, then we invariably see the DNA inside the nucleus. That doesn't mean it's integrated.

Now the recloning and also chromosomal association would indicate to me that perhaps very, very rarely it can be integrated. So the limit Warren has given us are perfectly agreeable with me, because it's a very, very rare event, if it occurs at all. But it's frequently in the nucleus.

So there's no question about its uptake and, once it's in the nucleus, of course, one doesn't know what might happen.
In the embryo we've also seen it in the nucleus, usually in more than one cell, cell type justice, which might indicate that the cells still divide after receiving some of the DNA, and then this pattern emerges after what we consider transplacental uptake.

DR. FRIED: Do you think you have to have cells dividing for the DNA to become integrated and, when they're injected into certain tissues, that the nondivided cells that DNA just persists and then goes away?

DR. DOERFLER: Possibly.

AUDIENCE PARTICIPANT: I want to follow up. Jim McDougall presented some data that I was not aware of, and that is that fairly small pieces of the herpes virus genome seemed to be associated with this hit and run mechanism.

I didn't count all the base pairs in those stem loop structures he had. It looked like to me somewhere between maybe 25 and 75, but I think the sequences you had that was about 500 base pairs, and I don't believe you looked at any smaller than that.

The question is whether you could chop away at those things and still show that that stem loop was associated with that type of event. But I
think the question that really comes to my mind is to
whether those stem loop type structures are present in
mammalian cell DNA, and if they are associated with
hit and run transmission of herpes viruses and, if
similar stem loop structures are present in mammalian
DNA, is that something that we would have to worry
about from a regulatory perspective?

DR. McDougall: Well, I think it's not
difficult to find stem loop structures in almost any
source that you go and look at. Their presence is
certainly there.

Did we cut them down any further? No.
The size that I showed was the -- That was the
structure that we could see in a piece of DNA that
actually was bigger than that, but that was the
consistent structure that was always present and was
capable of transforming cells. But again, you know,
I continually come back to what I know for sure.

That is, you have to use large amounts of
dNA to get this sort of transfection to work, and I
cannot believe that in any vaccine that's produced
there would ever be residual DNA at that sort of
level.

Now it's true that, you know, what you may
be looking at is a single event, and so if you
vaccinate 10 million people, you're likely to find one somewhere out on the end of the bell curve that gets a reaction to that. (But that's a price that you may have to pay in this situation.

The other -- While I'm on, the other thing that I do feel is that a decision whether or not to go ahead with this sort of -- this type of cell line that's been immortalized or transformed by a DNA virus, by papilloma or SV40 or adenovirus or whatever -- I still think that that sort of decision should only be made on the question of what residual DNA might not be in the vaccine, and not on the basis of whether a cell is partially moving toward neoplasia or completely moving toward it; because I think the truth is that, once you make a cell genetically unstable, it is going to accumulate more changes, whatever you do.

So the one thing I keep coming back to is how much DNA is going to be in one dose of a vaccine, and I cannot see that that will ever carry enough material to actually be damaging.

DR. HOEKENOF: I'm Ray Hoekenof of Intergene in Holland. I collaborate with Alex von Raab who is in the forum.

I'd like to add one thing to the discussion whether there is good cells or bad cells.
I think there was a comment earlier this afternoon that there is no evidence of any correlation between the neoplastic potential of a cell and its potential to transfer that neoplastic potential to the vaccine. I think there is one example that was not mentioned yet of the potential of transfer of such potential, which is the mechanism of homologous recombination.

I think the 293 cells which are generally used for gene therapy vectors but have also been mentioned for production of vaccines provide a clear risk that you can generate or can transfer elements from the cell line to the vaccine simply by homologous recombination. In 293 cells the 5 prime ITRS is present. The encapsulation signal is present.

So, for instance, if you would produce adenoviral batches on that cell line, you can very quickly generate recombinant adenoviruses that have taken up parts of the 293 cell line by homologous recombination, a phenomenon known as RCA.

I think there will be a paper presented later this week by Fritz Fallaux from van der Eb's lab to present cell lines that eliminate that risk, and I think homologous recombination between a neoplastic cell line and a vaccine is something that should be
kept in mind as a risk factor for transfer of
neoplastic characteristics.

DR. FRIED: So you would think all cell
lines that had transformed by viruses -- I mean, but
293 doesn't have -- It only has a couple of genes
there. Is that --

DR. HOEKENOF: That is correct, but --

DR. FRIED: So you think when they come in
with the ElA minus vectors, they're going to recombine
with the endogenous genes?

DR. HOEKENOF: Oh, in 293 that is not
something that you expect, but it is a fact. You very
quickly start to generate revertant adenoviruses which
result from homologous recombination between the cell
line and the vector.

So the point I'm trying to make is, if you
want to produce a vaccine on a cell line, make sure
that there is no homology present between your DNA on
the vector and the cell line, and maybe van der Eb can
comment on that.

DR. VAN DER EB: I agree with that.

There's little I have to add.

DR. RUSSO: Carlo Russo from Merck. Are
you referring to recombination with viral genes, not
with cellular genes?
DR. HOEKENOF: Well, in this case I'm referring to recombination with viral genes, of course, but any homologous recombination can lead to uptake of cellular DNA into your vector. So I think you should avoid that.

DR. FRIED: But again let's not drift away. We're asking in neoplastic cells, is it any different than, say, diploid cells, and are these phenomena that are specific to neoplastic as opposed to diploid cells?

DR. RUSSO: I think what we need to clarify is this concept of malignancy, because it seems to the FDA, at least to a couple of their representatives here have this concept that there are different degrees of malignancy, and perhaps the cell substrate should be identified, high malignancy, low malignancy, medium malignancy.

I think we should have a consensus here whether this is considered to be true or independently, as somebody suggested, the only concern should be on the final. What is the residual DNA that we're going to have in our vaccine, independently from which cell line the DNA came.

DR. FRIED: Do people on the panel have anything to say on what's malignant, how we would
DR. COOK: Well, we've covered this in bits already, it seems like, in three parts. One is: How do you measure this? And we've already talked about quantitating the malignancy of the neoplastic potential or the tumorigenicity of the cell.

The second is, once you have one cell line of a given kind of tumorigenic potential, what's the likelihood that something from that cell line could be transferred through residual DNA in such a way that it would be expressed functionally to lead to that in a recipient of some sort?

I think those are probably quite different questions. You can measure the first, once you define the parameters, and we can talk more about that if you want to at some point. But the second issue, I think, requires experiments like were referred to that are published in literature that I don't know about where you put in large amounts of tumor cell DNA into immunodeficient recipients, and you find no evidence of pathology, whether it's tumor development or some other kind of illness in those animals.

If you can't prove that transfer of that information, even though it's all sheared and chopped up, is going to do anything to the recipient host,
it's kind of hard to translate the information from
the virulence of the cell, if you will, to the risk of
that cell being used to convey something, if there's
no evidence that it's conveyed and if it's unlikely
that you're going to get the right combination of
genes put together after the sheared DNA is carried
over into the recipient.

I mean, that's where the question is. How
do you test that? You can test the first, but does it
have any relevance for the second; that is, the
transfer phenomenon, and what happens in the recipient
-- human vaccine recipient?

DR. FRIED: Is it worth testing it in the
transgenic models? I mean, which would be the best
that people who presented on the transgenes?

AUDIENCE PARTICIPANT: Well, I think the
question that we sort of have to get after is whether
it's worth pursuing and how far or how hard we should
pursue the need to ask the second question.

We can define the first and, in fact, as
you know, the issue of whether cells that have very
low TPD50 values on the order of one to a few hundred
cells compared to cells that have very high TPD50
values of, say, in the order of 105 or 106 -- the
question is whether we should be more worried and
pursue with some -- oh, I can't think of the word I want to use right now, but with some eagerness to look at that question with cells that have that.

Now in order to make that decision, the first thing you have to have is data that says there is a cell that this particular substrate of this cell that's being proposed for this particular substrate falls into that category of cells that are highly neoplastic or highly tumorigenic, if you will.

Neoplastic in this situation, I think, is not quite the right word, because you can't -- I don't know how you measure neoplastic. You can measure tumorigenicity, but the other thing is a more nebulous term when it's a tissue culture cell.

So if you have a situation in which you have a very aggressive or a highly tumorigenic cell line, should we in fact be concerned about pursuing whether the components of that cell represent more of a risk, and then we should in fact worry about how to measure whether those risks are transferable or not, I think, is the heart of our question.

We can measure the one, but we're not quite sure we know how to measure the other, and when you look at the models that are available for that, there aren't very many of them, especially the tissue
Now I don't know, with regard to looking at DNA from cells that are highly aggressive or highly tumorigenic versus cells that are weakly tumorigenic, whether those types of studies have actually been done. Dr. Nichols is talking about a lot of data. Unfortunately, I'm not familiar with that data. So I don't really know whether he's looked at cells that have very low TPD50 values, comparing it with cells that have very high TPD50 values in his models or not. But I think that's one of the things that we could think of doing, if we need to get at that kind of data. So I think the question to the panel is whether we in fact need to get at that kind of data, and if so, you know, is there any collective wisdom among you all that says how we should go about doing it?

DR. SISTARE: Two questions, I think. One is, if we're concerned that the cell is going to be surviving some processing and the cell is going to remain intact in the vaccine, then we got to approach it a certain way. There, the question of this neoplastic potential in the cell is a very real one.
If, on the other hand, our concern is not the cell -- we don't think the cell is going to survive the processing of the vaccine, and it's not going to be carried over, and what we're concerned about is the DNA itself, that's a different question, and I don't know that it's been addressed yet.

It strikes me that some of the experiments that Dr. Doerfler referred to and Dr. Onions referred to were newborn hamsters, I believe it was, and newborn young animals. They were injected or gavaged or whatever.

I don't know how the stuff was given, but they were given DNA in some form, and these animals came down with tumors, if I understood your statement correctly. It wasn't a primary part of your presentation, but you referred to it, some old data.

In a sense, the newborn mouse model, the neonatal mouse model that we use for testing pharmaceuticals is that same model. It's a single hit or double hit kind of a model with a long latency, a lifetime -- you know, just a one exposure, wait a lifetime and then see if a tumor develops. That might be the kind of model to ask the kind of question, neoplastic DNA versus primary cell DNA.

Now another thing that Dr. McDougall
brought up -- and it's another reason why this is a
very interesting experiment -- If you remember
correctly, he indicated that these small pieces of
palindromic DNA seemed to be doing the work, seemed to
be causing the problems, and it was the more mature
cell line the tumor actually lost these pieces of DNA.

So one might guess that the early phase of
the tumor might be the more pathogenic, the DNA from
that, as opposed to the later, the more mature, more
"tumorigenic" DNA.

So you don't know how the experiment is
going to turn out. If this palindromic DNA is really
the important stuff, it's going to go in and not
express -- if expression is not the most important
thing, but disruption of normal expression of the cell
is the key thing, then these are all kinds of
questions that could be addressed, and there are
models that we could do this with.

I don't know if the data is out there
already for these kinds of things. I don't know. An
we keep talking about efficiency of transfer and
looking for expression. That's not what we're trying
to do here, I don't think, is ask the question about
necessarily whether oncogenes are going to be
transferred.
I think the question we have to ask for is the rare event. If there is transfer, and we know it's going to be very efficient, when that transfer occurs is it going to integrate into a part of the cellular genome that's critical to the function of that cell? And if it's only one cell, and it gives it some selective advantage, then it's a concern. So it can be a rare event that we have to concern ourselves with.

DR. FRIED: Any other comments?

AUDIENCE PARTICIPANT: Given what you all know as experts on neoplastic cells, suppose you have a cell which is neoplastic for which you don't know the mechanism by which it became immortal. What is the relative pretest probability as compared with other types of cells that these cells will contain an abortitious agent, either infectious or latent, known or unknown?

We'd sort of just be interested in, in the sense of the panel, whether it is reasonable or unreasonable to be more worried about the possibility of abortitious agents being in these kinds of neoplastic cells.

DR. FRIED: I guess, if you passage things more, they may be there, and it depends on what
species we're looking at. I mean, I think Harry Rubin said this morning something about if they came from chicken, it probably wouldn't infect humans. Then I think John Coffin said maybe it would.

So I don't know. I mean, I guess agents -

- I mean, there's just the normal tests looking for viruses and nuclease resistant DNA in particles and PCRing up, as mentioned in the document. I don't know if the panel has any other ideas.

AUDIENCE PARTICIPANT: I was actually going to address a different issue which was mentioned earlier and has been mentioned a couple of times so far, which has to do with the damage due to integration of DNA in disruption or activation of genes.

In the very best models that we have for that, which is infection of chickens or mice with certain retroviruses, where very reproducibly every single infected animal will come down with a tumor, if you actually look in cases where the tumor is being caused by insertional activation and not expression of preexisting oncogenes, if you actually count the numbers of cells that are infected as compared to the ones that actually become transformed in this way, the ratio is probably a million to 10 million to one or
something like that.

These events are extremely rare at the cellular level. The introduction of foreign DNA has a very, very low probability of doing any harm to a cell in terms of induction of malignancy. So we have to keep that in mind. We can't think that introduction of DNA is per se a very damaging event to a cell.

You have to do it a lot of times. You got to do it a lot of times in an infected animal in order to get a tumor to appear.

DR. FRIED: You think it's preselecting a cell already is halfway there?

AUDIENCE PARTICIPANT: Well, there might be some of that. There's certainly a multi-step event to it, and it's certainly true that only certain cells in the body are primed for this.

It may not be because they're abnormal. It may just have to do with something about the differentiation, say, of that particular cell, but you can infect many cells with avian leukosis virus, for example, in a young chick, but you will virtually always get a B cell tumor that arises in the bird. So presumably, because there are some particular features about those cells that prime them, not necessarily
mutagenically, but in a differentiation specific way for the kinds of events that can then lead to oncogenesis.

DR. FRIED: The same with the P53 mice. I mean, they all get tumors by 200 days, but they don't get tumors on day one or day ten. Other events have to happen, and there's also a story of BRCA-1. There's a woman in Scotland in a little village where everybody knows everybody, and she was actually a knockout. She had had both BRCA-1 genes knocked out, and she didn't get a breast tumor until 39. So again, other events have to happen.

AUDIENCE PARTICIPANT: The rarity of these, I think, is a very important thing to keep in mind.

DR. TEVETHIA: I'd like to get back to the issue of the degree of tumorigenicity. I think it's a very important point for the cell line you're going to consider making in vaccines. Seems to me that we seem to be using this word rather more loosely, and I think the question really becomes that, if you look at any of the DNA virus transformed cells, they are transformed, but they don't metastasize. Yet you express a gene in transgenic mice
as metastasizing. So, obviously, we're looking at a
lot of artificial situations when you transplant a
cell line into a tumor. The cell may grow rapidly.
It may look like a tumor.

One really has to define actually what the
tumor looks like, at the blood supply, how soon the
blood supply is established, and other factors that
are produced.

So I think just because the cell line 107
cells end up producing a nodule doesn't necessarily
mean that is a very high cell line to make vaccine in.

DR. FRIED: Okay. Is there anything more?

DR. McDOUGALL: I'd just like to make one
observation on that observation. That is that human
papilloma viruses actually do create a tumor that's
capable of metastasizing, and this is an event that's
just initiated by a DNA virus infection.

So it's not -- Although in vitro and the
sort of lab experiments we do it's true that these
cells don't metastasize, in vivo they certainly do.

AUDIENCE PARTICIPANT: Yes. I have some
unpublished data on the ability of a lot of these
cells metastasize, and it's, in fact, quite striking.

If you look at SV40 transformed hamster
cells, it's the size of the tumor that depends on
whether the cells metastasize or not. They won't --

You can't begin to see lung metastasis in animals with
SV40 transformed cell tumors, hamster cells, until the
tumor is probably 30-40 millimeters in diameter. Then
the lungs are studded with metastasis.

We found the same thing for BHK 21 cells.
Now the interesting thing is that adeno-12 transformed
cells, which have the reputation of being highly
tumorigenic -- we've never seen a lung metastasis in
animals, even though the tumor is half the size of the
animal.

So there are differences there. We've not
looked -- made the same observations in mouse models,
but the idea that DNA virus transformed cells,
especially SV40 and some of these types of viruses,
won't produce metastasis, I think, is based on the
fact that you really haven't looked very hard, and you
have to wait until the animal gets around to
development of lesions. They're probably microscopic,
and you can't see them.

DR. COOK: I would just agree. I think
that the way these experiments are done is usually to
inject a large number of cells that causes tumors in
a fairly short period of time, and it creates an
environment, like Tev was saying, where you develop
localized tumors that don't look like the metastasize.

If you put in smaller numbers of cells and wait a very long period of time, like Dr. Lewis' experiments, we've seen the same thing in the BHK model in newborn nude rats inoculated with small numbers of adeno-5 E1A expressing cells that I wouldn't have intuitively thought would make anything once they sat around in this nude mouse to the time that they were an adult. But they can have lungs full of metastases if you let them sit for a very long time, and the cells are put in under the cover of immunodeficiency.

So again, this relative question about how tumorigenic a cell line is is interesting, but whether it has anything to do with the risk of that cell, I think, is another question.

DR. FRIED: Yes?

AUDIENCE PARTICIPANT: If I may suggest, you still haven't addressed that question. That's what we want you to address.

DR. COOK: Well, I want to go back to your comment, and that is, if we wanted to do number 2, which is to ask about the transfer of DNA and how could that be tested, because we're all talking about religion here -- I mean, there should be an
It seems like to put together your question with Mike's suggestion earlier, has anybody done a simple experiment like take DNA, like polyoma DNA that's infectious, run it through a vaccine production schedule of some sort, and find out whether that DNA which is -- I mean, you need a positive control for these experiments. We could all create a lot of experiments that would result in no tumors and say that the residual DNA in this vaccine prep didn't do anything bad. What about some DNA that we know can cause neoplasia, run it through a vaccine preparation, put it into an animal that's known to be sensitive? If those animals don't get tumors when inoculated with spiked preparations that have been processed, then I think the idea of working backwards from a positive control to what's likely to be a negative experiment might be more logical.

AUDIENCE PARTICIPANT: Well, I think that the question -- One of the questions that you're asking is, in fact, something that we thought about a little bit. That is the possibility of using -- to get around this whole issue of the problem of DNA is to use the same concept that was applied to the
business of viral clearance.

That is the concept of DNA clearance. In other words, you would spike DNA with DNA containing defined or known viral oncogenes, if you will, and then show that the manufacturing process actually clears that DNA to a certain -- with a certain efficiency, and then using those numbers, you can then make a prediction as to what else would be removed during the same procedure.

So that's one way that you can think about possibly getting around this business of testing. The other thing that comes to mind when you're thinking about risks associated with elements that are components of vaccines is the fact, if you -- and we're talking about tumorigenesis -- but the fact, if you worry about what is in the tumor cell and the possibility that the tumor cell might contain an adventitious agent or, in face, is induced by an adventitious agent -- and I think one of your examples, that's a ras sarcoma cell -- you infect ras sarcoma cells in chickens, and the chickens get ras sarcomas, but they also get ras sarcoma virus.

So the fact that a cell line makes a tumor could be obscuring -- The tumor itself could be caused by some sort of oncogenic agents, especially if you're
dealing with an unknown cell line.

The tumor could be obscuring the presence of an oncogenic agent. The oncogenic agent could be there whether you get a tumor or not, simply because the animal model you're using is not sensitive to that.

So if you wanted to go out and begin to poke at the issue of how to assess the transfer of a neoplastic event or some type of infectious event, then you need to compare the tumorigenicity of the cell line with the possible tumorigenicity of the X rac and things like that.

So there are models that -- things that you need to do, but I think the question is how can you do those quantitatively and at what point in time do you need to begin to define where you go in that direction; because this is not a small amount of work.

DR. FRIED: Okay, thank you very much. I want to thank everybody here. I think we've overrun our break. I feel I should summarize, but maybe we'll leave it to the last session.

I think we're much more amenable that DNA is not as big a hazard as we might have thought in the beginning.

(Whereupon, the foregoing matter went off
DR. ROSENBERG: If everyone could take their seats, we would like to get started. We're going to be shifting gears only slightly, I think, in this afternoon's session, and then continuing in the same vein tomorrow morning to a discussion that will highlight for you issues that relate to retroviruses and ways in which retroviruses are able to alter particularly the growth of cells and ways in which retroviruses can change during their replication in cells.

I want to very briefly introduce this by showing a couple of slides, and these will just highlight for you -- I think if we could have the lights down, please -- some of the aspects that will be coming up in the talks this afternoon and this morning.

Certainly, you're all aware that retroviruses, unlike some of the agents that have been discussed already, have as a natural part of their life cycle the ability or an obligate need to integrate into the DNA of the host. As a consequence of this, we appreciate that there are a number of things that can occur.
These are illustrated here. Here we see an insertion and an activation of an oncogene, leading eventually to the development of a mammary tumor in this mouse.

Other things can happen as well. While we may be focusing on these events, I think it's important to remember, these are not the only consequences. Two other inactivations, one an activation rather seen here, results in the hairless phenotype in this mouse, and an insertion here results in up regulation of amylase, which has been suggested at least by some, including the people who made this figure, perhaps to explain our great love for starches, including some that were just consumed in the hall.

So there's a diverse set of things that can happen when retroviruses integrate. To illustrate this in perhaps a more mechanistic fashion, because of the cleverness, or at least to me the cleverness of these agents, a number of ways that the integrated virus, shown here, can impact on genes nearby, it's illustrated.

Here we have a circumstance where we have expression of the neighboring gene under the influence of promoters here.
Here we have a circumstance where strong enhancers contained within viral control elements are again affecting the expression of a neighboring gene, and we certainly appreciate, although the slide illustrates that this gene looks very close, that this can occur over reasonably long distance.

Here we have another circumstance where viral control elements identical to these but located at a different position in the viral genome again are affecting expression of the gene.

So these insertions don't have to be completely specific with respect to cellular genes in order to impact upon them. In addition, in circumstances where replication occurs, not only can there be effects on gene expression. Here we have the integrated provirus, just as in the earlier slide, and a cellular gene.

In this case we have transcription, as I showed before, mediated from the virus into the cellular gene, creating a hybrid viral cellular transcript, and subsequent, although all the steps are not diagramed here, packaging of this transcript into a virion, subsequent replication, and in this case incorporation of part of the cellular sequence into the virus, then having the capacity to be transmitted
to cells subsequently if this structure is contained
within a cell with a replicating virus.

Clearly, this is a very low frequency event, but not one that occurs at such a low frequency that we haven't been presented with enough examples of these types of viruses to employ many retrovirologists over very long periods of time.

Finally, another event that we will hear about today involves the ability of retroviruses to recombine, and this slide illustrates recombinations that go on in the generation of a particular set of pathogenic murine leukemia viruses. However, this slide could apply to other situations with other types of retroviruses as well.

Here we have acquisition of sequences in this region from endogenous viruses, and -- hopefully the colors show up, although I can't see them from here -- changes also affecting the LTRs of this virus. As a consequence of these, the virus tropism changes, and confers to this virus a highly tumorigenic phenotype. Other kinds of recombinations can occur as well and, again, alter both host range and tissue tropism.

This is an extremely important way in which viruses can infect new hosts or new cells, again
leading to diverse consequences.

So I think, as we discuss all of these and present model systems in which these are well characterize, we'll hopefully set the stage for the discussion that will follow these retroviral sessions.

With nothing further, I'd like to turn this over to Sandy Ruscetti, who will present the first talk.

DR. RUSCETTI: I'm going to move down here, because I understand you can't see your slides otherwise.

Thank you, Naomi. As Naomi mentioned, when one uses neoplastic cells as substrates for vaccine development, one can inadvertently get viral-viral or viral-cellular interactions that could have unknown biological consequences.

So the organizers here asked me today to talk about a model system in the mouse, and that's the generation of MCF retroviruses as a model for understanding how viral-viral and viral-cellular interactions can result in the generation of new viruses that can have pathological consequences.

Now mouse cells are just loaded with endogenous retroviruses of various subtypes. Some strains have ecotropic MuLVs in small copy numbers
and, when these are expressed, they are involved in
the development of leukemia in these mice.

The AKR mouse is an example of such a
mouse, but all strains of mice contain many copies of
xenotropic, polytropic and modified polytropic MuLVs.

Now these viruses really don't cause any problems to
the mouse or the mouse cells, but when they are
expressed with ecotropic virus or if ecotropic virus
is injected into the mice, recombination can occur
with these endogenous sequences, particularly the
polytropic sequences, and this can result in the
development of a pathogenic virus.

These viruses are called MCF viruses, and
what I'm going to do today is to give you a background
about MCF viruses in the mice, how they're generated
and what their characteristics are, and give you a
little food for thought about whether we should worry
about these in using mouse cells as substrates for
vaccine development.

Now these are just some properties about
MCF viruses. MCF viruses are recombinant viruses
between an ecotropic virus and endogenous sequences
present in mouse DNA.

These viruses contain ons genes. So they
are derived from polytropic or modified polytropic
MuLV sequences, and they contain oftentimes LTR sequences that are also derived from the endogenous sequences in the mouse or certainly modified so that they can be expressed at high levels in certain tissues.

Now one of the properties of MCF viruses that gave it its name is that they can induce unique changes, and they were called foci, but not transformed foci, in mink lung fibroblasts. But other studies have shown that not all MCF viruses have this property.

One of the interesting things about MCF viruses, and another reason why we're interested in them today, is that compared with ecotropic MuLVs, these viruses have an extended host range which allows them to replicate both in mouse cells and non-mouse cells. This is because MCF viruses use a different receptor than ecotropic viruses to enter cells.

Now when one looks at the host range of MCF viruses compared to other classes of murine leukemia viruses, one can see that it has a host range that's a combination of them. Ecotropic MuLVs are mouse-tropic. They can only infect mouse cells and not non-mouse cells.

Xenotropic MuLVs cannot infect mouse
cells, but they are very efficient in infecting non-mouse cells, including human cells. Polytropic or MCF viruses can infect both mouse and non-mouse cells. Now it's still unclear how infectious MCF viruses are for human cells, and I thought it was worth bringing that up at this conference. Working with the prototype MCF virus, AKR MCF 247, some studies have shown that certain human cell lines can be efficiently infected with this virus. Other studies show that they can't, and I think, importantly, four different primary human cell cultures could not be infected with AKR MCF virus. So it's unclear just how infectious MCF viruses are for human cells, and they are certainly less infectious than the xenotropic or even amphatropic MuLVs.

The prototype model for the generation of MCF viruses is the AKR mouse. Now these mice develop thymic lymphomas at a high frequency when they are about six to 12 months of age.

It was shown early on that these mice contain in their genome an endogenous ecotropic MuLV. It's called AKV or several copies of AKV, from which infectious ecotropic replicating MuLV is produced at birth. Now it's always been a mystery why these
animals who produce lots of this virus do not get leukemia for six to 12 months, especially since early studies indicated that AKV was certainly important for the generation of this leukemia; because you could take AKV and express it in a low leukemic strain, and they would become a high leukemic strain.

Well, in 1977 Jan Hartley discovered a new virus from these tissues of these diseased mice that provided an answer. What she and her colleagues were able to do were isolate this new class of MCF viruses from the pre-leukemic and the leukemic thymuses.

Further studies showed that the MCF viruses were recombinants between the AKV and endogenous MuLV sequences, and replication of both the AKV ecotropic and the MCF viruses in these thymuses were what were responsible for the development of the leukemia that occurred when the animals were six to 12 months of age.

Now how are these MCF viruses generated?

Studies have shown that there are three endogenous MuLVs that are involved in the generation of AKR MCF viruses: AKV, which is the ecotropic virus I just mentioned; VXV1, which is an endogenous xenotropic MuLV; and various copies of polytropic and modified polytropic MuLV sequences.
The generation of MCF virus in the AKR mouse is thought to involve several different steps. First of all, the AKV is thought to recombine with the om genes of one of these endogenous polypotent MuLVs, generating a new virus which looks basically like AKV, but it has new om gene sequences that are derived from the polypotent virus.

Then the second event occurs. This recombinant recombines with the endogenous xenotropic virus to generate a new LTR sequence. Now it's not clear which of these events occur first, but both of them apparently need to occur. Finally, alterations appear to occur in the LTR sequences that the viruses acquired from the xenotropic MuLV, which often involve the duplication of the enhancer sequence, obviously making this now a pathogenic virus in the AKR mouse.

Now the AKR mouse is not the only mouse in which MCF viruses are generated. You can generate MCF viruses in low leukemic strains of mice if you inject them with ecotropic MuLV. Two good examples of that are Frend and Moloney MuLV induced leukemia.

If you take newborn mice and inject them with Frend MuLV -- this is an ecotropic MuLV -- you get the generation of MCF viruses, and these animals
develop erythroleukemia several weeks later.

If you take Moloney MuLV and inject those into newborn mice, you also get the generation of MCF viruses, but these animals now develop thymic lymphoma. A lot of studies have been done with these two systems.

It's a little bit easier to do studies with the input virus, because one can inject that into the mice when it's not just relying on AKV sequences in the AKR mouse, and it was shown -- several things that I think are important.

The type of disease induced is determined by the input LTR. So if you have a Frend MuLV LTR, you'll get erythroleukemia. If you have a Moloney, you'll get thymic lymphoma. This was determined by making chimeras between the two viruses.

It's thought that perhaps the MuLV -- Frend MuLV LTR may replicate better in erythroid target cells, and the Moloney in T-cells, and that may be why you get that particular type of tissue tropism. But also, too -- Actually, Leonard Evans who is here in the audience -- he showed that Frend and Moloney ecotropic viruses actually recombine with different endogenous polytropic om gene sequences to generate MCF viruses.
So the particular sequences that they recombine with may determine the type of disease that's induced.

Another thing: Adult mice are resistant to leukemia development by injecting these viruses. You need to inject newborn mice, and it's thought that the reason you need to do this is because adult mice may either have fewer target cells for the virus or may have an efficient immune response that allows you to clear either the input virus or the generated MCF virus.

We showed a number of years ago with Frend MCF that you could treat adult mice to increase the target cells or decrease the immune response, and they would be susceptible as adults.

Finally, there are strains of mice that are resistant to the development of leukemia induced by these MuLVs, even if you inject them into newborn mice, and these strains of mice do not replicate MCF viruses; because they contain genes that suggest they are MCF genes which prevent the replication of the MCF viruses.

This makes the case even stronger that efficient generation and replication of MCF viruses in these mice are responsible and are necessary for the
development of the leukemias that follow.

Now the generation of MCF viruses after injection of Frend or Moloney is a little less complicated than in AKR mice. Just showing this same slide with AKR, with this being the ecotropic MuLVs.

The ecotropic MuLV does recombine with endogenous polytropic MuLV sequences to generate a new om gene. Again, particular polytropic sequences they recombine with are different, but usually the viruses do not need to acquire a new LTR.

The ecotropic Frend and Moloney LTR seems to work fairly well to allow this virus to replicate in the target cell. Now sometimes there have been observed some changes in the enhancer region of the LTR, and these may be to allow the particular MCF to replicate to high levels in the target tissue.

One point I wanted to make was that both in AKR mice and in MuLV infected mice, one may generate lots of different types of MCF viruses, and maybe only a certain subset of those MCF viruses are actually pathogenic, ones that have acquired all the right changes to cause disease.

Now these studies have all been done where MCF viruses are generated in situ in the mouth. Now what happens if you take the MCF virus from the
diseased tissue, purify it away from the ecotropic

virus, and inject it back into mice? Nothing really

happens.

That's because MCF viruses need to be

pseudotyped with ecotropic MuLV in order to cause
disease when you take them out and try to put them
back in. For example, AKR MCF virus will accelerate
leukemia in AKR mice, but it will not cause leukemia

in the low leukemic NIH mouse unless these mice

express an AKV ecotropic virus as a transgene.

Okay. Frend MCF virus will not cause

leukemia in mice unless it's pseudotyped with

ecotropic MuLV. Also I put this in here, because we

had showed a number of years ago that, if we infected

a packaging cell line inside two lines with Frend MCF

virus, we could get a virus preparation that would

cause leukemia, erythroleukemia in NIH Swiss mice.

So this suggested that the ecotropic MuLV

may not have to replicate in the mouse once it gets

the MCF there, and we actually showed that we did not

regenerate an infectious ecotropic MuLV from these

studies.

Now it's thought that pseudotyping may

prevent the inactivation of MCF viruses by maybe serum

factors complement or it may allow the virus to bypass
an effective immune response that might be against the
MCF virus.

So how to MCF viruses cause leukemia?

It's not completely clear, but there have been several
hypotheses, and it may be actually a combination of
several of these.

It's possible that the MCF viral envelope
proteins may interact with cytokine receptors on
hematopoietic cells and alter their growth and
differentiation. Also integration of MCF viral genome
into the host DNA may lead to activation of certain
proto oncogenes whose inappropriate expression in
hematopoietic cells may also lead to the alterations
in their growth and differentiation.

Also, since MCF viruses use a different
receptor than ecotropic viruses, and since both
viruses are present in the diseased animal, that would
allow two MuLVs to get into the same cell and increase
the changes of an activation event due to viral
integration.

So the data with studies of MCF viruses in
mice indicates that you can generate in mice
recombinant MCF viruses, and that these are really
crucial for the development of leukemia in these mice.

But the question that's more relevant for
this conference is can these viruses be generated in vitro, and if they can, is that a problem when using mouse cells as substrates for the development of vaccines?

Also, even if these could be generated, could they cause a biological effect if they could actually replicate in humans? I think this will be perhaps addressed by some of the other talks in this session, and I think certainly will be a topic of discussion when we have our panel tomorrow, late tomorrow morning.

So if you have any questions in general about MCF viruses and how they are generated and how they are used as a model for viral-viral interaction, I'll be happy to answer those for you.

(APPLAUSE.)

DR. EVANS: I'm Leonard Evans from Rocky Mountain Labs.

I wanted to clarify about the recombination with distinct sequences. Those studies were done where the viruses were initially selected on mink cells.

We found subsequently that the viruses -- many more viruses that could be found on mouse cells than on mink cells, largely because they were
serotyped. When we analyzed the larger population of
viruses, we found there was a significant overlap.
Frend does not specifically recombine with something
different than Moloney.

There are differences in the preponderance
of the viruses, but not in the identify of those
viruses.

The second point I wanted to make is that
we can inject Frend MCF into NFS mice, and they do
develop erythroleukemia.

DR. RUSCETTI: In the absence of eco?

DR. EVANS: Yes. Markedly cloned and
transfected.

DR. RUSCETTI: Well, I know one of the
questions that always arises: When you purify an MCF
virus out of a diseased tissue, are you purifying the
pathogenic one or not?

Possibly, some of the reasons why you
might not be able to induce disease with a given MCF
virus is because you haven't actually cloned the
pathogenic MCF virus. But I know our studies with our
MCF virus, we were never able by itself to, but I
agree, that doesn't mean that you always have to have
that. That's generally a problem, though, with MCF
viruses replicating once you take them out of the
animal.

DR. EVANS: Yes. It's also been shown that there's a tremendous range of infectivities for different heterologous cell lines like mink rhesus mouse. It can be vary over several orders of magnitude in terms of the ratio, the relative infectivity, and there's such a heterogeneity of polytropic viruses that to generalize that they do not infect human cells is probably a leap, you know. They don't readily affect them. Maybe someone --

DR. RUSCETTI: All right. Now I wasn't trying to say that.

DR. EVANS: No, no, I know.

DR. RUSCETTI: I just think that people might have an impression that they are very infectious for human cells, and that may not be the case.

DR. EVANS: Some of them might be.

DR. LINIAL: Maxine Linial, Hutchinson Cancer Center.

So there are a whole bunch of other endogenous retro elements in mouse cells like DL-30s which, at least in one defective virus, have been shown to be recombined, and there are mouse endogenous viruses.
So has anyone looked in replication competent MCFs, maybe not the ones that grow out but in the pool, to see if they have acquired other endogenous retroviral sequences?

DR. RUSCETTI: Not that I'm aware of. I don't know. Somebody else might be able to address that, because I'm really not working in that field.

DR. ONIONS: David Onions. Hi, Sandra.

I didn't quite understand your last point when you were suggesting that the -- the possibility that the pseudotyping with ecotropic envelope was necessary to evade the immune response or perhaps to avoid a complement factor.

I would assume, as in other pseudotyping situations, the envelopes are chimeric with both ecotropic and MCF type sequences. So wouldn't that make them susceptible to complement, and wouldn't it also make them susceptible to the immune response?

DR. RUSCETTI: Oh, no. It would just be the MCF genome that's packaged in an ecotropic coat would be able to get past those inactivating factors in order to infect the cells that it needed to infect to cause disease.

DR. ONIONS: So you think that pseudotyping is absolute, as in there's a complete
ecotropic coat there?

DR. RUSCETTI: Right. Well, as Pug mentioned, that may not be absolute, but certainly it appears to be the common event that occurs, that you do get pseudotyping, but it's actually -- It's a complete ecotropic coat. It's not a chimera between the eco and the MCF.

DR. ROSENBERG: Thank you. The next talk will be by Clive Patience on MLV packaging systems.

DR. PATIENCE: Okay. Firstly, I'd like to thank the organizers for inviting me to be here to present some data. If I could have the first slide and the lights down a bit, it would be great.

The work I'll be presenting was primarily performed in Robin Weiss's laboratory in cancer research in London. However, recently -- it does not account for my jetlag, which I don't have -- I've moved to BioTransplant, a xenotransplantation company in Boston.

Now when I was invited I made the assumption that I'd be talking to a diverse audience, ranging from those which know very little about retroviruses up to those which are astral heights of editors of retroviral textbooks that should remain nameless.
So what I'll try and do is give you a story which I hope everyone will be able to follow, and it's going to deal with the murine leukemia based virus packaging systems, gene therapy models, and basically estimation of potential recombination.

Okay. For the uninitiated, this is the basic system, which I shall try and get in focus for you somewhat. I think that's as good as we're going to do. Basically, there's three components.

You have the packaging cell line at the top here. The function of the packaging cell line is to create the virus. There's generally three components, the gag and pol expression plasmid which make the body of the virus, and the envelope which makes the outer covering of the virus which determines what sort of cells you will ultimately infect.

Into that virus, rather than packaging the normal virus genome, you put in your therapeutic vector. You make your virus, and that virus then is used to infect your target recipient cells. Where the RNAs converts to DNA, integrate it and your gene is expressed.

A little bit more detail on the actual structure of a packaging cell line: Basically, we use the immortalized cell line HT1080 for the particular
cell line I'm going to describe.

We compared this human cell line in comparison to a 3T3 based murine based system. It's called FLY. It's got nothing to do with little insects. It's actually the initials of the discoverers, or I should say the inventors.

Okay. Firstly, you put in your gag pol expression plasmid, which basically produced the core of the virus. You then cotransfect in your envelope plasmid. So you can then make a complete virus particle with all the machinery required for retroviral integration.

You then insert your vector of choice, and basically that can be a therapeutic vector or in the laboratory a reporter vector. The particular plasmids which I'm going to discuss here, you can see, is diverged somewhat away from its wild type parent.

It has a Moloney murine leukemic virus, 5 prime LTR, as well as Moloney gag and pol. It then has basically a gap through here after the stop code on drug selection. So basically you get a read-through here.

About five percent of ribosomes will read through this stop code and go on to transcribe your drug resistance gene. So if you select the drug
resistance, you should, and indeed you do, select for
very high expressors of the gag/pol proteins and,
therefore, high levels of virus.

    Top down then we have a polydentylation
signal which I believe was SB40, if I remember right.

The envelope comes out very similar, 5 prime
retroviral LTR, no packaging signal. This region here
determines whether the RNA construct will actually be
packaged into virus particles. The envelope is
expressed up here, and again SV40, and your reporter
vector -- the important part here is the presence of
a packaging signal. So it should be efficiently
packaged into virus particles.

    Okay. We were trying to get some sort of
feeling for the degree of recombination that could
occur, and our logic was that it would be greatly
increased if there was any generation of replication
competent retroviruses in the packaging system.

    Now the recombination could either come
from the -- The viruses -- sorry -- could either come
from a combination of DNA present in the packaging
cell lines, and you may have noticed that all of the
plasmid constructs have quite a number of regions
deleted or mutated. Very elaborate cell lines have
been produced now over many years.
The alternative is that the recombination events could actually occur during reverse transcription of the RNA in the virus particle. For that to occur you need expression of the unwanted RNAs in the packaging cell line, and that in turn will be enhanced. This is significant similarity between the vector and the endogenous sequences.

Some bad news to you people. About one percent of your genome is retroviral. Yes, we're derived from apes, but we're also derived from retroviruses. Basically, so about one percent of your genome is retroviral. It probably represents the ancient remains of past retrovirus infections where germ line cells have become infected. Over the millions of years, most of these copies of the various viruses, which range number from one up to thousand, have become deleted and mutated. So they can no longer encode for infectious virus or, in most cases, even proteins.

Of particular interest or those which have received particular attention are the HERV-H or RTVL-H family of viruses. There's approximately a thousand copies of these in our genome, about 200 full length, about 800 deleted versions, very highly transcribed in
general terms.

HERB-K, another family, has been the focus of a lot of attention, primarily because it has pretty well conserved open reading frames. It still has the ability to encode envelope proteins, gag proteins, pol proteins, and there's been a lot of effort, people trying to grow this guy and correlate it with disease, etcetera, with not too much success compared to the amount of effort which has been put in.

So this is the basic scenario we were looking at. We were looking at the packaging cell line, which was human. Normal situation is that you would like for your therapeutic transcript to be packaged into the virus. We were looking to try and determine whether the human endogenous retroviral transcripts could also be co-packaged and get into your virus particle.

So the first scenario or the first requirement is that you have HERV expression in your cell lines. Now we compared two cell lines, the old and inverted comers M-12 and murine 3T3 based system and the human based system which we've developed in the laboratory.

This is basically an RT PCR. For those of you who are not familiar with PCR, basically a bright
band means a positive. A lack of band is a negative.

Therapeutic -- so that what we were looking at was actually reported back to LacZ, and you can see there it's expressed in both the human and the murine cell line.

We performed a degenerate PCR approach, basically using PCR primers designed to conserve regions in the virus and try and pull out as much as we possibly could and identify as many different sequences as we could.

In the human cell line we managed to identify sequences which showed similarity to the B and B-type retroviruses as well as the RTDL-H family, which I have mentioned earlier.

In the murine cell line we again managed to pull out the D type sequences and also another retroviral sequences called BL-30 which is an endogenous retroelement of mice which has been shown to be packaged in murine cell lines, not by myself but by Damian Purcell and others maybe -- and others.

We basic scenario we then went to do was we had confirmed RNA expression in the cells. We then wanted to try and identify whether this got as far as the virus particles. And if it gets into particles, it may be available for recombination events.
So we took these from the various packaging cell lines and spun it through a sucrose density gradient to purify and concentrate the particles with respect to their density. What we could then do was analyze the various fractions where I've put up the enhanced RT assays or PERT assays to identify the fractions with the highest virus content, and then also examine exactly those fractions for the presence of unwanted retroviral transcripts.

This is a typical section across a gradient where you see banding of virus particles at a density appropriate for mature retroviruses.

Okay. If we look at the old system, to start off with, the RT assay here identifies a concentration of particles, at one point 174 approximately grams per mil, which is appropriate for virus particles. We see, as expected, the reporter transcript as it possesses a packaging signal co-purifying or co-concentrating in the same fraction.

What we also see is significant levels of the endogenous retroviral element packaging in the particles.

When we looked at the human system, again we get a RT fraction, positive fraction, here with high levels of beta galactoside as reporter vector.
However, in comparison to the murine system we don't detect any packaging of endogenous sequences, RTVL-H family, BLD type sequences or use in pan-retroviral sequences.

We do see a faint band or found a faint band when we were looking with C-type primers, and I'll come back to the identity of what this product was a little bit later.

So this phase basically identified RNA expression in the cells, and we had in some cases shown that some of the endogenous retroviral sequences could be packaged in particles.

The next thing we really had to do was try and put the numbers on this. Really, a plus and a minus wasn't very informative. So the methodology which we undertook was some in vitro transcription whereby we cloned some of the PCR products' retroviral sequences, cleaved before your 5 prime end, effectively your ATG, if it were there, and synthesized RNA from the transcripts.

We could then use this RNA to actually determine accurately the sensitivity of our RT PCRs, and that would allow us to eventually estimate the concentration of RNA in the virus particles.

Shown in this slide here is the
sensitivities that we found for our different PCRs,

and it was really -- We were glad we did this, because
we got a huge range of sensitivities down from
approximately 1 femptogram reaction way up to in the
500+ femptograms a quad in the RT PCR to get a
positive signal.

For the reporter vector, which was the
least sensitive, it didn't appear to be due to
primers. We varied primers quite considerably and
didn't really see any improvement in the
sensitivities. It was probably due to the actual
sequence that you're trying to amplify any reactions.

So we then basically determined the
sensitivities of the various PCRs, whether it be
through reporter or for the different families of
endogenous sequences.

What we then went on to do was to perform
dilutions of the peak fractions. Basically, once we
had the sensitivities, we could combine these with the
dilution series of the fractions to determine the RNA
concentration.

If we look at the murine system at the top
here, the beta galactosidase, twofold dilutions,
looked to be in very similar concentration to the VL-
30 endogenous retroviral elements.
In the human based system we saw significantly more beta galactosidase present in the fraction, which is reflected in a higher titer of virus, and relatively little C-type product.

So basically, that allowed us to estimate the ratio of the desired therapeutic or recorder transcript in comparison to the unwanted endogenous retroviral transcripts in the cells.

The peak fraction of the murine based system contained $6.3 \times 10^3$ femptograms and nearly 900 femptograms of the endogenous retroviral sequence. If you do the mathematics, convert these to molarities, that meant, basically speaking, for every seven virus particles which contain the LacZ genome, which you want, you have one virus particle which contains the endogenous retroviral sequence.

Obviously, that means for every integration, every cell that you treat, one in seven is going to be the wrong molecule being integrated into the genome. In fact, it's slightly more complicated as the retro-particle actually carries two copies of viral RNA, but the mathematics is still basically the same.

When we looked at the human FLY packaging system, you see we saw much, as I had mentioned
earlier, higher level of reporter vector and
basically, looking at the endogenous retroviral sequences, these were much cleaner than the seven to one which we saw with the murine system.

For the C type product which we saw, we had calculated that about one in 19,000 virus particles would contain this retroviral sequence. We went on to identify what this was.

We cloned and sequenced it and, actually, it turned out to be on sequencing identical to the gag/pol expression plasmid which we were using to actually create the particles. So we inserted that as representing basically the packaging of random cellular transcript. There's no real way of getting around this. You will get random RNAs incorporated into viral particles at a certain, albeit low, degree.

What I'd like to -- Oh, sorry, I should state that, since performing this data, we've gone on to try and address the potential problem with this approach, whereby we're revising our sequences to known retroviral sequence.

What we've done is to actually perform a random PCR on CDNAS made from the virus particles, to try and identify unknown viral families which may be being packaged and other viral sequences.
I don't have any slides, but a summary of the results would be that in the murine based system
the predominant sequence which we identified was VL-30, as you might expect, because we could even detect
it with this system.

In the human based system, again we identified the gag/pol expression plasmid and a whole
range of various, apparently random, in many cases unidentified RNA transcripts, very few of which had
open reading frames, and most of which were quite small in size. So the significance of that data I'd have to come back to in perhaps a year's time or
whenever.

Regards another potential risk of retroviral gene therapy, we tried to address or what we wanted to address was whether, once you had treated
a cell, if the endogenous retroviruses in that cell were expressing gag proteins, whether they could pick up the RNA transcripts from your integrated
therapeutic vector, package them, and potentially immobilize them and bounce them around the genome.

So this would be a disadvantage due to insertional mutagenesis, and there are some mechanisms which we had talked about earlier.

In our bodies there are very few cells
which express significant levels of retroviral particles. One major exception is the placenta which is absolutely heaving with BD type virus particles butting from the few syncytial trophoblast layer into the cytotrophoblast which forms basically the barrier between the mother and the fetus.

There are all of these particles as yet to be determined. It's a lot of research going into that. So the cell line which we analyzed to try and assess this case was one of the few cell lines where we do get lots of virus production, HERV-K based BD type virus.

What we did was to look for the -- There's a Gh cell line for those that would like the information -- again, performed sucrose gradients on the virus particles. We could see indeed endogenous retrovirus particles being produced of the B and D type sequence, which were banding at densities appropriate for BD type viruses.

Interestingly, in those cells we could also detect the packaging of RTVL-H sequences, another endogenous family of sequences. The significance of that we're really not sure, and haven't taken this work any further at the moment. But the reassuring point was the murine leukemia based virus vectors
which we had introduced into these cells did not
appear to be packaged into these particles.
Absolutely blank. So that was somewhat reassuring.

So in conclusion, I think we have to, and
will always have to, put up with the problem that gene
therapy packaging cell lines, whether they be derived
from human or murine systems, dog, you name it, will
express endogenous retroviral sequences which can
potentially interact with your gene therapy vectors.

The FLY line, human baseline, and probably
other packaging cell lines seem to produce virus
particles which are significantly less contaminated
with endogenous retroviral sequences and do murine
cells.

As a result, these are probably less
likely to produce replication competent recombinant
retroviruses which could be a problem in such
therapies and, encouragingly, again human endogenous
retroviral gag proteins, core proteins, only interact
weakly, if at all, with the NOV based viral RNA
transcripts.

Once again, thank you for the invitation
to speak, and I'll welcome any questions.

(APPLAUSE.)

DR. COFFIN: It's an easy one. I'm John
Coffin from Tufts. It's also been reported that MLV
based vectors produced on certain packaging cell lines
ex situ contain very high levels of an MLV related
RNA, from Jim Cunningham's lab some years ago, for
example. Did you see any of those -- any of that?

Did you look for that?

DR. PATIENCE: We didn't look, actually.

It should be done, actually.

DR. KAPPES: John Kappes from UAB. You
said you identified that the gag/pol transcript was
derived from the plasmid you transfected. Through
your sequencing, were you able to gain any information
that helped you understand how it recombined?

DR. PATIENCE: I don't think it was a
recombination at all. I think it's basically a
nonspecific packaging of RNA transcripts, which you do
see in retroviruses. You can pick up viral sequences.
I don't think it was recombination there.

DR. KAPPES: If you looked in infected
cells for evidence of recombination -- Have you looked
in infected cells for evidence of recombination?

DR. PATIENCE: Oh, yes, sure. We've
looked for both the transfer of the gag/pol construct
and rescue of -- Basically, we targeted cells with
envelope only, introduced our high titer virus, and
looked for rescue of a reporter vector by the
envelope, which we had already put in, and the
gag/pol.

We could detect very, very low levels of
recombinant viruses -- very, very low levels.

DR. PURCELL: Damian Purcell. With the
endogenous viruses that you've detected packaged, the
human elements, have you been able to establish
transmission and expression for those endogenous
elements?

DR. PATIENCE: We haven't seen any co-
packaging and, therefore, we've been unable to detect
any transmission. We did, unfortunately, do the
experiment back to front where we infected cells and
then looked for human transcripts, and couldn't see
any, which I guess again is even more reassuring, but
nothing is getting across.

DR. ROSENBERG: Thank you. The next talk
is by Leonard Evans on, again, mixed retrovirus
infection.

DR. EVANS: Okay. What I want to talk
about today are mixed retrovirus infections between
two different types of viruses that have already been
discussed. That's the polytropic viruses and
ecotropic viruses, and these are experiments that
we've done in mice.

I guess the take-home message from this is that it's really going to be difficult to try to anticipate what the effects of a mixed retrovirus infection might bring.

The initial experiments I'm going to -- How this actually started out was not look at mixed retrovirus infections. What I wanted to look at was recombination, and I wanted to look at the recombination of a polytropic virus when it was inoculated into a mouse and see whether or not it underwent further recombination.

The reason we wanted to do this is because we saw that the population in the individual mouse of recombinant viruses that came up was plural. There were a lot of different types of viruses, and we wanted to know whether this was a sequential situation or whether or not they were independent events.

Now the virus that we chose is a virus called Fr98, and Fr98 was constructed in John Portiss' lab or Rocky Mountain Labs, and it's constructed by taking the envelope of the polytropic isolate and sticking it into a backbone of Frend murine leukemia virus, and in that way it was advantageous for this, because we knew that there were no other alterations
in the genome other than the envelope and part of the integrase gene.

The other reason we chose Fr98 is that it replicates very well in mouse genes. So we thought that -- I mean, if we're looking for recombination, we definitely have to have something that's replicating.

The other had to do with the antigenic properties of this virus. That's shown -- I'll explain that on the basis of this slide.

Okay. This slide describes the monoclonal antibodies that we used in these studies. There's four monoclonal antibodies here. The first three of these react specifically with polytropic viruses, and the last one reacts with the Frnd ecotropic virus, which is the ecotropic virus we used in these studies.

The first two antibodies here, hybridoma 7 and 516, define two antigenic subclasses in these viruses. Almost every virus that we ever looked at, including our isolates and other people's isolates -- virtually all of them react with one of the two of these antibodies.

They're mutually exclusive, and they map to the same position on the Su proteins and alternate amino acids. We've also looked at endogenous sequences, many of them, not all of them, and all of
the endogenous sequences, including the modified and
the regular polytropic viruses, correspond to either
this one or this one in terms of the amino acid.

Now what was unique about Fr98 is that it
was a virus that didn't react with either one of these
antibodies, although it did react with 514 which
reacts with all polytropic MuLVs.

S for our purposes, it was really a nice
virus, because any emerging viruses we thought that
would arise by recombination would either react with
hybridoma 7 or 516. So we would be able to watch
this.

Now another property of Fr98 is that it's
a neuropathogenic virus. It causes a neurological
disease in IRW mice, but at the doses we were going to
use we didn't think we would probably see a disease
for about 30 days. We weren't using IRW mice. We
were using NFS mice.

When we inoculated Fr98 into NFS mice, we
were able to actually follow the disease or follow the
mice for the generation of new polytropic viruses that
react with hybridoma 7 or 516 for about three or four
months, because it turned out that these mice didn't
get neurological disease with Fr98. They developed
erythroleukemia with a latency somewhat longer than
During the course of that time, we didn't see anything that suggested any emerging viruses with hybridoma 7 or 516. Everything was reactive with the 514, which is what the Fr98 is reactive with.

It occurred to us that maybe what was happening is, because we're putting in a polytropic virus, even if we generated new polytropic viruses, because of the vicinity that we're apt to be generating this in and it would be a high level of polytropic infection, that there some interference so that we would never see the virus, because it was never, ever able to spread.

Then it further occurred to us that, if this actually was happening, then maybe if we inoculated an ecotropic virus with the polytropic virus, that we could suppress the development -- the de novo development of new polytropic viruses.

So we co-inoculated Fr98 with Frend MuLV, and that's the next slide. Okay. Now I expected there might be -- You know, I hoped there might be a little suppression. What I didn't expect was the complete turnoff of any de novo polytropic viruses.

Normally, when you inoculate Frend MuLV, you get -- It's predominantly hybridoma 7, but there's
some -- two or three percent that are -- of the polytropic viruses that are reactive with 516.

We got about 60,000 here on the average of 12 days, and we got absolutely nothing, no hybridoma 7 and no 516. We have -- In fact, in any co-inoculation experiments, we have never seen any of the normal -- any hybridoma 7 or 516 polytropic viruses coming out.

We did, however, see a huge amount of -- This is the sera. We saw a huge amount of virus that corresponded to Fr98 that's reactive to 514 only. This is nearly 10^7, but there was a second observation that was really striking, and that's showing on the next slide.

We found that the mice developed -- The reason those experiments only went for 12 days is because that 12 days after, the mice are gone. We found out that, when you inoculated Fr98 with fMuLV, we got a very rapid development of neurological disease, very similar to what's seen in IRW mouse except for the onset. At this dose in IRW mouse, they would normally come up about here.

The mice appear really normal for about ten days, and when they're suckling, there didn't seem to be any problem. Then from 11 to 15 days these mice
get sick. They become ataxic. They fall over. They
have tremors, and then they die.

It's so rapid that, I mean, if -- The
range here appears to be about a week, but in fact,
when the first mouse in a litter develops symptoms,
usually within a few hours all of the mice will
develop those symptoms, and within 12 hours they will
all be dead.

Okay. John Portiss looked at the brains
of these mice. He could find no spongiform
encephalopathy at all. There's no hemorrhagic
differences. John is a pathologist at our lab. So --
which is very similar to what you see in IRW mice. In
fact, a higher levels of virus inoculum -- and this is
with Fr98 alone in IRW mice -- you can actually
shorten the time of incubation here to come very close
to this curve.

Well, we wanted to know what was going on
in these mice. So we had a hint from the fact that
the viremia was 107 at 12 days. So we decided to
follow the virus load in these mice, in mice that were
inoculated with Fr98 alone, with MuLV or with the
combination.

So we looked at both ecotropic, compared
the ecotropic with the co-inoculation, and the
polytropic virus load and the co-inoculation. Next slide shows those experiments.

This is the ecotropic virus. This is off course here a little bit, but basically with ecotropic virus we could see no differences in the -- This is a serum. This is the spleen, and this is the brain, which are the tissues we looked at in these mice.

We could see no difference in the ecotropic virus replication in these mice. It was the same, whether or not it was inoculated by itself or whether or not it was inoculated in combination with the polytropic virus.

Completely different situation, though, with the polytropic virus. If I can get the next slide. Okay. Here in the serum there is about a two-to-four hundredfold difference in the level of viremia between the co-inoculated mouse in the red and the mouse inoculated with simply Fr98. It's even more in the spleen in terms of infectious centers.

These are cell free viruses. These are infectious centers. A little less pronounced in the brain, but still a ten-to-twenty-fold difference in the level of infection in the brain.

So it appeared that the viruses were, obviously, interacting in these mice, and one of the
things a person might suspect would be pseudotyping

between the viruses, even though they were inoculated
as separate -- well, as a mixture of stock, but they
were inoculated as separate viruses.

So could I have the next slide? We looked

at pseudotyping in these mice. There's a couple of
things I've got to point out about this, a couple of
striking things.

One is -- that I'll come back to in a
second -- the fact that the polytropic virus appeared
to be pseudotyped completely, almost through the whole
course of infection, and pseudotyped by the ecotropic
virus. These assays are done by comparing the titers
on 3T3 cells versus the titers of the virus on 3T3
cells that are infected with fMuLV.

So that if it's pseudotyped because of
viral interference, it won't score on the fMuLV
infected 3T3s, but it will score on the 3T3s that are
not infected, because both eco and poly will go into

those cells.

The first striking thing here is the
pattern of pseudotyping in the brain. The pattern of
pseudotyping changed abruptly right before the onset
of disease, and this is one of the few instances where
we see polytropic viruses that are not pseudotyped by
ectotropic virus, and the simplest explanation of this, which we're currently trying to resolve, is that
the polytropic virus has spread to a new population of cells, and either that population is not coinfected
with ecotropic virus or that population of cells has a different property in that it does not promote
pseudotyping of the polytropic virus by the ecotropic virus.

The other puzzling thing here is that -- I can now extend these curves clear back to here for all this -- is the level of pseudotyping of the polytropic virus by the ecotropic virus -- the level of pseudotyping is nearly 100 percent here, and at this point in time the level of infection is very, very low. We're getting around 1000 titers.

So the very first viruses that we're detecting in these mice are, in fact, pseudotyped. Well, the only way you can get pseudotyping that I know of is to have co-infected cells. So this suggested then that we actually have a very small population of cells that are infected by both viruses, which is kind of remarkable, considering they both use different receptors.

It occurred to me that maybe this was because of the route of inoculation, because we were
mixing the viruses and sticking it in a compartment, actually in the peritoneum of the mouse. So we thought that possibly it might be that. So what we did was to inoculate the viruses subcu. as different stalks of viruses. So Fr98 is inoculated in one spot subcutaneously, and then like in the right haunch and the left shoulder, the ecotropic virus was inoculated.

When we did that, we found the same thing, complete -- almost complete pseudotyping by the time we could get a good measure of the virus. Well, that suggested to me that it might be a mobile population of cells that was actually being infected by these viruses. So -- and that at some point in time a person should be able to find cells that were not pseudotyped, so that you could find cells that were not co-infected. So we did a -- God, more experiments than I want to know to look at this at very early times. That's shown on the last slide. We could, in fact, find a time during the infection where the pseudotyping was minimal. This computer moved this curve. So it's supposed to be like this. But basically, at about a tenth of a percent of there cells infected we could find free
polytropic viruses.

After that there was a significant level of pseudotyping. So it looked to us then that there must be a very small percentage of circulating cells that are the initially infected cells by both of these viruses. I guess one of the messages here that this implies is that just because the virus's dose and infection is a quite low dose, it does not preclude fairly immediate interaction of the viruses.

I suppose I should say something about the possibility of this interactions in humans, not these interactions but the possibility of interactions of different types of viruses.

There have been some examples of co-infection in different types of viruses in humans. I mean, it's well documented, HtLV and HIV, but there is no good documentation, even though there was a lot of suggestion earlier, that HtLV may accelerate HIV.

To my knowledge, looking at larger cohorts there, there was no evidence of that, and also SiV and StLV infections in an experimental situation didn't show any synergism. But definitely, the tools for a mixed retrovirus infection exists.

I mean, we talked a little bit yesterday about cells -- or viruses that could infect humans.
There's a lot of viruses from a lot of different sources that can infect heterologous cells, and in fact, that may be the rule instead of the exception. With the murine system, the only ones that have never been, to my knowledge, demonstrated to infect human cells are the ecotropic viruses. So we know there's -- from the bovine, from the feline system, obviously from the higher primates, there's all kinds of viruses that can affect human cells. So that's the one prerequisite for setting up such a mixed retrovirus infection.

The other prerequisite is for these viruses to interact. Now they're somewhat restricted in the interaction in terms of packaging, but even that is not a foolproof situation. C-type viruses appear to package C-type genomes more easily, but there is still -- Moloney has been demonstrated to package the genome of HIV and also the unspliced -- or the unspliced and the spliced messengers.

So it may not be a specific packaging, but it definitely was found within the virion, although it wasn't shown to be passed, and it wasn't tried in those particular experiments.

In terms of utilizing glycoproteins and incorporating surface proteins in different
retroviruses, they're very promiscuous. Again, you have to search for places where it doesn't happen. It doesn't happen very easily with HIV and MuLV, because -- presumably because of the long cytoplasmid material of HIV, although SIV will incorporate into -- which also has a long cytoplasmid tail, will incorporate into MuLV particles.

You also have to be concerned about heterologous pseudotyping between different kinds of viruses. It's been shown that fowl plague virus, which is an influenza virus, can pseudotype MuLV. Sendai virus, both proteins in Sendai virus can pseudotype MuLVs. E. bola virus can, but that's probably a moot point.

So I think that the potential for a mixed retrovirus infection is definitely there. Whether or not it can be easily avoided is a question. Thank you.

(APPLAUSE.)

DR. ROSENBERG: Are there questions?

Thank you very much.

The next talk is by Damian Purcell.

DR. PURCELL: Can I have my first slide?

So we've been introduced to gene therapy and the complications of co-packaging between multiple RNAs,
two RNAs into the retroviral particle.

What I want to talk to you about today is some studies analyzing some of the early gene therapy vectors and packaging cell lines that would -- using what are now fairly unsophisticated reagents and the vectors based on Moloney murine leukemia virus and the N2 vector containing the packaging signal of Moloney and the helper constructs based on the amphotropic MuLV 4070A; and they have deletions in the packaging signal, but these constructs have regions of hemology where two recombination events can regenerate replication competent retrovirus.

So the emergence of these replication competent retroviruses was observed, and the safety of -- potential risks from those replication competent retroviruses in primates has been studied. I'll just review quickly the studies that have been published.

There are essentially three of them. The first one by Coronetta, et al. in 1990 examined a replication competent retrovirus coming from sax retroviral vector producer clone, which is essentially the same as the one on the previous slide.

So in this study there was an intravenous infusion 7 by 107 focus forming units of this replication competent retrovirus or RCR, and though
that entity wasn't really well characterized molecularly, presumably it had an ecotropic Moloney MuLV gag/pol with the ampitrophic envelope. Four rhesus macaques were infused intravenously, three normals and one immunosuppressed with prednisolone and cyclosporin, and in these studies the virus was cleared in 15 minutes with, obviously, no detectable pathology after 15 year follow-up.

So even within this study it was recognized that in these four animals infused with replication competent retrovirus produced in a murine cell line that probably a xeno antigen effect led to virolysis by complement where the enzyme alpha 13 galactosol transferase adds the alpha gel residue onto the glycoproteins of the virus particles, and antibodies that are raised in humans, petrophile antibodies, recognize this activate complement and lead to a lysis of these murine cell derived viruses very quickly.

So two ways to avoid this is, one, to reduce the virus in human or primate cells where you don't have the enzyme, and you also acquire passively into the envelope of the viral particles molecules that protect against the activation of complement such
as CD46, CD55 and CD59.

The other way is to introduce the replication competent retrovirus in vitro into autologous cells and then implant the infected cells. This is what was done in this study where 6 by 10^7 fibroblasts from a rhesus macaque were infected with the recombinant retrovirus, both subcu. and IP, and also for good measure there was 3 by 10^8 focus forming units of the prototypic amphotropic virus MuLV 4070A injected IP into a single macaque. This animal was immunosuppressed. The animal was viremic for two days, and virus was recovered from PBMC for several weeks. PCR could detect the presence of the amphotropic MuLV envelope up to 200 days, but there was no detectable pathology after five years of follow-up.

In the second study, also published by Coronetta et al. in '91, the following year, they took the examination of the safety of these replication competent retroviruses derived from the mouse and murine system in primates one step further where they examined the transplantation of replication competent virus infected autologous bone marrow cells, in this case four cynomologous macaques.

So in this protocol 1 by 10^6 focus forming
units of the replication competent retrovirus, again
from a sax derived vector but carrying the ADA gene
this time, were used to infect bone marrow, and they
used supernate directly from these producer cell
clones.

The animals for the purpose of bone marrow
transplantation received very strong immunosuppression
with 1000 rads of total body irradiation, gamma rays,
immediately prior to transplantation. On follow-up
there was no detectable viremia at early or late time
points, no detection of the amphotropic envelope by
PCR for up to three years, after long term follow-up
no detectable pathology out to seven years.

So on the basis of those studies, the
replication competent retroviruses are probably
thought of as being fairly innocuous in primates. So
a large study was done in the lab of Art Niehise at
the NIH examining the transduction of autologous bone
marrow in a group of ten rhesus macaques.

In this protocol, using the vector
packaging system on the first slide, they had a new
twist to increase the titer of virus introduced into
the bone marrow cells, and that was an amplification
technique that they termed the ping-pong amplification
where they basically bounce the vector between
ecotropic and amphotropic packaging cell lines in a co-culture system.

This increased the titer of the N2 neo-vector carrying neomycin marker gene in the study, and also produced low levels of replication competent retrovirus, which they knew existed at around -- up to 10^3 focus forming units, although the number does bounce a bit between the papers.

So if we describe the largest number, 10^6 focus forming units of the replication competent retrovirus, this was infected into 10^8 CD34 selected bone marrow cells in vitro in a system that cultured the bone marrow cells with the vector for 80 hours, and then these were infused into ten rhesus macaques.

There was immunosuppression supplied prior to harvesting or just after harvesting the bone marrow, 5 FU for nine days, and then one and two days prior to transplantation there were two doses of 500 rad of total body gamma irradiation, so total again of 1000 rads of irradiation.

So the surprise was that chronic retroviremia appeared in three animals, and these animals were noted not to have antibody against the GP70 or P30 capsid proteins of replicating competent retrovirus.
These three animals went on to develop T-cell lymphomas after 200 days, and these lymphomas were, obviously, rapidly fatal.

So just to summarize the animals there and the data that I've just described to you, we had an opportunity to follow up in particularly two of these animals the types of viruses that were administered through this protocol.

One animal in particular had very well preserved samples, this 15445, the other 88049, had suffered a degradation post mortem, but we were able to use these samples from animals to see what had happened -- what had generated the problem here.

So the first thing we looked at was the replication competence of these viruses in diploid rhesus cells. We wanted to avoid selecting particular murine viruses by culture on rodent cells, and we could find that readily infect this rhesus lung fibroblast cell line with -- directly with plasma or with virus pelleted out of plasma.

What was the surprise when we took that virus harvested out of the rhesus cells, along with virus similarly cultured in rhesus cells, and that virus being the 4070A amphotropic retrovirus -- so the prototypic amphotropic retrovirus -- normalizing
inoculant, reinfected that back into rhesus lung fibroblasts, and the virus that was present in monkeys had a much greater capacity for replication in the rhesus cells compared to the prototypic amphotropic retrovirus.

So when these viruses with the same inoculant were applied to Morsoduni fibroblasts which are very permissive for the growth of murine leukemia viruses, this growth differential was not evident.

So summarizing the properties in terms of infectivity of these viruses in various cell types, reverse transcriptase activity was directly detectable in the plasma of these animals. As I've said, the rhesus lung fibroblasts were regularly infected, bronchus fibroblasts. Human helo cells were also infectable, and rodent fibroblasts and Morsoduni cells.

What were not infectable after many attempts were rhesus PDMC, which we had activated with THA, and this is a bit of a pity, because the PDMC are obviously the easiest cells to examine from animals or patients for replication competent retroviruses, but may probably provide a poor source of deriving virus back.

So we next undertook a molecular
characterization of the types of virus present and transmitted and expressed in the host animal. We chose to use an RT PCR protocol and focusing on two areas of the virus, the envelope region which, we have already heard, determines tropism of the virus, and also the LTR, particularly the U-3 region containing the promoter and the enhancer sequences, because these also determine the tropism of the murine viruses, as we've heard.

So we extracted the RNA directly from the tumor tissue, made poly-A RNA from it, synthesized CDNA. Then we chose, after a lot of sequence alignments, sequence primers that would amplify as many of the murine leukemia viruses that we could pull out of the database and stuff into an alignment.

So we're essentially going for full length envelope sequences here, and we were successful using different samples of RNA from the rhesus tumor and amplifying full length envelope CDNAs, and we amplified no bands from the rhesus lung cell line.

I guess what is important to point out in this slide is that what amplifies is mostly the full length envelope, not other shorter products that might indicate the transduction with cellular components, as we might find after passage of the virus in the rhesus
lung fibroblast cell line.

So we sequenced -- or we cloned those PCR products and sequenced the clones that we derived. Essentially, three types of envelope structures were observed, the amphotropic envelope which was directly the same as that derived from the packaging cell construction 4070A with clear recombination back into Moloney right here around the R protein region of Moloney.

There was a variance within this type of envelope that had deleted six base pairs in a key region determining the tropism that's in the VRB or variable receptor binding domain of the envelope protein, and this led to an amino acid sequence change within that crucial amino acid loop structure.

What was a surprise in this animal was to find a new player, an MCFY envelope where the bulk of the envelope sequence had come from the Frend MCF strain with sequence towards the junction with the 4070A amphotropic sequence, resembling a cluster alignment with xenotropic envelopes.

So this was in one animal. When we looked in the other two where we had less good starting material to do an RNA analysis, looking instead at DNA, it was clear that all of the animals, all three,
had the amphotropic envelope sequence present, but only the one animal had the either MCF sequence with primers within the MCF region only or the recombination between the amphotropic and MCF sequence. None of these animals amplified with xenotropic primers.

The recombinant amphotropic MCF virus could be traced back to the producer cell clone and its emergence appearing between the 22nd and 25th week. So it seems that its derivation was from the packaging cell line.

So next we examined the LTR structures within the various players here. So firstly, I draw your attention to this line here, which is amplification across the U-3 region of the LTR. So we're looking at the structure of the core enhancer and the promoter region.

The amphotropic virus has a single core enhancer, giving rise to this size fragment here. The A2 producer cell line has predominantly the Moloney LTR which drives the expression of vector and also the recombinant retroviruses from the previous slide, and Moloney has a duplication of the core enhancer, and we see a larger size PCR product. Also evident was another faint band which
was further duplication of the enhancer element being
expressed within RNA in the producer clone.

In the two rhesus samples that we
examined, the predominant forms of LTR expressing RNA
were those that had the three copies of core enhancer
and four copies of core enhancer was also evident in
these cells.

We infect the same virus into the Mosduny
cell type, the indicator, it more closely resembles
the expression seen in the A2 producer. So this
greater addition of enhancer sequences seems to lead
to preferential expression in rhesus.

So just to give a cartoon version, this is
the amphotropic type U3 region. This is what Moloney
has. These forms here seem to be expressed in the
tumors.

So knowing the structures of the LTRs, we
did PCR analyses that would match the envelope types
to the LTRs and, as we expected, the three envelope
types characterized earlier, each had the LTRs that I
described, each having duplications of the core
enhancer. But when we used a specific primer for the
MCF type LTR, the U-3 region, we asked whether this
was also being expressed at very low levels in this
specific PCR for the MCF endogenous virus itself, we
indeed found expression of this in the tumors.

So I just wanted to point out that these viruses here, the MCF viruses, we couldn't detect infection in either the Mosaduny indicator cell type or the diploid rhesus lung, but these do grow in mixed cell lines.

So if we look at the genetic structures that we pulled out, so that the main player is the recombination between the vector and helper virus. So it's integrated into the DNA and does express RNA in the lymphoma.

Also, we find recombinations between endogenous retroviruses in two types. We see the MCF endogenous envelope represented in this main replication competent entity, but also we see just the expression of the endogenous retroviral elements themselves as RNA.

As Clive Patience mentioned earlier, we also looked for VL30, and we found lots of VL30 DNA present in the rhesus tumors, but no RNA expression from these endogenous elements. We also looked for the co-packaging of cellular RNA species or DNAs, which is not that easy to do, and we didn't find anything.

So if we want to summarize the factors
that might account for the pathogenesis of the replication competent retroviruses in three of the ten monkeys in this study, first we would have to question the use of mixed cell populations or the co-culture technique that might have fostered additional rounds of replication to generate novel replication competent entities.

The high level of post-immunosuppression is something that's also being pointed to, although this has been debated quite a bit, whether that was particularly high or more significant than previous studies where this didn't seem to contribute to the emergence of pathogenesis.

Certainly, the lack of response or lack of antibody response to the presence of the replication competent retroviruses in those three animals is another thing that seems to indicate a problem for that particular animal, and probably also associated with the emergence of the chronic retroviremia. But the adaption of the replication competent viruses is also, we think, a very important feature.

So the genetic factors, particularly, the recombination events that arise and are associated with elements from within the packaging cell lines, particularly the expansion of the core enhancer
of the BRB domain is a consistent feature between the animals, and we're unsure of the relevance of this, but probably enhances the infectivity to rhesus cells, and also the recombination with endogenous MCF is also an interesting thing, but doesn't correlate with each of the tumors in all of the animals.

Epigenetic factors, the alpha gel, so the lack of addition of the 1-3 galactose on the carbohydrates when virus is derived out of primate cells in this final study compared to the first one, clearly makes the difference here. So the viruses that emerge out of the autologous cells are resistant to complement lysis.

There was a very multiplicity of integrations after the prolonged in vitro infection, the 80 hours of exposure of the bone marrow cells to the vector and RCR mix.

So the implications are to select packaging cell types with the fewest endogenous viral RNAs, that the properties of the replication competent virus derived that emerge that combine the endogenous elements may not be totally predictable, are certainly not the same as prototypic viruses that go up to make the elements placed into the packaging cell lines, and
this has implications when selecting the indicator
cell lines for detecting the presence of RCRs in males
that have implications of prime transfer across
species.

Certainly, the immune compromising or
immunosuppression of recipients for gene therapy
protocols would place them at greater susceptibility
to spreading infection with RCRs.

Now because the organizers have put
implications of this work for viral vaccines, I've
included this slide here to try raising some of these
points.

Endogenous retroviral elements that are
present in many neoplastic cell lines, and
particularly the murine cell lines, as we've heard
earlier in this session, might receive help function
from the expression of functional viral proteins which
may be a component of a vaccine producing a functional
viral protein that might interact with elements, be
they RNA or protein, in the cell line.
The expression from viral genes may lead
to repair of replication competent retrovirus, and
that really is the really nasty element that is the
most obvious thing to guard against, the greatest
danger, if you like.
RCR might have novel cell tropism, an enhanced infectivity in vitro compared to prototypic retroviruses due to modifications in the envelope and LTR. Live attenuated retroviral vaccines may recombine with endogenous elements during production in cells expressing these endogenous retroviral elements, and mixed cell populations may facilitate rounds of replication that foster the emergence of a replication competent retrovirus.

So whether this argues maybe that some cell lines may be better because there's definitely a clonal population, that's another way of interpreting this.

I just wanted to thank my co-workers who helped with this work while at the lab of molecular microbiology at NIH, Norton Martin's lab, Elio Vannen from Gene Therapy, Inc., who did another similar study also published, and Art Niehise who is now at St. Jude's. Thank you.

(APPLAUSE.)

AUDIENCE PARTICIPANT: Have you looked at the integration sites of those lymphomas?

DR. PURCELL: That was -- Some studies were done with that, but it was appreciated that in the lymphoma there were up to 35 integrants from the
replicating competent retroviruses, and it was considered to be at that point too difficult, too many integrations. So that's clearly the undercurrent of the pathogenesis, the insertional mutagenesis, from having so many integrations into the genome.

DR. COFFIN: John Coffin from Tufts. Your talk and several before emphasized a very important but not often completely appreciated point about retroviruses, and that is that their really very large genetic flexibility allows them to respond to all kinds of different selection pressures in all kinds of remarkable and surprising ways.

So when one is thinking about handling systems that have retroviruses in them, one should think not only about the genetic variation, the mechanism of genetic variation that you undergo, but also and probably much more importantly the kinds of selective pressures you're putting on these viruses to enhance their growth.

For example, in the AKR mouse it's really remarkable that every single animal in lockstep virtually undergoes the sorts of changes that we saw in slides earlier, and the same kind of remarkable changes that you see here, and there are many, many other examples of these kind of things in
So I think we always have to be very careful in our thinking about how this goes, not only for understanding molecular mechanisms but also for understanding selective pressures that we're putting on the viruses and the cells to produce them.

DR. PURCELL: I couldn't agree more. I mean, also an interesting component of this things is when you put these viruses together in vitro where you don't have immune selections and you're greatly enhancing the start of the virus to recombine and make a whole host of new entities, do we immune suppress the animal? That gives it a new environment in which to make changes and get going.

DR. ROSENBERG: The last question, because we are running and want people to stay and hear our final speaker.

AUDIENCE PARTICIPANT: I'll speak quickly.

Two very short questions.

Damian, do you know if anyone actually measured the levels of natural antibody in the primate recipients of the various viruses?

Secondly, have the complement inhibitors ever been directly shown to protect viruses produced from potentially sensitive cell lines?
DR. PURCELL: There are studies published, I believe, that look at the lysis of murine derived retroviruses by human complement, and ascribe that activity. But I'm not sure that it's been conducted in molecular rigor.

AUDIENCE PARTICIPANT: Those studies certainly do implicate alpha 1-3 gal as the culprit molecule, but I don't think anyone has ever shown that the complement inhibitors work directly on the virus particles by accidental packaging of the inhibitors.

DR. PURCELL: Yes, I have to agree with that. I mean, my slide there was more deduction of the data rather than a good study to explain those results. So, yes, more work needed.

DR. ROSENBERG: Our final talk this afternoon is by Paul Jolicoeur on defective retroviruses.

DR. JOLICOEUR: Yes. I should thank the organizer for giving me the opportunity to talk. I'm going to talk mainly about pathogenesis of defective viruses, as I was asked to talk about. The first slide, please.

What are defective retroviruses? This is illustrated here. They are a shorter genome and, in general, they have deleted or mutated genes.
Therefore, they cannot replicate by themselves, and they do need a nondefective, which we call helper retrovirus for full replication. Some of them, however, are not a gene in general, which I think capture many of the oncogenes, and we'll see a little bit later what they are. These defective viruses can be expressed normally, once integrated, and we count helper proteins. This is important for pathogenesis of some of them; and if expressed, they can also be rescued by other nondefective viral proteins, even those encoded in other defective viruses.

In other words, in helper free stock you can generate one-cycle replication, and you heard about that earlier, in the packaging system where two defective viruses complement, in a way, each other. Why are there concern about defective retroviruses in vaccine preparation? Again, you heard about it from the previous speaker, that we do have several copies of endogenous defective retroviruses which are either nondefective or nondefective retroviral sequence, including in humans where most of them, if not all of them, are defective. These endogenous sequences participate in recombination events, give rise to novel viral
entities, novel viruses, again as you heard from previous speakers. And several strains of defective retroviruses have been shown to be pathogenic.

There are broadly two classes of defective viruses, the one which essentially encode only viral genes such as this one, and the best known examples are the Frend virus and the duplan strain virus or the MAIDS virus on which I will talk just a little bit more; and obviously, the one which encodes V onc, which have captured a nonviral cellular gene, and from which we have learned most about oncogene and from which the first oncogenes were derived from and labels on the FBJ, the RAS Harvey and Kersten and so on, and where you do have a V onc. which has been captured in the middle of the defective viruses.

The SFFV containing only viral sequences is defective in the sense that it has gag and pol -- several gag and pol mutations. It has a complete and open reading frame, but its end sequences are a form of two types of sequences, noncrotropic and ecotropic recombinant, plus an insertion, a one base pairs insertion here, giving rise to this new protein which is able now to recognize a structure which normally none of the parents recognize, that is, the CD EPO receptor.
The GP55 which is encoded by this Frend virus would stimulate the EPO receptor and give rise to this massive proliferation and erythroleukemia. This is an example of a virus which is defective and is able to participate in tumor formation very easily.

I have listed here some of the characteristics of defective retroviruses. We are going to go through some of them, and I will illustrate many of them in a model which we call the MAIDS model.

First, defective viruses can be generated in a lifetime of an animal, and this has been the hallmark of the V onc. defective viruses which all occur in one animal and were isolated in this animal, although very rare genetic events are required to generate in general these viruses, sometimes two or three recombinations of them.

Defective and recombination retroviruses appear to be generated more easily in vivo than in vitro. Defective viruses or pathogenic viral genome can emerge from nonpathogenic viral sequences.

As I said previously, two defective retroviruses can complement each other. Individual retroviral sequence expressed in absence of replication in defective systems, such as the
packaging system, can be pathogenic or, in the context of transgenic where you express only one gene.

In general, those viral genes interfere with -- or participate in cell signaling. We also know that defective viral sequence such as VL30 can enhance V onc. activity or such as gag in gag oncogene fusion protein. It has been shown, for example, with a gag Abelson fusion protein where gag is important for myristylation and location in the membrane.

Finally, defective viral genomes are as effective as nondefective viral genomes as insertional mutant, and we'll see some example of that.

Just to show a cartoon here, how can rare events -- very rare events, in fact, get -- occur in a live form of an animal as a capture of an oncogene where it has been alluded before by Naomi. First, it has to -- This is a model which has been postulated by many labs, including Mike Bishop, and has been reviewed here.

You have to have -- It is postulated that there is an insertion in front of a C onc., that you do have deletion which by itself is rare. First event is rare. Second event, this deletion is relatively rare, and you do have a transcript.

You make a packaging sequence which has
been conserved here so that you can package this with an infection of a new cell where you do have transcription and a jump, which is also a relatively rare event, where you do have a jump to capture this sequence and put it between two LTR in chicken and mice, mainly in these two species. It has been well documented that you do see that in the lifetime of a single animal.

I will not go through that, because many people have talked about the packaging system where two defective genomes finally can be produced in a one-cycle event.

Let me focus now on an animal model which is called MAIDS, which has been induced by defective viruses and where we can see the plasticity of what these viruses are.

MAIDS stands for murine AIDS, essentially a lymphoproliferative disorders with splenomegaly and lymphadenopathy where you do have severe immune dysfunction of TNB cell lymphocyte.

There is massive lymphoproliferation in these mice. You can have as much as two or three grams of lymph node tissue, due to this massive proliferation. It's basically caused by defective viruses which has a single open reading frame in the
gag region with severe deletion in pol MRV regions.

So essentially it's only a viral gene, and one gene -- one viral gene has been kept open. The origin of this virus is interesting. It arose after successive passage of cell-free extract for mix-free induced thymomas in C67 blacks.

The crude stock themselves contain several substrains of MuLV initially, and it is likely to originate from endogenous proviruses. In fact, some of the parents have been partially identified. One is a family of endogenous MAIDS related virus, MRV, identified in the mouse genome -- In fact, there are five members of that -- by Neil Kaplan, where the gag is intact and where a deletion of pol MRV is present, very much like the MAIDS virus.

The gag region is homologous to the MAIDS virus and harbors some additional mutation, however, and the LTR is this thing. The MRV is a xenotropic LTR, while the maze virus is an ecotropic LTR.

There is another family of expressed genome, EDV, where the gag is homologous to that of the MAIDS virus if you allow a frame shifting.

Therefore, it appears MAIDS is a recombinant virus, probably of at least three distinct endogenous retroviral sequences, which again occur during passage
of irradiated tissues.

It encodes a single protein, which is a

gag protein, which is not cleaved in matrix or capsid
or nucleocapsid proteins. It is not cleaved. This is
a peculiarity of this gag protein.

It is myristylated and well produced in
cells, and our hints that this system could be
interesting to study the pathogenesis came when we, in
fact, used a packaging system to produce helper free
stocks of these defective viruses and to show that, in
fact, these helper free stocks, which are replicating
incompetent except for one cycle, would cause disease
to a high frequency -- this is a Type 2 virus here --
in fact, at 100 percent as compared to a replication
competent helper virus.

This is remarkable, because when you think
about it, when you showed this prep in IP, it gets
into the peritoneum, and the very first cell it's
going to see and infect will be the last one, and
these cells will cause the pathology you just saw.

We are talking here of very low titer. In
the range of 10³, 10⁴ will give rise to this disease.

So 1000 cells to 10,000 cells in the body at maximum,
if we don't account for the loss of infectivity going
through the peritoneum, are responsible for the
What's happened when you do that and look in the spleen, for example, or lymph node, you do very early see little clones of proliferation which are independent. So a little polyclonal proliferation in distinct nodes and lymph nodes which eventually, as the time goes on, will fuse and get massive polyclonal proliferations.

We have been able, through different techniques -- I'm showing only one here; this is mainly a typing by immunocytochemistry to show that these cells are B cells. This is a B220 staining as opposed to the control, different controls in lymph node or a spread of cells here. This is a positive cell, because you see the blastic-like inside two positive cells, and the noninfected, more mature B cells which are smaller. So that you have the features of blastic, and they are B220 low.

Another interesting feature of this growth is that it's thought in many mice as polyclonal. This is a blot where we look at integration site, on the junction of integration site, and you can see that different nodes in the same animals -- in this animal, for example, or this animal -- you do have a polyclonal or different -- not polyclonal but distinct
integration. But in some animals the same clone has
taken over the whole animal here.

That is to say, a single clone here, a
single cell really, took over and appears in these
lymph nodes.

Looking at that, we hypothesize that in
fact not only the gag was providing a growth advantage
to these cells -- that is quite obvious, because you
can see it polyclonally and very quickly -- but also
the genome itself was an insertional mutagent.

We test this hypothesis by cloning some of
these integration events and asking whether these loci
were occupying a certain percentage of the tumor, and
we did find at least two loci like that which we call
Dis-2 where we did find common integration.

We have not yet identified the genes
involved here, but the Dis-1 -- and you can see here
the rearrangement and different tumor or lymph node
enlargements, if you wish, in the band of the Dis-2 or
Dis-1 locus here.

The other locus that we have identified is
the PU.1 locus which is a transcription factor which
is specific for B cells in macrophage. You can see in
a certain percentage of tumors that the integration
occurs at the same place where there were initially
identified in erythroleukemia and the Frend's systems.

Therefore, this defective virus participates in growth of these cells, not only by providing the gag protein but also by serving as an insertional mutagen, and this is an example of a defective virus genome which can serve as an insertional mutagen.

Now we did the structure function of that, and again this illustrates a point. When we do point mutation in this protein very early at the subcode down here within a few amino acid of the beginning of gag, disease capacity of that virus is lost.

If you do other point mutation along the protein or the sequence, you do see that this viral genome is pathogenic, but at the same time we found recombination in these viruses.

So, in fact, these are nonmutant, because they are revertant, and you will see what kind of version we found. So the only two viruses we could use where we found loss of capacity to induce disease was this early mutant stop coder and a myristylation negative mutant, as you can see here.

Surprisingly, despite the fact that we had a single point mutation in the middle of this sequence, the type of reversion we got was not a
reversion of that mutation, but in fact was a recombinant.

As you can see here, this is the virus we put in with the capacity to encode now a very short protein as opposed to P63 the encoder protein of P21.

This is in fibroblasts where we produce a stock of virus.

In every mouse inoculated with this virus where we looked in every lymph node, what we found is a recombinant having the molecular where it closed to PR60, and you can see they act differently in each other.

So again this illustrates the point with virology that John was coming at a few minutes ago, that those are very plastic and even, surprisingly, with a point mutation like that, what you achieve is you achieve a highest recombination event presumably with other endogenous sequence, and we know this was not -- we know it was not generated in vitro.

The myristylation negative mutants tell us something about that. First, it tells us that myristylation itself is required. So, therefore, attachment to the membrane is important for the pathogenicity of that virus, and this suggests that PR60 molecule is the only protein required for disease
induction.

Two, it suggests that the processing of that protein or that antigen, if you wish, by B cell, which are APC, is not required for this effect, although some people could argue about it; and PR60 is used as a docking side.

The way we see it now is that you do have this protein here, and presumably, by interacting with some protein, you do send two messages. One is proliferation, as illustrated by the very quick, within a day or two -- very quick proliferation of B cell polyclonal and, two, by reprogramming of these B cells the sense that B cells which proliferate will not induce total anergy of T-cells, as we do find in these mice.

So, therefore, there is something special about these cells which make them energize T-cells which are not themselves infected, and there are some motif, especially in the P12 domain of this protein, which is a very proline rich protein as opposed to either P12 protein of other MuLV, and where you do create pyridivous H-3 finding site, and as well some H-2 phosphotyrosine binding sites, as you will find in signaling molecule.

So to search for a putative factor binding
to PR60 gag, we use the yeast-two hybrid. As a bait we use the PR60 in the LXA system, and the very first clone we got was an interesting clone. It was the only small clone containing the SH-2 - SH-3 domain of C-ABL.

We did some coimmunoprecipitation and other studies that I don't have time to go through here, showing that this interaction was -- could be seen in vitro and in cells infected with the virus itself, as you can see here with an anti-IPA both, for example, and coming back with anti-CA.

Therefore, we do see this molecule, PR60 gag, as a docking molecule where protein or a signaling molecule such as C-ABL will dock to, or other putative molecule to send a single proliferation and reprogramming of these B cells.

So I will conclude with that, to state again that this is probably a docking site for C-ABL through the SH-3 domain, and the translocation of C-ABL to the plasma membrane in this system may release its block on the cell cycle progression and may give it access to novel target substrates for tyrosine phosphorylation.

The way we see the pathogenesis of this virus is summarized here in a cartoon where the
defective viruses, whether with a helper or without a helper, as we have shown, can infect a B cell which will start proliferating locally in the germinal center -- I forgot to mention that these cells are located -- these B cells are located in the germinal centers -- which eventually will progress, and at the same time influence other cells, either to progress or attract them.

I should mention that the infected cells in the huge lymph node sometimes represent only five percent of the cells of the node, a little bit like you will find with the Reed Stanford cells where the majority of the node is composed of normal cells. This is also the case here in most of the time, which is by itself an interesting biological feature of the system.

Consecutive to the growth of these cells, you do find in the body a defect in non-infected cells, which are themselves T-cells where you do find total anergy of all the B bearer T-cells, as well as defect of D cells. Obviously, the infected B cells are themselves un-normal as well.

So again, I'm just putting the same slide you saw before, which illustrates some of the characteristics and, in fact, you find many of them in
I'm not going to go through all of them here, but again to point toward one important aspect is that defective pathogenic viral genomes can emerge from nonpathogenic viral sequences, and that this viral gene products are involved in cell signaling and are able to interfere with the cells.

I will stop there, because time is running short, and I will thank my collaborator who has worked on this system. Thank you.

(APPLAUSE.)

My question is about set of data you mentioned on the insertional mutagenesis with this virus, and has relevance to what we've been discussing earlier about transfer of DNA.

If I get it right, you said that one mechanism also involved -- of the disease involves insertional mutagenesis, despite the use of very small virus inoculum. Right?

DR. JOLICOEUR: Yes.

DR. HENEINE: And you mentioned -- What is the number you mentioned about those viruses that you inoculate with -- the number?

DR. JOLICOEUR: Number of virus particle?
Infectious virus particle are in general $10^3, 10^4$.

I should stress here that the insertional mutagenesis is not an essential component of the disease. In other words, you do have disease when you do have a polyclonal growth of many clones.

The fact that some of these clones emerge and take over is not a feature -- is not a necessarily feature of the disease. In other words, to anergize T-cell, you don't need this novel event, if you wish, but it does occur presumably because there are so many cells being in proliferation that some of them are at an advantage over others.

DR. HENEINE: So it has no causal relationship, you're saying?

DR. JOLICOEUR: Not for the disease itself, no.

DR. HENEINE: Not for the disease.

DR. JOLICOEUR: No, but it occurs.

DR. HENEINE: Thank you.

AUDIENCE PARTICIPANT: I was very interested in your apparently pathology of B cells from the peritoneum into the germinal centers of the lymph nodes and ultimately the systemic tolerance of anergise in the T-cells.

Did you look at all in the bone marrow to
see if any of the B cells eventually resided, as I say, into the marrow?

DR. JOLICOEUR: If the B cell, the infected B cell?

AUDIENCE PARTICIPANT: Yes.

DR. JOLICOEUR: We take over the whole body. We know that initially it's not a B cell from the bone marrow which is infected. It's a B cell which is localized at the site of inoculation. We have done time course.

If you inoculate IP the very first cells are within the mediastinal lymph node. If you inoculate in the foot pad, it's in the right or left, whatever you inoculate, lymph node and so on.

So the target cell is localized at the draining lymph node.

AUDIENCE PARTICIPANT: Right. But do you know if any of the B cells from the germinal center in the lymph nodes eventually get back to the marrow where they could have --

DR. JOLICOEUR: They do, yes. They take over the whole body. Oh, yes, all over, including -- and quite infiltrating. Some of them infiltrate the liver, infiltrate the brain and so on. Some get quite aggressive.
DR. ROSENBERG: Thank you.

I'd like to thank all of our speakers -- Oh, one more? Okay.

AUDIENCE PARTICIPANT: What is there -- I'm Carlo Russo from Merck.

What is the receptor on the B cells?

DR. JOLICOEUR: Oh, this is the eco-receptor. We use -- In each case the helper has been the Moloney helper or the ecotropic helper. So, therefore, the cell that would get infected is infected through the eco-receptor.

Other group have used the amphotropic receptor for entrance, and they get the same disease.

AUDIENCE PARTICIPANT: And the mechanism of T-cell anergy, you think, is mediated by what?

DR. JOLICOEUR: This is a big mystery. One theory has been that it is a super antigen effect, but I think this has been quite ruled out by our group and other groups now.

It's totally mysterious. It's mysterious whether you need the contact with B cells to get this or whether it's a cytokine type of release from the B cells.

DR. ROSENBERG: If there are no more questions, we'll adjourn, I think, until eight o'clock
tomorrow morning when there will be more presenters.

(Whereupon, the foregoing matter went off the record at 5:30 p.m.)
Evolving Scientific and Regulatory Perspectives on Cell Substrates for Vaccine Development

Thursday, September 9, 1999

The workshop took place in the Plaza Ballroom, Doubletree Hotel, 1750 Rockville Pike, Rockville, Maryland, at 8:00 a.m., Naomi Rosenberg, Ph.D., Kathryn Zoon, Ph.D., and Keith Peden, Ph.D., Session Chairs, presiding.
PRESENT:

KATHRYN ZOON, Ph.D., Session Chair
NAOMI ROSENBERG, Ph.D., Session Chair
KEITH PEDEN, Ph.D., Session Chair
JOHANNES LOEWER, M.D., Panel Chair

STEPHEN HUGHES, M.D., Panel Chair
RON DESROSIE, Ph.D., Speaker
BEN BERKHOUT, Ph.D., Speaker

HSING-JIEN KUNG, Ph.D., Speaker
MAXINE LINIAL, Ph.D., Speaker
JOHN KAPPES, Ph.D., Speaker
JOHN PETRICCIANI, M.D., Speaker

DONALD BLAIR, Ph.D., Speaker
RUTH RUPRECHT, M.D., Ph.D., Speaker
EUGENE MAJOR, Ph.D., Speaker

HAIG KAZAZIAN, M.D., Speaker
BERNARD MEIGNIER, Speaker
GIRISH VYAS, Ph.D., Speaker
ALEX VAN DER EB, Ph.D., Speaker

THOMAS BROKER, Ph.D., Speaker
PAUL, SANDSTROM, Ph.D., Speaker
JAMES McDOUGALL, Ph.D., Speaker

BRIAN VAN TINE, M.D., Ph.D., Student
Speaker

SANDRA RUSCETTI, Ph.D., Panelist
PRESENT (Continued):

CLIVE PATIENCE, Ph.D., Panelist
LEONARD EVANS, Ph.D., Panelist
DAMIAN PURCELL, Ph.D., Panelist
ARIFA KHAN, Ph.D., Late Breaker
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Chairperson Rosenberg: Good morning. Please take your seats. We would like to start the session so that we try to stay on time.

This morning's session is going to continue. I think you'll hear many similar issues to those that we heard addressed yesterday afternoon. So we'll continue discussing retroviruses.

However, this morning we'll move more toward the complex retroviruses with presentations addressing issues relating to lentiviruses.

We'll start with our first speaker, Ron Desrosiers.

Dr. Desrosiers: Thank you very much. My laboratory -- excuse me -- my laboratory uses simian immuno-deficiency virus, SIV, infection of rhesus monkeys as an animal model for AIDS, and I'm not exactly sure why I'm here, but I think I'm here to --

(Laughter.)

Dr. Desrosiers: -- I think I'm here to give some examples of retroviral recombination from my experiments or where there may have been opportunities for retroviral recombination in our SIV system and
where we have not seen it.

So I only have three slides in the carousel, so I'll try to give some explanation of each of these three, and I should finish well ahead of time.

One of the things we've been doing in this SIV system is knocking out the so-called auxiliary genes. These are genes that are not absolutely required for the viruses' ability to replicate, but do contribute to the virus' ability to replicate in monkeys and to cause disease.

So we've done such experiments with the nef gene, the vpr gene, the vpx gene, and the vif gene.

When we introduced a premature stop codon into the nef reading frame to eliminate the ability of nef to be expressed and used that nef stop virus to infect rhesus monkeys, there was very quickly -- by two weeks after infection of rhesus monkeys, all we could detect was revertant virus, that is the premature taa stop codon had universally reverted by two weeks of monkey infection to a stop codon, indicating that there was strong selective pressure for open functional forms of nef in vivo, and that such revertant viruses, although they might be rare,
appear rarely as a rare event. The strong selective advantage of such a virus can very quickly result in that virus being the vastly predominant virus.

We've also made forms of virus with a large, gaping deletion in the nef gene, and infection of monkeys with such nef deletion virus shows that this nef deleted virus is clearly an attenuated virus. It replicates much less than the parental virus, and is much less prone to inducing disease.

So one of the things that we've done is to look very hard to see whether or in which ways a virus, such nef minus virus, could regain pathogenic potential.

We've looked both in cultured cells producing this nef deletion virus and in rhesus monkeys infected with nef deletion virus for possible recombinant viruses.

In discussing use of such strains as experimental vaccines, the question has been raised whether such viruses could possibly acquire cellular sequences in an analogous way, the way the Type C retroviruses can acquire oncogenes or other such genes to bring back nef function or to provide some additional oomph to the virus to replace the loss of nef.
And despite extensive efforts looking for such recombinant viruses, where there would be strong -- so in the absence of nef, there would be selective pressure for capture of any gene that created a selective advantage, and we should be able to see it in vivo because of the strong selective advantage that could impart.

And despite extensive efforts looking for such viruses, we've never found such recombinant viruses that have picked up extraneous sequences.

We've taken animals infected with nef deletion virus, and we've serially passaged virus in rhesus monkeys, and we have been able to increase the pathogenic potential of such nef deleted SIV by serial passage in monkeys, and the increased pathogenic potential is not due, at least in the two lineages we've looked at, is not due in either case to capture of cellular sequences or extraneous sequences, but is actually due to compensatory changes in SIV elsewhere in the genome that allow the virus to make up for the loss of the nef gene.

So similarly using nef minus virus, vpr minus virus, vpx minus virus, and vif minus virus, we've not seen any examples, despite extensive efforts looking for it; we've not seen a single example where
the virus has captured an extraneous sequence and made it increase selective advantage.

Now, a few years ago we published a paper where we attempted to directly look for -- we designed an experiment to see if we could directly demonstrate retroviral recombination in an infected monkey. So this is now -- we're not talking about recombination with cellular sequences, but recombination between two different SIV strains.

So what we did -- how do I go backwards here? -- so we used two different SIV strains for this experiment. We used one strain with the deletion in nef, and the second strain was deleted in both vpx and vpr. That is about four kilobase pairs away.

So we inoculated the nef deleted virus in one leg, and we inoculated the vpr/vpx virus in the other leg. Both of these are attenuated viruses.

By two weeks after infection, as reported in that Journal of Virology paper, by two weeks after infection all we could detect is wild type virus resulting from a recombination between these two different strains.

For recombination to occur, one needs infection of the two different retroviral strains, infection of the same cell, and one can easily achieve
that in cell culture, but there had been some question
how likely an event that would be in a whole infected
organism with the massive pool of cells, and obviously
at least initially a very, very low multiplicity of
infection.

For such recombination to occur, as I just
said, you need infection of a single cell with both
strains, and although at least theoretically there are
a number of or several different mechanisms by which
retroviral recombination can occur, the major way such
recombination occurs relates to the fact that virions
contain two RNA molecules, and when you have two RNA
molecules in a single virion of the two different
genotypes, when a cell is newly infected through the
reverse transcription process and the copy choice
mechanism in the reverse transcription process, one
can then get recombinant viruses, in this case
representing a wild type sequence and fully
pathogenic.

So these experiments illustrated two
things. They illustrated the ease with which
recombination can occur even in vivo in the large pool
of infected cells in the rhesus monkey.

And the second thing that they illustrated
was the enormous power of selective advantage and how
selective forces can very quickly selected for strains
of virus with a growth advantage.

And the final thing I want to talk about
is a virus deleted in the vif gene. So although vif
is generally considered an auxiliary, nonessential
gene, which it certainly is with HIV for growth in
some cell types, it turns out that vif in most cell
types for both SIV and HIV is very important for the
virus' ability to replicate.

In fact, for the strain of SIV that we
work with, the only way we've been able to grow a vif
minus virus is in a vif complementing cell line.

So this shows the growth curves of vif
deleted SIV, which has been produced in a vif
complementing cell line. Growth of this vif minus SIV
in the vif complementing cell line, viv CEMX compared
to growth of the same virus stock, same amount in the
parental line CEMX 174.

Now, the vif complementing cell line
contains the full vif open reading frame, and the vif
minus the deletion in the vif gene in the virus that
we used is within the vif reading frame. So it is at
least theoretically possible that there could be
recombination in this complementing cell line between
the vif minus viral sequences and the vif gene.
present, integrated in the host cell genome used for
growing the virus.

And obviously if there was a recombination
for vif-plus virus, that virus would have an enormous
selective advantage both in cell culture and in
monkeys, and we could quickly, quickly see it.

So we've made a number of vif-minus virus
stocks and vif complementing cell lines. We've
infected at least six different monkeys with vif
deleted SIV grown in vif complementing cell lines, and
we've not seen a single example of virus regaining the
vif sequences from the cell line that it was grown in.

That's basically the sum total of my
experiences involving recombination either with viral
sequences or cellular sequences in the SIV system.

So I'll stop there and take questions.

(Applause.)

DR. KUNG: Hsing-Jien Kung, UC-Davis.

Hi, Ron.

DR. DESROSIERS: Hi, Hsing-Jien.

DR. KUNG: So much of the -- in terms of
detection of the recombinants, usually you need a
selection. So I was asking you in the nif experiment,
did you grow the virus on just wild type non-
complementing virus in an effort to pick up -- to
actually pinpoint the selection of that virus?

DR. DESROSIEERS: Well, the --

DR. KUNG: The vif, the last experiment you were talking about. You said you didn't see the pick-up of the host gene.

DR. DESROSIEERS: That's correct. So vif minus virus does not grow in CEMX 174 cells or other cell lines, but the vif plus virus does. So we've transfected vif minus SIV DNA into the vif complementing cell line to make virus stocks. We've expanded those virus stocks in the vif complementing cell line.

If the vif minus virus had picked up a vif gene, it would now grow in a non-complementing cell line, and we've repeatedly tested our virus stocks, undiluted, large amounts. We've taken undiluted virus stocks and put it into six different monkeys, two different stocks prepared independently. We've never seen -- there there was clearly the enormous selective advantage of minute amount of vif plus virus would quickly grow out, and we've not seen it in the examples we've done.

MR. BROKER: Tom Broker, UAB.

In that last experiment, how much flanking homologous sequence might you have had, and can you
consider expanding that outward to ask whether you'd favor recombination with enough flanking homologous things allowing crossover?

DR. DESROSIERS: Yeah, I don't remember off the top of my head exactly how much, but it was not a lot. It was maybe 100 base pairs or so flanking on each side, but you're right. I think if one wanted to investigate it, I think that would be a pretty good system to do it.

MR. BROKER: It would be great.

DR. DESROSIERS: could expand out further and further and see under what conditions one might see it.

MR. BROKER: Okay.

DR. DESROSIERS: I think in the experiment when there was recombination between two retroviruses, I've always kind of hoped that people would have used that system a little bit more, too. One could actually -- we never went to the trouble to try to look. One could actually use that system to look at where the recombination events were occurring by putting in third base changes. You could actually map where the crossover, quote, crossover events occurred, and we never took the trouble to do that.

But I think anyone who's interested in
studying recombination in retroviruses, that would be
a pretty nice way to do it, but, no, we've not done
that experiment.

It was a short stretch of sequence. We
were actually -- for the experiments we wanted to do,
we wanted to avoid getting recombinant virus, and so
we kept the length of overlap short, but if one wanted
to study it, exactly right. You could just expand the
length and see if you had a much larger length of
flanking sequences, whether you had increased chances
of seeing recombination

PARTICIPANT: It's really an extension of
the same experiment. We know that retroviruses will
promiscuously package all sorts of cellular messages.
Do you know within the virion population that's coming
out of the complementing line whether you actually do
get vif message actually packaged in the virions that
might be a substrate eventually for a strand jump
recombination?

DR. DESROSIERS: Yeah, that's a -- we
haven't actually looked at it. My mind is turning in
theoretical terms, what that means. It's generally
believed that vif minus virus in a noncomplementing
cell line is capable of a single round of infection
events.
PARTICIPANT: Sure.

DR. DESROSIERS: It can get into one round, but then not pass. I think there's really only room in the virion -- some of our retroviral experts here can correct me if I'm wrong -- but there's really only room in the virions for two RNAs, and I think if a virus did package cellular RNA and that would leave room at most for a single viral RNA.

I think that would result in a defective particle, would not be infectious, but still could be propagated as a defective virus conceivably anyway in the presence of a helper.

CHAIRPERSON ROSENBERG: I need to remind the questioner to give their names.

DR. LINIAL: Maxine Linial, Hutchinson Center.

About your second point, we have shown with ALV that you can package a full length genome and a cellular RNA and get recombination. So it can occur.

But my main question was: how much replication did you get with the delta vif virus, and if you did see any, in what kind of cells was it replicating in the monkeys?

DR. DESROSIERS: The vif minus virus?
DR. LINIAL: Un-huh.

DR. DESROSIERS: The only way we were able to replicate the vif minus SIV is in the vif complementing cell line. So the vif minus virus, we've tested it. We can clearly generate high titred stocks in the complementing cell line, and we've used that to infect a variety of cell lines, primary rhesus PBMC, primary macrophage cultures, and inoculated into monkeys.

And in cell culture, the only way -- the only system we detect any replication is in the vif complementing cell line.

In monkeys, the vif minus virus is at the very most -- the very least the most attenuated strain we've seen. The only thing we've been able to measure in the delta vif inoculated monkeys is very, very weak antibody responses.

CHAIRPERSON ROSENBERG: Thank you.

The next speaker is Ben Berkhout.

DR. BERKHOUT: Okay. I would like to continue on some of the themes that have been introduced by the previous speaker, and I guess we can quickly go over the introduction.

Of course, most of you should be familiar with the idea of live attenuated viruses. These
entities should replicate and elicit supposed immune
response that confers lifelong protection against
challenge with a pathogenic wild type virus.

Of course, the concept has proven to work
in several systems, for instance, vaccinia, polio, and
measles, and for HIV-1 good results have been
described in SIV infection models of macaques by
several labs, including that of the previous speaker,
and it has been proposed recently by some groups that
one should start clinical trials on humans with the
HIV-1 versions of live attenuated viruses with
multiple gene deletions.

But there are still several safety
concerns. One of them is the induction of a fulminant
infection in immunocompromised hosts, and second, and
that's the one I would like to focus on today, is the
potential reversion of an attenuated vaccine strain to
a virus variant that can replicate fast and that
potentially can cause AIDS.

Now, all of the studies I will present are
done in tissue culture settings. So we will only look
at replication potential of viruses, and therefore, of
course, we don't have any measurements of
pathogenicity.

Now, let me start out with a purely
hypothetical graph. The idea here is to delete genes. As you know, HIV-1 has nine genes; simple retroviruses, only has three. So the idea is that we can probably removed a couple of the accessory genes and thereby we will credibly decrease the replication potential of such a virus. Probably that also has a consequence for the pathogenicity of this virus, and I did draw parallel curves. There's no evidence for that, but I think it's reasonable for this graph at least. Probably also the immunogenicity of these viruses will slowly drop off, and the idea is to reach a level that is below a threshold for the pathogenicity such that this virus will not cause AIDS. However, of course, replication should still suffice to induce a good immunogenicity, and of course, these thresholds are, again, hypothetical, and we don't know how big this window is. Even if you managed to identify such a virus that has these properties, one of the key questions is how stable is this virus and will it perhaps not be able to regain replication capacity and thereby going over this threshold. Now, why should we release genes? We
already heard in the previous talk that when you put stop codons in, for instance, the nef gene, it's repaired in two weeks. Even small deletions or substitutions will be removed by this virus very rapidly, and that was shown early on in a paper published in '95 using the SIV system and virus evolution in macaques.

A four amino acid deletion was introduced in nef, and that's shown here. It was still present two weeks after inoculation. However, after 17 weeks, the virus managed to repair this sequence, at least to fill in this gap, by duplicating the sequences that are located over here.

Officially at this point in time, you do not have the wild type sequence back, but credibly after 25 weeks, we do see a wild type tyrosine appear at this position. It's like the wild type, and after 45 weeks, we also have an aspartic acid here, and we are almost back to the wild type sequence demonstrating the enormous repair capacity of this virus.

So small deletions will not suffice and we, therefore, have to make bigger deletions.

Now, the system we are going to use is a tissue culture evolution system, and I will use one
slide to introduce the system. The method we have
called forced evolution. Basically we start out with
a molecular clone of HIV-1 in which the deletions in
accessory gene products have been introduced.

We start with a massive transfection. Ten
to 40 microgram of molecular clone is electroparated
(phonetic) into cells, and then initially the cells
are cultured, but when there is a sign of virus
replication, it's seen in either syncytia or GAG E24
production. We start to passage the culture
supernatant onto fresh and infected cells.

Initially these large inocula up to 1 mL,
later on credibly reducing the amount of virus that is
transferred.

So in Section A we will be able to pick up
revertants, and I guess it's important to realize that
this reversion or evolution is a two-step process.
One needs mutations, and those will be introduced
primarily by the first transcriptase enzyme, which is
error prone. Of course, these mutations will be
introduced in a random manner, and then will get
selection of those virions that are better than the
input virus.

So its evolution, as I guess it must be
proposed by Darwin, although he used different terms.
It's variation and survival of the fittest.

Now, because I think a presentation may not be complete without seeing an RNA stem loop structure, I actually show you one example of a study that we performed on a stem loop structure. That's the so-called TAR hairpin that is found at the extreme five prime and three prime end of HIV-1 genomes. This is the wild type situation.

This is a mutant that is completely replication defective. Here we have open to the lower left-hand side of the stem, and after about six months of culturing initially the cells, later on the virus passage, we ended up with this revertant. It has one, two, three, four substitutions. It doesn't go back to the wild type sequence, but what this revertant is telling us is that this hairpin structure is critical. Sequences are less critical, but one needs a base pair lower region here, and of course, we also have some idea of what this hairpin is doing. It's critical in transcription regulation of this virus, but also because it's present in both ends, it plays a role in the mechanism of reverse transcription.

So we obtained a rather complete set of deleted HIV-1 genomes from Ron Desrosiers, and I will today only focus on one of these deletion virions, and
that's the so-called Delta 3 virions with three

deletions.

One deletion is in the accessory vpr
protein, and this five prime half of the genome was
combined with this one, which has a deletion in the

nef gene. Then some of the sequences here at the
border of the three prime LTR are still present
because they are important for replication.

And then there is a second deletion in the
three prime LTR.

Now, it's important to realize that this
deletion in the LTR will be inherited in the progeny,
also in the five prime LTR, and the five prime LTR, as
you probably know, is the transcription promoter at an
untelldish (phonetic) virus.

Over here I've indicated some primers that
will be used in subsequent PCR analysis to actually
check whether these introduced solutions are still
present.

One more thing. We tried to do efflution
phonetic) in tissue culture in primary cells. That's
indicated over here, but in particular, for this Delta

3 variant we found that the replication, while that
much delayed, that it probably will take years to
really obtain the fertent (phonetic) viruses.
So to solve that problem, we are going to use a transformed T cell line, in this case Sup. T1 T cell line, because there the replication defects of this kind of mutants are less feared (phonetic). There certainly is a replication, delay in replication, but it's much less feared than in primary cells.

So the virus with three deletion was passaged for up to four month in the Sup. T1 T cell line. Samples were taken at day 14, 53, 83, 125, and we analyzed the samples with similar input virus amounts in a parallel infection, and as you can see over here, the initial virus or the virus present after two weeks has a hard time replicating, and then you see a gradual increase in replication capacity and a big jump actually is seen already at day 53.

So we were interested to find out what has happened with this virus. So we performed PCR essays, PCR analysis. PCR analysis across vpr gene didn't show any insertion or deletion of sequences, and in fact, the sequenced region, nothing has happened.

This is a PCR across the nef and LTR deletion, and as you can see over here, something happens around day 55. We see the appearance of a variant that is about 14 base pairs longer, and
apparently this variant is much more fit than the
input virus because it out competes the input virus
within a couple of weeks.

So at this point we got excited. We
thought, well, perhaps there is an insertion of
perhaps cellular sequences in these deleted virus, and
of course, we sequenced this region.

It turned out that the two introduced
deletions were maintained. The nef deletion was there
and also the deletion in the U3 region was perfectly
maintained.

There also was no insert of cellular
sequences, but what has happened is that in the core
promoter region, and as indicated here this is a
transcription start (phonetic) side, three binding
sites for the constitutive transcription factor, SP-1,
and a tandem repeat for nf kappa B binding. The
deletion that was introduced is indicated over here.
Nothing happened over there.

What we did see is a gross duplication of
all the three SP-1 sites. In addition, there are
seven nucleotides of unknown origin, and we have seen
an onser (phonetic) duplications.

Yesterday in one of the talks, and I must
say that this apparently is a very popular theme in
mutant retroviruses, they easily change the number of repeat elements in their genome, and in fact, we probably know why that is so, because this duplication is probably introduced during the first inscription by a mechanism called slippage realignment.

So RT enzyme has probably copied the sequences over here. Then RT and the cDNA is partially removed and it reanneals back to the original sequences, and in one step one gets a duplication of three SP-1 sites.

So we end up with this promoter. Does this make a better transcriptional promoter?

(Unintelligible) in transient LTR reporter assays, and what we compare is the wild type LTR, the one with the U-3 deletion, and then the one that's the duplication of SP-1 site. So as you compare the last two bars, we tested that in the absence of the transcriptional activate of protein TAT and also in the presence and then in cells that were not more activated by PMA and PHA.

In the absence of TAT protein, we, in fact, see that the LTR promoter does not gain function by duplicating of the SP-1 site. In fact, it gets a little worse.

However, in the presence of TAT protein
and, of course, in virus replication in this system, the TAT protein will be around. We do see a partial restoration of the LTR function, and in this case actually it's becoming a little better than the wild type.

So the transcription promoter is, indeed, improved in the presence of TAT protein.

Does this single mutation also improve the replication of the delta 3 virus? Well, indeed, it does, and that is shown here. So we reconstructed a molecular clone with three deletions, and then in addition the six SP-1 sites. So here is the wild type virus.

If you only delete vpr there is a small replication. So this is the input virus with three deletions, and if you in this virus then introduce the six SP-1 sites, there is a rather dramatic increase in replication capacity. In fact, this revertant virus replicates much faster than the virus with a single vpr evolution.

Now, we thought it was also of interest to test the replication of this virus not only in the Sup. T1 T cell line, in which it's evolved, but also in primary cells, and that's shown here, and there we do see a different picture.
This is the wild type virus replication in primary cells, and over here we see that there hardly is a difference in the replication of the original delta 3 virus and the one with six SP-1 sites. So it seems that the SP-1 site duplication is beneficial only in the Sup. T1 T cell line that was used for the evolution experiments. That may have to do with the pool of transcription factors that are present in that particular T cell line.

And, for instance, it has been reported that Sup. T1 cells have an extremely low amount of nf kappa B, and one can imagine that perhaps, therefore, SP-1 is more important for this virus. So just to sum up, we show that this delta 3 strain is genetically unstable. It retains replication capacity in tissue culture, and we have seen duplication of SP-1 sites.

Now, this is all tissue culture, but I think there are some additional evidence to suggest that these viruses are unstable. The Ruprecht laboratory has recently demonstrated that some of the viruses, the delta 3 viruses, do eventually cause AIDS in infected monkeys, and there's also some evidence from cohort studies in humans where people infected with a nef deleted and, therefore, attenuated virus
strain do over time show a decline in CD-4 T cell numbers and, therefore, perhaps they are at risk for going on to AIDS.

So the conclusion will be that these deleted viruses are safe.

Now, in the context of this meeting, I should say that the one example I showed you today we did not find any incorporation of cellular sequences in these -- in these deletion virions, and like the previous speaker, in all of the evolution experiments that we have done so far, we have not come across any insertion of cellular sequences in these virus strains.

Now, finally, I will discuss a putative route to perhaps a more safe vaccine. What I showed is that this deleted and attenuated virus is able to regain replication capacity, and that's indicated by this arrow.

Now, perhaps this virus may be a nice starting point to remove additional functions, thereby again going down on the replication letter. Perhaps then again we can try to evolve a faster replicating variant, and by repeated cycles of gene deletion and subsequent evolution, we perhaps may end up with a virus with only three to five genes that is able to
replicate efficiently, and that perhaps will be more stable in genetic terms.

And with that I would like to stop.

(Applause.)

CHAIRPERSON ROSENBERG: Is there a question?


This may be a silly question for someone --

CHAIRPERSON ROSENBERG: Be sure to identify yourself clearly.

MR. FALLEAUX: Yeah, Frits Falleaux, right.

This may be a silly question for someone from working with adenoviruses and also HIV, but did someone think of trying to make an attenuated to HIV which is, in part, replication efficient and in combination with, for example, a useful promoter that dries (phonetic) nef so that you have partial replication in the presence of the inducer, which you take away after the introduction of protection?

DR. BERKHOUT: People have worked along these lines. For instance, one has introduced the constitutive CMP promoter in the context of a replicating HIV-1 virus.
One of the problems with introducing exogenous sequences in this final genome is that in most cases the virus doesn't accept them, and they are over time kicked out of the genome. So the only way to safely introduce exogenous sequences in this final genome will be to introduce elements that are absolutely required for replication, and then I think, indeed, it would be of interest to study better and inducible promoters, but that hasn't been tested so far.

DR. EVANS: Leonard Evans, Rocky Mountain labs.

Did you try to put that promoter, the duplicate promoter back into the wild type?

DR. BERKHOUT: We did, and in straightforward replication curves, there is no difference in replication. If you do fairly sensitive competition assays so the wild type virus, and the wild type has six SP-1 sites, you do see that in the wild type context, in fact, the fitness goes down a little bit. So --

DR. EVANS: It goes down?

DR. BERKHOUT: Yes, it does down a little bit.

DR. EVANS: Okay.
DR. BERKHOUT: But that difference is marginal. So you have to do very sensitive competition assays with the two viruses to find out. So six SP-1 sites are clearly beneficial in the context of this deletion variant, but they don't make wild type virus much better.

DR. EVANS: Okay. Thanks.

CHAIRPERSON ROSENBERG: Thank you.

The next speaker is Hsing-Jien Kung.

DR. KUNG: Thank you.

So in today's talks and yesterday's, you have heard that retrovirus have significant potential to recombine with another retrovirus or recombine with host genes under selective pressure. Today I'd like to discuss with you about experiments that I would summarize as experiments regarding recombination between retroviruses and the herpes viruses.

If I can have the first slide.

Let's consider the following scenario. In co-infected cells with the retrovirus and the herpes virus, retrovirus copying to RNA, then into DNA, RNA copying to DNA, and has a choice of going into the cell or the genome or has the choice to go into the herpes viral genome if in the co-infecting cells. We can argue what is the probability.
What is the chance? Well, based on the most simplistic view, the simple calculation, simply based on the mass ratio, you would argue that if the herpes virus -- and let's take the worst case scenario. It's only present as one to ten copies -- one copy, let's say, in the latent state. Based on mass ratio, you would calculate that in every 10,000 integration into the host genome, you would have one into this size of herpes virus.

Now herpes virus sometimes will also replicate. So it can reach -- so okay. Let's take that calculation and say you'll have massive infected cells, about a million cells, in fact, with retrovirus. Then there should be 100 integrants if herpes virus is in latent state.

Now, herpes virus, of course, also can replicate, let's say, 2,000 copies of 10,000 copies. If it were 10,000 copies, then every integration into the host chromosome, there's a chance into the herpes virus genome.

So if you calculate, you say this is not too rare, but, on the other hand, you can make the argument that herpes viral replication usually are combined in certain subnuclear structure, and they may not be as successful to the retrovirus integrative
complex, and they may be encapsulated very quickly,
and so all of these theoretical considerations may
also argue against genotypic mixing or recombination
between these two.

Okay. So I think only experiments will
tell.

Next slide, please.

So today I'm going to talk about
retrovirus integration into herpes virus. The system
under study is avian retrovirus using reticular
endothelial virus as a model system, REV. It's a
typical nonacute Type C retrovirus that cause T cell
lymphoma and the B lymphoma in chickens, and we show
before that the mechanism of oncogenesis is through
insertion or activation of C mixing.

Avian herpes virus, Marek's disease virus,
is very, very prevalent. Until 1970 that was the most
important economic loss in the poultry industry, until
the live attenuated vaccine was developed.

Now, both viruses infect T cells, infect
the same T cell types. The tumors are derived from
very similar T cell types. So co-infection did exist
and has been observed and reported in many papers, and
in fact, many of the retroviruses were isolated from
Marek's diseased chickens, including the chicken
syncytia virus strain of the REV and avian myeloblastosis virus, some of you probably know, that carries the mip gene.

So there is a preponderance of evidence that these two viruses coexist in chickens.

So the implication of the study -- I will show you some experimental evidence, but the implication of the study is, of course, that you can generate a hybrid virus with altered gene expression patterns, which I will show you, and the phenotypes, emergings of new pathogens, if you will, and can certainly sometimes broaden the host range because some of the herpes virus, indeed, can carry retroviral information.

So with that in mind, let's first tell you a little bit more about the herpes virus, this Marek's disease virus. This virus, although it's lymphotropic, really the structure is very much similar to alpha herpes virus with the repeating sequence flanking the unique, long reaching, and repeating sequence flanking unique short.

And just in a typical alpha herpes virus, you are reaching and encode mostly structural and replication enzymes. In fact, this virus, now Lucy Li has entire sequence and, indeed, show a strong
correlation with herpes simplex virus.

Unique small region also encodes some of the genes, however. They are not important in the in vitro replication.

Now, the interesting part are usually confined in the more, but divergent region, in the repeat region, and we now know that in the Marek's disease virus that we have worked in the past few years, that it encodes a protein that we can call it oncogene, called the mac (phonetic), which is in the Joan Foss family of loosing zipper protein, and if you remove this, the virus becomes nononcogenic.

Okay. So in this sense the virus is similar to herpes simplex virus, but it's not, due to some of the coding sequences in the repeat region.

Okay. But for our purpose, I'd like to point out this virus is extremely oncogenic, probably most potent oncogenic herpes virus. It causes tumors within four to six weeks in experimental animals, and these tumors are of polyclonal origin. Okay. So it smells like this oncogene does do something in a direct way.

But today we'd like to talk about how this virus can be used as a template for retroviral insertion and what happens after that.
Just to give you a sense, this virus is probably, again, the most successful live attenuated virus vaccine against oncogenic virus. I'll tell you a little bit about the vaccine.

The serotype 1 is oncogenic strand, the JM MD11 and GA stand, but serotype 2 and 3 are vaccine strands. They share about 70 percent homology with serotype 1. However, they are not oncogenic in chickens.

Now, there's another way of making this virus attenuated virus, by taking serotype 1, okay, and simply passage. It's a mysterious way, but it worked every time. You passage for a long time. It could be five to ten years experiments, but you would get attenuated virus.

People still do not know exactly what happened, but there is a correlation of expansion with certain repeat sequence in the region. We now know these attenuated viruses actually still maintain the mac oncogene. So what happens most likely is that these viruses do not replicate very well. Therefore, they cannot induce T cell lymphoma in chickens.

In vitro, however, after long passage viruses tend to replicate quite well.

Okay. So we said that moving to the real
experiment. Now, our story began with this particular
experiment. They were intended to study our REV -- at
the time it was still retrovirologist -- REV insertion
of the T cell lymphoma.

        So Bob Isford took some of the T cell
lymphoma generated by Marek's disease virus and looked
for whether REV virus is present or not.

        It turned out REV viruses are not present,
but there was some surprise in that in the low passage
of serotype 1 virus, not serotype 2 or 3, that he
actually could detect hybridization. This
hybridization was under 30 percent mismatched, but he
could detect hybridization against REV or TR.

        And this suggests to us there are some
sequences related to REV, and we call it ALTR remnant
present in the present day serotype 1 viruses. So
this virus has never seen REV recently, but you can
see that they do have some sequence.

        The fact that they show specific bands and
they can map in the band D and band F region, which is
close to the repeat sequence, convinced Bob Isford at
that time that these are not a fluke. These are
probably real homologous sequence even though it has
diverged significantly.

        There are the high passage one now,
attenuated one. You can see that the sequence begin
to diverge because of expansion of the repeat
sequence.

However, in this, this is something else.
I will come back to this. This GM high virus has
multiple LTR related sequence. I'll come back to
this.

So then Bob Isford began to sequence of
them. So just to give you summary, indeed, there are
several patch homologies ranging from 70 to 81
percent, with some nucleotides ranging from 22 to 33
nucleotides.

Now, if there's only one site, you can say
this is very skeptical, but with all of these sites
clustered together in the right order and many of them
diverging at the junction of the retroviral genome,
and that suggests to us this probably is real.

So we took that as LTR remnants. There
are also some sequence related to the retrovirus, but
the LTR sequence was most, most prevalent.

And this stretch of sequence is most
interesting. This stretch of sequence turned out to
be that it's in the enhanced region of the REV LTR.
This sequence now is present as in the enhancer region
of the herpes virus alpha tif or the VP-16 equivalent
of the Marek's disease virus, and it's a T cell tropic enhancer.

So with that we figured that in nature this had happened, and let's see what happens, whether in recent -- this is what we call the ancestral insertion. We like to see whether in recent time whether there was any evidence of recent insertion, and we recall this one, the same picture.

This one has a multiple one, and now again, this is a virus derived from low passage, simply by culture them for five years. Okay? This is a passage of 211, and it's completely attenuated, and they can be potentially used vaccine, but this is a vaccine experiment that went exactly as intended.

So what happens once we discover there's some retroviral insertion, and these LTR hybrids under stringent condition and still stick to the filter paper. So we know these are more recent insertion.

And Dick Witter, our collaborator, collected all these viruses. So he then did the following smear by cloning this separately. So with that he actually was able to separate these clones and these LTR sequences of segregate, indicating indeed they are part of the viral genome, and they are genetically stable because they have been passaged for
about 200, 200 passages.

And so what happened is that during this long term propagation you'll feed DEF, duck embryo fibroblasts, the primary cells. We talked about it two nights ago, that DEF, and it turns out in passage about 87, that DEF they used for fuller infection by Marek's disease virus turned out to contain reticular endotheliosovirus. So during this evident of clone mixing fraction, that retrovirus integrate that, but now they become very stable.

Now, you would argue why do they persist for so long. Well, when Dick Witter compared the replication rate with the wild type, they all in vitro replicate much better. We do not know whether that's due to LTR insertion. However, the LTRs seem to be a persister, and the LTR integrants seem to dominate the culture at passage 211.

So this was a five year experiment ten years before we began the study. We could not control. We could not add any control. So Dick Witter and I think that if this happened nature, on an evolution or scale; if it had happened in the ten years prior to our experiments, can we do a more controlled experiments by doing a co-infraction (phonetic), and can we make this work in five weeks or
So the experimental protocol turned out to be very straightforward, that you mix the MDV, infected DEF with REV. MDV is cell associated virus. So it's actually a little bit difficult to do experiment, but if you mix them together, you passage them every week, and then by feeding fresh DEF, okay, and then we took individual passage mass culture, isolate MDV, and in fact just isolate the cell and look for MDV mini chromosome imposed fiogel (phonetic), and the free retroviral DNA should run out of the gel, and then you probe with REV LTR to monitor the kinetics of integration. It turned out it's not difficult at all. Okay. We have repeated this several times now. Basically after, in fact, five weeks you can see a little bit. This is a southern blot. So it's not a most sensitive method, but you can see that retroviral LTR integration increase, okay, after passage 16. It's a huge amount radioactively. This is just a load to show you that MDV DNA were loaded at about the same amount. Now, if you do TRP CPCR, you could actually detect within one to two passage. Okay. So the integration certainly can be very efficient,
although as I said, this is radioactive. You really do not know what is the population of the integrants versus no integrated one.

Now, this increasing intensity, of course, are due to two reasons. One is that REV is still present. It can infect more herpes virus, but, secondly, of course, it may be the herpes virus with LTR, in fact, replicated better in some fashion.

There is no selective pressure except in vitro replication. So this may actually contain the LTR integrate certain places that can enhance the replication.

So what Dan Jones did and Rhonda Koss did was -- were to actually look at the insertion sites, and then something rather interesting was revealed.

So by looking at -- this is 17 kilobase genome, but they looked at the integration sites. Integration sites are tightly clustered with two insertions in the GD region, but others are tightly clustered around the -- close to the boundary between repeat and the unique sequence.

Now, at Alsets (phonetic), if you look at the sequence, okay, just very briefly, you found actually these are the individual integration sites. I do not mean to have you look at the sequence, but
look at the arrow indicating that there's no sequence facility. It's a regional facility.

Okay. Now, at the outset we knew the integration should not be totally random because we were selecting viruses, replication virus. So any insertion in the essential genes would disrupt its ability and we may not be able to pick up. So, again, this underscored the importance that if you want to study some recombinant, that the selection pressure turned out to be an important one.

So we figured that maybe these are the sequence, the regions. There are no coding sequence.

Therefore, it integrates better, but that still cannot fully account for this tight cluster because the U.S. region shown by Robby Morgan and Marc Purcell can be completely deleted, yet in vitro replication, and still very viable.

So we then began to think this actually also looked very much like insertion of mutagenicity oncogene that we studied before, especially the RB oncogene in avian erythroleukemia in terms of the tight clustering.

So we began to entertain the hypothesis maybe this can activate some of the genes near the boundary.
But before I say that, before I show you the data that I have, we have to isolate the virus in order to do the experiment. This is a simple whole cell PCR mapping insertion sites. We did not have the virus yet.

So in the past few years Dick Witter was able to isolate the virus. This was a heroic effort because the retrovirus that we used had no markers, no selection marker. It's a wild type retrovirus. So it depends on how prevalent these integrations are in the whole mass population.

So Dick was able to isolate several, and I'm just going to talk about one, quote, RM-1. For the first retrovirus, the REV MDV hybrid virus number one, and this is very interesting because it contains a solo LTR. No other retrovirus sequence are present, and it integrates at a hot spot, the tight cluster area.

And this shows the retrovirus duplication of the MDV genome. So it's authentic integration. It has a solo LTR, our first in this region, and then it homogenized to the other region as well.

Now, the most interesting is the phenotype of this virus. This virus, okay, RM-1 -- these other
two are the clones -- had everything. Everything else
is very similar to the wild type in that it can
replicate very well inside bursa, inside T cells in
the chicken.

The only thing different is it has no
oncogenicity. Okay? So this is the viral clone that
did the test, in vivo test, and this is the wild type,
okay, the oncogenicity seven out of eight, five out of
eight, eight out of the eight, and this is the
attenuated one. Of course, after long passage it also
has no oncogenicity.

But this virus N and attenuated virus
differ significantly. This virus can replicate very
well, whereas the attenuated virus does not, do not
replicate very well in vivo.

Okay, and as a result, when Dick Witter
did the challenge protection experiment, so in fact,
it was RM-1, then challenged with the virulent, very
virulent MDV, it turned out this has much better
protectivity than the other attenuated strand.

And this is showing here -- maybe we could
just locate the protections. So this was done by
taking the RM clone, infect the first as a vaccine.
Then you challenge it with the very virulent virus,
and it turned out the protection is 100 percent,
whereas the current vaccine virus is 34 percent, which
this is the Basta (phonetic) vaccine virus, about 78
percent, and so on and so forth.
And the reason is that it replicate very
well inside the chicken, the only things that cannot
cause oncogenesis, and I'd be happy to speculate to
the reason why that's the case, but also because it
can spread very well. So it serves as very good
protection.

Okay. Now, I don't mean to say this is
the vaccine virus because it causes other associated
diseases. Other than oncogenicity, it does cause
thymic atrophy, like the wild type. So it cannot be
a vaccine yet, but this certainly gives us some clue
as how perhaps to manufacture better vaccine for MDV.

But in the context of this discussion,
this shows that LTR insertion can change the phenotype
of the virus. We look at other regions, whether
there's any gross change. There's no gross change,
but we cannot rule out point mutations.

So now, at the molecular level, we'd like
to see whether it activates anything. Indeed, this
RM-1 has a solo LTR integrate in there, and these are
the northern blot to show, indeed, there is LTR
insertion in here, and there is a transcript with a
link to LTR from here to here. That would transcribe polysystronic message carrying soft 2 open reading for US-1 and US-10.

Since this is the closest to the five prime end, we think this product may be relevant. We don't have evidence to show that this is relevant. We're just beginning to do that, but I can tell you a little bit about it. This is a novel sequence, unique to the oncogenic herpes MDV strain virus. The sequence is novel. So we do not know the function, but it does share some homology with US-22 gene family of this cytomegalovirus as well as HHV-6.

In the case of HHV-6, this particular protein, it's not the same protein, but it shows some similarity. It has been implicated in the transactivation of HIV.

So we think it may be a co-factor of the transcriptional factor. Indeed, recent experiments show that by Hous Chan's lab that this is a nucleoprotein.

I cannot tell you more about this simply because we don't have much data about this protein yet. So let me give you a conclusion.

So what I have shown you is that we have shown retroviral insertion of herpes virus at least in
this system at several levels. We show there's ancestral insertion; that avian retrovirus REV LTR remnants are present in the serotype 1 MDV, indicating that infection into the herpes virus in nature probably happened after the divergence between the vaccine virus and this virus.

I also show you in the acute co-infection experiments REV insertion into MDV detectable as early as second passage. REV insertion sites are non-randomly distributed. REV LTR insertion of mutagen of MDV genes demonstrated MDV with altered pathogenicity in the phenotypes also generated.

I'd like to take this in a broader context and tell you about what happened since our original discovery. Now this has been repeated by several laboratories, and that demonstrate that herpes retrovirus insertion to herpes virus.

First, that our own actor (phonetic) has extended REV insertion into the oncogenic strand experimentally to the vaccine strand, herpes virus of turkey, and we can see its insertion with no problem, again within one to two passages.

But here there's a very interesting clone. One of the clones -- I'm sorry. So this you saw already, but this is REV insertion into HVT.
There's one clone here that actually carries the full length of REV. In fact, we were able to show this can produce virus after transfection. So this is not anything specific for the oncogenic strand of MDV.

We also showed that rav 1, rouse (phonetic) associated virus, can also integrate in the MDV or HVT. Okay. So, again, there is nothing special about REV.

This is probably very significant. From Japan, Hiria's group about two years ago showed that this MDV virus isolate from chicken actually carry endogenous REV 0. Now, this experiment did not see in vitro culturing at all. So it was an in vivo isolate, and REV 0 very nicely integrated MDV, again, at a hot spot that we show for REV.

Furthermore, there are two more laboratories that I actually did not update this slide, show last year that the avian erythroblastosis virus LTR also integrates into MDV and another REV 0-like sequence integrated into MDV. So this has been repeated in the four laboratories independently.

Recently Eric Davidson in Israel did in vivo experiments to see whether in vivo recombinant can be detected or not, and they used PCR knowing that
the cluster region -- so they designed PCR primary
that can easily detect recombinant, and they were able
to detect about 20 of them and were helping them
analyze it.

Now, a year ago this paper is rather
profound to us. It's not retrovirus insertion of
herpes virus, but this group found that REV can
integrate into fowlpox virus, and the pox virus is a
vaccinia virus group.

This is profoundly significant to us
because this shows retroviruses can integrate into a
virus that only has a cytoplasmic life cycle. They
may not need the DNA pk. You may not other things in
the nucleus to do the job, but you can.

And again, I emphasize this was an in vivo
isolate. The virus never sees in vitro culture. So
its recombination in vivo.

Then finally, George Miller's lab found in
EBV infected cells there is a fusion of cDNA. They
did an isolated virus. So we do not know the fate of
the virus. They do find a junction that carried both
retroviral LTR and EBV sequences.

So this may happen in other systems as
well, and I do not -- I have no reason to believe it's
a special for chicken, but I think, again, selection
is very important. If you do not have selection, this kind of integration come and go.

I think I will stop here. Thank you very much.

(Applause.)

PARTICIPANT: I have a comment and a question. I think for the sake of the audience it's also worth noting that there is evidence for ancestral capture in a number of herpes viruses, ancestral capture of cellular genes by a process involving retroviruses.

So, for example, if you look at the members of the gamma-2 herpes virus group, they -- from new world primates, old world primates, and humans -- those herpes viruses have genes for dihydrofolate reductase, cyclin D, and a few other genes, in all cases lacking introns (phonetic), the lack of introns suggesting it's acquired by a process involving reverse transcription, and that's likely to have been acquired by a process involving co-infection of cells with a retrovirus.

My question has to do with why do you think -- it looked to me like that many of your examples of capture were pieces of LTR and not whole LTRs. You showed one example of a single LTR. Why do
you think there are so many -- I mean, how does that happen, and why does it just have small pieces or a single LTR, and is there any evidence in any cases that these LTRs or LTR pieces are actually driving expression of some viral gene?

DR. KUNG: Okay. First, I did snow in RM-1. I just went through so quickly. I'm sorry. The LTR is promoting insertion activate transcript. Okay?

Secondly, in terms of why it's LTR, I think that what happens, LTI/LTR direct recombination which especially in herpes virus, that you have the flip-flop of the R region. So the recombination is very, very acute. You have direct recombination between LTI/LTR you would delete the sequence of. In fact, the full length sequencing, the herpes virus genome, it's not very stable. It's about ten kilobase. So the virus has a tendency in my mind to spit out the extra sequence, and LTR seems to be harmless at least. Yeah.

MR. MINOR: Philip Minor from NIBSC. I'm completely ignorant about retroviruses. The integrations that you described were all with co-infections, I guess. Is it possible that you could get an integration if you were just looking at an endogenous retroviral sequence that was
maybe being transcribed? Can you pick it up in that way?

DR. KUNG: Yeah. Thank you for asking the question.

Again, I went so quickly. There was an endogenous virus REV 0 integration into the herpes virus shown by the Japanese group, but also more importantly is that the retrotransposon, popping -- I guess later the speakers will be talking about that -- it's very frequent.

I give you one example, not related to herpes virus, but you probably know the bacula virus, the autografa califonia nuclear polyhedral viruses, that the Freezen (phonetic) and Miller and their colleagues did a beautiful study, show that the TET, it's a transposal element, number four, integrating to the bacula virus genome transcribed the gene and contained the entire sequence.

So, yes, my feeling is it's -- all we're talking about is selection. If you have a selection, I think you will be able to detect those endogenous transposable -- retrotransposable like, and I think that's also related to Ron's comment.

Many of the herpes viruses captured, especially like the KSS-3, Kaposi's sarcoma, capture
a lot of cellular homologs, and many of them are
internalist (phonetic) even though herpes virus are
known to be able to splice out intron.

So my feeling is that some of them may not
be entirely due to retrovirus, but could be due to

retrotransposable.

DR. PEDEN: Keith Peden, CBER.
You may have said, but does the

integration site have the hallmarks of genuine
retroviral integration, the duplications at the ends?

DR. KUNG: Oh, yes, yes.

DR. PEDEN: It does?

DR. KUNG: Duplication at the end, yeah.

MR. COFFIN: John Coffin, Tufts.
Just to clarify a little bit your answer
to the previous question, I think it was asked whether
the endogenous viruses could actually be moving
without an infection cycle, and the answer probably
is no, for all that we know about endogenous

retroviruses. You still need to get -- in order to
move them from one place to another, you still need to
-- they still need to undergo a complete replication
cycle.

DR. KUNG: Yes.

CHAIRPERSON ROSENBERG: I think we need to
move on because we are somewhat behind, although I
don't want to cut this short.

The next speaker is Maxine Linial.

DR. LINIAL: Okay. Today I'm going to
talk mostly about foamy viruses, but I wanted to start
off raising a couple of issues, some work in my lab
about packaging of avian retroviruses.

Okay. So for many years my lab has been
interested in defining the minimal packaging region of
the avian retroviruses, and this is a schematic of
what from the literature appears to be defined as the
minimal packaging regions of NLV HIV and the ALV
viruses, and we had previously defined a packaging
region of about 160 nucleotides from the five prime
end of the genome, which are sufficient for packaging.

That is, you can place this region on any heterologous
RNA, and that RNA will be packaged with a high
efficiency into retroviral particles.

And in fact, we find that such a small
region on a neo or a hygro RNA is packaged only about
2.7 times worse than the intact ALV genome. So we
believe this is a sufficient packaging region.

More recently we've done a series of
experiments on this 160 nucleotide region and have, in
fact, found that we can delete off the entire three
prime end, leaving an 82 nucleotide region with this
set computer predicted secondary structure that has
several stem loop regions, and by mutagenesis RNAase
protection and looking at a variety of viruses, we
know that there is one, two, three stems that are
important, and that possibly the region that's
involved in protein binding and recognition of this
RNA may be only a four nucleotide loop.

So we're getting very close to
understanding what the structure of the packaging
region looks like for this virus.

And one other point I want to make is that
retroviral packaging seems to be a hierarchy of
sequences. The retrovirus most avidly packages
sequences with its own psi regions, but then, of
course, we know that vector RNAs containing psi can be
packaged with very high efficiency, and probably after
that the retrovirus is packaged cellular RNAs or other
elemental RNAs containing psi-like sequences, as we
heard for MLV. VL-30 sequences have psi-like
sequences and are avidly packaged, but they also
package random cellular RNAs.

And work from my lab and a variety of
other labs has shown that such RNAs can be a player in
packaging cell lines, and we did a lot of work on a
quail packaging cell line which contains a single

integrated RSC lacking psi, and the unique thing about

this packaging cell line is that the provirus is

exceedingly active and produces about 100 times more

virus than any of the other packaging cell lines that

we looked at, and because it made so many particles,

we could so that what was packaged was random cellular

RNAs, and that we could also show that these cellular

RNAs could be reverse transcribed in new cells and

integrated into the genome with detectable frequency.

And we could find about 100 using neo as

a cellular RNA. We could find about 100 transductions

of the neo gene in an infection with this virus, and

we propose that this kind of event can occur with any

packaging system if it's looked for, but this kind of

a looking for integrations in the new cell is not

generally assayed in any of these packaging systems.

So now I'd like to turn to foamy viruses. This is one of the seven genera of retroviruses, the

spuma retroviruses. They're a tightly knit family

that's fairly divergent from all the other groups of

retroviruses.

One aspect of the virus that's very

interesting is that unlike, for instance, the gamma

retroviruses which are know -- can be pathogenic in
their native host with long latency, the gamma

retroviruses or the alpha retroviruses are unlike the
lentiviruses which are known to be pathogenic and
accidently infected hosts but generally not in their
natural host.

The spuma viruses or foamy viruses are not
pathogenic in any host but spar. So that there is a
whole variety of these viruses, and their life long
infections in their host, and there's no
pathogenicity, and when there's an accidental
infection, for instance, several of these simian
viruses are known to infect people.

Again, there appears to be no
pathogenicity in those hosts, and this is a very
interesting question, is why the life style of this
virus is so different from those of its other
retroviral cousins.

The genome of the foamy virus is very
similar structurally to other retroviruses, has gag,
pol, and env genes. It also has several open reading
frames and two known products, the transactivator
protein called tas, which is absolutely required for
transcription from the LTR promoter, and uniquely to
this group of viruses there's a second promoter in the
envelope gene, the internal promoter, which seems to
be responsible for transcription of the accessory genes, tas, self, and a second gene which is a spliced variant from tas and bel 2 called bet (phonetic). Bet is a major product of this virus. It's completely dispensable in tissue culture, but is believed to play some important role in vivo, although we have no idea what the role of this protein is.

Some features of this virus that make it very different than other retroviruses is, first, the pol protein is not made as a gag-pol fusion. It's made from its own splice pol message. This makes it more similar to the hepadenoviruses that goes to the retroviruses. Again, it has an internal promoter unlike all the other retroviruses.

And a third feature is that you cannot get particle egress from the cell with gag alone. You must have the envelope protein. Again, we're similar to the hepadenoviruses like HBV than to the retroviruses.

Foamy viruses have been isolated from a variety of species. It's extremely prevalent in cats, both domestic and wild cats. Recent studies say about 70 percent of individuals cats are infected. It's highly prevalent in many bovine flocks.

Recently a virus has been isolated from
horses, although the prevalence of this virus is not clear at the moment, and from a variety of primate species, in fact, every primate species that's been studied does have a foamy virus.

And in some groups of primate in primate centers, essentially all of the individuals infected -- Jonathan Allen at Southwestern, I think, has shown that all of the baboons there do have foamy virus. So although there aren't so many studies in the wild, these viruses have been isolated from wild animals, as well.

There have been several isolates from human, and I'd like to speak briefly about that. The type species is called HFV, human foamy virus. This was isolated from a nasopharyngeal carcinoma cell culture from a patient from Kenya many years ago.

Recent studies, however, from the group at CDC have clearly shown that this human virus is basically a chimpanzee virus, and interestingly, it clusters very closely with isolates from Schweinfurthiae chimpanzees, which is the only chimpanzee that's present in East Africa where the HFV isolate came from, which clearly suggests that HFV was in this culture because the patient might have had contact with the chimp and acquired the virus through
a zoonotic infection.

And all the other isolates from people are clearly linked to having been bitten by a monkey or a chimpanzee.

So one interesting thing about HFV is despite the lack of pathology in vivo, as far as we know, in culture you get two types of infection. The first and most dramatic is a cytopathic infection of fibroblast, and this was what gives the foamy virus their name because these cells become highly multinucleic and syncytia, multi-syncytia form.

But there's also a second type of infection, a long term, persistent infection, and this is seen and our lab has found it in a variety of human cell lines, T cells, erythroid cells, monocytic cells, et cetera, and in these long term, persistent infections you see absolutely no CPE. The cells become infected. They grow perfectly normally, and you would not know that they were foamy virus infected without doing PCR or assaying the virus.

So this is clearly a problem when one deals with material from primates. One needs to assume that the primates are probably infected with foamy virus, and although many isolations of cell lines from primates lead to cytopathicity and clearly
show their foamy virus, since we know very little about the cells in vivo that are actually infected, it's very possible that you could have cell lines from primate cultures that are infected with foamy virus without CPEs.

This is an example. This is an indicator cell line where we have LTR driving betagal in a hamster fibroblast line, and when you infect these cells with foamy virus, you can see that that turns on the betagal expression from the tas transactivator. You get highly cytopathic cultures. And here's an example of a really giant multi-nucleus syncytia that can occur at high multiplicity. On the other hand, when we infect a variety of human cell lines, these cell lines grow for years. This only goes up to 40 weeks, but we've grown these cells. They continually produce virus, but they are never cured of the infection, and there's no CPE. Here we show that in this experiment we could not infect a V cell, but this is not a problem of infectivity. This is a problem of viral replication. In fact, now that we have vectors marked with gfp and work from other laboratories as well shows that there are basically no vertebrate cells that are immune to foamy virus infection. Everything
from fish on upwards, and in fact, in humans no cell
types to be seen to be immune to foamy virus. So
whatever the receptor is, it's extremely widespread.

So the life cycle of foamy virus is very
similar to that of other retroviruses with a couple of
striking exceptions. As I mentioned or I will mention
again, the virus, most of the viral budding is through
the endoplasmic reticulum, and although some virus
does bud from the plasma membrane, again, in order for
the virus to bud, there must be glycoprotein.

If you have an M minus mutant, virus does
not bud from the cell, and this tends to be a
cytopathic event. If there is no envelope, the cells
die very rapidly.

The other important thing to mention is
that work from our lab, as well as another lab,
strongly suggests that reverse transcription in this
virus is a late step in infection, and that means that
the functional genome in foamy viruses is really DNA
rather than RNA.

And this is done using AZT as an inhibitor
and show that the stuff that is sensitive to AZT is a
late step rather than an early step in the life cycle
of other retroviruses.

Another point to be made is that there are
huge numbers of intracellular particles. In fact, most foamy virus is intracellular. Only about one to five percent of the virus buds from the cell. The rest of it is cell associated, and this has suggested that perhaps like hepadenoviruses, there could be some type of recycling step where some of these intracellular particles get back into the nucleus, and I'll get to that in a moment.

So in terms of the HFV genome, by doing very sensitive PCR and RT PCR, we found that about 25 percent of the particles released from cells contain apparently full length, double stranded DNA. The AZT experiment strongly suggests that the functional genome is DNA so that even though there are a large number of RNA particles, we don't believe that these are infectious, and they're probably remnants of abortive reverse transcription events. And we've also been able to show that if you extract DNA from extracellular particles, it is infectious if you put it back into cells with lipofectamine.

Despite the fact that the foamy virus functional genome is DNA, what is packaged is RNA, and this is RNA's protection experiment using wild type human foamy virus or a deletion mutant in the pol
gene, looking at RNA's protection.

And I haven't shown you the controls here for particle numbers, but when you look, and this is RNA's protection that really only just looks at RNA in the particles and not DNA. If we RNA, we don't see any nucleic acid in the particle, and the same is true for the pol mutant.

And what we found from this study when we compared the amount of nucleic acid packaged in the pol mutant and wild type, it's exactly the same when it's normalized to the number of particles.

So you don't need polymerase to get genome into the particle. Of course, this is completely dead. So what's packaged is RNA, and DNA just occurs sometime during assembly or egress from the cell.

We also believe there are large numbers of particles in the intracellular particles that contain DNA. So when you look at a copy number of foamy virus persistently infected cells or acutely infected cells, you can find hundreds or thousands of copies of DNA per cell, and this has made it very difficult to look at integrated genomes in these cells.

This is a schematic of what we think the foamy virus looks like. Instead of having an RNA genome, it has a DNA genome.
Another feature of the foamy virus which I didn't mention, but which is also very striking is that the gag polyprotein is not cleaved except for four KD at the C terminus. So you never get maturation to caps at matrix and nuclear caps as in other retroviruses. Basically we believe that this gag protein is multi-functional, that the carboxy end, which has many basic residues, probably behaves like the core protein of hepadenovirus and interacts with the DNA genome in the particle, and that probably the amino terminus behaves somewhat like matrix and part of this protein also behaves like tapsin (phonetic).

So we were interested in looking at integration, and to do this we used a chronically or persistently infected H-92 cell line, and we cloned out single cell clones of this virus, and in these experiments, these southern blot experiments, we cut with NAG-1, which cuts ounces in the genome, and use the bet probe.

And if you do such an experiment, you can see a large number of -- a large amount of viral DNA. This is the cut DNA here. This is the small amount of DNA that was not cut.

And in many experiments this obscures the background of integration. So what we do is we treat
the cells for several weeks with AZT, and this gets rid of all the intracellular DNA in the particles, and then you can easily see the integrated copy number in these single cell clones, and what's very striking is that there is a large number of integrated DNAs.

And we've counted upwards of 20 bands per cell of, as I said, single cell clones of foamy virus infected, which were leukemia cells. We've confirmed that these are single integrations by cloning out the junction fragments and showing that they're not duplications or repeats.

These are bona fide new integrations.

We've also done FSH analysis. This isn't a very good slide, and the arrows are not pointing to all of the integrated copies. You'll just have to take my word for it. There's a rough correlation between the integration number that we see by a southern blot and by FSH analysis, and we do see up to about 20 integrated copies per cell genome of this virus as well.

And we have been very interested in the mechanism of these multiply integrated pro viruses. I'm not showing you the data. There is, in fact, a NLS sequence within the gag genome foamy virus which Axel Rethwelm's lab shows behave as an NLS. So after
infection, much of the newly synthesized gag protein goes back into the nucleus.

And by using a mutant in the NLS, so that if you delete the NLS the virus can grow fine in tissue culture, it does have a slightly lower titre, and we've shown that by deleting the NLS we prevent accumulation of all of those multiple integrated copies, and we now have clones of cells that only have one, two, or three copies per cell instead of ten or 20 copies.

So this implicates that there could be some kind of intracellular recycling mechanism that's responsible for the high copy number. However, what complicates this is that we've recently found that the foamy viruses also have another unexpected feature, and that is we have made a vector in which we have replaced the bet gene with GFP, and in this case we also put in the RSV strong promoter.

And when we use a vector to infect either BHK cells or these erythroleukemia cells, we get a high infectivity. This is shown with the GFP fluorescent, but surprisingly if we compare infectivity of either these uninfected H-92 cells or this single cell clone that has about 20 integrated copies, we find there's basically no difference in the
ability to reinfect the uninfected -- the infected
cells versus the uninfected cells.

So at least in these persistently infected
cells that have huge copy numbers, they're not immune
to super infection, and this is very surprising for a
retrovirus.

So, therefore, we can't say whether we're
getting accumulation of all of these integrated copies
by an intracellular pathway or an extracellular
pathway, and this also suggests that even if a cell is
infected by foamy virus, it would not be immune to
superinfection by another foamy virus or more foamy
virus.

The other point about foamy -- foamy
viruses are not very well studied. There's very
little information about the packaging sequences of
these viruses. Several groups have tried to make
viral vectors, and so far this isn't an attractive
genome for viral vectors.

For one thing, the virus, as I said, is
probably not pathogenic in either accidental or
natural hosts. It has a very large genome, greater
than 11 KV. It has at least one gene that we believe
we can delete without -- at least in vitro -- without
any untoward effects on the virus. It has two
promoters, so it's a very flexible genome.

And another point is work from Germany has shown that in infected monkeys you can find foamy virus DNA in every organ in the body, including the brain, although there's very low viral replication in vivo.

So it might be a good gene delivery target to evoke a wide variety of organs. So in vector development several groups have found that, in fact, there are probably at least two packaging or two sys acting regions in the RNA, and one is at the five prime end of the genome where you would expect a psi sequence to be, but surprisingly there's also a region in the pol gene that's required for transfer of vector sequences.

Whether this is another packaging sequence or has another sys acting RNA function is not known. For instance, since pol is not made as a gag-pol fusion protein and the method of incorporating pol into the particles does seem to require a gag-pol interaction, but we can't rule out that perhaps it also needs to bind to the RNA, and so this region of pol could be a pol binding sequence. We don't really know.

So the packaging regions of this virus are
not at all well understood at all.

So in summary, all retroviruses have packaging signals. Unfortunately those of the foamy viruses have not yet been delineated. Foamy viruses package RNA, although the functional genome appears to be DNA.

In some cells, at least in tissue culture, foamy virus infection leads to multiple integration.

This is an interesting point because one would think that if such a thing occurred in vivo, and we have absolutely no evidence for it, that foamy viruses would be all set up for promoter insertions and inductions of tumors in infected animals. Yet this has never ever been seen.

So it's possible that the replication in vivo is so meager that the virus never really does multiply integrate, but nobody has ever been able to or nobody has ever looked in vivo for foamy virus integration sites.

Nothing is known about the recombination between foamy virus and other retroviruses. Clearly, experiments that are done in monkeys, infecting them with other viruses such as SIV or viral vectors, there is probably foamy virus in all of those animals. So it would be very interesting to know something more
about how foamy virus interacts with other types of retroviruses.

And also any packaging cells derived from primates, bovine or feline species need to assess the effect of foamy virus as well.

And I think that's all I have to say.

(Applause.)

CHAIRPERSON ROSENBERG: We need to keep the questions, I'm afraid, brief because we are seriously behind. So I believe we're supposed to be at the break, but we still have one more speaker.

PARTICIPANT: Maxine, does anybody know if vaccines have been checked for foamy virus contamination?

DR. LINIAL: As far as I know, no.

PARTICIPANT: You mean nobody has looked or as far as you know?

DR. LINIAL: I don't know. There are very few reagents. I mean, there are reagents for the so-called human or chimp foamy virus, but as far as I know, there are no good antibody reagents.

PARTICIPANT: There are.

DR. LINIAL: There are?

PARTICIPANT: There are? They are checked. Okay.
DR. LINIAL: Are they checked for all of the simian foamy viruses?

PARTICIPANT: No.

PARTICIPANT: By PCR? Is that --

PARTICIPANT: (Inaudible.)

CHAIRPERSON ROSENBERG: Could someone repeat this so that --

PARTICIPANT: I think it's by a combination of tests, including PCR and infectivity tests; is that right? Okay.

DR. LINIAL: One problem is that these monkeys do get cross-infected with other foamy viruses. So, you know, I don't know how many you're looking at.

PARTICIPANT: Since foamy viruses seem to be breaking all of the rules, I wonder if it's worth asking or is it known whether integration is required for replication, whether there's significant -- since there are so many copies of DNA, et cetera, whether there can be significant expression and replication in the absence of integration.

DR. LINIAL: My lab, as well as a lab in Germany, have made a DD35E integrate mutants, and at least in tissue culture it's completely dead. So we believe integration is required.
CHAIRPERSON ROSENBERG: I think we need to move on. The last talk in this session is by John Kappes.

DR. KAPPES: Just a momentary delay. Technology, MacIntosh, a little slower in booting.

Perhaps I'll begin a short introduction.

The focus of my work -- there we go -- has been on the possible use of lentiviral vectors for gene therapy. Off to a bad start. That is the second slide.

The principal concern for using lentiviral vectors for gene therapy is that they may recombine to produce replication competent retrovirus, and underlying the concerns for replication competent retrovirus is genetic recombination.

There have been a number of different types of -- and I'm going to focus really just on HIV-based vectors, although I'll probably refer to them many times as lenti -- but there have been a number of different HIV-based lentiviral vectors produced to minimize the pathogenic properties of any RCR that could emerge, and those include deletions of most of the accessory genes, deletions of even the TAT and REV regulatory genes, the lesions in U3, and while I won't focus on a lot of these details, I will focus on,
again, what I think is fundamental to understanding the risks associated with generating replication competent retrovirus, and that is genetic recombination.

The assays which have been used thus far include, that is, to measure recombination or, if you will, really more because of the way they've been applied measurements of RCR, include gag transfer — oh, TAT transfer it should be — gag transfer, and DNA mobilization of marker rescue assays.

It's unlikely, and I'm sure they have not been suggested by the authors who published on these assays to be adequate indicators of the risk associated with these viruses or these viral vectors in vivo. Especially that's true in the long term.

So today I will present data from an approach that I devised to understand the risk, if you will, of using HIV-based vectors through an analysis that focuses on genetic recombination.

First, I'll present one slide, the unique difference that I've used to enable the detection of recombinant viruses and their analysis, and then several slides I'll show the detection and characterization of these recombinants, both biologically and genetically, and finally, in one of
two slides I'll show how I have further disarmed or
dismantled or split the functions of the lentiviral
based vector to improve safety.

This depicts the three component systems
for HIV-based vectors, not necessarily analogous to
what might be thought of as third generation, but the
important point is that there is a packaging
construct, a vector construct, and an envelope
construct, and just for this one slide, I wanted to
point out in particular the TAT gene because my
recombination assay is based on TAT.

That is, if recombination occurs between
the vector and the packaging construct, and if TAT is
included, I will be able to select for the recombinant
using this approach.

If lentiviral vectors are generated
through transfection of 2-9-3 T cells, which is what
I will show in every case, genetic recombination can
occur during reverse transcription to generate an LTR
TAT containing structure. It could contain gag; it
could contain the full packaging construction, but
minimally if it contains TAT and TAT is expressed, it
could confer resistance to puromycin in the cell line
that I call Hela-puro.

This cell line was transduced with this
construct, which confers resistance to puromycin

selection when the cells infected with a virus or a
vector containing and expressing TAT.

A couple of other points worth noting for
this slide because it's really what I will use in
terms of the components that generate the vector and
the data I'll show in every slide, except for a couple
at the end.

The packaging construct is driven by a CMB
promoter. It contains gag-pol-vif. Vpr, vpu, mpf
(phonic) are deleted, and also importantly nef is
deleted, importantly because there is no overlapping
sequence at this end of the genome, which will become
important for reasons which hopefully will be obvious
later.

So to generate the HIV vectors, I
transfect these three constructs into 2-9-3 T cells,
and that's depicted here.

Through transfection bioparticles are
generated, and these bioparticles can be used to
infect the Hela-puro cell line.

If TAT is expressed, the cell should be
resistant, and that's exactly what is shown here.

These are the colonies stained with crystal violet, I
believe, about nine days after puromycin selection,
and what's shown is that there is approximately 1,000 colony forming units per ten to the seventh infectious particles.

Also, importantly, if this infection is done in the presence of a niverapine, there are no resistant colonies detected, implying that it's mediated through the HIV-1 reverse transcriptase, and in particular, I chose niverapine because it's specific for HIV-1 RT.

As I suggested, recombination could occur through any region of the packaging construct. As long as TAT was picked up, resistant colonies could be produced. So to confirm TAT was actually present, we designed primers to amplify the first exon, and that's what's depicted here in what I call the lenti pool.

This is the lenti pool. After the cells were expanded, the high molecular DNA was extracted, and PCR shows detection of a 219 base pair fragment similar to that detected in proviral DNA.

Because or at least in part because data had been published that showed there was no TAT transfer, no gag transfer, no marker rescue using lentiviral vectors, I wanted to address each of those points.

And the first series of slides I just
showed would suggest that there is TAT transfer if the system is sensitive enough to detect it. This slide detects a slightly different approach for looking toward gag transfer. This is the path I just described for TAT transfer. Because recombination can occur in many ways where an infectious virus would not be generated, I chose this pathway for gag transfer because I was really looking for an open gag reading frame. So the difference is instead of going at the heel of puro cells, I infected the virus particles that were generated by transfection into 2-9-3 T, and in 2-9-3 T, if a recombinant forms such that you had an open gag-pol rating frame, you would expect that particles could be generated, and if those particles were pseudotyped by transfecting VSVG into the 2-9-3 T cells two days after infection, then they could infect the Hela-puro cell line and confer resistance, and that data is shown here. We detected 540 resistant colonies when the virus derived by 2-9-3 T cells was used to infect the Hela-puro cell line, suggesting both DNA mobilization and gag transfer. Another way of looking at DNA -- oh, let me point out that we also confirmed from the
supernatants of the puromycin resistant cells the presence of gag protein, both in culture supernatants and in viral pellets.

Importantly, the amount of gag detected was increased by about tenfold if TAT was transfected into those puro resistant cells, suggesting that the LTR was truly linked, that is, expressed in sys, with the packaging construct.

Another experiment, which was just a further extension of what I've already shown related to the gag transfer, to more specifically and more clearly differentiate between artifacts and true DNA mobilization is the depicted here.

The recombinant, which I referred to as mobilizing gag or gag transfer is depicted here. We don't know at this point whether this is the exact structure, but it should contain an LTR gag-pol reading frame, and certainly tat and rre. If this structure is respent in the Hela-puro line, it's possible that when it produces particles because the Hela-puro cell line contains puromycin and a packaging signal further puro RNA strained, that it, too, could be packaged into these particles, mobled to heal a TAT line in this case, and confer resistance to puromycin. And indeed, that's the result shown here.
So the data indicate by three assays recombination of the lentiviral vector components, in particular, recombination between the gene transfer vector and the packaging construct. So all of the data I've shown just far are really just biological evidence, and now I would like to turn to the genetic and structure data, that also supports these conclusions.

If a combination truly occurred between the packaging construct and the vector, you'd expect the two to be linked, and if I used primers in the U3 region and at the end of gag, I would expect to amplify a product from this lengthy viral pool, which is what it's called. This was the product of what I call gag transfer.

I would expect to amplify a PCR fragment of about 2,000 base pairs, and indeed, that is what's shown.

If this PCR product was cloned and sequenced, and if there was recombination into the packaging construct, what would be expected is this sequence, that is, the initiation codon for gag and a sequence depicted here, and in ten out of ten recombinants, this is what we found.

This sequence, it's also worth pointing
out, is different than that of the original vector sequence. Remember the vector includes part of gag because it's important for packaging. There are clear differences between these nucleotides. So this is further evidence that recombination between the vector and packaging construct occurred.

Looking instead of here on the three-pronged end of the genome, we used primers that would span from TAT, shown by this yellow bar, into the U3. By PCR we amplified three, maybe four small bands, and this one here, which corresponds in weight to what we expected, was cloned and sequenced.

And that analysis is shown here, and I'll show this in more detail on a couple of later slides, but the important point is that by sequencing through this entire region, what we found is that there is genetic evidence for recombination similar to what we found on the five prime end on the three prime end, and in fact, genetic recombination or different recombinants were found.

There were six recombinants that were of this type, one of this type, one of this type, and one of this type, and what are these types? This is what I show on the next one of two slides.

Also depicted here is how I suggest this
recombination is occurring. Remember reverse transcription is going to start up here somewhere, and the growing DNA chain will anneal to the RNA on the three-prong end, and as it's extended, in one of the situations I show I would suggest recombination occurring into the poly A tail of the packaging construct, in another case, into the poly A tail at a different position, and in another into the poly A tail at yet another position.

What these recombinants have in common is that all of them extended up into 108 base pairs past the end of U3 or the beginning of U3, depending on which way you're going.

The other type of recombinant detectable is one which went in at yet a different position, in position 45 or it contained 45 As downstream of the poly A signal, but it only contained 32 base pairs upstream of the U3 sequence.

This finding was unexpected, and I thought for a long time about how it might be occurring, and while I still can't explain it, here's a possible rational model based on the limited information I have.

If this is the RNA tail, poly tail, from the packaging construct, and this is the DNA which is
growing from reverse transcription, what I noticed in all cases just prior to where the recombination even occurred -- and remember eight of the recombinants jumped from this position into various areas of the poly A, and one of them jumped from this position into I think it was number 47A downstream of the poly A signal. What they all have in common is a sequence of Ps, which was suggested could bring those structures together to allow recombination. So based on this data, I think there are several safety concerns which I could point out.

One, the regeneration of envelope deficient recombinant lentivirus requires recombination of only two genetic elements. That would be the packaging construct and the vector. The LTR packages signal, gag-pol LTR recombinant contains all of the necessary viral replication machinery for replication minus envelope. The long term risk of stably integrated structures that might look something like this is unknown. Lentiviral vector recombinants may infect nondividing cells if envelope is provided in trains, and I think this might be particularly worthwhile to consider because unlike what we've experienced with
Maloney, the lentiviral vectors, if this structure exists in vivo, and it can be mobilized to nondividing cells.

I think especially this issue is important in cells transduced or CD-34 positive stem cells transduced with lentiviral vectors since it could represent a reservoir for ongoing gene expression.

Okay. Quickly just a couple more slides.

One possible approach to minimize the risks associated with genetic recombination between the vector and the packaging component I would depict, I think, in just two slides, and this is really based on a study that Wood, et al., published in EMBO in 1997, where instead of incorporating reverse transcriptase and integrate as part of the gag-pol precursor protein, these genes refused to vpr and expressed in trans with an RT integrate minus provirus, and what was demonstrated was that the provirus or that the RT integrate was packaged. It was processed, including into mature integrate and RT, P-51 and P-56 components, and they were fully functional, so provided an opportunity to further reduce the basic components of the HIV vector system by deleting RT and integrate from the packaging construct, and that's what's depicted here.

So now the packaging construct of what I
call translentiviral vector looks like this. It's broken apart into RT integrate infused with vpr, provided separately from gag-pro.

And one slide to show data, although there are others that show very similar results. If the 2-9-3 T cells are infected with translentiviral vector derived from transfection of all the vector components, this structure can produce viral particles, but upon infection of the Hela-puro cell line, there are -- no resistant colonies are generated, and that's due to a lack of reverse transcription and integration machinery because even though infection might occur in the absence of reverse transcription of the genome, no resistance will be conferred.

So finally, in conclusion, recombination does occur between the packaging construct and the gene transfer vector. This structure is regenerated, which contains all the basic replication machinery minus envelope.

LTR containing recombinants integrate into the chromosomes of the target cells. The recombinants express viral proteins, including tat, gag, and the entire gag-pol precursor protein.

The expression of the integrated gag and
pol genes produces virus particles which can be mobilized if envelope is provided in trans.

Recombination within the RNA poly A tail may represent a mechanism by which genes without homologous sequence can be mobilized, including oncogenes, which we discussed at some length yesterday.

Separating RT and integrate from the packaging vector controls the regeneration of a functional gag-pol structure which is absolutely required for RCR and DNA mobilization.

Thank you.

(Applause.)

CHAIRPERSON ROSENBERG: I don't think it's on. Switch on the bottom maybe.

DR. KUNG: I just want to comment that, in fact, in support of your data there are two --

PARTICIPANTS: Can't hear you.

CHAIRPERSON ROSENBERG: Why don't you just repeat his question after or you can come up here?

DR. KUNG: Okay. I just want to comment that in two naturally occurring avian retrovirus that carry oncogenes; one is avian rhesoblastosis. One of the isolate recombination takes place at the poly A tail. Okay? In that case it's 2-lycine. Is there a
crossover point?

Okay, and the other is fips (phonetic).

I think Mike Bishop published that. Recombination also takes place at the poly A sequence.

So there are two naturally occurring oncogene carrying.

DR. KAPPES: I was unaware of that. The data were very surprising.

CHAIRPERSON ROSENBERG: I think this should be the last question because we are actually even beyond the break.

DR. LINIAL: What kind of titres are you getting from the last where you break up RT into PR and RT integrate? What kind of titres of vector are you getting from those constructs?

DR. KAPPES: Let me be sure I understand the question. What kind of titres are we getting when we separate RT and integrate? Consistently, and maybe this isn't the best answer, but it's important I believe, consistently about three to fivefold less than what we derive from the lenti. Under our best conditions without concentration we can get near ten to the seventh per mL from the lenti. So under best conditions, maybe five times ten to the sixth with the translenti.
CHAIRPERSON ROSENBERG: I'd like to thank all of the speakers. Hopefully there's time for a short break before the panel.

(Whereupon, the foregoing matter went off the record at 10:12 a.m. and went back on the record at 10:32 a.m.)

DR. HUGHES: I would ask everyone to please come and take their seats.

Mindful of the fact that we are what stands between you and lunch, we're going to try to do this in a timely way and conclude the session as it was originally scheduled.

I think in keeping with the spirit that the conveners of the meeting suggested for us, we need to consider the issues that are before us in the context of the proposal to use neoplastic cells as vehicles for generating vaccine strains.

And I think in that context, it's probably important to actually give consideration to the differences in the types of vaccines that people might wish to generate, for example, the difference between live vaccine, kill vaccine, or sub-unit vaccine.

And what we've heard in the last couple of days are a reasonably good description of the manifold interactions that viruses have with each other and
with their cellular hosts, and I think we need to give
consideration to that in terms of how we would think
about detecting interactions between viruses and other
viruses or viruses and their hosts so that the
vaccines that would be produced would be as safe as
possible.

And as Dr. Lewis suggested when the
meeting was opened, we need to think in terms of what
we mean by being safe. We need to think about the
problems of detecting adventitious agents, and we also
need to understand and think about precisely the kinds
of tradeoffs that exist between understanding issues
of safety and issues of the benefits that are provided
by affected vaccines.

And I think with those remarks, I would
open the discussion both to the panel and to the
audience. Are there comments from the panel? Does
anyone have any remarks they'd like to make to begin?

No. The audience, are there questions?

We may get to lunch early.

(Laughter.)

DR. HUGHES: Surely the prospects of the
to
must have provoked some thoughts that are relevant to
this issue of vaccine development.

Please.

MR. KRAUSE: Just to get things rolling,
I'm Phil Krause from the FDA.

What does the panel see as the likelihood
that these kinds of interactions or risks or potential
risks might be greater with neoplastic cells than with
other types of cells?

And also, as you say, what kinds of viral
vaccines does one have to be more concerned about?
You know, I can imagine based on what we heard that we
might be more concerned about these kinds of things
happening if one is dealing with larger DNA viral
vaccines or with retroviral vaccines than with, you
know, small RNA virus vaccines, for instance, but I
would be interested in what people have to say on
that.

DR. HUGHES: Ladies and gentlemen.

DR. EVANS: Well, I'm not sure that
there's much difference between a transformed cell
line and a regular cell line, especially like a
primary cell line where you've got a lot of
differentiated cells there at least initially, but
like I said, I'm not sure. I don't know. I don't
know if there's elevated copies in certain cell lines of endogenous sequences that might be packaged and transferred, or I don't know if there might be change when you infect that cell line with some other agent because you could be inducing sequences and promoting transgeneric information.

In terms of the types of viruses that -- types of vaccines you might make because of the degree of heterologous pseudotyping between different types of viruses in terms of retrovirus transfer, I think you've got to -- anything that has an envelope protein that directs the tropism of that virus you're going to have to be concerned about in terms of transferring the vector.

In terms of that having a deleterious effect or whether or not you could detect it, you know, and eliminate it by monitoring is a different question.

DR. LINIAL: In terms of foamy viruses, I would say that the real danger there is using things that aren't cell lines because presumably you can easily assay cell lines, and probably most of them won't make it to cell lines if they have foamy virus, but you know, using primary explants from monkeys, you probably run a higher risk of having foamy virus in
DR. PATIENCE: I think with respect to the endogenous retrovirus sequences, I think basically we have to consider every potential cell line on an individual basis. As far as I'm aware, at least, there's no correlation between a neoplastic cell type and an ordinary cell type. It really has to be looked at on every cell.

With respect to the types of gene therapy packaging systems, there's never been any reports of lentiviral related sequences in the human genome, and with respect to the leukemia-based virus systems probably the closest family is the RTV LH system, which we could see no cross-packaging by MuLV gag.

MR. FRIED: Mike Fried, ICRF.

From what we talked about yesterday, what's the chance of the capture of a cellular gene or an oncogene? Has anybody done experiments where there's an activated rash and then take it and try to transfer it or an antibiotic gene?

I mean is it easy to pick up or not?

DR. HUGHES: The best data as far as I know suggest that those events happen so rarely that they're not seen in cultured cells, if we're talking about situations in which there's no homology between
the cellular component and the viral component.

You're talking about the straight acquisition.

What can be made to happen, depending on
the systems, and perhaps Dr. Kung would comment for
us, at reasonably frequent -- reasonably frequently in
infected animal systems.

So I think there is a substantial
difference in terms of the history that we have for
cells in culture and infected animals.

Hsing-Jien, you might want to speak to
your experiences with respect to the herpes system.

DR. KUNG: Okay. I guess, again, all of
this will vary because of selection pressure. So I'm
talking about still in terms of tumor selection.

In the case of recombination generates so-
called acute oncogenic containing virus, the
efficiency vary tremendously, but one of the, I guess,
champion is the avian erythroblastosis virus that
Harriet Robinson reports about 50 percent; you can get
50 percent of the tumors that are released oncogenic
virus.

And there, again, I think the reason is
because you select for the tumor, and another reason
is that in the case of RB activation, the retrovirus
integration utilized the five prime LTR. I think
Naomi talked about it a little bit.

Five prime LTR integration, therefore it's five prime LTR. It goes through the packaging signal, then splice into the RB gene. In so doing, you do not need any deletion in between, and I think John Coffin earlier recalled that this reach through type of transcriptional activation can be as high as 15 percent.

So the provirus position could very well to pick up a packaging sequence linked oncogene, and I think that is the theoretical base why the RB gene captured by retrovirus had been very frequent, and I think that's a pretty general phenomenon.

In our lab the frequency of generating those recombinants are very high. At Harriet Robinson's lab, she reports about 40 percent. So it is very high, but again, this is selection, you know, selection tumor.

I guess. I don't know. Maxine, you have done something that's without selection, just look at packaging and recombination, right?

DR. LINIAL: Well, we were using selection for a juggling marker, and you if you select for anything with retroviruses, you can basically find it at some frequency. How this would occur in the
absence of selection is unknown.

DR. KAZAZIAN: Haig Kazazian from Penn.

I just wanted to make a comment with regard to the last question on endogenous sequences and whether there be a difference in their movement in differentiated cells versus undifferentiated cells.

I'll be speaking this afternoon about retrotransposons, and these sequences are transcribed pretty much specifically in undifferentiated cells. So they're transcribed in tumor cells, and they're not transcribed in differentiated cells. So you can't get retrotransposons to move in differentiated cells, but you can very easily in undifferentiated cells. So that is a difference that these endogenous sequences have.

MS. HOROWITZ: I'm Jill Horowitz from Wyeth-Lederle Vaccines.

And I'd like to know if anyone either on the panel or in the audience is aware of studies aimed at determining whether any of the millions of recipients of life vaccines have currently or have in the past harbored viruses that have resulted from some of these live vaccine undergoing dangerous alterations.

DR. HUGHES: Would anyone care to comment?
DR. DESROSIIERS: I'm not aware of any specific examples, but I'm going to take the opportunity to express an opinion, and it seconds something that Maxine just mentioned.

In terms of adventitious agents or unwanted virus populations, I worry much, much more about the use of primary African green monkey kidney cells or any such substrate used for production of vaccines than I do about a tumor cell line or an established cell line that may be extensively characterized in terms of potential adventitious agents or unwanted agents.

In terms of things like what might come from African green monkey kidney cells that could be problematic, I don't worry so much about the agents that one can test for. I worry about the agents that you can't test for, that you don't know about. So even if there are no errors and no problems and all the agents that you know to test for it can be tested for and excluded from every lot of every vaccine, I worry about the agents that you don't know about. And I'll be honest and say that I'm surprised that primary African green monkey kidney cells continue to be used, and I'm a little bit disappointed that FDA and whoever is involved had not
had a more serious effort to move away from primary African green monkey kidneys.

We all know that there are a number of neurodegenerative conditions and other conditions where viral etiologies have been suspected for years and no viral agent identified. Maybe they're caused by viruses, but maybe they're not.

I think despite the fact that there may not be any specific examples of viruses having been introduced, more and more examples are accumulating of the dangers of cross-species transmission either naturally or through other practices, and that's something I think we need to really worry about that can be minimized by going to established cell lines.

PARTICIPANT: Walid Heneine (phonetic), CDC.

In response to your question, we've been looking at transmission risks of the chicken retroviruses that now we know now contaminate the measles, mumps vaccines and the yellow fever vaccines, and to the two types of viruses that were implicated, there are the endogenous type avian rhekosis (phonetic) viruses and the endogenous avian viruses which you will hear about in later sessions.

So far we have not seen any evidence of
transmissions to vaccine recipients that we have studied by molecular and serologic means.

PARTICIPANT: You know, I think you raised a number of very good points, Dr. Desrosiers, and I would like to explore them with you a little bit further in the sense that suppose one has decided then that for new vaccines one is not going to use primary cells, but then that neoplastic cells represent potentially a reasonable alternative.

If I sort of follow your comments to their logical conclusion, it sounds to me like you would say then that cells which come from non-human species, even if they're neoplastic, might potentially be slightly more dangerous than those from humans in that sense, and that one might also have to worry about unrecognized adventitious agents in those kinds of cells depending on their history.

Would you comment on that?

DR. DESROSIERS: What's the question again?

(Laughter.)

PARTICIPANT: The implication of what you just said was that the species from which a cell comes matters, whether it be primary or nonprimary, primary or neoplastic as one might consider as an alternative,
and there's the further implication raised by what you said that, in general, one tends to worry about in this context more about unrecognized adventitious agents than recognized one, since you can test for the recognized ones.

I just was wondering whether that was a reasonable sort of conclusion based on what you said.

DR. DESROSIEUX: Yeah. I wasn't referring so much to species of origin. I was referring to the fact that I think that it's much easier to extensively characterize and analyze established cell lines for what's in them and what's not in them than it is a fresh kidney every time you want to do something. That's what I meant.

DR. ENGLER: Dr. Engler, Walter Reed Army Medical Center.

I just wanted to make a comment and ask a broad question to challenge the panel, speaking from the perspective of a clinician and an educator, trying to translate the contents of meetings like this and the recent Thimerosal meeting for regular folks, whether it's providers or the patients.

And the complexity alone, and when people say, "I worry about the unknown agents," we have to worry about foamy virus transmission, lentivirus
insertion into herpes, and that all translates into,
for the common man, woman, and child, is there a bad
disease with chronic consequences that can result from
immunization.

And the Hepatitis B vaccine, the original
vaccine, which from my understanding and experience
with it was one of the purest vaccines ever made, but
could not really be sold very effectively to lots of
people, including the highest risk folks that should
have gotten it, like surgeons and doctors, and as you
all are thinking about these things and trying to help
CBER and the FDA, the question you have to ask
yourself is: if I choose to use a cell line or use a
process to make a vaccine, how do I respond to those
concerns?

You all may think it's a minimum concern
compared to jumping out of an airplane, but we're
dealing with a public right now that is extremely
phobic and growingly so about vaccines, and
congressional representatives who have children who
were adversely affected by one of our old vaccines and
truly believe that this is the cause of a problem.

So you know, yesterday the comment was
made, "Well, why don't you do the vaccine process and
spike it with your worst fear and see how it comes out
at the other end?"

Now, that's something I can explain to a patient or a nurse or a doctor. An awful lot of what you all have discussed is very hard to translate, but what's left is, "Gee, I'm not sure that this is safe," from your mouths, and so I would just caution before large investments are made in building a vaccine that you consider the audience of who you have to deliver it and how do you write the vaccine information sheet.

DR. HUGHES: I'm certainly not by training or persuasion clinically oriented. I'm a basic researcher, but as I understood the charge that we were brought was to try and bring out the issues that have to do with real safety, with the concerns that I think the public should have, that the vaccines that are being made are as safe as intelligent humans know how to make.

And I would like to be able to say to you, I would like to be able to say to the people in Congress who in some sense pay my salary, and I'd like to be able to say to the public that you have no concerns; that everything that you're going to see in vaccines and in your life will be completely safe. However, that would be false, and I think the best job we can do under the circumstances as they
exist in the real world is to try to have a frank and
honest discussion of what are, in fact, real concerns
and dangers.

And as Dr. Lewis pointed out, I believe,
in the opening, his opening remarks, we're dealing
with a situation in which there are costs and benefits
and risks and benefits, and I don't believe that's
avoidable.

The average American, as far as I know,
the most dangerous thing the average American does is
to jump into his car and drive to work every morning,
and by contrast, as far as I know, the administration
or taking of any vaccine strain that's now approved in
the United States has minimal risk.

Now, I'm certain that people don't
understand that, but that seems to me to be a problem
that is distinct from the problem that, as I
understood it, we were given, which is to try and
figure out how to make it as safe as possible and to
discuss the potential risks as clearly as possible.

And certainly if there were some way we
could make your job easier of explaining, I would be
happy to try and do that, but I think any discussion
that isn't frank and isn't complete and doesn't have
some clear discussion of the potential risks is doing
a disservice to exactly the people we're trying to help.

And I'm sorry it's complicated. I wish it were not, but I don't think that's an avoidable problem, and I think that if there is an answer to your ultimate concern, it's to try and see that there is some larger and better scientific education in general in this country, which I think is appallingly bad, but I don't really think that's our charge here, although it's a charge that I think we all must in some sense want to participate in and have as part of what we do every day.

Would anyone else care to speak to this? Is this different or the same? Are we done here on this one?

MR. ONIONS: David Onions, Glasgow.

I just thought really perhaps to echo the last speaker and really to echo your comments that I think that the FDA is to be congratulated because I think all regulations should be based on good science, and what we clearly heard in the last two days is some excellent science.

If I would just summarize where I thought we were, I think yesterday we put -- I hope we put -- oncogenic DNA to bed. I think perhaps there are
better assay systems that could be used as I articulated, but I really don't think that's a risk. I think tomorrow we're going to discuss, which I think is the risk, as Ron Desrosiers said, adventitious agents. I think that's still our big concern in any new cell substrate.

But I think the novel thing that we heard today, and I'd really like to put this back to the panel, perhaps the novel thing we heard today was is there any concern for the genetic stability of our vaccines given the kinds of work we heard about today, for instance, retroviral insertions or retrotransposon insertions that might occur into vaccines, other possible recombinations between retroviral sequences and other sequences.

So should we be looking at other mechanisms in our final lots, for instance, for the genetic stability of our vaccine? Is that an issue or is it not an issue?

That's really all I had to ask.

DR. HUGHES: Comments from the panel?

DR. PETRICCIANI: Well, I guess my comment would be that there is a lot of plasticity for a lot of the genetic elements that we've been discussing, particularly retroviral elements, but it's quite clear
that the active replication of these viruses for the,

you know, reverse transcription is a process that you
want to avoid at all cost because that is the major
contributor to the plasticity of the genetic element.

So if we can eliminate that from a

vaccine, that would be a major advantage in

stabilizing the genetic content.

PARTICIPANT: I think just to try to

follow up on what Phil was trying to say, I'm Hanna

Golding (phonetic) with CBER, FDA.

And the issues that we are really

concerned with is the unknown. We are dealing with

new cell substrate that are transformed. We don't

know their history. We don't know what's the

etiology. We don't know what's the multi-event that

occurred, and the question whether some of the

processes or mechanisms that you have described

related to retroviruses might have been participating

in the transformation event of these cells that occur

in vivo.

What we are afraid of, that we not dealing

with new cell substrates; that we don't know what

caused their transformation, whether they're viral

related, whether oncogenes related, whether

combination of both, whether some transformation of
cellular genes into a retroviral type of elements or not.

The question is: was the knowledge that you had from your labs -- do you think at least there is a way to start and design certain assays that would allow companies and sponsors, CBER, to try and test these type of cell lines beyond the current testing that is currently available?

DR. HUGHES: Well, one possibility is rather than to try and deconvolute the rather complex history, that many cell lines, many permanent cell lines, transformed cell lines have is to simply create anew the kind of cell substrates that Dr. Hayflick, I believe, described in his talk at the beginning of the meeting, in which while you can't be sure that there isn't some retrotransposition of that, you certainly have an understanding of the way in which the cell became a more whole and if it is transformed, transformed, because it is something done in the laboratory, and you can reasonably exclude under those circumstances that it was a viral agent if a viral agent is not employed to make the cell permanent.

Now, if the FDA chooses that path, it does exclude a number of the standard lines that people routinely use. I think given the long passage history
of some of the lines, it will probably be hard to --

you can certainly look at a number of genes in these

cells. You can look for certain known viruses, but I
believe as you articulated, and in a sense a cell line
reflection of what Dr. Desrosiers said, it may be very

hard to rule out the sort of unknown agent if the

history of the line is not well known.

Anyone else?

DR. DESROSIERS: Yeah, I'll comment. In
terms of any cell line that's being considered, in
addition to screening for all known viral agents in
such a cell one, one can use differential display, RDA

type techniques comparing the cell line to equivalent
normal cells to look for the presence of new sequences
or additional sequences not present in normal cells.

For example, that is how the presence of
Kaposi's sarcoma herpes virus DNA was detected in
Kaposi's sarcoma cells. It was detected as a new DNA
not present in normal cells of the same type.

So one can use those sorts of technologies
to look for the presence of additional unknown DNAs in
the cell line.

DR. HUGHES: Dr. Hayflick.

DR. HAYFLICK: Hayflick, UC-SF.

Several speakers have referred
continuously to neoplastic cell lines, and I have the feeling that there is a belief that all cell lines are neoplastic. I'm sure that most of the audience knows that that's not true, and we must be very careful because there are continuously propagable, immortal cell populations that do not produce neoplasia in any test that has been conducted.

It seems to me that the decision to use CHO cells for the production of non-vaccine biologicals, which was based on the experimental data that those cells, although they might produce neoplasia, do not produce malignancies, was a very wise decision.

But I think it's very important to understand that there are continuously propagable cell populations out there that are not neoplastic. Several, in particular, I mentioned the other night, and they are cell populations that have been transfected with H TERT (phonetic) and are immortal as fas as we know. There are well over 400 population doublings at this time, and do not show any signs of neoplasia when inoculated into proper experimental animals, which brings up another final point that I would like to make.

And that is the belief that immortal cell
populations have advantages over diploid cell strains,
for example, that have a limited capacity to replicate
might not be important in the sense of the presumed
advantage of immortality, and the reason for that is
that the numbers of population doublings that you can
get from a diploid cell strain, if limited to 50
population doublings only, yields about 20 million
metric tons wet weight of cells, which I think is
ample for the production of most biologicals needed on
this plant for the next decade or two.

And the answer to that is that WI38 still
exists. It's been used for the production of vaccines
for 37 years now. There's an ample supply of these
cells.

And furthermore, one must also be aware of
the fact that when immortal cell populations are used
for the production of biologicals, there is a window
of population doubling during which they are permitted
to be used. So despite the fact that they're
immortal, there is a limited window of opportunity
from the working cell bank to the production of the
final product during which population doublings the
cells are permitted to be used, despite the fact that
they're immortal.

Thank you.
DR. HUGHES: Dr. Coffin?

DR. COFFIN: One point, that one of the problems with the more limited cell strains is that not every agent that you necessarily might want to be growing in a vaccine will grow on these. If you wanted to grow HIV, for example, WI38 would not be a suitable choice, and you really have very little choice if you want to grow that except to go to either primary human cells or to some immortalized, almost certainly tumorigenic cell line. And so certainly in some instances it will be essential -- will not be possible to use these kinds of cells to do what you'd want to do for all kinds of vaccines one might want to make. The actual point I stood up to make was to actually return to the issue that David raised a few minutes ago which had to do with endogenous elements and genetic instability of vaccines and the possibility of endogenous viral contamination of products. We now know quite a lot about the endogenous virus, the endogenous retrovirus composition of certain species, some birds, mice and humans, in particular. And there's a famous phenomenon that actually hasn't, much to my surprise,
been mentioned yet, but which I should mention in endogenous retrovirology, and that is that if you take a human tumor line and pass it through nude mice, a very high fraction of the time this tumor line will come out producing very large amounts of a very good looking retrovirus.

As chair of IBC, I have had people walk into my office showing me these Grade EMs and saying, you know, "Look what we found."

And I say, "Yes, that's a very old phenomenon that has been described many times," and it's because many strains of mice contain an endogenous provirus. We know which one it was. It's a provirus called BXB1 that produces a replication in competent xenogropic virus and which can readily infect human cells, particularly under the sort of co-cultivation circumstances one gets in putting tumors into nude mice.

It would seem very obvious that cells that contain this provirus would be very good ones to avoid for the production of biologicals. That actually has not happened. Most monoclonal antibodies that are produced are produced in cells that come derived in some way from BALB-C mice and actually contain this provirus and are not unlikely to contain noticeable
1 amount of xenogropic MLD contamination, which could
2 easily infect the recipient if not very carefully
3 removed, or if they were used to grow vaccines, say,
4 DNA virus vaccines, could, indeed, contribute to the
5 sorts of genetic instability that Dr. Kung mentioned
6 earlier.
7 And in fact, this is at least a
8 theoretical possibility with virtually all mouse cells
9 which contain very large numbers of proviruses, many
10 of which have not been shown to be replication
11 competent, but which could in theory at least give
12 rise to some.
13 So I think there ought to be a lot of
14 consideration as to the species of origin of the cells
15 that are used.
16 On the other hand, for example, human
17 cells have not been shown ever to give rise to
18 replication competent endogenous viruses despite
19 considerable effort to try to find them. Some cell
20 lines give rise to particles and so on, but not
21 through replication competence.
22 So I think the species and the strain of
23 origin in the case of mice ought to be given very
24 serious consideration in deciding which of these cells
25 to use to avoid some of these issues.
MR. ONIONS: I was just going to make --

David Onions -- I was just going to make one point. Ron mentioned using RDA. Representation difference analysis is one of the ways for looking for perhaps new viral sequences in cell lines, and I wanted to come back to that tomorrow in the panel discussion on adventitious agents.

But just to make a comment on it briefly now, I think in many ways it's an excellent idea. I think one of the problems is that finding the partner to use as the sort of direct hybridization partner is a real problem with these kinds of cell lines, and when you do these kinds of experiments -- and for my sins I've done them -- you tend to find that, of course, any rearrangement that's occurred in those cell lines, and of course, there are rearrangements in those cell lines, will also come out in the RDA analysis.

So it makes it a very messy and also very labor intensive approach, but I think it nevertheless does have merit, and I'd like to come back to that tomorrow.

I think one question I would just like to ask the panel was we touched on pseudotyping in several context, and usually when a new cell substrate
is examined, one of the requirements by the FDA and
other regulatory authorities is we look for retroviral
sequences, for instance, by the kinds of analysis that
Joerg will talk about later, the pertile (phonetic)
assay for retrovirus.

Then if it's positive, we look and see
whether those viruses are infectious for human cells,
and that's part of the equation that's used to
evaluate the safety of that cell substrate.

But what is not usually evaluated is is
there any possibility of pseudotyping with the actual
vaccine virus that you're putting in. We now know, of
course, that you do get pseudotyping, not just
retroviruses to retroviruses, but of course with VSV,
but also with paramixaviruses, with flaviviruses, and
so on and so forth.

So the question I'm putting to you is:
should that now be part of our safety evaluation, that
we actually look at the potential of pseudotyping
between any retroviral sequence and the substrate and
the viral sequences that we are actually trying to
make a vaccine from?

DR. HUGHES: Comments from the panel?

DR. PATIENCE: I think with respect to at
least the gene packaging therapy cell lines which are
being used, that has already been addressed. I think
probably your comment was addressed more at other
viruses and other types of vaccine.

DR. HUGHES: It also brings up, I think an
issue with at least respect to the first part of the
comment that may be one of the more vexing problems,
and that is I think modern technology, particularly
nucleic acid technology is sufficiently robust that
it's relatively easy to determine that any agent we
know and understand can be found and, if we're
dealing with a cell line, avoided.

I think the greater concern is how do we
find the at least potentially adventitious agents that
we have yet to learn to recognize, and I'd be curious
to hear more about that tomorrow myself.

Please.

PARTICIPANT: I just wanted to comment on
the experiment proposed yesterday to spike cell
substrates with tumor virus DNA and look for

clearance. This was recently just referred to as one
of the most understandable parts of this meeting.

I also feel it's one of the things that
brings the greatest concern. I think asking
manufacturers to spike their materials with a
dangerous tumor virus DNA poses much more risk than
any potential endogenous things you're worried about
in these cell substrates.

So it's a bad idea from a public safety risk perspective.

This same experiment, by the way, I think is in the CBER draft guidelines. So I think it's very relevant for discussion.

Besides being a bad idea from a risk perspective, it's also not necessary from a scientific perspective. It's much more likely that a spike -- first of all, you could use a spike for a harmless DNA sequence instead of a tumor sequence, and also it's much more relevant to use PCR analysis of endogenous sequences versus a spike because a spike might clear differently than the endogenous sequences.

Thank you.

DR. HUGHES: As a comment, when I heard the statement made that you're referring to, I thought the proposal was to add something that was easily scorable, and I thought it was either plasmid or lambda that was proposed.

I would also say that it was not clear to me in the discussion yesterday that that was not a proposal to show that the process of clearance was valid, as opposed to a manufacturing process that was
MR. COOK: I made the comment. Jim Cook, University of Illinois. I think that you've mistaken the point. The point was that an experiment could be done to find out how the clearance process works. This wasn't proposing to put this in some kind of vaccine, run it across into the population and see if polyoma DNA causes tumors in man, which it doesn't. (Laughter.) MR. COOK: So I think doing experiments to find out whether some kind of process is safe or not is perfectly valid. That doesn't mean that that has to be put into some kind of production schedule. I think it's still a good idea, and I'd like to see the experiment done.

MR. LEWIS: Andrew Lewis, FDA. I'd like to sort of take the panel back to some more generic concepts, and I just think based on what we heard this morning the question I'd sort of like to have a poll of the panel would be as regulatory people do we really need to be worried about viral-viral and viral-cellular interactions. It seemed to me from the data that was presented this morning that there is reason to be
concerned about these types of thing, but perhaps I'm
wrong. Perhaps this is something that these are
experimental conditions, and maybe we don't have to
worry about them in the world of generating vaccines
in neoplastic cells or in primary cells.

So I'd like to have a sense of how you all
think about that because this sort of helps us in
deciding how seriously we should go about taking these
concerns.

DR. HUGHES: Well, I'll begin by saying I
think it would certainly, given the plasticity of
retroviruses, be worth paying attention to. I would
also say that if you're asking -- if the question
really has to do with neoplastic versus nonneoplastic
cells or permanent cells versus primary cells, since
the cells of many species, whether they're transformed
or not, whether they're permanent or not, have
endogenous retroviruses, and certainly things from
primary culture can have all sorts of exotic exogenous
as well as endogenous viruses; I'm not convinced that
there is an enormous difference that is engendered by
making the cells either permanent or neoplastic, but
that doesn't take away from the larger question of
whether or not that's a real issue.

Shall we just go down the panel?
Dr. Desrosiers?

DR. DESROSIERS: Pass.

DR. HUGHES: Ben?

DR. BERKHOUT: Well, regarding SIV and HIV, we haven't seen so far an example where a defective fire resistivity (phonetic) parrots by picking up cellular sequences.

On the other hand, limited number of experiments have been done so far. So I think we still should do more analysis, in particular, of the monkeys that were infected with live attenuated SRV strains to see if this really is impossible.

You know, it may need some more time to actually say that is impossible.

DR. KUNG: I guess I have to take a practical point of view. I think the theoretical potential is there. It's almost impossible to avoid, and I'm actually thinking we have no choices in the sense that I think these things will happen, to pick up DNA from retrovirus, and there are a number of endogenous viruses, some of the unknown ones we don't even know. So it's hard to even detect them.

But you know, in all likelihood, without any particular selection, those events will come and go, and so if I take a practical point of view, my
feeling is that it's not a serious problem. That would be my feeling. There's no other choice. If you had better choices, that would be fine.

It's very difficult to even identify those things, and so I don't know what the practical solution to that. It does happen. I think all on this panel would say it does happen.

DR. LINIAL: I guess there are two issues. The first, I agree with Hsing-Jien and Steve Hughes, and that is the problem of retroviral contamination or contribution to other viral vaccines. I think clearly it's a possibility, but it sounds like, you know, all the common ones are being screened for, and people are doing the best they can.

The other issue is using retroviral vectors or retroviruses as vaccines, and I think that's something that really needs a lot more discussion. We've heard a lot of evidence here that none of these things are really safe, and I have a lot of concerns about using retroviruses to put into people, especially possibilities of defective lentiviruses, which we know can cause disease, or even in the case of foamy viruses until we know a lot more about what happens when they're widespread in the human population, which they aren't now.
I would be very cautious about using retroviruses as agents as opposed to worrying about what retroviruses are doing to other agents.

CHAIRPERSON ROSENBERG: I guess I would make two comments. I agree with what some of the other panelists have said. There's nothing that really convinces me that there's a difference per se between using a neoplastic or an immortal-like cell for a substrate. I think it more reflects the properties of the particular cell, not whether it's normal or neoplastic or falls into one of these intermediate categories.

Another point I think with regard to the retroviruses. I think it's very difficult to really accurately assess the risk, but we've seen examples presented here, and certainly there are many others where situations that involve replication of the viruses or have the risk of replication of the viruses clearly can lead extremely rapidly -- we've seen several presentations that have emphasized how rapidly changes can occur in these populations.

So certainly circumstances that have a risk of replication, I think, must be avoided because the circumstances that can follow are quite unpredictable. I think that's another message that
has come through.

DR. KAPPES: Nava, would you like to say something?

DR. SARVER: Sorry, yes. Nava Sarver from the NIH.

I'd like to ask the panel and perhaps some people in the audience. There has been, I think, the stringentest (phonetic) case of potential risk with retroviral replication, has been in the case of gene therapy where they've been used extensively for delivering whatever therapeutic genes or replacement genes, and I'd like to know.

I mean, I'd like to know -- the FDA and the audience and the people in the panel that have been involved in gene therapy -- whether there are any documented cases where some of the concerns that are expressed here have been materialized, and from the limited knowledge that I have, I am not aware of any identified risk that has occurred in humans, you know, with the application of gene therapy.

So perhaps this could be taken as a case study and really exhausted and see what are the risks and are they meritorious. I mean, can we really rely on the concern that is expressed here when we extrapolate this to gene therapy and learn from that?
DR. KAPPES: I think we have no choice.

All of what we're speaking of is highly complex. Each individual question is complex and needs to be addressed individually.

I think my simple answer to whether we need to worry about these interactions: yes, and I think that's the obvious answer. No one would probably disagree.

Maybe that isn't exactly how the question was posed. The implications are tremendous. I don't see alternatives.

In the case of HIV vaccines, we have to continue to look at how the virus is different, for example, in comparison with what we have learned, Nava, maybe as you're referring to with Maloney.

One issue I pointed out that's clearly different with lentiviral vectors compared with Maloney is lentiviruses and the recombinants themselves are likely to have the capacity to infect nondiving cells. So if you have recombination and if that recombinant can be pseudotyped by superinfection or even normal cellular receptor ligand interactions, it's possible to mobilize that recombinant to adjacent cells, and that's very different than what you might see with Maloney.
It sounds hypothetical, theoretical, and certainly it is. So I don't have -- there is no -- I guess my answer is there aren't easy answers, and this is the platform by which I think the FDA, in particular, has established to try to address these important questions.

DR. PETRICCIANI: I guess my comment would be to compare vaccine use with gene therapy, is the issue with vaccines. You're involving far more people probably with the potential risk, and using a greater number of division of cells, and possibly the benefit is less for people than gene therapy. There's less personal benefit.

So it changes the equation in my mind.

My comment about the use of retroviruses is that replication is something that is to be avoided if possible, but you know, if that is present, then you're definitely going to transmit other genetic elements. That's something that will happen regardless of the cell type.

So the question between neoplastic cells or permanently dividing cells in my mind becomes less of an issue rather than the types of genetic elements, the endogenous elements that will be transmitted.

I think that John Coffin made a good point
about the species of selection. If you're going to administer a retrovirus to a human, perhaps you might want to think about using a human cell line where there's no new genetic element coming in from a different species. That might be more advantageous than choosing something completely different, like a mouse.

DR. SARVER: I'm not trying to minimize.

You know, I see the difference. I mean, it's definitely a vast difference between gene therapy and a case where you have the benefit definitely outweighs the risk, and the naive individuals that are going to be vaccinated.

What I tried to say is perhaps the gene therapy field can actually give a quantifiable number to the risk, to put the probability that something, an adverse effect can actually take place.

And granted we don't have that many number of patients in gene therapy, despite all of the hype that we hear, but nonetheless, it's a defined population of patients, and it can be used as a probability study. What are the chances and can we actually put a definite number on the probability that something risky is -- that something bad can take place?
This is what I was driving at, whether we can. I mean just to work up the numbers and to say are we talking with infinitesimal probability or is the probability really a quantifiable number.

DR. EVANS: One comment about that is we're going to have to wait for a long time after those experiments to see if there's any problem. I mean this could go on for 40, 50 years and something could come up.

PARTICIPANT: I was just going to make a comment on that comment from the last speaker. I really don't think it is a very good object lesson because, first of all, the numbers are small.

Secondly, a high proportion of the therapies that are being produced are in terminal patients. So there's a high correlation between having had the retrovirus vector and being dead.

(Laughter.)

PARTICIPANT: And that's not meant to be a flippant comment, but it's true.

And I think the third comment is that we know that a lot of these people have had delivered BLS-30 sequences. To my knowledge, there's not been a particular study that's actually followed up the consequences of having BLS-30 mainly because these are
terminal patients with other problems.

DR. PATIENCE: I've got a few points down here relating to this question and earlier with respect for gene therapy and the use of cell lines, such as WI38, it's not practical. Basically a lot of these systems need to go back to single cell clonings, and by the time you got through your 50 doublings, you just wouldn't have a useful packaging cell life.

With respect to do we have to be worried, well, I think we are looking at Maloney or at least murine leukemia virus, potentially lentiviruses. A lot of information is known about these viruses. So we can make the systems as safe as we intellectually possibly can and biologically possibly can. They're well understood, which may be reassuring.

Coming back to David Onion's point, with respect to the patient either dies or receives a gene therapy vector or another treatment, I think that really does touch on an important point, which is that we have to look at individual scenarios with respect to what species barriers we may be jumping. Retroviruses, for instance, their behavior when you cross the species barrier may be totally different to what you can see in vitro. You in very many cases just cannot predict whether they have any potential
And then another order of magnitude on from there is, yes, okay, there is a definable risk with respect to the recipient of a therapy, but does it not -- at least personally speaking, does it not become more of a public health issue if there's the potential for transmission? I think maybe that takes it on to a different level of assessment.

DR. RUSCETTI: Yeah, one slightly different point I wanted to make at least in relationship to using mouse packaging cell lines, you may generate recombinant viruses and there's some instances where that has happened, but it's not clear that that actually would be a risk, even if you did give it inadvertently to patients.

We know from studies of mouse retroviruses that there are very specific changes. These viruses have to have certain sequences that make them pathogenic, and you may generate lots of different recombinants that don't have those. Even to cause a disease in mice there really is very little evidence that you can generate pathogenic retroviruses in vitro.

For these then to cause diseases in humans I think are even less likely. These viruses have to
replicate at very high titres and certain cell types
and perhaps carry certain envoy genes that might have 
biological effect in human cells.

So I just wanted to bring up the point
that even though you might generate recombinant
viruses, they may pose no risk at all to humans, but
granted we don't know, and I think everybody will say
here we're not willing to say that there's no risk at
all for using these sorts of lines as substrates for
vaccines.

DR. BROKER: Tom Broker, UAB.
I wanted to kind of extend one
consideration Nava brought up, and that is there
really is a lot of other experience that I think this
particular concern today can be referred to, and one
of them is simply the blood bank system, the blood
transfusions that are very commonplace, the use of
clotting factors which have been produced in various
ways, and the use of interferons, for example, and a
variety of other proteins that are routinely delivered
to patients.

I think there is a lot of experience here
that could relate to the risk of transfer of agents
either from contemporary blood donors or through the
manufacturing process.
A second thing I wanted to bring up is that there is concern about, that the general public has, as well as the professional communities, about simply immune reactions to the very product that you're trying to transfer. In the case of vaccines, that's the objective, but in the case of contaminating components in a vaccine, the question is how many different things can you inject into a given person before you start getting negative effects.

Something I've experienced, I helped monitor an Internet site for laryngeal papillomatosis, and there is an extraordinary amount of what I call Internet paranoia that is not to be dismissed, patient support groups that absolutely go ballistic over the most elementary rumors warning people not to engage in a certain therapeutic protocol, for example. It's out there, and it's going to be unavoidable.

The fourth thing that I wanted to mention, which I'll talk a little bit about tonight and tomorrow, is that a number of cell lines that we have felt to be relatively stable, in fact, continue to evolve. There is a certain degree of genetic instability in cell lines that, while you may know what they are today, ten passages from now they may not be what you thought they were.
And so rather continuous monitoring of what you've got is going to probably be a necessity. Specifically, I believe this is related to the particular growth environment that the cell line has experienced in its most recent history. If the demands are to divide rapidly and produce large amounts of material, they will have a certain phenotype. If they are put into a more quiescent stage, it appears that they'll be able to alter their gene expression at least, if not underlying genetic changes, to kind of adapt to the demands of how much growth is expected out of them.

And the final point I wanted to make, it's a variety of different ones, of course, but that is the immune status of the recipient is going to affect the risk of anything that's transferred into a patient. For example, there's over 21,000 solid organ transplants a year, 25,000 bone marrow transplants a year in AIDS and a variety of other immunosuppressive disorders, and what works in the general population may not work for the several hundred thousand people in the U.S. who have compromised immune status where reactivation of a contaminating virus, for example, will be very different in that recipient compared to an otherwise healthy person.
So I've raised a lot of issues, but I think they all are relevant to the concerns of this session.

Thank you.

DR. HAYFLICK: Hayflick, UC-SF.

One of the panel members made mention of the fact that a limitation on the use of WI38 or other human diploid cell strains is the observation that they can only undergo about 50 population doublings which precludes their usefulness when cloning is necessary in order to produce a particular product.

That was true. It no longer is because WI38 and other normal human diploid cell strains can be immortalized by transfection with H TERT and undergo hundreds and hundreds of population doublings with full retention of their normal properties.

Thank you.

DR. HUGHES: All right. It is now slightly past 11:30, and unless someone on the panel or in the audience has an additional concern that can't be dealt with after lunch, we'll go to lunch.

Thank you very much.

(Whereupon, at 11:35 a.m., the meeting was recessed for lunch, to reconvene at 1:00 p.m., the same day.)
DR. ZOON: If we could get started, we will now enter into our next session, which is entitled "Cellular DNA as a Potential Source of Oncogenic Activity or Infectious Agents."

I am obviously not Rob Breiman. I'm Kathy Zoon, and I'm the Director of the Center for Biologics and happy to have an opportunity to chair this very important session.

I do want to announce that we are adding one additional speaker this afternoon. So our break will be delayed by about 15 to 20 minutes in order to accommodate an additional talk that was scheduled for tomorrow, which we are now having this afternoon. So I would appreciate your patience.

And also I will ask all the speakers, because we are having an extra speaker this afternoon, that please keep to your time so that we give everybody an opportunity to have their full time to present their information.

With respect to this topic, this is not a new topic that either the Public Health Service has been working with, and particularly the FDA, over a number of years, whether it's vaccine safety, blood
safety, or new therapeutic products, including gene
therapy, and obviously it's not without its
controversy over the years. Levels, acceptable levels
of cellular DNA has been a subject of many
discussions, all of which over the years have
developed into a position in many cases for a number
of the products regulated into scientifically sound,
I believe, estimates of risk involved with DNA, but as
we enter into new areas and looking at new types of
applications of cell substrates, we want to insure
that the very best science underpins our scientific
decision making and regulatory policy.

And we appreciate the opportunity of all
the participants in this conference in contributing to
those efforts.

So I now have the pleasure of introducing
Dr. James McDougall from the Fred Hutchinson Cancer
Research Center, and he's going to be talking to us on
designer cell substrate, telomerase activity and cell
immortalization.

DR. MCDougall: Okay. First of all, I
should thank the organizers for allowing me to talk
this afternoon. I have to get back to Seattle. The
sun's shining there.

(Laughter.)
PARTICIPANT: It won't be.

DR. McDougall: It won't be by the time I get there, no.

We really got into a real interest in telomerase and its inductions because we found out some while ago, maybe a couple of years ago, that one of the oncogenes that the high risk human papilloma viruses induces this enzyme in human epithelial cells, and this first slide just demonstrates the fact that using a trap assay to see the repeat sequences that are produced by the telomerase enzyme, that the 16E6 oncogene induces that enzyme, but the other oncogene of papilloma virus HPV16 or 18 does not do so. E7 does not induce it.

If we put the two together, we actually see an enhancement of that telomerase induction, and if we look at immortalized cell lines that come out of this sort of experiment, and these are in human mammary epithelial cells; if we look at a long term one, in fact, this one is human skin keratinocytes, passage for 84 times, we see that that telomerase level, if anything, increases in these cell lines.

One other thing that we were interested in in this context was does this do anything for the immortalized cell, for the ability to immortalize
cells, and does it do anything for maintaining the
telomere length in cells, which is the key point that
one's interested in here.

And if we look at clones of keratinocytes
that are just expressing HPB16 E7, you see that in
early and late passage, there is a loss of telomere
length in each one of these examples. The average
length is dropping. So the telomeres, as they do in
any replicating human cell, gradually shorten.

However, if we add E-6 to that mix, we now
see that they maintain their telomeres at a standard
length. So clearly E-6 induction of telomerase here,
and these are cells pre-immortalization or pre-
transformation, they maintain their telomeres quite
efficiently.

And the third thing about the human
papilloma virus situation is that as with many, if not
most, if not all cancers, they are very -- this is
cervical cancer associated with HBV16 or 18 -- they
are all positive for high levels of telomerase
activity. So the normal cells from the same patient
are negative, as one would expect. The tumor is
always positive.

So this was an observation that was of
interest to us in terms of HPV and led us into looking
more closely at the effects of telomerase itself, and
this is just a cartoon of the ends of the chromosome,
the telomere ends and the telomerase RNA template is
here, and this really should now be called with the
acronym H TERT, H-t-e-r-t, which is the catalytic
subunit of reverse transcriptase that is the active
part of the enzyme here.

So we set out to look at the effect of
this H TERT upon primary epithelial cells, but first
of all repeated the Bodner experiment from Gerund and
their associates, in which they put H TERT into human
fibroblasts, and what you see clearly is that those
become immortalized.

And as Dr. Hayflick pointed out, if you
had 20 tons of WI38, there's no reason why you
shouldn't have 2,000 tons of WI38 if you add H TERT.

One of the characteristics we looked at
was the presence of P16 in these cells, P16 which
always increases with senescence, and we looked at the
profile of P16 in these cells going through extended
life span.

Now, we've taken these up to about 300
population doublings, and what you see is a very
slight increase in the P16 levels from the earlier
picture, but it never reaches anything like the level
of P16 that we see in human keratinocytes.

By the time they've gone through 16 to 20 passages, they have very high levels of P16, and they senesce.

Now, the interesting thing that really came out of this and that we were clued into by the papilloma virus E6/E7 results was when we looked at mammary epithelial cells, we found that if you looked pre-M0, this first point at which mammary cells tend to senesce, we found that when you added 16E6 to these and immortalize them, you lose expression of P16 or it was greatly reduced, which was not the case if you put E7 in.

And the same picture occurs with the H TERT. So H TERT added to HMECs in order for those cells to become immortalized, you have to reduce the -- you have to have a reduction in the P16 level, and if we look at skin keratinocytes, the picture is exactly the same.

Where E7 is present, together with H TERT, you do not get a reduction in P16 level, but you don't need to because E7 is binding the retinoblastoma protein, and therefore, if you bind the retinoblastoma protein, you will actually -- sorry. Here -- you're actually achieving the same result as if you were
knocking out P16, but always in the case of H TERT alone in order for the cells to become immortalized, you have to down regulate P16, and that holds in a number of different types of human epithelial cell lines.

So the situation is that if you put H TERT into fibroblasts, then clearly they carry on, and there are no major phenotypic changes observed in those cells, but if you put H TERT into epithelial cells, human epithelial cells from any source whatsoever, they have to lose expression either of P16 or they have to have the RB pathway knocked out in order for those cells to survive and become immortalized.

Now, loss of P16, as I'm sure most and perhaps everybody here knows, is a major feature of many tumors. Up to about 50 percent of human tumors looked at have lost P16 expression in one way or another, and that's certainly true in a number of cell types, and I've just put a number of references to show this effect.

So the P16 locus is part of the N4A locus, and P16 is actually one of the two transcripts from this locus. So there's P16 and P14, and we were interested in the fact that when we looked at P16 in
most of the cell lines, P16 was lost, but P14 remained
transcribed and unaffected.

However, in more recent experiments that
we've been looking at in adenoidal epithelial cells we
now find that some of those have lost P14, but still
express P16. So it looks as though you can use one or
the other of these transcripts to allow this feature
to occur.

So, again, all of the H TERT immortalized
epithelial cells that we look at have lost expression
of one or other of these genes.

And as you know, these genes have very
important roles in the cell cycle pathway, P16 here
and P14 here, with its inhibitory effects on MVM2. So
these are extremely important genes.

Now, we were interested in how P16 was
being down regulated in these H TERT cells. One of
the ways we approached it was just to look for loss of
heterozygosity, and obviously we could do that in a
number of ways. This is just using comparative genome
hybridization where you compare the cell line that
you're interested in with a known normal cell line, a
competitive hybridization onto a normal karyotyp,
and when you do that, you should always see a line
that goes right down the middle here, showing that
there's no loss or gain of any sequences in either of
the cell lines that you're looking at.

    But what you see on here with a little bit
of noise on some of the chromosomes is a clear loss of
the short chromosome line, and in fact, this cell line
when we look more closely has an isochromosome of the
long arms of nine, but has lost the short arms, and
that's where P16 locus maps.

    So clearly loss of heterozygosity is one
of the mechanisms for this.

Now, a second mechanism that goes right
back to some of the talks yesterday is methylation.

Showing again a number of H TERT immortalized cell
lines from in this cases keratinocytes or some of the
adenoidal epithelial cells, and in every case when we
look at these, we see that at least one and sometimes
both alleles where there's not a lot of heterozygosity
are, in fact, the promoter is heavily methylated over
time.

    And so by the time any of these cells
become immortalized, there's clearly methylation of
the promoter of P16. So clearly two mechanisms are
operating in these H-term immortalized human
epithelial cells to actually down regulate P16.

    We haven't looked at P14, but I suspect
the same sort of results will show for that. Sorry.

I went the wrong way.

Now, one other feature that concerned us is that, as I said, P16 loss is very common in many tumors. What about the phenotype of H TERT immortalized epithelial cells as opposed to the fibroblasts where we have not seen phenotypic changes, and I qualify that by saying "yet."

One of the ways to ask this question is to look at organotypical RAFT cultures in which we put the epithelial cells onto a collagen and fibroblast containing substrate and allow the cells to differentiate and produce what amounts to essentially a normal skin.

Since we're putting these genes on with vectors, we also look to see what the vector, which is the LXSN retroviral vector that we use in these systems, and you can see essentially the result is the same. Even though this is a slightly smaller RAFT culture, basically the differentiation pattern is identical.

And just to show you with HPV, if we add E7 to this type of RAFT, we begin to see very definite changes in that there's not a clear difference between basal cells and surface cells, and there are obviously
replicating cells high in the differentiating surface, which is obviously abnormal and what you see in dysplasia, for example, in cervical carcinoma.

What happens when we look at the H TERT cells? Well, again, we can find divisions higher up in the level, but equally importantly, if we look at cytokeratin, in this case Cytokeratin 1, so here's a hemotoxin and eosin stain of a RAFT, and here it is with the TERT in it, showing that there clearly is a change in the phenotype here.

And if we look at the Cytokeratin 1 expression, you can see that it's different in the TERT RAFT. It's lost from these basal cells, and it's clearly very positive in these upper layers, whereas here it's much more evenly distributed.

And in one other example, if we look at an integrin, Alpha 6, Beta 4, we see the same sort of changes, where Alpha 6, Beta 4 is normally expressed in normal epithelial cells just in these basal and super basal cells. In the TERT immortalized keratinocytes, it's expressed right through the surface. So clearly a phenotypic change is going on here.

And my last example of the fact that there are clearly phenotypic changes in these cells is that
if we do a karyotypic analysis of a number of cell
lines, and this is just a subset of the ones we've
looked at, we find some that clearly are quite normal,
and as far as we can see, there is no karyotypic
change in these by any mechanism we've looked at.

But there are some cell lines that clearly
are changing. Here's the isochromosome 9-1 with the
P16. This nevertheless has lost P16, but has done so
only by methylation on both alleles, and here is
another H TERT cell line which has really quite
massive karyotypic changes in it, another one here
with an extra chromosome.

So clearly there are phenotypic changes in
terms of cell differentiation, and there are changes
in the keriotypes of these cells. So H TERT
immortalization is not completely benign.

So conclusions from this: telomerase,
whether by E6 or H TERT, is not sufficient to
immortalize human epithelial cells unless this pathway
is inactivated.

Secondly, we can get inactivation through
E7 binding of RB by methylation of P16 promoter or by
chromosomal deletion.

Thirdly, we can show that E6 activates H
TERT, but we're not clear about how that works.
Although E6 induces myc and myc activates telomerase in some cells, that doesn't seem to be the pathway for E6, since we know that E7 induces myc to quite high levels, but does not result in the activation of telomerase. So clearly there are two different pathways here.

We've found that epithelial cells immortalized by TERT can exhibit chromosomal abnormalities, and lastly, that epithelial cells immortalized by E6, E7 or H TERT with P16 loss have similar changes from the normal differentiation program seen in organotypic culture, and these are remarkably similar to what we see both in experimental systems and in cervical carcinoma.

Thanks.

(Appause.)

DR. ZOON: This presentation is now open for discussion and questions, and if I could ask each of the participants to identify themselves when they get to the microphone.

Dr. Fried.

DR. FRIED: Mike Fried.

What happens in the P16 knockout mice? Are the epithelial cells immortal?

DR. McDougall: I cannot remember. I
think they're perfectly normal actually. I don't really know the answer to that. So I won't speculate.

DR. FRIED: Okay, and what do you think is the difference between the fibroblasts and the epithelial cells when you add H TERT? I mean one goes and one doesn't.

DR. McDOUGALL: I do not know. Again, that's something that we're looking into and trying to think up the right experiments for, but we don't know.

DR. HAYFLICK: Is this working? Yes, I'd like to -- this is Hayflick, UC-SF.

I'd like to congratulate you on a series of very elegant experiments. I think, however, that one must emphasize that telomerase expression is necessary, as far as we know, for immortalization of cells, but insufficient for production of cancer in those cells.

Telomerase is an immortalizing enzyme, and as far as we know not a cancer causing enzyme, and the evidence for this is as follows.

Virtually all transformed human cell populations, certainly, have telomerase expression.

There are some exceptions, of course.

Furthermore, those cell populations have been transformed, as you so well demonstrated, in many
cases by oncogenic viruses and also by radiation and
carcinogens, and as a result of that immortalization
telomerase is expressed.

But even more to the point is the fact
that post fertilization of human sperm and egg,
telomerase is expressed for the first three months of
gestation in all cells. That expression then
diminished and remains constant in all of the stem
cell populations, that is, normal stem cell
populations, during embryonic development and then
during maturation and adulthood.

So that the mere expression of telomerase
is insufficient in itself to produce cancer, and I
think this is very important to recognize.

It's also been shown, as you demonstrated
here, that as the cells undergo many population
doublings after H TERT administration, there are some
-- there is aneuploidy developing, and I was happy to
see that you have also made this same observation.

Thank you.

DR. McDougall: Yes. Thank you.

Obviously I agree with everything you
said, except one thing, and that is that I draw a
parallel with what we see with E6, E7, and that
parallel is that E6, E7 also are not sufficient to
induce cancer. There have to be other genetic
changes, and my interpretation is that it's highly
likely that H TERT immortalized cells will eventually
produce those genetic changes, not because of H TERT,
but simply because that is a fact of cells as they
become immortalized and continue to replicate.

So I'm not suggesting H TERT is itself
sufficient, but I think the cells are certainly
capable of progressing.

DR. HAYFLICK: Yes, I agree. There are
downstream effects with P53, P16, P21, et cetera, that
are essential for the expression of malignancy or
mutagenicity.

Thanks.

DR. ZOON: Okay. Thank you very much.

Thank you.

Our next speaker I have a great pleasure
to introduce, and a long time colleague, is Dr. John
Petricciani. He's representing the International
Association for Biologicals, and he will be giving an
introduction to cell DNA issues, a subject which he
has become vastly familiar with over the past 20
years.

So, John, it's a pleasure.

DR. PETRICCIANI: Thank you very much,
Kathy. It's a pleasure to be here.

And as Kathy pointed out in her introductory comments, this is not a new issue. In fact, it's been around for decades, more decades than some of us would like to remember, in fact.

Because the DNA issue is so ubiquitous, it's already been discussed almost from the very first lecture here. So what I'm going to do is skip through a number of the introductory slides rather rapidly so as not to bore you and put you to sleep after lunch, and then we'll get into some of the other pieces of this where I think there's room for some discussion.

It hardly needs mentioning again that there is a rationale for being concerned about DNA, and it relates simply to the issue of safety of products that make contain even small amounts of DNA because of the theoretical possibility that the DNA in those products, once transferred to the patients receiving the product, may impose an oncogenic risk or some other type of pathology.

The risks, just to be a little bit more specific, could include, for example, if there were a complete viral genome in the contaminating DNA, possible at the extreme, possible uptake and expression of the viral genes to the extent that
intact virus could be replicated.

Of concern primarily in recent years to the DNA vaccine and the gene therapy communities has been the possibility of DNA causing mischief in terms of insertional mutagenesis, activation or inactivation of genes, up regulation or down regulations of genes.

So that's not an issue peculiar to cell substrates and has been primarily an issue, as I just mentioned, for those two other therapeutic areas.

The real area of concentration of concern over the past several decades in the context of this meeting has been on the possibility of tumor induction with very specific emphasis on the possibility of an oncogene being transferred from the cell substrate to the recipients of the product.

In addition, there has been some discussion of the possibility through residual cellular DNA of activating proto-oncogenes or inactivating tumor suppressor genes. Both of those possibilities, again, are not unique to this, but of special interest to the people involved in gene therapy and DNA vaccines.

Well, there's a lot known about DNA and the possible mischief that it could cause, and it goes back many decades. We know that -- and I'm not going
to dwell on this because some of these have already
been discussed, but even going back to 30, 40 years
ago, it's been clear that nucleic acid extracted from
phage can be infectious for spiroplasts under the
appropriate conditions.

So we know that viral nucleic acids for
many years can, in fact, be infective. We also know
that viral DNA can transform cells, and there are two
examples out of many up there on the screen, and as
was mentioned yesterday several times, cellular DNA
was demonstrated to transform 3T3 cells in a classic
experiment in the 1970s.

Two out of 26 human tumors formed foci in
the 3T3 assays, but complicating the interpretation of
those data is also the fact that normal human DNA also
causced foci in that assay system.

It's also well known that viral DNA can be
infective for animals, and we're going to hear a
little bit later about the SIV story. So I'm not
going to dwell on that.

And finally, in another classic
experiment, it was shown that viral oncogene, a viral
oncogene, v-src, could, in fact, cause tumors in
chickens.

Now, on the other side of the coin to all
of those positive things associated with nucleic acids and things to worry about are a bunch of negative results, and going back to the 3T3 experiment that I mentioned a moment ago, it's important to remember that 24 out of 26 of those tumors, in fact, did not cause foci in the 3T3 assay, and equally important is the fact that very high molecular weight, human tumor DNA, was required in order to get a positive, and the molecular weight was around 30 million, and that 20 micrograms was about a minimum that would give a positive readout in that system.

There's a recent report within the last couple of years of up to 250 micrograms of hybridoma DNA being negative in terms of any evidence of tumor formation in mice over a two-year period.

Also 100 micrograms of Hela and ten micrograms of T24 DNA have been negative in an anti-thymocyte newborn rat assay, and it's here important to point out that the T24 cell has an activated ras oncogene associated with it.

And then there's a study which was initiated actually during the last year that I was still at CBER, and that was an attempt to do a study in a non-human primate to try to assess the potential tumorigenic risk associated with tumor cell DNA, and
in that study we used T24, which as I just mentioned
has an activated ras oncogene in immunosuppressed
rhesus monkeys, and there were both chronic over a
two-week period, and acute inoculation of the monkeys
intramuscularly, intravenously, and intracerebrally.

And those studies were last reported in
terms of a follow-up of the inoculated animals about
four years ago, and that was after eight years from
the initial inoculation.

It's now 12 years since the inoculations
were done, and we're in the process of trying to
locate the monkeys in their colony down in Louisiana
for another follow-up.

And finally, I just would like to point
out that we do have a normal daily burden in all of
this of about one nanogram of proto-oncogenes that
result from normal cell death in our own bodies. So
it's not as if there's a zero background that each of
us experiences. There is a significant background of
DNA, in particular proto-oncogene DNA.

Now, it's been pointed out by us and a
number of other people in recent years, again, with
special emphasis on the DNA vaccine area that foreign
DNA meets significant physiologic obstacles when we
encounter it, and I'm not going to go through these
because they're well described in more detail in the

literature.

The point here is simply that there are a

number of hurdles that DNA meet ounces it gets into a

person or an animal.

Now, there is a little bit actually of

human experience with what one would consider

potentially dangerous DNA from products. The first

one, which I'll mention, is a -- actually it was

mentioned during the first day of the meeting by Dr.

Hayflick, and that is there was an experiment done in

the mid-1950s in which adenovirus vaccine was produced

in retrospect in Hela cells, and it was a crude

vaccine with an extremely high probability that Hela

cell DNA was a contaminant of the vaccine.

It was administered to only six people,

and a number of years ago we asked the Army, U.S.

Army, which is where this study was done, to conduct

a follow-up study on those six people. They were able

to actually find the people, locate them, and do a

medical assessment, and that was 25 years after the

initial inoculation.

And the result of that follow-up was that

all six people were alive and well and without any

evidence of neoplastic disease. A very small number
of people, but it's important in talking about potential risks to try to understand what data are available to try to put things into perspective.

Another piece of information which, again, is perhaps not as complete as one might want, but nevertheless is important to recognize, is a study that was done in the 1970s in which individuals that had received blood transfusion from donors who were later found to develop a neoplastic disease of the lymphoid system, either lymphoma or leukemia, were followed up to see whether or not there was an increased risk in the recipients of blood from those individuals who later developed neoplastic disease of the lymphoid system.

And the results of that study showed that there was, in fact, no increased risk for cancers of the lymphoid system seven years after the blood transfusions had been given.

Now, that's important also not just because of the potential for transmitting potentially oncogenic cells in the transfusion. It's important because recently it's been shown that human blood for transfusion contains a significant amount of human DNA, which obviously derives from cell death during the holding of the blood for transfusion in blood.
That amount of DNA is substantial. It can range from 75 to 450 micrograms of DNA. So the point here is that not only were potentially oncogenic cells transfused, but also the potentially oncogenic DNA in the blood packs.

Now, as was stated a number of times, these discussions are not new today, and there have been a number of previous discussions, and I won't do anything but highlight these very quickly.

The first significant one to tackle the DNA issue was 15 years ago in 1984 at an FDA and NIAID workshop, and at that point there was what's been attributed to that meeting, a ten picagram per dose outcome, and let me just show you the actual statement and then make a comment about it.

The statement of the working group on DNA stated that procedures for the production of biologicals must demonstrate that no cellular or other unwanted DNA molecules will be in the final product at a level which would have a biological activity, that is, activities which could induce changes of normal cellular processes.

I think that is really the point. It's not whether it's ten picagrams or 100 milligrams. The
point is that what we don't want in the product is DNA or other unwanted materials that are going to have biological effect, and that point is often in this because of the numbers, whether it's 100 picagrams or whatever it is, but this is really the issue.

A couple of years later, in 1986, WHO had another working group and reassessing the data in a more thorough manner than could have been done at the workshop, and that's where the 100 picagram per dose target came from.

More recently, Dr. Temin did some calculations which were published in terms of risk of DNA in products, particularly emphasizing DNA vaccines, and came up with a risk of activating a proto-oncogene of less than one in ten to the minus 16 per DNA molecule.

Now, I've translated that into the risk associated with 100 picagrams of DNA so that the numbers here are all normalized to 100 picagrams of DNA.

A couple of years later Dr. Reinhardt Kurth did similar calculations in terms of the risk that might be associated with DNA in terms of the probability of two independent activating events in a single cell, and translated into a 100 picagram basis,
the risk would be one in ten to the 12th, which is very essentially the same as Dr. Temin's calculation. Another group from the Netherlands estimated on a worst case basis for hybridoma DNA that a risk of an adverse event would be one in five times ten to the eight.

And then most recently, Dr. Krause and Lewis estimated in a published paper that if cellular DNA were to encode an entire viral genome, that there is a possibility that it could become infectious during the production of viral vaccines. They estimated that if there were 100 genome copies in the cell substrate and that if product contained 100 micrograms of cell DNA, then there would be a risk of about one in 400,000 to the recipients of that vaccine.

However, if you normalize that to 100 picagrams as I've done here, then the risk becomes about one in four times ten to the ninth.

Now, that brings us up to the present, and I think the question here is whether there are issues on which there is still some disagreement and on which there should be additional discussion. One of them you've already heard about, and that is the question of whether based on phenotypic characteristics of the
cells there is a relative risk associated with them in
terms of one cell being more tumorigenic than another,
and we've already had some discussion about that.

Another issue or another difference is in
terms of the amount of DNA that may be in a final
product, and what we haven't heard much about is
puriﬁcation and downstream processing of various
products, including the live viral vaccines using
contemporary methods and how that may be applied to
reduce the amount of DNA to levels that are not
worrisome.

And then there is the issue of the size of
the DNA, and what are we really worrying about in a
product and is it of sufﬁcient size to even be a
cause for concern?

In my mind the real question is in terms
of the cells themselves, is not how well or how badly
a cell is behaving in a tumorigenicity test, but
really whether the cellular impurities in the product
carry a risk.

For example, I could argue that a highly
malignant cell may have one copy of three activated
oncogenes while a very mildly tumorigenic cell has a
100 copies of a single oncogene, and I don't know
which one is worse in terms of using it as a product.
And I think the real point is to understand what it is that's in the product and to understand what the characteristics of the cells are in terms of whether you have activated viral oncogenes or other genetic elements that you can focus on and be sure that they're not there in the final product or minimized.

Well, let me summarize here and just again reiterate. We do know that viral DNA can infect in vitro and in vivo. We do know that viral DNA can transform cells in vitro, and we do know that viral oncogenes can cause tumors in vivo.

But at least to my knowledge, we also know that tumor cell DNA has not caused tumors in any animal system of which I'm aware, and that makes people a little nervous because there's a disparity between all of those positive results and the fact that try as one might, there's been no evidence that tumor cell DNA can cause tumors in animals.

Another point to try to remember is that when we look at probabilities of adverse events, they are a direct function of the assumptions that you make that go into the calculations, and you can jiggle around the assumptions and make things look better or worse by orders of magnitude, and it's the assumptions
and the validity of the assumptions that are really important in making a legitimate probability assessment of adverse events.

Now, in the literature, when you try to look at some of the studies that have been done in the past, it's important to look at the details of those studies and not just use the numbers that come out of them, like 20 micrograms of tumor cell DNA causes foci in the 3T3 assay. The devil is in the details, and unless you look at what the things like the 30 million molecular weight that's required, whether you look at whether facilitators like DMSO or calcium phosphate precipitation are needed to get the result, then you're using things inappropriately and misleading when you try to use those numbers in doing a calculation.

Now, there are, I think everyone would agree, less than all of the data that we might need to make some really firm conclusions in this area. Unfortunately, the lack of data has been with us for 15, 30 years, and I think if there were ever a justification for a research based regulatory organization, this is an example of where it is because there are probably generic pieces of data, basic ones.
For example, dose response data could be very helpful in understanding whether, for example, there is a threshold below which we really don't have to worry about DNA, and those data don't exist. At least I'm not aware of them.

So there are areas where focused regulatory research could be very helpful so that we don't have to come back here 15 years later and go through the same thing again.

Now, one final comment, and that is I do think that there is one very special case, and that's been discussed particularly today, and that is live viral vaccines produced in tumor cell lines. It presents very special problems and needs to be addressed with the greatest of care.

But I think that that is one element in a very big array of possible products, and we shouldn't confuse the very special case with all of the other ones that are more easily addressed, and we shouldn't make the whole area very difficult when some of them can be handled relatively straightforward.

(Applause.)

DR. ZOON: Thank you for that very nice summary.

Because it ran over, I would ask unless
there's a burning question that we'll move on to the
next presentation.

If I could then introduce our next
speaker, Dr. Donald Blair. Dr. Blair is from the
Frederick Cancer Research and Development Center at
NCI, and he will be discussing evaluation of the
transforming activity and tumor inducing capacity of
tumor cell DNA.

DR. BLAIR: Okay. Thank you very much.

The subject of the transforming potential
of DNA from transformed cells has been something that
has come up frequently from the start of this meeting.

In fact, this morning someone made the comment that
the question had finally been or the danger or hazard
of DNA had finally been put to bed. If that's true,
then I am going to tell what is probably part of the
bedtime story, which is what I was asked to do was to
review some of the data from a series of experiments,
most of which were done over ten years ago in
evaluating the ability of transformed trell (phonetic)
DNA to induce foci and tumors in systems, in tissue
culture, and in animals.

So what I will try to do is to sort of go
through some of the techniques that were used in some
of these experiments and try to summarize the results
that we had obtained in some studies that we did, plus

adding some from some others.

Okay. So what I'll do is review this data

and some of our results, and the whole question of

d this is based on the need to have an effective way to

transfer mammalian DNA, transfer traits from DNA into

mammalian cells.

And attempts have been made ever since the

observations you could do this in bacteria were made

over the years, and in general, most of them were

relatively difficult to reproduce and difficult to

obtain. It wasn't really until the development of the

 technique by Graham and Van der Eb of using calcium

phosphate to transfer DNA that there was really a

reliable way of doing transfer of DNA into mammalian

cells.

Following that observation and using those

techniques, there was a rapid development of and

demonstration that one could transfer viral DNA. One

could transfer the oncogenes of transforming viruses

and induce transformed cells using purified DNA, but

it was in 1979 that the observations out of Weinberg's

lab and also out of Mike Wigler's lab that it was

possible to transfer the transformed phenotype from

chemically transformed mouse cells to recipient cells
through transfection and through the transfer of DNA and chromatin.

And following that, the demonstration, again, by Weinberg, Cooper, and Wigler's lab that one could do this from human tumors and that, indeed, the material that one was transferring was the identified oncogenes, such as ras.

These observations and this technique led to a great outburst of attempts to use this technique to try to evaluate whether all tumors or all DNAs were capable of doing this and whether one could use this technique to identify and isolate transforming genes, and as probably everyone is aware, this went on for some time with a varying degree of success.

The technique was based on the observation that if one transferred this DNA onto NIH3T3 mouse fibroblast, one could see foci of morphologically transformed cells, some better, some easier to see and some less easy to see. As we pointed out, occasionally normal cells would also transfer this activity.

But the crucial factor here in transferring the human DNA was the fact that within these foci, one could identify the presence of human genetic material using repeat probes, and furthermore,
that one could take these initial foci, take the DNA,

transfer this again, and retain a defined set of human
sequences which eventually could lead to the
identification of specific transforming genes
associated with those sequences.

So the initial assay was fairly simple
using the calcium phosphate procedure, transfecting
that onto NIH3T3 cells with various treatments to
enhance that DNA uptake, incubating those cells until
one sees foci of morphological transformation.

The initial results, however, were
relatively -- this process was relatively inefficient,
and so a lot of people set out to try and make this a
little more efficient to try to enhance it, and we and
Wigler's group, as well, devised an assay which was
called the nude mouse tumor assay to try and increase
the sensitivity of this kind of assay, and this simply
summarizes the sort of final version that we and a lot
of other people over the years have used, and that is
to do the transfection of high molecular weight DNA
from tumors or tumorigenic cell lines, in the presence
of a selectable plasmid, DSV2 neo, and to select the
cells which had taken up DNA by selection for neo
resistance to accumulate a population of cells, all of
which contained at least some human DNA.
Those pools of colonies could then be trypsinized, injected into mice to generate tumors, or could be replated and looking for foci. The advantage of the tumor assay at the time was that this was a more biologically significant endpoint. Furthermore, it appeared to be a bit more sensitive than looking for foci. It avoided a heck of a lot of tissue culture and time staring at microscopes, and it did pick up some transforming genes that couldn't be recognized by morphological transformation since they induce relatively poor morphological transformation. Again, the products of these would be reisolated, checked for the presence of human DNA, transferred to a second cycle in order to validate that, indeed, this was a genuine transfer.

The critical features of this that we found over the years were really, as has been alluded to, the state of the DNA. The DNA appeared you have to have it as relatively high molecular weight. Fragmentation of any significant amount destroyed the majority of the activity in most cases, although with the appropriate restriction enzymes you could retain activity with fairly small pieces due to the size of the genes, as it turns out, you were transferring. The recipient cells, however, in general
had to be 3T3s. They were ideal because they had a relatively low spontaneous transformation rate. If one used seed cultures and controlled the serum and a variety of other variables, one could retain their normal state.

However, they could be transfected stably to high levels, and this appeared to be critical because we tried a number of other cells, and although we could in some cases transfer genomic DNA, the efficiencies were low, and the amount of DNA the cells retained was generally much lower than what we saw in 3T3.

So in general, this was the cell line of choice that everybody has tended to use.

Now, in using the -- the problem both in foci and in tumors was that you had a background, and this simply sort of summarizes some data that we accumulated over the years of controls. Most of these were transfected either just with DSV2 neo or in many cases with human placental DNA as a control, and the percentage of tumors which arose within the first ten weeks after injection of around three million transfected cells back here turned out to be around ten or 11 percent.

The feature of these tumors is we could
never recover human DNA from these in cases where we
had transfected human DNA, and we were unable to
retransfect this and generate a second round. So
these appeared to be the kind of spontaneous
transformance that Dr. Rubin talked about earlier in
this meeting.

So with that, however, there were a number
of cell lines clearly, which everyone is well aware,
which contained transforming DNA where one could see
foci and one could get tumors in a tumor assay which
arose relatively early, and one could get secondaries
by taking the primary tumor of these cell lines and
retransferring them.

It's interesting that most of these, with
the exception of MNG HOS are ras. All of the genes
recovered and the genes apparently responsible for
this transformation were ras.

This was a cell line which we used as a
control. It's a single copy MOS transformed cell
line, and it allowed us to make an estimate of really
how efficient this was.

We knew if we took 100 micrograms of
genomic DNA we could generate about a single focus,
which gave us an efficiency of around ten to the minus
seventh to ten to the minus eighth.
If we did the reconstitution experiment with a clone plasmid DNA and at the maximum efficiency, we could get the same single focus with around ten to the minus fourth micrograms, again, roughly within certainly orders of magnitude, the same kind of efficiency.

But the efficiency is very low. It's a lot of DNA being put on these cells to get very few transformants, and given that we were pushing this assay and others were pushing this assay, it became clear that we could generate transforming genes out of things that weren't transforming genes in our original tumors.

In our case we took a cell line called OV CAR 3, generated an active transforming agent which, upon analysis and characterization, turned out to be a fusion between sequences on Chromosome 8 and sequences in the 9P23-24 on Chromosome 9. These sequences were formed during the transfection. They could be continuously transferred, but they were not, indeed, transforming sequences in the original tumor.

And as more and more people pushed this assay to many of the genes, which were recovered from this kind of transfections, turned out to be fusions or transfection generated fusions or activations,
deletions of control elements, other aspects.

It's interesting that at least in our hands we only saw these kinds of things with DNA from tumors, not with human placental DNA, which we ran extensively as controls. Whether that has something to do with the methylation state, which was referred to earlier, I don't know, but the generation of sort of spontaneous activation seemed to be more prevalent in tumors.

However, in terms of what was negative, which was part of why I was originally asked to do this, because we had a lot of negative, a number of cell lines pretty capable of forming tumors were repeatedly negative in our assays, in addition to what we expected the human fetal lung, human placenta, and others have shown that cell lines like VERO and MRC5s are also negative or have been negative when pushed into these assays and assayed in this way.

However, if we summarize all of the analysis that we did, we looked at about 27 cell lines and 52 primary tumors and recovered a total of around nine positive transfections when all was said and done. Most of those are ras, five of the cell lines, both of the tumors. One of them was nep and one ovc, a gene which we called ovc.
I also added, because this recently appeared in National Journal of Cancer a summary from Dr. Janssen, where he had summarized a similar set of experiments using a relatively similar assay protocol, 129 tumors and cell lines, most of which turned out to be ras. A number of others were genes that he also identified. A large percentage of these were transfection generated recombinants and activations.

So the conclusion, I think, from our data, from the data of others over the years was that this was not an easy thing to do, to transfer 3T3 cells with genomic DNA, and while it does detect oncogenes, it does so at relatively low efficiency and most of them are ras.

Now, as we look back now, from what we know about the situation, it is perhaps not surprising that that's the case because as we all know and as we've heard, it takes a number of changes to make a cell become a transformed cell, and this is simply taken from the recent paper in Nature by Weinberg in which he essentially took a normal human fibroblast to a transformed cell by making the addition of telomerase, large T antigen, and ras in order to make the escape from senescence to get through crisis to an activated series of tumor suppressors, and finally to
activate the mitogenic pathways.

The cell line that we used for most of these studies, NIH3T3, appears to have a P16 inactivated, and certainly that might tend to bias what genes we were able to detect, but in any case, the detection of transforming genes and the ability of DNA to transform 3T3 cells was relatively low even in the situation where we were talking about optimizing our conditions and optimizing the system in order to detect transforming DNA.

So certainly a fraction of tumorigenic cell lines were able to transform 3G3 cells. A large number of them, however, a large percentage, probably 70 to 80 percent were not.

Rearrangements during transfections, however, could also generate transforming genes, and they've contributed to this, but the frequency even there, again, was fairly low.

So even under optimal conditions for transfer, for getting DNA into cells, the ability to transfer this DNA into transformed cells is very low, and since there's multiple genetic alterations in rodents and human cells in order to generate these cells, that's probably responsible in part, despite the fact that we were getting as much uptake as we
could, why there's a relatively low frequency of

transformation.

And so the basic conclusion seemed to be

that, yes, the DNA was transforming, but, no, it's a
difficult thing to do even when you try and bias the

system in any way, and when you go to primary cells

and go to reduced levels of DNA, reduced levels of

uptake, the frequency became at least in our hands

and, I think, everyone else's as relatively

undetectable.

So I will stop at that point.

(Applause.)

DR. ZOON: Thank you very much for that

excellent overview.

Please identify yourself.

DR. FRIED: Mike Fried.

It's interesting. We note today that

NIH3T3 cells are not only missing P16, but they're

missing this P14 arf, which Jim McDougall referred to,

and arf is a very interesting protein because it's

upstream of P53, and it's actually only induced by

oncogenes, by myc, by ras, E1A, and others, it then

turns on P53.

So if the arf NIH3T3 cells are arf

negatives, and as most cell lines partially have a lot
of changes, but without arf, it allows oncogenes to be

expressed.

Well, in normal cells if these oncogenes
came in, P53 would be turned on. The cell would go
through apoptosis and be taken out as a defense
mechanism. So I think that explains why NIH3T3 were
so sensitive for those detecting oncogenes, and most
of the cell types aren't.

DR. BLAIR: No, I think that it's clear
that each cell with the pattern of inactivations and
3T3 having the particular set that it did probably
made it possible to do this, although I think the
point that they do incorporate a large amount of
incoming DNA and then take some up also contributes to
the fact that you were able to do these experiments.

DR. FRIED: Sure, but I'm just saying that
most cells picking up oncogenes would turn on P53 and
be taken out of the population.

DR. KRAUSE: Phil Krause, FDA.

Don, have you done experiments in which
you directly injected DNA from tumors into animals?

DR. BLAIR: We didn't try to directly
inject the DNA. We did some experiments directly
injecting cloned DNA, and that in our hands was
relatively clone V MOS DNA at the time, and those
experiments were not terribly efficient, and so we
never really tried with genomic using tumor DNA
directly into mice, no.

DR. KRAUSE: And I think I and a lot of
people are intrigued by the Weinberg experiment that
you summarized. Would you care to speculate? What do
you think would happen if you tried to inject those	hree DNAs directly into an animal?

DR. BLAIR: I mean, I guess my feeling
would be that the probability of getting all three
into the same cell might be probably fairly low, and
whether you could see the responses that he sees in
cell culture, I would think it would be unlikely, but
you'd have to do it.

DR. KUNG: I'd like to make a -- oh, go
ahead.

DR. ZOON: Please identify yourself
though.

DR. KUNG: Oh, sorry. Hsing-Jien Kung
from UC-Davis.

I'd like to make a comment because from
Dr. Petricciani's talk, he mentioned the injection of
V-SARC DNA can cause tumor, and in fact, I did that
experiment, and had I known that I would be invited to
this FDA meeting 18 years later, I would have kept
that chicken longer, but I didn't.

(Laughter.)

DR. KUNG: But the one point I'd like to make is that that experiment in that paper, we also said showed that the tumor actually regressed. All the tumors inject with V-SARC DNA, tumor regressed, and we felt at that time was due to immune response because we did not have nude chicken. We had the immunocompetent chickens there.

And so it's really not a cancer in that sense. All the tumors regress. Whereas if we injected the entire rsv DNA, okay, doing direct injection, then the tumor will, in fact, cause mortality of the chickens. Okay? So there is a difference.

And I'll finish the story. We never published because it was a negative result. So we did inject C-SARC, and C-SARC never caused tumor. The assay was very sensitive in the sense that if you have a point mutation 527, the C-SARC point mutants, that can cause tumors.

And so the assay was very sensitive, but it would pick up something that I would not call it cancer. Okay?

DR. ZOON: Thank you for that comment.
The last comment/question.

DR. BERKOWER: Yes, Ira Berkower.

One of the problems is I see a contrast between what you said and what Dr. Petricciani said, and that is the relative risk between tumor DNA and normal DNA. You had no transforming events with normal DNA. So can you put an upper limit on the relative risk due to the tumor DNA versus normal or lower limit maybe?

DR. ZOON: Could we have the mic, please?

DR. BLAIR: I think it works now.

(Laughter.)

DR. BLAIR: I think, you know, what we saw was that when we did normal DNA, placental DNA and at least one or two human normal cell populations, we didn't get any tumors or foci that contained DNA that we could transfer. We were doing on the order of 300 micrograms of DNA at a shot. We probably did the placental DNA ten or 20 -- maybe ten times.

So in that sense, we never saw anything at that level that we could verify that there was a focus or a tumor that was due to the transfer of the DNA.

So that's the only limit I could put on it.

DR. BERKOWER: But would you say tumor DNA is at least one log more tumorigenic? It seems to me
that it is just from what I've seen in here.

DR. BLAIR: Well, if you talk about the active ones, yes, but if you talk about the other ones that are not active in our hands, no, they were no more active than -- we didn't see anything. So --

DR. BERKOWER: And, secondly, you know, yesterday we were trying to get at this question whether some tumors are more worrisome than others.

It would also seem from your data, as opposed to what was being said on the podium yesterday that some tumors are much more worrisome than others. Others are almost not worrisome.

This would be towards getting a molecular discriminator of the risk.

DR. BLAIR: Well, I think, you know, this assay clearly is biased, and so one could argue that a tumor that has an activated ras gene would be more likely to be detected in this assay. In that sense, tumors that had activated ras genes were more readily detectable.

In some of those there were amplifications that may have helped to increase the sensitivity of the assay, but this is, I think -- you know, this is within a very specific set of conditions designed to optimize the detection of, you know, morphological
transformation of NIH3T3 cells.

DR. BERKOWER: And lastly, one thing I used to take comfort in was this requirement for very high integrity, high molecular weight DNA, but in fact, that may be entirely spurious. That may be almost an artifact, again, of the method because if there is more than one step needed, all you're really measuring there is how far those two genes are apart from each other. You just want to get them into the same cell. You're not actually measuring anything about the DNA. You're measuring something about the genes, that they're far apart. So they need to be on the same Ps or something like that.

DR. BLAIR: Well, the 20 kilobase size, 20 to 30 kilobase size, is pretty small on the genomic scale. So they're pretty close together. I don't think the linkage is probably something that you would -- that would be a problem in this particular kind of experiment.

DR. ZOON: Okay. If we could have one last comment, and then we need to move on.

DR. HUGHES: Yes. Let me respond by saying that you need to take into account that even though the DNA comes from an abnormal cell, if the transforming principle you pick up is not present in
the cell DNA that you began with but was created by
transfection, those are events that will happen with
normal DNA, and the reason that those are seen with
DNA from tumor lines or cell lots and not with normal
DNA represents the very large additional number of
experiments that occurred.

But those are certainly events that will
take place with normal DNA if you do enough of it.

The other important point is in trying to
put some sort of form or assessment of risk, what
you're really measuring in these assays is the risk to
3T3 cells, which is not the same thing as the risk to
cells that are not immortalized and don't have lesions
in them which do not make them necessarily oncogenic,
but which certainly make them immortal and, as a
consequence, easier to get into a state in which you
can measure tumorigenicity.

So I don't think you can reasonably use
this as a measure of either the safety of normal DNA
or the danger of DNA from a transformed cell.

DR. ZOON: Thank you very much for that
comment.

And thank you, Don.

Our next speaker I'm very pleased to
introduce. We've introduced her many times at
workshops, is Dr. Ruth Ruprecht, who is from the Dana-
Farber Cancer Institute, and will speak to us this
afternoon on infectivity of retroviral provirus DNA.

Thank you for coming.

DR. RUPRECHT: Thank you for inviting me.

DR. ZOON: The lights, please.

DR. RUPRECHT: I'm speaking about the
infectivity for retroviral DNA. My laboratory came
upon this issue in a somewhat surreptitious way.

PARTICIPANTS: Microphone.

DR. ZOON: Switch it on.

DR. RUPRECHT: Is that better?

Our experiments started with a mutant of
SIV made by Ron Desrosiers' laboratory called SIV
Delta 3. This mutant is built on the backbone of SIV
MAC 239 and has three big deletion, one in the vpr
gene, and two in the nef gene. The third deletion
also overlaps with the LTR, the negative regulatory
element in it.

When Ron's group used this virus in
juvenile and adult rhesus monkeys, it was attenuated,
and it turned out to be protective in about 50 percent
of the recipients upon challenge with wild type nef-
plus virus.

We wanted to develop a neonatal macaque
monkey model, and we gave these neonatal animals the
live SIV Delta 3 orally. As you know, we have a
rather surprising result which we reported in 1995.
These animals developed AIDS that looked for all
intents and purposes exactly like the disease induced
by the wild type virus.

Follow-up data then showed that the SIV
Delta 3 was 100 percent pathogenic in the initial
cohort. We were obviously concerned that we may have
had a contamination reason, immunosuppressive virus,
and we ruled out any known immunosuppressive virus in
this initial series of animals. Therefore, we knew
that there was no wild type SIV, and we also knew that
there was no simian retrovirus Type D present in the
animals to explain this result.

But we couldn't test for an adventitious
agent that may have been present in the initial CMX-
174 cells, and we also could not test for an
adventitious agent that may have been present when we
grew up the virus in rhesus macaque PBMC.

Therefore, we decided to go to pure DNA,
and we felt that the truth would be in the DNA.

The initial four animals are shown here.
The first three got cell free virus. The fourth one
got infected blood.
We decided to directly clone the virus out of animal 94-1 for 80 weeks post inoculation. At this time this infant already had immunodeficiency. We were able to clone virus out without any intervening amplification in vitro.

So what we did was we took PBMC and cloned the provirus genome in two halves, the five prime half and the three prime half, and then reconstructed a replication competent virus.

In doing so -- next slide, please -- we realized that 90 percent of the proviral halves that we cloned out were actually defective, and only a small minority of the seemingly clones of right sides were actually giving rise to replication competent viruses.

Now, in order to continue studies with proviral DNA, we needed to first conduct a positive control experiment. We needed to make sure that wild type SIV MAC 239 would yield similar virus replication kinetics and disease in rhesus macaques.

Afterwards our plan was to then take proviral DNA of SIV Delta 3 of the virus that we cloned out of the animal and compare the pathogenicity.

Earlier this year we published the results
that we obtained with the parental SIV MAC 239. I will go through these results really quickly.

Intramuscular inoculation of 400 micrograms of DNA in plasmic form into one infant, and 500 micrograms of DNA into two adults resulted in systemic infection, persistent viremia, and death eventually in all three animals.

The viral loads in the infant and in the adult were actually reasonably similar to what you're seeing when infections are conducted with cell free virus.

The same goes for cell associated proviral DNA load. The kinetics are similar to what has been published by other groups with cell free virus, and the same statement also holds for infectious cells in the PBMS.

So intramuscular inoculation of super coiled plasmid DNA and coding full length SIV MAC 239 is, first of all, reproducible. We were able to maintain the provirus stably in a single plasmid. The three animals became viremic and they all succumbed to AIDS after longer term follow-up, and after this series of experiments, we felt that intramuscular inoculation of cloned proviral plasmid DNA is reproducible and would be a wonderful tool to
study viral pathogenesis because if you rely on pure
proviruses in plasmid form, it can easily quantify
your inoculum, and if you're looking at viral mutants,
you can eliminate any parameters that may affect the
infectivity of your inoculum. Standardization, in
other words, is very easily achieved with super coiled
plasmid DNA.

So after this series of controlled experiments were finished, we then took the provirus
that we got out of infant 94-1. This provirus is
called SIV Delta 3-plus. We inoculated a series of
newborn rhesus macaques and adult macaques with this
SIV Delta 3-plus in DNA form and compared it with SIV
Delta 3, the parental vaccine strain.

First of all, infant VR-6 had high virus
load and died of AIDS. So the proviral clone, SIV
Delta 3-plus, is not only infectious and replication
competent in vivo, but it is also pathogenic, and it
shows very clearly that the progeny virus that was
isolated from the vaccine recipient is a pathogenic
virus. There was no other adventitious agent present,
and even with very sensitive PCR analysis, we've made
sure that there was no full length nef gene present.

Another interesting set of data when it
compared virus loads in adults inoculated with SIV
Delta 3-plus proviral DNA in comparison with adults given the parental SIV Delta 3 DNA. It's actually somewhat of a shocker. The adult animals given the parental vaccine virus DNA are shown in the dashed line. The peak viral RNA levels were approximately 10,000 copies per milliliter. The three animals then were able to control the virus and were not viremic in long term follow-up.

The picture in the adults given the progeny virus that evolved in that long infant (phonetic) is quite different. First of all, peak YLRNA levels are two logs higher, and secondly, the animals are persistently viremic a short time later.

This shows very clearly that the virus has become much more aggressive as it adapted in the infant, in the initial infant, and that what has been happening in vivo was selection of the fittest. Even though virus isolation in adult vaccine recipients of SIV Delta 3 is negative, we have to know that the vaccine strength persists in the lymphoid tissues. The virus actually continues to replicate at all times and uses the faulty reverse transcriptase to do so, and as John Coffin has published and other groups have published as well, lentiviral reverse transcriptase is
a lousy, error prone enzyme that generates a number of

mutants.

The virus is then selected for optimal

replicative capacity, and over the years a more

aggressive virus emerges, and then natural selection

in the host environment eventually will compensate for

the initial attenuation and replicative capacity that

was engineered into the vaccine strain.

I should say that the peak viremia that we

saw in these three adults is actually in the same

order of magnitude as the peak viremia seen with SIV

MAC 239.

Now, in terms of infectivity of DNA, a

number of questions should be addressed for which we

don't have experimental answers. First of all, what

is the minimal infectious dose for intramuscular

inoculation of cloned SIV MAC 239 plasmic DNA?

Second, which route of inoculation is the

most infectious?

And, third, what is the infectivity of

genomic DNA isolated from an SIV MAC 239 infected

animal?

Now, there are some indirect answers to

some of the questions regarding the infectivity and

the amount of DNA received to achieve systemic
infection. In 1991, Norman Leitwin and his colleagues have published a series of an experiment that showed the infectivity of a lambda clone containing the provirus of SIV MAC 239.

Four cynomolgus macaques were inoculated intramuscularly with 200 micrograms of this lambda clone. Three out of the four animals were infected, but actually long term follow-up data were not provided, and we don't know whether these animals went on to develop disease.

Now, 200 micrograms of the lambda clone is equivalent to about 50 micrograms of our plasmid DNA. So we were approximately ten times above the amount of provirus that's used by Norman Leitwin in our own experiments, which were conducted with 500 micrograms of proviral DNA plasmid.

A number of publications have appeared over the years showing the infectivity of DNA in animals, and I've just summarized some of them for you here. The references are in the Liske, et al., "AIDS Research on Human Retrovirus," a paper that appeared earlier this year.

I would like to focus on the SIV experiments by Elizabeth Spreter because there is some more quantitative information available in that paper.
But, first, polyoma virus. There is some information about infectivity of plasmid DNA as compared to cell free virus, and the infectivity of the super coiled polyoma plasmid DNA is about four to five logs lower than that of cell free virions for a given route of inoculation.

For SIV Elizabeth Sparter and colleagues have looked at intramuscular and intradermal inoculation at various doses. The lower dose of 30 micrograms per animal was no longer fully infectious, but we don't really have a threshold from these experiments either.

To conclude, there is actually a positive note also to the experiments I've described. If there ever is a nonpathogenic, avirulent, live attenuated AIDS vaccine, the way to go would be through infectious proviral plasmid DNA because, first of our, our experiments have shown that cell free virus can be replaced by infectious plasmid DNA. This would bypass the need for preparing virus stock in human neoplastic T cell lines. Standardization is easy and is very reproducible.

Systemic infection can be induced very reproducibly. I can tell you that with 500 micrograms in rhesus monkeys, given intramuscularly, 18 out of 18
animals became systemically infected. So this is highly reproducible.

And lastly, infectious proviral plasmid DNA is stable and can be stored very easily.

(Applause.)

DR. ZOON: Thank you, Ruth.

This presentation is now open for questions, discussion.

Yes.

DR. MURPHY: I'm Brian Murphy, NIAID.

Have you actually taken SIV that's integrated into genomic RNA and into genomic DNA, taken the genomic DNA itself and not taken the provirus out, clone it up, put it into lambda, put it into provirus, and seeing if you can initiate an infection with genomic DNA containing a complete copy of SIV?

DR. RUPRECHT: No, we have not done this yet, and to my knowledge, there are no reports of it.

DR. HUGHES: A brief comment to clarify a misconception.

DR. ZOON: Please identify yourself.

DR. HUGHES: I'm sorry. I'm Steve Hughes from ABL.

And that is the role of RT in engendering
mutations in any retroviral life cycle is undefined.

It is unlikely that -- there are three enzymes that are involved in the replication of the genome: RT, the host DNA dependent polymerase, and the host DNA dependent RNA polymerase.

While it's unlikely that the DNA dependent DNA polymerase makes a significant contribution, the relative contributions of RT and the host DNA dependent RNA polymerase is completely unclear, and although it doesn't really matter here, it's led to some important misconceptions in terms of the drug treatment for HIV.

DR. RUPRECHT: Well, what is known though is that the mutation rate of lentivirus is orders of magnitude higher than the mutation rate of DNA polymerases. So that gives the virus a chance to adapt, and as you have seen, it adapts rather quickly over 80 weeks.

DR. HUGHES: The question is whether or not it is RT that is responsible for the errors, and it's entirely possible it's a cellular RNA polymerase that's responsible for many of those errors.

DR. RUPRECHT: I think that's unlikely, but that's my personal view.

DR. LINIAL: Maxine Linial.
Did you compare the sequence of the Delta 3-plus the whole cDNA and the Delta 3? And if so, how many different changes were there in the genomes?

DR. RUPRECHT: We have sequenced the whole Delta 3-plus, the entire genome, and we found some very intriguing mutations, but right now without sequencing more progeny viruses, you can't really see yet which of the many mutations that we have seen are the important ones, but that's what we're studying right now. Stay tuned.

DR. PURCELL: Damian Purcell from Macfarlane Burnet Center, Australia.

We did very similar experiments which I'm going to present tonight, and I was curious with your Delta 3 mutation, the U3 deletion particularly, did you build that into the five prime end of the construct?

Because when we failed to do that, we find reversion of the virus very quickly, presumably a contribution from the presence of high level of plasmid DNA containing this element.

DR. RUPRECHT: Well, during the in vivo replication, the deletions in the three prime end were followed with PCR in all animals, and what we noticed in every animal that went on to progress to disease
was an increasingly large deletion in the area of the
delta nef and delta nre. So, in other words, the
virus actually lost additional sequences.

Now, through the process of retroviral
replication and the mechanisms of the reverse
transcriptase, any deletions in the three prime LTR U3
region will automatically be copied into the upstream
element. So they will be present also in the five
prime LTR in the next round of replication.

So overall the Delta 3-plus virus lost 180
base pairs in the LTR, which then will also obviously
be lost in the five prime.

DR. ZOON: Thank you very much. We very
much appreciate it.

We’ll now go on to the next paper by Dr.
Eugene Major of the National Institute of Neurological
Disorders and Stroke, and the title of his paper is
"Cells and Their DNA Containing Integrated DNA Viral
Genomes: Differentiated Phenotypes of the Human
Central Nervous System."

DR. MAJOR: Thank you.

I feel a little bit like Monty Python, I
think, standing here because I'm about to say, "And
now for something maybe not completely, but just a
little bit different."
I've been sitting here since Tuesday night, and what I didn't want to do is to be too redundant about talking about virus systems that I think we're somewhat familiar with, but I would like to talk to you a little bit about the human polyoma viruses and the nature of the integrated DNA that we can find in sequence in cells that they transform, and so that would be a new virus we haven't talked about yet.

And also then talk about -- and if we could have the first slide, which is what it says on this slide, and talk about cells we haven't talked about yet, which are cells from the human central nervous system, and a particularly unique cell line. At least we think it's unique, that probably would fit into that category of a design cell substrate, whether it's useful for vaccine development or not I'm not sure, but certainly it's one that has unique properties, which was established by the introduction of a viral immortalizing gene.

So we'll talk a little bit about copy number of the human polyoma viruses in cells that it transforms, and the ability to rescue the integrated viral DNA from these cells. We won't talk about integrated lentivirus in the human central nervous
system. We won't have time for that, and then we'll
go on to talk about a specific what we think is an
interesting cell line which we made a number of years
ago from the human fetal brain.

Just a few comments then. These

particular bullets here can describe SV40 DNA
integrated in human immortalized cells, and it's
relatively true, generally true for human cells

whether they are myoblasts, muscle cells, epithelial
cells, fibroblasts, central nervous system cells.

Generally speaking, and it's also true for
BK and for JC, the human polyoma viruses in cells that

it transforms, not human cells, but rodent cells.

And I should say just parenthetically here

that the human polyoma viruses are pathogenic agents

in the human population. DK itself is associated with
kidney infections. JC is associated with a
demyelinating disease. Both of diseases or both of
those viruses are associated with infections in

reasonably severely immune compromised individuals.

JC, for example, causes neurological
complications in approximately ten percent of all AIDS

patients. So it's a rather substantial infection.

In most of these cases, there is a low

copy number of the viral DNA which is integrated, and
frequently what we find in human cells, that it's
integrated in tandem copies, either head to head or
head to tail, and one to two viral copies.

It's usually located at a unique
chromosome. There aren't a lot of duplications there,
and it's been reported in the literature that SV40
DNA, for example, will integrate in human Chromosome
17, 12Q23. I'll show you evidence of 2Q35. Usually
it's a single integration site that takes place.

Integration -- this actually should say
does not select for a cell phenotype, and what I mean
here is that integration in a particular chromosome is
not pathomnemonic for a particular type of cell. In
other words, you won't get integration of these
particular DNAs. If it's a fibroblast in a particular
chromosome; if it's a myoblast it will be another
chromosome. That's not what we mean here.

The integration site, however, during the
life span of the cell line is generally stable. We
have not seem translocations, for example, of
chromosomal content from one chromosome to the other.
From very early passages to much later passage, the
site of integration of the chromosome remains the
same.

In all of these cases, if it's SV40 or if
it's JC or VK, the T protein is synthesized, and it
binds P53, the retinal blastoma gene products, and of
course, it interacts with the cell cyclins, and this
has been discussed quite a bit at the meeting already.

SV40 immortalized human cells, as Leonard
Hayflick has reminded us a number of times here, is
nontumorigenic in the conventional ways in which it
has been tested, in introduction into immunodeficient
rodent models.

Now, BK NJC, the human polyoma viruses,
will induce tumors in rodents, hamsters particularly,
rats and mice. JC itself, if inoculated
intracranially, will induce gliomas, Grade 4
glioblastoma multiform. In New World monkeys it's
perhaps the only good primate model for gliomas that
really exists.

But SV40 immortalized human cells is
generally nontumorigenic, and personally I like to
make the distinction as we discussed here in the
terminology between neoplastic, which is cancer
causing, and immortalized, which is a term that at
least we use to simply indicate that it has an
unlimited life span.

this is an archive slide. I had to dig
this out from the archives in my own collection here,
SV40 T protein and its immortalized human cells. All of the cells contain T protein, and these can be cloned out. As you can tell, this is a pretty old slide because these are immunoprecipitation experiments, simply demonstrating that the SV40 T protein or JC or BK will immunoprecipitate along with P53.

Now, let's turn our attention to, for example, the way that we can look at the nature of the integrated DNAs which are present in, let's say, BK virus, the human polyoma virus, transformed hamster tumor cells.

Now, before the advent of gene amplification techniques and PCR, what we used to do to be able to get these DNAs out of the transformed cells is do cell fusions, and this is a technique that we used to use a decade ago or so.

So if we wanted to take a look or even grow BK virus, for example, what we would do is do an experiment in which we would take BK virus transformed hamster cells, and we'd physically fuse it in co-cultures with a cell that BK is very permissive to, which is human embryonic kidney.

And when we do that, you form Heterokaryons and synkaryons, and you'd be able to
rescue the viral genome by excision, DNA replication,
and then the virus would grow, and in many cases a
long time ago, this is the way we used to propagate
these viruses.

When the fusions begin to form, for those
of you who may not be too familiar with that, you'll
get a heterokaryon that's formed, which is a single
cytoplasm which contains two nuclei, one of the
transformed cells, for example, and one of the
permissive cell.

Then as those two nuclei begin to fuse,
what happens is that the viral DNA which is integrated
in a particular single chromosomal location site is
excised. We don't know what the mechanism of that is,
but here you see actually a synkaryon, which is a
fused cell in which you have the nuclear content, the
chromaton from two of the different cells, and viral
DNA begins to replicate after it's been excised.

Now, we measure replication or we detect
replication here by using biotinylated DNA probes
that's specific to the viral DNA, and the chromogen
here is diaminobenzidine, and in the presence of strep
avid and horseradish peroxidase at areas where the DNA
is replicating, which is, of course, in the nucleus,
then you'll get a brown precipitate from the oxidation
of the chromogen, which is diaminobenzidine.

We've quantitated this technique so that we can tell when we look at these particular kinds of nuclei that we have several thousand, if not more, copies of the viral DNA which is present in the nucleus of these cells, and it's a technique we've used quite a bit for diagnosis of viral infections in the human brain.

Now, what happens with, for example, if we do this experiment now with BK, but let's say JC, and JC grows very much more slowly even in a fused culture, that actually the nuclei in which the DNA has now been excised will replicate and begin to divide. The cell begins to divide even before virions are made, and that's what you see here, a very high copy number.

These fused cultures will undergo cell lysis with time, and we can go into these cultures and we can go ahead and look at the viral DNA which is present there, whether it's BK, for example, or whether it's JC. These are simply the genetic maps of all the primate polyoma viruses that are before you with JC.

And interestingly enough, at least for us in looking for BK and JC which has now been excised
from the transformed cells, that the DNA that we sequence is perfectly excised from whatever region of the genome that it was integrated, and integration I should say within the viral genome is random also.

Now, in order for transformation to exist, to take place, the integration usually takes place in sequences obviously that are not involved with the T protein coating region, the early region of these viral genomes. So they'll be in the late region, in the capsid coating regions here or, for example, in here for JC or BK.

However, and they're linear obviously if they're integrated within the chromosome. However, upon excision, replication and virion production with these DNAs, what the DNAs that we get out in the nucleotide sequences are exactly the ones that we put in. We don't really find any cellular chromosomal DNA.

Now, we usually take extracted virion populations so there could be a selection for encapsidation of these human viral DNAs that come out, and it's possible that there could be free viral DNAs present in these particular kinds of fusion cultures that have chromosomal DNA, which we wouldn't see by the particular kinds of assays.
If, in fact, however, we take these viruses and we grow them in permissive cells in culture, there are a lot of rearrangements that take place in the genome, and in nature this takes place as well. There's a lot of genetic changes that take place in BK virus as it grows in the human kidney or in JC virus as it grows in the kidney or grows in the brain.

But if the DNA remains integrated in one of its transformed cells, there doesn't appear to be any rearrangements that take place.

And that's also true if we take plasmids, conventional colicin derived E. coli plasmids and we transflect these DNAs into cells that are very permissive, JC, into cells from the human brain, BK, and to kidney cells.

Without excising the DNA before we do the experiment, if we simply transflect these DNAs that are infectious, even if the plasmid DNA is still present, we just take the whole nine KB or ten KB vector that we made, retransfect it into cultures. We allow lytic infection to take place, for example, BK and HEK cells, and then we sequence the DNA when it comes out, and whatever that mechanism is for excision, there are no plasmid nucleotides left as
well. So it's a very specific kind of mechanism.

So we're certain, of course, that the DNA that is integrated into these particular transformed cells is wild type DNA, and we can determine what those nucleotide sequences are.

Now, I'd like to turn your attention now to a particular kind of cell line that we made because we needed to have in the laboratory a consistent source of cells that we wanted to work with that would be a cell line or an immortalized cell line, but wouldn't have the probability of being able to excise its DNA.

And so we were able to get a hold of one of the origin infected mutants of SV40, which Jasha Gluzman had used and he had created some years ago in the creation of a series of cell lines called COS cells.

And that I'll describe to you is really the human fetal brain counterpart of the COS cell, and the advantage that we thought we would have at the particular time is to make an immortalized line of a reasonably difficult cell to work with in the laboratory derived from human fetal brain, that we would not have to worry about viral DNAs excising and replicating.
So we used an origin infected mutant of SV40. It cannot replicate. The immortalizing gene is SV40 T protein. The cell lines were established from the CNS, and I'll talk to you about the differentiated phenotypic properties.

I'll spend just a couple of slides here to show you the way we handle these kinds of cultures, and we have been culturing cells derived from human fetal brain for quite a long period of time now as targets for viral infection for human CNS diseases.

What you see here is a heterogeneous population of cells from an eight week gestational aged human brain. There are cells here which are neuronal in definition, in glial, as well as progenitor cells.

We have ways to be able to physically separate out the different population of these cells so we can grow glial cells which are predominantly astrocytes and progenitors for the oligodendrocyte (phonetic), which is the myelinating producing cell with CNS. The astrocyte performs a variety of functions in the human brain. It performs part of the blood-brain barrier. It biochemically modulates the number of neurotransmitters. It produces chemokines, cytokines. It's a real work horse.
And actually the human brain consists predominantly of the astrocyte. Fifty-five percent of all of the cells in the human brain is the astrocyte. About 35 percent are neurons. These are a neuronal population.

This is just an Omarski optics of a phased contrast, viable cells of the human brain. Here are the cells that have attached to plastic, at the glial cells, the cells here that have the round body and the process bearing cells are progenitor neurons.

And viruses, different kinds of viruses will go ahead and infect different cell types, what's here. This is just another view of the same kind of culture, and we phenotypically characterize these cells as either being fliel or neuronal by different markers.

And what you see here is a digitized image of phenotypic markers in these cells that we grow. The astrocyte, for example, in glial cells will express an intermediate filament called glial fibrillary eosinic protein, and that's what's marked in the green here. The red actually in these cells is marked by an antibody which we recently made to another intermediate filament called nestin, which is a marker for progenitor stages of differentiation.
It's not unique to the brain, although the nestin that we used as an antigen to make this antibody we closed from the sequences from the human developing train.

We can actually identify cell types here that have both markers, have both nestin, for example, and FGAP, and we know that these cells are in a differentiated pathway to either go to a glial cell or in other cases to go to a neuron, and so we characterize these cells this way.

So we have this availability in the laboratory. We were fortunate enough to be able to get one of the origin defective mutants from Jasha Gluzman, and for those of you who may not remember, but what was done is that there was a restriction at the nucleus site in the replication origin of the SP40 DNA. This is just in comparison to the human polyoma viruses and the deletions made in that origin.

The one we used to introduce into human fetal brain cultures had an 11 base pair deletion in the origin here. So the virus was dead. It could not replicate its DNA. It would never make proteins.

So we took that construct. We inserted it into a 12 week gestational age, mixed culture of human brain, and this is what we got. This simply goes ahead and gives you something of an idea of the time
frame within which we did this from the initial time
of the transfection that was taking place. We did not
close these cells. We simply collected those cells
which survived as a matter of growth.

And after a period of about five passages
or so in culture, they passed through, I guess, what
would be called the replicated senescence period or
M0, perhaps M1. All of the cells became T protein
positive. Those were the cells that grew out. They
did enter a crisis phase later on in passages 31 or
38. This would represent perhaps 80 cell doublings in
tissue culture, and then from then on, developed into
a permanent or immortalized cell line.

And these are just phenotypically what the
cells looked like. They have some interesting
biological characteristics, however.

All of these cells, regardless of whether
they're at this, the early stage, or the post crisis
stage are anchorage dependent for growth. They're
absolutely non-tumorigenic in rodents or in non-human
primates at least under the systems in which we have
inoculated them. They're contact inhibited for
growth. They're somewhat serum dependent for growth.

For our particular purposes in the
neurobiology of some of these cells, they are quite
interesting because they produced nerve growth factor,

brain derived neurotropic factor. They produce a
great amount of the vascular endothelial cell growth
factor. They're Class 2 positive, Class 1 positive,

CE-4 negative.

But when you look at the susceptibility to
virus infections, these particular cells are
susceptible to JC virus and to BK. They're also

susceptible to HIV-1, HTLV-1 as well, to all of the
herpes viruses. Well, we haven't tested EBB, but
certainly CMV, herpes Type 1 and Type 2, varicella
zoster virus, which isn't on this list, as well as

vesicular stomatitis virus, which can be pseudotyped
into HIV lentiviral vectors. They're actually
exquisitely sensitive to those.

So all of these cell types or these cell
types are sensitive to a variety of different kinds of
viruses.

Now, we cloned out -- the fact that it was

non-tumorigenic, there was nothing we could do with
these cells to make them tumorigenic. So we thought,
well, there might have been mutations in the T protein

casting region of the DNA that was inserted into the
chromosome of these particular cells. So we cloned
out just the T protein coating sequences, and that's
what you see here in this particular slide.

And we found actually there were five nucleotide changes in the DNA in these cells compared to the DNA that we initially put into the transfection experiment, and some of these nucleotide changes would predict differences in amino acid calling for the protein, but we haven't sequenced the protein, so we're not sure.

But they don't really fall in locations which could tell us much about any differences in the biology of what T protein may be doing in these cells, but at least we know that, and we do know that in FSH staining, which was done by Tom Glover at Ann Arbor -- you probably can't see that very clearly here -- that there is a single integration site of the SV40 DNA in Chromosome 2Q35 location, here, and by sequencing analysis, we know the location along the SV40 DNA, which that integration had taken place, and we also know that it's in a direct tandem type of copy.

Now, we began to be interested in this cell line for a whole variety of other purposes here, and in collaboration with many other laboratories that actually have this cell line, because we made this cell line back in 1985.

I will go through this relatively quickly
If we continued to grow the SV40 T protein immortalized human fetal brain cell line, which we call SVG, in standard laboratory medium E-MEM in fetal bovine serum, it's phenotypic characteristics are that of a glial cell, and these are simply phenotypic markers that we use by flow cytometry or staining in order to be able to define the type of cell that it is.

They have fragments of cholera toxin, cholera toxin and tetanus toxin, another surface marker, which is gliactocil sulfatide called A2B5 on their surface, and this really defines a type of cell which is either terminally glial or it's a progenitor to a glial cell.

However, if we treat this cell with molecules that induce cyclic EMP, for example, like forscalin (phonetic), and on the next slide I'll show you that, or if we take these cells off of serum and put them in medium which we use in the laboratory as well as other laboratories use that are selected for neurons, that we get a shift in phenotype.

And what happens is that we lose the A2B5 population. We increase the cholera toxin population, and we also increase the number of cells which are
cholera toxin/tetanus toxin positive in the neuronal phenotype of these cells.

Cholera toxin/tetanus toxin denotes a cell which is in a neuronal lineage, and this was something of a surprise to us because we had thought that after so many passages in culture, that in fact this would be a stable phenotype of these cells, and the presence of the SV40 T protein would lock those cells into a certain time in which they were immortalized.

DR. ZOON: Could you please summarize?

DR. MAJOR: Okay. I'll skip that then, if I have to summarize this, and I'll say, for example, these are simply cells that we can treat to force them into a neuronal phenotype. This is what the cells look like, for example, if we put them on neural basal medium. These are the cells as they look like if they're in glial medium. In the presence of serum, of course, they will go ahead and divide.

If we infect these cultures with JC, for example, you can't see that, but JC will infect these cells into glial phenotype because JC is tropic for glial cells, and it's at a molecular level.

However, if we take these cells and we induce the neuronal phenotype, this is the same parent cell; then infect those, then JC will not infect that,
and it's not a matter of attachment or entry because we've already shown that, in fact, susceptibility to JC infection of these particular cells is at the level of transcriptional control, a very much different type of cell.

So let me then summarize by saying at least that here what is surprising to us is that after many passages in culture, 50, 60, 70 -- some other laboratories have passed this out 150 times -- what we seem to have been able to do is to immortalize a population of human central nervous system progenitor cells.

That these cells still are multi-potential for the different phenotypes of the cells that we can grow either in a glial lineage or in a neuronal lineage, and we use these now back and forth in this particular lineage patterns to ask questions of neurobiology, to ask questions of cell susceptibility to the human viruses, and for a variety of other kinds of questions that can be asked.

And, again, the surprising thing is that even in the presence of SV40 T protein, which is continually made in these cells, there's still multi-potential for differentiation, and they respond to signals that the human brain responds to also in
lineage pathway commitments as it develops.

And so this is actually from page 19 of the information that Andy Lewis had sent out to all of us, talking about perhaps getting to know a little bit more about the progenitor nature of the cells and designing specific kinds of cell lines that may or may not be useful for certainly vaccine development, but certainly cell lines that are extremely helpful for us in understanding other kinds of questions at least in neurobiology.

Thank you.

(Applause.)

DR. ZOON: Thank you. Thank you very much.

If we have time for one quick question, comment? No. Thank you very much. I appreciate it.

Our last presentation in this session is by Dr. Haig Kazazian of the University of Pennsylvania School of Medicine, who will speak to us on mobile elements in mammalian genomes and their implications for cell substrate safety.

Thank you for coming.

DR. KAZAZIAN: Thank you.

Well, I'm going to say some things about endogenous DNA. I thought I'd start off by telling
you a little bit about retroelements and L1 biology,
and then I'll tell you a little bit about an assay for
retrotransposons that we use, and something about the
number of active human retrotransposons, and I'll try
to give you an estimate of the insertion frequency in
germ lines and tell you what I think we know about the
potential for insertion frequency in somatic cells.

So let me go ahead and start.

DR. ZOON: Do you want me to get this slide?

DR. KAZAZIAN: There we go. Retroelements
are sequences in the DNA that have been put back into
the DNA by reverse transcription, and there are a
number of different types of retroelements, but
retroelements probably make up something on the order
of a third of human DNA. So a large fraction of human
DNA.

There are non-LTR, non-long terminal
repeat, or poly-A retrotransposons. They have no long
terminal repeats. They have a three prime poly-A.
They usually have two open reading frame, and they're
reverse transcribed by an unusual mechanism, target
prime reverse transcription or nick and prime
mechanism.

And an example of these is mammalian L1 or
line elements, and we'll be talking about those.

Long terminal repeat retrotransposons, we don't have any of these in humans that we know of. They have long term repeats. They have two open reading frames. They have reverse transcription prime by a TRNA, so very different from this group of retrotransposons as far as their reverse transcription goes.

An example of this group is TY1 of yeast. Retroviruses, as you've been hearing a lot about, have long term repeats. They usually have three open reading frames. So they have an envelop gene that gets them to be infectious. There's no envelope gene in these retrotransposons.

Reverse transcription in retroviruses is, again, primed by a TRNA, like that of LTR retrotransposons. A good example is HIV-1 of human. There are non-autonomous retroelements in humans. These are transcribed, but contain no functions for retrotransposition. Examples of these are alu elements, small nuclear RNA pseudogenes and process pseudogenes, and so they need reverse transcription and help by other elements, perhaps non-LTR retrotransposons.

So what we're talking about is
retrotransposition which is an element being
transcribed into an RNA, reverse transcribed, and
integrated at a new site in the genome. So this is a
copy and paste mechanism, duplicative mechanism for
actually expanding the genome.

And the major retroelement, the major
player in the human genome is the so-called L1 or line
element, and when I made up this slide, I said
approximately 100,000. There are new estimates that
say there are up to 400,000 or so of these line
elements in the human genome. These are database
estimates, which are probably more accurate now, now
that we have something like 12 to 13 percent of the
human genome in the databases.

The majority of L1s are truncated at their
five prime end. That means they're short, and they
have mostly three prime end sequences and not five
prime end sequences, but about 3,000 L1s are full
length, and some of these can retrotranspose.

So what does a full length L1 look like?
Here's the structure of that element. It's six
kilobases in size. It's got at the five prime end an
internal promoter sequence which is relatively poorly
defined at the moment, but is mostly present in the
first 100 base pairs.
It has a first open reading frame which makes a protein which is likely to be an RNA binding protein. It has a second open reading frame, which is quite long, most of the element, and within this second open reading frame is an endonuclease domain, a sequence which encodes an endonuclease; a reverse transcriptase domain which encodes a reverse transcriptase, and a conserve domain at the C terminal end, which may be a zinc knuckle sequence.

It's got a very short, three prime untranslated region, and then it ends in a poly-A tail, and it's usually flanked by target site duplications.

So in the early 1990s, we were able to isolate the precursors for a couple of insertions that occurred in humans, and these full length precursors were good candidates for active retrotransposable elements.

So in the mid '90s, John Moran came into the lab, and he devised this assay for L1 retrotransposition. So in this assay then a full length element has in its three prime UTR inserted a marker cassette or a retrotransposition cassette which will tell us when a retrotransposition event has occurred.
And this particular cassette has a backwards neomycin gene which is split by a forward intron. So here's the spliced donor site and the spliced acceptor site of that intron, which is in this transcriptional direction.

A cassette is also flanked by a promoter for the neomycin gene and a poly-A signal for the neomycin gene.

So when transcription occurs from this promotor, we go through this intron in this direction. We can then splice it out, and then with reverse transcription, we can have this neogene now transcribed and put back into the genome in the right orientation now with no intron disrupting it and driven by its own promoter, and having its own poly-A signal, we can get G4 and 8 resistant cells.

So this, as it turns out, is the only way you can get G4 and 8 resistant cells, is with a retrotransposition event using this reverse transcriptase.

So we put this receptor into a piece of plasmid and a nice receptor zone goes into the cell and into the nucleus and has about 20 or so copies, and we infect Hela cells with the constructs by lipofection, and we have on this P-sep (phonetic)
element a hygromycin resistant gene. So we hit the

cells with hygromycin.

And so we end up with cells that are expressing the marked L1 that have been transfected,
and we outgrow and plate dilutions in medium containing G41A, and the G41A resistant cells then contain an L1 retrotransposition event.

And here is an example of the kind of assay that we get when we put in an active element.
It turns out this was a precursor of an insertion into a Factor A gene causing hemophilia in a child.

So you can see that this is with plating out something like a million transfected cells and getting something on the order of 1,000 positive retrotransposition events. Each one of these blue specks is a colony of cells which contains an individual retrotransposition event, and that was proven by cloning a number of these events and showing that they look like the retrotransposition events that are seen in an endogenous human situation.

Here is a construct that has a big deletion in the element, and here is a construct that simply has a point mutation in the reverse transcriptase domain of the element.

I should say that we now have on the order
of nine of these active human elements in the laboratory, and the extent of retrotransposition varies considerably. So with some of those elements we get much less retrotransposition than is seen here, and with others we get much more.

So we have one element in the lab now that will retrotranspose at a frequency of about one in every ten transfected cells, a very high frequency.

I should also say that in other experiments we've shown that the site of retrotransposition is relatively unbiased. That is, the insertions into introns and genes is about the frequency that one would expect for the size of intronic sequences or the proportion of intronic sequences within the genome.

So it turns out that there have been on the order of 13 natural L1 insertions in humans causing disease, and it turns out that 12 of those 13 have come from a very small subset of L1 elements within the genome.

And so we decided to see if we could make an estimate of the number of active human L1 elements in the genome, and we got Gary Swyrbl to do a count of the number of full length elements of this subset in the haploid genome. He came out with a number of
We isolated a number of these subset elements specifically from the genome and found out that roughly a quarter of them were capable of retrotransposition. So from that we could make an estimate that the number of active human L1 elements in the genome was on the order of 40 per diploid genome, and so you could make a little range on that estimate and say perhaps 30 to 60 active elements in the genome, and yet now we're sitting with hundreds of thousands of copies that have built up over evolutionary time. So I just wanted to give you an idea of the number of recent retrotransposition insertions in humans and mouse. I mentioned that L1 insertions, the number here was 12. I know of another one now. So let's say it's 13, and there have been a number of non-L1 insertions, particularly alu insertions, in humans, and we think that those are driven by L1s, reverse transcriptase, but there's no direct evidence for that yet. But in any case, if we add those up, we get on the order of 30 or so human insertions that have been seen that cause disease. That's the only way we would know of them, and as you'll see on the
next slide, that makes up something on the order of
one in every 600 mutations.

In the mouse it is very different.

Spontaneous diseases that have been characterized in
the mouse are on the order of only 200 or so, and so
the number of insertions or the frequency of
insertions causing mutations in the mouse is on the
order of ten percent, very much higher than it is in
humans. So I guess we're lucky in that regard.

So when does L1 retrotransposition occur?
Well, L1 RNA and the orf-1 protein have been found in
primary spermatocytes in leptotene and zygotene
stages. This is mitotic prophase, and this is mouse
work. This is not human work.

Timing of retrotransposition events is
really unknown, but it's thought to be early or during
germ line production.

So we can make an estimate of the
frequency of retrotransposition insertions in the human
genome. As I said, the number of known insertions is
on the order of 28. This was back in March I made
this estimate. It's now 31 that I know of, but the
number of mutations in the database is higher.

So the number of mutations in the database
when I made this calculation back in March was 16,650.
Okay? That was from the human gene mutation database.

So that gave us a fraction of mutations that are insertions of 28 over 16,650 or about one in 600. So most of the mutations in humans are point mutations, but occasionally you get an insertion, one in 600 insertions.

So one can then go back and see, well, what's the frequency of insertions in the average genome, and one can do that by a couple of calculations.

The estimated frequency of spontaneous mutations in man is about ten to the minus nine per nucleotide per year. So we can go from there. There are three times ten to the ninth nucleotides per genome, and I used here a generation time of 30 years for another calculation. I said it was 25, but let's say it's 30. It's a little long, but 30 years, and times ten to the minus nine per nucleotide per year. That's this number.

And that equals about 90 mutations per haploid genome per generation, or let's go to that and say it's 90 mutations per sperm producing an individual upon fertilization. That's total mutations, not insertions, total mutations. Okay? Ninety per sperm.
So the frequency of retrotransposon insertions per sperm would be one in 600. Remember I said one in 600 is the frequency of insertions per all of the mutations. One in 600 times that 90 or about one in six. So about one insertion per six sperm.

If you use the 25 number for the generation time, you get one in eight sperm. Okay. So that's about one insertion, new insertion, in every six haploid sperm.

Since these insertions are random, less than five percent of the insertions should be deleterious because exons comprise one percent of the genome. Other sequences important for gene expression likely account for maybe another one or two percent of the genome, but it should be that most insertions shouldn't cause a problem.

Okay. So how about somatic insertions and what do we know about endogenous somatic insertions? And the answer is not much.

Okay. So I know of one L1 insertion that's likely to have been involved in the etiology of cancer. There was one L1 insertion into an exon of the APC gene, adenomenous polyposis coli gene reported by Nakamura and his group about five years ago, and that particular insertion was only seen in the tumor,
not seen in the constitutional tissue of the normal colon, so the colon cancer tumor.

There was one alu insertion into an MLVI-1 or 2 gene which was associated with leukemia. It was in a cell line. As I recall, the patient was not available for study. So it's not clear whether that was a somatic event or a germ line event.

Okay. There have been a few germ line events in tumors of alu insertions, but they're not somatic events.

Okay. We do know that L1 transposes at high frequency in Hela cells. I showed you that. All right. So that's something.

And we do know from the work of Tom Fanning that L1 is expressed in ovarian, testicular, and breast cancers. Okay. So it's expressed in cancers, and not all, but in some cancers.

So that about ends what I know.

Retrotransposon insertions occur in tumors with some frequency, although their observation has been pretty rare, and clearly retrotransposon insertions occur in cell lines. We've seen that in the laboratory.

The incidence of endogenous somatic retrotransposon events is unknown as far as I'm concerned at this point.
That's all I have to say. Thank you.

(Applause.)

DR. ZOON: Thank you.

Go ahead, Phil.

DR. KRAUSE: Phil Krause, FDA.

That was a very nice talk. One mechanism by which people would like to consider testing lines for the presence -- cell lines for the presence of adventitious agents is obviously highly sensitive reverse transcriptase assays. I guess two questions which are related.

One of them is if you were to test a cancer cell line which you say might be more likely to contain L1 elements, would that cause it to be positive in a highly sensitive RT assay?

And I guess the next question is: is that a good thing or a bad thing? On the one hand, does that tell you that the cell line is more dangerous? On the other hand, does it prevent you from detecting or does it then make the assay less valuable because that's a potential alternative explanation for a positive RT result?

DR. KAZAZIAN: Okay. First of all, you should do the assay in such a way that you might detect an L1 reverse transcriptase so that it's going
to be different from a -- I mean you could do a
specific assay because the reverse transcriptase with
L1 is going to have different properties than a
retroviral reverse transcriptase. Okay? So you
should try to set it up as a relatively specific assay
for the L1 reverse transcriptase.

Secondly, you're going to have a hard time
finding it because people have had a very difficult
time finding endogenous L1 reverse transcriptase.
Okay?

So it's very likely that if you find it,
it's probably, you know, with your assay, without
looking for a specific L1 reverse transcriptase, that
it's going to be a retroviral reverse transcriptase.

DR. ZOON: Johannes.

DR. LOEWER: Johannes Loewer, Germany.
Perhaps I missed the point. Is the
transposition activity of the construct you have
described restricted to human cells or does it occur
in every cell?

DR. KAZAZIAN: Okay. I didn't mention
that, but we have shown that it is functional in mouse
LTK minus cells, even though it's a human element.
You can put the human element into mouse cells. You
can also put the mouse element into human cells and in
mouse cells. It's functional in LTK minus cells.

It's functional in hepatocarcinoma cells.

I heard from Maxine Linial's lab that it was functional in quail cells? Okay. So, yes, it's functional in quail cells. It's functional in Chinese hamster ovary cells from another lab that has it.

However, we have not been able to get it functional in differentiated fibroblast type cells.

Okay? That's what I mentioned this morning.

Yes.

DR. ONIONS: David Onions.

I really enjoyed the talk. I've got a quick comment and then a question.

If I remember correctly, I think Don Cohen showed that there were line elements in a tumor called transmissible venereal tumor, which is a canine tumor, and I think he's got a line item in the upstream of myc, which is in the --

DR. KAZAZIAN: Yes, that's correct. Okay.

DR. ONION: But just another example.

DR. KAZAZIAN: Right.

DR. ONIONS: But the question was that the remarkable transposition frequency that you're seeing in Hela cells. Did you follow the fate of any of these clones that you had? And ask the question: was
there retrotransposition? And if so, what was --

DR. KAZAZIAN: Okay. I'll tell you what we have done though. We haven't done that because all of the insertions that we saw were highly truncated insertions.

DR. ONIONS: Okay.

DR. KAZAZIAN: So they were missing then their five prime end, and so they would lack promoter activity. Okay?

DR. ONIONS: Okay.

DR. KAZAZIAN: But what we have done, an experiment where we've looked to see if there's, quotes, heterogeneity of the Hela cells. So that we have taken cells that have had a retrotransposition event and compared those and looked at the frequency of retrotransposition in those cells and in another group of Hela cells that have not had a retrotransposition event to see if the frequencies were different, and we did not see the heterogeneity. We did not see a different frequency.

So the one group of cells was not any better for retrotransposition than the other.

DR. ZOON: Thank you, Haig.

We're going to take a 15 minute break. So we're going to reconvene shortly after 3:45.
Thank you.

(Whereupon, the foregoing matter went off
the record at 3:34 p.m. and went back on
the record at 3:54 p.m.)

DR. LOEWER: Good afternoon. I think we
should continue with the program, the panel-audience
discussion.

First what I want to do is to ask the
speaker to come to the podium.

So I think we are now complete.

I would like to welcome you again and
shortly to introduce myself. My name is Johannes
Loewer. I'm Deputy Director of the Paul-Ehrlich
Institute in Frankfurt, Germany.

Paul-Ehrlich Institute is an institution
which has more or less the same duties as the Center
for Biologics Evaluation and Research. I'm,
therefore, also a regulator, and I will lead this
discussion from the point of view of a regulator, not
of a scientist, which is not much like exclusive, I
guess.

The organizers of this meeting have
prepared quite a number of questions which I have
posed to the chairs of the different panels, and
because the issue we have to deal with is really very
complex and all of the discussion that we have had so far show that there are many different facets which influence each other.

So to be as constructive as possible, I thought it is maybe helpful to follow the questions which have been posed directly to me, and copies of this question have been distributed here.

And I would also like to try to simplify the questions in order to be able maybe to come to clearer answers wherever possible.

So I have made a number of slides from these questions, and these slides should guide us through the discussion. I would like to introduce the question shortly, and then we could open the discussion, which should not exclude if there are other urgent questions and contributions.

So the first question posed to the panel here is, again, of course, the risk of residual DNA, residual DNA which comes from neoplastic cells which would be used in viral vaccines, and there are a number of questions.

Is there a special, I would say, infectious risk event, a special neoplastic risk event, and is there a risk for epigenetic changes?

So we should keep these questions apart,
I guess, and should maybe focus on the question of infectious risk event. As we have discussed during the last two and a half day, one and a half day or two days, there are different types of infectious events maybe.

There may be extraneous agent, and I would say there is not a different risk between neoplastic cells and diploid cells for extraneous agent, but we should ask the question: does transformation increase the risk for infectivity for maybe a broader range of viruses or is this risk for extraneous agents really similar for diploid cells or not, or does it really depend on the virus?

That's one question we should discuss.

The second one is there may be, of course, endogenous virus or endogenous element as we have heard, and is there an increased risk for the activity in neoplastic cells compared to diploid cells, and perhaps we could discuss this question first.

First I would like to invite members of the panel to give their comments to the questions I have just posed.

DR. KAZAZIAN: As far as retrotransposons go, there's no infectious risk. They don't go from cell to cell. They're stuck in the cell in which
they're transcribed.

Neoplastic risk, hard to know. I would
guess it would be low, but I don't think we have
enough data on that as yet. We'll probably have more
data in the next few years.

And the third, again --

DR. LOEWER: Let's come to the later
question.

DR. KAZAZIAN: All right. Go ahead.

DR. MAJOR: I'm sitting next. So I have
to answer this, too, I guess.

I think, you know, as I tried to point
out, of course, if you have an integrated viral DNA,
particularly in a cell line, it can be excised. You
need to have a functional original of DNA replication,
and in many cases perhaps adventitious viruses don't
have the complete genome present there. So whether
they're there or not, I think you have to ask the
question whether that DNA integrated is functional to
potentially look at an infectious risk.

I think also something we probably should
be mindful of, too, even though we're looking at these
kinds of risk factors here, is that, for example, in
the viruses I talked about, the human ones, BK and JC,
you're very widely prevalent in the population
worldwide. All of us in this room have come in contact with these agents. We have antibodies to them, and the vast majority have these type of agents already latently infected and potentially, as far as we know, integrated in some fashion or other in certain types of organs, as well.

But it seems like my impression is the consensus seems to be that cell lines or neoplastic cells do not represent that much of a risk here.

DR. RUPRECHT: I would concur with that last statement. The data I presented were generated with exogenous primate lentiviruses for which probes do exist, and neoplastic human cell lines could be screened to rule out exogenous lentiviral infection.

One issue though that maybe should be reiterated, we presently do not know what the minimal infectious doses for lentiviral DNA is in the animal models. So that would be something that's relatively straightforward and information that maybe should be obtained.

We also do not know whether or not infections can be induced with chromosomal DNA taken from infected animals. I did mention that most of the profile sequences that accumulate during the course of a lentiviral infection in the case of this SIV is
actually defective. So the chance of getting replication competent virus out through contamination seems to me relatively remote, but we should get this baseline information.

DR. BLAIR: I think as far as the infectious agents, I think I would agree that although you don't know the minimal dose, it would appear that the efficiency uptake is probably fairly low so that if you reduce the level to the kinds of residual levels that people talk about in viral vaccines and things, that this would not pose a risk.

I think in terms of the neoplastic risk, I think clearly evidence is that to induce a neoplastic event in a normal cell of any lineage takes multiple events, some positive, some negative, and the attempts to do this even under optimal conditions requires a fair amount of effort and fairly large amounts of material.

So, again, the risk would seem to be minimal with the kinds of residual DNA that you find in vaccines.

DR. LOEWER: John.

DR. PETRICCIANI: Yeah. There's very little that I can add to what people have already said. Just let me remind you the paper that was
recently published by Krause and Lewis, taking a worst
case analysis for an infectious event, if you
normalize that down to 100 picagrams of DNA, it falls
right back into the same order of magnitude of risk as
the oncogenic events and is really no different from
that.

The other thing that Ruth mentioned a
moment ago and I mentioned in my talk I think really
is important. If as a result of this conference
people feel that yet more information is needed, and
that's debatable, but if more information is needed,
I agree with Ruth 100 percent that one of the most
important elements is some dose response data because
we're operating in Never-Never Land without that, and
whether it's in the model that she was talking about
or if it's in the 3T3 assay or whatever, I think
that's a basic, fundamental piece of data that's been
absent for decades.

And if we're going to pursue this as an
intellectual discussion, we really should have that
background data.

DR. LOEWER: I have a specific question to
Dr. Ruprecht.

You mentioned it's not known with
chromosomal DNA which have a complete retrovirus is
really infectious or not. Would you accept such a
cell line for production of vaccines?

(Laughter.)

DR. LOEWER: Even if the possibility is
rather remote?

DR. RUPRECHT: Well, I would probably try
to get the non-provirus containing equivalent of the
cell line.

DR. LOEWER: I think this should be clear.
It's obvious, in my opinion, at least, cell lines
which have a complete virus, genomes are not good
candidates for production of any biologicals. I think
I could mention a degree on this.
The question is whether these cell lines
contain part of whatever this, for example, oncogenes
or other parts, but this still would exclude -- sorry?

PARTICIPANT: (Inaudible.)

DR. LOEWER: Right.

DR. RUPRECHT: Exogenous virus.

DR. LOEWER: Yeah, I guess they have only
sometimes part of DNA viruses integrated, not these
retroviruses maybe.

DR. KRAUSE: Dr. Loewer, how would you go
about proving that a cell line does not contain a
single copy of an infectious genome?
(Laughter.)

DR. LOEWER: I think, of course, you can test directly for certain virus you know. I think every cell line should be well characterized in the cell bank, and especially the use of cell lines over the years, I think, proves to some extent that they contain or do not contain any extraneous viruses. So I think one can be rather sure about well characterized cell lines, that they do not contain complete viruses.

DR. KAZAZIAN: Phil, I gather that if you PCR'ed it up and found that it was negative, you still couldn't be sure. Is that what you're getting at?

DR. KRAUSE: Well, what primer would you sue?

DR. KAZAZIAN: Yeah, right.

DR. KRAUSE: Right. So my answer to Haig is what primers would you use.

DR. LOEWER: Okay. I think this question is not really new and maybe not specific for neoplastic cells and all people dealing with regulation of biologicals deal 50 years with this problem.

DR. KRAUSE: I agree with you, of course, and again, for the transcriber, I'm Phil Krause of the
But I guess the question comes up if there's a cell line that's neoplastic or that's tumorigenic and we don't know why it's neoplastic or tumorigenic because of an incomplete history or maybe it came from a person or something like that, the question is then: does that make us think that it's more likely to contain some of these latent viral sequences or does it make us a little bit more suspicious, that being one of the mechanisms by which a cell like that might have become neoplastic?

DR. LOEWER: Of course, this is an always open question. To test for the unknown is always rather difficult.

DR. RUPRECHT: Maybe if I could add one thing here. If we knew from a known animal experiment what the minimal dose is of chromosomal DNA, say, from an SIV infected animal, and if it turned out that it is ten micrograms intramuscularly, you know that you never have ten micrograms in a vaccine dose.

If it turns out that 100 picagrams of chromosomal DNA will never, you know, even if you test it on 200 animals, never lead to systemic infection, even if the DNA were derived from a known viremic animal, then at least you would have some sort of
DR. KRAUSE: I agree with you. That makes a lot of sense. One think that makes me pause a little bit is the slide that you showed and that I showed a couple of nights ago that appear to demonstrate that polyoma virus DNA, for instance, is quite a bit more infectious than retrovirus DNA. And you know, the list of viruses whose DNA has been tested for infectivity, and in fact, the study that Andy Lewis and I published was based on retrovirus infectivity, DNA infectivity, when we submitted that, we actually weren't aware of the polyoma virus data. But so the question is: are there other kinds of DNA from other families of virus which haven't even been looked at which are quite a bit more infectious? And so how does one really determine what that baseline is?

DR. RUPRECHT: The answer to that is yes. I showed a slide that summarized a number of animal experiments with other agents, including Hepatitis B in chimpanzee, HTLV-1 in rabbits, FELV, murine leukemia virus, just to name a couple of the others. Those response curves though are not always available.
DR. BLAIR: It seemed to me though that the polyoma data was in the system where the polyoma replicated, and in a sense, that's biasing. I mean, if the virus can replicate in the tissue, in the thing you expose it to, it's going to -- you're going to much more easily pick it up than if it's something that has to go in, you know, and multiply infect many cells just directly as DNA.

DR. KRAUSE: I guess, except you're not putting the virus in to start with. I'm not sure how that's different from the SIV mac situation where you're also putting the DNA, which also then presumably once you get a virus out of that also replicates in that animal.

PARTICIPANT: The yield of the polymer is about a million particles per cell. So I think it's a lot more than the retrovirus.

DR. MAJOR: As long as we've talking about polyoma, I'd like to bring it back to the human again.

JC DNA is not infectious in animals, in the kind of situations that it causes tumors in non-human primates.

BK, on the other, is. The BK DNA is infectious, and animals will not only make virions, but they'll make antibody to the BK DNA and that
antibody will also be specific to chromosomal DNA as well. We don't know that in humans, but certainly in animal situations it is.

DR. LOEWER: Yes.

DR. HEINEINE: Walid Heneine, CDC.

No one has mentioned how much DNA we now have in the licensed vaccines. I mean, how much are we being exposed to? Do we have any idea in the viral vaccines, like yellow fever, measles, mumps, how many? Do the regulators have an idea from the manufacturers, how much DNA there is?

DR. LOEWER: I have probably no idea.

Nobody that I know has mentioned it.

Paul?

DR. KRAUSE: I promise to sit down for a while after this. This actually came up when we were licensing the chicken pox vaccine, an it's part of the safety data that was submitted for that vaccine. The manufacturer, Merck, did a number of studies quantifying the amount of DNA in different vaccines and submitted those to us. I noticed that there are people from Merck here if someone from that company would like to describe those results, which are in the public domain already.

(Laughter.)
DR. KRAUSE: But the answer to the

question is that --

DR. SHEETS: I do have an answer. This is
Becky Sheets from CBER.

I think that the vast majority of licensed
vaccines, U.S. licensed vaccines, have not been tested
for residual DNA. Most of them are manufactured in
primary cells and diploid cells and nobody worried
about it.

The few that have been tested are the ones
that have been licensed in the last few years,
including varicella and Hepatitis A.

DR. KRAUSE: The one that has the most
that I'm aware of is varicella, in fact, which has a
little over a microgram per dose of MRC5 cell DNA, and
in that context several other vaccines besides the
ones that Becky mentioned were also tested and were
lower than that, but that gives you a range of what
one potentially could be dealing with if one is
talking about a live attenuated viral vaccine that
doesn't grow particularly well in tissue culture, for
which you simply can't do anything to get rid of the
DNA without risk to the virus that you're attempting
to deliver.

DR. HENEINE: So, I mean, the whole thing
could be an issue and may not be a big issue for some
vaccines, and it could be an issue for some other
vaccines where we know we have some DNA.

We have some premier data from our lab.
We've looked at the MMR vaccines, and we amplified the
chicken endogenous retroviral sequences basically in
an attempt to type these loci in these vaccines, and
what we found out in the cef produced vaccines,
there's very little genomic gain anyway. We are able
to amplify some sequences from them, but there's not
very strong signals.

And other vaccines like yellow fever, for
example, there's we found much higher amounts of
genomic DNA and much higher amounts of viral proteins,
as well, which you can immunoprecipitate very easily.

So it looks like there's a wide spectrum
of residual DNA and proteins that may be present in
these vaccines.

DR. LOEWER: I think this is now known for
a couple of years that Dr. Schuepbach, also in the
audience and I guess he will address this question
tomorrow during his talk, and your findings are, I
guess, repeated by a number of laboratories which also
find AMB and EAB sequences as well as DNA in such
vaccines.
But to my knowledge, at least in our laboratory we have not quantified the amount of cell-wide DNA in these vaccines.

DR. HENEINE: Yes. The relevance in those vaccines that have large amounts of DNA is that we have full length proviruses that are intact that would be present there, and that could be taken up by the cells like the mechanism we've been talking about these two days. The dose would be enough to initiate infections.

DR. LOEWER: Yes, but the point mentioned earlier is that these DNAs derived from normal cells, therefore, didn't create any concern so far, and at least to my knowledge there are no adverse reactions in the millions of vaccinated people which could be attributed to the presence of this DNA.

That's the point. There's a question here, indeed. Do we expect the same with neoplastic or with DNA derived from neoplastic cells or not?

That is the question we cannot really answer so far.

DR. MAJOR: Yeah, let me ask something, maybe a naive question here. I'm not sure I understand exactly what the nature of the state of the DNA that is the contaminant in these vaccines. Is this cellular DNA which is encapsulated into virion
structural particles so that there would be a
difference between enveloped --

DR. LOEWER: No.

DR. MAJOR: -- versus -- or is this DNA

which is just carried along with the purification

process? Is that what we're talking about?

DR. HENEINE: Yes. I mean, if you take
the vaccine, you resuspend it, and you extract it, and

you can amplify endogenous retroviral sequences from
it very easily, and so it must have been cellular,
genomic DNA that got purified with these particles.

Now, also you can find particle associated

RNA. That's something else, but in addition to that

you also find genomic DNA.

DR. LOEWER: But one has to realize that

there is not much purification for live viruses.

DR. HENEINE: And that's not true, like I

mentioned, for all viruses. Maybe the yellow fever

vaccine, the way it is produced explains why you have

higher levels of genomic DNA in it.

DR. ONIONS: Johannes, I wonder if I could

just ask for a clarification because rather as Ruth

was talking about the titration of DNA proviruses to
get a take, to get an infection, we can push FALV down
to around about ten micrograms. You don't get 100
percent take at that level, but you do get takes.

And you just said -- and could you clarify
this? -- you just said that you got full length
proviruses; is that correct? You've actually
amplified the whole --

DR. HENEINE: No, no, we have not tried
that. The experiments were originally designed to
type the endogenous ALV loci in the vaccine. In other
words, if the locus -- are we dealing with defective
loci or full length infectious loci?

So we've used the vaccine itself as a
source of DNA which could be a good representative of
the cell substrate in which it was grown to amplify
and type by PCR based methods the different ALV loci.

DR. ONIONS: Okay. I'm sorry.

DR. HENEINE: But while doing those
experiments, we found out that some vaccines have very
little DNA, but others have much higher levels of DNA,
and the concern is whether those that have high levels
of DNA, whether there is DNA with full length loci
that are infectious, that are known to be infectious
loci like EV2, EV18, EV19, very known, and ALV that
can be taken up by cells and initiate infection.

DR. ONIONS: I mean that seems to be a
central point, is the state of sharing of the DNA in
all of these examples, and again, not just making an
average estimate, but actually looking at the
distribution of size I think is something that would
be relevant.

DR. HENEINE: I guess the other experiment
to do is to see if we can have any evidence of full
length providers that are associated with the
vaccines.

DR. HUGHES: If we're talking ALV related
avian viruses, rav 0 and its cogenants, they are not
infectious for any mammalian cell on two criteria:
the envelope, subgroup E does not recognize an
appropriate target on mammalian cell, and the
machinery of the virus even if you transfec in the
intact provirus into a mammalian cell does not produce
an infectious virus.

So while I'm not recommending having those
types of full length proviruses present necessarily,
I don't think they prose any particular threat to
human.

DR. LOEWER: Any further question or
comment?

DR. HAYFLICK: Hayflick, UC-SF.
I think it's important to keep in mind
that it's not only the cell substrate that might
contribute DNA and RNA, but in those instances where serum and trypsin are used, there is a logical expectation that it could come from one or both of those sources despite the fact that the final product might be diluted heavily or no serum might be used in the final product, but be present in the former population doublings that led up to the production.

I don't think that this has been addressed, and I'm wondering whether anyone has, in fact, determined the residual DNA content or RNA content in serum.

DR. LOEWER: Not to my knowledge at least.

DR. SHEETS: I think there is an answer to that, but I'm not the one that knows it unfortunately. This is Becky Sheets, FDA.

I wanted to respond to an earlier question regarding how purified are live viral vaccines. Most -- I'll just generalize -- many, maybe most live viral vaccines are made by inoculating virus into tissue culture or into the allantoic fluid of chicken eggs, and once infectious virus is produced either by cell lysis or budding, then the harvest is made of the supernatant or the allantoic fluid, and oftentimes this harvest is filtered, and that's your viral vaccine.
So that we're all on the same page, that's a live viral vaccine. It's not impure or unpurified, as has been said, but it is minimally purified, often simply by filtration to get rid of cell debris and bacteria and fungi that might be present, but it is not purified to the extent that a single protein in a therapeutic biologic is purified.

DR. LOEWER: Thank you for this clarification.

Now I would like to ask a regulatory question to Dr. Kazazian. As he mentioned, the activity of retrotransposons is highest in some type of tumors. For example, you mentioned testicular tumors and, I guess, ovary tumors. The same is true for other endogenous retrovirus, and I show a Northern Blot from our laboratory which shows a number of different tumor cell lines and diploid cell line. This is a terata carcinoma cell line, amnio chorion carcinoma. I guess this is a raptomycin sarcoma cell line. There's a vero cell line, and one endogenous human retrovirus, HERV-K, for example. It is expressed only in the testicular tumors, and we know that other endogenous retrovirus, for example, HERV-H, is also very well expressed in testicular tumors.

Would it be your opinion that this would
exclude such cell lines for the production of

vaccines?

DR. KAZAZIAN: Well, first of all, I think

that there are testicular, ovarian tumors of similar
type that don't express retroposons, at least, and

what I guess I'm saying is not all tumors of the same
type will express. I don't know whether that's the
case with HERV-K or not, and I think that certainly

there are those that don't have the expressions

excluded.

That's a tough question. That's a tough

question because I don't know that expression is

related to retrotransposition. Clearly we don't know

that with HERV-K. We don't know that HERV-K is

retrotransposable at this point, and I don't know that

there is a correlation between expression and

retroposition for sure.

DR. LOEWER: Now that you mention it,

expression is a prerequisite for transposition.

DR. KAZAZIAN: Yes.


DR. KAZAZIAN: It is requisite, but I

don't know that it completes it.

PARTICIPANT: Regarding endogenous viruses

like this one and the ALVs, I don't think it's been
discussed much so far that these viruses as virtually all of them that's found in the genome are very extensively methylated and expressed at very low levels. The endogenous provirus that gives rise to the infectious rav 0, in the form in which it's inherited seems to be expressed at a level of maybe a tenth of a copy of RNA per cell; if you then allow that to be replicated, it goes up by a factor of 1,000 or more over that.

So that actually provides -- if you're worried about these things, that actually provides a margin of safety because that's the form in which these kinds of things at least will be passed in recipient cells if they contaminated the vaccine products.

DR. KAZAZIAN: And presumably that's also what's holding down retrotransposition of line elements of methylation.

PARTICIPANT: Presumably. It's been argued that this may be a mechanism to protect the genome against these.

DR. KAZAZIAN: Right.

PARTICIPANT: But if so, it's doing a pretty lousy job.

(Laughter.)
PARTICIPANT: In fact, it probably allows their increase because it reduces the counter-selective effects.

PARTICIPANT: As a bit of a follow-up to an issue I raised this morning about how you culture the cells may affect genetic events within the cell, have you ever compare the activation of lines, for example, in stationary cells versus rapidly cycling cells?

A lot of, for instance, prophage will activate as E. coli approach the stationary, and there may be very, very big differences between the activation of events in cells depending on how they're being handled at that time.

DR. KAZAZIAN: Yes. We have not, but Karl Schmid has looked at both alus, principally looking at alu transcription. This is only transcription. It's not retrotransposition. So looking at alu transcription, and he's looked at line transcription, and it goes up, as I recall now, in serum starvation, goes up in heat shock, goes up in adenovirus infection. That's what I can tell you.

PARTICIPANT: Yeah. That's sort of what I kind of expected.

DR. KAZAZIAN: So it's way up.
PARTICIPANT: A stress like environment where somebody wants to bail out of the genome, that's the perfect trigger for these things to start thinking about moving.

DR. KAZAZIAN: Thank you.

DR. LOEWER: So let's go on in discussion to come to the neoplastic risk event, to leave the infectious risk event, and we had quite a number of talks and discussions already on this issue, and I do not know whether somebody on the panel would like to comment again.

We know from the 3T3 assays that there is a neoplastic risk rendered at least in tissue culture. I had the impression from the talk today that NIH3T3 test is mainly a test for activated ras and to a lesser degree for other oncogenes.

But I think what is intriguing is that is difficult or impossible to find a neoplastic risk by injecting animals with neoplastic DNA.

So what about epigenetic and genetic changes, since this is an issue, I guess, mainly to the people working on methylation? Can the methylation pattern -- I think that's the background of this question -- can the methylation pattern maybe change by transfection or by transfection of
neoplastic DNA?

And is this a risk for the recipient cell with respect to development of a tumor? Are there data available in this respect?

DR. DOERFLER: Walter Doerfler from Institute of Genetics in Cologne.

Why you probably approach this question partly to me at least, it's difficult to answer. Of course, we have pursued this possibility very systematically in some of the transfected cell lines that we have produced and see changes in methylation at quite distinct places. Whether this is a general phenomenon, of course, we haven't amassed enough data.

On the other hand, I feel one could probably predict for the foreseeable future that a lot of people will study this or similar issues with the increasing availability of DNA chip (phonetic) technology because DNA methylation is one thing, but changes in expression patterns is not so difficult to do.

Say I make a lambda transgenic cell line and a non-transgenic cell line or take a lambda DNA transgenic mouse and non-transgenic mouse, same organ, same mouse strain, and I compare cDNA hybridizing back to a huge DNA panel, say, having 20, 30, 40,000 DNA
dots on it, which technically is not impossible to do.

It's actually quite simple to do. It's probably expensive right now.

So I would expect a lot of work in this direction will be done within the next few years.

Certainly we plan to do a lot of that sort of work. Whether this will answer the question is another problem, of course, but we will get at least an overview, and we perhaps will be able to predict in a more defined and a more scientific way what's going on in these cells.

And I suppose this will also help evaluate some of the other because if you found quite distinct differences, well, maybe your suspicion would be stronger that also oncogenic events might ensue.

I understand, although I've only heard it in talks and never seen the data myself, that there are a number of labs now who have looked in transgenic in knockout mice, yeah, I think in knockout animals, and they do find changes in genes other than the one knocked out.

I cannot swear that this is true because I haven't seen the data, but I have heard from, as one says, reliable sources that this is so. Perhaps other people have information on that. It would be
interesting.

DR. KAZAZIAN: I would just say that I'm sure that you all know that with expression analyses, that when you do these kinds of things, I've heard -- again, I haven't done them myself -- but you get changes in hundreds of cDNAs both in the up direction and in the down direction, and it's a very complex analysis.

PARTICIPANT: Or a viral infection.

DR. KAZAZIAN: Or in viral, right.

PARTICIPANT: I liked your approach of asking the questions about what kind of cell line could one rule in or out, and I wasn't sure that there was a complete answer to this question of specifically if there were an intact viral genome of some nature that could be defined by any of you, would that rule out the use of that cell.

And likewise you asked Dr. Kazazian whether he thought that the expression of HERV-K would rule out the use of a cell, but I wasn't sure that there was a clear consensus on that.

DR. KAZAZIAN: Well, I said I didn't think so because I don't know of any HERV-Ks that are active. Okay?

PARTICIPANT: Okay. So maybe there is a
DR. LOEWER: But the same is true, for example, for IAPs when, for instance, atag (phonetic) particles and cell lines producing IAPs are accepted for the production of biologicals, but not for vaccines insofar as CHL cell (phonetic), for example.

Yeah.

PARTICIPANT: We've been thinking about producing live attenuated virus vaccines in vero cells for use by the mucosal route. Give it a couple of drops in the nose, and we were wondering and I'm wondering whether you feel that it is safe.

These live virus vaccines will have cellular DNA in it, vero cell DNA. I think, Dr. Blair, you had mentioned that vero cell DNA was given in your assay and was not found to be tumorigenic in the 3T3 cells, and I think as far as some of the examples Phil has been using, if you take polyoma virus DNA and give it parenterally, it is not only infectious, but it is tumorigenic. That same DNA when given mucosally has no biological activity, you know, when you give it the maximum amount that you can grow up and actually give to a mouse.

So would it be reasonable to conclude, based on these kinds of data that do exist, that
certain continuous cell lines, such as VEROs, which are poorly tumorigenic would be perfectly safe as far as the DNA issue in terms of transferring infectivity or neoplastic risk, would be perfectly safe to use via a mucosal route?

I personally believe that it absolutely would be, and I believe that there are other neoplastic cell lines that could also be considered for this route. What is the feeling of the group in this first cell line such as this?

This is a very important point because there are vaccines for very important viruses can be made in this cell line and almost only in this cell line.

DR. LOEWER: So as a member of a regulatory authority, it wouldn't be wise for me to give you a clear-cut answer.

(Laughter.)

PARTICIPANT: So you're being asked as advisors to the regulatory --

DR. LOEWER: The members of the panel, of course, are free to answer what they believe is correct.

(Laughter.)
PARTICIPANT: Maybe in support of what you said about it's difficult to give a clear-cut answer, I think we have the practice. We make oral polio vaccine, as I will tell later, on vero cells, and it's purified, and the amount per dose is probably in the range of one to ten picagrams.

Now, having said that, it doesn't say that for another virus systems one could reach such figures because the virus system, the processing, the downstream processing will probably greatly affect, and the cost that you want to pay for that downstream processing, for the losses that were incurred during the purification process will probably decide how much residue of DNA will be there.

Besides, that should be put in parallel place in parallel to the basic decisions of whether we could or we could not be happy with, say, the presence of cDNA. It's not clear-cut. It's not black and white. It's really a quantitative issue.

PARTICIPANT: (Inaudible.)

PARTICIPANT: Can't hear.

PARTICIPANT: Thank you.

I want going to question the assumption that live viral vaccines necessarily have to be impure because modern methods of production should blend the
possibility to provide even conventional whole
particle or live vaccines in a purified form, and
we've heard the answer to that, the vero cells.
Now, over 20 years ago I produced
experimental cold adapted in flame devirus (phonetic)
vaccines in chick eggs, and these, in fact, were
purified products. It was then purified.
The reason we were only purifying them at
that time wasn't safety. It was to enable -- we had
more control over the product. I could adjust the
titre, clinical trials, but there's also a practical
reason. It enabled me to have product which had
tropic sucrose in it, which improved the infectivity,
but it also improved the palatability for the
volunteers, and I very soon found out that the
volunteers did not like having allantoic fluid put
down their noses, and a purified product was much more
acceptable.
So I just risk the question, the
assumption that the live virus vaccines need to be
pure. They couldn't be purified.
DR. LOEWER: So personally I am not
absolutely sure even with new technologies it would be
possible to purify live vaccines or live virus much
better because this may be true, for example, for
naked viruses, polio or so, but with enveloped
viruses, maybe herpes viruses or even rabies, it may
be more difficult, and they may even incorporate
similar DNA or nucleic acids which cannot be purified
away.

It's at least a possibility which has to
be checked.

So CBER, of course, quite a number of
questions this group, and this is the next question
they asked. Consider factors that might contribute to
increased safety concerns associated with residual
cell substrate DNA, and one of the question is -- this
was posed several times during this meeting and
wasn't, I guess, really answered -- was aggressiveness
or tumorigenicity of tumor cells is a factor which
increases safety concern.

That means the more aggressive, the more
risky or isn't it true?

So my personal view to say it, having
learned that the NIH3T3 test really recognizes only a
certain spectrum of oncogenes, the question would be
whether these tests in animals also select only for
certain aspects, for certain oncogenes. The question
is how well it models the situation in human.

And, therefore, personally I would think
it's difficult to extrapolate from the data in animals to the possible risk in humans. So that would be my argumentation, but I would like to hear other opinions. So if there are no opinions, I guess --

(Laughter.)

DR. PETRICCIANI: Johannes.

DR. LOEWER: Yeah.

DR. PETRICCIANI: I think with regard to the aggressiveness or the degree to which a cell is tumorigenic in one or another animal system, as I mentioned during my presentation, I don't see that as an important criteria with regard to whether or not a cell should or should be not more or less acceptable. I think the real issue is as we've said many times. The issue is what cell contaminants are in the final product. Whether they came from a very aggressive cell or a less aggressive cell probably doesn't make any difference.

The example that I gave was you could have one cell that's very aggressive with a few activated oncogenes. You could have one that's much less aggressive in those systems for whatever reason that has 100 activated oncogenes, and I don't know that you can say one is more or less better than the other as
far as the cell substrate, just as one example.

I think using that as a criteria is fraught with problems.

DR. LOEWER: Dr. Hayflick.

DR. HAYFLICK: Hayflick, UC-SF.

I'd like to return to the point I made earlier relevant to the use of serum and trypsin in many, but certainly not all of these vaccines, and that is the likelihood that there can be carried over into cell culture sources of DNA and RNA, for example, from live virus vaccines that are given to most cattle these days.

There are a number of vaccines that are used worldwide, certainly in this country, to prevent a number of virus diseases of cattle. Those virions are carried over in the serum that is then used for the manufacture of vaccines. In addition to that, most cattle in this country and in other countries are given anti-helminths and other chemicals that are known to have breakdown products identical to known carcinogens that are also carried over in the serum to a source of product development or manufacture. And I think very little attention has been given to this. As long as we're talking about
aggressiveness and potential tumorigenicity, I think that this matter should be seriously considered.

Thank you.

DR. LOEWER: Thank you.

I think we have already discussed the next two questions here, the presence of proviral DNA, the presence of latent/occult viral genomes, and there is a question whether there are other factors which could increase the concern.

I have here a list which was put together already 15 years ago or so. One is amplification for oncogenes, which John already mentioned. Then there is maybe the risk for enrichment during purification, especially when the sequences are incorporated into the viruses themselves.

And there may be a certain risk from substances which could enhance the uptake. For example, so far as I know nobody has tested the effect of adjuvants on the uptake of DNA itself, but of course, one has to realize that live viral vaccines do not contain adjuvants. Only inactivated viruses, and this inactivation of viruses alters the DNA, may be inactivated.

So these are other factors which have been considered at the beginning of the '80s at least.
I don't know whether somebody wants to add some other factor or some other possible risk. So we will shortly continue.

This was also discussed so far that the amount of residual DNA, if it's as low as possible, which may contribute to a lesser concern.

Then there is mentioned the possibility of the physical state of residual DNA, whether incorporated in viruses or not, but it's of high molecular weight or it's cut in pieces, and personally I'm not sure what the influence of the physical state really is.

The larger the DNA molecule, the higher the probability that a complete genus is coded for. It contains a complete gene, but maybe less effectiveness of incorporation, and also some less possibility of integration.

And the shorter the pieces, and we have heard yesterday that even very, very small pieces can integrate and cause tumors; that means the smaller the DNA, the more single DNA molecules, the higher the risk for indication maybe. So there may be two sides of the coin.

And the question of DNA clearance removal was also addressed yesterday, I guess, and we have
discussed it shortly here.

Is there any further comment from the panel or from the audience to these questions?

So it's not the case. So this was a question, I guess, which was not addressed in our session or the session as the members or the speakers have presented their data, namely, the usefulness of the model system, and so I will switch this, and we'll come to maybe as a last point of this discussion to the quantitative aspects.

And you may have read the paper by Krause and Lewis with some considerations with respect to the quantitative issues, and the first question here is: would it be acceptable that in a frequency of one pair, one million doses, if it is an acceptable level of risk? In other words, would it be acceptable that one of one million vaccines developed a tumor because of the tumor DNA, which may be --

DR. KAZAZIAN: By the way, how are you going to prove that? How are you going to prove that, you know, one in a million is due to the tumor -- I mean due to the vaccine as opposed to a natural cause?

DR. LOEWER: You are completely right. I will not defend this. I just mention what he has written.
(Laughter.)

DR. LOEWER: And indeed --

DR. KAZAZIAN: So who wrote that anyway?

(Laughter.)

DR. LOEWER: Indeed, that is the major question. This would never be detected in the background of tumors. That's absolutely clear, and it may be only a theoretical value, which could be a goal to reach. It will never be detected in --

DR. KAZAZIAN: The problem is that the jury might pay the suer for that one million even though we don't know. It's part of the background.

DR. LOEWER: So, indeed, I think this highlights the problematics or the problems with all of these theoretical calculations. It's questionable whether it's worse to do so.

Phil, you make a comment?

DR. MINOR: Well, I'm not sure you wouldn't pick up one in a million per vaccine dose.

When you think how many doses children get and you're talking about in my country it's like half a million children a year get this stuff, and you can pick up adverse reactions at one in 100,000, and vaccines get withdrawn for that kind of reason.

DR. LOEWER: But it depends.
DR. MINOR: But, yeah, there are things like meningitis is huge, as well, and you can pick that up, and you can withdraw vaccines on the basis of that.

DR. LOEWER: It may depend, I think, on the patient period. If it takes 40 years to develop the tumor you will never detect it, but if it takes only two years, it may be possible.

DR. MINOR: Well, I think alternatively you might get a very nasty surprise in 40 years.

DR. LOEWER: Yeah.

PARTICIPANT: Yes.

(Laughter.)

DR. LOEWER: Okay.

PARTICIPANT: I think the idea is to try to think about these issues conservatively based on the idea that if one is going to start manufacturing vaccines in new cell substrates, you would like to be able to assure the public that you've considered the risks down to a very low level, and so even if it occurred at this level we would not be able to detect it against the background, that doesn't mean that we as a regulatory agency don't have an obligation to the general public to be able to tell them --

PARTICIPANT: At least tell them that.
PARTICIPANT: Well, we'd like to know
that, too, that the risks are at negligible levels.
DR. LOEWER: So usually the figure one to
one million is more or less accepted figure in
biologics. For example, the risk for transmission of
HIV by blood donations is also in this order, and it's
more or less accepted or the risk of polio OPV
associated, poliomyelitis is also a disorder.

PARTICIPANT: On the other hand, just to
follow up, I'm not sure that right now the public is
willing to accept one in a million risk of vaccine
associated paralytic polio, and I'm certain that the
public is not willing to accept a one in a million
risk of HIV infection among healthy children who are
just coming in to get their routine shots for school.

DR. LOEWER: Okay.
DR. LEWIS: Yeah, I think that those
figures are based on what we could possibly generate
from an experimental or a validated test system, not
what we're going to expect in the population at large.
In other words, the issue is how to come to grips with
estimating the risk or the perceived risk, not
necessarily the risk, because at this point in time
I'm not sure we have any risks here, but these are
perceived risks, and if we're going to go out and
convince people to give these products to their
children and to their patients, then we have to have
some level of information upon which to make a
decision.

At least from my own personal perspective
to say "I think" or "my opinion is" in a situation
like this is not going to be good enough. We have to
have data.

Now, the question is: how do we generate
that data and what do we use that data to do?
And I think an estimation like this is
based on a simple attempt to come to grips with how to
estimate this level of risk so that when we try to
convince ourselves as a regulatory body that it is
safe and we want to put the stamp of FDA approval on
this or, to quote myself the other night, the FDA says
this is okay, we need to have a concrete set of
information upon which to make that decision.
Otherwise it becomes a simple exercise or article of
faith.
And at this point in time I don't think
I'm willing to sign my name to something that's an
article of faith. We need some data, and the question
is: how do we generate that data?
And what we're, you know, hoping to get
from discussions like this is how to proceed to get
that information.

                DR. LOEWER: Dr. Hayflick.

                DR. HAYFLICK: Hayflick, UC-SF.

                We already have a baseline for the
exception of what I read up there to be infectious
risk events in respect to the fact that we are living
today with an attenuated polio virus vaccine that, if
my memory serves me well, has resulted in one
acceptable case of paralytic polio per three million
doses or something in that range.

                And, in fact, there are more cases of
paralytic polio occurring in this country per year
from the vaccine than from the wild type. We already
have such a baseline.

                DR. LOEWER: Thank you.

                I would like to come to a final point
here, and this will address as the problems with all
of these calculations, and what I've shown here is the
calculation for the activation of, quote, oncogenes by
intracellular foreign DNA, and this calculation was
published by Reinhardt Kurth after some discussions in
our institute with Phil Minor sitting over there and
starting to laugh.

                It demonstrates, I guess, when you run
into problems by all these calculations, and I would

like to go through very quickly to the difficult
aspects here.

The calculation starts with a quantitative
aspects of the confection of the zarc gene (phonetic)
in the wing of chickens, which are already mentioned
here and the probability to create a tumor with
respect to the per molecule of DNA or three times ten

to the minus 12.

And the efficiency of the cellular uptake
was taken from some studies to be ten to the minus
nine. That means that the probability that an
intracellular DNA may integrate in the chromosome at
three times ten to the minus three, but anybody can
easily recognize that there's many assumptions, and
I'm not sure how representative these data really are,
especially the uptake of injected DNA in the cell as
more attachment.

The probability that if an integration
occurs it's integrated into or close to a portal
oncogenous, easier to calculate from the size of the
genome and the size of the portal oncogene, and this
is in the range of ten to the minus five.

The probability that this integration
leads to an activation of the gene is taken here as
ten to the minus two, and this, I guess, is pure
judgment. It's not based really on data, but now you
can multiply all of these probabilities, and you will
end up with the probability of a single intracellular
DNA molecule to activate a single portal oncogene in
a size range of three times ten to the minus ten.

It's pretty low, but we all know that the
way -- that is a multi-step way to tumor information,
and this calculation it was assumed that two
independent activation events are required, and this
leads to a number of ten to minus 19.

And if you'll look in the recent
publication of Nature, we have seen the picture, their
claims that at least four different steps would be
necessary, and then we apply this figure to this
calculation, and we end up with ten to the minus 38
here in this case.

And then one starts to wonder why tumors
at all occur if this is such a rare event, and this
again shows that all of these calculations are very
difficult, and one has to take great care to take
these figures and to draw conclusions from this.

And please also, my problem for you if you
want to find figures to convince the public on the
risk, it will be always difficult to have figures and
to rely on these figures. They may lead to a false
sense of security or knowledge.

So I would always be very care of these
type of calculations.

Comments?

DR. BROKER: Tom Broker, UAB.

I want to kind of approach these kinds of
numbers in a very different way, and that is from the
perspective of human papilloma virus infections that
lead to cervical or penile carcinomas.

One of the more remarkable facts of this
family of diseases is that since 1980 more people have
died of HPV disease than have died of AIDS. I mean
this is a disease that takes out three-quarters of a
million people per year.

Now, the kinds of calculations, they just
don't seem to square with what we know is the reality
of that disease process. For example, vast numbers of
people, hundreds of millions, harbor HPVs, and the
process through which neoplasia occurs is, first, the
establishment of the viral genome in the infected
tissue, followed some time later in a small
percentage, usually one percent of the patients, where
an integration event takes place, and that's where I
think we're interfacing with this kind of table.
The integration, it turns out, is somewhat selected. It's going into an actively transcribed region of the genome. There's further selective events for the active expression loci, which then leads to an up regulation of genes that foster somewhat more rapid cell cycling.

In the presence of mutagenic agents, and over a period of time, probably ten years or more, these genes get more and more activated. There's a little bit of increase in P53, of E6 taking out P53, and so forth, and it's a progressive neoplastic process.

And so I think you can't have instantaneous numbers, but the bottom line is three-quarters of a million people die every year of this family of diseases.

DR. LOEWER: I personally believe that such type of calculations cannot be applied for virus, tumor viruses. In the case of HPV, of course, virus produce actively oncogenes, E6 and E7, and this is not taken into account in all of these calculations. These were just calculated, passive activation of an oncogene.

PARTICIPANT: Johannes, I was just going to re-echo that last point. I mean the assumption
here is that they are independent events, and I don't think that is the case necessarily in transformation events.

For instance, if you introduce nat and myc (phonetic) into a cell, the probability that that cell population then goes on to cumulate the other hits is heightened because we're into a replicated advantage. So I think that final assumption is just not correct. Once you've initiated a process, you're on a cascade of other events are required, but they're not necessarily independent anymore.

DR. LOEWER: That's a chronic observation, yeah.

PARTICIPANT: Maybe I can ask you to clarify. Are you rejecting the use of a quantitative approach? Because as Andy just came up here and pointed out, the alternative to at least attempting some kind of a quantitative approach is to simply go based on your gut feeling, and our question is: is that good enough?

So while maybe these numbers give us too much reassurance, which I think is what you're implying, the question is if these numbers came out differently and this number were ten to the minus five or something like that, wouldn't that mean we need to
go back and work a little harder?

DR. LOEWER: So that's really my personal opinion. I would be very careful with numbers in this respect because we are so many ambiguities, and I personally prefer more qualitative approach to this question, but this, of course, can be discussed.

John.

DR. PETRICCIANI: I think the issue of trying to quantitate to get a more firm fix on a given situation is attractive and it's seductive. The problem in applying a quantitative approach in this area to me lies in the uncertainties of all the things that go into that final number so that when you come out with a number, it's mushy because you have so much uncertainty in virtually every variable that you put in it.

It doesn't mean that it's of no value, but I think at least in my mind what you'd come out with is a qualitative impression of whether you're on one end of the spectrum or in the middle or at the end.

But I agree with you, Johannes, that in this arena I'd be pretty reluctant to use a number because it doesn't have enough meaning to it.

PARTICIPANT: That may very well be right, but then the implication of that is that if you can't
say that you think these risks are very low, less than
one in a million or something like that, but you all
say, "Well, these risks exist. You know, we see these
things happening in tissue culture," there's a chance
that this stuff can happen. What you're really saying
then is if we don't have a way of getting a handle on
what these risks are, that really if the best we can
do is qualitative, then can we take these risks?

DR. PETRICCIANI: Well, let me go back to
two points that I made in my talk. Number one is that
in this analysis and all of the other ones, they are
absolute worst case. They're not best case by any
stretch of the imagination, and if you used what I
think is perhaps a more relevant piece of data, and
that is human tumor DNA as opposed to viral oncogene
DNA, you'd have zero up on the top because there isn't
a shred of evidence that I know of that human tumor
DNA causes tumors in any animal system.

The other point is that it would be
helpful -- you're getting tired of me saying this --
but it would be helpful to have some basic generic
pieces of information like DNA dose response to help
actually get a better handle on those quantitative
questions.

PARTICIPANT: Yeah, I was going to save
this for tomorrow, but I'm getting more and more

irritated in a sense over actually the last ten years
when I last attended a meeting to discuss these issues
at which this kind of calculation was done by both me
and Howard Clemens' calculation. The same thing was
cited using Hsing-Jien Kung's data, and here we are
ten years still using Hsing-Jien's classical data when
for more than ten years the basic experiments to get
a handle on some of these numbers have been obvious
and doable.

They're not rocket science. It is really
very simple, basic stuff of taking this and that DNA
and treating it this and that way and injecting it in
this and that animal. Any one of us in this room
could design a suitable set of experiments, but nobody
has done it for ten years.

Why not? Why not? It could probably be
done on less than 1,000 mice and probably for less
than a million dollars spread out over three or four
years.

That may be a lot of money to the FDA, but
to the NCI this is what you ought to be supporting,
this kind of experiment. This is a very small amount
of money.

(Appause.)
DR. ONIONS: So again really echoing --

David Onions -- echoing what John has just said, I mean I do think those systems do exist, and I made a case yesterday for a variety of transgenic models, and I think those do offer the prospect of giving you the kind of sensitivity that you need and to give you the kind of dose response curves that John has referred to. I think that is important.

I mean, you can force a system rather like the V-SARC experiments and take the worst case, but then you can go and do the experiment that I think John has wisely said. That is, you take tumor DNA from a tumor cell line or cell lines where you actually use the substrates, inject those in, and you're using systems that are highly primed to develop neoplasia, and because of the time scale and because of the sensitivity, you don't have to go into very high numbers either.

So I mean, I think those systems exist.

You might have to use multiple models, but I think they exist, and I think the reason they have not been done is actually because the academics like me who develop these systems do it for other reasons. We're looking for new oncogenes. So that kind of experiment just gets in the way.
But, I mean, if somebody now said that's a useful experiment to do, then we should do it.

PARTICIPANT: I'd just like to say one more thing about the numbers and the models. I'm a very poor person to be talking about mathematics because I can't add two and two, but I think from a simply perception point of view, as an experimentalist and now as a regulator, to get at the issues that Dr. Petricciani was mentioning, we have to start somewhere. We have to think about how to approach these things from a quantitative perspective because that's where the future is.

If we continue to do this on an ad hoc, believe type basis, we're not going to make any progress, and 15 years from now we're going to be right back here addressing the same questions again. We have to start somewhere.

As crude as the numbers may be, if we begin to make the attempt, we can find out what we know. We can find out what we don't know. We can pose the questions to ask to improve the numbers as we go along.

If we don't make that attempt, we're not really making progress, and I think that has been the sort of thing that has been driving us for the past
five or six months, is we've tried to struggle with
how to come to grips with thinking about these issues
and putting together some sort of approach that made
sense.

This is simply a beginning, and we're
going to make mistakes, but the thing that is, we have
to be very careful about the kind of mistakes we make.
We can make mistakes in calculations, but if we make
mistakes in delivering products that are unsafe, the
consequences of that are going to be incalculable,
both for the regulatory process and for the public
health and for the companies that are unfortunate
enough to be associated with that.

So I would, again, I would make the plea
that our job is to try to figure out the best models
we can at the time, realizing that they're not
perfect. They probably will never be perfect, but the
job is to start somewhere and attempt over time to
improve them as information becomes available.

DR. LOEWER: I didn't want to say that
quantitative considerations wouldn't be helpful. I
think they are definitely helpful to find out and to
evaluate the different factors which may influence the
whole process, but I have to admit the same ideas like
John came into my mind.
These issues have been discussed in the early '80s, and in the meantime the number of experiments which help to clarify these issues is very limited, and maybe one of the outcomes of the quantitative approach may be to design adequate experiments to perform and to find solutions to the questions we have posed.

I think we are already half an hour late, and I think I shall conclude this afternoon's panel-audience discussion.

I would like to thank the speakers again and also all the members of the audience who have contributed.

Thank you very much.

(Applause.)

(Whereupon, at 5:10 p.m., the afternoon session was concluded, to reconvene at 7:00 p.m., the same day.)
EVENING SESSION

(7:08 p.m.)

DR. PEDEN: Good evening. I think we'd better get started since we have to be out of here by ten o'clock.

This is a miscellaneous session, and the topics are diverse, and at the end of the formal presentations of the program, there are going to be some shorter presentations, but so we'll see how we go, how many we get through. We have to be out of here by ten.

And I'm going to ask the speakers this time to abide by the lights that now are going to appear on the podium. So I've brought it down to 15 minutes. So perhaps we can get everything done by 15 minutes.

So I'm going to use the Chairman's prerogative, although I have one other thing to say. This is a very special day today. It only occurs once every decade, right, and the question, I suppose, is where were you when it last occurred. So you can be thinking about that.

What I want to do first, if I may have the first slide, I'm going to take the Chairman's prerogative and -- is someone over there? Could I
have the first slide? All right, and the lights down
a bit.

Because part of the thing we have to deal
with is quantitation of things, what I want to do
first is just give a couple of slides briefly on
methods that we've been developing at the FDA on two
topics.

The first is the sensitive reverse
transcriptase assay called PERT that Joerg Schuepbach
coincd as the product enhanced reverse transcriptase
assay, and we've adapted that to the real time
quantitative PCR machine, the tag man machine, and
just go on and give another example of an assay we're
developing to look at primate polyoma viruses as an
example of just what sort of quantitative assays we
can do.

Since it hasn't been covered yet, this is
the quantitative real time PCR assay, and it's a very
convenient assay, and what it depends on is a reporter
and a quencher, and when these are on the same probe,
the reporter does not fluoresce.

But then after PCR, with the tag DNA
polymerase, it gets excised because of the five prime
and three prime nucleus activity. It releases the
quencher, releases the fluorochrome, and the machine
registers that. So it's a real time PCR, and the assay detects the cycle at which the product is detected.

So in the first example we use the PERT assay, and since, again, no one has presented this, I just run it through briefly. It's the standard RT assay where the reverse transcriptase either comes from a retrovirus or purified RT, and the presence of a known RNA sequence, for example, MS2 RNA and a primer, you get a cDNA synthesized, and then the taq polymerase by PCR, you can amplify this sequence since it's known, and you get double strand cDNA products, and then you can assay it by various methods.

And what we're talking about now is the taq man PERT assay, the TM PERT assay, and I just want to show you what this assay can do.

When we look at three different purified reverse transcriptases from AMV, Maloney murine leukemia virus RT and HIV RT, this assay -- and make dilutions -- this assay, as you can see, is linear over about six orders of magnitude, and when you use equal amounts of the RT according to the manufacturer, these two enzymes fall on the line, and the HIV, in fact, is off the line, indicating that the assay, in fact, if the units are correct as determined by the
manufacturer, is probably more sensitive on the MS2 RNA than it is on the synthetic templates.

But in any case, the assay is highly sensitive, and it's linear over, let's say, about six or seven logs, and we can detect, going down here to make the calculation, we can detect the equivalent of RT of a single virion, not infectious virion, just a single virion.

So the sort of things we want to do or use this assay for us you can assess the retrovirus contamination in cell substrates, biologicals, and vaccines. You can use this assay, and we're testing to see how efficient this is, to monitor retroviral clearances from such products as monoclonal antibodies, and also detect and identify unknown retroviruses.

So it's a very useful assay. The problem with this assay, as everybody who's worked with it knows, is it's so sensitive and it detects DNA polymerase's activity. So you're never sure that the assay you're detecting is, in fact, pure retrovirus, a true retrovirus or DNA polymerase.

And we have not been able yet using this assay to discriminate between those two. We're still working on that.
In the second example using the taq man,

we've designed primers and probes for the primate
polyoma viruses for 40 BK and JC-V, in this case in
the late region of the genome, and then when we show
an example of this, we're using dilutions of SV 40 DNA
down to about in this case you can register ten
molecules of SV 40 DNA. This is a cloned SV 40 DNA.
Viral DNA is the same.

And in these blue dots are dilutions of
cos cell DNA which carries a single copy of SV 40
genome, and this should be one copy, two copies, ten
copies, and 100 copies, and as you can see, it falls
on that line. So from this we can conclude that, in
fact, cos cells do have a single copy of SV 40 DNA.
So what we've done so far is test the
sensitivity and specificity of each of these primer
sets. With SV 40 we can detect about one to ten
molecules reproducibly. With the BK-V primers we're
only at the moment at about ten to 100 copies per
assay, and in the JC-V it's about one to ten. No the
JC-V primers pick up also BK-Vs. So now we have to
both increase the sensitivity of this assay and try to
improve the specificity of this assay.
So just in summary, we can say that the
developed primers for the three primate polyoma
viruses, it can detect reproducibly between ten and
100 copies and sometimes down to one. The reaction is
linear over at least seven to eight logs, and primers
so far specific for SV 40 and BK-V, and JC-V primers
cross-react with BK-V.

And using a series of SV 40 transformed
mouse and hamster lines, we've shown that, in fact,
the assay can detect different quantities of the SV 40
genome per cell.

So this is just an example of sort of
quantitative assays that are being developed and are
going to be very useful in determining the level of
quantitation -- the level of contamination of various
products in cell substrates and vaccines. So that's
what I'm going to say, and other people will cover the
assay a bit later on.

I think we should move on to the first
talk, and that's by Bernard Meignier from Pasteur
Merieux, according to the program, and he's going to
talk about industrial experience with live polio
vaccine prepared in the VERO cells.

And, again, I would try to ask you to keep
to the 15 minutes.

MR. MEIGNIER: Can I have the first slide,
I would like to thank the organizers for their invitation to share with you this example of the use of -- no, that's not the one. I'm Bernard Meignier -- so to share with you that example of the use of continuous cell line applied to the manufacturing of vaccines.

When we have the slide, I wish also to acknowledge the colleagues of mine that helped me assembling that presentation, and I would also like to say as an introduction that I was not personally part of the development of the VERO cell system, nor of the manufacturing of those vaccines.

However, I will take the liberty of using the "we," "we do this," "we don't do this," "we did this," or "we did that" --

(Laughter.)

MR. MEIGNIER: -- just to express how collective an activity could be. It's just the liberty of language, nothing else.

Forward. It looks like this time ring.

Good.

So the VERO cell story starts in 1962.

The two Japanese scientists there on March 27th, one to establish a system, a cell system where they could grow SV 40 virus in a lytic system to work more easy.
They could establish one line. From that line there was a derivation of various other sub-lines in Japan, and then two years later Simizu brought one of those branches to NIH, and again, in the States there were various lines that were spread among some laboratories.

One of those branches, LT3 and VDOT (phonetic) ATCC was deposited by Drs. Hann and Riem at pass 113, as you can see here. ATCC, characterized at cells scale, the top (phonetic), and two, 121, and from there can supply cells -- you have the code number there -- supply cells at the level of per such one in 24 (phonetic), and that's where in 1979 Institut Merieux, which then became Pasteur Merieux Connaught for various mergers, that's where we started; they started at the time.

And from that passage 124 derived as shown here, primary cell bank, and then from there working cell bank, and established a scheme of production, production being done at the passage one and 42. In parallel to that, one bank was established at the level of the working cell branch that was made available to WHO. That bank is deposited, in part, in ATCC, in part at ICAC, and is available or could be supplied upon request through
Now, these cells, just a few words on the technology. These cells are grown on microcarrier cells. Microcarrier is a small particle on which the cells are grown.

There were cells encourage dependent. They would not grow unless you got them -- especially they could not grow in suspension. So in our case, the microcarrier is made of DEAE dextron with an optimized charge. The brown (phonetic) name is called cytodex, and it's made by Pharmacia.

And what you're seeing here is one of those small particle sizes, about, say, 100 microns or slightly more, and actually you don't see the microcarrier. What you do see are the cells. This was taken from a culture, and you do see the cells that are entirely covering the beads.

The prime scale at which we are operating is 2,000 liters, and a full fermenter contains when it's ready for being effective, contains only half a dozen trillion cells, which is a fairly large amount.

Now, those cells, to maintain the safety subjected to quality control and all the activity of development of the banks and all of the QC that goes with them and all the characterization that was done
was established in relationship -- I should say in collaboration -- with authority BWHO that has the asset of regulations and guidelines with national authorities, including FDA in this country.

The sets of QC that you do see here, it's a busy slide, but just to say that some of QC are just done once in a while. We are still working with the same primary cell bank from the beginning. We make seven working cell banks, while some other controls are done routinely on each and every batch of production.

And as you can see from the list -- I won't read it -- but most of those assays which are done both on the cell themselves and on -- I'm sorry -- on the supernatants; most of those cells really have to do with looking for adventitious agents, be they bacteria, be they fungi, and mostly be they viruses.

The basis is hem (phonetic) absorption inoculation to animals and inoculation to cells. ADC means human diploid cells. The PMKC are primary monkey cells, and NPCC is cell, a continuous line from monkey kidney.

Attention is paid also to assays to look for tumorigenicity as shown here and here, and that's
done only theoretically when we make a working cell bank. The cells are assayed, of course, on the cell bank itself and are also assayed on a special pool of cells that is made ten passage doubling after the production level. That is to optimize the chance or the probability that we have to pick up tumorigenicity. And, by the way of tumorigenicity, out of the number of assays that were done, I selected this table because it fits with things that were said and discussed on several occasions during this meeting. The test here consists of injecting newborn rats with various amounts of cells, one of ten million for rats, and keeping the rats immunosuppressed by injection of antithymocytic antibodies. And they are then monitored for the development of nodules at the site of injections or metastases at the draining lymph node or metastases in the lung. And what you can see, there are two messages basically. One is that -- it's not unexpected, by the way -- but one is that the amount of cells that are injected do actually influence the results. This is really typical here of the sort of
limit of positivity of metastases, this one here, and that always causes the question for the sort of arbitrary design that you can take for a test. That was said before.

And the other message is that clearly something happens. The cells change with the level of passages, and something happened between that level of pass -- the passage that was tested and that level of passage in the sense that before the subcu. nodules were -- there were no metastases and histological -- it's not historical. It's also historical, but it was histological. The pathologist could see here regressing signs on the lesions, on the nodules wide beyond that passage or from that passage on, they do see progressive nodules, and you do see metastasis.

That's not really original. It's been said before, but it's always interesting to see numbers. What I showed is really the set of pre-T controller assays (phonetic) that are mandated by regulations or guidelines or points to consider. There are specifications attached to them, and they have to pass for the banks to be qualified or for the production to be accepted for release.

Now, in addition to that, a number of assays were done on the banks at various levels using
one of several of the techniques that are shown here
to look for viruses or for viral sequences.

And the next slide shows the set of
viruses that were specifically searched. Most of the
QC assays are rather broad. You go for the unknown.

In that case, those studies were looking specifically
for viruses of simian region. For obviously reasons
we are dealing with simian cells and for some human
viruses that, say, were causes of concern for us and
for health authorities. All of those searches were
negative.

I did not mention that there is another
set of analyses that goes with your in-process
analyses, like the monitoring, the visual inspection
of the cell 12 passages day after day, et cetera.

That also brings useful information as far as the
status, the cleanliness, if you wish, of the cultures.

A few years ago, Florian Horaud published
in 1992 his attempts or his -- his attempts to look
for the expression by Northern Blotting of some of the
oncogenes. He was really trying to -- he wanted or he
wished at the time to try and explain how come that
those cells have, let's say, a normal profile and,
however, have an indefinite life span as far as we
know. In his analyses, there was no signal that was
beyond the background.

Now, just a few words on the way we use those cells for the manufacturing of OPV. We choose OPV for tonight because we also make IPV, inoculated polio and rabies with the VERO cell system, but those have been described several times before, published. So it's a bit more fun with this one.

In principle it's very simple as manufacturing. You take the VERO cells, and as was mentioned from the working cell bank, say, about 200 million cells through five passages. The cells are just trypsinized like regular culture from the beads and then plate seeded, if you wish, on the more beads.

You go in five passages to the level of use for the production. Once the cells, the culture is established, it is seeded. It is inoculated with one or the other of the virus types taking from the working seed that has been also controlled for being clean, what it claims, et cetera, and you just keep the culture for a couple of days until the cells are lysed and progeny viruses are released, and that constitutes the harvest.

And there is a set of steps to concentrate and purify that virus. That's a code for filtration, two steps of concentration through pettycon (phonetic)
and ion exchange per chromatography.

The purpose of that is to concentrate so
that industrially we have manageable volumes of virus
suspension and purify so that we have clean material.

This suspension is tested or titred for
infectious titre, and based on the result of that and
knowing what regulation asks to be put in the
monovalent vaccine or in the trivalent vaccine, then
there is dilution to adjust the potency. It is
filtered, mixed with other valences and that is the
vaccine.

Again, the viruses or virus suspension are
subjected to a number of assays, and here, again, it's
the same pattern. There is a set of assays that are
done during the process as in-process control that
contain things that you will not see here because
these are the ones that are asked or requested by
regulation.

For example, in-process control has -- we
monitor the amount of DNA. It's not requested
officially, but it is monitored as a way to follow the
efficacy of the purification. We do monitor the
content of protein. We do monitor the mayput
(phonetic) test, et cetera.

The set of QC assays that were done, I
will just focus on the prediction level. We are not really dealing with the virus itself tonight -- has a set of analyses to show that we have the right virus, what we claim we have, in terms of its polio on the right site.

It is also to determine that we have the right quantity, and then there is a number of analysis to show that we have no more than what we claim.

There is no adventitious agents, and there is the two analyses, RCT4 and level virulence in monkeys. The purpose of those analyses is to show or to be sure that throughout the manufacturing process the polio virus, which is a live attenuated virus, again, did not change in the pattern of attenuation.

Another important feature in the development was to show that the virus, the vaccine that was produced on VERO cells was equivalent to the vaccine produced in primary monkey kidney cells, and that, again, was done through a variety of approaches, the clinical monitoring antibody references and the pattern of isolations.

Just the last or the one before last, this slide is just to show the power of that technology which enables us now to produce 440 -- more than 400 million monovalent doses per year. That's the number
for '98. The number for '99 will be in the same
range.

More important than that, at the scale
that we operate, 2,000 liter scale, we have a capacity
for about ten times more. That will be impossible to
run with monkey primary cells.

And the last thing I would like to add is
that beyond suppressing monkeys for the production,
using that scale, using the scale that permeates the
VERO systems reduces also the use of monkeys for QC
because the neurovirulence test can be applied to much
larger batches. We need to use less monkeys on the,
say, dose or median dose basis.

And I think I will stop here. Thank you
for your attention.

(Applause.)

DR. PEDEN: Thank you.

Does anybody have a question they wish?

David?

DR. CASHMAN: Neil Cashman from Toronto.

A trivial question. Is the entire batch
of vaccine per year -- does that come out of the 2,000
liter production hit?

MR. MEIGNIER: One fermenter makes one
batch. That's the practical and the regulatory rule.
DR. CASHMAN: How many fermentations do you do a year?

MR. MEIGNIER: In principle one a week. One week provided you have the right set of fermenters to feed the final fermenter.

DR. ONIONS: David Onions. Bernard, incredibly impressive performance in producing those number of doses.

MR. MEIGNIER: I tell them.

(Laughter.)

DR. ONIONS: But as this meeting is about, you know, concerns of perhaps new adventitious agents, could I just ask? We now know that lots of serum batches have contained fetal calf serum batches and calf serum batches contain bovine polyoma virus, and we know that VERO cells are permissive, and there's also good serological evidence that that virus is potentially zoonotic.

Do you do any testing for that virus now or what was the position?

MR. MEIGNIER: Yes. (a) It's the sort of multi-level process. (a) The calf serum is obtained from donor calves that are monitored for their health and that fermenting (phonetic), as we say, hervs or frons (phonetic) with sanitary status that is
These batches of serum are tested for a number of agents, BVD. I'm not sure for you -- BVD testing. They have a set of --

DR. ONIONS: Yeah.

MR. MEIGNIER: -- bovine viruses that they look for, and third, the calf serum is irradiated before being used.

DR. ONIONS: Okay. Oh, sure.

MR. MEIGNIER: We hope that if you go down that way, it turns out without problem.

PARTICIPANT: Could I ask very quickly what your seed virus is grown in, and is that in monkey kidney or is that VERO cells?

MR. MEIGNIER: That's a tough one.

PARTICIPANT: I mean the point is if you're going -- if you have a VERO cell system, you're introducing whatever is in your monkey kidney cells if that's your seed. On the other hand, if you have a VERO cell seed, you've undergone a couple of passage levels from the Sabin strains. So it's of interest from two points of view.

MR. MEIGNIER: I must say I do not remember. I believe, but I'm not sure. If you want the exact information, I'll be glad to --
PARTICIPANT: Okay.

MR. MEIGNIER: -- convey the question.

I believe it's done on -- it was done -- new burns (phonetic) were prepared on VERO cells, but my recollection is also that they kept the same level of passages of the virus because of the concerns for changes in givens (phonetic).

PARTICIPANT: Bidion (phonetic) from Provirus.

Have you tried looking to see how much of the virus which is produced by the VEROs are active?

MR. MEIGNIER: Active in terms of?

PARTICIPANT: In terms of nucleic assay and also particles.

MR. MEIGNIER: No, I don't think that was done.

PARTICIPANT: It was not, and have you tried an NCO-free medium for the production?

MR. MEIGNIER: I missed the question.

PARTICIPANT: NCO-free medium for the production?

MR. MEIGNIER: No. The cells are grown in serum, in a medium that contains serum, and serum is removed for the virus production.

PARTICIPANT: And comparing the adduced
matter with like using eggs or other kind of systems
for growing, how would you compared those, in vivo and
in vitro?

MR. MEIGNIER: I don't think eggs were
ever used for the polio production.

PARTICIPANT: Or any kind of --

MR. MEIGNIER: If you compare the sort of
standard that other companies or other institutes
still use are primary monkey kidney cells or human
diploid cells. Now, the comparison is clearly in the
scale. In the capacity -- the main feature of the
system is that using the VERO cells of microcarriers,
one can apply the ferment of the technology so that
you can keep the cells because those particles are
small enough; you can keep them stirring culture
medium, and you can scale up relatively easily I would
say, not to say that it's easy, but relatively easy,
while future in retrovirals, for example, is extremely
tedious, and when you want to scale up, you just have
to multiply the number of bottles, the broken
incubators (phonetic), the people to handle them, all
by robots, but still it's tedious, cumbersome.

PARTICIPANT: So this is showing that all
of the virus which is produced is active more or less?
MR. MEIGNIER: I really cannot -- I don't
have data to really answer that one.

PARTICIPANT: Okay. Thank you.

PARTICIPANT: Do you have any evidence that you have an interseception (phonetic) in oral polio vaccine?

MR. MEIGNIER: I missed the question.

PARTICIPANT: Interseception rate in children that received the polio vaccine.

MR. MEIGNIER: The immunization rate?

PARTICIPANT: No, interseception is adverse event in vaccination regard.

MR. MEIGNIER: I don't think it was looked specifically at the time. The only thing I can say is we have, as mandated by law, there is a special department that recalls side effects that all may be related to vaccines, and despite the high number of doses that are sold and used -- I guess they were used if they were sold -- no record was made of side effects associated with those vaccines.

But I don't think they ever -- there was a specific study to look to address that question.

DR. MYERS: Martin Myers, the National Vaccine Program.

Relative to what we're discussing here, do you have any long-term follow-up on a cohort of
children specifically looking at malignancy attack

rates?

MR. MEIGNIER: No, there is no follow-up.

DR. EGAN: Bill Egan from FDA.

That was also my question about what the

clinical safety profile was that was studied with the

vaccine, sort of in keeping with this current meeting.

MR. MEIGNIER: Well, at that time the only

follow-up that there is is the regular market -- post

market, if you wish, surveillance and the reports that

doctors may make that they found one or the other

strange association.

And I agree that it does not account for

possible putative evidence that occur not only in

people who received the vaccine ten, 20 -- ten years

ago or more.

DR. EGAN: Okay. Thank you.

DR. PEDEN: Last one.

DR. MERTEN: Merten from Genethon in

Paris.

When I worked in the institute in Paris,

I developed different media for different cell lines,

and I want to respond to the question concerning cell

free (phonetic) media.

The virus that grow rather easily in cell
free media and stay attached as cells, NCO-free media,
and so you can grow them in serum free medium and
they stay always attached and you can grow them in
microcarriers.

And the titres you will get out with polio virus, for instance, are more or less the same as when
you use a class production process from -- from area
(phonic) for instance. The titres are about, for
the Type 1 virus, about ten to the eight P few
(phonic) per liters.

DR. RUSSO: I think we need to discuss the scale of your growth because in our experience in
Merck -- I'm Carlo Russo from Merck -- the VERO cells in the serum free medium, large scale, are now growing very well.

PARTICIPANT: Okay. This was a laboratory scale because in Pasteur Institute and in Paris we had only laboratory scales, and in the scales of 1.5 liters, using five grams per liter of cytotech 1, cytotech 1, microcarrier, they attach very well, and they grew very well. We had no problems with this.

DR. PEDEN: All right. Thanks.

Do you want to respond?

MR. MEIGNIER: The only comment is we have tried to, too, to use the medium, and the difficulty
still, as you know -- I really wasn't aware -- the
difficult is still the passaging of the cells.

It's true that there was some good growth
in cell free medium, but in the industrial scale, the
kind of handling, the passaging or associating the
cells and passing them still would be difficult to
really master, if you wish.

DR. PEDEN: Okay. Thank you, Dr.

Meignier.

So the next talk is by Girish Vyas on the
proteins of replication-incompetent virions for HIV
vaccination.

DR. VYAS: This is a cartoon of the famous
virus, HIV. There's a lot more known about it because
our government has been spending almost $1 billion on
everything that we can think of about biology, the
structure of the virus.

Three things have been learned in the last
ten years. First is that this grows in unlike
Hepatitis B and Hepatitis C virus -- this virus can be
cultured both in cell lines, as well as in normal,
peripheral blood mononuclear cells, or PBMCs.

In fact, all of the structure of proteins,
particularly the envelope proteins, the gp120 and
gp41, these two proteins are critical in design of
vaccines, and the first generation of vaccines have been designed with cloned proteins which have an inherent problem of antigenic radiation that results from traditional mutations that occurred in gp120 or all replicating RN viruses have a propensity to mutate. So that's the built in handicap with some of the recombinant vaccines.

The background is that limited progress in the development of a safe and effective AIDS vaccine has been accomplished. The structure of HIV-1 envelope and some antibody neutralizing epitopes has been defined. A successful AIDS vaccine should in this, both CTL, cytotoxic T responses and neutralizing antibody responses against primary virus isolates and not laboratory virus isolates, and finally, expanded studies of the vaccine's alarming progress in non-human primates and in Phase 1 and Phase 2 clinical trials. These include vaccination with DNA and attenuated pox viruses which induce virus specific CTL in non-human primates, and in combination with the booster immunization with recombinant subunit vaccines which induce neutralizing antibodies against primary isolates.

Again, this background some naive guy like us from blood banking field though that perhaps it may
be a good idea to have a perfectly human blood based vaccine as an alternative, and let me just recapitulate what are the ideal requirements for an AIDS vaccine.

First is the efficacy in preventing transmission by mucosal and parenteral routes.

Secondly, safety in profile with minimum risk of adverse reactions, even unscreened, real world populations.

Third is long-lived protective effect of many years after successful immunization. This is a wish list.

Low cost, allowing widespread vaccination in developing countries.

Stability and ease of administration, facilitating mass immunization campaigns in developing countries with minimal infrastructure.

And finally, protective immunity against diverse virus isolates, preventing need for many virus isolate specific vaccines.

Now, we thought it's rather simple to take advantage of the biological property of replication in competent viruses or what John Holland used to call defective, interfering particles, or DI particles. Any RN virus each time it replicates makes 100,000
times perhaps more noninfectious viruses than a replication competent virion, and this replication incompetent virion or RIV is an acronym that we have given predominantly all of the replicating virions in all viral isolates.

So prepared, whole virus vaccine is our approach, and triple inactivation of this primary isolates of prevalent HIV strains, pooled and expanded in vitro by full culture with peripheral blood mononuclear cells as the cell substrate, and I emphasize the word PBMC as a cell substrate.

As you know, each time you get a unit of transfusion, four million American get 14 million units of blood and blood products, and each time you get a transfusion, you get basically 400 micrograms equivalent of human DNA because these cells disintegrate. They are perfectly useless and, therefore, some time in near future blood bankers are going to be actually excluding the peripheral blood mononuclear cells from blood for transfusion.

So these cells as a substrate becomes cheap, available, widely donated, and blood bankers can uniquely do this. My own background is actually in blood banking.

Secondly, instead of fetal calf serum in
culturing the virus, we have shown that human AV serum
works equally well and, in fact, probably slightly
better than fetal calf serum in HIV cultures. So it's
human blood based vaccine.

The prestored leukodepletion (phonetic)
makes sense because immunological and virological
hazards of transfusions are minimized, and quality
control systems are now in place in blood centers in
the country. Ten percent of blood donated in this
country is leukodepleted. I understand that in
England, they are going to start routine depletion of
leukocytes from blood for transfusion.

So this is a ready resource that's
available for using culturing virus.

Let me just focus that, please.

Okay. Well, I want you to focus your
attention on the fact that any virus isolate is
composed of the replication incompetent virions or in
vitro virions and the natural virions of the
replication incompetent virions, and for the purpose
of the vaccine, the most important things are gp120 on
the surface and gp41, the trans-brain antigen.

Both antigens are present in the
replication incompetent virions as well as in
replication competent virions. The built in advantage
of making a blood based vaccine is that in a given
unit of plasma, for every virus that is replication
competent, there are 100,000 virions of this type that
are replication incompetent, and by in vitro expansion
of this virus each time that we do the expansion, we
are creating more of this replication incompetent
virions in vitro, and that's really the principle that
we thought would be an advantage in making a blood
based human vaccine or a blood based HIV vaccine.

And the genetic analysis can be done with
prototype relevant strains by interlooplets (phonetic)
analysis, and this would be done at Uttering
(phonetic) Memorial Blood Bank in San Francisco.

The principles are that plasmas is
procured as the primary source of HIV, negative for
HCV and HBV, and the PBMCs -- I'm sorry. I'll come to
that -- plasma pooled and concentrated for viral
expansion in peripheral blood mononuclear cells
prepared from CMV negative blood donors and AB serum

is used as a supplemental medium.

So this is basically the principal
components of the culture system. The HIV is
cultured, is concentrated from primary virus
inactivation using a compound called Sino-wiring N
(phonetic).
Now, blue-green algae is a bacterium which has an 11,000 Dalton protein that it makes, and this has been cloned and sequenced and large quantities are being produced by National Cancer Institute. Its unique property is that it interacts with gp120 almost irreversibly, but it only selectively inactivates the replication incompetent -- I mean, sorry -- replication competent virions.

So after the expansion is achieved, the Sino virion is bound to magnetic particles used Steptoff (phonetic) in biotin. That is, this is biotylated. It's bound to Steptoff and encoded, and it removes the infectious virions so that the leftover virions, which are lot of viral protein, is actually culture negative in vitro.

This work has been presented. Some of you who may have been at the meeting that we organized in San Francisco in March on blood safety know this data that were presented there.

The secondary inactivation is specific for inactivating residual RNA that is still present in some of the replication incompetent virions, and this can be achieved by nucleophilic means or with dyes, such as dimethyl methylene blue or psorlens. Psorlens is now in advanced area clinical files, and perhaps a
preferred compound that does not bind to any proteins, but specifically binds to the RNA.

And then finally, the purification of the inactivated virus from all the components in the culture system, as well as the inactivating chemicals is achieved by molecular C ring (phonetic).

After this is put into final containers for terminal viral inactivation, the pressure cycling technology, this is basically a hydrostatic pressure that is repeatedly applied to the virions in the final container that disrupts the virions and disintegrates into nonprevalent bond proteins.

So basically that's where the word or the title comes, that HIV proteins are produced through this pressure cycling technology.

After that we put the adjuvant and the immune response tested in mice shows that such antigens are quite immunogenic.

Safety and efficacy testing is going to be done in chimpanzees, and finally this is what I say we are going to be doing. CGMP facilities for manufacture of the RIV vaccine, as we call it, in an FDA approved facility, and finally Phase 1 and Phase 2 clinical trials in humans are planned.

And finally, I want to show the
investigators at UC-SF are myself; Cliff Lawless, Chief of Immunology; Manish Gandhi is a pathologist and transfusion medicine specialist at our place.

Mike Boyd at NCI is the one who cloned and provides the Sino virion N.

Mark Manak at the BBA Biotech is the one who has the pressure cycling technology for inactivation of the final product.

Mike Bush and Eric Diewart were both responsible for purifying for genetic analysis of the pool against standard strains of HIV.

And finally, the safety testing in chimpanzees will be done in Southwest Foundation by Chris Mouffy (phonetic).

And finally, I want you to make any thoughtful suggestions you can make so that this project has the maximum benefit of peer input.

Thank you.

(D applause.)

DR. PEDEN: Thank you for keeping on time.

Are there any questions?

PARTICIPANT: Can you say something about where you HIV is actually going to come from Girish?

I mean, it seems to me that the blood banking people have spent the last 15 to 20 years getting rid of
intravenous drug abusers and HIV infected gays, and
you're going to be out there hunting for them again;
is that right?

(Laughter.)

PARTICIPANT: I mean, are they saying thank you to you for this?

DR. VYAS: Well, actually we have more than adequate resource of plasma that is HIV positive.

Yes?

DR. BROKER: In the protocol, you had mentioned possibly putting psorlens into the preparation near the end; is that correct?

DR. VYAS: That's correct.

DR. BROKER: That's a potent mutagen. It seems like you're kind of going backwards on this in terms of safety.

DR. VYAS: Well, there are three alternatives, and any one of the three, which is RNS specific inactivation otherwise. That is, there is RNA there. Nobody would simply accept final virion as a primary inactivating agent as adequate. Certainly my colleagues at FDA would not approve of it.

So we wanted to be sure that we include one step which is specific for nucleic acid and does not in any chemical way alter the proteins, and either
it can be a means that has been used for foot-mouth
disease, virus, for many, many years or it could be
psorlens.

Now, we have not completely made a
decision as to which of the three alternatives we are
going to go, but putting inactivation of HIV has been
successfully done with psorlens, and then in the final
stage, they are able to remove the free chemicals to
molecular series.

DR. BROKER: Yeah, I just would point out
that there have been some adverse effects in using
psorlens in dermatology for psoriasis. It tends to be
used only in the extreme. It's an interpolating dye,
and if it for any reason passes through to the
patient, I'd be quite concerned.

DR. VYAS: Your point is well taken. In
fact, the amount of psorlens that's found to the
residual nucleic acid is so exquisitely small that it
has no adverse effect so far, in fact, much less than
a unit of blood.

The clinical trials for virally
inactivated blood is in Phase 2 and Phase 3 clinical
trials, and those trials have not had any adverse
reactions as far as I know. So I'm not discouraged by
all the points that you made. We are aware of it, and
I think that since we are removing the chemical inactivating agents, we probably are safe. And in the amount that is there in the residual inactivation, it's probably trivial.

DR. BROKER: Okay. Thank you.

DR. VYAS: Sure.

DR. PEDEN: That question was by Tom Broker.

Please give your name when you ask the question. In the back.

DR. RUSSO: Carlo Russo from Merck.

How stable is your inactivation? How stable do you think it is going to be? And you know, in the context of this meeting where there is lots of concern in the way we validate the product that we're going to use for a vaccine, how are you going to validate your release assay for your preparation?

DR. VYAS: I'll come to Merck for doing that work.

(Laughter.)

DR. RUSSO: That's not a bad idea. We have a terrific validation procedures, but I would like to have an announcement from you.

DR. VYAS: I just want to mention that the
primary work of this nature has been done in my lab. The major NIH support for this study is impending, and if that gets approved, then the R&D work will be done at the University of California and BBA Biotech.

The validation issues and the manufacture issues are tertiary, and we have not yet come to grips with those issues yet.

DR. LEWIS: Yeah, Lewis, FDA.

Have you given any possible thought to the use of human stem cells as a source of substrate? It might be less of a problem. You might be able to generate them in large volumes and from a very limited source that would be a more amenable testing than would be pooled human peripheral blood mononuclear cells.

DR. VYAS: Our major consideration was actually having the Third World countries' ability to make this vaccine. I think the stem cell biology and propagation in vitro is still not available in the majority of Third World countries.

So leukofiltration is one way of getting rid of leukocytes, and we have actually been able to get leukocytes out of the filters and been able to culture them and grow HIV into that. So that's a first step.
The point out stem cells is a very good point, and I think ultimately that would be one of the ways that we want to go about it.

DR. JOHN LEWIS: Lewis from Merck.

I'm curious to know if you know what the physical product of your high pressure dissociation is and whether you have quality control assays that would assure the consistent manufacture of that.

DR. VYAS: Unfortunately you were not in San Francisco in March when this work was presented by the BBI people, and they have the protect technology and the quality control in place for pressure cycling technology and validation that introduces six logs of HIV infectivity.

As such, after the primary inactivation, in vitro we cannot demonstrate any infectivity in the vaccine. The second inactivation with psorlens or MEs is an added, and I call it tripolene (phonetic) activated with special cycling technology.

But the immunogenicity is the main criterion we are using in the animal model, and as long as it is immunogenic and it produces neutralizing antibodies, those are our standard criteria for useful product.

DR. PEDEN: Okay. Thanks. We have to
move on.

The next talk is by Alex van der Eb,
unusual response to apoptin of diploid fibroblasts
from cancer prone syndromes.

DR. VAN DER EB: I would like to talk
about apoptosis producing protein that we call
apoptin, and which some of you may have heard, and the
unusual effects of apoptin on diploid cells of certain
cancer prone individuals.

However, before I do that, I have to
summarize some of the properties of apoptin, and if I
can have the first slide, please, apoptin is a
protein of the chicken virus, chicken anemia virus.
The chicken anemia virus is a small avian pathogen
that causes a lot of economic damage in the poultry
industry. It causes clinical signs in young chickens,
although older chickens are immune for the virus.
Anemia, it causes anemia, and by
destruction of the erythroblastoid cells,
immunodeficiency by depletion of the thymocytes.
And in the early stages of the work, which
was actually intended to try and make a vaccine
against the virus, we found that CAV causes these
disease symptoms by inducing apoptosis.
In order to clone the virus in the final
DNA was a circular, single stranded genome which encodes a single mRNA, and on this mRNA there are three open reading frames, coding for what we call VP1, VP2, and VP3.

So we asked the question: which of these three open reading frames causes the apoptosis? And we transfected cells with each of the open reading frames and found that it was VP3. So the smallest of the three proteins causes apoptosis.

So how does VP3 cause apoptosis? There are many pathways of apoptosis, and the next slide shows you the major pathway that is mediated through P53, when P53 is properly activated will cause a signal that causes activation of the CAV spaces. IS is one of the CAV spaces, and that will then eventually lead to apoptosis.

There are several proteins that regulate this process, like VCL2 and BAG1, which are negative regulators.

So we asked the question which does apoptin, as we call this protein, that is encoded by VP3 -- does that need VP3? Is it inhibited by VCL2? Does it need half spaces for apoptosis induction? And the answer was initially rather surprising. P53 is not needed. Also cells without
P53 go in apoptosis, VCL2 and BAG 1, which are strong anti-apoptotic proteins, do not inhibit apoptin induced apoptosis, and a number of CAV space inhibitors, including CrmA, did not prevent apoptosis induction by apoptin.

So that was surprising and indicated that apoptin had an apoptosis pathway of its own. However, recently we found that the P35 gene, an anti-apoptotic gene or maculovirus and which is an inhibitor of CAV spaces, of the downstream CAV space also inhibit apoptin, indicating that apoptin also activates the CAV spaces.

When we did these studies, we realize that what we had tested so far, what we had tested in initial studies was a number of cell lines that all went in apoptosis, and all of these cell lines were actually neoplastic cells.

And so what about the non-neoplastic, normal, diploid cells? And then came a surprise. It turned out that the normal cells, diploid, fibroblast endothelial cells, T cells, smooth muscle cells, et cetera do not go in apoptosis. They are completely resistant.

That was a surprise, and why would apoptin not cause apoptosis? Well, it turns out to be the
localization probably of the protein.

The next slide shows you a tumor cell, an osteosarcoma cell which is transfected two days ago with apoptin, and you can see here the protein, the fluorescence of the protein in the nucleus, and it is just present in the nucleus throughout the nucleus, and the DNA staining of the same cell shows that everything is still normal.

However, the days after this second day, an increasing number of cells appeared that have a condensed and fragmented fluorescence as you can see here, and if you look at the DNA staining, you can also see that it is abnormal, and this cell is an apoptotic cell.

So this happens in cancer cells. So what about the normal diploid cells? Well, it turned out that the apoptin is not present in the nucleus, but stays in the cytoplasm, and even if you looked many days after transfection, it still stays in the cytoplasm.

They even have transgenic mice now that express apoptin in a number of tissues and everything seems normal. So it is not toxic for normal cells.

Now, what would happen if you transformed these cells with SV 40, for example? We have the same
cells formed by SV 40, and then we are completely sensitive to apoptin. So what would happen if you co-
transfect normal diploid cells now with SV 40, the antigen and transforming gene that completely transforms the cell?

And then it turns out that these cells become sensitive. So here is the diploid VH10 fibroblasts that are co-transfected with apoptin and the SV 40 P antigen, and here you see that the cells that were transfected with apoptin 3T3 alone and neofactor did not go in apoptosis, whereas cells that were transfected with VP3 and actually 4T, large T, here went in apoptosis and will eventually reach the 100 percent if you wait long enough.

So this means that also a transient expression of an oncogene can cause sensitization of normal diploid cells to apoptin.

The next question that we asked was: what would happen if you treat normal diploid cells with a carcinogenic agent? And since we have an irradiation program in the lab, we irradiated with ultraviolet light or with actually ionizing radiation, and we found that if you do that, the diploid cells still remain completely insensitive to apoptin, and this is actually what was expected because a single radiation
exposure will not transform the cells, of course, in
a single step, and maybe you induce a few mutations
and no more than that.

So up until recently we have found a
category of diploid cells from cancer prone patients
that do react with apoptin, and before I go into that
part, I have to briefly introduce something about
irradiation or radiation program or radiation
research.

Oh, incidentally, this is what happens if
you transfec t with T antigen a normal cell, T antigen,
and apoptin, and this is two days after transfection.
In the normal cells after one day the apoptin is still
present in the cytoplasm, but after two days -- and
this is after two days -- there is a kind of a
transfection. You see that it moves into the nucleus.
So it is actually the movement of the protein, the
transport of protein to the nucleus that may be
essential, and one day later, everything is in the
nucleus.

And now radiation. It is, as you probably
know, that radiation of cells, particularly UV
irradiation, causes a large number of responses, most
of which are transient, such as activation of plasma
membrane, activation of genes that are normally
induced by growth factors, secretion of growth factor, stabilization of P53, and so on and so on.

Also, ultraviolet light induces so-called SOS-like phenomena, such as a reactivation, an aus (phonetic) mutagenesis, ER and EM. They are called like that because these phenomena they resemble, the SOS responses that were first described in E. coli in response to irradiation.

Now, we asked the question, and this is several years ago: do these, in particular, EM somehow contribute to carcinogenesis? And we decided to try and study that.

First I would like to show you what ER and EM are. ER, an ounce (phonetic) reactivation, and EM are both measured with viruses. They are either measured with DNA viruses, like HSV, or actually for virus and ER, an ounce (phonetic) of reactivation, is the phenomenon that your re-irradiated virus survives better in your re-irradiated cells than in unirradiated cells. In fact, it survives about two times better. And that means that ER by definition is two.

EM also makes use of the virus, but in this case the virus is not irradiated, and if you allow it to replicate this unirradiated virus and you
re-irradiate it or unirradiated cells, it turns out
that the mutations that are accumulated in the virus
are about two to three times more higher in your re-
irradiated cells, indicating that your re-irradiated
cells have a kind of mutator activity, and that is
about two or a little more than two.

The effect is transient, and here you see
ER, an ounce reactivation, in a cell. It returns
after a few days going back to one, and EM also
returns to one, and they have a maximum expression of
one day after irradiation.

So do any of these phenomena play a role
in carcinogenesis? So we decided to measure ER and EM
in cells and diploid cells from cancer prone
individuals, and we have measured two different cancer
prone individuals, and in one of which we saw
abnormalities, and not with EM, as we expected, but it
was with ER.

And ER turned out to be extremely high in
diploid fibroblasts from individuals who are cancer
prone as a result of a germ line mutation in a tumor
suppressor gene, and here you see, for example,

Aniridia cells from an Aniridia patient, just normal
diploid cells who have a mutation in one of the
Willems tumor genes, and as you can see here, the ER
is extremely high.

Now, this is an only single point, but you can believe me that this is reproducible, and the correct slide with more points in the curve I couldn't find, and it's always disappeared in the lab.

So surprisingly this was ER and not EM that was correlating with cancer prone. So we then -- this is a list of the syndromes from which the diploid fibroblast show the extremely high ER phenomenon, ER super plus, as we call it. Retinal blastoma, Li Fraumeni, Euro Frondromitosis (phonetic), Willems tumor, et cetera, et cetera, et cetera, and we have measured now more, and all of them show this abnormally high ER phenomenon.

And then we decided to test these cells also for their sensitivity to apoptin without and with radiation, and we have -- and this is quite recent -- we have three of these cell strains in culture: Li Fraumeni cells that have one inactive LEU (phonetic) and one wild type LEU, and they are ER super plus.

A Lynch Type 2 syndrome familiar in breast and ovarian cancer, ER super plus. The gene is in this case, as far as I know, still unknown.

And there was a dysplastic Nevus syndrome, a familiar melanoma, which is also ER super plus and
which has mutated P16.

Each of these cells, these two cell lines, we have two different individuals from the same family, and for the Li Fraumeni syndrome, we had one patient who was affected and one individual from the family who had two wild type alleles.

The first experiment already showed that the normal irradiated fibroblasts are completely resistant to apoptin. This was actually not unexpected, of course, but after irradiation with ultraviolet light, we found quite a surprise, and that was that these cells become completely sensitized to apoptin just as tumor cells.

Here you see the reaction of the PH10 normal diploid cells, which remained resistant to apoptin, and they remain so much longer, as long as you can keep the cells alive.

However, this is a Li Fraumeni cell, and that is the Lynch Type 2 syndrome, ovarian/breast cancer syndrome. They become completely sensitive within four to five days.

And the next slide shows the more complete study of all the cells together, and in the left panel you see the cancer prone syndromes here, fibroblasts from the cancer prone syndromes, and here first the
cells, the normal cells, which have nothing special or
the Li Fraumeni patient, the Li Fraumeni individual
who had two wild type, family member who had two wild
type P53 molecules, genes.

You can see that these remain completely
resistant to apoptin after re-irradiation, whereas the
other ones go completely in apoptosis as if they are
cancer cells.

In contrast, if you transfect and you re-
irradiate the cells with a desmid control gene instead
of apoptin, then all of the cells remain completely
resistant and do not go into apoptosis, indicating
that the effect is specific for the 3T3 or apoptin
gene.

The next slide shows just a dose response
and where you can see that at ten Joules you already
get the maximum reaction of the Li Fraumeni cells in
this case, whereas at five Joules there is still
nothing.

If you transfect with desmid, again,
nothing happens, and if PH10 normal cells, even at 25
Joules, show no reaction whatsoever compared to

transfection with desmid.

And then the last slide shows you here
that the effect on these cells from cancer prone
individuals is transient, like many of the other injury induced phenomena. It completely disappears again after four to five days and returns to zero, to the same level as the PH10 control cells.

So in conclusion, the diploid cells of these cancer prone syndromes that have one allele of a tumor suppressor gene have two abnormal properties and have lost one allele tumor suppressor gene, have two abnormal properties.

First, they show very high ER response, and, second, they express what are also found in cancer cells and which render them susceptible to apoptin, which is a cancer specific protein. And essentially it means that apoptin is allowed to be transported to the nucleus, and both properties, irrespective of the tumor suppressor gene because it's found in many different tumor suppressor genes now also irrespective of the type of irradiation.

We are trying now to identify what is induced by radiation in these cells and that is different from what is caused by irradiation in normal cells.

(Applause.)

DR. PEDEN: Thank you.
And we have time for a couple of questions.

DR. FRIED: Did you say you had a transgenic with --

DR. VAN DER EB: Yes.

DR. FRIED: Does it get cancer?

DR. VAN DER EB: This is something that we still have to study. The transgenic mice exist, and it is expressed particularly in the lymph nodes and other lymph organs, and what we have to do is see what happens if you, for example, try to induce lymphoma by leukemia viruses. They should be resistant.

PARTICIPANT: That was Mike Fried.

Could you give your name, please?

DR. CASHMAN: Neil Cashman.

You've adroitly avoided any mention of the sequence of this gene.

DR. VAN DER EB: Right.

DR. CASHMAN: Can you comment at all?

DR. VAN DER EB: Yes.

DR. CASHMAN: Are you free to comment?

DR. VAN DER EB: I have the slide with me, but of the protein sequence of the gene. It's the small protein, 120 amino acids. It does not resemble any other known protein in the cell bank. It has two
nuclear localization signals, in the right-hand C
terminal part, and it has a nuclear export signal in
the internal part, and that's about it. There's
nothing else, no.

I have it. It's published also.

DR. COFFIN: John Coffin.

Ordinarily one thinks of induction of
apoptosis as an antiviral mechanism.

DR. VAN DER EB: Yeah.

DR. COFFIN: Can you speculate on why it
might be of value to the virus to do such a thing?

DR. VAN DER EB: I have no idea. This is
a good question.

One possibility is that this virus is a
very simple virus. It has only three genes, and it
has no other means to get out of the cell, and then
the best way to get out of the cell or to infect other
cells is to induce apoptosis. Then the cell fragments
and is taken up by other cells, and maybe that's the
way it's expressed. I don't know. No, I don't know.

DR. PEDEN: Thank you, Alex.

The next speaker is Tom Broker from UAB,
and his title is "Human Papillomaviruses: a Window
into Eucaryotic Cellular DNA Replication Mechanisms
and Regulation."
I'd like to share with you several things regarding papilloma virus DNA replication, and what I'd like to do is start by very briefly reviewing two aspects of HPV biology.

The first is that the virus infects a squamous epithelium through a wound allowing direct access of mature virus particles from the environment to the germinal layers of the skin, typically the basal cells or the parabasal cells.

The virus would be harbored in these cells and maintained more or less indefinitely by replicating in synchrony with the normal cell cycle, and it would be latent in that capacity in most cases. Occasionally induction of viral early, as well as late gene expression can commence at about the third layer up where it becomes so the so-called spinus epithelium with the delayed early, late transcription, viral DNA replication occurring here, and assembly into mature virus particles, and then naturally a release through desquamation of the cornified envelopes.

Now, periodically the genes that normally affect the onset of reentry into S phase can inadvertently become expressed in cells that have not
yet withdrawn from the cell cycle, and that can lead
to dysplasias, and through an accumulation of cellular
mutations and probably some viral mutations, you can
progress over a period of years toward higher grade
dysplasias and carcinoma in situ.

I want to emphasize that there are several
modes of replication of HPVs. They're in the wound
healing phase when there is literally a stripped off
epithelium. There is a re-epithelialization across
the wound bed that originally allowed access in the
virus to these layers, and at that time there's a
transient up regulation of viral oncogenes E6 and E7.

However, once the full thickness skin has
been reestablished, E6 and E7 transcription stops at
this level, and I'll show you that, and the E6 and E7
oncogenes only are transcribed from this layer up in
a full thickness epithelium.

So we have establishment replication
during the first phases of wound entry; secondly,
maintenance replication here; and third, vegetative
replication there higher up.

This is the HPV18 genome. It's pretty
typical of most viruses. The proteins that I'll be
discussing today are the origin binding protein E1 and
E2. What you'll also see is E2 plays a role in
transcriptional regulation at the promoter right there, and that the E1 is the only enzyme encoded by this virus and is the preferred target, therefore, for drug discovery. It is a helicase with some remarkable properties.

It together as a multimer binds to the E6 promoter region, which is synonymous with the replication origin right here. E7 protein, which I'll talk about a bit today and a bit tomorrow, interacts with the retinoblastoma protein and triggers S phase entry, and as many of you know, the E6 protein interacts with P53 and blunts some of the repair responses and also can blunt movement toward apoptosis, allowing the virus time to replicate.

Now, in a normal, full thickness, squamous epithelium, in this case cervix, without any viral infection, I want to point out that the only DNA replication that the normal cells are engaged in are the parabasal or transid amplifying cells, as marked by PCNA stain.

All cells above the parabasal layer do not have the oxyribonucleotide triphosphates, DNA polymerase, topoisomerases, PCNA, or any of the other replicative enzymes. Yet the virus replicates up here, and that is the dilemma the viruses have to
face. How do they reactivate S phase entry and the
reinduction of all the replication machinery to
support their own replication?

This is one piece of evidence from a
natural laryngeal papilloma virus from a child in dark
field illumination, and the probe we used was 40-67
transcripts, and as you can see, they're basically
excluded from the basal cells. This is a dermal read,
and the signals are in the spinus layer of the
epithelium for E6 and E7 once the wound healing has
been complete.

Okay. Now, I very briefly want to remind
you of the normal cell cycle and that it is controlled
by the retinoblastoma protein being bound to the E2F
enhancer protein, which itself is in complex with a
cofactor called the DP1 or DP family of E2F cofactors.
That would be the normal state in the G1
phase or G0. However, upon mitogenic signals, cyclin
dependent kinases, primarily cyclin D and DCK4, can
phosphorylate RB, causing it to be released from E2F,
and then E2F DP1 complexes can up regulate the
transcription of the replication enzymes and certain
cell cycle progression proteins like cyclin E.
Conversely, the papilloma viral E7 protein
can disrupt this association in the absence of the
kinase activity, and therefore, the virus can trigger the up regulation of the necessary enzymes for its own synthesis.

And this is shown here in a couple of examples. This is a genital condyloma, and I want you to see in this cross-section of the papilloma that, again, the basal cells do not have PCNA. The parabasal cells do. Then the lower spinus cells do not have PCNA, and the upper spinus cells, once again, do have PCNA.

So you have this natural cell cyclin here, and the viral induced S phase reentry toward the upper layers of the epithelium.

This phenomenon is also seen with other important enzymes, in this case DNA polymerase alpha in the natural laryngeal papilloma; again the tritium or S35 silver grains are predominant in the upper layers of the skin, and I want to particularly emphasize that there's a massive over expression of the polymerase, as there is with PCNA, in the upper layers. That will become particularly important.

It's something I'll have to say probably in tomorrow morning's talk.

Now, the second thing Andy asked me to comment on is some of the model systems that we have
for investigating in an experimental way. This is the epithelial raft culture, and very briefly, one makes a collagen matrix, imbeds dermal fibroblast in this, and then you can take either dispersed keratinocytes, primary human keratinocytes or established immortalized keratinocytes, and spread them on the surface, and at that point you can raise the entire thing up into the air.

Think about your own skin. You have an air surface, and you have a blood surface, and when you do this, the exposure to air sets up a capillary action, calcium gradients and other gradients of probably mytogens, and so forth, and you quickly develop a stratified and differentiated squamous epithelium.

To show that this actually can work not only with disbursed cells, but with punch biopsies, we've been able to take skin from any anatomical site in the body, place it on the raft, which is right there. This is a punch biopsy from a excess cervical epithelium, and these cells will grow out across the collagen surface and very rapidly stratify and differentiate appropriate to the tissue of origin.

You can do the same thing with papilloma itself, and you always recapitulate the type of skin
you started with. In this case, the natural laryngeal
papilloma will grown out and become an in vitro
papilloma.

The third thing we've been able to do is
introduce papilloma virus genes or reporter genes into
the keratinocytes through retrovirus mediated gene
transfer process. Denise Galloway and Jim McDougall
did this in which they placed the papilloma virus
E6/E7 oncogenes directly under the control of the
upstream LTR.

With Louise Chan in my laboratory, we
chose to place the reporter gene or the E6 or E7 genes
under the control of their own promoter at a rather
downstream location, in which case we were able to
very exactly recapitulate the differentiation
dependent express of the E6 promoter.

Parenthetically I would note because it
reflects a change in thinking, people had always felt
that the loss of an E2 gene upon HPV integration would
invariably lead to the up regulation of the E6
promoter, which it can repress in basal cells. This
construct has no E2 gene in it, and indeed, the E6
promoter driving LAX-E (phonetic) in this case is only
in the upper layers where we expected it to be.

So E2 has absolutely nothing to do with
the repression of the E6 promoter in these types of
cells, unless other cellular changes have occurred,
which in this case they're PHKs, and they haven't
engaged in those mutations yet.

To show that this system works and that we
can do genetic dissection of HPV, we have an empty
vector here. We have an E7 expressing vector here,
and as you saw in the natural papillomas, polymerase
alpha is up regulated; PCNA is up regulated; and
bromodeoxyurasyll incorporation, all occur in the upper
compartment of the full thickness skin, which
developed in just one week after the viral infection
and lifting of the rafted air surface.

And notice again the basal cells tend to
have low grade expression of these induced genes.

They're highly up regulated in the differentiated
compartment.

What we noticed upon doing a variety of
analyses is that while many of the cells do have up
regulated PCNA, only a subset of them are capable of
incorporating bromodeoxyurasyll in response to these up
regulated genes. This will become important for
tomorrow's talk, but notice that we always compare a
natural papilloma with an E7 raft culture, and only a
subset of the superficial cells become competent for
DNA replication. In other words, all warts, as well as rafts, are a mosaic of cells that are capable of engaging in DNA synthesis and S phase entry, and others which appear to be inhibited for reasons I'll discuss tomorrow.

Now, I want to give the bottom line of what we now know about the assembly of the replication complex so you know where I'm going with several of the data slides.

Very briefly, the viral E2 protein is the main ORI binding protein, and as I'll show you, it associates with the nuclear matrix. It then recruits the viral E1 protein, the helicase, and one of the most remarkable observations that we've made recently in collaboration with Doug Seer and Jack Griffith is that the assembly of this complex depends on heat shock protein 70 and heat shock protein 40, and in fact, heat shock protein 40, the co-chaperon for HSP70, in fact, remains a permanent component of the active replication complex. We presume it's true also in general eucaryotic replication, but it certainly is with the virus.

The E1 protein, in turn, can recruit DNA polymerase alpha, and when that assembly has occurred, cyclin E, CDK2 moves in, and as you'll see
phosphorylates this complex.

I would like to show you some of the data. Very briefly, the E1 protein has a series of domains. At this end is an ATPase, which is essential to the helicase action. There's a DNA binding motif, and as you'll see, very, very critical, a cyclin E binding motif right here which has great bearing on our understanding of Hela cells.

The E2 protein has three basic domains, the DNA binding domain, a hinge, and a transacting domain. As I'll show you in a moment, the hinge anchors this entire complex to the nuclear matrix.

What we did is put green fluorescent protein tags on each of the domains of the E2 protein, full length protein and the various combinations of one or two portions of the E2 protein. We were able to show function was retained despite the GFP tag, and bottom line is the hinge unexpectedly was the thing that targeted the E2 protein to the nucleus.

If you didn't have the hinge domain, as in this column, the proteins remain cytoplasmic. If we did have the hinge, they got nuclear. If we had the N terminal domain and the hinge together, we began to see nuclear foci. If we had the whole protein, the E2 protein went to the pods or nuclear domain 10s of this
And we were able to identify the anchor to the nuclear matrix as a basic motif in the center of the hinge that's highly conserved among all papilloma viruses. If we mutated that domain, we got a cytoplasmic distribution. If it was wild type, it went to the nuclear foci.

And this is the model then. The hinge anchors the E2 protein to the matrix. The C terminal domain binds to the viral DNA. The N terminal domain associates with the matrix and also helps recruit the E1 protein into the complex, as you can see here.

E1 protein and the bromodoxyurasyld replication foci are coincident.

Now, I want to show you one really remarkable thing about the nuclear domain, about the assembly, yeah, yeah, and that is that the DNA J protein of E. coli and K protein are the things that help the lambda ONP proteins assemble at the lambda ORI. DNA J and DNA K also help the assembly of the E. coli replication components, and they're highly conserved all the way up to eucaryotic and human cells.

This domain right there helps the helicase assembly. This was done with Jack Griffith's lab at
UNC. In the absence of the J protein, the E1 protein by itself can form a hexamer ring around the DNA, either a super coil or an open circle. You'd throw the HSP40 in, and you get a dihexamer, which we believe is a bidirectional helicase.

Secondly, the E2 protein helps recruit the RPA, the single stranded binding protein, and we feel this assembly of E2, E1, the J domain right here creates the preinitiation bubble at which you end up with four replication strands, two leading strands, two lagging strands, and the complex bound with RPA. I think I'll stop at this point, and I'll be able to show you the role of cyclin E tomorrow.

Thank you.

(Applause.)

DR. PEDEN: Thank you, Tom.

We'll have maybe one question.

Well, I guess we have to move on. So the next talk is by Paul Sandstrom, "Facilitated Detection of Adventitious Agents Using Genetically Engineered Cell Lines."

DR. SANDSTROM: What I'm going to present tonight is somewhat of a revisiting of some previously published data in an attempt to try to develop it in the direction of the theme of this meeting, and that's
the detection of adventitious agents which may be
present in cell substrates that's used for vaccine
production.

Largely speaking, it's a proof of concept
model which demonstrates approximately a practical
application of a cellular phenomenon that's received
considerable attention over the last ten years, that
being program cell death.

I'm not sure what I'm pointing at here.

Okay. This project was done in the
laboratory of and under the direction of Dr. Tom Folks
from the HIV and AIDS and retrovirology branch of CDC,
and I myself have the, I guess, notoriety of being, I
think, probably the only Canadian citizen who's
employed by the American Public Health Service and who
is working for the Canadian equivalent of the Public
Health Service and stationed in Canada with all of the
rights and privileges that come with working for the
two largest bureaucracies in the Western Hemisphere.

(Laughter.)

DR. SANDSTROM: Okay. It's readily
obvious, I think, to probably everybody here that when
you go about trying to isolate a potential
adventitious agent, that the failure to isolate
doesn't necessarily mean the agent isn't there. It
just means that possibly the culture system wasn't
sufficient adapted to allow for the agent to be
isolated.

So what's needed for isolation is, number
one, it would be good if you had an adventitious
agent, or at least good depending on which side of the
fence you're on, that the virus has the ability to
bind, fuse, or otherwise gain entry into the cell;
that the wiring and forming of the cell are such that
it can support a complete cycle of virus replication;
and of particular importance or I should say of equal
importance, I guess, is that the cell is a good host,
and it stays alive long enough for the virus to
complete its replication cycle.

Apoptosis or program cell death is
involved, in part, not exclusively, but in part as a
first line host defense against pathogenic viruses,
and efficient and rapid apoptosis followed by viral --
following viral infection possibly acts to restrict
virus replication and, if unchecked, will ultimately
result in an aborted viral infection, and this is sort
of the ultimate goal that the cell had in mind when
developing the programs.

So as a result of this intense restrictive
pressure which apoptosis places on virus replication,
many viruses have evolved to develop their own repertoire of borrowed proteins, which act to block the cellular programs.

It's this that I think we're interested in here, that when trying to isolate an adventitious agent, along with all the other elements that I identified earlier, you're always going to be running up against the possibility that the cells that you're trying to isolate it in are attempting to undergo apoptosis and, therefore, limiting your ability to isolate the virus.

So to get around and to take a look at this, what we did is to use the, I guess, historical adventitious agent of HIV, coming from the HIV and Retrovirology Branch, and we took a cell line which was exquisitely sensitive to support HIV replication.

This is a sub T1 cell line.

One of the reasons we chose sub T1 is that it is, or at least the clones that we were using lacked expression of the very strong anti-apoptosis gene, VCL2, and so what we did, quite simply, was to take VCL2, place behind a constitutive promoter, and to transfect it into the cells, and then go on to select clones.

So what we have here is our positive
control. This is a Western Blot, positive control, and two control clones which lack VCL2 expression, which are paired with two clones which express significant amounts of VCL2.

And we went on to analyze these and characterize these in terms of the ability of VCL2 to block apoptosis in sort of the nonviral, but traditional, apoptosis models, and we were able to identify that the expression of VCL2 in this case was associated with enhanced resistance to program cell death.

Now, intuitively it would appear as if you over express a cell, a protein which would block apoptosis, that you would, in essence, increase the viability of those cells. If anything you would increase the viability of those cells upon infection.

What we found, in fact, was the opposite, and this graph illustrates viability which was characterized by using a probe for mitochondrial function. So it's not actually measuring cell lysis in this case.

And what we saw is that if you gave these -- infected these cultures at the low MOI of infection, and this is about one in every 10,000 cells, although we were able to go significantly lower
than this, what we saw is that for about the first four or five days everything appeared pretty much the same in the control and the VCL2 cultures. However, at about five days, there is this precipitous drop in viability that was observed in both of the VCL2 forms, whereas the control forms kept on going out past ten days before they, too, started to die due to viral mediated cytopathic effects.

And this effect on cell viability was recapitulated on what we saw with regard to the effect on viral replication, where both of the VCL2 clones that we were looking at seemed to support a much more robust viral kinetics, peaking significantly in advance of what we saw in the case of the control clones.

This larger spike in virus here is probably due to just more cells being present in the culture at that point. So it's somewhat counter intuitive until you sort of sit back and look at what was going on in the culture, and what we observed was that -- this doesn't show up all that well -- but these two clones here represent -- all four of these cultures are infected with the same low MOI of HIV, and what we saw was in the two control clones you had a very nice
confluent lot of single cells, no evidence of viral
mediated cytopathic effects taking place, whereas in
the case of the cells which were over expressing VCL2,
we had these very large syncytia appearing.

Just to show that it wasn't due to a cell
specific effect, we carried out similar experiments on
another cell line, this being the A301 T cell line,
exact paralleling the experiments I just outlines, and
once again saw the exact same effect, that the VCL2
expressing clones showed a significant enhancement and
cytopathic effects going on in the culture.

What was significant though was the nature
of the typopathic effects. Using a slightly different
model, and this one here was an attempt to get away
from the issue that comes up with viral kinetics that
would happen during a spreading infection, what we did
was we took a cell line which constitutively expressed
HIV. This is the A301 cell line, which is quite
commonly used in HIV research, and this cell lines
throws off just gobs and gobs of virus and expresses
on its surface viral proteins, and we cultured that
with our sub T clones, and what we found is that all
the clones would roughly make about the same amount --
that in all of these cultures we could identify about
the same amount of syncytia.
However, in the case of the control cells, and the black represents viability here; in the case of the control sub T cells, that most of these syncytia were dead, and this was done initially by just trypan blue, whereas in the case of the VCL2 clones, syncytia were maintaining their integrity. Their membranes weren't being disrupted. We also ran a routine for -- the cell line itself is RT negative. So what we did is use other measures for virus presence in the supernatant, and found that in both of these cultures. We've seen roughly equivalent amounts of virus.

Now, if we examined -- this is the tunnel method for measuring apoptosis, which basically is an in situ measurement of DNA fragmentation. What we found was that red cells here represent cells in which the DNA hasn't defragmented, whereas this green appearance is through a number of steps is representative of cells where the DNA has been fragmented through apoptosis.

What we found was -- and these are syncytia here -- in the case of the VCL2 cultures we have these nice, health looking syncytial masses, whereas in the case of the sub T clones we've seen that these cells are undergoing apoptosis. So this is
probably more graphically represented.

In the next slide, if we look at actual EMs, and once again this isn't really showing up all that well, but this is a syncytia, and if the reproduction was a bit better, what you'd see is that the cellular nuclei are complete intact. They're sort of wedged together, crushed together inside of the cell, but each nuclei generally represents what you'd see in a single cell, whereas in the case of the control cell, we see this very characteristic condensation and marginalization of the chromatin, which is diagnostic of apoptosis, as well as vacuolization of the cytoplasm and membrane webbing going on.

And this below just represents sort of a more general view of the field, showing that we're not just looking at one cell that in the case of VCL T have multiple, health looking syncytia with no apoptosis, whereas in the case of the control cultures, most of the syncytia that are present are undergoing apoptosis.

And this is just to add in that no matter whether you have VCL2 expression or you don't, if the syncytia reaches a significantly large size, what happens is you start to witness necrosis taking place.
with rupturing of the cellular membranes, release of

the cytoplasmic content, swelling of the nuclei.

So are the effects that we're looking at

here the enhancement of HIV replication and
cytoplasmic effects strictly due to syncytia

formation? Apparently not.

If we take a look at -- in this case here

what we did is we went back to our original sub T

clones, and these are our two controls, which lack

VCL2 expression, and these are our two clones that

have enhanced VCL2 expression and effective MOI of

one, and this essentially means that every cell in the

culture receives an infectious hit, and 48 hours later

took a look at the level of viral replication, and

what we see is in the case of the VCL2 cultures we

have significant -- and this is a Western Blot looking

for the HIV protein and the cell associated HIV

proteins -- that we have significantly higher levels

of HIV protein present in the clones, in the VCL2 over

expressing clones.

So overall the point that I'm trying to

make here is that in the case at least for HIV and

possibly other lentiviruses, we have looked at other

retroviruses and see similar effects; that the

engineering of cell lines which over express in this
case VCL2, although there is a range of anti-apoptosis change, both cellular and viral, which probably gives similar effect and, in fact, we have seen this exact same phenomena with other cellular genes which are VCL2 mimetics, such as glutathione peroxidase, as well as just throwing in chemical mimetics of VCL2, but we see a similar phenomenon taking place.

So the engineering of cell lines, the changing of cell culture conditions in a way to block apoptosis may allow facilitated isolation of adventitious agents' presence in cellular substrates.

The other point that sort of came out of this, and I had forgotten about it until I was actually putting the slides together this afternoon, was that when it comes to designing a cell substrate for the purpose of vaccine production and deciding what components need to go into the recipe, it may be wise to look at apoptosis and apoptosis inhibitors.

Just looking at the data set which I presented, the over expression of VCL2, we see remarkably higher levels of viral replication, and in addition, we see significant inhibition of DNA fragmentation, which are two issues which I think are of interest at least from this afternoon's talk.

So that's it. Thanks.
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(Applause.)

DR. PEDEN: Thank you, Paul.

Any questions?

PARTICIPANT: Very interesting data.

Indeed, it's fascinating. I have a couple of questions.

One is when you say that the over expression of VCL2 render the cells resistant to apoptosis, what stimulus do you use for apoptosis besides the virus?

DR. SANDSTROM: I have to think about this. I know that we use serum starvation, and we used a flavonoid dose called Genestine, which is -- I'm trying to think of the mechanism by which it produces apoptosis. I think it has something to do with mitochondria.

But with these VCL2 clones, we have looked at a number of other cell lines that people had engineered, but the effect is just remarkable, these VCL2 clones, and I think what was most remarkable is that Genestine would inhibit cellular proliferation in the control cells. You'd see the same pattern.

Cellular proliferation would cease. Control cells would die.

The VCL2 expressing cells would just
continue to live or at least stay intact in culture,

and this would go on for days or longer, weeks with
cells lysing and releasing their components.

PARTICIPANT: Yeah. My question was how
generalized is this observation. I assume those are

T cells, and perhaps using mechanism of apoptosis that
are specific to T cells, such as engagement of CD3 of
the T cell receptor may allow you to dissect whatever.

This is a generalized phenomenon that will apply at
different cell lineages or it's specific to T cells
and perhaps to HIV virus. Do you --

DR. SANDSTROM: Yes, BCL2 isn't specific

for T cells.

PARTICIPANT: No, no. I know the BCL2,

but the T cells that you use in HIV are T cells and

the virus that is tropic for T cells. So I wonder if

you have other information that will allow to
generalize this information to other cell lineage.

DR. SANDSTROM: No. The only cell line

that we've personally looked at was T cells.

DR. LEWIS: Lewis, FDA. Am I on?

The development of syncytia in cultures

infected with retroviruses, lentiviruses, whatever you

want to call them, is very much reminiscent of the XE

phenomenon that was discovered by Rowe in Wally's
laboratory back in the late 60s. Do you have any
evidence that there's any proportion dilution with
these syncytia?

DR. SANDSTROM: I'm sorry?

DR. LEWIS: Do the syncytia develop in
response to the dilution of the multiplicity of the
HIV that you put on there? Have you looked at that?

DR. SANDSTROM: No, we didn't. I'm trying
to think if maybe I serendipitously looked at it, but,
no, not specifically.

DR. LEWIS: Gosh, I mean, the question
goes through my mind of sort of being of the classical
oralgia (phonetic) modes, whether you finally have a
CPE assay for HIV infection.

DR. SANDSTROM: Oh, I see. Yeah. No, I
think the only thing I could say to that is that in
our own experience we would see better syncytia at the
lower multiplicity of infection, but I always think of
it just as a more controlled infection, a more
controlled spreading infection rather than at the high
ones that just went too fast.

Does that answer your question?

DR. LEWIS: Well, no. I guess I would
poke at you a little bit and say suppose you went
below the lower multiplicity that you're using because
if the titre is ten to the seven or ten to the eight
in this assay and you're looking at ten to the four,
then you know, you're up here.

    DR. SANDSTROM: Right.

    DR. LEWIS: You haven't looked at the

    range.

    DR. SANDSTROM: Yeah. One of the problems
is that just because of the culture when you get down
too low, you reach the mathematical endpoint. There's
no virus for the number of cells that you have there.
So I think we were able to bring it down to somewhere
in the range of about, so to speak, five viruses in a
culture class, and we would see the effect.

    The effect actually is sort of -- the
differences between those clones was enhanced at the

    lower multiplicity of infection.

    DR. LEWIS: Well, I guess if it's not, if
the virions are not doing it and it's not proportioned
dilution, then what is it?

    DR. SANDSTROM: Yeah.

    DR. PEDEN: It's certainly proportioned to
the dilution, Andrew. It's certainly proportional.

    Is it pressing?

    DR. COOK: Jim Cook.

    So you're putting BCL2 into the cells.
The nuclei remain intact. The cells die early, and you said your indicator of cell death was a mitochondrial marker of some sort. It sounds a lot like cell necrosis.

Did you look at anything in terms of mitochondrial function, mitochondrial respiration, and BCL2, as you know, is a mitochondrial membrane active agent.

DR. SANDSTROM: Right.

DR. COOK: And the question is whether somehow or other this is sensitizing the cells to injury induced necrosis rather than apoptosis, and that's why you get early cell death.

DR. SANDSTROM: I don't think that's what's going on. I mean, we were using MTS, which is largely -- I mean, basically that measures mitochondrial function, and --

DR. COOK: So if the mitochondria are not functioning, the nuclei remain intact. That's almost the definition of necrotic cell death, right?

DR. SANDSTROM: Well, I think it's a lot of things. I mean, number one, necrosis and apoptosis are endpoints on a Gray scale. So there's a certain amounts of overlap, certain features that they have in common.
But largely speaking, when we looked at it in terms of the lack of chromatin, fragmentation, marginalization, and the fact that the cellular membranes stayed intact would to us say that it was apoptosis that was going on or the blocking of apoptosis that was taking place.

DR. PEDEN: All right. Thank you.

The next and last of the formal program is by Brian Van Tine. I hope that's the correct pronunciation. "In Situ Transcriptional Analysis of Integrated Viral DNA."

DR. VAN TINE: I'd like to introduce myself. My name is Brian Van Tine, and I work in the laboratory of George Shaw in the Center for AIDS Research at the University of Alabama.

I work on a technique called FICTION by tyramides, and FICTION stands for fluorescence immunohistic chemistry and in situ for the study of neoplasms, or in our case for the study of RNA DNA in proteins in a single cell at the same time.

Briefly, what I'd like to go over is just a method which is I realize basic for most people in here, but is highly informative. You pick a target, whether it's a protein, an RNA or a DNA. We design either an antibody or an RNA or DNA probe to it; come
in with a detection scheme, which leads to an HRP;

come in with a fluoro-4-tyramide and cause a covalent
deposition to the slides that we're interested in, and
why this is important is because you can kill that HRP
and start over.

So if you're interested in studying, say,
your HIV-1 DNA, the RNA it produces, and some of the
proteins it's associated with, you can now do this in
a single cell.

This work was done in collaboration with
NEN because the experts that went to microdetections
were there, and as a result a collaboration was
involved with Phillip Moen and a papilloma project was
developed.

And so in general, this research that I'm
presenting today is on papilloma HPV16, which
predominates in cervical cancers. It's, in general,
widespread and prevalent, and it's a causative agent
in both benign papillomas and condylomas.

It's multiply spliced, and the early
proteins that we'll be concerned with today are E6,
E7, E1, and E2.

What this shows is a general map of HPB16,
but what's important to note is it's only 8 KB, and if
this virus is to integrate not in tandem, but to be
just a single copy, it is very, very difficult with
any sort of sensitivity to detect a single copy by
normal methods, and that's why this method becomes
important.

I'd like to introduce our cell line
players for today. The CaSki cell line has
approximately 600 copies of HPV integrated into
various places in a very complex genome.

The SiHa cell line has one to five,
depending on the literature, copies of HPV, both on a
Chromosome 13, and we have designed probes either to
E6, E7, E1, all the way through E1, and we have a
probe just to E2. We also have full length probe
specific for each cell line.

Can we make it any darker in here?

What we are -- what I'm demonstrating in
the top figure here is the CaSki cell line, and this
is a hybridization for DNA and in tat cells, and as
you can see, there's many, many sites in each and
every cell, but down here in the SiHa cell there are
two cites that grow up on both 13 chromosomes in this
genome.

What we did was to ask the question, you
know: are all the copies in the SiHa gene on? And
what you do is you begin with a hybridization for RNA.
What you can see, we have two RNA centers. This is a G2 doublet indicative of cells in G2. This is a bit brighter in intensity and is probably an overlap G2 signal.

We then RNAase the slides, denatured them, and detected the DNA, and what you end up with is you can see that both copies of the HPV associated with SiHa cells are both on.

This is a metaphase analysis of the CaSki cell line. It has approximately 78 chromosomes and approximately 16 integration sites ranging from what is probably 100 down to, based on HIV size comparisons, one copy of HPV.

What became interesting is we then proceeded in the CaSki cell line to ask which of the DNA integrants were active, and surprisingly it was one. Here is the DNA in situ. This is a single cell. It was pre-extracted, fixed, and hybridized for RNA, then denatured, and the DNA was detected, and we find that this minor site right here, an emerged picture down here, is the only site that is transcribed in this cell line.

We then went on an exploration to see if we could discover exactly which of the sites was active, and if it was the same. So we did this by a
variety of paints where in a yellowish color on this slide we have a Si-3-tyramide to HIV and we have a chromosome paint in this case to nine.

We at the end of the day came down with three minor sites, one on Chromosome 3, one on Chromosome 14, and one on a Chromosome 21 and 22. We haven't, because of the paints, decided which one it is.

We then went in, back to an interphased intact cell, and just to show you that there are many colors of tyramides. If you work with NEM, there's now about eight.

Here we have in blue an integrated DNA. Red, we have the RNA, and in green we have Chromosome 13 territories, and we were looking for association, and we found that there's an association between Chromosome 14 and the active integrant (phonetic). We have run controls on chromosomes known to contain larger tandem repeats and not see that association, and on some of the few chromosomes that didn't have an HPV integration and found that uniformly we find that only the integrant associated with Chromosome 14 was active.

So that led us to our next question, which was: is there something special about 14 and its
position in nucleus that would suggest that it be specifically transcribed?

And so we began by looking at an SC35 splicing complex oligo to RNA, and we didn't find an association there.

We looked at a U2SN RNA and did not find an association, but what you see here in these darker regions are nucleoli, and consistently we have an association between this being turned on and the nucleoli.

We also looked at coiled bodies and found no association, but we're not quite sure to this point yet why there's only one copy turned on in the cell line.

We also found in our process of running controls that not only could we detect the sense transcript in CaSki cells, but there's also an anti-transcript which has been previously reported in the literature. Just to show you the sensitivity and the specificity of this technique, this is the E6/71 riboprobe in the SiHa cells, and we did a riboprobe here to the E2 protein which is missing in the SiHa integration to show you just how clear and specific this technique is.

What becomes important is we have now gone
to a cervical carcinoma, and this is actually a

formalin fixed tissue section where in red we detected
DNA and in green we detect RNA, and we found that
we're seeing the same effect in vivo where we have
nuclear -- we have DNAs that are both on and off, and
we're starting to dissect exactly if there's an
association between position and being on or off.

So, in general, in SiHa cells, HPV16, DNA

and RNA, they're both on, and they colocalize.

In the CaSki cells, the RNA colocalizes
only with a single integration site, and at the time
the slide was made, we weren't sure exactly what it
was, but we now know it to be 14.

The RNA and the DNA does not associate
with splicing factor domains. The RNA and the DNA
does not associate with coiled bodies, but they do
tend to localize near nucleoli.

It was initially assumed that all
integrants were transcribed and that all transcription
occurred in the same direction, and the CaSki cell
line shows evidence for transcription from one minor
locus, and we detected the E2, E6, and E7 transcripts,
were detected from both strands -- I believe that
should be E1 -- and we also have anti-transcriptions
seen in vivo.
And going with the themes that we've picked up in the meeting, we have suggested some mechanisms for the HPV silencing, which may either deal with methylation, the chromosome positioning effects, or maybe just histos.

And so with that I'll conclude.

(Applause.)

PARTICIPANT: Can you -- what is the sensitivity? The viral promoter may be very strong. Can you use this to detect the cellular RNA?

DR. VAN TINE: Cellular RNA, yeah. If it's colocalized in one spot, you can probably, if you talk to Robert Singer, who does really fancy deconvolution, you can see individual copies of RNA and map the direction.

PARTICIPANT: Is that right?

DR. VAN TINE: That was reported in Science about six months ago.

PARTICIPANT: I was not too clear about the fixation. Why do you not see RNA in the cytoplasm?

DR. VAN TINE: They're pre-extracted before they're fixed. So the only RNA that remains in the cell is that associated with the DNA as it's being transcribed.
DR. FRIED: Mike Fried.

If you put in five As of cytidine, do the other sites come on?

DR. VAN TINE: As I recall they don't.

DR. FRIED: So you don't see inspotsylation (phonetic)?

DR. VAN TINE: Not on the first experiments that we've done. They're still in process.

DR. PEDEN: Okay. Thanks very much. That ends the formal presentation. Now, as far as I know, there are a couple of people who want to present -- what's this? Just one? Yes.

So Dr. Arifa Khan will present first, and I think Johannes Loewer will present after. Is that right, Johannes? Do you still want to present? It depends on the stamina of the audience.

DR. KHAN: Can we have the first slide, please?

DR. PEDEN: Press it.

DR. KHAN: Oh, Dr. Khan.

DR. PEDEN: It's K-h-a-n.

DR. KHAN: Although my talk was to make it very short, but I think it's going to get longer than I --
DR. PEDEN: It's the one with two people

in it, Peden and Khan.

DR. KHAN: I just wanted to give a brief
presentation of our recent work on protein analysis of
chemically induced k BALB 3T3 cells and to present
this as a potential strategy for the protection of
infectious retroviruses in vaccine cell substrates.

Okay. The k BALB 3T3 cells are derive
from BALB 3T3 cells. These are Christian Maloney
sarcoma virus transformed. BALB 3T3 cells are non-
producers, and the reason that I selected this
particular cell line is that extensive work has been
done on this cell line actually by Stu Aaronson's
group on the NIH campus in the '70s, and the
endogenous retroviruses that can be induced from this
cell line have been well characterized biologically.

I should also mention that in terms of
retrovirus induction, extensive studies have been done
on mouse cells, and most of the studies have been done
by labs on the NIH campus in the early '70s, and in
terms of optimizing conditions for other cells, very
little, as far as I know, has been done, and basically
the conditions that have been optimized and determined
for the mouse cells in most cases are just directly
applied to other cell lines from other species.
The purpose of initiating this work was to optimize -- to understand the kinetics of retrovirus induction in this well characterized cell line, and then to extrapolate to see whether the results from this study can be used to investigate other vaccines, potential vaccine cell substrates from other species.

In terms of the amount of the BALB 3T3 cells, it's known that these cells contain or con produce retroviral particles of three types. One is the IAP, which are the defective Type A particles which are present in numerous copies in the mouse genome, and these are defective sequences that are associated with internal Type A particles. In addition, there are two proviruses that can be induced as infectious virus from these cells. They have been designated as BALB virus Type 1 -- BALB Virus 1, which is an entropic, ecotropic Type C virus, and BALB Virus 2, which is a xenotropic virus based upon infection on rat kidney cells, which is also a Type C virus.

So these two viruses, Virus 1 and Virus 2 have previously been shown to be induced from these cells.

So what we wanted to do was to revisit these induction studies, applying the highly sensitive
PERT assays to study the kinetics of retrovirus induction from the k BALB cells.

There has been extensive work to investigate a variety of agents in terms of their ineducability in mouse cells and in some cases even in avian cells.

Yeah, it's from your book, John. It's your table, and you're right here. Good. Any comments, direct it to Dr. Coffin, please.

(Laughter.)

As you can see, there's a variety of agents that can induce retroviruses, chemical agents, especially halogenated pyrimidines, IBU and BRDU, also other mutagens, and a bit of the protein synthesis, neurological agents, and even viruses can induce retroviruses.

So the reason I've listed this is just so that one should be aware that during the production of a vaccine, you know, there may be a concern that under certain circumstances the use of certain agents that may be useful for producing a high vaccine virus titre may inadvertently also be activating a retrovirus.

And, therefore, it is very important to be able to detect very early on if the cell substrate has a potential infectious retrovirus using sensitive
techniques.

We investigated using IDU since it is a potent retrovirus inducer, and extensive studies have been done with this, some of them.

In this study, the k BALB 3T3 cells were exposed to various concentrate -- to 20 microgram or 30 micrograms of IDU at 24 hours or at 48 hours, and the control cells -- this is using the taq man PERT assay that Dr. Peden has described earlier.

The control cells were negative in the assay.

As you can see, the 30 micrograms produced high RT activity early on. This is day two time point. This is day four, and this is day six here. So early on the high dose, even the 24 hour post induction resulted in high RT production, whereas the lower dose at either 48 hours or even 24 hours, did produce low levels, but they were not detectable at the later time points.

On further culturing, we saw another P come up later on on day 17 with the higher dose and was not detectable by the PERT assay with the lower dose.

I should mention that these two peaks, an early peak and a later peak, has previously been
identified, induced from these cells using co-
cultivation and marker rescue experiments. So we
confirmed that we could, you know, repeat these
studies, and using the PERT assay.

However, the additional data is that the
higher dose was necessary to be able to pick up the
virus early on.

I should also mention that in the earlier
studies done by Joe Anderson's group they were able to
also detect RT using the traditional assay. However,
they had to use samples that were 100-fold
concentrated, whereas in our case this represents one
microliter from a T-75 flask. So this demonstrates
the sensitivity of the assay.

And in this case, the supernatant was
harvested daily up to seven days, and then after every
three days.

To further investigate the particles that
were induced, we also analyzed some of the time points
by EM. This is the untreated sample. At day two
there is your typical Type A particle, which is at low
levels in our induced cells.

At 20 micrograms, even at 48 hours at day
five the only particles that were detected by EM were
the A particles. We did not detect any C particles,
although we know that C particles are present at this concentration because we did detect it by the PERT assay. The As would not be released into the medium. So we would only be detecting exogenous C. So this is just the sensitivity of the assay.

With the 30 micrograms at 24 hours at day two, we saw the As. In addition, we could see a few Cs being produced, as you see this one budding out, but there were very few Cs at 24 hours, whereas it was increased at 48 hours.

However, I want to mention that the higher concentration and the long time of treatment, as you can see, there was cell toxicity. So, however, to assess that the RT that we were detecting was due to retroviral particles and not due to cellular lysis, we also have tested lipid in the PERT assay, and the RT levels that were detected at this time point were threefold above that of cellular background, concentrated cell lysis. So this RT -- this doesn't go back? -- anyway, so the RT that we were picking up were associated with the Type C particles and not cell lysis.

Okay. So in summary, spontaneous release of virus was not detected in the untreated k BALB 3T3 cells. However, it has been shown that under, I
guess, certain culture conditions this can occur.

However, we were careful not to overgrow the cells or stress the cells to avoid that.

Induction of endogenous Type C retrovirus was dose dependent, and we found that evaluation of several early and later time points was necessary to completely analyze all of the inducible retroviruses.

And in conclusion, I would just like to propose this strategy for consideration for detection of endogenous infectious retroviruses in cell substrates used for production of biologics.

The first step would be to optimize the induction conditions, which would be to identify and use a potent general retrovirus inducer or it may be more than one, I think, depending on your cell substrate and what you might expect from it.

To determine the optimum dose and exposure time, you want to achieve high viral induction with low cell toxicity.

Then you want to combine this with sensitive detection of retroviruses from induced cells. The PERT assay is very useful in this aspects, and you would want to test several early and late time points.

And of course, if you have a positive PERT
result, it then would be necessary to further identify
the source of the activity by EM or by co-cultivation
to see whether you have an infectious retrovirus.

I would just end with that.

(Applause.)

DR. PEDEN: Thank you, Arifa.

Any short questions?

Dr. Coffin.

DR. COFFIN: I would suggest adding to
this some attempt to characterize the genome of the
virus that's induced. Do you know what you're
actually seeing from the BALB C cells? I mean, there
are 60 or so proviruses that might give rise to this
activity.

DR. KHAN: Well, the BALB C cells, I
think, is very well characterized because, you know,
the two proviral genomes that give rise to these
viruses, I think, are known in terms of genetic --

DR. COFFIN: Those are the infectious
ones.

DR. KHAN: Yes.

DR. COFFIN: But the particles that you're
seeing may actually be coming from any of a number of
other ones as well. It would be of some interest
actually to look at the envelope genes and see what's
going on.

DR. KHAN: As you know, we've been both
down this path in terms of, you know -- I think for a
long time -- in terms of trying to characterize all of
the endogenous retroviral sequences, and I think the
majority of them are defective, and the question
actually, a broad question I get with your -- which
you could present is that, you know, in terms of RT
activity or in terms of particles that are produced,
what is the real public health concern, and I think,
you know, would it be a defective particle? Would
it --

DR. COFFIN: We don't know that. I mean
to be fair, we don't know that for most of the
endogenous C type viruses that you can see in mouse.
We know that you don't see whole virus that
corresponds to most of those, but we don't know that
they're defective --

DR. KHAN: No, I agree.

DR. COFFIN: -- in any real way.

DR. KHAN: So in this case, the next step
that we need to do was to do infectivity studies to
see whether those peaks correspond to any infectious
virus, and the reason I did not -- you know, I went
ahead and presented this data is that for this
particular cell line, these two time points, day three
and day 20, are associated with infectious virus. The
early time point is with the phenotype, BALB Virus 2,
and then they have infectious BALB Virus 1, endotropic
virus.

But in general, for a novel cell
substrate, if you had RT activity, the next would be
to see whether there's infectious virus.

DR. COFFIN: But that echovirus has to be
the product of recombination or mutation because the
proviruses themselves are defective.

DR. KHAN: Right. That's why you see it
over a long period of time. So I think --

DR. COFFIN: There must be other -- there
may well be other defective things inducing that might
contribute biologically to the final product.

DR. KHAN: Right. You would propose that
once you have RT activity, then you need to follow it
up with infectivity experiments? Yes?

DR. COFFIN: Well, I would do some PCR and
see what sort of sequences you get in there. It's
fairly simple with oligo probes and so on to just the
PCR amplify envelope genes and see what's being made.

DR. KHAN: Right. You can do it, and
then, you know, wraps it and stuff, yeah.
DR. PEDEN: Okay. The next speaker is

Damian Purcell.

Is Purcell's carousel in the -- it is.

Thank you.

DR. PURCELL: Okay. This is going to be fairly fast since half the slides have been presented and duplicates in some ways a story we're already heard from Ruth Ruprecht with slide differencing in some of the outcomes which I'll show you.

So what we've done here, as Ruth did, we evaluated the logistical problems of delivering a live attenuated HIV vaccine strain, and we saw that this would be difficult with the virus strain in our minds being at the time a strain of HIV that emerged in Sidney, Australia, know as the Sidney Blood Bank Cohort, which is essentially a nef deleted virus, which for a long time appeared to be attenuated and not causing disease in patients, but just recently some of the cohort of that group of patient have begun to lose immune function. So it's probably following the same story as the simian cases that progressed to full pathology.

Anyway, we saw that the possibility of having to culture the isolate of this virus strain that we had in the lab by necessity in PBMC, we
thought that this would be a problem rather than an opportunity as other people have found, and the necessity to produce large lots even if we could find a human cell line would cause problems in terms of the approval of the vaccine, a live attenuated vaccine.

So we figured that the best way of dealing with these issues would be to deliver a truly attenuated virus, if this was one, as provirus DNA.

So we thought to model this in the SIV system, and we took the sequence of the Sidney Blood Bank Cohort patients and identified the minimal deletion, which is a region in U3, and the patients had other deletions in and around nef and NU3, but we chose to take the minimum region of the deletion.

We made an alignment against the SIV MAC 239, which through this region is not a very strong alignment, and we made a deletion that sort of represented roughly the maximum amount of sequence deleted out of the Sidney Blood Bank viruses, and just as a comparison against the equivalent deletion, which I guess we're more familiar with, the second of the two deletions in SIV made by Ron Desrosiers. It sort of accommodates half of that second deletion.

Now, we made two forms of this proviral strain, one bearing the deletion in just the three
prime end, which we call SIV SBBC Delta 3. So the

deletion is 105 base pairs, and after some coercing
with the provirus, we managed to make the double
deleted form with the deletion also in the five prime
LTR with the provirus.

We're unsure when administering these
proviral constructs into the animals whether it would
be necessary to retain the five prime U3 promoter
function, but even with the deletion in the three
prime end, as we've heard in the meeting earlier, RNA
transcription commences at the R region, so effective
excising the first of the U3 region in this singly
deleted form, so that after one round of replication
of the virus, there's a duplication of the deletion,
and all progeny virus then will have the double
deletion after one round of replication.

So our design was to administer plasmid
DNA into eight juvenile macaque Nemestrinas. We've
already done one titration point. Compared to Ruth
Ruprecht here we're down. She used 500 micrograms.
We used 300 micrograms IM.

We've also taken the gene gun and
administering 15 micrograms with wild type SIV and
with the two different attenuated strains, the Delta
3; so that's with the just three prime deletion in U3.
We did an IM 300 microgram injection of this one, and with the double deleted version, we loaded this up on the gold beads and administered 15 micrograms of DNA by gene gun.

So the wild type delivery in each case was successful. What we're looking at is plasma RNA.

Three of the animals -- well, this one actually died at ten weeks. This one was euthanized here with very low CD-4 counts, as you'll see, and the other one also was euthanized with very low CD-4s.

But what I want to draw your attention to is with the singly deleted form of the construct that we administered, one of these two animals or both of them scored high virus loads, but then by between five and ten weeks one of these animals, Monkey 16, had a high level of virus coming back in the plasma.

The time points we have on the double deleted virus were short. They haven't had this virus in the monkeys as long, but it dwindles down very rapidly. It's certainly detectable, to begin with.

Just, again, looking at the CD-4 counts in the wild type monkeys, they lose their CD-4 very rapidly. So it's a very efficient way of administering virus, just as Ruth Ruprecht showed.

And this Monkey 16, as well as retaining
high viral load, has begun to lose CD-4 cells.

So just following up, the changes to the sequence in the two monkeys, this is the one that does not regain high viral load nor lose CD-4 counts. This is the kind of changes that we commonly see when you administer genuine virus with deletion. You see extra deletions within the nef to increase fitness, little duplications of sequence.

But at week five we did find a wholesale duplication of 63 base pairs from the wild type part of U3 into this, but it was lost through time, but the other monkey, Monkey 16, by the third week we found a complete replacement of the U3 here in a small fraction of the clones, and as time went by, all of the clones had reverted to wild type.

So the question is: how did this event occur? It's a different form of reverse to what we typically see, and we strongly suspect the presence of the complete U3 within the DNA, the plasmid that was administered to the animals as a bolus of 300 micrograms.

So just in conclusion, wild type SIV DNA is certainly pathogenic. As low as 15 micrograms of DNA administered by gene gun is also very efficient even with the attenuated double deleted form of virus.
Probably the most interesting thing is that the evidence of the reversion in one of the animals implicates the DNA as a potential source of recombination with the virus. And I'll leave it at that.

(Applause.)

DR. PEDEN: Thank you, Damian. Any brief questions, comments?

DR. PATIENCE: Damian, thanks a lot.

DR. PEDEN: Clive Patience.


I don't know if I saw it through a haze. It's getting late for me, but did you see quite a severe dropoff in the intradermal administration of your variants after one week in the CD-4 count? I think it was just in the intradermal if --

DR. PURCELL: Yes.

DR. PATIENCE: And if so, if I did read it right, do you have any explanation why the CD-4 count went down about fourfold, I think it was?

DR. PURCELL: It was probably the way -- this was not numbered, the CD-4s. We were measuring the percent of CD-4s. So it's a percent of the pre-
inoculation percentage, if you like. So rather than

counting total CD-4 numbers, we're looking -- we run
the samples through the fax (phonetic), and we look at
the ratio of CD-4s at the time point that we examine
and compare that back to the pre-inoculation time

point.

So it's really looking at the ratio more

than the actual numbers.

DR. PATIENCE: Okay. Do the real numbers
follow the same sort of pattern as well or is it
disturbance of the ratio of your CD-4 --

DR. PURCELL: At the time we started this,

we didn't have the technology to count the numbers in
the primates, but we'll have to look at that in the
next set of animals, yeah.

DR. PATIENCE: Thanks.

DR. BERKHOUT: I guess the --

DR. PEDEN: Berkhout.

DR. BERKHOUT: Ben Berkhout, yeah.

The unusual recombination event that you
see may still have occurred through RNA, although, you
know, I guess there are two ways. One is that perhaps

the transcription is fired from your plasmids starting
at the three prime LTR, and of course, you can also
have transcripts that are started at the five prime
LTR, but you get read through transcription.

DR. PURCELL: The possibility that we're initiating transcription from here and reading through an RNA transcript to here, which would then be terminated, polyadenylated without requiring an action.

DR. BERKHOUT: But if polyadenylation is not efficient then --

DR. PURCELL: True, and the other probably more likely possibility is a read through the polyadenylation site here down to this, the second one that appears, making it, you know, quite a long RNA, which might be code packaged.

So we haven't completely excluded it. We need a second experiment, I think, but certainly the possibility is there, and I think that the selective pressure we're picking up, that piece of DNA would be greater than any other cellular DNA.

DR. PEDEN: All right. Thank you, David.

I think Johannes wanted -- do you still want to speak?

DR. LOEWER: I would, but I guess --

DR. PEDEN: Did you turn off the machine? So if the audience is able to take one more talk.
DR. LOEWER: What I wanted to do is to present very quickly the most recent experiments we have performed to understand and moditate (phonetic) molecular biology of the human endogenous retrovirus families, K family. At least to my knowledge, it's the only retrovirus, human endogenous retrovirus family with codes for all structural viral proteins.

These proteins are expressed with the zone (phonetic) virus family, which is able to form wider particles which you can see here on the micrograph. This virus is expressed mainly in terata carcinoma cell lines, as I've shown this afternoon. This is a Northern Blot describing as full length and singly spliced N message in addition to these four messages. We see also two. There is more messages, which is very unusual for endogenous retrovirus. This resembles the more complex retroviruses. Analysis shows that one of the smaller length RNAs is doubly spliced, encodes for a small protein, which is located in this area which was called because of this localization central open reading frame.

And this protein resembles to some extent the left protein from HIV or the next protein from
HIV-1 in the sense that it has nuclear localization signal, as well as a domain which resembles the (unintelligible).

And the question we ask, of course, is that if this is a functional protein which enhances or allows the export of full length simply spliced RNAs, to study this we used a system developed by Barbara Felmer and Charles Papalakus. It's a construct which expresses HIV only if there is a responsive element like the RRE and if ref or a corresponding protein is provided in (unintelligible), we have replaced RRE by different parts of the three prime part of the K genome and have also provided the C orf protein in trans.

The HIV expression was first -- or what I have to say, we have checked here several pieces, and what turned out is that there is a responsive element. It's a three prime LTR which we called RcRE, analogy to our XIA from HIV-1 because it has very similar localization.

And this is shown by more fluorescence data here, for example, only in the presence of what we call RcRE HIV that is produced with other parts of the nonresponding fragments. You can only see as a C orf protein localized in the nuclei.
This can be quantified by P24 antigen measurement. Here is RRE, the HIV system, and if raf is present at RRE, you get a high expression of P24, and the same is true if you have the responsive element for C orf and C orf is present, but raf can also go up to some extent on RcRE, but C orf cannot work on RRE and other parts of the genome are not active.

Next, we looked at the different domains. We mutate nuclear localization signal and also mutated the positive nuclear export signal, and we check the shortened -- if the C terminal shortened, C orf would also be active. In this case, all this construct, they were used to keep lost protein (phonetic).

And these are the effects of the different mutations. This is wild type. This is C orf localized in the nuclei, and P24 in the cytoplasm with mutation of the nuclear localization signal. The C orf cannot enter the nucleus. It's found only in cytoplasm, and there's a mutation of the nuclear export signal as you can expect. The mutant is localized in the nuclei as well as the glial line (phonetic), but no P24 is formed. The same is true with the one which is a protein which is truncated.

Shortly, the next question of course is
whether the same export pathway is used for HIV. HIV uses the CREM pathway, and this can be blocked by a type called leptomycin B, and this is also active in the RcRE orf system, for example, in the presence of six nanomolar leptomycin, no P24. In this case it's the major core protein of rev K itself, is formed, no N protein in this construct, but C orf is still produced and present in the nuclei and in the cytoplasm.

So in summary the endogenous retrovirus family, we have K codes for protein which is equivalent to ref and rex. That means that the nuclear export pathway was not only detected by reasoned lentiviruses or reasoned HIV-1 BLV viruses, but already 30 million years ago when this family most probably was active.

What also makes this family interesting, observations by Nicholas over lunch and his group in Germany, he has indications that this virus may be orf'ed in oncogenesis and only in situation where C orf is also present.

So the next study in the end, to find out whether this look like nuclear export protein may play a role without the conditions in carcinogenesis.

Thank you.
(Applause.)

DR. PEDEN: Thank you, Johannes.

I think because time is very short and we have to get out of the room I'd like to express my gratitude to the audience for staying so late, and especially to the speakers.

So I think we should terminate this evening and be back tomorrow.

Thank you.

(Whereupon, at 9:55 p.m., the meeting was adjourned, to reconvene on Friday, September 10, 1999.)
The workshop took place in the Plaza Ballroom, DoubleTree Hotel, 1750 Rockville Pike,
Rockville, MD, 20852, at 8:00 a.m., Regina Rabinovich, M.D. and Martin Myers, M.D., Session Chairs, presiding.
PRESENT:

Regina Rabinovich, M.D.       Session Chair
Martin Myers, M.D.            Session Chair
David Onions, Ph.D.           Panel Chair
John Coffin, Ph.D.            Panel Chair
Philip Minor, Ph.D.           Speaker
James Robertson, Ph.D.         Speaker
Joerg Schuepbach, M.D.        Speaker

Jens Mayer, Ph.D.             Speaker
Thomas Broker, Ph.D.          Speaker
Neil Cashman, M.D.            Speaker
John Sedivy, Ph.D.            Speaker
Frits Fallaux, Ph.D.          Speaker
Michael Fried, Ph.D.          Panelist
Stephen Hughes, Ph.D.         Panelist

Johannes Loewer, M.D.         Panelist

Also Present:

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CHAIRPERSON RABINOVICH: Good morning. I would like to welcome you back to Session 6, Adventitious Viral Agents in Cell Substrates, and congratulate all those that were here until 10:00 last night for the latest part of the show, including Mr. Harris, who put in a grueling 18-hour day in yesterday, our visual aides person.

I would like to introduce the first speaker, Dr. Phil Minor, from the National Institute of Biological Standards and Control, who will be giving us an introduction to adventitious agent issues, both reviewing the past and current experience with adventitious agent contamination of biologicals in vaccines.

DR. MINOR: Thanks. Thanks very much. Can I have the slide on, please, or do I just press it here?

What I am going to do is to review firstly all of biologicals, if you like, from an adventitious agent point of view. So it won't just be vaccines. In particular, I will be talking about the range of source materials that people have used in preparing biologicals. There will be a clear message that comes
out of that, which is that the more you use well-
characterized cells, the better.

I will also be talking about the SV40
story in some detail, which has been gone through a
number of times, but I will be going through it from
a particularly regulatory point of view because again,
there is a message there which says that if you get it
wrong, you will still be working on it 40 years later.

Finally, I will get onto the continuous
cell line bit right to the very end.

So there are a variety of source materials
that you can use if you are preparing biologicals.

They are sort of listed here, if you like. There are
biological materials which are made from whole
animals. That would include things like blood and
blood products. I will describe that in a moment. So
you can just go to a whole animal and take something
out and make your biological from that.

You can use your whole animal as a
substrate for growth. I will discuss that in the
context of things like influenza vaccines and the
like.

You can grow material on primary cells.
This was the main starting point for things like polio
vaccines in the early days, where the SV40 issue
arose. Finally, you can grow materials on well-characterized cell preparation.

The further down the list you go on this thing, probably the happier you are from the adventitious agent point of view.

This shows some examples of contaminants which have arisen when whole animals have been used as source materials or the origin of the source material. Most of these will be human rather than anything else, but really an awful lot of the serious adventitious agent problems that have arisen have arisen because of materials sourced from whole animals or using pooled preparation.

The first one on this list here is CJD, Creutzfeldt Jakob Disease, which was transmitted by growth hormone. The growth hormone was produced from human cadaveric material. A very unpleasant disease. It's almost impossible to detect the agent other than by standing back and waiting for the incubation period to go.

In France, there are still a large number of cases coming through as a result of this. It may well be that around 10 percent are recipients of human-derived growth hormone, will actually wind up going down with CJD in France.
Dura mater is another one. That should be one T, not two Ts. Again, this has been shown to transmit really quite readily when pooled materials are actually used. Almost impossible to detect. Clear, very, very serious kind of consequence of it.

Scrapie was first shown to be a transmissible agent by the use of a TBE vaccine, which was grown in the brains of sheep. TBE being tick-borne encephalitis, which then transmitted scrapie to a large number of the sheep that were actually inoculated with it. So again, this is a whole animal source material, if you like, that had quite serious consequences, especially if you were a sheep.

Over the last 15 or 20 years or so, one of the best examples of serious or disease-causing transmissions of infectious agents has been through human blood and blood-derived materials, clotting factors in particular. In all of these things, the entire alphabet soup of hepatitis viruses has been transmitted by blood product.

In the early days at least, some of these were really regarded as really a hazard, if you like, of being a hemophiliac. So, for example, hepatitis C, in the days when there was a non-A, non-B hepatitis, it was really regarded as an inevitable consequence of
using factor 8 to treat hemophilia. I am not sure that that is an acceptable way of actually doing things any more. I am sure that hemophiliacs would agree with that. B-19, paravirus B-19 is still transmitted by clotting factors.

Finally, this one down at the bottom here is a classic example of a transmission by a vaccine, if you like, where hepatitis B was transmitted by yellow fever vaccine back in the 1940s. The hepatitis B actually came from the stabilizers of the albumin that was actually put in there to keep it stable.

There is a story that Fred McCallum, who is head of the Public Health Service in the United Kingdom tells to the effect that he basically won the war because he prevented Winston Churchill having a yellow fever vaccine when he was going off to talk with Stalin around 1944.

So most of the serious consequences really come from whole animal source materials, if you like.

You can use whole animals as substrates. I'm using the term "whole animals" in a fairly broad sense. Eggs in the definition of the Animal Regulated Use Act in the United Kingdom count as an animal because they are embryonated.

For many years, rabies vaccines were
produced in mouse brain or sheep brain. They have quite serious consequences, but not necessarily associated with adventitial agents. You can get encephalitis as a result of immune responses to the non-invasive protein.

The Japanese encephalitis vaccine, which is used for travelers in the United Kingdom, is still made in mouse brain. So it's not an unusual source of material, if you like. Smallpox for a long time was made on the scarified flanks of calves. Like I said, isn't any more. However, while these things seem really quite primitive, in terms of how you make vaccines nowadays, you still have a number of vaccines that are made in eggs. Yellow fever is the classic example, and influenza.

Yellow fever is not required to be grown in avian leukosis-free eggs. The reason for that is that there are a number of sites at which it was manufactured throughout the world, where yellow fever is a really very serious problem, such as Nigeria, for example, South America, whatever, where SPF eggs, avian leukosis-free eggs even, were really not freely available. So yellow fever can in principle at least be made in avian leukosis containing eggs, and in fact is. I think there's no evidence that this has an
adverse consequence. But on the other hand, you
wouldn't necessarily want to have a virus in there
that you didn't know about.

Influenza is an actuated vaccine. Again, it's not made on SPF eggs, that is, specified
pathogen-free eggs. They are avian leukemia free, but they are not free of all the other variety of
pathogens that you would choose to screen for measles vaccine production system, for example.

So even today then you have to bear in mind that a large amount of vaccine that's made is
made on really quite crude materials, from an adventitious agent point of view. It's not a trivial
usage. In fact, when you go through and consider what vaccines are actually made on these days, they are
quite primitive, if you like, in some respects.

Primary cultures as been described previously around here, are really cultures that are made directly from the animal. So they are not one
pass. They are directly from the animal, if you like.

Here are a number of examples where agents are actually being found or at least located in these kind
of cultures.

SV40 is one that I'm going to talk about in some detail in a minute. This was in polio
vaccines in the 1950s and very early 1960s, probably,
a source from rhesus monkey kidney. Polio vaccines
are still made on monkey kidney, though they are not
usually on rhesus monkey kidney. It would be
cynomologous or something like that, for reasons which
I'll describe in a moment.
Nonetheless, a great deal of vaccine is
still made in primary monkey kidney cells. There are
reasons for that. There's a deep conservatism I think
about changing the vaccine production process if you
have a vaccine that works, largely because you are
dealing with a prophylactic material rather than a
therapeutic material. So you don't want to mess about
with anything if it's reasonably safe and effective.
I'll mention very briefly the defective
retrovirus story in chick embryos. I think Jim
Robertson will probably mention this in more detail,
but I will mention that just as I go by. Finally
recently, the FDA released a talk paper on a
preparation of urokinase, which is used in treating
the heart. This material was grown from primary
cultures made from aborted fetuses. I think it was
aborted fetuses or miscarriages, or whatever. There
were quite a variety of infectious agents were
actually found in this. I believe this one has now
been suspended.

The point is that there are still a large number of materials which are made on really quite basic culture systems, if you like, where adventitious agents are a serious consideration, if you like. So it's not all continuous cell lines versus the rest. I mean there are -- most of the vaccines that are made in the world probably come from other primary cultures or eggs or things of that nature.

I will now talk about SV40. I'm sure in this audience there are people who know far more about SV40 than I do. But nonetheless, I'll talk about this from what you might call the regulatory adventitious agent point of view, if you like.

So it's a very common polyoma virus of old world monkeys, and particularly rhesus macaques. The difficulty with this was that when the rhesus macaque monkeys are sacrificed and a primary monkey kidney culture is made from him or her, as the case may be, a silent infection is set up. So there is on evidence of infection just by looking at the cultures. In fact, these cultures can throw out as much SV40 as they do polio, when you start infecting it with polio. So you wind up with a culture that's just stiff with adventitious agent which you really don't want.
It's able to transform non simian cells in vitro, and it can be tumorigenic if you have the right kind of animal that you put it into. Between 55 and 62, probably at least a third of all the vaccines that were made on these kind of cultures, because they were pooled and the like, were almost certainly contaminated with SV40. It wasn't a trivial contamination. It was really quite a serious contamination.

Because it was mainly an activated polio vaccine, there wouldn't have been that much live SV40 in it perhaps. But SV40 is more resistant to formalin than polio is. So almost everybody who received the shot of inactivated polio in the 1950s, which would include me, would have received live SV40 in some form or another.

So the concern is really summarized here, which is basically that everybody, I mean this is my own take on it, that everybody -- I mean you can argue that it might not have been sort of everybody, but I think it probably was. But almost everybody who received the full course of polio vaccine between 1955 and 1965, also got live SV40 stuck into them. That's millions of people basically.

There were epidemiological studies that
were done at the time which really didn't cause much

concern, but they can all be criticized. Some of the

studies were really quite short-term, about two or

three years or so, looking to see if there were cancer

effects basically, as a result of SV40. It may be

that two or three years is not enough to actually find

such an effect, if it actually exists.

The longest which was assumed was over a

period of about 19 years. Most of the individuals

involved in that study would have been oral polio

vaccine recipients rather than inactivated polio

vaccine recipients. So they have had it by mouth

rather than by injection. Again, you could argue that

that might not be the right cohort to actually be

looking at.

So while the studies were reassuring, the

most reassuring thing was that there was no sudden

surge of cancers that you can actually trace back to

polio vaccine usage in the United States or in Europe

where these things were used in a big way. So it

really did seem that in the long term, over about 19

to 20 years, there was no real cause for alarm.

However, in 1992, Michaili Carboni and

colleagues and others, a number of others, including

Janet Butelle down in Texas and the like, identified
SV40 sequences which were present in a variety of relatively rare tumors. So mysathelia, which is the asbestos tumor, osteosarcomas, pendymonas, actually the young chorioid plexus tumors of children, these sequences do appear to be genuine SV40 sequences.

Where they come from is really not quite clear. Part of the argument was that you could get similar types of tumors in experimental animals, like hamsters. I think that is probably the only example where a hamster is cited as a good model for a human being perhaps. But who knows? In fact, this might actually be an argument that this has got nothing to do with it.

So the question then arises as to where did the SV40 sequences come from. Of course the classic response really would have been it must have come from the polio vaccine because why not?

Now SV40 was discovered around 1961 or 1962 or thereabouts, 1960 perhaps. Directly it was discouraged. There were precautions put in place to exclude it from polio vaccines, because it was known to be a tumor kind of virus, if you like. These were the kind of things that were put in place. They are listed in WHO requirements from about 1962 onwards. They reached their final fully flowered form, if you
like, by about 1965. A number of countries certainly
had put this in place before that.

The first thing you can do is to use
seronegative animals as the source of cells. So you
can use animals that have no evidence of SV40
infection as your source. That really is something
which is now very firmly in place, which manufacturers
now do.

The second thing is, you remember I said
that it was the rhesus macaques with the problems.
The problem was that the cell cultures didn't show any
sign of having defect, when they were actually
infected with SV40. What you can do then is you can
use species, such as cynomolgus or pattus monkeys,
where the primary monkey kidney culture cell, when
infected with SV40, will actually wrinkle up and die
on you. So at least you know you have got something
nasty and you can throw it away.

Finally, you can test your control of
production cultures for SV40 by the same kind of
procedure. That is why using sesetral cells to see if
anything comes through.

Around the period that this was taking
place, wild caught monkeys were being used extensively
in vaccine production. Up to a half of the cultures
would have been thrown away because of adventitious
agent contamination, mainly foamy virus, but certainly
other things as well. I think that just illustrates
the kind of lack of control, if you like, over the
source materials that was going on, and the extent to
which adventitious agents are really a serious problem
in finding monkey kidney cultures or primary cultures
in general.

An alternative way of doing this is to
actually use a validated cell bank. Certainly many
manufacturers use MRC5, and Mary of course used vira
cells, as we heard last night.

Nonetheless, a significant, if indeed not
a large proportion of the world's supply of polio
vaccine is still made on primary monkey kidney cells,
which should really fit this kind of criteria for
excluding SV40.

One of the questions that then arises is
were these precautions good enough? What we did at
NIBSC, because we happened to have about 150-odd
batches of vaccine archived from the years, was to go
back and look at them by PCR. PCR of course is the
cat's pajamas. It's really the best technique that
anybody ever invented in terms of sensitivity. It's
probably about as good as infectivity, at least in our
hands anyway.

But nonetheless, we went back and we looked by PCR at 133 preparations of polio vaccine which had been used in the United Kingdom between 1966 and about 1997. What we had done was looked at all batches of vaccine which had been used since 1980, and all of those were free of SV40 sequences. So that gives you some reassurance that these precautions were actually appropriate.

In fact, the only preparation which had any SV40 sequence in it at all was a seed virus which was used by a manufacturer for making vaccine from. The amount that was in there was around two logs worth of genome as opposed to seven logs of genomes in a really full-fledged infected preparation. So there wasn't that much in there. The manufacturer had also treated this stuff with toluidine blue, which is supposed to kill of SV40. This was done on the advice of Albert Sabin back in 1960-something or other.

But nonetheless, it does seem to me that it's rather a foolish thing to have a seed that's got SV40 sequences in it at all. I think the WHO requirements have now been changed so the seed has to be checked to see if it does have SV40 sequences in it or not.
This particular seed was not infectious SV40. We did some quite serious studies on it, like transvecting the DNA into cells to see if it would work, infecting monkeys with it to see if we could actually get seroconversion. There was no seroconversion. So there was no infectious virus there that we could actually detect. But nonetheless, the seed did have material in it.

If on the other hand you look at materials from around the 1960s or from other parts of the world a little bit later than that, you can pick up SV40 sequences quite easily. So the method would have picked it up had it been there.

So our conclusion from that was then that really as soon as these kind of precautions were put in place, no SV40 would have been present in all polio vaccines used, at least in the United Kingdom and I would guess in the United States as well, because it's after the same kind of precautions were put in place.

So the precautions were adequate. Which means that SV40 exposure of the population through polio vaccines would have stopped around 1962.

So what you then have is the problem of the chorioid plexus and appendinoma tumors, which occur in children who are around two years of age or
maybe less. You have to say well how did they get a
hold of the SV40 sequences? One possibility, which is
mooted with some enthusiasm is that maybe you are
getting passage of SV40 from parents who did receive
the SV40 contaminated polio vaccine to their children.
So how this stuff gets around is quite important.
One of the things that we have been
involved in is doing serological surveys of
populations to see who has got SV40 antibodies and who
hasn't. It is about a five percent seropositivity by
the assay that we're using at least. It seems to peak
at around age 10 or thereabouts, and doesn't arise
after that.
So what you could argue then is that you
are seeing vertical transmission from parents down to
their children. What you could also argue is that you
are not picking up SV40 specific antibodies at all,
and they could be other human polyomas like the BK or
the JC, and it's cross-reacting antibodies that we're
picking up. I think that is still a thing that needs
to be resolved. This is how we were trying to resolve
it.
We have access to a number of sinomorgous
breeding colonies. One of them at least is absolutely
riddled with SV40. It's chronically infected. They
are all infected basically.

So this is just four examples of this particular colony. There's about another 50 or so. This happens all the time. The mothers here are highly sera positive to SV40, all of them. What happens is that the mother and the baby stay together for about six months until the baby is weaned. Then the babies are taken off, no longer being babies of course. They are all banged up together in one gigantic sort of teenage squabbling colony.

At the time of weaning, the babies are uniformly negative. So despite the fact they have been on the mother for six months, they have not sera converted to SV40. Almost immediately you bang them up together, or at least within about a month or so, they sera convert. So we actually have a sera conversion panel here, if you like, with about 50 or 100 or so sera, where the babies actually were seronegative and then become seropositive.

My view on this is probably that the babies don't get infected until you bang them up together. But it may be that they are infected, but they are just not seropositive. So what we have to do here is to fish out the virus from these animals here, and see if it looks like the mother's virus or if it
looks like the other babies' virus.

The point about this long story which I have just been telling you about SV40 is that SV40 was a problem between 1955 and 1962, and it's now 1999, and we still don't really know what was going on. So if you actually make a mistake, it's really quite serious. It may keep you occupied for the rest of your working life.

One last quick thing or two last slides here. One is about reverse transcriptase of vaccines. Dr. Schuepbach will be talking later and Jim Robertson will be talking in a moment about detection of reverse transcriptase in chicken cell grown vaccines, such as flu or yellow fever or measles, mumps, rubella.

This appears to be due to the presence of defective non-infectious particles. There are sequences from EAV and ALV both in these things, a ratio of about nine to one as I understand it. It does seem to me that you are not really quite sure what the AV sequence is in there and what ALV sequence is in there. It's probably going to vary from chicken to chicken in so far as these chickens have not been bred. In other words, every egg is a new experiment. You are really not quite sure what you are dealing with in that. I think that is quite an unfortunate
Finally, this is my last slide, and this has to do with characterized cells. The issues that I have been dealing with really have been to do with primary cells and primary cell problems where the virus comes in direct from the animal origin. I think there is no doubt in my mind that that's the main source of concern in terms of human health.

Nonetheless, there are clearly problems which also arise with characterized cells and the continuous cell lines, in particular. We have some down here.

Now the regulatory authorities in the room will be well aware of a large number of other examples of this type which don't actually get published. I think that's not so good. I think this stuff really should be out there in the public literature. But nonetheless, these are the ones which are well known, I think.

CHO viruses, CHO cells have defective retroviruses. Manufacturers take a great deal of care to actually get rid of them in the final product. So they are endogenous.

There are examples of things like BVDV contaminating cells which are growing in culture, and
also other bovine viruses contaminating cells in
culture, particularly when they are grown on a very
large scale. Whether or not that poses a hazard is
another matter, but clearly there must be methods in
place to actually detect them.

The classic example here was the minute
virus in mice, where the tpa had been grown in CHO
cells on a 10,000 litre stove essentially, and then
tiled up for an effect with minute virus of mice. Now
this was on the order of eight logs, as I understand
it, of virus per mil, and yet a 10,000 litre fermenter
culture. This is probably more minute virus of mice
in one place on the planet than has ever been the case
before. You might want to think how you actually get
rid of it actually.

This is a question of actually getting the
cells infected while they are actually burning in
culture. So while family cells are clearly a major
problem, and while whole animal sources, if you like,
are probably the biggest hazard which is likely to be
raised in terms of human health, biological aspects to
do with well characterized cell banks, where viruses
may be introduced from biological materials or they
may be introduced by mice walking across the top of
the fermenter or whatever, are nonetheless a
significant matter. It really is not totally clear whether these things have an implication for human health. But I think you would be wise to make sure that they are not actually present.

That's where I stop. Thank you.

(Applause.)

CHAIRPERSON RABINOVICH: Please identify yourself.

DR. COFFIN: John Coffin of Tufts. That was a really nice summary actually, Phil. But some caution might be called for in translating the results of vertical transmission experiments from monkeys to humans.

As far as we know, simian immuno deficiency virus in monkey populations are not transmitted vertically. Yet HIV-1 is transmitted with reasonable efficiency vertically in human populations. So there may be some underlying biological difference that perhaps a very subtle one, that promotes this kind of transmission in people, where you wouldn't see it necessarily in monkey models.

DR. MINOR: Yes. I take you point. We are doing the studies for two reasons. Firstly, to look at natural transmission to monkey on the grounds that it might be a model, although I take your point
entirely. But also to supply serum conversion panels so that we can try and sort out specificity of immunological reactions as well. I take your point entirely.

DR. ONIONS: David Onions, Glasgow.

Phil, when people switched to cynomologous monkeys, and I can see the reason because you can pick up SV40. That's very clear. But how do you know that at the same time, you have not invented a new problem, that you have got another polyoma virus in that species that you are not detecting. I mean has anyone done redundant PCR to look?

DR. MINOR: I think I would choose to look at a polyoma man to answer that question. Anybody?

DR. MAJOR: Gene Major, Bethesda. In the monkeys that we have used for the human polyoma virus studies, we have screened all those animals for the conventional SV40, but not have addressed the question of whether or not there's other additional polyoma type viruses that are present there.

Clearly by this time, PCR technology has advanced to the point that perhaps if something were there whose sequences were somewhat similar to the ones that are currently expressed in these animals, we may have picked it up, but we certainly haven't found
anything yet.

DR. LEWIS: Phil, I take it that seroconversion is by neutralizing antibody?

DR. MINOR: Sorry? Say that again.

DR. LEWIS: I take that seroconversion is by neutralization?

DR. MINOR: That's right. Yes.

DR. LEWIS: Have you had any chance to look at monkey breast milk to see how long they may be treating antibodian, so that the newborns can be passive immune?

DR. MINOR: Right. The answer is no. We have discussed, for example, getting the urine out of these monkeys after they are banged up together. I am told that actually chasing them around the floor is insuperable. I'm not sure you can actually pry a baby monkey from her mother long enough to actually milk her. It's a worthwhile question. I think we'll have another go and see if we can do something about it.

There may be some resistance, however.

DR. BROKER: Tom Broker, UAB. I wanted to follow up on that exact question. We are facing the same problem with potential vertical transmission of human papilloma viruses. I'll mention it later in my own presentation, but briefly, it does appear there is
some protective immunity during nursing. On the other hand, removal of an infant, say through adoption to another family, is the highest risk factor for a child acquiring laryngeal papillomatosis later in life. So a two to three year delay.

DR. MINOR: So is the assumption then that the infant is infected, but it's not infected properly then?

DR. BROKER: It's infected vertically, presumably perinatally, perhaps just before or during delivery, but receive sufficient passive immunity by nursing that gives it life long protection.

So what I would propose as a potential experiment is to literally take the, if possible, take the baby monkey immediately away from the mother, and don't allow it to nurse, and then just have different lengths of time of nursing to see if this onset of seroconversion is affected by a timing mechanism. Alternatively, don't ever let that baby monkey be housed with other baby monkeys in the daycare center, and keep it with the mother even if it's not nursing, and see if it fails to seroconvert.

So the question is, is it getting infected from its playmates.

DR. MINOR: That's right.
DR. BROKER: Or is it receiving a period of important passive protection from the mom.

DR. MINOR: I figured we could do that by looking at the actual strain of viruses the monkeys get infected with. We have a number of different gang rooms, if you like. If you get a different strain in each gang room, but it's the same strain within a gang room, then I think that will answer the same question.

You can also go back to the mother and see what kind of strain she's got too. But it's a valid point.

AUDIENCE MEMBER: I would like to reemphasize one of the important points that you made.

I know you didn't have time to expand on it, but I think it is extremely important. That is the need for those organizations who discover a new virus or some contaminant, cell population used for vaccine production or in a production run, to make that publicly known.

I think that the declaration by Genentech, who has published this information under their name, that an NBM contamination occurred in a 10,000 fermenter is an act of great courage. I think that that kind of courage, this declaration by other companies in this field, is very necessary for the health of this industry.
I understand from some of the remarks that have been made that there are others that are known to a small coterie of people here that have not been publicly declared. I urge all of you to think about this seriously because it can and will have a great impact on this industry. Thank you.

DR. MINOR: I agree totally with that. It does seem to me that sooner or later the information will leak out. I think the industry looks very bad.

DR. VAN DER EB: Van der Eb, Leiden. Did I understand it correctly that ferrisfaruses were found in human embryo material that was used for urokinase production?

DR. MINOR: I think the FDA can answer this one better than me, yes. But I mean that was my understanding of it. It's out on the net.

DR. VAN DER EB: But where does it come from?

AUDIENCE MEMBER: I think it's a rea virus.

DR. MINOR: It's various rea viruses, plus others.

DR. VAN DER EB: I see. Okay.

DR. FRIED: Mike Fried. Was any of the old vaccines from the 1960s that were contaminated,
were they PCR'd up to show that the virus was the same as being found today? Because it's also possible that we all have a latent SV40 type virus which likes to grow in tumor cells, and that's why you find it. It's a passenger. But I mean since there's polymorphisms in the sequence, if you can go back to the 1960s and then find out if it's the same thing that we find today, it would be helpful.

DR. MINOR: We looked at, when you say the 1960s, I have to emphasize this is very early 60s. Certainly the things that we have got which came out positive weren't used in the UK, or they might have been used somewhere else.

We had a Russian SV40 and we had an American SV40, and we had an SV40 of unknown origin all from the 1960s, and they were all different basically. They were different from the 7-7-7, you know, the cos kind of sequence as well. So they were all unique basically, in terms of the region we were looking at, which was C terminus of t antigen.

CHAIRPERSON RABINOVICH: Last question please.

MS. MARCUS: Carole Marcus Sequora from Bassey Consulting.

I just wanted to clarify that urokinase is
produced from cells. It's not aborted fetuses. It's newborns who did not survive. Just for the record.

DR. MINOR: Thank you.

MS. MARCUS: It was rea virus.

DR. MINOR: I'm sorry about that.

CHAIRPERSON RABINOVICH: Thank you. Our next speaker is Dr. Jim Robertson, speaking on experiences with retroviruses in avian and mammalian cell substrates.

DR. ROBERTSON: Good morning. For those of you who don't know, NIBSC is CBER's cousin from across the pond in the U.K. What I am going to do is pick up where Phil left off and concentrate on the retrovirus aspect of viral contamination. Initially I will look at say biologicals in general, but ultimately focusing down on the vaccine issues.

So I will begin with some direct information regarding retrovirus situations with biologicals. I will go onto look at how some of the regulatory guidelines deal with the issue of retroviruses. I will go into look at RTase testing, which is a reasonably current them just now, and finish up looking at the recent situation of the finding of retroviral-like particles in avian cells.

So to begin with, here is a short list of
the incidence of retrovirus contamination found in biologicals in general, not just vaccines. I have sub-divided these into two groups here. You see this upper half here, this is where we have in the past had overt adventitious contamination by a retrovirus of a biological. For instance, being mentioned earlier, ALV, that causes virus in yellow fever vaccine by virtue of producing the vaccine in embryonated eggs infected with the virus. The other one that was mentioned earlier by Phil, HIV and blood products.

The bottom half here is a quite, somewhat separate type of contamination. In fact, you might find it equaler to call it contamination or not. Certainly these are not adventitious situations. These are situations in which an endogenous retroviral-like particle is present in the manufacturing process.

In the first instance here, it's established that murine hybridomas used in the manufacture of monoclonal antibodies produced, secretes C type particles. These have been tested in a variety of other mammalian, including human cell lines, and generally are not infectious.

The titre can be very high for these types of particles. You can get 10 to the sixth particles.
per mil. I've even seen 11 particles per mil in one instance. So you can have a very high burden of direct viral particles.

It is also well established that CHO cells, which are used for producing biopharmaceuticals, secretes C type particles. You also get intertestinal type particles from these cells. These are probably much more characterized, a bit more work has gone into describing the particles from CHO cells, sequence information from the endogenous elements within the CHO genome, which is producing these particles, give some ideas as to why they are defective. The reading frames are incomplete. There are stop signals. So you don't get a proper infectious virus from these endogenous elements.

The latter type is the only type here that's dealing with vaccines, even dry vaccines produce either an ovo or cef cells. I'll come back to that in a few minutes.

From a regulation point of view, how do we deal with virus contamination and retrovirus contamination? There are a couple of guidelines I would like to bring to your attention. The first one here is an ICH guideline, which looks at viral safety
evaluation. Admittedly it is only for biotech products. The scope of the guideline does comment that this is not, this guideline is not applicable to vaccines. But I think it is worth looking at what it says about virus contamination.

Within the document, it describes five different cases of potential contamination, starting from the most desirable case, where you don't have a virus present in the process in any way, down to the worst scenario where you know you've got a virus, but you haven't a clue what it is.

The guideline goes on to state what is acceptable and what is not acceptable in the manufacturing process. The only two cases which are generally acceptable of the first two cases, Case A, where you have got no virus, and Case B, where you have got a non-pathogenic retrovirus. The other cases are only exceptional. Generally you don't want one at all. The manufacturing is not allowed when you have got a virus contamination.

So for Case B, really what you have here is a murine retrovirus is probably the only contaminant acceptable in the bulk harvest. If you remember this guideline is applicable only to recombinant products and not to vaccines in general,
and these recombinant products are highly purified.

The other guideline is the WHO requirements which came out recently for use of animal cells as in vitro substrates. That does include vaccine production.

When it comes to testing for retroviruses, this guideline has several -- many other guidelines in the past have indicated, the types of assays being used for retroviruses, specific infectivity assays, electron microscopy and transcriptase assays, are the three general approaches for checking for retrovirus contamination.

There may be the use of specific antigen detection as is in some particular cases, but these are the generally recognized methods of going about picking up retros.

If I can concentrate now on the RTase assays. The traditional type of assay involves incorporating a nucleotide precursor, a labeled precursor of some kind into an assay using a rather synthetic type of template. Then more recently of course we have the PERTs, PB RT, AMP RT type of assays, which includes a PCR amplification step, with the result that these type of assays are incredibly more sensitive than the more, as I can call it,
traditional type of assay, and what is often quoted as up to a million fold times more sensitive by virtue of incorporating a polymerase chain reaction.

Now using this type of assay, the cat was set among the pigeons. When this paper came out, I might even say that the fox was set amongst the chicken coop. Detection of reverse transcripted activity in live attenuated virus vaccines. This quite naturally caused a bit of concern as to what was going on here. The vaccines indicated, the one common feature was that ovine produced in eggs of some kind, measles vaccine out of CEF primed cultures, similarly mumps. Yellow fever and influenza in ovo. But not measles vaccine out of human diploid cells or rubella vaccine out of human diploid cells. So the common link here seemed to be the CEF, the chicken source used in the production of the vaccine.

We joined in the boat here and started looking at this issue. Every type of hen fluid that we have looked at, CF fluids or an type fluid from a variety of different strains of hen have all been positive in the assay for reverse transcriptase.

Summarily, quail, jungle fowl, and pheasants are positive.

The types of sources of fluids which have
been negative for reverse transcriptase are listed here. Some species are not positive, turkey and duck cultures, quite a range of human cell lines. Simian rabbits have been tested and found to be negative. So the clear source of this RTase that was being picked up in the vaccines is quite clearly of chicken avian origin.

We would want to look at -- I should add that this RTase was known at the time to be particles associated and appears in the supernate of the cells. We are going to look at this particle to see if it would pick up any infectivity. In all, we looked at 10 different cell lines, mainly human, but including rabbits and turkey. Over 21 tests and 116 passages.

In each case, in every test and at every passage level, the cultures were negative for reverse transcriptase activity. There's absolutely no indication that this particle is infectious. Since then, CBER and CDC have also come up with similar data, including use of PBMCs. No infectivity associated with these RTase containing particles.

Where might these be coming from? Presumably they are derived from endogenous retroviral-like elements in the chicken. The information to date regarding such elements in the
The chicken genome are quite well characterized EV loci, which are related to the avian chosis virus family, and more recently discovered about 10 years ago, EAV-0 family, which is an older element than EV, and then older still, ART-CH and CH-1 elements.

The information at the time and pretty much where it still exists is that we knew that there's a line of chickens which was negative for EV. It had been eliminated from the genome. This line of chickens, the culture fluids were positive for RTase. So we knew that it had to be at least one of these elements producing RT activity. At the same time, you couldn't eliminate the fact that EV might also be producing RT activity. The best bet was EAV-0, given the sequence information that was present at the time.

More recently, in the last year or two, Joerg Schuepbach's laboratory has produced a good evidence for the presence of EAV-0 derived RNA associated with the RT particles secreted from CEF cells, and then this year, Walid Heneine, CDC, also produced the presence of EAV and ALV RNA. When I say ALV, I mean derived from the EV loci and not exogenous ALV contaminating RNA.

So what can happen here retrovirus-like particles, defective particles being produced from
endogenous elements both from EV and EAV-0 family of endogenous elements. The presence of the RNA and relsconstrictase in a particulate fraction leads one to come to the conclusion that we have retroviral like particles in the CF fluids of the chick cells, which is present in the vaccines measles and mumps.

The absence of infectivity in the current genetic information, sequence information that we have on EV loci and the EAV-0 family of endogenous elements strongly indicates that these particles are defective viral particles. The only question mark that remains from the regulation point of view, but also scientific point of view, the possibility of pseudotype formation during vaccine manufacture. The current evidence suggests the particles that are defective in the envelope-like protein and so there's a particulate of pseudotype formation with the glycoprotein of vaccine viruses being grown in the CF cells.

So to summarize a couple of these issues then, from the practical point of view, testing for reverse transcriptase as an indicator of retroviral contamination, these assays are evolving, changing all the time. One has to take into consideration the strength of the assay and the validity of the assay. There may be different requirements within an assay
for different sources of RT. It may be necessary to
use some other sort of method to assess the
significance of any RT detected because we know that
RT activity can derive from other enzymes. Telomerase
is or DNA polymerase, cellular DNA polymerase is.

These features are not specific to the more recent
sensitive type of assays involving PCR, the parents,
and the PBRT. These features were also factors that
had to be considered in the more traditional types of
assays.

It is often quoted that the RT levels in
chick cells is very low, given that it was detected by
a very, very sensitive assay, and has not been
detected by the more traditional type of assays.
Certainly some preliminary data that I have got
suggests that it is not quite as low as we first
thought. Really this RT activity in chick cells and
ultimately in vaccines is only just below the level of
detection of the more traditional type of assay. In
fact, this was a relatively novel phenomenon
discovered just a few years ago. It was in fact first
reported 20 years ago in the late 1970s by Berne and
Hofschneider at the Max Planck Institute in Munich.
They reported the presence of a novel type of RT
enzyme in chick embryos and in chick cells. That was
in the days before PCR.

So the level -- I certainly believe the level of RT and the level of these particles is actually quite high in chick cell fluid. Ultimately I think what we have to do is look into the need for standards, standard materials in some way to assess on a quantitative basis the level of RT activity in chick cells, in measles vaccine, in mumps vaccine, in order to come up with some meaningful conclusions regarding it.

So to look at the RT issues from a regulatory point of view, a couple of comments I would make. First, that these are state of the art technologies. When these highly sensitive assays first came about, it posed very useful from a research point of view to what use are they in a routine manufacturing validated type of assay.

I think the time has come where yes, you would say that these are state-of-the-art techniques and can be and should be used for detecting the presence of RT in your manufacturing process. However, when it comes to, for example, chick cells, and until we have a greater understanding of what the levels might mean, and until standards are available, there is really -- it is difficult to justify any
requirements to perform RT or PBRT assays on systems,

and basically here I am talking about chicks, which
inevitably will be positive. We know they are going
to be positive, that there's no great need to actually
require any manufacturer to do these assays. But

certainly there is a still a strong requirement to
provide evidence for freedom from retrovirus
contamination. This will have to derive from other
data. Thank you.

(Applause.)

CHAIRPERSON RABINOVICH: Thank you, Dr. Robertson.

Any questions?

AUDIENCE MEMBER: Just a comment. For
known endogenous avian retroviruses or exogenous avian
retroviruses, of the cell lines, of the cells that you
tested for infectivity, only the turkey cells would
have given a positive result. I would urge for avian-
derived -- urge the use of those cells, and a PERT
assay is a sensitive readout, for detection of perhaps
unknown agents in these vaccines, end products, as
being the most sensitive, at least for avian-derived.

DR. ROBERTSON: Yes, yes. The turkey
cells are sensitive for rav, because it's virus, but
apparently not for the RTase. I have no idea, duck
cells are also negative, but I have no idea if duck
cells are susceptible to the --

AUDIENCE MEMBER: They are not as good as
turkeys. Turkeys themselves actually are not
sensitive to all exogenous ALVs, but to most they are,

and all endogenous ones.

DR. ONIONS: David Onions. I really
enjoyed that, Jim. I just want to make a comment on
your comment about standards. I think as we heard
from Keith last night and what we're doing, and I'm
sure George is doing too, using the tac man technology
where you can actually quantitate the PCR product.

Then if you actually do EN counts of virus particles,
dilute these out, you can actually quantitate your
assay system and actually determine the number of
particles you can detect.

Now it seems to me that that is a useful
kind of standardization, and that you can then relate
that to if you like, a consistency of your starting
material, in this case the egg.

So I think in that case, applying those
techniques does have value, because it gives you a
kind of lock-to-lock consistency of your materials.
So that if something goes out of spec, then perhaps
something odd is going on in those materials.
DR. ROBERTSON: Whatever we want to approach this standardization, one would have to do it on a quantitative basis. I was sensitive earlier to quote any actual figures, but to quote you some figures that I have got so far, in one chick cell preparation, the culture fluid, there was the equivalent of the order, and this the first of investigation, 10 to the 4 focus forming units of rav in uninfected chick cell fluid.

A large current high level is when your typical infection goes up to 10 to the 6th, I believe, focus forming units. You are only talking about 10^4 drop, lower value. So you are not far away. It is going to be difficult. If you have got an overt contamination going up to 10 to the 6th, I think that will be quite clear on a quantitative basis. But once you drop down a bit, it is difficult to say whether you have got an infection or whether it's just background level of endogenous RT-derived activity that you are picking up.

DR. MYERS: Martin Myers from National Vaccine Program. As I sit and count the number of immunizations that various populations receive with these particles in it, repeated immunizations with it, I wonder if there is any data on sero responsiveness
DR. ROBERTSON: Well the reason I am looking over your shoulder, we have Walid Heneine from CDC. I'm not sure if you are going to say something along those lines, but serologically, there is no real evidence for reaction to ALVs. Epidemiologically, when it first came out, we also, not ourselves but epidemiological colleagues, to provide information. There is no evidence again, for any increase in the incidence of childhood cancers since the onset of measles, mumps vaccination.

Walid, were you going to say something?

DR. HENEINE: Yes, I have. Regarding transmission risks, so far the data we have where we have looked at the presence of antibodies to avian leukosis virus by western lot, I'd say we developed as well as presence of ALV sequences and EAV sequences in the peripheral blood lymphocyte from vaccinated kids as well as in plasma. So far, the results have been all negative for both viruses.

I have just one comment regarding referring to these viruses in general as defective.

My comment is that the evidence we have so far on those we have studied in a couple of vaccines between in Europe and in the U.S. suggests that those, for
example, ALVs we're dealing with could be defective because they come from loci that have deletions. However, this may not be true for all the contaminants from it we might find in other vaccines, because these contaminants reflect the particles expressed from these loci in the different cell substrates that Phil mentioned in his talk, that the nature of these particles and their phenotypes would vary depending on the presence of the particular loci in these substrates.

So just a comment, not to generalize that we always should expect to have defective particles. We might or we might not in certain cases.

DR. ROBERTSON: Yes.

DR. SCHUEPBACH: Joerg Schuepbach from Zurich. I would also like to make a comment regarding seroconversion. We have done two vaccination studies, one with yellow fever vaccines, where about 120 individuals were tested actually for reactive antibodies against HIV. The reason for this was that in 1991, there have been reports that false positive HIV reactions were found in people that have been vaccinated against influenza. Influenza vaccine is one of the vaccines which contains the EAV.

So we found that statistically highly
significant the vaccinees which have received the
vaccine which have the higher content, about 80 times
higher than the other vaccine which we used to use
too. That these patients actually had highly
significantly elevated antibodies to HIV 1 and 2,
although none of these actually became sero positive.
The serious bonds was highest in those individuals
that also had a history of measles vaccine.

In the second study, this was an influenza
vaccine where we tested two different brands, a split
vaccine and a rather crude vaccine. We also had a
response to HIV bond two in the third generation tests
in the vaccine which contained more in the crude
vaccine, which contained more of the EAV protein, and
again those individuals who had a history of yellow
fever vaccination had the highest type, the highest
increases.

We also tested yellow fever vaccinees by
PCR, RNA PCR and DNA PCR for EAV-0 sequences. We
found one out of 180 individuals in which both these
tests were positive as plasma on PBNC. At the moment,
we cannot exclude that this was the result of a
contamination, but we are working on that. So I think
that the matter is actually not as clear as has been
presented by others.
MS. SHEETS: Hello. I'm Becky Sheets from
FDA. How would you recommend that avian-derived
products be tested for retroviruses? EM is not very
sensitive. The conventional test is often inhibited
by the allantoic fluid, and therefore, is not
necessarily a valid test. How would you recommend, if
you don't use a PCR-based RT des?

DR. ROBERTSON: At the end of the day, it
would have to be an infectivity assay. There are also
some antigen, ELISAs for the viral antigen. I don't
have experience with those. I don't know the
sensitivity of them. Ultimately you are looking at an
infectivity assay which can be performed even on chick
cells, which are positive for RTase. One could assess
for after several passages on chick cells, looking at
an increase in RT activity or increase in antigen.

MS. SHEETS: When you said infectivity
test, were you talking about those specific for ALV or
were you talking about general tests to detect any
kind of retrovirus?

DR. ROBERTSON: Well, it would have to be
an avian retrovirus if one is performing the assay on
chick cells. Propagating the material, the test
material in chick cells but using either RTase in
general or an ELISA specific for ALV test for
increased presence of either RT or for the presence of ALV antigen.

CHAIRPERSON RABINOVICH: Dr. Coffin, the last question?

DR. COFFIN: Yes. I'd like to actually address Dr. Schuepbach's comment. Did I understand that you were basing your sero assays on the rationale that there might be cross reactivity between ALV and retro viruses in HIV? There is no rational basis for that. There's virtually no amino acid the same between those two viruses, except for some extremely highly -- you know, three or four in pol and some other places. Did you actually assay directly for seroreactivity against ALV? It would have been a much more straight-forward experiment.

DR. SCHUEPBACH: We agree that there is no sequence homology on the nucleic acid and on the protein label, but these are the results which we found. We have to find an explanation for them. We don't have at the moment.

CHAIRPERSON RABINOVICH: Thank you, Dr. Robertson.

Our next speaker, Dr. Jorg Schuepbach, from the Swiss National Center for Retrovirology.

Induction/activation and detection of occult viral
agents that are present in mammalian tissues.

DR. SCHUEPBACH: May I have the first slide, please? Okay, from previous remarks, I heard that I was expected to talk about these avian retroviruses as well, but actually I was asked to talk about the induction activation of occult viral agents. So I will just have a few remarks on this other stuff. So occult viral agents are agents you don't detect or at least do not easily detect. They may include two groups: a group that includes known agents which are present at two low concentrations for easy detection. The reason for these may be latency; The other group consists of unknown agents. Since we do not have good detection methods for these, they may be present at low or also at higher concentration.

Viruses known for their latency or various types of the herpes virus, true, they are latent in various types of non-permissive cells such as neurons, B cells, monocytes, PBLs, and others. They are activated from these latent stages by various kinds of stimulation of their host cells by differentiation, agents by the differentiation of precursor cells, to more mature cells. Again, by other activating agents. Other viruses could be considered in addition to the herpes viruses include the adeno
viruses, the adeno-associated virus and the pathyloma and polyoma viruses of which we heard yesterday, and will hear more in a subsequent talk.

Regarding the RNA viruses, I might discuss the measles viruses and of course the retroviruses.

When we look at the mechanisms by which we can activate these various viruses, it is mostly by activation of their host cells, by cell stimulation, by induction of cell differentiation of these cells, and then by co-cultivation with cells which are permissive for replication.

Now since we have different viruses and host cells systems, these methods vary greatly among the different viruses. If you have unknown viruses, you really don't know what to do.

So the effect of such activation would be that from a lonely latently infected cell, by inducement of replication, a virus would spread throughout the culture, resulting in virus gene amplification in production of viral proteins. So this would make of course the detection easy. You might also have some pathogenicity which is easy to detect.

However, our goal is actually not detecting any possible virus that might be present.
The principal goal is to provide a virus production system which is free of such agents. It is suggested here the easiest way of achieving this is actually cellular cloning. Because if you have an agent that is present in only a minority of the cells, the chances that you derive a clone that is free of these agents is very high.

If by chance you hit an infected cell, the descendants of that cell will all carry along the virus and of course then we come into a situation which makes detection of unknown viruses and also known viruses much easier because either all of the cells will be infected or none at all.

So cellular cloning, if we hit an infected cell, has actually a viral gene amplification effect which is comparable to virus induction activation if it's successful. Most importantly, it is a procedure that works for all the latent viruses except endogenous retroviruses, but these are present anyway in all of the cells.

So going on to the detection methods for these agents, let's first talk about known viruses. Since all the cells will be infected, we actually do not need the most sensitive procedures. We do not need procedures that detect the single viral copy.
What we need is broadly reactive methods which go
detect all the different members of a certain virus
group.

So I think techniques, old-fashioned
techniques like hybridization techniques on the low
stringency or if we want to use PCR or nucleic acid
based methods, we should take care that we take a lot
of different probes, use data generated primus,
multiplex PCR and so on.

Of course in addition, we should also do
the classical methods, doing cell activation and co-
cultivation as permissive cells, the routine detection
methods of broadly reactive antibodies which detect
all the different members.

For those who think that what I have told
so far is rubbish, and that we actually do need very
sensitive methods, I offer the mega PCR, which has
also been named catcher PCR by others. The purpose of
this method is to take very rare sequences among a
very high background of DNA or RNA. So here we
convert the samples of up to 500, maybe even 1
milligram of DNA or respectively RNA.

The principle is very simple. We use
biotinylated capture probes which bind to these
sequences inquest. We isolate these complexes on
coded beads, wash the rest of the DNA away, and then amplify these by PCR with primus which are located outside of this capture probe.

The advantage of this is that we absolutely do have no carry over because the amplicons are selected against when we do the capturing. It is this type of test which I would actually like to have been seen when testing in the question of xenotransplantation where the PERV sequences can be found in humans which have received pork material. I think this will be the test, to test these questions.

Now using this method, it's actually very sensitive. You can detect a single copy here of HIV DNA. We still have double positive signal, is about one copy. This serial dilution was done in the proper range here. The fact that in these two, three last dilutions only one of the two duplicates was positive clearly demonstrates that we are in a Poisson distribution. So we can detect the single copy with this method in 100 microgram.

DNA, we have actually demonstrated that there's 95 percent probability we can detect three double standard HIV copies in 100 micrograms of DNA.

So now going on to the exclusion of unknown viruses, and I will talk about retroviruses
later, we can actually use the same procedures as I have already described previously. We just have to take care that we really have broadly reactive methods. This is true for molecular based tests as well as for the more classical procedures.

Now coming to retrovirus detection, of course also of cell cloning, here we have two situations, the exogenous retrovirus may not be present in none of the cells or in all of the cells. The endogenous retroviruses were always present in all of the cells. The known exogenous retroviruses are detectible by tests for conserved sequences. Of course you might also use universal pool primers for unknown retroviruses -- because of the endogenous retroviruses. Not all of which, or very few of which are actually harmful.

So I think it is better at this time to switch from the analysis of cells to the analysis of particles. This is best done by the PERT assay which has been mentioned before by several speakers.

Now when we devised this test in 1992, we devised it as an anti-family of related tests which would have in common that reversed inscriptase present in a sample would be used to create from a template primer combination and nucleic acid that is to be
unamplified.

Now in most instances, this will simply be the cDNA. There are other possibilities as well. You can take any nucleic amplification procedure, not just PCR. You may also use ligase chain reaction or NASPA or you can make use of auto replicated DNAs or RNAs in order to generate amplification product, which can then be assayed by different methods.

So since we have provided for all these different methods already in 1992, we do not think that it is necessary to invent new names for these current assays.

Now this test is actually very sensitive. This experiment in comparison to classical RT assay. It occurred as six to seven orders of magnitude more sensitive, and in a direct comparison with -- in the case of HIV, where we compared the method with RT PCR, detecting one copy of cDNA, we had the same dilution endpoints for two different samples.

Actually as others, we can detect only a few particles in the case of HIV. We believe that in some cases we can detect even less than one particle.

Now this is one of the theories taken from the Joerg Koenig paper in 1996, where we demonstrated that the measles vaccines, the mumps vaccines, the
yellow fever vaccines, and the MMR vaccines all contain activity which is about three orders of magnitude higher than the background here on other vaccines, and were negative. Now in order to identify the viruses behind these activities, we along with the PERT assay, developed the method for the identification of unknown retroviruses. It is based on three properties of old retroviruses, namely, that they all are polyadenylated, that R sequences are repeated at both ends, and that cDNA synthesis has started here at the primer, binding site, and that for primers, tRNAase are used and the use of such tRNAase is actually very much restricted among the various retroviruses. For example, is just four PRNA primer equivalence. You can start cDNA synthesis for all exogenous retroviruses known today. So what we do is that we bind the retroviral RNA to poly t coated beads. Then we start here, the synthesis of the cDNA with one of the various t RNA primers, synthesizing the strongest of DNA. Then adding a tail here, and then with anchored TCR, we can amplify this sequence and submit the sequencing directly. Actually this method has also been used by
the group of Dr. Loewer at the Paul Ehrlich Institute, and even published before us. But we have somehow optimized this procedure, so in general we need less than one-thousand RNA sequences, sometimes as few as 20 or 40, 50, in order to generate this sequence here.

As soon as you have it, you actually know whether you are dealing with a retrovirus or not. When you deal with a retrovirus, you have to R sequence and then you can check with the other anchored PCR. Where there is R here, it's repeated at the three prime end. If it is, you can then amplify the entire genome with a little bit of luck by long PCR.

So this is what we use to identify this EIV-O sequence. We have also done some other work. For example, we investigated the NIH 323 cell line. This was negative by convention RT tests, but positive by PERT assay. We had a nice band in sucrose, and then radiant. Using this procedure which we call parar, we identified 23 different products, 15 of these were actually retroviral sequences from four different groups. Three of them were unknown sequences, at least at that time. So far we have not further characterized these sequences, but this is still awaiting.
Now staying with retroviruses, as Dr. Coffin pointed out yesterday, sometimes if you have a cell line here, you are dealing with melanoma cell lines which were found to be highly percipated by PERT assay. We analyzed what was in there. It turns out to be endogenous murine leukemia virus, and later we were told that these cell lines have actually been passaged in mice.

If you have low titres of activity, then that becomes a little bit more complicated. This is the analysis of primary samples from a patient with MC cor cultures. No actually not cor cultures, just cor cultures which were found lowly positive in the PERT assay with activity in the order of two, maybe three times above background.

Here the patterns is a little bit more complicated. You have here a small peak that might correspond in density to ritualized particles. This one might correspond to cor particles. You have another identified -- unidentified peak here. It will certainly be a challenge to find out what this stuff is.

Next, please. This is another example of a primary culture where we have a very short peak at the higher density. This might be for particles,
could be a different retrovirus, a different virus, or
just a subcellular particles containing some cell or
enzymes.

Now you will say that this test of course
detects only retroviruses that are released. We are
also worried about retroviruses that are inside the
cells, so stimulation may be necessary. Actually I
think one important question is or one possibility is
that actually the vaccine virus we would like to
produce in such a cell might activate latent
proviruses. So I think it is important that we
actually do not just test the virus production systems
while uninfected, but also when this seed virus has
been added, and then we harvest the virus.

Now in some cases, as in the measles virus
or so, this has proven very easy. We had quite a good
specificity. But in other cases, it might be more
difficult as indicated in this example, where we
tested a vaccine, experimental vaccinia, recombinant
vaccinia virus vaccine against melanoma. This was
found highly positive by PERT. It had actually been
produced by just the lysing, the infected cells by
ultrasonication.

What we now find is here in black, is the
vaccinia virus DNA two peaks. We have here a major
peak of RT activity which does not coincide with the
vaccinia virus peaks, and also is not characteristic
of retroviruses. So I think in this case, we can rule
out the presence of a retrovirus.

Now it may also be interesting to find out
whether upon induction, viruses might come out. So
this would add an increase of safety to the vaccine.
As retroviruses are regulated, you have the promoter
in anti sequences in the upstream LTR in the U3
region. Depending on the cell type, activation state
of the cell and the differentiation, you have various
sets of transcription favors interacting with this
enhancer regions.

In addition to this balance of positive
and negative transcription factors, you may have
positional effects as the chromatin structure or the
DNA methylation. You may now try to influence this
balance by tipping it by either inducing mitosis cell
differentiation by substances that lock inhibitors or
by alleviating the negative positional effects, again
by inducing mitosis or by inducing DNA de methylation.

The number of induces have been described
in the past. The most important ones are listed here
at the top, allogenated pyrimidins, the azacytidine,
which only both of them working only in infected
cells. I will not mention the others because of the lack of time.

Now it depends a lot on the virus whether azacytidine or the deoxy pyrimidine is preferable. For example, in experiment in cell line where two types of different retroviruses are produced, several type A particles here. The azacytidine is certainly better. But in C-type particles, these cells produced IdUdr. Yes, the IdUdr is better. So you might have to use a combination of these two drugs.

So in conclusion, I think induction activation certainly serves to amplify latent viruses for which improved detection. I think it is more important that we early in the process of selecting virus production systems be cloned B cells, and sub-cloned, because this will amplify, because this really facilitates detection very much.

In consequence of this, we do not -- I think this is very important. We do not need the most sensitive procedures. What we need is broadly reactive procedures which will detect all the different agents.

I also think that at the end, the only important thing actually when dealing with adventitious agents, not just with DNA, which might be
infectious, is that the vaccine is free of these contaminant viruses and for retroviruses I believe that this can be verified by the PERV assay. Thank you.

(Applause.)

CHAIRPERSON RABINOVICH: We'll take just a couple of questions because I would like to leave the rest for the panel discussion.

DR. COFFIN: John Coffin. I would agree that if you get preparations of vaccines that are negative by all these assays, you can have a pretty good level of confidence that they are not contaminated with retro viruses. The problem is, if you do these enough, it may well be that no vaccine will pass these tests.

What I think is very important to add to this would be one more level to your last slide. That is an infectivity step. As in the example we saw before when one perhaps collects a panel of cells or cell lines which are pert negative, and there seems to be reasonable numbers of those, and then test the vaccine, the induced stuff and everything else by infectivity and induction of pert activity on those cells.

I think that would be a much more useful
and reliable test for the presence of viruses that
might be problematic than simply looking at the pert
activity in preparations with cell soups.

DR. SCHUEPBACH: Yes, I agree with you.
I actually thought that was included in those
conventional methods which I have listed for the known
viruses. Of course you should also do some studies
for retro viruses.

AUDIENCE MEMBER: You mentioned results
associated with particles from supernatants of primary
human materials. Did you try to find retro virus-like
sequences in these particles for para assay?

DR. SCHUEPBACH: Yes. These are very
recent results. We are in the process of doing that.

DR. KRAUSE: Phil Krause, FDA. One of the
issues in testing vaccine products is obviously what
tests are available and have been validated and that
we understand the sensitivity of. So I guess in the
context of thinking of highly conserved sequences to
which we might develop primers that could detect a
broad array of viruses, including some unknown related
viruses, what can you say about the current state of
the art? How good is that? How well has that been
validated? Is that something which if we decided
tomorrow we wanted to apply that to new vaccines
producing neoplastic cells, we could simply say "let's do it" or is more work required?

DR. SCHUEPBACH: I'm actually not very familiar with other viruses than retro viruses. But I think these things, however they exist, should clearly be developed.

CHAIRPERSON RABINOVICH: Thank you. We will go onto our next speaker, Dr. Jens Mayer, from the University of Pennsylvania. The status of HERV in human cells.

DR. MAYER: Okay. My talk will deal with -- can I have the first slide, please? Okay. My talk will tell you something, I hope, about the status of these human endogenous retrovirus regarding the coding capacity and the expressions. Just again, it was mentioned before already what is actually an endogenous retro virus. HERV is created by the germs of infection of an exogenous retro virus. This leads to radical inheritance of this newly created virus following generations. In the course of the evolution, it will be also inherited to newly arising species.

The human genome, like all mammal genomes, and also some invertebrates, invertebrate genomes where it has been shown, contains several families of
elements and so on. It has been estimated that about one percent of the human genome of such retro origin. These elements antiquated already several million years ago through the genomes of human predecessor species. Some present for at least 30 million years. Some have been shown to be present for at least 40 million years. We have several indications of different various families. So they were independent of several exogenous retro viruses. Some of these elements that are now present in the human genome existed. Single copy, and some have copies, copy numbers up to 1,000, per haploid genome.

But as I said, most of these sequences were already present for a long time. Therefore were targets for mutations. Most of these families then became coding deficient or they do no longer encode for retro R proteins. However, even if they are coding deficient, many of these families are still transcribed in several human tissues. Some have been discolored just by virtue of their expression. It also seems that the expression of these sequences is regulated in certain tissues and tumors, so we heard that there might be an deregulation of families. It seems possible that that deregulation mechanism is not present in certain tumor tissues.
Just a word regarding the nomenclature of these sequences. The tRNA that was originally used in the priming of the transcription process, the life cycle of the exogenous vaporized, and according to the amino acid and tRNA codes for, and this single code for the amino acid stands dependent. This is just one possible nomenclature of perts. It's still very confusing.

I said that most retro viruses are coding, HERVs are coding deficient. However, there are some good described examples, especially some new examples of coding in intact HERV sequences. At least there are some intact genes. We have already known for a long time the so-called ERV-3 sequence that belongs to the R-family. This, we agreed, pro-virus, or pro-virus sequence encodes, and 1.9 KBN open reading frame. That open reading frame is highly regulated to the transformation of trophoplasts into sensitio-trophoplasts in the placenta. So we have here clearly an up-regulation during a developmental stage.

We have for instance, you have H-family and we have about 1,000 copies of that H-family.

Among them are 100 copies that are still intact regarding the pro-virus structures. They have an LTR gag pol env, LTR structure remaining 900 lack N gene.
There has also been reported that this HERV-H families are expressed in various cell lines. We see the highest expression for these elements has been reported in cell lines that are derived from germ cell tumors, and germ cell tumors I guess you will hear some more about germ cell tumors later on.

Just this year, Lindeskog, Mark Lindeskog reported the isolation of an intact HERV-H env gene.

So it is now clear that there is within the human genome one intact HERV-H evn gene. It's not know so far whether there are any among these many sequences, whether there are any intact gag of pol sequences.

I would like to mention the new discovered HERV-W family that has originally been reported, has been isolated from retro virus by particles from multiple sclerosis patients. It has also been reported that these HERV-W sequences are up-regulated in the placenta. Joni Blanc also reported this year the isolation of an intact HERV-W in the genes. It is also not known whether there are intact gag pol genes.

I would like to in the second part of my talk, report about results for our family of clearly outlines from our other HERV families in the coding capacity. This is the so-called HERV-K HML-2 family. This is quite complicated.
The human genome contains several families that use lycine primer binding site or TRNA for primer binding. They were named human MMTV-like sequences, one through six. The family that we are talking about is reported in more detail by Ono and co-workers and the original sequence was the so-called HERV-K 10 sequence, which is by the new nomenclature is the HERV-K HML-2 sequence.

We have reached about 25 to 50 copies of that HERV family is present in old world monkeys, but not in new world monkeys. One concludes that family is present for at least 30 million years in the genomes.

In the past, there have been reports of isolation of the isolations of intact HERV HML-2 sequences. So there were reports about intact gag sequence and intact protease sequence has been reported, that is able to process that HERV-K gag protein, intact pol sequences with RT activity, with endonuclease activity, and have been reported and also intact mRNA has been reported from the group from Johannes Loewer. And also what we heard yesterday evening, there is also an additional splicing product from the N gene, the so-called C-ORF that still has a rav-like function.
What is known already for a longer time is that these particles or the cell lines are derived from germs of tumors or typically testicular tumors of the young man. These cell lines do produce with rav particles. Boller and coworkers could show that these particles are encoded by the HML-2 gag protein, labeled antibodies, and recognized that gag protein.

If we look at patients suffering from germ cell tumors, we also have some surprising results regarding that HML-2 sequences. Namely, if we look at the antibody status of these patients compared to controls or other non-germ cell tumor types, we see that mixed germ cell tumors and here especially, seminomas, these patients have very high antibodies directed against HERV-K gag and HERV-K N proteins.

These tumors or these antibody titres are already very high if the tumor is clinically detected. From other results, we also know that the precursors of these tumors, the so-called carcinoma in situ, also expresses already on the RNA level these HERV-K HML-2 sequences.

We were interested to see or to find out where in the genome are these impact genes located that are responsible or that cause finally the production of these gag and env antibodies. As I
said, it has previously already been reported that they are intact genes, but it was not possible because of the high copy number of these sequences to isolate or to at least chromosomally assign these intact genes. We, therefore, tried to chromosomally assign these intact sequences using a combination of the so-called protein truncation test and using a monochromosomal hybrid panels, or panel of human rodent fusions, fusion cells.

We were able to show that there are at least, still at least eight intact gag genes within the human genome, and at least three intact env genes. We did not publish that. There are also several intact pol genes within the human genome.

I just want to show you how we got these numbers. This is the protein truncation test that has been described by Roest and coworkers in 1993. So it was originally developed for the detection of APC gene carriers that carry it, the APC gene. So the APC lesion is characterized by trends or not completely translated APC proteins. It is almost like the 3 prime terminus.

So we have three possibilities. One is that both are intact, both genes are intact. The carrier will carry one defective APC gene. The
defective person would carry the two defective genes.

The principle of the test is that the coding sequence is PCI amplified, where the protochomo contains the T-7 promoter and the translation initiation sequence. So if this PCI product is then in vitro transcribed and translated and impressed and radiolabeled amino acid, electrophoresed, and then auto radiographed, you will see according to the status of these donors that you will have only full-length proteins, the carryover also show an additional shortened protein and defective people will only produce defective proteins.

We in principle used the same test because we in principle have the same situation. We have some defective gag genes within the genome. There must be at least one gag or env gene because we have the antibodies. So we put -- in principle used the same test.

What we did was we are looking for the presence of full-length gag genes or env genes on the human chromosomes and then tested the PCI product we got from the chromosomes for their coding capacity. This is the result for the gag coding capacity. So gag protein would result in a protein of about 73 kilodaltons. So these are controls that give the respective proteins.
You see that there are several human chromosomes that contain or produce a full-length protein. There are eight human chromosomes that contain at least one gag gene that contains four full-length proteins.

I also would like to mention that we are also able to demonstrate the defective gag genes if we see here, these proteins that are just smaller than expected. These are very likely the gag genes that are defective. Stop codons within the coding sequence.

We did the same for the HERV-K env genes. We see here that three chromosomes produce a protein of about 76 kilodaltons. These are the chromosomes 7, 19 in here on the chromosome.

What we also see in the gag experiments is that there are additional env genes that are only on the almost intact. We have here a protein that is about four kilodaltons more. So this actually could also be considered as an intact reading frame.

So we have several human chromosomes that still contain gag and env genes. We have three chromosomes that contain both intact gag and env genes, the chromosome 7, 19, and the Y chromosome. We were interested whether these chromosomes or the
intact genes on these chromosomes are derived or
located within one provirus or within several or
different positions within the particular chromosome.

I would like to report or tell you
something about what we found out for the chromosome
7. We were using for addressing that question, we
were using a chromosome-specific, chromosome-7
specific cosmid library. We were screening for clones
that contained both gag and env sequences.

What we finally found out, that we
isolated the so far least defective human endonuclease
on chromosome 7. We were able to characterize the
proviral sequence within one cosmid clone that still
has intact LTRs. So they regulate to the elements.
They are able to transcribe, as you will see. We have
an intact gag gene. We have an intact protease gene
that protease is able to cut itself from a gag
protease, polymer precursor protein, and is
furthermore able to process encoded gag proteins. So
it's typical retro-ized protease.

We know just from sequence comparison, one
can deduce that the endonuclease within the polymer
genes also acted just by sequence comparison, no
significant changes compared to recently described
active K in the nuclease. We have an intact env gene.
This intact env gene sequence has already been described by Johannes Loewer's group as an MRA, which also shows that this sequence is actively transcribed. So this is actually an expressed provirus.

We have spliced on the inceptors sides the corresponding position that would allow to splice an M on A, and what we heard yesterday also, to splice an additional soft M RNA.

What we see is that this proviral sequence is only defective in the RT domain. It has a single-based permutation within the YXDT motif. So very likely, this highly important catalytic motif is -- so only in reverse transcription function this probably missing from that proviral.

Okay. We have here almost intact proviral sequence. But now regarding infectivity, we had that already several times I guess before. We have HERV-encoded retro of particles, several cell lines, even in tissues, the placenta tissue for instance. We find HERV-RNA in these particles.

We have no infectivity so far shown for any of these HERV sequences. We do not really know why. There are several reasons that can be mentioned for the HML-2 family. It has to be reported that the env protein cannot be cleaved into the auto membrane
transmembrane domains. It is conceivable that they are defective genomes that are packaged into these particles, so only if they would be able to get a new cell, they would only deliver defective genomes. It is also not clear whether the receptors, that they were once used by that, retro families are still present and would still be used. So what you should take home I guess is that human endogenous are expressed in several tissues tumor types that are highly up-regulated in certain tumor types. Several HERV families are still able to encode proteins, and among them, the HML-2 family that still encodes all essential proteins. We have almost intact HML-2 provirus within the human genome. Thank you.

(Applause.)

CHAIRPERSON RABINOVICH: I think we will hold questions at this point. We are going to take a 10-minute break now. We are going to come back and finish with the last two speakers. I need to figure out how to catch up time, and yet leave the time for the panel discussions. I ask you to do two things. Check-out time from the hotel is 12:00. You should know that. They have already called in a bunch of the taxis so that if you
need taxi arrangement, please let them know so they
can do that for you. Ten minutes we will start again.

(Whereupon, the foregoing matter went off
the record at 9:55 a.m. and went back on
the record at 10:10 a.m.)

CHAIRPERSON RABINOVICH: Is Dr. Broker
here? Great.

If you could take a seat please. The next
speaker is Dr. Thomas Broker from the University of
Alabama at Birmingham speaking on viral latency-
papilloma virus model.

DR. BROKER: Thank you very much. I would
like to deal with two subjects under this topic. The
first is a study of the prevalence of HPV in the
general population, and then following on Dr. Mayer's
pattern that you just heard, a study of some
endogenous sequences in papilloma virus transformed
cell lines with some surprising results.

We have done some inside 2 hybridization
studies of the expression of human papilloma viruses
in biopsies from women with HIV/AIDS who were
moderately immuno deficient. This is one example, but
fairly typical.

What you are seeing is a full thickness of
across the cervix. The various probes that we used
reveal the expression of one of the major early transcripts of papilloma virus, the E4, E5. You are seeing it here in bright field illumination and dark field, matched pairs, basal layers right there. As I indicated yesterday evening, papilloma transcription is differentiation dependent and occurs typically in the upper half of the skin. E6, E7 messages, the delayed early oncogenes are hard to see in bright field, but fairly easy to see in dark field. You can see they follow a comparable distribution. The capsid component, L1 or L2, again, is right at the very top of the last live layers of the epithelium. Also to the point, the vegetative amplification of viral DNA is in the upper half of the epithelium. Papilloma infections of the genital tract in fact have been designated an official AIDS-defining illness in the syndrome because of the significant upregulation of HPV gene expression in women who have AIDS or other immuno deficiencies. With that knowledge in hand, and pictures like this, we undertook the following study. We decided to investigate the prevalence of HPB in the population by focusing on immuno-
deficient groups. The three that we have chosen so far are: women who are in enstay renal failure and in need of a kidney, and most clearly ill; those then who get a kidney and are pharmacologically immuno-suppressed beyond their underlying illness; and those with AIDS.

The strategy that we're using is an extension of the techniques Steve Wolinsky and I developed really 12 or 13 years ago when we first proposed the use of degenerate primers for looking at related genomes. The pair that's most commonly used in the papilloma field is our original design called MY911, but Louise Chao and I moved right next door. We found that this region is a little too long to use in form one fixed tissues, and this particular pair has some wonderful restriction fragment polymorphisms available that will allow us to do genotyping after amplification.

So basically we start with the nested PCR approach, outer primers and inner primers. The starting material is cervico vaginal lavage, which harvests cells from throughout the lower genital tract of the women. We amplify and then we put it through several different assays. Initially, agarose gel electrophoresis to look for a 278-base amplimer.
Secondly, restriction fragment polymorphisms which usually can tell us which genotype is present. But if it's a pattern that we cannot recognize, we will put it through sequencing. As you are going to see, about half of the fragments that we amplify we need to sequence.

The results of this study, I am going to summarize. It's absolutely mind-boggling. Seventy-four percent of all women in the AIDS cohort have clearly identifiable HPVs. We have managed to type over 85 percent of these so far. Fifteen percent are still under investigation. In more than half the cases, the individuals yield multiple HPV types.

The study is longitudinal, and has been going on for three-and-a-half years now. Many of the members of the cohort have been sampled two up until seven different times at six to 12 month intervals.

So that's our biggest cohort.

These are the renal transplant cohort. We have statistically significant numbers. I would like to point out that in instage renal failure, but no pharmacologic suppression, about 59 percent of those women have detectible HPV. Again, quite a few, a high percentage have multiple infections. This carries over to that portion of this group who go onto actual
transplantation.

Some of the remarkable outcomes of the care with which we undertook the genotyping is the following. In the pre-transplant population, the prevalent types are those that are commonly seen in the general population as causing disease, namely HPV-6, 11, and 16. Those types persist in those women who were pharmacologically immuno-suppressed.

We see a scattering of other types, but the common types from prior studies are those that predominate in the renal transplant cohorts.

In contrast, those women who are in various stages of immuno-deficiency as a result of AIDS, do not show the same genotype profiles. The only member in common is in fact most common of all genital HPVs, HPV-6. What we see instead are niche homologs of the common types. For example, HPV-45, as you are going to see, if a close relative of HPV-18, which is often cited as a common virus. But we don't see that in the AIDS cohort.

HPV-52 is our most common virus. It is a close homolog of HPV-16, which we don't see amplifying in this cohort. Most notably are the ones that I indicated by stars, which are a very rare detection within the general population, but in fact are most
common viruses in the AIDS cohorts.

In particular, we have identified 13 new HPV types based on less than 75 percent sequence homology to each other or to any other known papilloma virus. They are all members of what has been designated group A-3, which appear to be an AIDS-defining subset of HPVs.

These can be at least considered in the context of phylogenetic trees based on sequence alignments in the L1 region. So, for example, HPV-16, the main cause of cervical cancer in the world, is seen in the renal cohort, but a very close relative, 52, is seen in AIDS.

Six and 11, that cause benign genital warts and laryngeal papillomas are here. One of the main groups coming up in AIDS is this group of cousins of these guys.

HPV-70 is one of our most common types, as well as 45. They are in the HPV-18 family, but represent new members of this niche. The group I just mentioned, A-3, that is so commonly seen in AIDS, include our members jyn 2, 3, 4, all the way up to 13, MM8 and 61, 72, and 83. That cluster seems to be an AIDS defining group. The other ones that we have seen abundantly are 51 and 53 in this arm.
Overall, in the Birmingham and generally Alabama population, every virus types seen with the star we have found one up to 23 times, indicating that we have universal presence and also detectibility of all of the known viruses within our immediate population.

While this is up here, I also wanted to point out the very large huge group of epidermal dysplasia formus viruses that other labs have studied. Again, it is a very rare group of illnesses, in fact, only defined a few hundred times in all of medical history in terms of individual patients. However, there is this huge ramification of somewhat related, but clearly distinct genotypes that comprise the family or subgroup of viruses responsible for EV.

It is known that these patients all have particular cell-mediated immune deficiencies. Again, suggesting that particular arms of the immune system are responsible for either containing or failing to contain different subgroups of the papilloma viruses.

As we look at these women over a period of time through these six month or so samples, what we also find, and other labs have exactly the same results, is every time we sample, you may or may not see the type you saw before. It may switch. For
instance, we have this patient who had 6 plus 16, and
then 11 plus one that was minor and we couldn't tell,
then jyn 2, and then type 40, and then we had a type
53, but the others disappeared.

Everyone's experience in the field has
been that the viruses rise above a detectibility
threshold, stay there for a while, days or weeks or
months, and then fall below detectibility, only to be
replaced by a different HPV type. These are not new
infections. They are basically cryptic or latent
persistent infections that fluctuate in their levels
of replication and detectibility. Pretty much anybody
is showing that flexibility.

What I want to state at this moment before
showing the correlation with disease may sound
controversial, but I will stick by it. We have found
a brand new HPV type for every 10 people that we have
looked at. Philodelius and Ethel Michelle Diveres and
zur Hausen and Shamen in European study of tutanias
papilloma viruses have found a new papilloma virus for
just about every other person they have looked at when
they use the combination of nested PCR and DNA
sequencing.

Robbie Burke's group, Jill Polefski's
group, have very comparable experiences looking at
anal papillomas or female genital tract.

It is my contention right now that instead
of 80 HPV genotypes or 150 that have been officially
named, that there probably are millions of variants,
virtually a continuum. We feel that basically
everybody has their own personal microflora, that
these are passively acquired or vertically acquired,
not necessarily sexually, but certainly possibly
sexually, and that they simply are part of the human
condition as are microflora, just as we have
microflora composed of bacteria and many other
viruses, and that they basically are utterly
ubiquitous. I will come back to that point in a
moment.

We did try to correlate the various other
medical parameters in these cohorts, especially the
AIDS cohort, with CD4 count, HIV virus load, other
infecting known STDs like herpes, chlamydia,
trichomonas, so forth. The one correlate that held up
and not surprisingly at all, was that the degree of
pap smear abnormality from normal, abnormal cells of
unknown significance, low grade dysplasias or high
grade dysplasias, is with CD4 count.

The medians, these are all the people who
had multiple infections, a high risk virus type, a low
risk, no virus at all, and had either normal or these various abnormal pap smears. These bars here are the median CD4 count in each of these groups.

The one place where we saw active disease, low and high grade dysplagia, these by median, is when people fell below the CD4 count of 200 cells per cubic millimeter.

In summary of that data, we found that it's very very possible to have negative pap smears, but definitely have HPV infections. We feel these are people who have not yet reactivated long enough to have resulted in cytologic change as a result of infection. We have on the other hand, the people with overt disease by biopsy or by cytology, and the higher the grade lesion, the more likely it is to see either single infection or especially multiple virus types present within that patient at that time.

So the more that we can detect the virus, that is, the more it has replicated or amplified throughout the population, the more cells that are shedding the virus in effect, the more likely we see disease.

So to summarize this part of the talk, I feel that they are virtually ubiquitous. they are typically sub-clinical, persist in or latent
infections. There are staggeringly large number of
genotypes if we take the care to look. I might say
that the reason these are typically not found is that
people use generic cross-hybridizing probes or have
cut off their probe sets. If you're not probing for
something, you are not going to see it.

Most of the viruses in this number 60, 70,
80 and above, are not even present within the
commercial probe sets. So if you aren't probing, you
are not going to see them, and you are going to get
lower numbers.

They can be found throughout the genital
tract in 60 to 75 percent of the people that we have
looked at who are admittedly good yielders, because
they are immuno-compromised, but I think this simply
represents the general infection in the population.
They can be found in oral and esophageal
mucosa. Utaneous types persist in hair follicles.
There's a wonderful study from Amsterdam by Tershaget
and Ingebor Boxman. She plucked hair follicles, both
eyebrow hairs and pubic hair, and 60 to 70 percent of
all people harbored EV viruses or other rare virus
types in their hair follicles. No disease, it's just
part of the human condition.

I believe they are vertically transmitted
perinatally, mother to baby. Some of them are clearly
pre-natal infections. As we know, there's long-term
maintenance that requires viral replication in concert
with host replication in the cell cycle.

So what I would like to do now is tell you
a little bit about a very unexpected observation we
made in Hela cells. This goes back to last night's
talk regarding the structure of the replication
complex of HPVs.

As you know, cyclin E is one of the key
checkpoints or entries into S phase. Ectopic
expression of cyclin E can speed up entry into S
phase, and it can even bypass the need for some of the
RB phosphorylation by cyclin D. It's simply one of
the key steps that needs to follow the induction of
the DNA replication enzymes.

HPV E7, the viral oncogene that in fact
binds RB and can help bypass that step, among the E2F
enhancer protein regulated genes is cyclin E itself.

In other words, HPV infection upregulates cyclin E.

So we asked whether the induction of
cyclin E is essential for the reactivation of
unscheduled cellular DNA synthesis in the upper
stratum of squamous epithelium that differentiated
caratinocye. I'll just summarize that data.
I got you to the point last night where

the El diheximer, the double helicase held together by
the HSP-70 co-chaperone protein, is there.

The next thing that loads in the study we
did with Theresa Wong at Stanford, is the recruitment

of the cellular DNA polymerase, and showed direct
interactions between the helicase and the catalytic
sub-unit of pol alpha, P-180, as well as its P-70 sub-
unit. This was the first indication of what P-70 does
in the four sub-unit complex of pol alpha, which
includes two primary sub-units. The answer is, it
brings the polymerase to the ora itself.

The next thing that comes in is cyclin E,
CDK-2 complex, that critical S phase entry point. AS
a result of that, what happens is upon cyclin E

finding an appropriately assembled pre-initiation
complex, five target proteins are phosphorylated.
They include: the E2 protein, which appears to be
displaced by that event; in addition, P-70 helps
displace E2. So the loading of this and the
phosphorylation kicks this guy out.

Secondly, El is phosphorylated. These two

sub-units of preliminary salpha that bind directly to
El are phosphorylated. When all four of those have
been successfully modified, the kinase phosphorylates
cyclin E itself, which is displaced and degraded by ubiquitination. That enables the pre-initiation complex to convert to the elongation complex.

In studies with Wade Harper and Jien-Ling Ma at Baylor, two things were done. The first is together we found that there's a cyclin binding motif that the amino terminal have at the El protein, which in fact is shared with a number of other things that bind the cyclin E. That motif involves an RXL. That is, an arginine something leucine motif right there.

In addition, their candidate phosphorylation cites, the series of serine, serine, serine, and threonine, mutation of any of these, the motif or any of the target phosphorylation cites, diminishes the capacity of cyclin E to convert the pre-initiation complex to an initiation complex. So the functional requirement for phosphorylation has been verified. But keep in mind this location. We'll come back to it in a second.

So we assumed that the consequence of upregulation of cyclin E by E7 gene expression would identify those cells that are capable of supporting papilloma replication. To our amazement, we found the opposition. This is our epithelial raft model. We have done the same in natural papilloma lesions. Here
we monitored cyclin E expression, over expression in
the tissue. Here's bromo deoxy uridine incorporation
or PCNA upregulation. These are the match.

What we found is the cells that had high
cyclin E could not replicate. In fact, they are
mutually exclusive with those capable of supporting
DNA synthesis. Conversely, PCNA, which is upregulated
by papilloma 7 and cyclin E do co-localize. But we
see a number of cells where PCNA is present and there
is no cyclin E. So we have a reciprocal pattern to
what we expected.

I am just going to very briefly tell you
that P-21 cip, one of the inhibitors of cyclin D and
cyclin E, is also upregulated by E-7 expression in
natural condylomas or in our E-7 expression raft
cultures. You can see those signals in the upper
strata again. So we have P-21 upregulation, again, in
a subset of cells.

When we look in rafts or in natural
papillomas, we see that those cells that have high P-
21 are mutually exclusive from those capable of
supporting either viral or cellular DNA synthesis.

When we did the third pairwise combination and looked
at cyclin E and P-21, we found perfect colocalization
of those two.
So ironically, the cells that have high cyclin E also have high P-21 and do not support replication. This was really perplexing, except we did know this inhibited that. But we assumed cyclin E was in the licensing factor for engaging in replication.

So what we came to feel is the following model: that in the course of unscheduled DNA synthesis reactivation, if cyclin E appeared in the appropriate timing or sequence or amount, once a pre-initiation complex formed, you would successfully phosphorylate the target proteins, polymorases and E-1 and E-2 proteins, and successfully engage in elongation replication.

Conversely, if too much cyclin E appeared and it appeared in an untimely fashion, its inhibitor, P-21, would recognize misassembled complex. They would cross stabilize. They would both pile up to high levels, and those would be defective in engaging in elongation.

Now we put this all together by asking how does this play into the establishment of immortalized and transformed cells and cancers. What Wade Harper had found is that when he did pull-down assays with cyclin E to ask in hela cells what binds to cyclin E,
almost all the things that came down in the assay was that E-1 protein from the resident HPB-18 in the cell lines. Up until that point, people had thought the E-1 gene was deleted from hela. In fact, it's present. The entire length of the E-1 gene is still present in hela. In fact, is expressed.

Now the functional assay that our lab did was that we found that hela cell extracts could not support HPB replication in our cell-free system, that there was a missing factor in hela that the extract needed. We could put 293 cell extracts or any other cell line that we could find, they would easily complement papilloma replication in vitro. But anything from hela cHa caski or any other HPB transformed cell line could not support it.

The upshot of the whole thing is that every papilloma transformed cell that we studied expressed a full length E-1 transcript, but in all cases, the transcript had an either frame shift or a stop code on partway through the gene or miss sensed mutations in this vicinity, so that in tact E-1 could not be made. But in all cases, it made the RXL portion that interacts with cyclin E.

So we added a little cyclin E back to hela cell extracts and immediately restored full
complementability to those extracts, establishing that the missing thing in hela cells was cyclin E.

So our conclusion is the following. I think we feel very confident about it. In the process of immortalizing cells, either natural cancers or attempts at making cell lines, substrates in effect, it's good to have all the upregulation of DNA polymerase, topasomerases, PCNA and so forth, that help rapid cell cyclin. But the one thing that E-7 upregulates you don't want, is cyclin E.

So what these natural experiments did, is figured out a way to sequester part, but not all, of the cyclin E by putting in retaining fragments of E-1, capable of mopping up that one product that's upregulated that you don't want to have. That is, cyclin E.

Brian Van Tine, last night, also indicated there's evidence of some antisense in other papilloma lines like cHa caski, which would again, modulate the amount of E-1 that you could translate from the messages that are clearly there.

Together we believe that to establish these cell lines, whether you make them in the lab or whether nature has made them for you through cancer, you need to diminish the amount of cyclin E to achieve
an equilibrium where you have enough cyclin E to 

support cellular replication, but not too much so that 
it's an unsuccessful high level. Thank you very much. 

(Applause.) 

Oh, I did want to acknowledge -- could I 

have the slides for one second -- a very, very large 

number of collaborators. I'll leave them up here. 

But we're very grateful for our own students and 

collaborators at UAB, Jeff Engler, Doug Seer, Sean Van 

Tine, Kim Towns. At Baylor, Wade Harper's lab, UNC, 

Jack Griffith's lab, who did the things with the HSP- 

40, and at Stanford, Theresa Wong, and our 

collaborators at the Free University, who did a lot of 

work on some transcriptional control that we 
collaborated on for several years. Thanks. 

CHAIRPERSON RABINOVICH: Thank you. Are 

there any questions? 

DR. FRIED: How could you be so sure that 

with the evolution of the HPVs, are not due to new 

infections, but to pre-existing sequences to cover? 

DR. BROKER: I think one is the repetity - 

- 

DR. FRIED: Mike Fried. 

DR. BROKER: With which these viruses are 
appearing, especially in women who at least declare
they are not engaged in much, if any, sex. A number
of the epidemiologic studies have traced either the
frequency of recent sexual activity. Everyone who has
looked feels its an emergence of pre-existing
subclinical infections. There is some evidence in new
infection, but an awful lot of it simply appears.
Women in that stage renal failure, for
example, who are 38 years old and married basically,
are not suddenly acquiring new infections.

CHAIRPERSON RABINOVICH: Back microphone,
please. Why don't you come up to the front.

DR. RUSSO: Hi. Carlo Russo, from Merck.

Very interesting talk.

I have a couple of questions. One is, how
can you be sure that what you are sampling by PCR is
really an infective virus, it's not just a transient
presence with a virus due to the fact that you can't
control sexual behavior, and perhaps the woman has
just been exposed to a virus?

DR. BROKER: Well, I think one way is that
in a very high percentage of the people, there are
clear dysplasia, low and high grade. All the women
who have any degree of dysplasia are also biopsied,
and the inside 2 hybridization, as you can see, is
showing clear effects in the tissue.
So I don't think there's surface adventitious contaminants. These are within, at least a fair number, if not all, are within the cells, and causing various degrees of actual overt illness.

DR. RUSSO: I may have missed the data.

Did you show the types that are associated with high grade lesions, the HPV types? I didn't see on the table.

DR. BROKER: Yes. Well basically, in this immense spectrum of what's now 37 different viruses that we found, those that are most typically associated with low and high grade dysplasia, the actual diseases, are the higher risk types.

DR. RUSSO: So you are not suggesting that if you want to prevent cervical cancer, we should focus on different types of the one already identified?

DR. BROKER: Well the real problematic thing for any clinical management, either vaccination programs or small molecule drugs, is this absolutely exploding number of virus types.

The one thing that I think is going to --

and I commented a day or two ago that in the U.S. alone today, there are over 250 to 300,000 people immuno-suppressed just due to organ transplants,
steroid use, or bone marrow transplants or AIDS. So there is an immense reservoir of particularly high risk patients.

Nonetheless, most of the diseases are still being caused by a handful of viruses like 16, 18, 52. So I think, at least the ones we have to worry about today, are still manageable in number.

AUDIENCE MEMBER: I would like to ask a little bit about cell substrates. Considering that hela has multiple HPV integrants, I guess, are any of those infectious?

DR. BROKER: No.

AUDIENCE MEMBER: Can you get them back and make them infectious?

DR. BROKER: No.

AUDIENCE MEMBER: What are they missing?

DR. BROKER: They are all truncated within E-2, at best. Although there are 30 to 50 copies, depending on the hela sub-1. All the integrated copies are truncated.

AUDIENCE MEMBER: Would that DNA be transforming, even not infectious?

DR. BROKER: They do contain the E-6 and E-7 genes. Expression of those genes, as shown by studies primarily in zur Hausen's lab, must be
maintained or you no longer can cycle hela cells.

That is antisense to E-6 and E-7 in hela makes them not cycle any more. So the driving force of hela is the overt expression of E-7.

AUDIENCE MEMBER: Would hela cells qualify as an example of a cell that should never be used to make a vaccine?

DR. BROKER: I don't know that I would go that far. It's obviously wonderful as a producer of all sorts of biomedical products.

DR. MURPHY: I actually wanted to ask you the same question, but I would phrase it in a different way.

(Laughter.)

DR. MURPHY: This is Murphy from NIAID.

That is, do you see any reason why, you know, having an intimate knowledge of hela cells and human papilloma viruses, that hela cells should not be used as a substrate for making live attenuated virus vaccines?

DR. BROKER: I don't know of any evidence of these genes being transduced out or in any way posing a risk. I was going to save it for the panel, but it occurred to me last night, I had challenged one of last night's speakers about the use of psorilins as
a cross-linking agent. It gives me great concern that it is a known carcinogen. However, Brian and I were talking. Brian van Tine and I were talking last night, and he reminded me that there are biotinylated psorilins.

So for all the debates regarding how to remove contaminating DNA, one strategy in principle is to throw in a biotinylated psorilin, cross link it, and pass the whole thing over avidin magnetic beads or batch subtraction of the DNA.

So in fact, that strategy may actually help you deplete adventitious contaminants very, very readily. So it is an alternative at least.

AUDIENCE MEMBER: Can I just ask you about hela again? We learned last night that not every HPV, if there's 30 to 50 copies, are not all active, I mean in caski only one was active.

DR. BROKER: Yes.

AUDIENCE MEMBER: What is the state of hela? Are they --

DR. BROKER: Very, very few are active.

We and Wade Harper's lab are both sequencing all the transcripts. This actually was done by Elizabeth Schwartz and others in zur Hausen's lab in 1985, and a variety of groups since then in Japan and elsewhere
have looked at the expression loci in copies in hela.

There appear to be three or four different transcripts made from different positional integrants, but the majority are silent. A few of them are active. But so far, all the ones that are active have truncated E-1s. They have the cyclin E binding motif, but they don't have their normal carboxy terminus.

CHAIRPERSON RABINOVICH: Thank you very much.

Let's go on to Dr. Cashman. Thank you for being so patient. Transmissible spongiform encephalopathies: vaccine issues.

DR. CASHMAN: It worked. My friendly A/V guy explained how to do this.

I am Neil Cashman. I am predominantly at the Center for Research and Neuro Degenerative Diseases at the University of Toronto. I have a special interest and a long-term research effort in the expression and function of the normal cellular isoform of the prion protein.

I am also obliged to mention that I am the chief scientific officer one day a week of a little biotechnology company in Montreal called Caprion.

I want to spend a few minutes talking about prions and prion disease. We had a speaker
yesterday who said "and now for something completely different." Well, how does a genomeless infectious agent grab you?

Creuzfeldt-Jakob disease is the most common human prion disease that we run into. I do want to spend a few minutes talking about this so that we are all on the same page with regard to public health risks. Creuzfeldt-Jakob disease or CJD is basically a disease you wouldn't want to wish on your worst enemy. It is a completely untreatable uniformly fatal disease resulting in death within six to nine months of presentation. Survival over a year is recorded, but it is not very frequent.

The presentation is usually that of a kind of Alzheimer's-like syndrome, with problems in memory and intellectual function, but it can also present as a disorder of gait and balance as well. Most people have mild clonus, which is twitching of the muscles, sufficiently forceful to move a joint. Other features of the neuro-degenerative syndrome are reminiscent of other neuro-degenerative diseases like Lou Gehrig's disease and Parkinson's disease. Basically it's like having every neuro-degenerative disease at once, telescoped into an unmercifully short period of decline.
Fortunately, it is rare. Sporadic Creuzfeldt-Jakob disease occurs at about one per million population per year. Also, somewhat fortunately, it's not a disease of children. The average incidence of CJD is in the 60s.

There are three recognized forms of CJD. The most common being sporadic. This is a spontaneous onset of CJD in an individual for which we have no clue why they have developed it. There are familial variants, which seems to be passed as an autosomal dominant in families. That constitutes about 15 percent of the cases of human prion disease that occur. There are iatrogenic prion diseases, which are caused essentially by treatments and surgeries, well-meaning, but nonetheless transmitting the disease.

Of course the transmissible spongiform encephalopathy that even my kids know is bovine spongiform encephalopathy, or so-called mad cow disease. Since the early 1980s, this disease has affected about 200,000 cattle in the U.K. and Republic of Ireland, and a few hundred across continental Europe. About 2 million cattle have been killed in an attempt to stem the epidemic. This culling, as well as change in policies, such as feeding ruminant to ruminant -- we turned cattle into neo-cannibals -- is
resulting in a rapid decline of new cases, predictions being that the epidemic in cattle may be essentially stamped out in the early part of the new millennium. I won't even say the new century. I'll say the new millennium.

Unfortunately, this disease is unique, unlike every other known naturally occurring prion disease. It doesn't seem to obey species barriers, or at least obeys them to a much lesser degree. There is an outbreak of feline spongiform encephalopathy in house cats. There is spongiform encephalopathy in zoo animals, including primates. The primates that we are most concerned about are also vulnerable to this disease.

To date, 44 people have developed a new variant of Creuzfeldt-Jakob disease, which is clinically and pathologically distinct from classical CJD. The statisticians predict there will be somewhere between a few hundred and maybe 80,000 cases. This does not include the chicken little predictions of the extent of the epidemic.

The disease unfortunately seems to strike the young. There have been teenagers involved. It is a relatively slower progression than classical CJD. There are clinical features that are distinctive, but
I won't bore you with them this talk. The pathology is also absolutely distinct, including a preter natural accumulation of PrP Sc, which is this abnormal amyloid protein that's been linked to infectivity. This occurs both in the brain and in peripheral lymphoid tissues.

Well, before we leave the clinical stuff about CJD and prion disease, I want to kind of set the stage with a sobering statistic, which is there is iatrogenic transmission of this group of diseases. Considering the penetrants and the young age of vaccinees, this is a scary possibility. This would dwarf every other iatrogenic transmission known to date.

In humans, basically a few hundred cases have been attributed to iatrogenic transmission, from hormones extracted from cadaver pituitaries, from dura mater transplantation, which is the tough lining of the brain. But incredibly, the largest iatrogenic transmission known to date, also the first documented, was that in passage with a vaccine, which was a vaccine for looping ill of sheep. Formal and inactivated brain preparations passed sheep scrapie to about 1,000 sheep. So hopefully this will not be a pattern with human vaccines.
Well here is the prion hypothesis. This has gone from being an object of ridicule to the middle of the road interpretation of prion infectivity. It has been sanctioned by the Nobel Prize committee, garnering the prize for Stanley Prussiner, the investigator whose ferocious work with this group of disorders and with this agent has given him, in my opinion, a well-deserved Nobel Prize.

The basic tenants of the prion hypothesis are that there's a normal cellular protein, which is called PrPC, which has been cloned and recognized. It's expressed by just about every organism down to drosophila. It is a very old gene. It's incredibly well conserved in evolution. It is predominantly alpha helical in secondary structure.

Now this normal cellular protein can adopt an alternate confirmation, which is rich in beta sheet structure. When this protein is in this alternate confirmation, it acquires many unique physical chemical properties. It becomes partially protease resistant. It tends to aggregate. It's very poorly soluble. Plus, it then seems to act as a catalyst for recruiting more confirmational copies of itself.

Now whether this occurs by a kind of enzymatic confirmase activity or whether this is kind
of a biological crystallization phenomenon is being actively investigated. But it is clear that this abnormal confirmation isoform of the protein, called PrP Sc for scrapie, is capable of recruiting more confirmational copies of itself from the normal cellular isoform.

So onto vaccines. There are some concerns about vaccines. I will mention three areas that need to be considered. I will dwell most of the time on cell substrates, which is nice of me considering this is a cell substrate meeting. I will also talk briefly about potential prion infectivity coming over in media supplements for those cells, and in excipients, which are compounds used to stabilize vaccines in their final formulation.

In this case, luck seems to be at least partially on our side, because it's not easy to infect cells in vitro. It is possible to infect primary neuronal cell lines neural blastoma. But there is not much infectivity, and basically each successful infection of a cell line is worth a publication or 10.

This may be due to the fact that cell lines have very little PrPC, which is the precursor for PrP Sc. The conversion of the protein from PrPC
to PrP Sc forms occurs at the surface or a post-
surface compartment. So in general, cell surface
abundance of the protein correlates with infectivity.
Most cell lines in my own laboratory, including hela,
express no more than one-tenth of the amount of cell
surface PrPC that a primary neuron does.

It has also been thought that the very act
of cell division itself can kind of sterilize a
culture because cell division can out pace the
relatively slow conversion and processing of PrP Sc.
So if you have a couple units of infectivity, they get
progressively diluted by having huge numbers of cells
that bear no infectivity. Finally, those cells may
die, the infected cells.

There is also a poorly quantifiable role
for cell biology, which I put in quotation marks here.
Things that we really cannot quantify at this point,
like proper trafficking, post-translational
modifications of PrPC that are important in
conversion, and even sub-cellular distribution. The
protein seems to accumulate in this glycosal
phosphatindinol rafs at the cell surface. Some cell
lines don't seem to support these sort of rafs.

Now to make some kind of estimate about
the spontaneous development of prion infectivity in a
cell culture, especially a vaccine cell culture that may have hundreds of trillions of cells, I am going to back up and try to explore some assumptions about the spontaneous development of prion disease in humans, which is the species for which we have the best numbers.

According to the prion hypothesis, an occasional accidental mis-folding of PrPC to PrP Sc is what triggers the recruitment process which proceeds on an exponential basis. Each molecule that's converted converts to more, da, da, da, da, da, da, which happens on a post-translational level. No genome involved. "Look, ma, no genome."

But sporadic disease in humans is incredibly rare, one per million people per year.

Humans have something on the order of 100 billion neurons. So one can make the kind of interesting calculation that a productive infection arising from a single neuron, you need about 10 to the 17th neurons, 100 million neurons across a million people in order to develop spontaneous scrapie.

But of course that's not the only way one could potentially develop spontaneous CJD. Discovered by familial CJD and familial prion diseases, there are mutations within the open reading frame of the prion
protein that apparently predispose to this accidental
misfolding, such that somebody with a prion protein
mutation that actually results in an amino acid
substitution is basically guaranteed of developing the
disease over the course of a lifetime if he or she

lives long enough.

So could this occur in vitro? Could
certain cells in vitro acquire a somatic mutation
which is then propagated to infect an entire culture,
again, on a post-translational level? Well, let's run
some numbers on this one.

The mutation rate in man is about one per
billion basepairs per year. I thank Dr. Kazazian for
yesterday for pointing me to this reference. Thank
you very much.

The prion protein open reading frame is
really less than 1,000 basepairs. It's a relatively
small protein that's all contained in one exon. This
gives rise to a kind of pseudo calculation that a cell
can develop a mutant prion protein gene, a cell in
vivo, and a human can develop a mutant prion protein
gene in about one out of a million cells per year, if
you take one out of a billion and multiply it by
1,000, that's one out of a million.

This, just as an aside, this gives a rise
to a kind of startling calculation that all of us in
the audience are generating about 100,000 prion
mutants in our brain per year.

However, there must be a safety factor
here because the rate of prion disease arising from
somatic mutation cannot exceed the calculation of
prion disease arising from individual neurons that we
just went through in the last slide, which is 10 to
the 17th neurons per year.

So incredibly, somatic mutation is a
seriously flawed non-efficient process for producing
prion disease. Something on the order of one out of
100 billion mutations are productive of infection.
This may give us some comfort when we turn to the in
vitro scenario.

So let's talk about cell substrates. Is
it possible that spontaneous prion infectivity could
arise de novo in a culture? I told you that substrate
cells have usually less than one-tenth PrPC than
neurons. So if we run these calculations, by
misfolding one substrate cell per 10 to the 18th
years, and I think that is a quadrillion or something
like that, it's certainly comfortably larger than the
projected age of the universe.

Substrate cells, however, are less
genomically stable than primary neurons. If one says
that there's say 1,000-fold greater rate instead of
one out of a billion basepairs, one out of a million
basepairs can be mutated per year in a substrate cell,
this gives rise to a calculation suggesting that you
need 10 to the 14th substrate cells per year in order
to have one productive, i.e. spontaneous infectivity
arising in a culture.

Now this number looks incredibly large,
but when we think about the numbers we heard last
night about the production of polio virus from vero
cells, according to my calculations, 300 million cells
are used per year to generate all those vaccine lots.
Ten to the 14th is only 100 -- sorry, 300 trillion,
and 10 to the 14th is only 100 trillion. Did I get
that right? Please forgive me and correct me if I
didn't get it right.

So perhaps it is possible, considering the
enormous scale of substrate cell culture, that prion
infectivity could arise through somatic mutation in a
substrate cell, and could contaminate a vaccine
destined for human beings.

Well, there are some things to talk about
with this model. If this is so, how come we haven't
seen any vaccine transmissions yet? One of the
factors is that very few human cell line vaccines have
gone into humans, certainly not on a scale of vero
cells or primary cells that are used for culture.

Another unsettling thing is that if indeed
there is somatic mutation in a culture of human
substrate cells, would we ever detect it? We are
talking about something that would occur in one out of
a million cells or even one out of a thousand cells,
would ever be able to find by PCR or SSEP or anything
you could think of, a mutation at this level.

So aside from substrate cells, I did want
to touch upon a few potential sources of infectivity.
The media coming in contact with substrate cells are
potentially carrying prion infectivity. Bovine serum,
fetal calf serum, and newborn calf serum is used as a
supplement for proteins and growth factors and
hormones for most cell lines. Some cell lines are
also supported by human serum albumin. I am not aware
of a vaccine cell line that's supported in this
manner, but many recombinant proteins are supported
with human serum albumin.

There's also potential prion infectivity
in excipients, this last compound that's added to the
preparation to keep it stable before use. Many
childhood vaccines are stabilized with pig skin
gelatin. Pigs don't seem to be a species which
spontaneously develop prion disease or a species that
can catch prion disease via the oral route, although
deliberate intracranial injection of BSE infectivity
can produce a prion disorder.

Human serum albumin is also an excipient
in measles, mumps, rubella, and rabies vaccines. I
would like to spend just a few seconds talking about
human plasma proteins before I close, and give my
final advice, such as it is.

Human serum albumin of course comes from
humans. Of course it's a plasma fractionation
product. There has been a great deal of work trying
to identify potential risks of transmission of CJD
from human to human through blood or blood products.

Suffice it to say that population studies, case
control studies, and cohort studies have proven
universally negative. There is no documented
incidence of human CJD, classical CJD being passed
through blood or blood products.

There are of course case reports of people
getting a transfusion and developing CJD, but one
should not expect that transfusion or administration
of a blood product is protective against CJD. The
incidence of CJD in the transfused or treated
population is the same as in the non-transfused or
treated population.

However, we're in more difficult territory
with variant CJD. This is, I told you, an unusual
agent. It seems to cross species lines with impunity.

There are other features that are quite scary with
regard to human blood and human plasma products,
including albumin that might be used as an excipient.

The agent starts of course in the
periphery through oral exposure, suggesting a
prionemia. There is a huge accumulation of PrP Sc,
our only surrogate for infectivity, our only
biochemical surrogate for infectivity, in not only
brain, but in lymphoid tissue.

The agent itself has odd properties. It
is stable across species. It may in fact be
specialized or selected. I realize that these terms
are not often applied to a protein. I'm borrowing
terms from agents that contain a genome. It suggests
that this agent may be more virulent, especially with
regard to peripheral exposure. In other words, one
unit of classical CJD infectivity will not cause
disease when injected intramuscularly. One unit of
variant CJD infectivity may very well. There is no
data.
Another thing that has been noted is the young age of onset of variant CJD. This has been attributed to kids eating hamburgers and all kinds of weird meat products. But it could also be attributed to host factors which would promote infectivity in the young. Since vaccinees are usually young, we have to take this in mind.

But our greatest risk factor here is that this is a new disease and we have no data. So how do we minimize the risks from vaccine transmission? Basically there's three ways that I can think of. I'm sure that other people can think of more. It is important to add prion validation to the list of agents and microbes which are tested for in vaccine lots.

This could be done two ways. The biochemical marker of infectivity is PrP Sc. This technology is in evolution. It appears that capillary electrophoresis, some types of optimized immunoblotting, and even ELISAs are reaching the point where one unit of infectivity will be detectible.

Another important way of assay for infectivity is called the bio assay in the field, in which selected samples are injected into a species which is capable of supporting that infectivity. That
would be non-human primates, and again, a technology
in evolution, transgenics engineered to express human
and perhaps bovine PrP.

There is also the possibility of trying to
prophylax cultures, substrate cultures with chemical
agents. This is also in evolution, but the classic
molecule in this regard is congo red, which not only
seems to bind to PrP Sc, but seems to dissolve
infectivity in vitro. New discoveries out of Byron
Kohe's lab that tetrapyrrole, including porphyrins and
phthalocyanines, can block infectivity. Perhaps some
of these compounds can be utilized at appropriate
concentrations to use as a kind of antibiotic for
substrate cultures.

Finally, the slam dunk in this area would
be to develop a cell line that lacks a prion protein
gene. The prion infectivity, whatever the hell it is,
seems to be absolutely dependent on the presence and
expression of PrPC. So if one were able to ablate the
prion gene out of a substrate cell, that didn't come
with 300 other bad pathogens, this may be a strategy
of obviating any prion infectivity in vaccines.

So I would like to summarize by saying
that it is possible, although not favorable, for
substrate cells to be infected with prions. It is
possible, considering the huge bulk of cells that are
cultured, 300 million a year for one vaccine, that
prion infectivity could potentially emerge by
misfolding and/or somatic mutation in vitro.

I will note that prion components and
excipients may contain prion infectivity. Although
this is an old story with regard to classical CJD, we
don't have the information for the BSC variant CJD
agent. We should worry, at least for the time being.

The remedies for this are selective
sourcing, avoid animals and people that could
potentially be brewing prion infection, biological
manipulation in vitro, including anti-prion agents,
and maybe ablating the prion gene, and then validate,
validate, validate. Prion infectivity should be added
to the list of infectivities that are excluded in
vaccine lots.

I thank you for your attention, and I
would be glad to answer any questions.

(Applause.)

DR. KRAUSE: Phil Krause, FDA. In keeping
with the idea that one presumably wants to find cell
substrates which carry the least risk, I guess if one
presumes that tumor cells have a greater risk of
genomic instability than non-tumor cells, are you then
implying that there's a greater sort of spontaneous
mutation than prion risk from tumor cells than for
instance primary or diploid cells?

DR. CASHMAN: That's a good question. I
guess this is basically not quantifiable. If one
takes a rock solid cell that enjoys all kinds of DNA
repair mechanisms then yes, that is less likely to
give rise to the mutation in the prion protein gene.

One area which should be investigated, I'm
realizing from this meeting, is to take some cell
lines and look at 1,000 clones a piece and see if any
of the prion copies have acquired mutations. So this
would be a piece of data that we could use to actually
discuss this issue. Right now, I don't have any.

AUDIENCE MEMBER: Along the same lines, I
guess the implication would be that if somebody wanted
to produce a vaccine in cells that are derived from
neurons, given the fact that they are making a lot
more of this, you have the potential for greater RNA
polymerase mutation rates, and perhaps also greater
risks?

DR. CASHMAN: Well, neurons are the best
factoring for making infectivity, both in vivo and in
vitro. Part of that is due to high levels prion
protein. Part of it is due to factors that haven't
been identified as of yet, prion receptor, trafficking
of prion protein. So I guess one would want to stay
away from neurons unless they came from an animal or
human that had the prion gene locked out or not
transcribed or translated.

CHAIRPERSON RABINOVICH: Dr. Egan?

DR. EGAN: As Dr. Minor mentioned before,
Japanese encephalitis virus is made in mouse brains.

Can the PrP SC of a mouse catalyze the conversion of
human PrPC to PrP Sc?

DR. CASHMAN: That is an extremely good
question. I neglected to mention in my talk, my
oversight, that there is a very prominent species
barrier between most prion agents, something like one
to a thousand, one to a million, even higher for
generating infectivity, especially between widely
differing species.

Now as we move to human cell substrates,
we will no longer be protected by the species barrier.

Even non-human primates have a sequence similarity to
convert human PrP and vice versa.

So yes, I think that answers it. The
exception to that is of course this new variant agent
which we're all frightened about because it doesn't
seem to obey species barrier.
DR. ONIONS: David Onions. Could I just ask the converse of the question that you posed for vaccine substrates, which is I think one that has been discussed. That is, the idea of knocking out the PrP gene. We know that PrP mice are viable, so it looks like perhaps an interesting way to go.

But can you also engineer cells over expressing normal PrP and use those as substrates for infectivity? You mentioned that one of the problems was the low level of PrPC in most of the cell lines you have used. Can you not over-engineer cells so that they become susceptible?

DR. CASHMAN: The only -- that's never been done. The only data that really pertains is transgenic mice. In fact, if you have knocked out the prion gene, you are absolutely resistant. If you have one normal copy, you have disease with a longer incubation and a shorter rate of -- or a longer rate of progression. If you have two normal copies, you have disease at the normal time. If you have transgenic 10, 20, 100 copies, then the disease presents at an earlier age and is more rapidly progressive.

As far as I know, nobody has moved that observation to an in vitro paradigm.
I would like to challenge, to some extent, your calculation on the risk of mutations, because they do not take into account the similar biologies of prion protein that are secreted from the cell, as they can spread to other cells. That there's really a short time induction of new PrP Sc.

For example, at least to my knowledge, if you infect so to say cell cultures who use prion via PrP, you get another multiplication of infectivity. It stays more or less. If it disappears, it may be more or less there's no real spread.

The question I have is, you are asking for validation studies. What would be the material you would recommend to spike? Should we spike with scrapie brain material? Is this relevant for purification in these cases? What would be the ideal spike?

DR. CASHMAN: I would very much appreciate some challenge from my numbers. They are new numbers, so don't hold back.

The question of spiking, there have been experiments performed at least with a purification of albumin and other plasma proteins. Paul Brown and Robert Roewer published an article last fall in
Transfusion detailing the amount of infectivity if you start from a high amount in whole blood, what you end up with in albumin. In fact, there's at least a 10 to the 6th loss of prion infectivity if one follows the normal protocols for purification of albumin.

The question is whether a single infectious particle is there. The other question is what happens if this single infectious particle is variant CJD. If the single particle is classical CJD, nothing would happen. The disease would not take hold. We don't have enough information about the virulence of variant CJD to be able to answer that question with any confidence.

Did that answer your question? I have a feeling it didn't.

DR. FRIED: Mike Fried. I think your numbers also don't take into account modifier genes, which could be just random mutation.

For instance, I understand that all the people with the new variant CJD have a polymorphism of one type in the PrP, the protein. Is that not true?

DR. CASHMAN: In fact, that is partially right. There is a normal polymorphism in the prion protein open reading frame at codon 117. One can either have a valine or a methionine. To date,
everyone -- well, there's a nice distribution in the
normal population, about 50 percent are heterozygotes
and about 25 percent are met met, and 25 percent are
val val.

In a new variant CJD, everyone who has
contracted the disease to date has been met met. But
that in fact may be due to the fact that the met mets
are more susceptible to the agent. It may not be due
to the fact that val vals or heterozygotes are
resistant.

DR. FRIED: Sure. I am just saying that
that goes into your numbers, whether there's
modifications.

DR. CASHMAN: The point of modifier genes
is a very important point. I tried to -- 129, I'm
sorry. That's David Asher who was keeping me honest
in more ways than one. It's the polymorphism is at
codon 129.

Now what was I going to say? I was going
to say something.

CHAIRPERSON RABINOVICH: Modifier genes.

DR. CASHMAN: Modifier genes, yes.

Modifier genes have of course been proposed from
animal studies of infectibility and experimental
scrapie. Dr. Prussiner has hypothesized a protein X,
which may be a receptor or may be a chaperon that somehow modifies susceptibility of an animal to prion diseases. There is also a protein Y that Dr. Prussiner has hypothesized.

I agree with you from the bottom of my heart that there will be modifier genes affecting susceptibility to prion diseases and the propagation of prion infectivity in vitro. But we don't know what they are yet. At the crude operational level of being able to infect cells, yes. We can infect cells in vitro. So at least some of those modifiers have to be there. Did that make sense?

CHAIRPERSON RABINOVICH: Dr. Hayflick, final question.

DR. HAYFLICK: Hayflick, UCSF. I was intrigued by your observation that the species barrier for prion transmission is less, is reduced between non-human primates and humans, which would raise some additional concern about the use of primary tissue, and particular, primary monkey kidney tissue for the production of human virus vaccines, because contrary to popular belief, that tissue and any primary tissue does not contain -- and I'll use primary monkey kidney as an example, only cells that are derived from a particular part of the kidney.
A primary monkey kidney culture consists of an enormous variety of differentiated cell types that compose the vascular system and neurons. So that monkey neurons do play a part in the production of polio virus, for example, derived from monkey kidney. So that I think that it's important to mention that neurons are not only a part of brain tissue in considering various cell substrates.

Also I was wondering whether there's some reason why you omitted the mention of trypsin as potential source in your discussion of substrates or media supplements for prion transmission. I didn't see trypsin as a component. Was there some reason for that omission?

DR. CASHMAN: Just blanking out. So thank you for adding that to the list.

DR. HAYFLICK: The final question I have is I may have misunderstood your slide in which you were addressing the question of utilization of human cell line, that a human cell line had not been used for the production of enormous numbers of doses of vaccine, for example, in order to support one of your contentions.

Did you mean it in that respect, a human cell line that is defined to be immortal and
transformed? Or did you mean any human cell population?

DR. CASHMAN: Perhaps you could educate me. Has any human immortal or neoplastic cell line been used to develop large penetrants, large population vaccines?

DR. HAYFLICK: No. Not as you have defined that cell population. I was interested particularly in normal human cell populations, which have been used for the production of up to a billion, with a B, doses of virus vaccine. But you are not including in that?

DR. CASHMAN: Say it again. I'm sorry.

DR. HAYFLICK: Normal human cell populations have been used for the production of about three-quarters of a billion doses of human virus vaccine world wide, but these are not continuously propagable abnormal heteroploid cell populations. These are normal finite lifetime cell populations.

DR. CASHMAN: So these are cell strains?

DR. HAYFLICK: Yes, as I defined them. I realize there's a problem in understanding these terms.

DR. CASHMAN: Which vaccines?

DR. HAYFLICK: Virtually all pediatric
vaccines, polio, rubella, mumps, measles, rabies,
adenoivirus, some rhinovirus vaccines, are all produced
on a semi-continuous human diploid cell strain like
WI38 or MRC5.

DR. CASHMAN: Thank you.

CHAIRPERSON RABINOVICH: I think you
better clarify.

DR. LEWIS: Yes. To my knowledge, there
are really no nerve cells in the kidney. The nerve
cells lie on the spinal cord and porosises go down
there. I don't believe there are any nerve cells in
the kidney. Even if they are, once the nerve cell is
differentiated, they basically do not grow on tissue
culture. I think that needs to be corrected.

CHAIRPERSON RABINOVICH: Okay. I would
like to move rapidly to bring the panel members up to
the podium, and to invite Dr. Onions to come over and
run things.

DR. ONIONS: I notice that we now have
actually 45 seconds for discussion according to the
program.

(Laughter.)

Brilliant as this panel are, I don't think
they could do that. So could I have some guidance on
when you would like to close this panel session?
Perhaps somebody could give me some guidance.

CHAIRPERSON RABINOVICH: Forty five minutes.

DR. ONIONS: Forty five minutes. Thank you very much.

Okay. We were charged in this panel to answer a number of questions. I'll come to those and try and go through and cover the areas that the panel will discuss. I would be very grateful for as much participation from the audience as possible.

I thought it was just useful to pick up two strands that I think came out of some of the comments yesterday. One I think that's important to make, and that is that vaccine production is a very pragmatic process, and that once there have been lots of theoretical objections to particular cell substrates, particularly autogenic cell substrates, there are very practical reasons for the use of cell substrates that might be immortalized or neoplastic from a new generation of vaccines. I don't think we should lose sight of that. There are very practical reasons in scale up and use that I think we should bear in mind to produce therapeutic vaccines.

The second concern was that came out, and perhaps didn't get enough airing as it should have
done, and that is there clearly is a trend in society at the moment about concern in safety of vaccines. That perhaps therefore focuses particularly on the item we are going to discuss today, which is the possibility of adventitious agents.

The question that the FDA asked us to evaluate, or at least one of the first questions, and they would like the panel to take a look at is do neoplastic cells represent the greater equivalent or lesser risk for the presence of adventitious agents than primary cells, diploid cells, or non-chunogenic continuous cell lines.

I am not sure, given my previous comments, whether I think necessarily that you can answer that in a simple sense, but does anyone in the panel want to sort of pick that one up first of all?

What I could perhaps do is to prompt people, is perhaps to put up those which is just my suggestion, of some of the factors that might influence the risk of adventitious agent testing in a variety of cell substrates.

DR. MINOR: I am personally convinced that they are going to be better than primary cultures. I am not sure whether they will be necessarily better than human diploid cells or anything else if we even
get a decent banking system going. It seems to me that when you get to that kind of stage, it's the concern about how you find what's in there rather than anything else. I think the actual extent to which you can characterize them is clearly to me, it's very similar.

DR. ONIONS: That would be a generally universal statement that primary cells are likely to be more difficult to characterize and therefore, if you can use a cell line, that is probably the way to go.

But I think it's also worth making a countervailing point that there are still vaccine strains that are very successfully produced in primary cells. There are others that have been passed in primary cells and therefore change them into a cell substrate, the genetic stability of them. So there are nevertheless countervailing arguments.

I think the statement is correct. That is, adventitious agent testing is clearly going to be more in the region --

DR. ROBERTSON: Another point which one could consider. Where neoplastic cells might be considered more susceptible than primary cell cultures, in that the primary cells are derived
specifically for vaccine production. Whereas the neoplastic cells have probably been kicking around at least one, if not several laboratories, before being put into use as manufacturing of vaccines. Because of that, they may well have picked up something that you don't want to be there. Nothing to do with the cell type or the origin of the cell. So a virus of some different species all together which you really have got to check for.

So if you are actually setting up a cell bank of a neoplastic cell, you shouldn't just be considering species of origin of that particular cell, whether it's porcine, human, murine whatever. You have really got to consider any virus under the sun. We know there have been instances of this happening.

This morning there was the comment about I think it was a human endogenous retrovirus which was actually of murine origin, been picked up from passage somewhere.

DR. ONIONS: There is a good example in a commercial product. That of course was the Glaxo Wellcome's novalma cell line which was used to produce interferon, which in fact contained SNRV, and probably picked up SNRV in George Kahn's lab at some point during its history, I think was the general consensus.
But clearly that was unknown and the whole system was used in the production for several years before it was realized that perhaps this was contaminated by an adventitious agent that you just would not expect in this cell line. So I think that is a very good point.

There are issues about tumor cells. One of the things that occurs to me is that -- actually it does concern me quite a lot about adventitious agent testing. That is that it is rather traditional in character still. It is not very directed in terms of its specificity in looking for certain viruses. That is changing I think, but I think until recently that has been the case.

For instance, we have known that in certain tumor cell lines, that viruses that we have only recognized in the last decade are certainly found. For instance, well HHV-6 isn't a transforming virus, but there are cell lines that carry HHV-6 that have been used in the lab for many years. The same is true for HHV-8, which is a transforming virus.

So there is a concern that we may have cell substrates that are contaminated by other tumor viruses.

Tom, would you like to pick up?

DR. BROKER: I think we have actually a
wonderful opportunity for a so-called natural experiment. That is solid organ transplantation. It turns out, as we all know now, virtually all kinds of organs, not only the corneal we have just heard about, but kidney, liver, pancreas, part of the intestine, heart, lung, so forth, have all been transplanted. I think the opportunity is that the recipient is invariably immuno-suppressed until the transplant takes, and then they are slowly weaned off the drug like cyclosporin.

Yet on other occasions, the transplant fails for one or another reason. One could go back into failed transplants to look for the reactivation of agents that came from all these different tumor or tissue types I mean.

One example I could cite that we recently encountered in the course of our kidney transplant study is a pair of kidneys that went in from a five-year-old boy to a 19-year old female. Within a few days, the kidneys had completely become destroyed, necrotic.

It turned out -- they suspected CMV infection, but it turned out to be adenovirus. The presumption, and I'm being completely hypothetical, is the five-year-old boy who had died in a bicycle
accident, the donor, probably was in the age bracket
where adeno was just a natural infection in his
airway, and that these cells say from his tonsils or
adenoids, which were in the midst of processing the
adeno, became circulating, were in the kidneys, and
the recipient female then acquired adeno-infected
kidneys, and upon transplant to her, the virus
reactivated and just wiped out the tissues. I might
also say the different individual who received the
boy's liver also lost the liver.

So presumably these were entering through
B cells that were in any of these remote organs.

Nonetheless, the basic opportunity to look at organ
recipients I think is the experiment to ask how much
infectious agent is being transferred.

DR. RUSSO: Carlo Russo from Merck. I
think as you indicated, these patients are profoundly
immune-suppressed. Therefore, is going to be very
difficult to assess where this agent came from. In
your case, it's very well possible that the woman was
exposed to adeno virus. Since she was immuno-
suppressed, that's the reason why she got the
infection.

DR. BROKER: Well, in that case, it's why
I did also point out that the liver who went into a
completely different boy also wiped out. But I agree
with you. One doesn't know whether it's endogenous,
but it does give some indication of infectious agents
in these organs.

DR. ONIONS: Could I just actually take a
backup actually? I was about to go back to the
primary cell issue again. Phil gave I thought a
wonderful presentation. I hadn't heard parts of this
before. It actually started to worry me a little bit
actually.

To what extent do you need now to control
the kinds of colonies of these particular primary
colonies? I'm not sure, I mean I don't know what kind
of testing goes on in these colonies for a range of
adventitious agents.

Can you maybe just comment on that? I
mean are we dealing with inverted SPF animals?

DR. MINOR: Well with respect to the
primate, you will certainly not. But they are
increasingly heavily monitored. It depends very much
on the manufacturer and how much monitoring they do.

One manufacturer, for example, has only
recently, well in the last four or five years I guess,
started using colonies of monkeys that were monitored
for foamy virus. The result of that is being
revolutionary in terms of the number of cultures that
you get surviving to production.

You would have thought you might have
started this a bit earlier perhaps. But you couldn't
call them SPF, but they are increasingly closely
monitored I think. Certainly some manufacturers have
them more closely monitored than others.

But one of the difficulties with the whole
of adventitious agent business of course is you only
really find what you are looking for. That's an
ongoing problem.

Things like chickens are a different
matter. I mean I think this would establish what you
need to do to make an SPF chicken colony. But
primates are much more tricky.

DR. ONIONS: There are other cell
substrates out there that are used, cells like primary
hamster kidney cells in JV vaccines and various other
things. So there are I think other vaccines out there
that are going to come to attention because they use
primary cells. I think we perhaps ought to start
thinking of the kinds of procedures that are needed,
like closed colonies and embryo derivation of these
animals in some cases.

Could I move to perhaps the third element.
That is, we have heard a lot about retrovirus.

Retroviruses always come back to focus when we deal with cell substrates. What is the panel and the group's feeling here in general about the concerns of using either immortalized cells or transformed cells, because frequently those -- well, that's not an accurate statement. Activation of transcription of endogenous genes is more frequent in such cells. Is that of concern or not a concern? Or do we have to go cell by cell, species by species, to answer that question?

John, would you like to make a comment?

AUDIENCE MEMBER: Well, I think clearly there are some famous cases of activation of transcription of endogenous retroviral genes and genomes and tumor cells. We heard about germal tumors. We have heard about recently in the news about mammary tumors and probably a variety of others. It is not entirely clear to me whether this actually represents activation of transcription of these cell lines or a fixation of a differentiated state which itself is what's activating the transcription. I am inclined to think probably both are true in different cases.

One of the things that we have learned, at
least from human tumor cell lines, is that none of these things that are activated have ever been shown to be infectious, despite the fact that as came out in the earlier talk, the probability for recombination between a large variety, for example of the HERV-K sequences, would seem to be rather high. If these were other types of retroviruses, such as MLVs where you do get that endogenous, you do get that kind of thing.

So there's probably something else that's protecting the people in the cell against actual infectivity in this particular case. We don't yet know what it is.

DR. ONIONS: I was just going to bring in, I was very struck by the HERV-K story. That is to a very pragmatic level. I take the point that it does seem to me that it doesn't much recombination or much adjustment to me, at least into a more functional virus. I mean should we simply be screening any human cell substrate for the expression HERV-K? Would that be something that would be useful to do?

DR. PALLEY: That's a problem, in that you will I guess you will have a hard time finding a human cell line that would not express any human retrovirus, so I report that it's the case for HERV-K, the special
HERV-K family.

DR. ONIONS: But that particular locus, I mean that is not expressed, as I understood you, in all?

DR. PALLEY: We do not know whether that particular locus is expressed in some cell lines. We know that if this, at least this HERV-K family, is activated for some reason, that there is very likely not only one locus activated within the genome, but that there are several loci that will be activated. So it's possible that this locus is also activated, but we also then have the problem that if that locus would not be activated, that there's some transcomplementation. So one gag, intact gag gene would complement another intact pol gene and so on.

So that could be a problem, but we see it then in several, even in normal peripheral blood lymphocytes, we have HERV-K expression. It obviously is not a problem.

So I wouldn't see so much a problem in that we have HERV expression if defective sequences are expressed, and that are not coding the intact.

AUDIENCE MEMBER: I think we have to focus on infectivity here, because if we just go looking for expression of defective stuff, you always find it, and
we all go home and won't be able to do anything. It might not be a bad idea perhaps, but --

(Laughter.)

AUDIENCE MEMBER: Sounds good to me. But just to push this point a little bit further on the HERV-K, you know, about two-and-a-half years ago, there was a report in Cell, which is a better journal than I usually publish in, which claimed that HERV-K env can act as a super antigen that then stimulates diabetes mellitus in some people.

DR. ONIONS: I think that story -- just before you go on, I could be wrong, but Johannes might know. Is that story being modified? I'm not quite sure that's -- yes. It's no longer supported.

AUDIENCE MEMBER: Okay. I think it would be worth explaining how it's been modified.

DR. ONIONS: I think the retraction is in fourth hit. So before you sort of put that out as a paradigm.

DR. LOEWER: I think the main point is that a couple of groups tried to repeat this data, and they were not able to repeat. So it seems not to be specific and even not effects on the T cell lines could be repeated. So let us depart from this idea.

DR. ONIONS: Can I maybe get your opinion?
I really would like to get some feeling because we have heard a lot, and it's scientifically really interesting by the expression of these human endogenous retroviruses. I think John has probably just summarized it. It looks like we're saying the list, this collected group here of retro, are saying that as far as we are aware, at the moment these are not of concern and uninfectious, and probably therefore there is not a great deal of point in looking for expression of these in cell substrates, the very pragmatic practical point.

Would that be your opinion too, Johannes?

Johannes is nodding. That's a "yes," I assume.

AUDIENCE MEMBER: From a research standpoint, it's absolutely worth pursuing to see if one can find these things eventually. But in terms of vaccine issues, I don't see how we could possibly deal with it now.

DR. SCHUEPBACH: Yes. I also would like to make a comment regarding that super antigen activity because we are coauthors in that paper. It is true that the presence of these sequences, RNA sequences in the serum we can not repeat, so it's not specific for IDDM patients. But to my knowledge, our
data regarding the super antigen activity and the
stimulation of VP cells has not been disputed by any
other group. So that is still around.

I think that the real important topic here
is whether these endogenous viruses actually give rise
to infectious particles. I believe that with the PERT
assay, we actually can contribute very much to this
question. I think, as I pointed out, of course the
easiest thing is to test the super natants for
reversed inscriptase activity. But I think with a
little bit of additional work, it should also be
possible to define that profile of RT activity and
cellular, DNA polymerase activities along the
different fractions of the sucrose gradient and tend
to recognize any abnormal pattern that might be
associated with infectious rate of viruses.

AUDIENCE MEMBER: Let me just go back then
because obviously the question comes up if the public
is asked to accept a vaccine that's made in cells that
express HERV-K. Even if one part of the story has
been refuted, the question comes up. You know, are
there potential immunological consequences of the
expression of antigens from these kinds of cells which
are not expressed in human diploid cells? Even if
this story is wrong, I guess, and it sounds like it
hasn't all been refuted, the question then comes up of what level of scientific data in the literature is necessary to completely refute it? I can imagine the outcry that could occur if people believe the story. If there appears to be some controversy about what part of this story has been refuted, then I think one might have a public confidence problem as well.

DR. HENEINE: David, I have a comment which could be redundant, but go ahead and say it. While thinking about all these questions from my simple mind, it looks like if you want to compare cell lines versus primary or diploid cells, the two questions that were raised is which ones transmit less adventitious agents or transmit less neoplasms to vaccine recipients.

What we have heard so far about the mechanisms of the neoplasms, many of those are mediated by viruses or viral-like elements. So it looks to me that the majority of the concerns are rising from the adventitious agent group rather than from other elements.

So therefore, in trying to make up our mind, based on the available data, which one is the more suitable substrate, maybe we can go very simply
with a checkpoint list on these different cells, targets, which one we can test for the presence of these adventitious agents known, unknown, and which can be better monitored, which can be for practical reasons of culture as well, and make up our mind, rather than jumping right and left with different issues.

If you can say cell lines, primary and diploid, and then go one by one, all these concerns that we have been talking about, and say which one is more suitable for each of these points so that we can conclude at the end. I mean it's one suggestion.

DR. SCHUEPBACH: I would like to come back to the human endogenous viruses. Since we all carry them and have them expressed in one or the other part of our body, I do not think that this presents a particular risk. Independent of whether you have a super antigen activity or not in some of them, I think is just the same as receiving blood from any person, because they also would have these endogenous retroviruses.

So I think the only thing that matters is endogenous retroviruses from other species, not from humans.

DR. LEWIS: This is a question. I think
we are sort of being faced with something of a dilemma here, because as you very correctly pointed out, the one way to look for a retro virus, adventitious retro viruses or endogenous retro virus, whatever, is by the PERT assay. Now if we take a situation in which our HERV-K is expressed and there's RT activity in there with the PERT assay, from a regulatory perspective, what do you do about that?

I don't think you can dismiss it. I don't think at this point in time that you could dismiss the use of the PERT assay for looking for adventitious retroviruses or any type of retrovirus activity in there.

So the question is going to be, when you find something, what do you do about it if the assay is positive?

DR. SCHUEPBACH: I think once you have activity, then you have to characterize what it is. Depending on whether this is exogenous or endogenous virus, steps will be taken. I think identification is important.

DR. LOEWER: I think we shouldn't continue to discuss use of this type of cell lines in absolute terms, because the other side of the coin of course is a product which could be made from it. This is always
to be judged in conjunction, in my opinion. For example, there's the question of endogenous sequences which may be active, just retro virus or line elements. So far as I know, this is not the same in all cell lines. It's mainly expressed in tumor cell lines, and so far as I know, there is so far no need to use these cell lines for vaccine production.

If somebody believes it's necessary, must be a very special virus which can only replicate in these cells, then perhaps we do not have another choice to use them.

DR. ONIONS: Could I just go to Arifa?

She has been waiting very patiently. Then Steve.

DR. KHAN: Yes, thank you. I think it is important to clarify the word "expression" in terms of human cell lines and human cells. I think we all expect that there will be some RNA expression in the human cells from endogenous retroviral sequences. However, I don't believe that you are going to get particle production in the majority of the cells under normal conditions. So therefore, I think the use of the PERT assay would be very helpful to evaluate particle production from human cells, which can then further be investigated for infectivity, as opposed to looking at RNA expression, which I think you would
find at some level in all human cells.

DR. ONIONS: I would like to endorse that.
I think really we're making it too complicated. I think it's very simple. You use that kind of assay system which has a very high sensitivity developed by Yumascript here in the audience. Then if you get a positive, then you go and look and see if there's something there that's infectious. It is a hierarchy of testing strategies, it seems to me.

DR. HUGHES: I am willing to take an even stronger line here, which is I think akin to the line that was espoused by John Coffin.

If your technology is sensitive enough, all cells from all vertebrates are going to have endogenous viruses in them, either intact or defective. The question really devolves down to not whether they are there, because they are. If we use the most stringent criterion, are they there, you are not going to be able to do anything. We'll be paralyzed in terms of making vaccines.

So the obvious criteria is not whether or not these agents are there, and in particular, I think not whether they would make physical particles, because my strong prejudice is if you look hard enough, you are almost certain to be able to find it.
There are almost certainly ways of looking even more stringently than we look now. In very large batches of material prepared for vaccines, if you look hard enough, you are going to find something. The question is whether there is an infectious agent which represents any kind of pathologic threat when its present in a vaccine. We have, as has been pointed out earlier, given from chicken cells, some of which were clearly contaminated with agents which are infectious for the chickens, if not for humans, that hundreds of millions of doses have been given, with as far as anyone can tell, no untoward effects.

DR. ONIONS: Okay. I would like to bring Jim in, because I am not sure that statement is -- well, it could theoretically be true. Maybe if you get a more sensitive technique, maybe you are correct. But there is a very strong distinction if you look at cell lines like MRC-5 and compare them to what you see in say AB, you get signals, if you're looking at MRC-5 you don't. If you look in tetra anacells, you get a signal.

DR. HUGHES: I would argue that is with a particular assay that has been tuned up to detect RT in a particular way. I would be willing to wager that
if we look hard enough, we could certainly find
evidence of particle production in any of these cells,
simply because they are full of endogenous viruses
that -- I mean the very fact that there's obviously
expression is RNA present.

DR. SCHUEPBACH: But the important thing
is I think the number of particles. You have very,
very low number of particles, and you do a test, a
PERT assay from a concentrated material where you
pellet virus, let's say, from one liter, and assay
that and find a very low activity. You know for
production of the viral vaccine, the cell harvest, the
vaccine harvest will be diluted 50 to 100. You also
realize that most of the particles even of infectious
retroviruses are non-infectious. Then you certainly
can come to some calculation which permits you to
establish a level of safety where you have a very high
probability that this vaccine is safe.

DR. HUGHES: But the safety is not
predicated on whether or not there are physical
particles. The safety is predicated on whether or not
the particles are infectious.

If the particles are infectious, a very
small number is very important. If the particles are
not infectious, in particular, if the particles are
not infectious for humans, the presence of a relatively large number of particles is probably also irrelevant.

DR. ONIONS: My only caution about that is that you can make assumptions about affectivity that also are not true. Since Clive Patience is here --

DR. HUGHES: I think you have to do the test. I don't think you can make assumptions about it.

DR. ONIONS: Well, the point I was going to make actually was that Clive's group and our group showed that you could actually infect cells with PERV, yet those experiments have been done 20 years ago, and been done by very good people, including George Tadai, and were unable to show infectivity. It's just the techniques have changed slightly and we could get infectivity.

So I think that you are making -- there is a straight yes or no about infectivity. That is not always the case with these retroviruses.

DR. HUGHES: I am not trying to suggest that the assays that we have for infectivity are necessarily always 100 percent accurate. But what I want to get away from is the idea that the presence of a physical particle is somehow a measure of safety or
lack of safety.

I certainly agree with you that there are numerous technical problems in determining infectivity. But what I believe we should focus on is better ways of doing infectivity assays rather than better ways of doing physical assays.

The physical assays can be very useful if they are coupled to infectivity. In fact, I believe that was a statement that John Coffin made. What I would propose is what I think John Coffin proposed, that we actually use these really wonderful sensitive techniques, but in the context of measuring whether or not the viruses are infectious, not whether the particles are physically present.

DR. SHEETS: Can I ask a very pragmatic question of Dr. Hughes?

DR. HUGHES: Sure.

DR. SHEETS: I'm Becky Sheets, FDA. What I hear you suggesting is that rather than testing for RT activity by a physical assay, as you called it, a PERT assay or a conventional RT assay, you think that it would be preferable to test vaccines for infectivity assays?

DR. HUGHES: The ability to transmit that RT assay to a reasonable recipient cell. I believe
this is exactly what John --

DR. SHEETS: The pragmatic question is that would you do this testing lot by lot on vaccines? For instance, if you were making a vaccine in a primary cell substrate, for instance an egg, would you test each lot of vaccine or each batch of vaccine for infectivity assays? Then the really pragmatic part of it is, if you are making a flu vaccine, where the timing of production, the timing of testing, and the timing of lot release is very tight, would you recommend these infectivity assays on lot by lot for primary source?

DR. ONIONS: We're running out of time. Do you want to answer that? You have been asked a question, do you test lot by lot?

DR. HUGHES: Very simply, if we're talking avian systems, I think there are reasonable ways of determining that endogenous avian viruses are not infectious. My personal bias, and I mean it no more broadly than that, is in the case of avian viruses, as long as you carefully establish that the avian viruses that are present are not infectious for human, that's not necessary. But that is my prejudice.

DR. SHEETS: That's fine for SPF situations. I am asking this question because this is
what sponsors ask FDA. So they want to know do we
need to do this lot by lot, or if a cell bank, you can
do a one-time characterization. Of a primary system,
you can't do it that way.

DR. HUGHES: Use SPF chickens and don't

ask.

(Laughter.)

DR. LOEWER: I would like to make a

comment to Dr. Hughes' comments. They are very sound
in a scientific meaning, but they face regulatory
problems, the main problem indeed.

Regulatory authorities have to show that

there is no infectivity and the proof of non-activity
is always nearly impossible in a scientific sense.
You will always find reasons to say you were not able
to find infectivity. Look at HIV. If you would use
MRC-5, for example, or a lot of other animal cells,
you would never find infectivity of HIV. The same is
true for many situations.

So there a fundamental problem is to test
for non-activity or noninfectivity.

DR. ONIONS: I would like to stop this

because we are running out of time and there are other
issues.

I am going to take the Chairman's
privilege and just say that I actually think you need multiple techniques, because I think as Johannes has just said, if you have complete infectivity, you will miss things as we would have missed cell lines producing ver. I think you really need to have a combination technique. So I think it's a belt and braces situation. That's a personal view.

What I would like to move onto, is were asked by the FDA also to consider species of origin. I think really you end up in very general statements here. You can argue that if you are worried about adventitious agents, then clearly there are species barriers to the transmission of some agents. On the other hand, other agents do go across species barriers, sometimes in abortive replication. They can be very nasty. We of course know that herpes B going across species barriers is actually lethal. Ad 12 in hamsters is oncogenic. Equine herpes veras, which is an alpha herpes veras is oncogenic in hamsters. There are natural examples of cross-species transmission, the ovine herpes veras 2 is innocuous in sheep, but it kills cattle. So there are examples of these heterolic transmissions being worse than natural infections.

Is there anything that we can say, the FDA
have asked us, in a general statement about species of
origin? My own view is I don't think you can, but
does anyone want to make a statement?

DR. MINOR: I think sometimes it is better
and sometimes it's worse.

(Laughter.)

DR. ONIONS: Yes. That's exactly what I
think. Thank you, Phil.

I would like to drop the discussion now,
because I think that sums it up. I'll turn the phrase
back on the edge and say it's a case-by-case, it seems
to me.

I don't want to trespass really on
yesterday's, but I think maybe just to come back to --
we're going to move onto assay systems in a second,
but I think one of the issues we're coming around to
in a second is latent viruses, because those seem to
be the real concern. It may be worth just remembering
some of the things that were partly discussed over the
last two days. That is, that the complementation of
defective viruses can occur. For instance,
adenoarectus can be complemented by HPV in hela cells.

We talked about pseudotype formation both
today and yesterday. I think just I would like to
make the point about pseudo formation. We talked a
lot about retrovirus retrovirus pseudotype formation,

but this can occur across viral species. For
instance, paramyxovirus is rather badly, but they can,
pseudo type retro viruses. So you could alter the
host range when endogenous agent is expressed in your
cells.

Of course there are recombinants. Some of
these recombinants, and we have got representatives
who did the work here, interesting recombinants like
SV-40 adnivos recombinants.

One of my concerns, we'll come on in a
second, I think is like polyoma viruses in cell
substrates and that potential for interaction with
other cells.

So if we can take that as a kind of

background, can we turn to a question of -- this had
come up and was discussed by several candidates,
unknown viruses. What are the potential candidates
and what kind of systems do we use to try and go
looking for those unknown viruses.

Anyone want to comment on what we should
be doing about novel cell substrates and you have got
a virus there that you don't know anything about.
What sort of technique should we be applying?

DR. PALLEY: I would like to make a
comment from that HERV field. I think if we talk about possible recombinations of HERVs with some other viruses that might be a little difficult, I think it could be conceivable.

We do not know, however, it's very hard to predict the outcome of whether it is possible at all. We basically have or we might have situations where a HERV is expressed where another virus, retrovirus is also present in the same cell. I think that has to be discussed or taken into consideration, that there might be possible recombinations between HERVs and some retrovirus status put into a particular cell.

However, I think it's very hard to predict whether there's any possible recombination and what the outcome of that recombination might be.

But I think there are examples where retroviruses indeed recombine with each other and produce some productive outcome. But it's very hard to predict what, in which ways HERV case for instance, or whatever HERV sequence within human genome could recombine with something else.

DR. ONIONS: I was just going to point out I think one of the things I have been very struck by by some of the talks here, particularly the polymerized talks, it does strike me, the comment I
made to Phil earlier about the use of cynomologous
monkeys and perhaps have these been screened for other
polyoma virus. That seems to be of concern. We know
that polyoma viruses are coming in as contaminants in
bovine serum into the primate cells, the bovine
polyoma virus. They worry me as potential
adventitious agents. I am just wondering again
whether we should be doing more in terms of redundant
PCR approaches to look for these agents in both
primate and non-primate cell lines.

Any views on that?

DR. ROBERTSON: This is potentially the
most important door, also the most difficult to deal
with. If you think back to what Phil was saying in
the first talk this morning, all these instances of
vital contamination, generally they occur with viruses
unknown at the time, viruses expected, the presence of
viruses in vaccines or biological preparations.

Potentially is not something we're talking
about today, it's not an endogenous virus or
recombination between an endogenous virus, but
something unknown that's going to leap up at us out of
the dark. Of course almost impossible to deal with.

But Joerg was saying this morning about
this is what we should be looking out for, the
unknown, and if possible using a more broadly reactive
type of assay rather than highly specific type of
assay to look for something.

If we knew to look for something, that's
fine, we can deal with it. It's what's not there
which causes the problem.

DR. SCHUEPBACH: May I add something to
this? I think our chances to detect such unknown
agents are really much better if they are present at
high concentration. So I don't know whether you
accept that concept of cellular cloning in order to
either get rid of these agents or to have them at the
very high concentrations so that their detection is
actually much easier. In the meantime, you can try to
activate the host cells by all kinds of different
agents. You do EM studies, you do serological
studies, use broadly cross-reactive antibodies. I
mean this is a wide field actually of methods you can
employ.

So I think using such an approach, we
should actually be quite capable of detecting such
agents.

DR. MINOR: Can I ask for a definition
here? What do you actually mean by a virus? I mean
what I would understand by a virus is something that
actually grows in the cell and increases in numbers.

If you think back to the very, very early days of polio vaccines, amongst others, there was an awful lot of effort put into trying to make sure that you put your supernatant or whatever, into all sorts of potential different systems where a virus might grow.

So you put it into different cell substrates and you look for cytopathic effect, where you can say maybe you have got a virus that doesn't cause a cytopathic effect. You put it into mice, you put it into eggs, you put it into everything. So it's like evidence of actual growth. You see?

I think there's actually quite a lot of effort that goes into trying to detect viruses that you don't know are there, but you suspect might actually grow in some system that you are going to check it on. If they don't grow, I'm not sure certain that you are worried about them, or even if they are viruses. So what do you actually mean by an unknown virus?

DR. ONIONS: Well, okay. Let me give you an example. I think one of the areas that is going to emerge as a concern are going to be circaviruses. The reason I say that is that TGV may be a circavirus. We now know of others. Thomas was talking about the kind
of normal flora of HPVs. It looks like we all have a

normal flora of these circaviruses. Certainly when we
started now looking in animals, we find these all over
the place.

So I think they may come up as potential
cell substrate contaminants, but also in our serum, in
our trypsin, and so on. Of course trypsin is full of
of course circoviruses.

What we know is if you look in a cell
substrate like PK-15, which has used in the pig
vaccine industry, that carries a circovirus genome.
It looks, it's possible semi-defective, but you can
introduce it as an infectious agent, but most of the
time it isn't inducible as an infectious agent.

So I think I'm not quite sure I take your
distinction. I think there are latent viruses there
that are reactivatable under certain circumstances and
are a concern. In fact, in the vetmurines vaccine,
you have to get rid of that virus.

DR. PALLEY: Yes. I just had sort of an
alternative. Virtually all the conversation has
revolved around using higher eukaryotic type cells as
the producer cell source. There is an alternative
that's being actively pursued in the papilloma vaccine
business, which is to make virus-like particles either
with a baculo virus expression system in insect cells
or even in E. coli.

Very briefly, what's done is the L-1 and L-2 capsid proteins of papilloma virus self assemble no matter where they are over produced. Four different groups are pursuing these now. I am sure they are engaged in some corporate relations.

But one of the neat strategies that John Shiller here at NIH and others have done, is to fuse peptide epitopes or even intact other genes to the L-2 protein at their n-terminus. It turns out that when L-2 assembles into the virus-like particle, but brings this n-terminal protein in with it, that other protein is on the inside of the virus-like particle. So it will ultimately be presented to the recipient of this virus infection. It's non-genetic, but it will be taken up by cells. So there is in effect no risk of some bacteria phage infection running rampant in our bodies. So it's a complete alternative.

DR. ONIONS: I think obviously there is a move to sub-unit vaccines in this form of vaccines. But I think it's still going to be a long time before our traditional eukaryotic cell production is going to be lost. But I take your point. That is a valid point.
Could I just ask perhaps questions about we had examples indeed from Thomas toady about using redundant PCR techniques. It would be feasible to screen for a number of viruses that are of concern, like herpes viruses, circoviruses, polyoma viruses, herpes viruses retro viruses by redundant PCR techniques. These are not in FDA terms, validated techniques. On the other hand, they are very powerful techniques. For instance, one of my colleagues just developed a herpes virus redundant PCR technique with 112 primer combinations that to date has picked up all of the herpes viruses that’s been challenged with both human and animal origin. So you could go looking for herpes viruses by that kind of technique. Robin Weiss' group in London picked up a new human herpes virus, HRV-5 by a redundant PCR technique. So do any of you think that these kinds of technologies should be implied to cell substrates? If so, which viruses should we use? Because it is a lot of work to do this kind of thing. So should we be doing this or is it not necessary? Can I have comments?

Jim, you look like you are about to say something, but not quite sure.

DR. ROBERTSON: One would not expect to
see this type of assay risen up in a pharmacopeial
recommendation of any kind. But certainly they do
have uses at the investigational level, especially
with some incredibly novel cell types.

    Everything is kind of going molecular
these days. All these assays are looking at things
from the very molecular point of view, and picking out
a signal say with your herpes primers, need not
necessarily say that you have got an infectious herpes
virus.

    So go back to this argument we have been
having this morning about it's infectivity which
potentially is what we're concerned about, and not
picking up a fragment of a genome.

    These are assays, again I mentioned that
Joerg mentioned them this morning, broadly reactive
molecular assays. Phil rightly pointed out that we
have had in place for eons broadly reactive
infectivity assays using tissue cultures, suckling
mice eggs, looking for signs of infectivity of any
kind.

    DR. ONIONS: But don't you think -- my
feeling about that is, I tend to share, I don't think
one would ever use these kind of assays on a routine
basis.
DR. ROBERTSON: No.

DR. ONIONS: They might be useful in establishment of a master cell bank or something, you know, the first one. But I mean I would criticize, I don't think that current infective assays do pick up everything. I think that's the whole problem. I think, for instance, that it would miss -- well, polyem virus has perhaps been used.

DR. ROBERTSON: At the end of the day, that's the weakness of anything, that you will not pick up something that's not designed to pick up. You will potentially miss viruses in an infectivity assay. But you also potentially miss viruses with a redundant PCR. If you don't pick something up, you can't say it's there or not there. It's a bit of a philosophical argument.

DR. ONIONS: That's why I'm just saying shouldn't you have an adjunct to these? I mean I think should you not have at least an adjunct in terms of broadening the kinds of assay systems that you are using?

DR. ROBERTSON: Sure. Oh yes.

DR. ONIONS: Does it have anything to do with the -- I mean there are people out there who have to do this for a living, rather than us who can just
sit here and pontificate about it. How does this go
don with the industry? What does the industry feel?

DR. PALLEY: Just one point regarding
again HERVs.

DR. ONIONS: Sorry?

DR. PALLEY: I mentioned in my talk that
HERV-W family that has been reported for the first
time this year and has been isolated from vitro by
particles from multiple sclerosis patients. It turned
out that it at least codes for an env gene. So it's
certainly worth -- the human genome I guess is among
the genomes that regarding endogenous retroviruses is
among the best characterized genomes besides mouse,
for instance.

I think it is certainly worth to continue
and even by such redundant PCR approaches and so on to
further characterize HERV sequences on endogenous
retrovirus sequences, and to see, to give then an
estimate whether there are any additional sequences
that could be harmful. So far, we did not find any
sequences, but it's certainly worth doing that.

AUDIENCE MEMBER: I think if you just kind
of look back at the history of biological products,
and maybe even going way back to when hepatitis B was
discovered, and then there became the ability to look
for hepatitis B in blood and blood products. There is always concern about what are you going to find, and what are the implications, and what are the costs, and all of those things.

But the bottom line is as technology evolved, then the discovery of reverse transcriptase, and when I was still here at the agency, we applied in a research setting to vaccines and first demonstrated RT in yellow fever. You get concerned about again, what are you going to find when you look at all these cell substrates, and then with the more enhanced sensitivities of these systems. You are always in the same muddle. That is, is this appropriate to apply across the board? Where should it be properly applied? Should it be done more in the initial stages of characterizing something versus a routine quality control test?

Those things, you can't sit here in a meeting or on a panel and give specific answers to those questions. I think the bottom line is as technology evolves, and it's going to continue to evolve with more sensitivity and specificity hopefully, it needs to be explored and it will find its appropriate place in the overall testing, whether it's in characterization or perhaps in some cases, if
it's appropriate, on a routine basis.

But I think the general principle of applying new technology as it begins to be available to look at these issues, particularly as they relate to safety and the presence of adventitious agents, is unassailable.

DR. ONIONS: I agree. Can we move on, because I would like to just cover TSEs just before we have lunch time. I'm desperate for some lunch.

If you have got something, sorry to inhibit you. If you can be brief.

AUDIENCE MEMBER: I agree completely with what John says, but would add that if we are faced with a decision of whether to approve the use of different types of cell substrates that are tumorigenic or derived from tumors from which we don't know the mechanism of transformation, we are faced with not only the question of should technology be applied, but is the technology as it exists today and can be applied today, good enough to permit us to say that it's okay to use these cells. So that is a very practical question which perhaps could be answered.

DR. ONIONS: Can we have a view on that?

That is, given the technology we have today, is it acceptable to use the kinds of substrates we have been
talking about, that is tumorigenic or immortalized cell substrates? Are we confident that with the technology we have, that we can use these cell substrates safely?

DR. BROKER: I would just basically say I think so. I think if we combine PCR with these microchip or microprobe arrays, DNA chip technologies, and we have the growing human genome base and the analogs and a number of other species, I think we have got the tools at a level of sensitivity far beyond what would probably would be more than adequate.

DR. ONIONS: Okay. I would just like to finish up, because we heard a really I think important interesting talk from Neil. While perhaps the risk of spongiform encephalopathies in the kind of cell substrates we are concerned with is probably extremely remote, the consequences of being wrong about this issue are potentially devastating. So it is certainly worth cautious consideration.

Really I think Neil in his talk, already summed up these key issues about the possible origins, are the mutations spontaneous or infection, and the kinds of cell substrates of concern might be, it seems to me, are the neuronal cells. Since it's recently shown that in the peripheral introduction of TSEs, the
B-lymphocyte might be important to carriage, then perhaps lymphoid cells, particularly B cells that are invariably used, and since we're looking at the possibility of using HIV and T cells, maybe that suddenly becomes an issue. Maybe we should be looking at lymphoid cells for the potential of there being spongiform encephalopathies.

Which brings you back to the question that Neil finished with. That is, what should we do? It did strike me that one of the possibilities was that there are now very good, very interesting new cell lines being based on retinal cells, which we heard from Dr. van der Eb and others, which look very, very promising for the generation of anti-viral vectors.

But as they have, as I understand it in theory, I mean just at the simplistic level, should not one thing be done and just sequence the PrP gene in that? The probability of having a key mutation seems to me extraordinarily remote. But then it's a relatively simple thing, cheap thing to do is to go and sequence the PrP gene. Is that something that we should do in that kind of a situation? Should we also do that in T lymphocytes? It's a trivial thing to do?

DR. CASHMAN: I would say in this case, you get what you pay for. It is a trivial experiment,
but if one out of a million or one out of a thousand
cells could be harboring a mutant prion protein gene,
the technology is a little more dicey. So yes.

DR. ONIONS: I was thinking of excluding
the origin of the familial form, which clearly occurs
to some inherited disorders. I mean I think you are
right. You can't cope with a somatic mutation.

DR. CASHMAN: Okay. You can't cope with
somatic mutation, I agree. But certainly one can cope
with a mutation that's in every cell, yes.

DR. ONIONS: Neil touched on validation
technology. I think that's important. I think there
are new techniques for doing validation of TSE
removal, but using a disrupted PrP protein. But I
don't think that's going to be applicable to quite a
lot of the processes that are used to produce vaccines
at the moment. It is by technology products, but not
to vaccines.

I just wanted to touch on testing because
I know that Neil has an interest in that area. It
seems to me that we were sort of rather optimistic a
couple of years ago, and indeed, there have been
publications by Bruno Esch and others on specific
antisera of PrP Sc. But those haven't held up. They
actually pick up aggregated protein and not, as I
understand it, strictly PrP Sc.

We can use, and we have been using, treatment of protease followed by immuno blotting. It certainly works, but it isn't that sensitive. The problem, it seems to me, is that we really don't have a specific test that's an in vitro test. The only thing that you are left with, at the moment, it may change, but at the moment is animal inoculation.

Would you like to comment?

DR. CASHMAN: I think that the bio assay, with no species barrier, can detect one unit of infectivity. It's the most sensitive thing we have to date. But in fact, there is new technology. Mary Jo Schmirr and her colleagues have developed a very sensitive capillary electrophoresis technique which is, if one can believe the papers, including one in press, is as sensitive as bio assay. So technology is evolving, and there may in fact be a specific and sensitive test for PrP Sc right around the corner.

DR. ONIONS: Okay. On that optimistic note, I am going to wrap the session up. I think we're all getting edgy for lunch. Unless anyone has got some burning issue that they wish to go into before we go.

If not, I think the panel members, and
thank you all for participating.

CHAIRPERSON RABINOVICH: Thank you, Dr. Onions. There is a light repast outside for those that have been so patient. I would like to get everybody back in here in 15 minutes.

For those of you who would like to avoid the wholesale garage sale that's going to go with your luggage, for those of you that haven't checked out of your room, I encourage you to complete that now.

Thank you.

(Whereupon, the foregoing matter went off the record at 12:33 p.m. and went back on the record at 12:53 p.m.)

CHAIRPERSON MYERS: Back to order. I would like to introduce a co-chair and a new person we are very pleased to have attending the meeting, Dr. Gary Nabel, who is the new Director of the Vaccine Research Center at NIH. So he is going to join me in chairing this session. I am probably going to disappear before the end of the session to make a plane.

The first night I got here, as some will recall, I came in a little late. I ran into a couple of you in getting a beer because it was after the time of the close of the meeting. The discussion ensued as
to what is a designer cell substrate. What do we mean by that? My first reaction to that of course was it's anything that I happened to have made. Clearly at this point in the meeting, it's not primary cells. I suppose from a strictly semantic perspective, it would be a cell substrate created with specific characteristics. It could be immortal or not. But I think over the last couple of days, at least my thinking on this and for the purposes of this discussion, by a designer cell substrate, we mean a cell substrate of defined origin and with a defined pedigree.

It is probably immortalized because it is likely to have been cloned. It will be validated as specific pathogen-free and at least specific pathogen sought and perhaps in certain circumstances, defined as non-infectious.

For the purposes of the next discussion, we are really talking about immortalization. Jim McDougall, as you know, presented his paper yesterday. So we'll start this session with the first paper by Dr. John Sedivy from Brown University, who will talk about differences in the capacity to immortalize rodent, primate, and human cells by tissue culture passage or viral transformation.
DR. SEDIVY: Thanks very much for the invitation. I am sorry that Jim gave his talk yesterday because -- well, maybe it will jive all together.

I was asked to give somewhat of a historical overview on the issues of replicated cellular senescence, and obviously the topic of cellular immortalization.

So from a historical point of view then, this is the Hayflick phenomenon. This experiment has been performed in numerous labs and always with the same result. This happens to be an experiment in my lab. You will see a number of slides like this from me today. What we're plotting here is replicated lifespan, the doublings of the culture versus days.

We see a culture growing and then reaching a non-proliferative plateau. This is what we define as senescence.

Really the interesting point here is that the correlation here of this plateau is with the number of cell divisions as opposed to chronological time. The question that has been plaguing this field ever since its inception is well, is this really some type of a terrible artifact. I don't really want to get into this discussion. It really revolves around
the issue of media and media artifacts, and have these
really been adequately resolved today. I don't think
they have, especially for some more specialized cell
types. I think they have been pretty well resolved
for keratinocytes, maybe breast epofelial cells,

fibroblasts, et cetera.

One really has to keep in mind that if one
sees a culture that is slowly declining in its
proliferation, this could simply mean that
increasingly a larger and larger fraction of those
cells are withdrawing from the cell cycle. This could
be perfectly explained by inadequate culture
conditions, such that eventually on the macroscopic
scale, the culture has ceased proliferating.

There are really three arguments that have
been used historically to justify the claim that
replicated senescence is a biologically interesting
phenomena. Here we are plotting, again very simply,
the mean-like span of a species versus fibroblasts
replicated life span in tissue culture. As you can
see, there is a rather striking correlation, such that
animals that don't live for very long don't have cells
that live for very long in tissue culture.

The next phenomenon that one often sees
cited is the age of the donor plotted against -- here
is the age of the donor, and the remaining life span of the cells, in this case fibroblasts taken from that donor. As you can see, the points are all over the place. In fact, more recently, this view has been challenged by a recent paper in PNAS from Vince Cristofalos, who actually claims that this correlation doesn't exist. But if you read the literature, you will see this coming up over and over again.

The one fact that seems to remain, at least to my knowledge, and that is if you look at these points down here, these are fibroblasts taken from individuals that suffer from premature aging syndromes. These are called progerias. Typically, these cells have a very short life span.

So this really is the issue here. How do we differentiate between senescence, quiescence, and differentiation. I think that for the purpose of discussion today, this is really not a point of major interest, but for historical reasons I'll go through it rather quickly.

Quiescence is defined as a reversible process. So what we are talking about here is essentially a cell cycle phenomenon. That is, we can have a culture that is cycling or contains a large fraction of cycling cells. Then these cells can
withdraw into the quiescence state. Then when they
are induced with the proper growth factors, and here
of course the key phrase is what are the proper growth
factors to elicit this phenomenon. At any rate, we
are talking about a reversible process. Whereas

senescence by definition is irreversible.

So then of course the very interesting
next question is how do we differentiate senescence
from terminal differentiation. I don't really have
answers here because in many cases, this is very
difficult to do in many specialized cell types. What
one would like to see in general is the absence of
features that are characteristic for terminally
differentiated cells. But this is not possible in
many cases.

So really this has given the impetus to a
search for molecular events. So then if we pose the
question are there molecular events that are unique to
senescence versus quiescence versus differentiation,

again, the picture is not very clear cut. I don't
want you to absorb this whole slide. Suffice it to
say that this is well, not all, but the major part of
the regulatory circuitry in G-1. Here you see the D-
type cyclance. CDK-4 and CDK-6 driving RB
phosphorylation, which in turn drives the second
phase, which is cyclin E production, activation of CDK-2. Of course there are a lot of modifying proteins here, CDK inhibitors, kineses that activate the basal CDK kinase, et cetera.

Now this is an area that is receiving a lot of attention. The general theme, at least to me, it seems that there's a high degree of overlap between mechanisms that regulate quiescence, senescence, and differentiation. I don't think this is really surprising because all these three states are characterized by the absence of cell cycle progression. In most cases, by an arrest in the G-1 or a G-0 state.

The one central theme is that the regulation of cyclin dependent kinase activity is necessary to achieve a physiological cell cycle arrest. In addition to the cyclins, which are the positive affecters, there is a number of CDK kinase inhibitors that have been shown to play a key role.

The two major inhibitory pathways that act on this basal cell cycle machinery are the RB pathway, shown here, and also the pathway regulated by the tumor-suppressor protein P53. In both of these pathways, CDK kinase inhibitors have been shown to play key roles.
So let me turn to the issue of immortalization. We all know that senescence can be overcome because quite obviously, there are many cell lines out there that are very immortal. So in a very simplistic and general sense, we can think of cell culture in three broad categories. We can have primary cells or cell strains that have a limited life span and senescence after several passages. We have a category of cell lines that are immortal, not necessarily by the 3T3 protocol, but in general, they display the characteristics of unlimited lifespan, non-malignant phenotype, and in most cases by the ability to become quiescent.

Finally, we have the large group of cell lines that are derived from either tumors or have been transformed by one process or another. These of course also have an unlimited lifespan, but they have a malignant phenotype as defined by one or more criteria. They also usually cannot become quiescent.

This again is the Hayflick plot. What I am showing here is a rodent culture, mouse in this case, and human. This little bump on the curve in fact is senescence for a mouse fibroblast culture. So it's been known for a very long time that rodent cells can overcome senescence spontaneously.
You can also see the great difference between the replicated lifespan in vitro of human cells that go on for a very long time. If this experiment here was continued, it would level off and you would see the typical Hayflick phenomenon. So the human plateau up here in fact is corresponding to this rather short plateau senescence in rodent cells.

So the relatively low frequency of immortalization -- I should point out that this doesn't really seem like a low frequency, but on a per cell basis, it actually is an event that has a frequency of 10 to the minus 5, to 10 to the minus 6. It's just that the X axis is plotted in days here.

The fact that this immortalization can be stimulated by mutagens has led to the hypothesis that this in fact is a mutational event in nature. This is supported by the existence of several viral genes, such as, and we have heard about them here, SV40 large T antigen, polyoma large T antigen, animal virus E1A, HPV E6 and E7, that can cause immortalization. In fact, when these genes are introduced into rodent cells, they are sufficient to cause immortalization in a single step. In other words, if you take a rodent culture and you put SV40 large T into those cells at this point, the curve would look like this. No
apparent senescence under the right culture conditions.

So what are these viral oncogenes doing to promote immortalization? Without going into a lot of detail, there is a large body of evidence that now indicates that these proteins interfere with the function of the P53 and/or RB growth inhibitory pathways. In agreement, there's a lot of data from knock-out mice now recently that has shown that the elimination by gene knock-out of a variety of negatively acting affecters can result in apparent one-step immortalization, as shown here for example.

To date, embryo fibroblasts from strains deleted for P53, P16 inc 4A, P19 arf 1 in P21 cip 1 have displayed this apparent immortalization phenotype.

So what happens in human cells? Normal human cells have never been observed to spontaneously immortalize. Senescent cultures do not give rise to sub-populations that resume proliferation as shown here. Treatment with mutagens has been shown to sporadically give rise to immortalized derivatives, but the frequency of these events is significantly lower than that in rodent cells.

Let me now talk a little bit about the
phenomenon of crisis. So what happens when we put, for example, SV40 large T or E1A into a human fibroblast? What we get instead of immortalization, is a phase of so-called extended lifespan. So here we see a primary cell, the initial proliferative phase.

This is senescence or the Hayflick limit. The introduction of a viral oncogene is going to cause an extended lifespan for variable duration, typically in human fibroblasts of 20 to 30 divisions. Then one sees a second proliferative decline. This has been designated as crisis.

Now this decline at the end of this extended lifespan which we call crisis, this word is somewhat ambiguous, because it has also been applied to rodent cells. These cells do not display a two-stage mortality process. So to distinguish more clearly between senescence and crisis, some groups have started to use the word "M1" for mortality stage one, and "M2" for mortality stage two.

Senescence is different from crisis. These are not just the same proliferative decline. The main distinction is that cells in senescence or M1 are truly non-dividing. Whereas in crisis cultures, the apparent absence of proliferation on the macroscopic scale is actually the result of ongoing
cell division combined with ongoing cell death.

This is an experiment that was performed in my lab. What we show here is that elimination of the CDK inhibitor P21 in a pre-senescent normal human fibroblast causes an apparent extension of lifespan that is equivalent in magnitude to that elicited by SV40 large T antigen. So also in human cells now we have been able to do ablative intervention. That is eliminate the activity of certain negatively acting affecters and cause an apparent extension of lifespan.

In terms of cell substrate design or the technology that would go into doing this, this was really strictly an aside, we have now developed methods -- these are really based on gene knockouts, homologies reculmination gene targeting, that can be used to delete entire genes, multiple genes in human cells, including normal human cells.

So let me now turn to my last topic, which is the molecular clock of aging. I think probably this is where I am going to overlap with what Jim has already said. As I told you, there are some older observations that correlated entry into senescence with the lap cell division as opposed to chronological time. Quite a few years ago, this has led to the proposal for the existence of some sort of a molecular
clock. Then one envisioned that the running down of this clock would generate a signal that triggered the senescence program.

Then the expression, for example, SV40 large T could either prevent senescence by overriding a signal from this clock or by what I think is more likely now in light of new evidence, actually interfering with the senescence machinery itself.

So as you know, the currently prevailing hypothesis is that the nature of the molecular clock is the attrition of telomeres. This is a slide by one of my dear friends, Chris Counter, who has fancifully imagined H-TERP, which is the catalytic sub-unit of human telomerase sitting here at the end of a chromosome end. So this is a telomere here. Then catalyzing the addition of the telomere heximer. You can see the telomerase RNA that acts as a template for that process right there.

Germ cells and some key stem cells are known to express telomerase catalytic activity while the majority of somatic cells lack this activity. The estimation of telomere shortening for one generation is in human cells between 50 to 100 bay spares. So that's 50 to 100 bay spares per S phase. This correlates reasonably well with the average telomere
length in a young human fibroblast of 18 to 20 kilobases and the length of 8 to 10 kilobases in the senescent fibroblast. I think it's an important observation that senescent cells in fact contain appreciable telomerase. So here we have a normal cell or a young cell. We get attribution of telomerase. At this point, the telomerase are maybe 8 to 10 kilobases in length. This generates a signal. If the cell is now driven into the extended lifespan phase, these telomeres will continue to erode because telomerase is not expressed in that state. Eventually one enters into a crisis which is caused by erosion at the end, genetic instability, et cetera, et cetera.

It is really the nature of this signal that I think is one of the enduring mysteries of the field. One can really now beginning -- we can start to see the process as being composed of a clock, a signal, and then the senescence machinery itself which is most likely composed of the same players, CDK cyclin inhibitors, et cetera, et cetera, that are used in other types of responses such as differentiation and quiescence.

The linguistic definition of senescence is the state of being or the process of becoming old.
This term has therefore been used to describe essentially any sort of age-related irreversible proliferative decline. In light of these new molecular insights, I prefer to use senescence in the more restrictive mechanistic sense to designate the response triggered in normal cells. I really believe that senescence is an active genetically programmed process that responds to an inductive signal. Perhaps telomere shortening, but that is not 100 percent clear.

How the signal is generated is not really well understood. One can argue that the ensuing growth arrest has the obvious advantage of preventing the cell from becoming grossly genetically unstable. In contrast then, I think of crisis as an unphysiological state. You have to do something to the cell to drive it to this point, and that it leads eventually to the catastrophic breakdown of chromosome stability, which is caused by critical telomere shortening on many chromosome ends.

So now this is really just a restatement of the two-stage mortality process. What I have added here now is telomere length in kilobases on the Y axis, the replicative age on the X axis. So here we have a cell in the beginning. If this happens to be
a germ cell or a stem cell, it will maintain telomeres because it will express telomerase activity. Most somatic cells will start down the slippery slope of telomere attrition, eventually entering into a physiological state of growth arrest, through which they can be driven by either the expression of certain viral oncogene or the ablation of certain inhibitory pathways that are intrinsic to those cells. The cells then enter into extended lifespan. They continue to erode telomeres. They enter into a state of crisis, which is characterized by genomic instability. Finally, at this point, one can attain a truly immortalized derivative in the key step here, is the expression of telomerase catalytic activity. I should also point out that telomerase need not be expressed at the final step. It has been shown experimentally that telomerase can be artificially or experimentally activated anywhere along this line, and that that will lead in some cell types, not necessarily all cell types, to immortalization. However, I think the large body of evidence suggests that at least in vivo, and by this I mean during the natural development of malignancy,
the activation of telomerase activity is a relatively late step.

So if crisis doesn't exist in rodent cells, and bypass of senescence is sufficient for immortalization, how does telomerase become expressed in somatic cells, rodent somatic cells? The bottom line here seems to be that telomerase is not very strictly regulated in rodent cells and tissue. A variety of rodent tissues have been shown to express telomerase activity. Telomerase negative primary cultures often become telomerase positive over time even prior to reaching senescence.

In contrast, telomerase appears to be regulated very stringently in human cells. Therefore, telomerase activation could occur in rodent cells that are undergoing immortalization either prior to or after the senescence bypassing event, and could easily occur in the subtle and gradual fashion so that no clearly apparent downturn in proliferative capacity of the ball culture would be observed.

In other words, one step immortalization that one sees so often in rodent cells may in fact require two steps, the obvious step of senescence bypass and very likely a second step that may be very subtle, at least in rodent cultures. That is, of
activating telomerase catalytic activity.

So I think that is about as good a summary as I can think of in 20 minutes. I will be glad to entertain questions.

(Appause.)

AUDIENCE MEMBER: Bill Egan, from the FDA. When you immortalize cells, you know, after they go into crisis or whatever, what becomes the length of the telomere? Does it go back up to 20 kilobases?

What maintains the length of that telomere at a fixed --

DR. SEDIVY: That's a very good question.

AUDIENCE MEMBER: Why doesn't it become 30 or 40 kilobases.

DR. SEDIVY: In fact, it seems that excessive telomere length is not good, at least in human cells. It's been known for a long time that many spontaneously immortalized human cell lines which we love and honor like 293 and Hela, et cetera, et cetera, have very short telomeres. These telomeres can be maintained at a length of one to two KB. These cells seems to be perfectly happy with that.

So I think it's more the maintenance of the telomere length rather than the absolute length of the telomere.
If you artificially introduce telomerase catalytic subunits into fibroblasts, what one typically sees is that the best clones are ones that build up telomere length to about 8 to 10, 12 KB and then maintain it at that level. It seems to be a function of the expression level of the H-TERP gene, because if one does this experiment, you see cultures that very slowly erode their telomeres. They will eventually senesce.

You see cultures that build up telomeres to maybe 20, 30 kilobase in length. That doesn't seem to be good for them because the rate of growth goes down. So really the best cultures are the ones that maintain at least in fibroblasts. So I think it's the maintenance rather than the absolute length.

DR. HUGHES: Would you please comment on Carol Greider's knockout mice?

DR. SEDIVY: Well, yes. I didn't get into that at all because that's at least for the time being -- there are some paradoxes here. Okay? The obvious paradox is that mice have extremely long telomeres, 60 KB on average. This is the laboratory mouse. Muskulorattus has perfectly normal telomeres, for example.

In fact, this is really the other way
around because these cells live for a very short time in tissue culture. When they undergo senescence, they undergo senescence with telomeres that are 50 KB in size as opposed to 60 KB in size.

So there are really two answers to that.

One answer is that if you look at individual telomere ends, you will see that there's a certain degree of heterogeneity in that in fact in all cells, including mouse cells, you always see a minority fraction of chromosomes that have very short telomeres. If the signal is caused by a perfectly short telomere that gives, for example, DNA damage-like signal, then you only need one per cell to give the senescence signal. So that's one explanation. I don't really know whether it's correct.

The other explanation is that mouse cells don't senesce. They neither undergo crisis or they undergo senescence. In fact, that plateau that we are seeing during the immortalization is not senescence.

It's a differentiation-like process.

There are people, including myself, that given this kind of loose distinction between quiescence, differentiation, and senescence, would prefer to define senescence now as a process that is triggered by telomere erosion. Obviously when a mouse
primary fibroblast culture undergoes senescence, it's
not doing it, probably not doing it because it's
receiving a telomere signal.

I don't know if that is -- does that make
sense?

DR. HUGHES: I had hoped you would comment
on the mice themselves.

DR. SEDIVY: The mice themselves? What do
you want to know about the mice themselves? They are
alive.

DR. HUGHES: Yes, I know. But in the
Greider experiment with telomerase knockout.

DR. SEDIVY: If you knock out telomerase
in mice, it takes six organismal generations to
observe lethality. Okay? What you see at each
generation is that the average telomere length. So
genation one, it's 50. Generation two, it's 40.
Generation three, it's 30.

If you take mouse and real fibroblasts at
any one of these generations, they senesce in vitro on
schedule. Is that what you wanted?

DR. HUGHES: (Inaudible.)

DR. SEDIVY: Well, I think I just offered
you one explanation for that. That is that what we
are calling senescence is not senescence. It's
something caused by some insufficiency in the median
that is in fact triggering a differentiation event.
Actually, Jim McDougall and I also don't quite agree
about what's happening in his keratinocyte cultures
because what he is calling senescence, some
keratinocyte biologists would prefer to call

differentiation.
DR. NABEL: Okay. If we could move the
questions along, maybe brief answers. Then we'll move
onto the next speaker.
AUDIENCE MEMBER: Alex van der Eb, Leiden.
You just already answered, I think, my question, which
was why do mice cells, mouse cells enter senescence
while they have such long telomeres? In fact, you
answered already part of that question.
Do these cells that enter a so-called
senescence have high levels of P21 or P16 or something
like that?
DR. SEDIVY: Yes, they do. Yes, they do.
AUDIENCE MEMBER: So there is a signal
then.
DR. SEDIVY: If you take a knockout mouse
for P21 that doesn't undergo senescence. It just
keeps going. But you know, that's what I was trying
to say. That is that op regulation of P21 is not a
molecular market for senescence. P21 is op regular
because of oxidated stress, osmotic stress,
differentiation signals. This is a very general
machinery that is used to establish cell cycle/rescence.

I, in fact, don't know of any molecular
marker that is specific for senescence. This includes
the famous senescence-specific betagalactocytis
activity. You know, you see a lot of people staining
cells, and they turn blue and they say it's
senescence. Everybody knows if you put hydrogen
peroxide on your cells, they turn blue as well.

AUDIENCE MEMBER: Just a brief comment for
those people who might be setting up assays that would
be monitoring P21 sip. We, as I showed, found that
P21 is elevated in a subset of the HPB infected cells.

We did three other related assays. One was to look
for P21 MRNA. It turns out it's abundant in all
differentiated cells. But there is a post-
translational control on the accumulation of P21.

It turns out what happens is that if there
is not a signal that unscheduled in a synthesis is
underway, namely, abundant cyclin E, then proteosomes
rapidly degrade the P21 that's translated. When we
put in proteosome inhibitors, P21 piled up in all
cells and all replication was blocked.
We went on to ask one additional question.

That is, how does P21 actually block S phase or DNA synthesis. Unexpectantly, it had nothing to do with blocking cyclin E activity. It turns out cyclin A, CDK-2 or cyclin A CDC-2, can phosphorylate DNA preliminary cell and all these other subunits I showed.

The one thing cyclin A can't do is bind to PCNA. But when the P21 sip piles up in these cells that have excessive cyclin E, the way the P21 is actually functioning is by binding to the PCNA and blocking elongation, not initiation.

DR. LEWIS: This may be a naive question. Is there any change in the activity of endogenous oncogenes in cells at about the time they are entering into senescence, especially rodent cells?

DR. SEDIVY: By activity, you mean level of expression? I am not aware of that.

AUDIENCE MEMBER: What happens in spragues? I mean are they different than muskulorattus? Do they have shorter telomeres that they go through?

DR. SEDIVY: What happens in spragues is exactly the same that happens in muskulor.

AUDIENCE MEMBER: So even though one has
60 KB and one has 2 KB?

DR. SEDIVY: I mean, you know, this kind of all argues that the length of telomeres has nothing to do with this plateau in mouse cells that we define as senescence. Okay? I think there's a result that's kind of floating around, which is also consistent with that. That is, we all know the wonderful experiment of expressing telomerase in human fibroblasts which causes immortalization. It doesn't do that in mouse cells, which also argues that the length of telomeres in mouse cells is not what is triggering this growth.

DR. NABEL: John, I am going to just end with one last question. You referred to the notion of program of senescence and it being dominant. I am just wondering, has anyone ever done a cell fusion experiment where you have taken cells approaching their limit and then fused to neo-natal cells. Is it in fact dominant?

DR. SEDIVY: Yes. Those are very old and classical experiments. In general of course, senescence is a dominant state.

DR. NABEL: Thanks. The last talk in this session is from Frits Fallaux. The title of his talk is using defined adenoviral genes and primary human cells for the generation of immortalized cell
substrates.

DR. FALLAUX: First of all, I would like to thank your organization for inviting me here. The subject of my talk will be on the generation and characterization of new helper cell lines for the construction, provocation, and protection of recominance replication effective adenoviral vectors.

In the past few years, the interest in vectors derived from human viruses. This is caused by the fact that from the many years of intensive fundamental research on human adenoviruses, it has been found that adenoviruses have several favorable characteristics, including high stability of variance. The variance is very easy to grow into pure with very high fibers. It has a very broad host range. Importantly, it has the capacity to transduce non mitotic cells. This makes adenovirus a very potent gene therapy.

It is known that it has very low kinisity, and there is there ample experience with adenoviruses as vaccines.

This slide shows a schematic representation of the adenoviral genome. It is a double stranded linear DNA molecular of approximately 36 KD, carrying several genes, flanked by inverted
herminal repeats. The genes are sub-divided in so-called early genes and in late genes, depending whether they are stressed early or late during the lytic infection.

This slide shows you a scheme of the classical methods to construct the common adenoviruses. All currently used adenoviruses carry a deletion in E-1. This renders the virus replication effective, and it also provides space to insert therapeutic genes.

Now in the old days, we used to isolate the DNA from wild type adenoviruses at 5 or S-2. In purified DNA, and I just -- the restriction enzyme cla-1, which puts ones in area region one. We then purify the large fragment.

In addition, it also needs the construction of an adaptor plasmid which carries the transcriptase unit, including geno-fenchrfras, but also the left inverted herminal repeat and a part of the adenovirus sequence which is also present in the large fragment. Pro-construction of these two molecules in so-called helper cells, and the helper cell is the 293 cell made by Frank Reim. Upon close inspection, another mination occurs, creating now the recombinant adenovirus, carrying the gene of interest at the
position that we want.

You can proficate these elongated viruses due to the fact that the helper cells complement the missing elong function.

Now despite the encouraging results of things so far with the use of recombinant adenoviruses, there are also several problems associated with the use of such vectors. These problems include the growth infectivity range. That is, you do not only infect the target cells, but also non-target cells. This may cause pathogenicity.

Also, the viruses are rather immunogenic. We only leave off E-1, and all the other viral genes are still present and can be expressed to low levels, resulting in numerous responses by the host, both humoral responses, antibodies, and cellular responses against new cells. The cells are killed and the therapeutic effect is lost within several weeks.

Another issue is the occurrence of replication competent adenovirus, abbreviated RCA. I will focus on this topic. There are various sources of replication competent adenoviruses. In a sort of infection, during the production of viral, or as an earlier stage, or during the construction of the recombinant factor, especially when you use classical
methods, if you use the large clavon fragments. If the digestion if not complete, you have RCA, namely the wild virus.

It has also been shown recently that you can generate RCA by homologous recrimination because
the factor and the helper cell carry adenovirus sequences that overlap. As a result, by homologous recrimination, you can get RCA. I will focus on this source of RCA.

Well, how does it work, homologous recrimination resulting in RCA? This is a scheme of a typical elongated factor. This is a scheme of the integrated adenovirus sequences in the helper cells.

The helper cell line is 293, and more recently, we made alternative cell line 911. Both helper cells carry the evon A, evon B in chorion regions. But in addition, they also carry sequences that enclose structural protein lines, downstream of evon B, and upstream of evon A, be left for determinal repeat.

Those sequences are also present in the factors. So that a sequence overlap 5 prime and 3 prime of the therapeutic gene. As a result, you can get homologous recrimination by which the recominance virus now trades its therapeutic gene for region E-1,
and becomes replication competent. Now what you can
do about this is to avoid the sequence overlap. We'll
come back to that later.

Well there are only a few helper cell
lines available when you work with recombinant
adenoviruses. We are fortunately in our lab to have
three of them, including the two in our free cell
line, recently an iomosa line, and even more recently,
the PER cell line. All three cell lines are obviously
of human origin. They are all derived from primary
diploid embryonic cells. 293 is derived from kidney
cells, 911 from retinoblasts, and PER C-6 as well.

Now when I started to work with
recombinant adenoviruses in our lab, which is the lab
of Professor van der Eb at the Leiden University, I
used obviously 293 cells, and I met with some
technical difficulties. Since we had a panel of adeno
virus transformed human cells, including cells of
kidney, lung, and retinoblasts, I decided to screen a
panel of cells in order to find an alternative for
293.

From this panel of cells, I selected one
particular retinal cell line. We named it cell line
911. The reason for this name was to get the
attention of our colleagues in the U.S.
Well, the construct we used to make the 911 cell line is shown over here. It carries the adenovirus sera type 5 nucleotides 87 to 5,788, including evon A and evon B.

Now I want to be short on the 911 cell line. The most important findings were that they performed very good in virus titrations. We also found that the virus use of 911 are up to three times higher with various viruses, also recombinant viruses, three times higher than obtained from 293.

Some other characteristics of this cell line are that they express very high levels of evon A and evon B, are highly transfectable, which is important when you want to construct recombinant viruses at the classical method. The use of the viruses are very high, as told, and they perform very well in titration assays. So we concluded that 911 is a good alternative for 293.

However, I have shown you the construct we used to generate the 911 cell line. We now have a situation which is similar to 293. Namely, and also in 911 cells, besides evon A and evon B encoding sequences, also sequences of the left inverted termin are repeat, and sequences in part encoding protein 9
are present. So again, there is overlap and you can create RCA.

So what we decided to do is start all over again and make now the cell line in combination with a so-called matched vector, now sequence overlap.

What we did was to make a so-called packaging construct carrying only the evon A and evon B encoding sequences in which evon A is driven by PGK, a heterologous promoter, and a heterologous poly and signal, and lay matched vectors that are deleted of exactly that elong region which is present in the packaging construct. Thus affording sequence overlap and thus eliminating homologous recrimination as a source of RCA.

This shows you one of the packaging constructs we constructed. Present are adenovirus sera 5, sequences four, five line to 3,511. Those are only the evon A and evon B encoding sequences. Evon A is driven by the human PGK promoter. Evon B is under its natural promoter, and directly flanking the evon B stop codon as the polyadenylation signal derived from hepatitis B virus.

Now before we decided to transfect in this vection construct into our retinoblasts, we decided to do some functional assays with this construct first.
We did this because first we only had a few frozen ampoules of the retinoblasts. Second, the packaging construct contains several PCR fragments. This slide shows you some of the functions or features of adenovirus evon A. The features are that domains 1 and 2 are involved in the regulation of expression of genes. Evon A is known to associate at the protein level with cellular proteins, P-105 RB, cyclin A, P-300. I think the list is growing. All these different features result in the transformation and immortalization.

Not shown is the feature or function of evon B. Evon B prevents the cells from growing into apoptosis as a result of the activities of evon A.

Now this is the actual functional assay we performed. At the left is shown the constructs we tested. At the right is shown the number of colonies we obtained upon transfection of one or five microprin of these plasmids into baby rat kidney cells. When we transfected in a construct expressing only evon A, we only found on average one focus or colony upon transfection of one microgram. This is very low.

This is caused by the fact that expression of evon A in primary cells, in the absence of evon B, is toxic, causing apoptosis.
Transfected this plasmid to come in plasmid in evon B, and we did obtain reasonable amounts of foci. Obviously also the construct we used to generate 911 resulted in focus formation.

Unfortunately, also when you are packaging construct, which we used to make the PER cells gave foci, thus indicating that the packaging construct allowed the functional expression of early region non-probenes.

So then we went to the actual experiment, transfected the packaging construct into primary human diploid retinalblasts. We could establish seven clonal cell lines. We tested these clones for first, expression of evon A and evon B proteins. Now we found that all clones expressed very high levels of evon A in both 55 and 21 K evon B, when compared to 293 and 911. We also looked at vector use. We looked at three clones, clone 3, 5, and 6. As you can see, we found that the three PER clones tested exhibited similar use of recombinant viruses, compared to 293 and 911. Since PER C6 played the highest use, we decided to analyze this clone in further detail.

Now of course the major issue for us was to test whether or not our approach to use the PER
cells in combination with matched adenovirus vectors would reduce or even eliminate the generation of RCA. This testing has been performed at the enzyme. What I did was to amplify an RCA free master stock of a typical adenovirus vector and amplify it to 293 or PER C6. What I found, I can summarize it for you, is that amplification of 293 resulted in RCA positive vector batches in approximately 50 percent of the places. Now for a clinical setting, that means that you might consider to throw away half of what you had made. In the case of PER C6, fortunately in none of the batches amplified on PER C6, we were able to detect RCA, not even in a large scale production setting. So we concluded that our strategy to make a PER cell in combination with new matched vectors severely reduced, maybe even eliminated the RCA problem, at least by homologous recrimination. The next two slides summarize some of the other features of PER C6 cells that contain three to five copies of the packaging construct, very high levels of evon A and evon B, comparable to 293 and 911. Good use of the different vectors, also similar to the other two producer cells.
The cell line was very stable. We have now come over passage 250 actually. So far we have not detected RCA, and the list of productions with different vectors is still increasing.

We have a master cell bank available for PER C6, also a working cell bank. Importantly, the PER cells were made on a GLP conditions, using certified U.S. bovine serum and trypsin. Currently, InterGene is doing all kinds of tests which were necessary for the use in the chemical setting, including mycoplasma and sterility testing. In the academic lab, you can simply draw the cells to standard medium.

Finally, I would like to thank all the people that are involved in this project. Number one, the PER cells were generated in the lab of Professor van der Eb at Leiden University, in the Applied Virology Group, supervised by Dr. Gugen. The packaging constructs were made by Edie von Frel of InterGene, and all the downstream processing, a lot of work is currently being performed by InterGene, supervised by Valeria in the Adenovirus group, supervised by Dr. von Laud. As I told, all the RCA testing was performed at Genzyme by Kathy Hay here.

Thank you.
(Applause.)

DR. NABEL: Thank you. We'll take questions.

DR. FRIED: Mike Fried. Do these cells form tumor in nude mice?

DR. FALLAUX: Actually we did not test that yet. However, we did test this for the 911 cell line, the weakly tumorogen in nude mice. So you might expect the PER cells would exhibit the same feature with respect to that.

DR. FRIED: What is weakly tumorogen?

DR. FALLAUX: Compared to some of the let's say adenovirus sera type 12 transformed rodent cells, they are much less tumorigenic.

DR. SHEETS: Becky Sheets, FDA. I had a similar question, but I have a couple of other. Does the PER cell stay diploid or is it aneuploid? Also, the individual from whom you obtained the retina, did they have wild type RB genes or were they -- you know, was this someone that died of retinal blastoma?

DR. FALLAUX: For your first question, they are no longer diploid. I don't know all the details, but we do find chromosome duplications I guess in PER C6 because we have tested recently.

Your second question. This may sound
silly, but we are currently trying to find out the
information of the donor. The cells were isolated in
the early 1980s, and we're now working backwards to
find out those details.

DR. NABEL: John?

AUDIENCE MEMBER: 293 cells have become
quite popular in the laboratory for reasons that have
little to do with their ability to support adenovirus
vector replication. Have you checked these other
cells, for example, high levels of trans vectibility in
being a good host for other kinds of viruses and that
kind of thing that make 293 cells so beloved by many
virologists?

DR. FAL LAUX: In fact, all adenovirus
transformed cells are as highly trans vectible as 293
cells.

DR. NABEL: Okay. If there are no further
questions, then I think we can just proceed onto the
panel discussion. John Coffin will chair that. If
the panel members want to come forward and get
started.

DR. COFFIN: By my calculation, we are
running almost exactly an hour late. A check with the
board outside reveals that we really probably can not
go much past 3:00 before we start to lose people quite
seriously. So we probably should shoot for an hour in
which we either have a lot or a little to do,
depending on the will of the crowd and our host.

This panel discussion actually as two
functions. One is a discussion of the last topic
covered. That is the designer cell substrates, two
talks we heard today and the one yesterday. Then
secondly, where we really earn the generous honoraria
that FDA is paying us, where we try to summarize and
hopefully answer some questions that might be useful
to our host in terms of development of policy, ideas
for further meetings, experimentation and so on.

Tentatively we'll plan to sort of split
the discussion half and half between these issues, but
I think we can play that by ear as we go along.

Again, I expect widespread audience participation,
particularly since these are topics that I myself am
not really actively working in and am familiar with.

The questions that on the first part, on
the designer cell substrates, that we were charged to
address are summarized on this overhead. Before I
turn it on, I want to apologize in advance for two
things. One is my handwriting is very bad, so you are
going to be subjected to that for a while. Secondly,
I was given a rather blunt instrument to write with.
Thirdly, of course I'm not well enough organized like some of the previous chairs who have prepared these ahead of time.

So this is a paraphrase, I hope an accurate one, of the issues that were raised, that are raised in the points to consider. The first regarding designer cell substrates is the issue of whether cells that are derived by the kinds of defined means that we have seen, and we have seen the example of introduction of telomerase plus or minus oncogenes, viral oncogenes, or viral oncogenes alone whose function among other things was to stimulate or inactivate genes that are involved in senescence.

Whether cells that are created in this way in fact offer significant safety, create safety issues relative to other cell lines, whether they offer advantages or disadvantages, whether we can go through the sort of defined risk algorithm that was given to us at the beginning of the meeting, to address these and anything else.

So if we could get onto the first point here. Are there significant safety issues relative to tumor or neoplastic cell lines? In other words, uncharacterized, what we should call it perhaps, uncharacterized cell lines. Cell lines that have just
been handed to us either in tumors or that have arisen by means we don't really understand very much about in culture.

Would anybody on the panel care to --

DR. SEDIVY: Yes. I would just like to make a very brief comment about designer cells. I was asked to talk about the history of immortalization. In fact, what my lab works on is more related to interventions, genetic interventions in human cells. So you know, obviously we have talked about putting an H tert and putting in various viral oncogenes. So really here the issue is can we make a cell line that is immortal and it has a particular spectrum of phenotypes that we want by absolutely defined genetic interventions. I think the answer now is yes. We can do a lot better than putting in SV-40 large T or E-1A or E-7, because as have heard even today, we don't really know exactly down to the last T what these viral proteins are doing to the cell.

So in fact, what we can do is we can delete cellular genes using gene knockouts, and produce very much the same effect. That is, we can really now contemplate really designing cells without the use of viral oncogenes. I think that putting an H tert is obviously a necessary step, but this is a
cellular gene. So I think that's probably okay.

DR. ONIONS: It's really a question of clarification from my point of view. But it seems to me that one of the advantages of cell lines that come from potential oncogenic background is they have some of the features from mass culture that industry.

That is, they can be grown in an anchorage-independent way. They can be grown in high density in fermenters.

What's the position with telomerase immortalized?

I assume actually they are mimicking much more the kinds of cell type that Dr. Hayflick would define as -- I forgot what he used, apologies -- the first stage of cell strains. That probably do not have those particular phenotypic properties. Do you know what the stage of those cells are?

DR. SEDIVY: Well, you know, we have a really limited experience. This game has only been played for a few months, maybe a year in some privileged labs. I think what we're really talking about here is proof of principle. In my lab, we're not interested in growing cells in fermenters. We are interested in cell cycle progression. But I think if somebody wanted to make a cell line that grows well in fermenters, I think it may be a good idea to contemplate some of these new approaches.
DR. ONIONS: Yes. The point I was trying to make was that if you start down the road of trying to produce new cells and immortalize them, and you try and use procedures that you think are of in a sense, the safest, whatever that means, those might not be the steps that you actually need to actually produce an industrially useable cell line regardless of the importance of the science. The practical end may not be what you want.

DR. FRIED: What else besides, you said you would knock out genes? I mean would this be in human cell lines or instead of using the viral proteins?

DR. SEDIVY: I think in general, the first thing you have to do is you have to immortalize the cell. For that reason, we put an HH tert. That is a technical feature that we need because to do the knockouts, we have -- you know, there's limited time to do a knockout. We can do two knockouts before a human fibroblast undergoes senescence. But if we want to do more, and we're obviously interested in doing a lot more, it is very convenient to put tert in there at that point.

If you don't want tert, you know, tert now comes flanked with lock sites, so that you can take it
out later on if you are interested in that.

DR. COFFIN: Can you be more specific about what you would knock out?

DR. SEDIVY: Pardon me?

DR. COFFIN: Want you to be more specific about what you are knocking out. Which genes have you knocked out?

DR. SEDIVY: Well what we are interested in doing is we are interested in dissecting the machinery that establishes senescence. So not in a single cell line, but at this point, we have P-21, cip 1, RB, B-53, and P-16 ink 4A knockouts in various combinations. So you know, the vectors are available.

One interesting point about human cells that is interesting to the technocrats I think is that until this time, we do not see the requirement for isogeneic DNA. So in fact, these vectors that we have made, and some other labs have contributed to this, can be used and essentially -- and have been used in any human cell with equivalent frequency of recombination.

DR. HAYFLICK: Yes. We have a lot of information about the biological properties of H tert transformed normal human cells now. There are the ones with which Choma is familiar, are now as I
indicated several days ago, approaching, and in one or
two cases beyond 400 PDLs. Many of them are beginning
to show some signs of aneuploidy. They are still
anchorage dependent. Their virus spectrum seems to be
identical to that with which we are familiar prior to
H tert transformation. Finally, the studies that have
been done on animal inoculation of these cells
indicates that they are not neoplastic. So that we do
know that much.

However, we are not talking about two
other classes of cell populations that are important
candidates to consider. I mentioned these in my
opening remarks on Tuesday night. Namely, normal
human diploid cells that can be immortalized using
repeated treatments of exposure to chemical
carcinogens. We published on this on a population
that that explanation defines about 25 years ago.
It's called SUSM-1. It's freely available. It is
from a normal human diploid fibroblast transformed by
multiple exposures to MMNG.

A second class of cells that also falls
into this category of normal human cell populations
transformed with something other than a virus, by way
of example, is KMSD-6 transformed by a former student
of mine, using multiple exposures to cobalt 60
radiation. That cell population was also described and in fact is used commercially today. It was described about 25 years ago. I think it is very important to realize that it's possible to immortalize well-characterized normal human diploid cells like WI38 and MRC5 and perhaps others, utilizing non-viral means.

I would also like to repeat what I mentioned the other day because it's been restated again, and it's not accurate. That is, that there are spontaneously transformed normal human cell populations. I will be happy to provide the references to whoever would like to have them.

DR. COFFIN: Any response to that? Do we feel in terms of this first issue that there are significant safety, differential safety issues of these kind of cells relative to the relatively uncharacterized lines?

DR. MINOR: From the point of view of the infectious agent side of things, I mean I don't see much difference between, you know, a brand new tumorigenic cell line that appears in your hand and one that's actually being designed to actually appear like that. It seems to me they are both uncharacterized and you would have to look at both of
them very carefully. I'm not sure that you have new
infectious issues simply because you designed it to be
transformed.

DR. FRIED: Something came up the first
day. Would it be worth getting new cells that really
grow well with defined media so we can avoid serum and
any problems that come with that as something one
might think about?

DR. COFFIN: If you avoid serum, I think
would be a highly desirable trait in vaccine
production if one could engineer that. Obviously
there are BSE issues, and that sort of raises what
might be the tip of an iceberg. Is that a practical
goal for production?

DR. FALLOUX: Actually, for the
production, we now can grow PER C6 in serum-free media
in suspension.

MR. LEWIS: Lewis, FDA. Are there any
restrictions to the telomerase immortalization? In
other words, there are some populations that you can
immortalize and others that you can't, or is this a
universal way of immortalizing all types of human
cells?

DR. SEDIVY: You know, I am by no means
expert on this, so I can, you know, basically restate
what I think is already out in the literature. That is that you can definitely immortalize human fibroblasts, pre-senescence fibroblasts by putting H tert in. That's been shown now in a number of laboratories.

You can also immortalize retinal pigmented epithelial cells as reported by the Texas group. I am not sure whether it's been published yet, but I have heard that you can immortalize T cells, CD8 positive peripheral lymphocytes.

Jim McDougall says that you can not immortalize keratinocytes unless he said something different yesterday, in that you need to interfere with the RB pathway in addition to putting H tert. There's also some indication that breast epithelial cells may need an additional step to become immortalized. That's all I know at this time.

DR. LEWIS: Since you can immortalize T cells and you suspect that those would grow in suspension like normal T cells, and you could grow them in any large suspension culture, that you would need to.

DR. SEDIVY: I don't think this work has been published, so I think I'm just telling you something that I heard at another meeting. So maybe
we should just cool it. But I would presume that if
they grew in suspension before tert, they would grow
afterwards as well.

DR. COFFIN: Obviously these immortalized
T cells would be of great interest to people who are
interested in growing attenuated HIV vaccine.

DR. FRIED: But I mean once you have
something that's immortalized, you could always select
for something that grows in suspension, or you could
try anyway.

DR. COFFIN: But when doing that, of
course as soon as you start selecting for these
additional characteristics, when you are introducing
new and uncharacterized genetic changes.

DR. FRIED: Right.

DR. COFFIN: That these are less important
than safety issues, than changes that might have led
to immortalization in the first place.

DR. SEDIVY: You know, I think I would
also like to second the point that was brought up
earlier. That is that sure, we can always make the
claim that we know exactly what we did to the cells
ourselves, but we don't know what the cells have done
on their own during those zillions of passages that
they are growing in my lab. It's definitely being
documented that H tert immortalized fibroblasts are karyotypically very stable. But if you passage them for long periods of time, you will find anemploy these. So I don't think that that's any different from any other established cell lines.

So it's really, you know what you did, but

DR. COFFIN: But you don't know what

happened. To bring a point to this, if one is concerned about issues of what might happen with DNA from the cells that was carried along, then it sounds like, it sounds from what I'm hearing like there may not be a great deal of difference between using these cells and using these kinds of cells as compared to using relatively uncharacterized cells.

Although there are very good reasons for making such cell lines, that this particular issue may not be the most important one.

DR. SEDIVY: You know, you could do some pretty neat things that under certain circumstances may be very advantageous. For example, if somebody wanted to knock out the endogenous PR gene, that could be done. You could make a cell line like that.

DR. COFFIN: You can also use factors that make these cell lines highly susceptible to viruses
you might want to be growing on them, and things like

that.

AUDIENCE MEMBER: I'll just make a comment

on one of the papilloma transform cell lines that

Margaret Stanley initially isolated. It harbors -- it

was from a cervical dysplasia and harbors episomal

HPV-16 from which E-6 and E-7 are expressed.

Paul Lambert sub-cloned an isolate that

carried generous, approximately a thousand copies of

this episome in a fairly homogenous state. The

problem with it, and it sounds very appealing to have

episomal maintenance of your E-7 gene. The problem is

it's dreadfully unstable, and nobody has been able to

keep it with the episome. It tends to integrate and

completely rearrange, and has gone aneuploid.

So any efforts toward trying to utilize an

episome are probably doomed to a degree of failure

because of the risk of -- well, you have to maintain

episomal replication in addition to your chromosomal

replication. It just doesn't seem to work.

DR. COFFIN: And this of course, this kind

of issue gets far more amplified when we're talking

about growing up 10 to the 13th.

AUDIENCE MEMBER: Yes, absolutely. We

can't keep this thing going for three months.
DR. FRIED: Also, every time you knock out an allele, you have to lock out the other alleles to get them both. So that means more passages, and they get away. I don't know whether that's good or bad. I mean if you finally end up with the cell type that has a lot of positive features, it may not really matter whether they are instability of chromosomal, and stable or rearranged.

DR. ONIONS: Just as a general principle about whether it's useful or not to engineer cells rather than go out and select a transformed cell, a pre-existing transformed cell from a tumor, it does strike me that again, that it's under control and that you have a number of choices.

The kinds of studies that PER C-6 has been involved in give you a very precise engineered system that's absolutely ideal for vector production. But it perhaps also highlights with respect to the mistake that was made. That is, that you had another possibility here where you could actually choose the cell. You could validate its origin. You could check the person. That is the other advantage of you being able to engineer materials, that you can actually pre-select the actual source of the material that you start with. That would have been an advantage that
was unfortunately missed in this particular case.

That's not to undervalue the value of these cells, but it does seem to me that that's what engineering cells can give you. It gives you control at each stage of the process.

DR. FALLAUX: Can I mention that it's nowadays rather difficult to take primary human, especially immuno material.

DR. ONIONS: It was -- I understand only too well. It's not at the end of the day a criticism of PER C6 success, which I think are excellent. But really just that where possible, that that should be done.

DR. COFFIN: Okay. So I think we have a consensus here that there's lots of useful things about such cells, but that we really don't know whether they enhance any particular safety issue or not. I think that's a sort of at least some sort of closure on that particular point.

The points we were asked to address also included the use of the defined risk algorithm that was mentioned at the beginning of the meeting, to evaluate these kinds of things. Andy Lewis had that on his slide, which I have asked him to put back on. This will also, I mean with this, we will
sort of segway into the general discussion as well I think, because these are the issues.

So the question is, can we go through and do this, and is it possible in this particular case, just using this as an example of this kind of procedure, to assess the level of risk posed by these issues, infectivity, infectious and so on, quantitatively. My own feeling right now is that we're no where near a position to do this, certainly for DNA issues. We might be able to put some sort of numbers on viral issues. It's a little hard to see exactly how because there's so many different ones, which could have a different contribution. But maybe we can get some further comments on these sort of issues from the panel.

DR. ONIONS: My only comment, and I understand why a defined risk approach was used, and it certainly makes you think. I mean that's one of its great virtues. I think one of the real intrinsic problems, that if you applied, you can give yourself a false sense of security. It would strike me that Phil's story about SV40, if it turns out that is the origin, SV40 in people, would have given you such a false sense of security, I think, because you might have come through that exercise in the 1960s. I'm not
sure how you would have predicted that that agent was there a priori. 

So I'm not sure that you can give guarantees that 1 and 10 to the 6th dose is one-half X, if you don't know what X you are looking for.

DR. SEDIVY: Yes. I mean I broadly agree with that. I think it's worth trying to do some sort of numerical calculations, so long as you don't believe the numbers that you get out at the end of it.

(Laughter.)

Because I think one thing it will do is it will tell you where you think you are confident, on what stage of the process you are actually confident. Then you can actually question whether your confidence is misplaced or not. But I think if you come out with a number, I think you are asking for trouble.

DR. LEWIS: Yes. I don't think that we discounted that. I think in sort of going through this thing, what we were trying to do was to figure out exactly where we can be reasonably confident of what we're doing and where we can not be confident of what we're doing. But we always recognize that when push comes down to shove, the bottom line is that we'll always -- we can never be sure.

So at some point in time, it requires a
leap of faith to say this product or this cell line, 
or whatever, can in fact be used. I think what we're 
trying to figure out a way to do is to make sure that 
when we get to the point or we have to make that leap 
of faith, that it's better to find than it would be if 
we did nothing at all. 

So the attempt here is to develop sort of 
a way of thinking about narrowing that margin of 
error, or at least to develop a margin of error that 
is better than it would be if we're just doing it on 
intuition. 

DR. ONIONS: I think that's absolutely 
right. I was taken by Neil Cashman's risk assessment, 
quantitative risk assessment today. I think again, 
what it did, although I think he himself didn't 
believe certain the numbers at the end of the day, it 
makes you think about the process. I think that's 
fine and I think I would agree with that. 

DR. HUGHES: One of the things that's true 
about the numbers that I've seen is no one has 
attempted to put what I guess I would call a 
confidence interval on the numbers. One of the things 
that makes me uncomfortable is that I think in some 
cases the uncertainty is as large as the number. I 
would feel a bit more comfortable with a calculation
with which I'm fundamentally uncomfortable, if I had

a better notion of how uncertain people were about the
assumptions they were making in generating the numbers
in the first place.

DR. ONIONS: I think that's what we're all

saying, is I think I started it off by criticizing the
whole approach. I think what Phil said is what I
think. I think Dr. Lewis said the same thing. That

is, don't believe the numbers. All it is is gives you
a manner of approaching what are the issues, really.
I think that's the way it should be treated.

I agree. I don't think anyone should

believe the numbers at the end of the day.

DR. HUGHES: I think it might help if when
someone put down a number, they at least put down a
range of numbers, and that would generate a range of
confidence at the end.

I think what people will see when they do
that, is that the ability to define the confidence

interval is going to expand when you multiply the
numbers together. I think that act may in a sense
help define how uncertain the number actually is.

DR. COFFIN: I think from a regulatory
standpoint, what often happens is that the far end of
the confidence interval on the worst possible side is
taken, and then that's propagated through. You never

see the other side.

DR. ONIONS: I don't want to just go into
an academic discussion about risk assessment because
I'm not really interested in it, in that formal sense,
but there are two other approaches that are used. The
engineering industry uses a form of analysis that
doesn't do risk assessment like that. It actually
looks for holes. It looks for what could go wrong.
In a sense, that's really probably what we ought to be
doing. Then there are four mechanisms of that kind of
analysis.

The other form of analysis is the one that
has become fashionable in the U.K., which is this
concept of the precautionary principle, which
ultimately, it seems to me, means you don't ever do
anything because you never know what might happen,
which seems to me completely dumb.

DR. COFFIN: So we're voting against the
precautionary principle. You can't be sure of
anything, but you can be sure of that.
Are there any other points anybody would
like to make about this? One could say that this is
a useful way to organize your thoughts on this
subject, but shouldn't be taken as giving you either
additional grounds or comfort or discouragement,

unless you actually had a situation where you had
measurable quantities.

Are there any other issues or questions
regarding the designer cell substrate issue that
anybody wishes to raise?

DR. SHEETS: Hi. Becky Sheets, FDA. I am
going to ask the whole panel what I tried to ask Dr.

Hughes earlier. That is, the kinds of questions that
sponsors ask us. One question I would ask is we've
heard a lot of people in this meeting say that the
oncogenic DNA issue has been put to bed. Has it?

DR. COFFIN: That segways us into the
next, into the general discussion, which is fine.
Before we go into that, can we see if

there are any other specific issues regarding cell
substrates?

DR. SHEETS: Any questions about the
quantitative?

DR. COFFIN: If we do that, we can turn
off that slide, put up my next one, and then you can
ask the question.

DR. SHEETS: There's one question.

AUDIENCE MEMBER: Jerry Sato from Merck.

I understand the reluctance to put a number on
something when you have such degree of lack of confidence and the assumptions that are going into it. But I do think that getting an order of magnitude of where we are is actually helpful in our thinking about what we feel comfortable going forward with or not.

When you have a lack of confidence in each of those areas, you also have to ask the question, what are the chances that all of your assumptions are wrong? In other words, are two of them wrong, three of them wrong, five of them wrong, seven of them wrong? Because you have to put a degree of that's not likely to happen. So if you multiply the lower end of the confidence interval for all those things, then you will never do anything. But that's not the way it works in reality.

So I think it would be useful for somebody from the engineering community, where they design bridges that aren't supposed to fall down and other things, to try and put a bit little more sophistication into this analysis, because in the end, somebody is going to ask our community, which is the regulatory community, the academic community, and the industrial community, for the number or at least what they thought the number was when they went forward with their act of faith. Because there is a certain
amount of common sense that goes into it, which is the
basis of the act of faith. Then there is whatever
to kind of quantitation we can put into it. It's the
combination of those two things that I think we are
going to have to reassure the general public about.

DR. HUGHES: I would recommend to you the
book Strategy and Conscience by Anatol Rappaport,
which attempts to deal with the issues having to do
with what was called strategic thinking, in which you
calculate, for example, the probability of some
unlikely event, such as thermonuclear war. Mr.
Rappaport does a very good job of making clear why
doing the calculations when you don't have the proper
data is in fact a very risky and misleading
proposition.

AUDIENCE MEMBER: I guess it might be
worth pointing out that there are, however, some cases
where you clearly do have the proper data. Right?
You know the sensitivity of a specific assay for a
specific adventitious agent, if in fact you choose to
figure out what it is.

So you can actually answer, based on those
kinds of questions, and based on that kind of data,
the specific question of how sure are you that
something isn't there. If a better assay comes along,
and it's still negative, then you can say by how much
more certain you are.

So just because you can not come up with
good estimates for some of the numbers, seems to me it
would be crazy to throw the baby out with the bath
water and claim then that you shouldn't attempt to
come up with good numbers for those things that you
can.

DR. RABINOVICH: Gina Rabinovich, NIH. A non-regulator asking a question from experience
learned this summer, in which we have been dealing
with using a quantitative number, i.e. the numbers
that the Federal agencies and the global agencies has
set for acceptable limits of mercury, i.e. methyl mercury, and then trying to attempt to understand what
those uncertainty factors mean for thimerosal and
vaccine exposure im.

The concern I have, and I think it has
been heard, is that these numbers take on a life of
their own. They become the standard against which
things are measured. So that that kind of concern
needs to be entered into, attempting to use the data
when those data do exist, but understanding the limits
to it.

DR. COFFIN: That's inherent in
regulation, that things become standard.

DR. LEWIS: Lewis, FDA. To follow up a little bit on what Phil was saying. I think one of the areas that we could approach with some confidence is the ones who saw Keith Peden's data last night using the tac man assay to detect JC, BK, and SV50 in human tissues.

Now if someone comes in with substrates they derive from a neuroblastoma, which we learned at the DNA tumor virus meeting a year or so ago, is it's usually contaminated with BK virus. We wanted to be sure that there was no BK virus in that substrate.

Then we could apply this assay with a fairly sufficient level of sophistication and say with some certainty, based on the volumes and things that were tested, the level at which that particular genome or that particular virus is absent. So I think that we sort of view that as a possible starting point for a quantitative approach.

Now obviously you can't do that unless you know exactly the probes and things that you are working with, and you define the limits of their ability to detect things. But I think this is one of the sort of examples that was going through our mind when we were thinking about how to do this.
So you start at the place where you might be able to generate some relevant data that's meaningful. The other stuff will fall into place as we get better.

DR. ONIONS: I mean I think that's exactly right. I think more and more that we move to assays, that we get good quantification on, we can validate them, and it's the sensitivity and limits of detection. I think all of that is absolutely essential. I mean I absolutely 100 percent concur with that. I think it does add confidence to those specific questions.

I think when you are asking specific questions, then I 100 percent agree. I think my concern is perhaps that where you try and make assumptions, for instance, of residual DNA. I mean I was the one who said I thought it should be put to bed. That is because all the evidence that I had heard didn't convince me that there was a risk.

On the other hand, I actually believe that no one has done the right experiment to actually convince me of that, in a formal scientific sense. So we're then dealing with the area of conjecture. That conjecture is based on non-quantitative data.

DR. COFFIN: We're leading into you,
Becky.

DR. SHEETS: I'm patient.

DR. COOK: I'm sorry. Jim Cook. As I was sitting there thinking about how you would describe these issues to a patient or to a group who is asking you about the wisdom of using a vaccine, it seems like in addition to trying to generate some logic about calculations of numbers and risks, that every opportunity that you have, it would be worthwhile going back to history and saying well, we have done virtually something like this along the way ever since vaccines have been developed, and the experience with this approach has been the following.

So maybe there could be some real numbers in a historical sense, used to color or give some more real meaning to these, what are otherwise theoretical things, to help communicate this to the public, as well as to provide some, a little bit more logic than just phenomenology to the calculations that are being made now. So if history is used to color the estimations, that might be of some use.

DR. COFFIN: How comforting is it to say--

DR. COOK: Say it again?

DR. COFFIN: How comforting is it to tell
patients that there are three or four cases of

paralytic polio?

DR. COOK: Well, I think you have to be
honest with them. You say look, you are one of 250
million people. The odds for your child is the

following, and I think it's a very good idea to use
this vaccine.

It is those kinds of conversations that

eventually lead adolescents into getting hepatitis B
vaccine. We're doing a miserable job of this, by the
way. I think as a culture, you know, if we bat 50
percent, we're lucky.

DR. COFFIN: That's clear in light of
things in the movies, because lately we're doing a
terrible job where these people show up with these
anecdotal cases of somebody's child gets vaccinated,
and then two months later is diagnosed with autism.
It's automatically due to the vaccine.

DR. ONIONS: But I think history can also

be a dangerous thing. I mean the British government
has been criticized, partly justifiably, but I think
partly unfairly for the problems of the way BSE

problem goes up.

But the issues concerning public health

were based on people were asked what is the risk, what
is the risk of the human population of the BSE outbreak, when we had a few hundred cases of BSE in cattle. Well, there were only a few hundred cases. The general assumption was, and it was a widely shared general assumption by those who were informing the area, the people who worked on scrapie and so on, well, scrapie has no evidence whatsoever of scrapie transmission to man. We have been eating scrapie-infected sheep for generations and it doesn't seem to have been transmitted to people. There is no evidence of that whatsoever.

The probable likelihood that BSE is of scrapie origin that's gone through the rendering process, because we have never seen a spontaneous spongiform encephalopathy in cattle, so that's probable origin ipso facto, you know, there's no problem.

It wasn't quite as glib as that because actually within a year, all the controls on human food were in place and so on and so forth. They were badly conducted, but they were theoretically in place.

So I think history can also be dangerous.

I'm not sure that we can always learn the right lesson.

AUDIENCE MEMBER: I get your point, but I
think there is a difference between an ongoing epidemic that's yet resolved, and the experience of X number of hundreds of millions of doses of polio vaccine.

So what I am saying is, if you are going to make a calculation about the likelihood of one in a million or one in 10 or 100 million happening with a vero cell or with a primary cell, you can say well, we've got experience making polio vaccine in vero cells, and there have been the following hundreds of million doses given, and the likelihood is that when the incidence of the real disease gets so low that you are finally going to see some background. That's the real --

DR. ONIONS: I'm sorry. You misunderstand. What you are saying, I absolutely agree.

DR. COFFIN: Do you want to continue this discussion or do you want to break into --

AUDIENCE MEMBER: Very similar aspect, although a little bit less scientific. As I perceive the discussion, of course there's this highly sophisticated, highly conscientious scientific community, and there's the general public on the other side.
The general public, to my perception,

consists of at least two sub-groups. One group that
is sort of generally benevolent and would believe
scientists. But there is a very strong group that is
not believing scientists. They are sort of using that
as a political tool to attract attention. In our
country, we have had this experience with the Green
Party, that has become very influential in the
European Union and maybe in other countries as well.

Now one thing I also, since we're ending
and coming to the end of this meeting, would like to
raise, isn't it the responsibility of scientists also
to do something to better educate the public? I know
this is a utopian goal, but at least if we could
increase enough of people in the general public who
are educated or better educated in science and biology
and biomedic issues, we would at least have a
political community that might support scientific
issues more valuable than we have had it so far.

You know, in our country at least, every
time when the Greens demanded to stop all biomedical,
all gene technology research, and it course never
happened. After they come to power, maybe they have
different outlooks on life. But I think as a
scientific community, unless we do something at least
for the future, we might be in a very difficult
	situation to defend certain issues.

                      If I confronted some of the violent
ideologically pure Greens in our country, because the

                      trick is, we have been discussing here, I'm sure they
would say "Shut it down because this is unsafe,
totally unsafe."

                      So what I am trying to recommend is we

                      have to do something to have more people in the
general public who can appraise and can assess the
difficulty and the uncertainty in any biological
research. We can never get down this figure to 10 to

                      the minus 80. So we have to raise understanding on
the other side.

                      DR. SHEETS: So have we put oncogenic DNA

                      to bed?

                      DR. COFFIN: No. I would like to use the
sort of summary -- I think that's in a sense, an
overhanging issue. We have talked about infectious

                      risks and measurements and so on considerably today

                      and in the past few days. I think an overhanging
issue is this oncogenic DNA issue regarding the

                      specific charge of the meeting, which is the use of
tumor versus other kinds of cells, tumor and

                      neoplastic whatever, transformed cells versus other
kinds of cells as substrates for vaccine production.

Although I think many of us here, perhaps all that's here, feel this is not a risk to be really concerned about in a scientific sense, I think many of us here might agree that the issue is not completely put to bed in the sense that we can't put any real good numbers on it.

So now if you ask your question.

DR. SHEETS: Okay. Has oncogenic DNA been put to bed?

DR. HUGHES: I will answer in two different ways. I will give you my opinion, personal opinion, and then I will tell you what I think should be done, which is slightly different.

Personally, and this would apply if you approached me to do something to myself, I am not concerned. However, it is my view that the data that we have, particularly for the consequences of putting DNA into animals, is not sufficient to satisfy me as a scientist. I am going to try to help my colleague, Dr. Coffin, and some of my colleagues at the FDA to try and organize a simple study that would be more satisfying to me.

I think I would feel more comfortable if we had more data that was of the experiments done on
a larger scale under more controlled conditions. I think that would give me a greater degree of comfort.

DR. ONIONS: I think I almost entirely concur with that comment actually. I did say a comment, and said it partly to be provocative, but on the other hand, I think I share that opinion.

I have seen nothing that would convince me at the moment on the data, which there's a singular lack of, or just from I suppose theoretical reasoning, to suggest that this would really be a significant danger. But on the other hand, there are the tools now, and I say yet again, but I think some of the transgenic models offer that possibility for testing, whether or not DNA is a risk. It can be done in a series of graded experiments from taking the worst case examples of actually simply just repeating -- of injecting oncogenes at various titrations into animals that are already primed with oncogenes as a transgenic, down to taking tumor DNA down to taking normal DNA.

I mean those experiments, they are pretty straightforward to do. I mean the interpretation might be a bit more complex, but they can be done. I think they are worth doing. It might help you to put some limits, broad limits on the thing we're all just
making conjectures about.

DR. MINOR: I don't think it's been put to bed either.

DR. COFFIN: For the same general reason?

DR. MINOR: Broadly speaking. I mean I think it's clearly a very, very complex issue about how you actually induce a tumor. So you do your 3T3 assays and you pick up H ras. Okay? I mean it's an artifact of 3T3s or was that just a question of how common H ras is.

If you go and put your DNA in intravenously, is that the same as putting it in subcutaneously, for example? I mean if you put it in because it's been picked up by an envelope virus, is that going to make any -- will it be picked up by an envelope virus? Will that make any difference?

I mean it seems to me that there are so many sort of loose ends to it that I don't think while there is no evidence that DNA is tumorigenic, and I buy that 100 percent, it doesn't seem to me that it's necessarily been dealt with properly. That is why I guess I am agreeing with what the previous two speakers said.

DR. LOEWER: So as I already have said, I personally believe that there's not a real big risk
with purely oncogenated DNA. Purely oncogenated means
three or five or six. But I realized that there is
still, I believe, lack of experimental data. This was
already mentioned by John Coffin, in saying that since
18 years, this question is on the table. Since 18
years, no additional experiments have been performed.

I would like to propose that regulatory
authorities, which are involved in regulation of these
biologicals, the major ones, that we should sit
together and to join the efforts and maybe decide on
experiments which can be done in the foreseeable
timeframe.

But I look forward to see what types of
experiments John may recommend.

DR. FRIED: I think most of the evidence
we have so far, which is limited, says at least
putting DNA into animals, we haven't seen anything
happen.

We have only seen in NIH3T3 cells, and we
now know that there is a defense mechanism in the cell
when it sees an oncogene. That is this P, this arf,
which is the alternative reading frame of P-16. So
it's like an immune system of the cell, specifically
for oncogenes. The radiation activation of P-53 is a
different pathway. This turns on, and arf activates
P-53, and P-53 then closes itself to go through apoptosis.

The only positive things are in NIH3T3 cells. They are the classic cell where the arf gene is inactivated. Probably that's why people have been using that for years. They are very easy to transfect because maybe even transfection kills the cells in terms of P-53.

But that said, I would like to see a lot more injection of DNA from different tumor lines into animals, and to really put it to bed.

DR. COFFIN: It's striking to me that one of the very few, if only successful experiments with injected activated oncogene DNA in chickens is the one that Hsing-Jien Kung did that was off-site, or in any animal, that was Hsing-Jien Kung. It was actually fairly efficient sort of transient transformation of cells, but transformation of cells is always transient. It's virtually impossible to immortalize them. They are much harder then human cells to immortalize. It's been done once, to my knowledge.

So there may be something very fundamentally different going on in that model, because there may be some fundamental difference in chickens as compared to mice, as compared to humans,
which already have important --

DR. EGAN: Bill Egan from FDA. I would certainly like to work with you and other regulatory authorities to try and design and our colleagues in PHS, to try and design these experiments and do these experiments, and get the data, and get away from the remark I quoted from Maurice Hilleman from 30 some-odd years ago about this debate being a philosophical or ecclesiastic debate because we simply don't have the data. Here we are 30 some-odd years later, you know, with the same question, with the same debate. It's still opinion.

I must say I also feel that personally, myself, I don't think there is a large risk from the DNA. But then again, the kinds of risks that we're talking about are very small risks, very, very rare events. Things like one in a million are not acceptable or in many cases are not acceptable. Those are hard data to get.

While I may not feel there is a risk to me, the bottom line basically for the approval of almost any of these vaccines, is would I put this into my children. There it becomes a much more conservative process. If putting it into my children is putting it into other people's children, it's the
same thing.

DR. HUGHES: If you want one more piece of data that should give you some comfort, it is the experience attempting to make antibody to the oncogene sarc, which involved putting an avian virus into a number of mammalian species under circumstances in which the virus absolutely does not replicate.

In adult animals, to my knowledge, no cell growth was ever seen. The only experiments that succeeded, to my knowledge, were those initiated by John Burge, in which he put enormous amounts of ras sarcoma virus of a subgroup that would infect mammalian cells. We are talking sort of 10 to the 10th infectious units. Into immunologically naive baby bunnies. In those animals very transiently, there were small nodules which regressed.

In those animals, you did see antibody to the oncogene sarc, implying that there was transient uptake of the DNA, at least probably permanent uptake of the DNA. But even under those circumstances in which the delivery of the DNA is extremely efficient and every copy of the DNA carries a known potent viral oncogene under circumstances where the DNA will not replicate but will insert, do you see any permanent transformation? Again, I'm not suggesting that this
is sufficient. I am one of the advocates of more experimentation. But the data that we have suggests that this is not a simple process.

DR. EGAN: No, but I mean these are the kind of data that start to put brackets around the numbers for the levels of risk.

DR. COFFIN: Of course you have enormous problems, including the fact that sarc is never seen. It's a human oncogene. For many years, the most popular viral model.

AUDIENCE MEMBER: I had a question. If given the unknowns, and given the data that was presented about hit and run DNA modification potentials, would the panel in the context of this type of vaccine development, and given the unknowns, give the vaccine to someone with a strong family history of malignancy or who was a cancer survivor who we know is at increased risk for a second cancer, if that was you or your family member?

DR. COFFIN: The question, to sort of focus that a little bit, the question is whether we would consider there to be a greater risk in certain sub-populations who might have sort of pre-activated oncogenes or some other fact of predisposing.

DR. ONIONS: I understand the question,
but it sort of arose -- I will go backwards, because
one of the things that used to concern me was when
people were doing clinical trials with rusvel vectors,
and they were using marker studies. That sort of
study did worry me, because actually what you were
there doing was putting something, inserting something
into somebody who probably already had a preexisting
oncogenic hit. That struck me as being dubious,
mildly.

In this situation, I would have thought
that unless you have got somebody with a Li-Fraumeni
syndrome or something, that you are dealing with
changes that are somatic changes in a few cells, even
if they are going to risk of a second cancer.
So the likelihood that you hit the right
cell is pretty low. So I wouldn't have thought it up
the risk -- I'm sure the risk has increased, but I
wouldn't have thought that risk has increased
significantly, unless of that sort where you've got

DR. MINOR: It would also depend on what
you are trying to protect them against too, wouldn't
it?

DR. SHEETS: Before we lose our entire
audience, I wanted to -- I think we have gotten a
pretty clear answer on the oncogenic DNA issue. I wanted to ask another kind of question that FDA has asked, before we lose our entire panel. That is, are there risks, additional risks that one perceives in using a continuous cell line such as vero cells, particularly vero cells, which is immortal but not tumorigenic at the levels that vaccines are made, past the level that vaccines are made. Is that worse in any way than diploid cells, for a live viral vaccine?

DR. MINOR: This is apart from the DNA issue?

DR. SHEETS: Well, in a continuous cell line, certainly there are — it may be aneuploid, but it's not tumorigenic in animals. So you can comment if you'd like about whether you think the DNA is oncogenic.

DR. MINOR: I would say that the DNA from vero is as questionable as the DNA from anything else. I mean John, with whom we discussed these matters, more or less said the same thing. I think that it doesn't matter how malignant it is. Maybe it depends on how many oncogenes you put in there. So I would have thought that a vero is as questionable as anything else, or is not as questionable as anything else.
DR. SHEETS: So you wouldn't suggest to make a live viral vaccine in vero cells?

DR. MINOR: I think it would depend on the live viral vaccine. I mean I think OPV clearly has been made in vero cells. You can scrub it clean. I

think you can more or less destroy anything that's actually hanging on the end.

I have more serious thoughts perhaps about things like a paramixovirus vaccine, because you couldn't clean it up so much perhaps.

DR. SHEETS: What about the sort of crude, less purified live viral vaccines, not the purified vaccines like we heard about last night with OPV, but the things that are just filtered cell culture supernatant?

DR. MINOR: Right. They might be figit.

I'll tell you that. Which is not to say there's any good reason for me to be uncomfortable with them.

It's just that they make me feel uncomfortable.

DR. COFFIN: We are very fast losing our audience, so I think --

DR. HUGHES: Isn't it partly the question do you know the life history of your cells as opposed to the state of the cells at the end?

DR. SHEETS: Vero cells are a bank that is
well characterized. The reason for the question is
that we have numerous live viral vaccines of the sort
I described that are being proposed to be made in vero
cells. Manufacturers prefer vero cells because one,
they can be characterized. Two, you get a high yield.

Three, they can be grown in the sorts of fermenter
culture that you heard about.

DR. HUGHES: I'm not particularly bothered

as long as I know that the sort of life history of the
cell. But I think the question is, if you have a cell
that's been in culture for a long time and has had a
complicated culture history, do you know that history?

AUDIENCE MEMBER: I asked the last panel
the same question. It comes down to the question
really is the adventitious agent issue put to bed as
well. Do we now have the assays in place that can
easily be applied to validate the freedom from
adventitious agents of these kinds of new cell lines?
The answer that Dr. Broker gave in the

last panel suggested that one could attempt to use DNA
chips and things like that, which to my knowledge
aren't assays that at least tomorrow I could go out

and do on a cell line and give me some confidence.

So my question to you is, sort of using

the standard assays that you are all aware of, without
developing further assays for this specific purpose,
do we have enough information to be sure that these
kinds of new cell lines are safe from the adventitious
gent perspective?

DR. ONIONS: That's another unanswerable

question, isn't it. I would just make the point that
I think you have to adopt somewhere between good
science and pragmatism. I mean you could

theoretically go and do representation difference
analysis on all these cell lines. Actually, I don't
think it's possible because you don't usually have the
partner. But theoretically you could do that. That's

not really a practical solution.

It does seem to me that we do know virus
types that tend to be latent in cells, and that it's

sensible to perhaps think of strategies of widening
the brief of detecting those agents, because I'm not
convinced that the kind of routine types of infecting
-- infectability assays when they work are as

sensitive as PCR, as just Phil pointed out. But I am
not convinced that always the right infectability
assay is present to actually detect certain agents.

So that you are probably relying on a combination of
things. Perhaps we do need to look at redundant PCR
for certain agents.
DR. COFFIN: I would think the producers would have a big attraction, is set up the same assay and use it for everything.

DR. MINOR: I mean I think you could also argue that you have used these assays for looking at human diploid cells and primary cultures, and all that sort of stuff. Right? What's the difference in principle in terms of adventitious agent contamination between those and the cells you are looking at here? I am not sure there's much difference. But are the concerns as big or as little.

AUDIENCE MEMBER: For one, I would like to thank Dr. Andy Lewis for bringing us together. This has been a very stimulating week. Last night Dr. Vyas showed us a picture of the thinker. But that actually prompted me to remember that Rodin placed that gentleman directly above the gates of hell.

(Laughter.)

The question I would like to pose right now is whether we're walking in through the gates or out.

DR. COFFIN: I would like to also second the thanks to the organizers for setting this up and bringing us here.

DR. LEWIS: Yes. On behalf of the
sponsors and those of us at CBER who worked on this,

we really appreciate the effort that the session
chairs, the panel chairs, and the speakers have put
into this meeting. When you attempt to put something
like this together, there's always a question of how
it's going to turn out. I think the success that we
have enjoyed here the past three days is a tribute to
the work, an incredible amount of work, that has gone
on on a very short period of time.

I think that I was very concerned when we
were trying to contact folks in May to do this by
September. For those of you who rose to the
challenge, I can't thank you enough on behalf of the
sponsors.

With that in mind, I hope everybody has a
great trip home. Get your papers in whenever you can.
Thank you.

(Whereupon, at 2:57 p.m., the proceedings
were concluded.)