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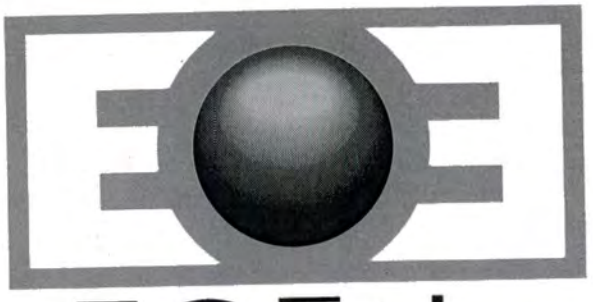
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Viricidal Effect of Polymorphonuclear Leukocytes on Human Immunodeficiency Virus-1

Role of the Myeloperoxidase System

Seymour J. Klebanoff and Robert W. Coombs
Department of Medicine, University of Washington, Seattle, Washington 98195

Abstract

Myeloperoxidase (MPO), H_2O_2, and chloride form an antimicrobial system in neutrophilic polymorphonuclear leukocytes (PMN) effective against a variety of microorganisms. Normal human PMN, when stimulated with phorbol myristate acetate or opsonized zymosan, are viricidal to HIV-1. The viricidal effect was lost when chloride was replaced by sulfate and was inhibited by the peroxidase inhibitor azide and by catalase, but not by heated catalase or superoxide dismutase, implicating H_2O_2. Stimulated PMN from patients with chronic granulomatous disease (CGD) were not viricidal to HIV unless H_2O_2 or glucose oxidase (which generates H_2O_2) was added, and the viricidal activity of H_2O_2-supplemented CGD PMN was inhibited by azide, implicating endogenous MPO. Stimulated PMN from patients with hereditary MPO deficiency had decreased viricidal activity unless MPO was added, and the viricidal activity of MPO-supplemented, MPO-deficient PMN was inhibited by catalase, implicating endogenous H_2O_2. The data suggest that when PMN are stimulated, MPO released by degranulation reacts with H_2O_2 formed by the respiratory burst to oxidize chloride to a product (presumably hypochlorous acid) that is toxic to HIV-1. Our findings raise the possibility that this viricidal effect of stimulated PMN may influence the host defense against HIV-1. (*J. Clin. Invest.* 1992. 89:2014–2017.)
Key words: neutrophil • chronic granulomatous disease • hereditary myeloperoxidase deficiency • AIDS • hypochlorous acid • HIV

Introduction

We have previously reported that the cell-free myeloperoxidase (MPO)[1]-H_2O_2-halide system is viricidal to HIV-1 and that H_2O_2-generating *Lactobacillus acidophilus* can provide the H_2O_2 required for this system (1). Since lactobacilli and peroxidase are present in the vagina of most normal women (2–4), the possibility was raised that heterosexual transmission of HIV-1 may be influenced by the presence and level of the peroxidase-mediated antimicrobial system in the vagina.

MPO is present in large amounts in cytoplasmic granules of neutrophilic polymorphonuclear leukocytes (PMN) and is released into either the phagosome or the extracellular fluid when PMN are stimulated by a variety of soluble or particulate stimuli (5, 6). Stimulation of PMN also results in a respiratory burst in which oxygen is reduced first to the superoxide anion (O_2^-) and then to H_2O_2. MPO, H_2O_2, and chloride form an antimicrobial system in PMN through the formation of powerful oxidants, namely, hypochlorous acid and related chloramines (5, 6). We report here that intact-stimulated PMN are viricidal to HIV-1, and provide evidence for the involvement of the MPO-H_2O_2-chloride system in this toxicity.

Methods

Reagents. The lymphadenopathy associated virus-1 (LAV-1) strain of HIV-1, kindly provided by Genetic Systems, Seattle, WA, was propagated in CEM cells (Genetic Systems) in RPMI-1640 (Gibco Laboratories, Grand Island, NY) containing 10% fetal calf serum (Gibco Laboratories), 50 U/ml penicillin, 50 μg/ml streptomycin, 0.01% DEAE Dextran (500,000 M_r, Sigma Chem. Co., St. Louis, MO) and 0.01 M Hepes buffer pH 6.8 (CEM growth medium). A stock viral preparation of 10^6 tissue culture infective dose ($TCID_{50}$)/ml in CEM growth medium was frozen at −70°C and on the day of the experiment was diluted 100-fold in 0.1 M sodium sulfate before a further 10-fold dilution in the reaction mixture (final concentration 1,000 $TCID_{50}$/ml). In addition to the components listed in the table legends, the reaction mixture contained all the components present in CEM growth medium diluted 1,000-fold. Catalase (CTR, bovine liver, 84,150 U/mg) was obtained from Worthington Biochemical Corp. (Freehold, NJ), phorbol myristate acetate (PMA), Histopaque-1077, superoxide dismutase (bovine erythrocyte, 3,150 U/mg) and glucose oxidase (*Aspergillus niger* type V) from Sigma Chem. Co. The catalase was dialyzed against water overnight before use and was heated at 100°C for 20 min where indicated. The PMA was dissolved in dimethylsulfoxide and the concentration of the latter in the reaction mixture did not exceed 0.01%. MPO was purified from human leukocytes (7) and assayed by guaiacol oxidation (8). Zymosan (ICN Pharmaceuticals, Inc., Cleveland, OH) was suspended in water by homogenization, boiled for 20 min, washed twice, and suspended in water at 10 mg/ml. The zymosan was opsonized by incubation with an equal volume of pooled normal serum for 20 min at 37°C with shaking, and the opsonized zymosan was washed twice and suspended in water at 10 mg/ml.

Isolation of PMN. Venous blood was collected from normal volunteers, two patients with chronic granulomatous disease (CGD) and one patient with hereditary MPO deficiency, just before the experiment, using 0.2% EDTA as anticoagulant. The PMN were isolated by dextran sedimentation and centrifugation in Histopaque-1077, and the erythrocytes removed by hypotonic lysis (9). The cells that always contained

A preliminary report of portions of this work was published in abstract form. (1991. *Clin. Res.* 39: 360*a*).

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1. *Abbreviations used in this paper:* CGD, chronic granulomatous disease; MPO, myeloperoxidase; TCID, tissue culture infective dose.

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greater than 97% PMN with an average purity of 98–99% were suspended in 0.9% sodium chloride at 5×10^7 PMN/ml. The final PMN preparation contained 4.9% eosinophils (average of five determinations) with the remainder neutrophils.

Measurement of viricidal activity. The components of the reaction mixture (see legends) in a final volume of 0.5 ml were incubated in sterile screwcap microtubes with sealing *O* rings (4.3 × 10.8 mm, 1.5 ml capacity, model 75.692.005; Sarstedt, Inc., Princeton, NJ) for 30 min at 37°C in a CO_2 incubator. The tubes were centrifuged to sediment the cells and 10 μl of the supernatant fluid was added to 48-well plates (model 3548; Costar, Data Packaging Corp., Cambridge, MA) containing 2×10^5 CEM cells in 1 ml of CEM growth medium. After incubation in a CO_2 incubator at 37°C for 6 d, a 200-μl aliquot was removed for measurement of HIV-1 P24 antigen by solid phase sandwich-type enzyme-linked immunosorbent assay (Abbott Laboratories, Chicago, IL). In general, culture supernatant levels of HIV-p24 antigen were either zero (< 10 pg/ml), indicating no replication, or very high, indicating unrestricted replication, with the level reached being related to the number of CEM cells (2×10^5), the volume of the reaction mixture added to the CEM cells (10 μl), and the growth period (6 d). In initial experiments, dilution and remeasurement of p24 antigen was not performed when the maximum optical density of > 2.000 (> 604 pg/ml) was obtained in the initial assay. These data are included in the tables under "No. with HIV Growth"/"No. of Samples," with an optical density of > 2.000 considered to indicate growth. In subsequent experiments, when an optical density of > 2.000 was observed, the supernatant fraction was diluted and remeasured. The data are expressed as the median p24 antigen level (picogram/milliliter) above background for those samples in which dilutions were performed when necessary. A positive HIV p24 antigen cutoff of 10 pg/ml was used. Median values were employed because of the general distribution of the values into two populations, representing no growth and extensive replication. Where indicated, statistical analysis was performed using the Mann-Whitney *U* test. Not significant (NS) $P > 0.05$.

Table I. Viricidal Effect of Stimulated Normal PMN on HIV-1

Supplements	HIV-1 P24 antigen: Median pg/ml of (*n*) samples	No. with HIV-1 growth/ No. of samples
None	493,480 (27)*	27/27
PMN	59,910 (14)*	19/20
PMA	300,250 (9)	15/15
PMN + PMA	<10 (32)‡§	4/36
Chloride deleted	390,390 (6)	6/6
Azide added	5,050 (9)‡	16/18
Catalase added	12,350 (9)§	14/18
Heated catalase added	<10 (12)	1/12
SOD added	<10 (12)	0/12
Ops zym	594,290 (5)	12/12
PMN + ops zym	<10 (29)	10/36
Chloride deleted	997,700 (5)	6/6
Azide added	209,780 (6)	18/18
Catalase added	215,160 (6)	18/18
Heated catalase added	<10 (8)	6/12
SOD added	<10 (10)	2/12

The reaction mixture contained 5×10^{-3} M sodium phosphate buffer pH 7.4, 0.128 M NaCl, 1.2×10^{-2} M KCl, 10^{-3} M $CaCl_2$, 2×10^{-3} M $MgCl_2$, 2×10^{-3} M glucose, 1,000 $TCID_{50}$/ml HIV-1 and, where indicated, 10^6/ml PMN, 100 ng/ml PMA, 1 mg/ml opsonized zymosan, 10^{-4} M sodium azide, 58 μg/ml catalase, and 10 μg/ml superoxide dismutase. In tubes in which chloride was deleted, isosmolar amounts of sulfate were added. The results are expressed as the median P24 antigen level in pg/ml and the ratio of the number of samples in which viral replication occurred over the total number of samples. * $P = 0.002$; ‡ $P = 0.01$; § $P = 0.03$.

Results and Discussion

Normal PMN, when combined with the soluble stimulus PMA or the particulate stimulus opsonized zymosan, were viricidal to HIV-1, as measured by an inability to replicate in CEM cells (Table I). No viricidal effect was evident when either PMA or opsonized zymosan was added alone, whereas a decrease ($P = 0.002$) but not loss of p24 antigen production was observed on incubation with PMN alone. The viricidal effect of PMA- or opsonized zymosan-stimulated PMN was lost when chloride was replaced by sulfate in the incubation medium. The viricidal effect was inhibited by azide, a potent inhibitor of MPO, and by catalase, but not by heated catalase, indicating a requirement for H_2O_2. Superoxide dismutase was not inhibitory, indicating that O_2^- was not required for the viricidal effect. These properties are compatible with the release of MPO by degranulation and the formation of H_2O_2 by the respiratory burst and their interaction, presumably in the extracellular fluid, with chloride to form an agent toxic to HIV-1.

Additional support for the involvement of the MPO-H_2O_2-chloride system in the viricidal effect of intact PMN on HIV-1 came from patients with a hereditary defect in PMN function. CGD is a rare genetic disorder in which PMN lack a respiratory burst due to the absence of a functional nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (10–12). As a result, the toxic products of the respiratory burst, including H_2O_2 and oxidants dependent on H_2O_2 for their production, are not formed. The MPO content of CGD PMN, however, is normal. When normal PMN were replaced by PMN from patients with CGD, toxicity to HIV-1 with either PMA or opsonized zymosan as the stimulus was lost (Table II). This loss of viricidal activity was reversed by the addition of H_2O_2 or glucose oxidase, but not by the addition of MPO. Glucose oxidase generates H_2O_2 in the course of the oxidation of its substrate glucose, and the involvement of the H_2O_2 so formed in the viricidal activity was indicated by the inhibitory effect of catalase but not heated catalase. At the concentration employed, H_2O_2 alone or combined with unstimulated CGD PMN (i.e., without PMA or opsonized zymosan) was not viricidal to HIV-1 (data not shown). The HIV-1 viricidal activity of H_2O_2 (or glucose oxidase)-supplemented CGD PMN stimulated with PMA or opsonized zymosan was inhibited by azide, implicating endogenous MPO released by stimulus-induced degranulation.

The PMN of patients with hereditary MPO deficiency lack MPO, but, in sharp contrast to CGD PMN, respond to stimulation with a respiratory burst which is greater than normal (6, 13). The viricidal effect on HIV-1 was greatly decreased when stimulated normal PMN were replaced by PMA- or opsonized zymosan-stimulated MPO-deficient PMN (Table III). In contrast to the findings with CGD PMN, the loss of viricidal activity was unaffected by the addition of H_2O_2, but was prevented by the addition of MPO. MPO alone or combined with unstimulated MPO-deficient PMN were without effect on HIV-1 (data not shown). The HIV-1 viricidal activity of MPO-supplemented, MPO-deficient PMN stimulated with PMA or opsonized zymosan was inhibited by catalase but not heated catalase, implicating H_2O_2 generated by the stimulus-induced respiratory burst.

Table II. Viricidal Effect of Stimulated CGD PMN on HIV-1

Supplements	HIV-1 P24 antigen: Median pg/ml of (*n*) samples	No. with HIV-1 growth: No. of samples
None	493,480 (27)	27/27
CGD PMN + PMA	366,700 (6)*	9/9
MPO added	44,260 (9)*	9/12
H_2O_2 added	<10 (8)	2/9
H_2O_2 and azide added	505,330 (6)	9/9
GO added	<10 (9)	1/9
GO + catalase added	279,710 (6)	6/6
GO + heated catalase added	<10 (6)	0/6
GO + azide added	502,830 (6)	6/6
CGD PMN + ops zym	309,270 (6)‖	9/9
MPO added	25,680 (9)‡	9/12
H_2O_2 added	<10 (7)	2/8
H_2O_2 + azide added	524,170 (6)	9/9
GO added	<10 (9)	0/9
GO + catalase added	51,380 (6)§	6/6
GO + heated catalase added	<10 (6)	0/6
GO + azide added	470,000 (6)	6/6

The reaction mixture was as described in Table I, except that CGD PMN were employed and 0.23 U/ml MPO, 10^{-4} M H_2O_2, 10^{-4} M sodium azide, 0.03 U/ml glucose oxidase (GO), and 58 μg/ml catalase were added where indicated. * NS; ‡ NS; § NS.

Table III. Viricidal Effect of Stimulated MPO-Deficient PMN on HIV-1

Supplements	HIV-1 P24 antigen: Median pg/ml of (*n*) samples	No. with HIV-1 growth: No. of samples
None	493,480 (27)*§	27/27
MPO-def PMN + PMA	98,110 (6)‡§	9/9
H_2O_2 added	59,270 (6)‡	9/9
MPO added	<10 (9)	0/9
MPO + catalase added	137,910 (6)*	9/9
MPO + heated catalase added	<10 (9)	0/9
MPO-def PMN + ops zym	336,670 (6)	9/9
H_2O_2 added	470,730 (6)	9/9
MPO added	<10 (9)	0/9
MPO + catalase added	503,860 (6)	9/9
MPO + heated catalase added	<10 (9)	1/9

The reaction mixture was described in Table I, except that MPO-deficient PMN were employed and 10^{-4} M H_2O_2, 0.23 U/ml MPO and 58 μg/ml catalase were added where indicated. * $P = 0.05$; ‡ NS; § $P = 0.01$.

In the MPO-H_2O_2-chloride system, H_2O_2 combines with MPO to form a complex which oxidizes chloride to form the powerful oxidant hypochlorous acid (HOCl) or its dissociated form, the hypochlorite anion (OCl^-) (5). The pKa of the dissociation is 7.53 so that both forms are present at physiologic pH. When the pH falls, HOCl predominates and HOCl can react with excess chloride to form chlorine (Cl_2). HOCl can also react with nitrogen-containing compounds to form mono- and dichloramines which retain oxidizing activity. Some of the chloramines are relatively long-lived, which provides a mechanism for the prolongation of the oxidizing activity of the MPO system and for the penetration of oxidants into fluids rich in proteins and other scavengers of the more reactive oxidants. HOCl/OCl^- (Clorox® bleach) is a well-recognized disinfectant effective against a variety of organisms, including HIV-1 (14).

Holmberg et al. (15) have classified the biological factors that affect sexual transmission of HIV-1 into: (*a*) the infectiousness of the HIV-1-infected donor; (*b*) the infectivity and virulence of the virus; and (*c*) host susceptibility to infection. Although the importance of these factors has been inferred from epidemiologic studies, the specific host defense mechanisms against HIV-1 are poorly understood. Our findings raise the possibility that among the factors that influence host defense against HIV-1 are the products of stimulated phagocytes. Destruction of microorganisms by PMN can occur intracellularly, i.e., within a phagosome, or extracellularly. Neutrophils lack CD4 and therefore the uptake of HIV-1 by this receptor would not be expected. The uptake of antibody-associated HIV-1 by Fc receptor-mediated endocytosis is possible. However, our studies were performed in the absence of specific antibody. It is probable that under our experimental conditions, stimulation of PMN resulted in the release of MPO and H_2O_2 into the extracellular fluid where they reacted with chloride to form agents toxic to HIV-1. Stimulation of PMN at mucosal surfaces or at sites of inflammation due to exposure to a variety of chemotactic factors, cytokines, or opsonized microorganisms would be expected, and toxicity to adjacent cell-free HIV-1 may occur under these circumstances. The precise microenvironment in which toxicity to HIV-1 by PMN may occur is not known; factors such as pH, protein concentration, and low molecular weight inhibitors may influence the viricidal effect in vivo. Cell-free HIV-1 has been demonstrated in plasma (16) and seminal fluid (17) and, thus, may be susceptible to attack by extracellular products of stimulated PMN in these and other locations. Provirus or intact cell-associated HIV-1 virions would be expected to be protected from these extracellular oxidants. It is not clear whether disruption of the cell-free pool of HIV-1 would influence the transmission of HIV-1 or the progression of disease. It is of interest in this regard that vaginal transmission of cell-free simian immunodeficiency virus (SIV) (18, 19) or HIV-1 (20) has been demonstrated in nonhuman primates, and that cell-free HIV-1 in plasma is a sensitive marker of the clinical stage of infection (16).

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December 16, 1992

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FOR IMMEDIATE RELEASE

Stephens Releases Test Data On AIDS Antiseptic.

Little Rock, Arkansas, U.S.A. Jackson T. Stephens, Jr., reports that the National Institutes of Health has just completed a series of pre-clinical tests that demonstrate Exact™ is effective in killing clinical isolates of HIV-1 in serum. Exact™ is the trade name for two enzymes-- Myeloperoxidase (MPO) and Eosinophil Peroxidase (EPO)--that have previously been documented to kill the AIDS virus *in-vitro* , and could be used in a vaginal or anal suppository, or cream to prevent the spread of HIV and other Sexually Transmitted Diseases (STDs).

"Just as important as the fact that Exact™ kills HIV-1 in serum, is that there was no cytotoxicity, and the animal studies indicated no inflammation or irritation. We are very pleased with the support and efforts of the NIH to help us through this initial pre-clinical test stage, and we hope to begin human clinical trials in the first part of 1993."

"We will take every precaution to insure the safety and efficacy of our product both here in the U.S. and abroad," said Stephens, commenting from his office in Little Rock. "All of our work to date has shown that Exact™ is both safe and effective. We are grateful for the government help in expediting testing."

Stephens said: "The low cost of Exact™ makes it affordable for consumer use in suppositories, topical creams, lozenges, or lubricants. Use of Exact™ enhanced

products could make a significant contribution in the prevention of AIDS and other Sexually Transmitted Diseases (STDs)."

Because of the prohibitive cost of manufacturing even low concentrations of MPO and EPO, large clinical studies and commercial applications of the enzymes have, heretofore, not been possible. ExOxEmis has developed a proprietary manufacturing process which makes wholesale distribution of Exact™ viable.

"Scorched Earth Versus Smart Bombs"

To date, other antiseptics and topical anti-viral agents are effective against infectious microbes only at concentrations that damage surrounding tissue and the immune system. In 1918, Alexander Flemming, the inventor of penicillin, documented this problem; Exact™ solves it.

Exact™ has a very high affinity for pathogens, but does no harm to surrounding tissue or helpful bacteria which naturally occur in the body. Stephens calls it the "smart bomb" of antiseptics.

Exact™ uses a chemical called singlet molecular oxygen which occurs during a high energy reaction. The "killing" properties of this oxygen are restricted by its short lifetime. The region of singlet molecular oxygen killing is about the width of a bacteria wall, so only organisms to which it binds are damaged.

ExOxEmis discovered and documented Exact's™ unique ability to bind primarily to harmful or pathogenic microbes without harming the normal flora.

More About MPO and EPO

Myeloperoxidase (MPO) and Eosinophil Peroxidase (EPO) are the two primary killing components of a group of white blood cells known as neutrophils and eosinophils. These cells are considered the foot soldiers of the human immune system because there are so many produced by the body and they are the first component employed by the body's defense mechanism.

For many years, medical researchers have known these enzymes were effective microbe killers. What was not known was their selective ability to bind primarily to pathogenic organisms. Exact™ employs MPO and EPO in this unique and previously undiscovered method of selective binding.

ExOxEmis has completed the research, development, patent filings and manufacturing scale-up to supply Exact™ for commercial use.

Exact™ Has Many Consumer Applications

The most immediate benefit of Exact™ to the general public can be its application in the prevention of AIDS and other STDs. When fully developed as a suppository, Exact™ can provide an effective barrier against infection. Exact's™ addition to donor blood bags to kill HIV and other blood transmitted diseases is a high devlopment priority with ExOxEmis.

Because Exact™ can kill a variety of pathogenic bacteria, viruses, yeasts, and molds, it has the potential for wide spread application in a diverse range of products -- these include: ophthalmic solutions; infant and enteral formulas; burn ointments; urinary tract lavages; feminine hygene products; surgical and consumer antiseptics; skin care products and cosmetics.

ExOxEmis is now offering commercial licensing and distribution agreements to major companies for Exact™, as well as bulk sales to academic and industrial researchers.

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

National Institutes of Health
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January 7, 1993

Jackson T. Stephens, Jr.
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Little Rock, Arkansas 72201

Dear Mr. Stephens:

As per our phone conversation of Tuesday, January 5, 1993, I want to re-emphasize that the importance of continuing research and development of HIV viricides, such as MPO and EPO, is acknowledged by us in the National Institute of Allergy and Infectious Diseases. I have discussed the present state of development with Dr. Anthony Fauci, Institute Director, Dr. Pat Reichelderfer, Program Virologist, and Dr. Chuck Litterst, Drug Development and Surveillance Chief.

As previously stated, we are committed to do animal efficacy studies in the guinea pig model of Herpes. We feel such animal model efficacy studies are a logical next step, and if these compounds prove positive in a Herpes model, would generate support and enthusiasm by the AIDS Clinical Drug Development Committee (ACDDC). We are prepared to initiate animal studies with an appropriate drug formulation provided by you that will ultimately be employed in clinical studies.

We are also in agreement with your Scientific Director, Dr. Bob Allen, that animal studies of local toxicity following vaginal administration are critical. As you are aware, increased transmission of HIV has been associated with conditions of abnormal vaginal epithelium. The pathology report on the rabbit toxicology studies has been completed and you should be receiving a copy of the report shortly from Dr. Nancy Alexander of NICHD. The report apparently describes considerable toxicity with the gel base alone. Dr. Alexander's group does not formulate compounds, but, Dr. Allen has stated that the product (Exact) could be formulated as a gel. It is unclear whether the formulation supplied to NICHD was tested in vitro for efficacy. Again, it will be important to perform toxicity trials with the compound and formulation to be used in the clinical trial.

Further testing can proceed as soon as we have received the revised compound. Once the various contractors have received this formulation, it will take approximately 3-4 weeks to complete the cell culture work, and 3-4 months to complete the animal efficiency studies. It is highly likely that the ACDDC will wish to evaluate the results of the animal efficacy studies before making a decision to proceed to clinical trials.

As we discussed, it is also important to continue the dialogue with the FDA to understand what other suggestions or requirements they may have prior to initiating clinical trials.

Again, I want to re-assure you that because this product and approach are very promising in preventing HIV-1 transmission, we remain committed to assisting ExOxEmis so that this concept and product are developed in a timely manner.

Sincerely,

Steven M. Schnittman, M.D.
Chief, Medical Branch
Division of AIDS
Clinical Research Program
National Institute of Allergy
and Infectious Diseases
National Institutes of Health

cc: Anthony S. Fauci, M.D.
Pat Reichelderfer, Ph.D.
Chuck Litterst, Ph.D.

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Chicago, Illinois 60616-3799
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since 1936

L06255
December 7, 1992

Steven R. Turk, Ph.D.
Developmental Therapeutics Branch
BRDP, DAIDS, NIAID
6003 Executive Blvd., Room 2C14
Bethesda, MD 20892

SUBJECT: Contract No. NO1-AI-05077
Report No. 38
Myeloperoxidase and Eosinophil Peroxidase:
Testing of Virucidal Activity

Dear Steve:

We have completed initial studies evaluating myeloperoxidase (MPO) and eosinophil peroxidase (EPO) for virucidal activity against Human Immunodeficiency Virus (HIV). As was requested in your letter dated October 29, 1992, these two peroxidase enzymes were evaluated for direct virucidal activity against HIV-1. Virucidal activity was assessed by the reduction in infectious titer of a standard virus sample. The details of this experiment are described below.

Porcine myeloperoxidase (lot # 1899201) was supplied as a 14μM solution; porcine eosinophil peroxidase (lot # 1929201) was supplied as a 28μM solution. Each enzyme was tested for virucidal activity at a final concentration of 50, 5.0, and 0.5 nM. Hydrogen peroxide was included in each reaction mix at a concentration of either 1000, 100, 10, or 1.0 μM. Enzymes and hydrogen peroxide were diluted to desired concentrations using acetate diluting medium (lot # 2889250). This diluting medium consisted of 50mM acetate buffer, pH 6.0, plus 100mM NaCl and 1mM NaBr, and was supplied directly to us by ExOxEmis, Inc. Hydrogen peroxide (USP 3%; 0.88M) was obtained from a local drug store.

The viricidal activity was tested using a high-titered stock of the G910-6 strain of HIV-1. This strain is the post-therapy isolate from patient AO18, and has decreased sensitivity to AZT. It was selected because it represented our HIV-1 stock having the highest titer of infectious virus. Tubes containing 2.7 ml of virus stock were placed in an ice bath, and a 150 μl volume of MPO or EPO (prepared as a 20x concentration) was added. After a ten

minute incubation on ice, each virus sample was split into five equal aliquots of 475 µl. To each of these samples was added 25 µl of hydrogen peroxide (prepared as a 20x concentration). All samples were then incubated at 37°C for thirty minutes, and placed back of ice. A virus sample, treated only with the acetate diluting medium, was processed in parallel as a control.

Each virus sample was serially diluted in RPMI-1640 cell culture medium using 10-fold dilutions. Starting with the undiluted samples, and for each dilution of each virus sample, 50µl aliquots were added to six replicate wells of a 96-well culture plate containing 10,000 MT-2 cells per well. MT-2 cells, a human T-lymphocyte cell line, were used because of their sensitivity to cytopathic destruction by this strain of HIV-1. The culture plates were then incubated at 37°C in a humidified, 5% CO_2 atmosphere. On the eighth day of incubation each well was observed for signs of virus infection. In addition, a 50µl sample of the supernatant medium from each well of the initial titration plates was added to a well of a second plate of MT-2 cells. This second plate served as a sensitive indicator system to identify all wells of the initial titration plate in which infection and virus production occurred. Each of these wells was scored microscopically for signs of HIV CPE after seven days in culture. For each sample the titer in terms of the 50% tissue culture infectious dose ($TCID_{50}$) was then calculated using the classical method of Reed and Muench.

The results from the experiment with MPO are shown in Table 1. A substantial loss (about 100-fold) in the infectivity was seen for the sample treated with 50nM MPO and 1000µM hydrogen peroxide. MPO at a concentration of 50nM in the absence of hydrogen peroxide had little if any effect of the titer of the virus sample, suggesting that the presence of hydrogen peroxide was required for this virucidal effect. At hydrogen peroxide concentrations of 100 and 10 µM, 50nM MPO showed some virucidal activity, though not as substantial as at 1000µM hydrogen peroxide. Treatment with lower MPO concentrations did not result in any convincing virucidal activity.

The results from the experiment with EPO are shown in Table 2. There was at best a suggestion of virucidal activity for samples treated with 50nM EPO; however, the effect did not seem to be dependent on the presence of hydrogen peroxide.

A follow-up experiment was performed to confirm the virucidal activity of MPO, examine the role of the hydrogen peroxide, and test EPO at a higher concentration. The basic experimental design was the same as used previously. Based on the results of the first experiment, a MPO concentration of 50nM and a hydrogen peroxide concentration of 1000µM were used in this evaluation. Virus samples were treated with enzyme and/or hydrogen peroxide as indicated in Table 3. Some samples were additionally treated with 10µl of a bovine liver catalase solution (lot # 3009251; supplied

directly from ExOxEmis, Inc.) for two minutes at the end of the 37°C incubation.

The results shown in Table 3 confirm the virucidal activity previously seen with MPO. The virus sample treated with 50nM MPO and 1000μM hydrogen peroxide was reduced about 100-fold in infectious titer. In this experiment, however, there appeared to be some virucidal activity with MPO in the absence of any added hydrogen peroxide. Although treatment of virus with hydrogen peroxide alone did not reduce the titer, a toxic effect was apparent to the cells treated with the undiluted hydrogen peroxide solution. When a similar sample was treated with catalase prior to addition to the cells, no toxicity was seen. Thus, it appears that hydrogen peroxide is toxic to cells, and that when residual hydrogen peroxide is present in a virus sample the cells treated with the most concentrated virus dilution are likely to be destroyed by the cytotoxic effect of the hydrogen peroxide. This explains the toxicity noted in Tables 1 and 2 for samples treated with 1000μM hydrogen peroxide.

In contrast to the low level of virucidal activity seen in Table 2 with 50nM EPO, when the EPO concentration was increased to 500nM substantial virucidal activity was found. The combination of 500nM EPO and 1000μM hydrogen peroxide reduced the infectivity of the virus sample 1000-fold (Table 3).

We conclude that both MPO and EPO have promising virucidal activity against HIV-1. We are continuing to perform experiments with these enzymes in order to further characterize the limits of the virucidal activity. If you have any question after reviewing these results, please don't hesitate to call me at (312) 567-4867.

Sincerely,

Louis E. Holland II, Ph.D.
Senior Virologist
Group Leader of Virology
Life Sciences Department

Approved by:

Peter T. Thomas, Ph.D.
Program Manager, Microbiology
and Immunology
Life Sciences Department

Enclosures

TABLE 3. FURTHER CHARACTERIZATION OF VIRUCIDAL ACTIVITY

			VIRUS DILUTION							
Enzyme	H_2O_2	Catalase	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Titer
50nM MPO	--	--	6/6	6/6	6/6	1/6	0/6	0/6	0/6	$10^{3.9}$
50nM MPO	++	--	T	5/6	2/6	0/6	0/6	0/6	0/6	$10^{2.9}$
50nM MPO	++	++	6/6	6/6	2/6	0/6	0/6	0/6	0/6	$10^{3.1}$
None	++	--	T	6/6	6/6	5/6	0/6	0/6	0/6	$10^{4.7}$
None	++	++	6/6	6/6	6/6	6/6	0/6	0/6	0/6	$10^{4.8}$
500nM EPO	++	--	6/6	0/6	0/6	0/6	0/6	0/6	0/6	$10^{1.8}$
None	--	--	6/6	6/6	6/6	5/6	1/6	0/6	0/6	$10^{4.8}$
None	--	--	6/6	6/6	6/6	6/6	1/6	0/6	0/6	$10^{4.9}$

Equivalent virus samples were first treated with or without enzyme as indicated for 10 minutes on ice, then treated either with or without 1000μM hydrogen peroxide for 30 minutes at 37°C. Two of the samples had an additional 2 minute incubation with catalase. Serial 10-fold dilutions were made of each sample, and six wells of MT-2 cells were inoculated with each dilution. The fraction of the wells actually infected is indicated for each sample dilution. The titer, expressed as $TCID_{50}$/ml, of the infectious virus remaining after treatment was calculated using the method of Reed and Muench. T = toxic; infection of wells could not be evaluated at this dilution due to toxicity of the test mixture.

TABLE 1. EVALUATION OF MYELOPEROXIDASE VIRUCIDAL ACTIVITY

	50 nM MPO					5.0 nM MPO					0.5 nM MPO					
Dilution	1000[a]	100	10	1.0	0.0	1000	100	10	1.0	0.0	1000	100	10	1.0	0.0	Virus Control
10^{0}	6/6	6/6	6/6	6/6	6/6	T	6/6	6/6	6/6	6/6	T	6/6	6/6	6/6	6/6	6/6
10^{-1}	4/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
10^{-2}	1/6	6/6	5/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
10^{-3}	0/6	2/6	1/6	3/6	4/6	4/6	4/6	4/6	6/6	5/6	4/6	6/6	4/6	5/6	5/6	6/6
10^{-4}	0/6	0/6	0/6	1/6	0/6	0/6	3/6	1/6	1/6	0/6	0/6	2/6	4/6	2/6	1/6	1/6
10^{-5}	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
10^{-6}	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
Titer	$10^{2.7}$	$10^{4.1}$	$10^{3.8}$	$10^{4.5}$	$10^{4.6}$	$10^{4.6}$	$10^{5.0}$	$10^{4.7}$	$10^{4.9}$	$10^{4.7}$	$10^{4.6}$	$10^{5.1}$	$10^{5.3}$	$10^{4.9}$	$10^{4.8}$	$10^{4.9}$

Equivalent virus samples were first treated with MPO at the indicated concentration for 10 minutes on ice, then treated with either 1000, 100, 10, or 1.0 μM, or without, hydrogen peroxide for 30 minutes at 37°C. Serial 10-fold dilutions were made of each sample, and six wells of MT-2 cells were inoculated with each dilution. The fraction of the wells actually infected is indicated for each sample dilution. The titer, expressed as $TCID_{50}$/ml, of the infectious virus remaining after treatment was calculated using the method of Reed and Muench. T = toxic; infection of wells could not be evaluated at this dilution due to toxicity of the test mixture.

[a] Concentration of hydrogen peroxide in μM.

TABLE 2. EVALUATION OF EOSINOPHIL PEROXIDASE VIRUCIDAL ACTIVITY

	50 nM EPO					5.0 nM EPO					0.5 nM EPO					
Dilution	1000[a]	100	10	1.0	0.0	1000	100	10	1.0	0.0	1000	100	10	1.0	0.0	Virus Control
10^0	T	6/6	6/6	6/6	6/6	T	6/6	6/6	6/6	6/6	T	6/6	6/6	6/6	6/6	6/6
10^{-1}	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
10^{-2}	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6	6/6
10^{-3}	2/6	0/6	1/6	2/6	2/6	3/6	3/6	5/6	2/6	4/6	4/6	6/6	3/6	5/6	5/6	5/6
10^{-4}	0/6	1/6	0/6	0/6	0/6	1/6	1/6	0/6	0/6	1/6	2/6	1/6	1/6	2/6	0/6	0/6
10^{-5}	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
10^{-6}	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
Titer	$10^{4.1}$	$10^{3.9}$	$10^{3.9}$	$10^{4.1}$	$10^{4.1}$	$10^{4.5}$	$10^{4.5}$	$10^{4.7}$	$10^{4.1}$	$10^{4.7}$	$10^{4.8}$	$10^{4.9}$	$10^{4.5}$	$10^{4.9}$	$10^{4.7}$	$10^{4.7}$

Equivalent virus samples were first treated with EPO at the indicated concentration for 10 minutes on ice, then treated with either 1000, 100, 10, or 1.0 μM, or without, hydrogen peroxide for 30 minutes at 37°C. Serial 10-fold dilutions were made of each sample, and six wells of MT-2 cells were inoculated with each dilution. The fraction of the wells actually infected is indicated for each sample dilution. The titer, expressed as $TCID_{50}$/ml, of the infectious virus remaining after treatment was calculated using the method of Reed and Muench. T = toxic; infection of wells could not be evaluated at this dilution due to toxicity of the test mixture.

[a] Concentration of hydrogen peroxide in μM.

Nancy J. Alexander, Ph.D.
CDB, CPR, NICHD
August 13, 1993

Path to a New Vaginal Product (spermicidal, microbicidal and virucidal)

1. Efficacy for spermicidal properties
2. Formulate in carboxymethyl cellulose
3. Rabbit irritation
4. Pre IND meeting with FDA
5. Absorption studies
   radiolabel
   HPLC
6. Formulate for final product
7. Rabbit Vaginal irritation (GLP)
8. Vaginal toxicity
   May take 3 mos to 1 yr in 2 species (rat, rabbit) but will be able to start Phase I clinical before the end of the toxicity study
9. Oral toxicity (may or may not be necessary)
10. Primate vaginal irritation (not necessary but will be good comparison with N–9 irritation with multiple use)
11. IND (2 months to prepare, FDA has 30 days to respond)
12. Phase I
13. Phase II Comparative with known spermicide, efficacy, multicenter
    Phase I and II will take 4 years.
14. Prepare NDA (1 year)
15. NDA evaluation by FDA (may take 2 or more years for FDA review)
16. Postmarketing STD prevention studies (since each indication for efficacy against an STD takes a study, such evaluations could take years and since many STDs have a relatively low prevalence, the studies will be difficult to do).

Brief Definitive Report

Viricidal Effect of *Lactobacillus acidophilus* on Human Immunodeficiency Virus Type 1: Possible Role in Heterosexual Transmission

By Seymour J. Klebanoff and Robert W. Coombs

From the Department of Medicine, University of Washington, Seattle, Washington 98195

Summary

Peroxidase, H_2O_2, and a halide form a powerful antimicrobial system in phagocytes and tissue fluids, and certain microorganisms can serve as the source of H_2O_2 for this system. H_2O_2-generating *Lactobacillus acidophilus* (LB^+) is present in the vagina of most normal women and peroxidase has been detected in vaginal fluid. LB^+ at high concentration is viricidal to HIV-1, and, at levels where LB^+ is ineffective alone, the addition of peroxidase (myeloperoxidase, eosinophil peroxidase) and a halide (chloride, iodide, bromide, thiocyanate) restore viricidal activity. LB^+ can be replaced by H_2O_2, but not by non-H_2O_2-producing LB, and viricidal activity is inhibited by azide and catalase. The survival of HIV in the female genital tract and thus the likelihood of transmission may be influenced by the activity of the LB^+-peroxidase-halide system in the vagina.

Heterosexual transmission of HIV is occurring with increasing frequency in the United States (1) and remains the most prevalent mode of transmission in Africa (2, 3). In heterosexual transmission, HIV released into the vagina by either partner survives the local defense mechanisms to infect sexual partners. However, the efficiency of sexual transmission is extremely variable, suggesting that there may be factors that can affect the survival of HIV in the vagina.

Peroxidases, when combined with H_2O_2 and a halide, form a powerful antimicrobial system which is effective against a variety of microorganisms (4), including viruses (5). Mammalian peroxidases effective in this way include myeloperoxidase (MPO) present in neutrophils and monocytes, eosinophil peroxidase (EPO) in eosinophils, and lactoperoxidase (LPO) in milk and saliva. A peroxidase with antimicrobial (6) and spermicidal (7) properties is also present in the uterine fluid of estrogen-primed rats. Although peroxidases are generally effective with several halides, the physiologic halide for MPO is believed to be chloride (4), for EPO bromide (8, 9) or the pseudohalide thiocyanate (10), and for LPO thiocyanate (11, 12). The source of H_2O_2 for the peroxidase-mediated antimicrobial system includes the respiratory burst of stimulated phagocytes and the metabolism of certain bacteria.

Bacteria designated as lactic acid bacteria, e.g., lactobacilli, streptococci and pneumococci, release H_2O_2 (13) which can be autoinhibitory (13) and, when mixed cultures are employed, can be toxic to other bacteria (6, 14), fungi (14), viruses (15), spermatozoa (16), or tumor cells (17), particularly in the presence of peroxidase and a halide. The ability of lactobacilli to provide H_2O_2 for a peroxidase-dependent cidal system is pertinent to the female genital tract, since the predominant bacterial species in normal human vaginal secretions is *Lactobacillus acidophilus*, and the vaginal overgrowth of a number of organisms in bacterial vaginosis is associated with a decrease in H_2O_2-generating lactobacilli (18). Thus, the production of H_2O_2 by lactobacilli in the vigina appears to be a nonspecific host defense mechanism which can be potentiated by peroxidases of leukocytic or uterine origin. In this study, we demonstrate the viricidal effect of the peroxidase-H_2O_2-halide system on HIV, and the ability of H_2O_2-generating lactobacilli to provide the H_2O_2 required for this effect.

Materials and Methods

Human Immunodeficiency Virus. HIV-1 (strain LAV-1 kindly provided by Genetic Systems, Seattle, WA) was propagated in CEM cells (kindly provided by Genetic Systems) in CEM growth medium (RPMI-1640 [Gibco Laboratories, Grand Island, NY] containing 10% Fetal Bovine Serum [Gibco Laboratories], 50 U/ml penicillin, 50 mcg/ml streptomycin, 0.01% DEAE Dextran [500,000 Mr; Sigma Chemical Co., St. Louis, MO], and 0.01 M Hepes buffer, pH 6.8). HIV in the cell supernatant was diluted to 10^6 tissue culture infective dose 50 ($TCID_{50}$)/ml in CEM growth medium and frozen in liquid nitrogen gas phase. Stock virus was diluted 100-fold with 0.1 M sodium sulfate prior to a further 10-fold dilution in the reaction mixture (final concentration, 1000 $TCID_{50}$/ml). In addition to the components listed, the reaction mixture contained the components of CEM growth medium diluted 1,000-fold.

Peroxidase. MPO was purified from human leukocytes (19) and

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Volume 174 July 1991 289-292

EPO from horse eosinophils (20). Peroxidase activity was determined by guaiacol oxidation (21).

Lactobacilli. Hospital cultures of *L. acidophilus* obtained from human vaginal swabs were initially tested for H_2O_2 production by exposure to air of colonies which had been grown anaerobically as surface colonies on agar containing horseradish peroxidase and tetramethylbenzidine (18). Colonies of H_2O_2-generating organisms (LB^+) formed a blue pigment, whereas organisms which did not form H_2O_2 (LB^-) remained white. LB^+ and LB^- cultures were maintained by aerobic growth on HBT agar plates (01-478 Remel, Lenexa, KS) at 37°C in a CO_2 incubator for no longer than 2 d. Before the experiment, the organisms were transferred to peptone yeast extract medium (22) containing 1% heat-inactivated FCS (Hyclone Laboratories, Logan, UT) and grown aerobically at 37°C for approximately 6 h with tumbling for LB^+ and 24 h in stationary tubes for LB^-. The lactobacilli were washed twice and suspended in 0.1 M sodium sulfate. H_2O_2 generation by LB^+ was determined before each experiment by the scopoletin method (23).

Measurement of Viricidal Activity. The reaction mixtures described in the legends to the figure and table were incubated in sterile screwcap microtubes with sealing O-rings (4.3 × 10.8 mm, 1.5 ml capacity #75.692.005; Sarstedt, Inc., Princeton, NJ) for 30 min at 37°C. The tubes were centrifuged at 2,940 *g* for 2 min to sediment the bacteria and 10 μl aliquots of the supernatant fraction were added to triplicate wells (48-well plate #3548; Costar, Cambridge, MA) containing 2 × 10^5 CEM cells in 1 ml of CEM grown medium per well. Following incubation at 37°C for 6 d in a CO_2 incubator (5% CO_2-95% air), a 200 μl aliquot was removed for measurement of HIV P24 antigen by a solid phase sandwich-type ELISA (Abbott Laboratories, Chicago, IL). If an optical density of >2.000 was observed, the supernatant fraction was diluted and remeasured. The data are expressed as the P24 antigen level above background. A positive HIV-P24 antigen cutoff of 10 pg/ml was used. In general, culture supernatant levels of HIV-P24 antigen were either zero (<10 pg/ml), indicating no replication, or very high (>1,000,000 pg/ml), indicating unrestricted replication, with the level reached being related to the number of CEM cells (2 × 10^5), the volume of the reaction mixture added to the CEM cells (10 μl) and the growth period (6 d).

Results and Discussion

H_2O_2-generating *L. acidophilus* (LB^+) alone at a concentration of 10^7 CFU/ml was viricidal to HIV in lactate buffer pH 5.0 as measured by a decrease in viral replication in CEM cells (Fig. 1). When the LB^+ concentration was reduced to a level where it was ineffective alone (2 × 10^5–5 × 10^6 CFU/ml), the addition of MPO and chloride resulted in a return of HIV viricidal activity (Fig. 1).

The HIV viricidal activity of high concentrations of LB^+ alone was variable, with complete loss of HIV infectivity observed in five of nine wells, giving a median P24 level of <10 and a mean of 123 pg/ml (Table 1). An anti-HIV effect was not seen when the LB^+ was heated at 100°C for 15 min or was replaced by *L. acidophilus* which did not generate H_2O_2 (LB^-). This toxic effect on HIV was inhibited by catalase, but not by heated catalase, implicating H_2O_2. Table 1 also demonstrates the viricidal effect of low levels of LB^+ combined with MPO and chloride, the requirement for each component of the system, and the inhibition by heat-treatment of LB^+, by the substitution of LB^- for LB^+ and by the addition of azide (which inhibits peroxidase) or catalase, but not heated catalase or superoxide dismutase, again implicating H_2O_2. Chloride could be replaced by iodide, bromide or thiocyanate ions, LB^+ could be replaced by reagent H_2O_2 and MPO could be replaced by EPO. However, chloride was less effective with EPO than with MPO.

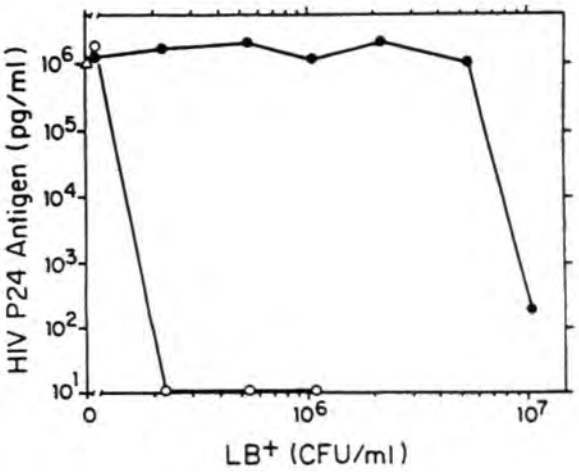

Figure 1. The viricidal effect of H_2O_2-generating *L. acidophilus* on HIV in the presence and absence of MPO and chloride. The reaction mixture contained 4 × 10^{-2} M sodium lactate buffer pH 5.0, 0.005% gelatin, 2 × 10^{-2} M glucose, 7.7 × 10^{-2} M sodium sulfate, and 1,000 $TCID_{50}$/ml HIV, either alone (Δ) or with LB^+ at the concentrations indicated without (●) or with 23 mU/ml MPO and 0.1 M sodium chloride (○) in a final volume of 0.5 ml. The results are the mean of 3–12 determinations.

The inhibition of HIV infectivity was not due to direct toxicity to the CEM cells. The evidence is as follows: (*a*) the reaction mixture was diluted 100-fold on addition to the CEM cells; (*b*) the CEM cells continue to metabolize and divide at a normal rate as indicated by acid production and increase in cell number; (*c*) identical results were observed when catalase was used to stop the reaction before the addition of an aliquot to the CEM cells; and (*d*) the toxicity of the peroxidase system is known to be strongly inhibited by protein and low mol wt components present in the CEM growth medium.

L. acidophilus has been detected in the vagina of most normal women with the level of H_2O_2-producing lactobacilli averaging 8.4 × 10^6 organisms per ml in one study (18). Under our in vitro conditions, the amount of H_2O_2 generated by 10^7 organisms was sufficient to inactivate HIV in the absence of peroxidase, and when MPO and chloride were added, concentrations of LB^+ as low as 2 × 10^5 CFU/ml were effective. Thus, lactobacilli appear to be present in the vagina in adequate numbers to exert an antiviral effect. Peroxidase has also been detected in human vaginal fluid (24). Of the halides, chloride is present in cervical mucus in high concentration (25) and iodide and thiocyanate are concentrated there following intravenous administration to humans (26). Their presence in vaginal fluid would thus be anticipated. The pH employed here is approximately that of vaginal fluid. Additional studies are needed to evaluate the influence of potential inhibitors in vaginal fluid (catalase, protein, low mol wt-reducing substances).

Table 1. *Properties of the LB⁺-Dependent Human Immunodeficiency Virus (HIV)-Inhibitory System*

Additions	HIV P24 antigen	No. with HIV growth/ No. of samples
	(pg/ml)	
None	1,202,604	9/9
LB^+ (10^7/ml)	123	4/9
LB^+ heated	2,063,677	3/3
LB^+ deleted, LB^- added	1,982,759	3/3
Catalase added	162,754	8/9
Heated catalase added	198	5/9
LB^+ (10^6/ml) + MPO + Cl	<10	0/12
Cl deleted	2,303,638	3/3
MPO deleted	4,368,805	3/3
LB^+ deleted	3,760,265	3/3
LB^+ heated	5,614,311	3/3
LB^+ deleted, LB^- added	3,685,807	3/3
Azide added	3,368,574	3/3
Catalase added	5,075,827	3/3
Heated catalase added	<10	0/6
SOD added	<10	0/6
Cl deleted, I added	<10	0/6
Cl deleted, Br added	<10	0/6
Cl deleted, SCN added	<10	0/6
LB^+ deleted, H_2O_2 added	<10	0/6
LB^+ (10^6/ml) + EPO + Cl	12,965	6/6
Cl deleted, I added	<10	0/6
Cl deleted, Br added	<10	0/6
Cl deleted, SCN added	<10	0/6

The reaction mixture was as described in Fig. 1, except that the additions were: LB^+ and LB^- at the concentrations indicated; 23 mU/ml MPO; 23 mU/ml EPO; 0.1 M sodium chloride; 10^{-4} M sodium iodide; 10^{-4} M sodium bromide; 10^{-4} M sodium thiocyanate; 10^{-5} M H_2O_2; 10^{-4} M sodium azide; 5.8 μg/ml catalase (CTR bovine liver, 84,150 U/mg, [Worthington Biochem, Freehold, NJ] dialyzed before use); 10 μg/ml superoxide dismutase (SOD, bovine erythrocyte, 3,150 U/mg, [Sigma Chemical Co.]). The LB^+ and catalase were heated for 15 min at 100°C where indicated. The heated LB^+ was collected by centrifugation and resuspended in 0.1 M sodium sulfate to the required optical density. The results are expressed as the mean P24 antigen level in pg/ml and the ratio of the number of samples in which viral replication occurred over the total number of samples. The results are the mean of 3–9 determinations.

We have concentrated on the destruction of cell-free HIV. HIV in semen is both cell-associated and free in seminal plasma (27) and it is not clear which form is responsible for male to female vaginal transmission. The infection of primates by intravaginal infusion of cell-free HIV or SIV, however, has been described (28, 29).

Our findings suggest that the presence and level of H_2O_2-producing lactobacilli in vaginal fluid may affect the heterosexual transmission of HIV by the release of H_2O_2, which can act either alone or in conjuction with a halide and peroxidase of leukocytic or uterine orgin to inactivate HIV. Normal individuals, or persons with sexually transmitted vaginal disease, in whom vaginal H_2O_2-producing lactobacilli are few or absent, may thus be at greater risk of infection and, as a corollary, may benefit from vaginal colonization with H_2O_2-generating lactobacilli. The systemic treatment of AIDS patients with orally-administered live lactobacilli has been proposed (30). The presence of peroxidase in other locations, e.g., LPO in milk and saliva, MPO in neutrophils and monocytes, EPO in eosinophils and extracellular MPO or EPO in inflammatory loci containing degenerating or stimulated phagocytes, raise the possibility that the peroxidase system utilizing H_2O_2 formed by microorganisms, phagocytes or soluble enzymes also contributes to the host defense against HIV at other sites.

We thank A. Waltersdorph, K. Chaloupka, K. Nelson, and D. Phinney for technical assistance and S. Larsen for the preparation of the manuscript.

This study was supported in part by National Institutes of Health grants AI-07763, AI-27664, and AI-05065.

Address correspondence to Seymour J. Klebanoff, Department of Medicine, SJ-10, University of Washington, Seattle, WA 98195.

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NATIONAL INSTITUTE OF ALLERGY
AND INFECTIOUS DISEASES

TELEFAX COMMUNICATION

DATE: December 22, 1992

CHRISTOPHER TSENG, PH.D.
ANTIMICROBIAL CHEMISTRY PROGRAM OFFICER
ANTIVIRAL RESEARCH BRANCH
DIVISION OF MICROBIOLOGY AND INFECTIOUS DISEASES
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES
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TO: Forrest Seale 512-491-0038
FAX NUMBER

REMARKS:

Forrest,

Attached is the letter from Phil with information on MPO and EPO. Phil told me over the phone that MPO showed no in vitro toxicity (IC_{50} > 500 µg/ml) both in the presence of and in the absence of virus. The data shown in his letter only referred to EPO in the absence of virus. In other words, only cells and EPO were incubated together. The EPO experiments have been repeated. Please let me know if you need more information.

Merry Christmas.

Christopher Tseng

December 16, 1992

Christopher Tseng, Ph.D.
Antimicrobial Chemistry Program Officer
Antiviral Research Branch
Division of Microbiology and Infectious Diseases
National Institute for Allergy and Infectious Diseases
National Institute of Health
Room 3A22
Solar Bldg.
Bethesda,
MD 20892

Dear Chris:

While myeloperoxidase (MPO; ARB 92-187) has no apparent inherent cytotoxicity, eosinophil peroxidase (EPO; ARB 92-188) apparently does. In our assay for cytotoxicity the following values were obtained:

Cell line	IC_{50} (ug/ml) Visual	IC_{50} (ug/ml) neutral red
HEp2	24	40
HEp2	48	48
HEp2	8	5
VERO	24	24
VERO	62	17
VERO	32	94
MDCK	16	3
MDCK	8	4

WE HAVE SUCH A Product!

WPost A-3 7/31 501-375-4171

Evidence of Women's Increased Risk of AIDS Pushes Search for Protection

By Boyce Rensberger
Washington Post Staff Writer

In light of growing evidence that young women are especially vulnerable to infection by the AIDS virus, scientists over the last year have been stepping up their efforts to develop a product that women can use—without a man's knowledge—to protect themselves against the fatal disease.

The hoped-for product, often called a microbicide or virucide, would be a foam or gel or a solid object that a woman could place in her vagina before sex. It would be undetectable by her partner and would emit chemicals that destroy the AIDS virus and perhaps other microbes that cause diseases that predispose a woman to AIDS.

"We desperately need something that gives women more control over their lives," said Penny Hitchcock, chief of the sexually transmitted disease branch of the National Institute of Allergy and Infectious Diseases in Bethesda. "There are many circumstances under which women have sex and for many of them abstinence is not possible, however much they might wish it. And for many women it is also not possible to negotiate condom use."

Hitchcock is among the leaders of an increasingly urgent effort that has been spurred in recent days by several new reports that AIDS is growing fastest among girls and young women, primarily as a result of heterosexual activity, and that the number of American women who came down with AIDS last year grew four times as fast as the number of men.

To encourage the effort, Rep. Constance A. Morella (R-Md.) has introduced a bill that would authorize the National Institutes of Health to spend $30 million a year to develop such a product. Sen. Paul Simon (D-Ill.) is planning to introduce a similar bill in the Senate, possibly next week.

Such "earmarking" bills are seldom expected to pass. But if they gain enough co-sponsors, they may become part of the "report language" that guides federal agencies as they implement the main budget bills.

The nearest things on the U.S. market to a microbicide are vaginal foams and gels that contain a substance called nonoxynol-9 (N-9). Although it kills sperm and is intended as a contraceptive, there is evidence it also destroys viruses. As a result N-9 products have sometimes been recommended to women for protection against HIV, the AIDS virus.

> *"We desperately need something that gives women more control over their lives."*
>
> —Penny Hitchcock, National Institute of Allergy and Infectious Diseases

But it is far from perfect, Hitchcock points out. For one thing, frequent use may have the opposite effect, making a woman more susceptible to HIV infection. The reason is that N-9 is a detergent that not only breaks up the membranes that enclose sperm and HIV but the membranes that enclose cells lining the vagina and the cervix. As a result, scientists fear N-9 may make it easier for the AIDS virus to get in.

Hitchcock said there is a need for more research to learn whether N-9 is truly protective against HIV. Several other spermicides not licensed in this country are also to be tested.

More promising are several classes of potential products based on natural infection-fighting substances. One group relies on small proteins called defensins that are present in the mucus of many mammals, including humans. They can bind directly to certain bacteria and viruses, rendering them unable to infect.

Another class would use large quantities of antibodies, the molecules that the immune system tailors to attack specific microbes. These would be manufactured in the laboratory.

The idea would be to put these substances into a foam or a gel or even a rubber ring that could be placed much as a diaphragm's ring is. The active ingredients would then diffuse and attack the virus.

The ideal product, Hitchcock said, would be one that is cheap, safe, easy to use as well as colorless, odorless and tasteless so that men could not object to its use. As it happens, the product would also protect a man if the woman was infected.

Hitchcock said it is also a goal to make a product that will prevent disease but not prevent pregnancy. "We're talking about a fatal disease," she said. "A woman shouldn't have to risk her life to have a baby."

She said there is also a need for more basic research on such questions as what volume of viruses an infected man might give a woman with each ejaculation, how long the microbes must remain in the vagina to infect, and exactly what the mechanisms of infection are.

Hitchcock said that even if a product could be developed that protected women only against other sexually transmitted diseases, that in itself would offer valuable protection against HIV. Preliminary evidence shows that women who do not have other sexual diseases have a much lower risk of getting AIDS.

CORRECTION

The phone number for information on tonight's performance by Ashe at Mount Vernon College is 202-625-4655. The number was incorrect in yesterday's Weekend section.

Stephens Enterprises, Inc.
111 Center Street, Suite 1616
Little Rock, Arkansas 72201
Tel : (501) 375-0940; Fax : (501) 375-4171

FAX-MEMO

TO: Carol Rasco
The White House

FROM: Jackson T. Stephens, Jr.
Stephens Enterprises, Inc.

DATE: August 4, 1993

****

I sincerely appreciated your getting back to us regarding our interest in meeting with the newly appointed AIDS Czar. I know how busy you are and I appreciate your taking time out to give us an update.

Gary Faulkner and I are scheduling some meetings in Washington during the next couple of weeks and I would like to introduce you to a new contact we have been working with at the NIH. Her name is Dr. Nancy J. Alexander and she is Chief of the Contraceptive Development Branch at the NIH. If we could have a brief meeting with you and include Dr. Alexander as well as Dr. Penny Hitchcock who is Chief of the Sexually Transmitted Disease Branch, I would greatly appreciate it. In a nutshell, Dr. Alexander believes we can be moving at a faster pace and has some ideas about how to proceed. I am including with this fax a copy of a recent Washington Post article that sums up the focus of their attention.

We could meet with you anytime the week of August 9 with the exception of the morning of August 12. We could also meet anytime on August 16 or 17. I believe we may have found in Dr. Alexander someone who can quarterback this process for us. If it is not too much trouble, I would appreciate us being able to have a short meeting with you.

Thanks again for all of your interest and help. My office number is (501) 375-0940 or you can reach me by fax at (501) 375-4171.

WPost A-3 7/31 501-375-4171

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Washington Post Staff Writer

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THE SECRETARY OF HEALTH AND HUMAN SERVICES
WASHINGTON, D.C. 20201

JUN 8 1993

Memorandum for Carol H. Rasco
Assistant to the President
for Domestic Policy

FROM: The Secretary

SUBJECT: AIDS Drug--Letter from Mr. Gary Faulkner

This is in response to your memorandum forwarding correspondence from Mr. Gary Faulkner of Stephens Enterprises, Inc.--the parent company of OxEmis, Inc.--expressing his concern over the postponement of the May 13 meeting of the National Institute of Allergy and Infectious Diseases (NIAID) AIDS Clinical Drug Development Committee (ACDDC).

I have discussed this matter with NIAID's Director, Dr. Anthony S. Fauci, and his staff and I want to emphasize that the NIAID is committed to continuing research and development of HIV viricides, such as EXACT™ (myeloperoxidase) developed by OxEmis, Inc. After careful review of the interaction between OxEmis, Inc. and the scientists in the Division of AIDS, NIAID, it is my understanding that there has been a regular exchange of communication, both verbal and written, between this company and the NIAID with the mutual goal of moving ahead in this important arena of AIDS prevention. However, all companies with promising agents seeking ACDDC review and evaluation are required to submit certain information prior to review.

The ACDDC was established by the NIAID to help evaluate HIV-related treatment and prophylaxis products with potential for study in NIAID-sponsored clinical trials. Because of the large number of products suggested by individuals and organizations each year, the ACDDC review and evaluation helps to ensure the most effective use of resources by recommending the most promising agents for clinical study. In order for the ACDDC to analyze a potential therapeutic candidate in a rational and objective scientific manner, standard guidelines for submission of data have been developed for all sponsors of agents. These guidelines, which have been well-received by the community, were provided to OxEmis, Inc. in a June 1, 1992, letter to Mr. J.T. Stephens (attached). Unfortunately, the ACDDC has not yet received a submission of the requested supporting documentation for EXACT™.

I have been informed that scientists at ExOxEmis, Inc. and the Division of AIDS have pursued various avenues of both *in vitro* and animal testing required for the clinical development of EXACT ™. Recent communication from the Division of AIDS (see attached letter of January 7, 1993 to Mr. J.T. Stephens) clearly outlined the type of preclinical animal model efficacy studies remaining to be done. Once the appropriate tests have been completed, these results and other pertinent information may be forwarded to the ACDDC Executive Secretary, Dr. M.A. Luzar, in order to schedule a review of EXACT ™.

In regard to the May 1993 ACDDC meeting, none of the companies with potential products were able to submit the necessary preclinical information required for ACDDC review, thus the ACDDC meeting was postponed until September 9.

The NIAID would be delighted to schedule an expedited ACDDC review of EXACT ™ or a related product of any manufacturer before the September meeting, provided that the required materials are submitted to the committee by a mutually agreed upon date. In this regard, I suggest that Mr. Faulkner contact Dr. Luzar at (301) 496-8213 to discuss the current status of their efforts regarding EXACT ™.

Donna E. Shalala

Attachments

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

National Institutes of Health
Bethesda, Maryland 20892
Building: Solar
Room : 2A02
(301) 496-8213

June 1, 1992

J. T. Stephens, Jr.
Chairman and C.E.O.
ExOxEmis, Inc.
Suite 1770
One Union National Plaza
Litte Rock, AR 72201

Dear Mr. Stephens:

Thank you for your interest in AIDS treatment research. Outlined below are the additional data needed to review your purified myeloperoxidase compound pursuant to our telephone conversation on May 29th. As the Executive Secretary of the NIH AIDS Clinical Drug Development Committee (ACDDC), I am responsible for reviewing submitted information on promising agents for AIDS that require further clinical development. The Committee was established by Dr. Anthony Fauci, Director, National Institute of Allergy and Infectious Diseases (NIAID), to advise the Division of AIDS (DAIDS) regarding agents to be studied in clinical trials sponsored by the AIDS Clinical Trials Group (ACTG) and by the Community Programs for Clinical Research on AIDS (CPCRA).

The ACDDC is composed of both Government and non-Government experts. Non-Government experts include ACTG and CPCRA investigators, thus providing continuity and enabling speedier movement of drugs into clinical trials. Drugs may be submitted to the Committee by sponsors in industry, academia, and/or Government. Following a thorough review of data on each drug, the Committee assigns it a priority rating. The criteria for evaluation include a rationale, laboratory evidence of activity, safety information in animals and demonstration of *in vivo* efficacy. The DAIDS decides which drugs will enter clinical trials it sponsors, taking into account the recommendations of the Committee.

Because of the large number of suggestions proffered by individuals and organizations regarding treatment of this disease as well as the substantial time and effort required to review the relevant data, it has become necessary to limit review of suggested therapies to those which have demonstrated scientific merit. The ACDDC has established suggested guidelines for the submission of data for review in order to analyze a potential therapeutic candidate in a rational and objective scientific manner which will assure optimal use of federal funds and patient resources. We strongly encourage the inclusion of the requested data (listed below) in your submission. Material which is imprecise will be of limited value to the Committee in fairly evaluating your purified myeloperoxidase compound as a potential therapeutic approach to AIDS.

Please submit five (5) complete copies of the data to be reviewed (suitable for photocopying); however, if your proposal contains colored charts and/or tabs we request that you submit 50 copies. This information is for the exclusive use of the AIDS Clinical Drug Development Committee and the Division of AIDS staff, each of whom recognizes the confidential nature of the submission and his or her obligation to restrict the use of this information to the deliberations of the Committee and the Division of AIDS staff. A copy of our confidentiality policy is enclosed for your information.

Please limit the packet of information that you send us to no more than 50 - 55 pages. If any of the submitted data have been published, the specific references should be indicated where the data are presented in the body of the submission, as well as providing a separate bibliography. You are encouraged to submit reprints.

The information submitted should include a brief summary of:

1. Theoretical background

   A brief description of why your purified myeloperoxidase compound should be a) effective, b) safe, and c) feasible. For what condition is the compound proposed? What is the purported specific mechanism of action? For example, if the agent has anti-HIV activity, does it inhibit reverse transcriptase/uptake, prevent release of virus from infected cells, etc.? Similarly, if the agent is an immunomodulator, specify the effect(s) on the immune system.

2. *In vitro* documentation of efficacy

   Ideally, data from two independent laboratories should be submitted. Please provide details of how the summarized studies were performed: the specific assays used, reagents, cell lines, isolates employed, parameters used to determine a positive or negative result (eg. the background level in a laboratory doing reverse transcriptase activity assays), positive and negative controls. Please identify the laboratories where the testing was performed. If you have been unable to obtain such testing we may be able to arrange for screening of compounds for antiretroviral activity by the NIH or one of its contractors.

3. *In vivo* documentation of efficacy

   Data from animal studies, if any, indicating the safety, tolerance, and efficacy of the proposed compound in conditions possessing some similarities to AIDS. Again, in your summary of these data, please provide details that will enable us to evaluate the agent: study design, species, dose, route and duration of administration, relevant pharmacokinetic data, length and frequency of observation, measurable parameters used to define outcomes.

4. Preclinical pharmacokinetics and toxicology

   Briefly summarize acute, subacute, and chronic toxicology studies; results of mutagenicity and teratogenicity studies; single and multiple dose pharmacokinetics in animals.

5. Results of Phase I pharmacokinetic studies

   Single and multiple dose studies; safety and tolerance. Describe patient population(s) studied and how outcome measurements were made.

6. Results of clinical trials (Phase IB or later studies)

   Summarized data should include: a) description of patient population, b) eligibility criteria, c) study design, d) objective parameters for both laboratory and clinical improvement, e) toxicity, f) concomitant therapies allowed and utilized, and g) patient status at the conclusion of the trial and at later follow-up, if done. Emphasis should be on measurable, quantitative parameters that can be summarized in tables and clearly-marked graphs. Be specific. Generalities such as "symptoms were reduced", "antiviral activity was seen", "opportunistic infections were reduced", or "lesions of Kaposi's sarcoma were reduced" are of limited value.

7. Product formulation and availability

   Data about the structure, formulation, stability-indicating assay, shelf-life, and availability should be provided to assist in determining the feasibility of conducting clinical trials with the proposed agent.

8. Cover letter delineating your overall development plan for the proposed therapy including the extent of AIDS Clinical Trials Group (ACTG) participation anticipated.

Your compliance with these guidelines will expedite the evaluation of your purified myeloperoxidase compound and will be greatly appreciated by the Committee. Information should be sent to me at the address below:

Mary Anne Luzar, Ph.D.
Executive Secretary
AIDS Clinical Drug Development Committee
Division of AIDS, NIAID
Control Data Building, Room 2A02
6003 Executive Blvd.
Rockville, MD 20852

Please note that future ACDDC meetings are tentatively scheduled for September 17 and December 10, 1992. We would appreciate receiving information on your agent four weeks prior to a meeting date in order for it to be considered for presentation to the committee. If a favorable consideration is given, you will be contacted by the Executive Secretary about arrangements to be made for a representative from your organization to appear before the committee for a formal presentation.

Enclosed for your information are a Committee roster and charter, as well as a set of guidelines for Biological Response Modifier Evaluation, the confidentiality statement for members and a list of ACTG and CPCRA principal investigators.

Please contact me at (301) 496-8213 if I may be of any further assistance to you.

Sincerely,

Mary Anne Luzar, Ph.D.
Executive Secretary
AIDS Clinical Drug Development Committee
Division of AIDS
National Institute of Allergy and Infectious Diseases

Encl. (6)

cc: Dr. Henry Masur

DEPARTMENT OF HEALTH & HUMAN SERVICES
Public Health Service

National Institutes of Health
Bethesda, Maryland 20892

Solar Building
Room 2C22
(301) 496-0700

January 7, 1993

Jackson T. Stephens, Jr.
Chairman, ExOxEmis Inc.
1769 One Union National Plaza
Little Rock, Arkansas 72201

Dear Mr. Stephens:

As per our phone conversation of Tuesday, January 5, 1993, I want to re-emphasize that the importance of continuing research and development of HIV viricides, such as MPC and EPO, is acknowledged by us in the National Institute of Allergy and Infectious Diseases. I have discussed the present state of development with Dr. Anthony Fauci, Institute Director, Dr. Pat Reichelderfer, Program Virologist, and Dr. Chuck Litterst, Drug Development and Surveillance Chief.

As previously stated, we are committed to do animal efficacy studies in the guinea pig model of Herpes. We feel such animal model efficacy studies are a logical next step, and if these compounds prove positive in a Herpes model, would generate support and enthusiasm by the AIDS Clinical Drug Development Committee (ACDDC). We are prepared to initiate animal studies with an appropriate drug formulation provided by you that will ultimately be employed in clinical studies.

We are also in agreement with your Scientific Director, Dr. Bob Allen, that animal studies of local toxicity following vaginal administration are critical. As you are aware, increased transmission of HIV has been associated with conditions of abnormal vaginal epithelium. The pathology report on the rabbit toxicology studies has been completed and you should be receiving a copy of the report shortly from Dr. Nancy Alexander of NICHD. The report apparently describes considerable toxicity with the gel base alone. Dr. Alexander's group does not formulate compounds, but, Dr. Allen has stated that the product (Exact) could be formulated as a gel. It is unclear whether the formulation supplied to NICHD was tested in vitro for efficacy. Again, it will be important to perform toxicity trials with the compound and formulation to be used in the clinical trial.

Further testing can proceed as soon as we have received the revised compound. Once the various contractors have received this formulation, it will take approximately 3-4 weeks to complete the cell culture work, and 3-4 months to complete the animal efficiency studies. It is highly likely that the ACDDC will wish to evaluate the results of the animal efficacy studies before making a decision to proceed to clinical trials.

As we discussed, it is also important to continue the dialogue with the FDA to understand what other suggestions or requirements they may have prior to initiating clinical trials.

Again, I want to re-assure you that because this product and approach are very promising in preventing HIV-1 transmission, we remain committed to assisting ExOxEmis so that this concept and product are developed in a timely manner.

Sincerely,

Steven M. Schnittman, M.D.
Chief, Medical Branch
Division of AIDS
Clinical Research Program
National Institute of Allergy
and Infectious Diseases
National Institutes of Health

cc: Anthony S. Fauci, M.D.
Pat Reichelderfer, Ph.D.
Chuck Litterst, Ph.D.

Stephens Enterprises, Inc.
111 Center Street, Suite 1616
Little Rock, Arkansas 72201
Tel : (501) 375-0940; Fax : (501) 375-4171

FAX-MEMO

TO: Carol Rasco
Fax # (202) 456-2878

FROM: Gary Faulkner
Fax # (501) 375-0940

DATE: June 8, 1993

****

We thought you would be interested in the following article summarizing the highlights of the Berlin AIDS Conference.

It was especially interesting to us that the participants placed an urgent priority on the "development of some substance or device that would enable women to protect themselves from infection without the consent of male sex partners. Possibilities include a microbicidal jelly- ideally one that also would kill bacteria and parasites-"

As you know, this is exactly what we have developed at ExOxEmis. As soon as we can get it through the bureaucracy it could be made available to the public.

Thanks for your continued interest.

****

$2.5 Billion a Year Urged to Curb AIDS Cases

Officials at World Conference Cite Explosive Growth of HIV Infection in Some Countries

By David Brown
Washington Post Staff Writer

BERLIN, June 7—For an investment of $2.5 billion a year, the world community could cut in half the number of new AIDS virus infections expected to occur by the turn of the century.

That was the prediction offered by a World Health Organization (WHO) official today as the Ninth International Conference on AIDS opened with nearly 16,000 scientists, public health workers, activists and journalists.

The $2.5 billion, said Michael H. Merson, an American epidemiologist who heads WHO's global program on AIDS, "is an investment on which the returns would be huge."

WHO estimates that about 13 million adults and 1 million infants have been infected with the human immunodeficiency virus (HIV) since the disease was first identified in the early 1980s. Without the added funding, about 20 million more cases will develop in the Third World by the year 2000. With more intensive promotion of condom use, treatment of venereal diseases and other low-tech interventions, new adult infections could be reduced to 10 million, Merson said.

"But can the world afford the necessary $2.5 billion? Well, $2.5 billion is scarcely one-twentieth of the $49 billion spent on Operation Desert Storm. It would hardly buy one can of Coke for every person in the world," Merson said.

The hopeful exhortation came with a catalog of grim statistics.

Growth of HIV infection is "explosive" in Southeast Asia. In Thailand, the number of those infected has grown from 50,000 to 450,000 in three years. In Burma and India, the prevalence of infection in drug addicts has gone from 0 percent to 50 percent in a slightly longer period, Merson said. The disease is spreading with similar speed in Latin America, where half a million Brazilians may be infected by the end of the decade.

The worst numbers are from Africa. In the Ivory Coast city of Abidjan, for example, about 11 percent of all adults are infected; in southern Sudan nearly half of all female prostitutes are HIV-positive; and in Zaire the rise of a generation of "AIDS orphans" is becoming a major social burden.

But in terms of worldwide threats to life, AIDS may not be the most severe, according to an official of the World Bank, who previewed a report on development policy and health that his organization is scheduled to release in about two weeks.

"I would submit that the mortality from AIDS argues against (its having) any degree of priority relative to smoking, TB and control of diarrheal diseases," Dean T. Jamison said.

Nevertheless, he added, the World Bank has scrutinized WHO's estimates and concurs that increased spending now on AIDS prevention would be useful, in part because of "rapidly declining cost effectiveness" of health interventions once infection occurs.

Others criticized the World Bank's analysis.

June F. Osborn, a professor of public health at the University of Michigan and chairwoman of the president's AIDS commission, called it a "sad understatement" of the importance of the infection, which she called a "wild card" among diseases because it preferentially strikes the young and is so difficult to control.

Elhadj As Sy, a resident of Dakar and an official of the International Council of AIDS Service Organizations, said the disease's impact in Africa is not told by numbers alone. The two chief engineers of a major public works project in one African country were recently diagnosed as HIV-positive (presumably threatening the project's completion), and in another country 30 percent of the medical personnel are infected. He did not name either nation.

There was general concurrence by international AIDS officials here that a particularly urgent priority is the development of some substance or device that would enable women to protect themselves from infection without the consent of male sex partners. Possibilities include a microbicidal jelly—ideally one that also would kill bacteria and parasites—or some version of the cervical cap.

This is very important for teenage girls, said Peter Piot of the Institute of Tropical Medicine in Antwerp, because their physiological immaturity makes them more susceptible to infection. In many places this age group constitutes the leading edge of the epidemic.

The conference so far has been without the clamor of social protest or scientific speculation that marked some sessions in the past. In Amsterdam last summer, reports of a small number of patients with AIDS-like illness who were not HIV-positive fed rumors that a new, mysterious epidemic was underway. Those cases were later determined to have different causes.

There has been a concerted effort to bring persons and organizations to this conference who might not otherwise have attended. The steering committee spent about $3 million sponsoring 2,000 persons who could not afford the registration fee. A consortium of nongovernmental organizations paid the travel costs of another 200 attendees and found them housing with families in Berlin.

Karl-Otto Habermehl, a German professor of virology and the chairman of the conference, said he did not know how many HIV-positive persons were among the registrants, but he added, "it is certainly a large number."

THE WASHINGTON POST
TUESDAY, JUNE 8, 1993 A17

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

National Institutes of Health
Bethesda, Maryland 20892
Building 6100 Executive Blvd.
Room Room 8B 13P
(301) 496- 1661

May 3, 1993

Mr. Forrest Seale
ExOxEmis, Inc.
18585 Sigma Rd.
San Antonio, Texas 78258

Dear Mr. Seale:

As you are aware, we conducted for you a vaginal irritation study of Eosinophil Peroxidase Acetate Jelly (CDB-3663), Myeloperoxidase Acetate Jelly (CDB-3664) and Control Acetate Jelly (CDB-3665) at our Biological Testing Facility several months ago. Recently we reexamined the results. The pH of these formulations as well as that for Ortho-Creme® along with their mean scores of microscopic findings are summarized in the table below.

CDB No.	TEST ARTICLE	MICROSCOPIC	pH
3663	Eosinophil Peroxidase	8.7	4.2
3664	Myeloperoxidase	11.9	4.3
36655	Control Acetate Jelly	8.1	4.2
-	Ortho-Creme®	4.9	5.5-6.0

The pH of the rabbit vagina is close to neutral (7.0-7.6, mean 7.3, in intact New Zealand Rabbits and 6.7-7.2, mean 7.0, in ovariectomized animals in our laboratory) and introduction of acidic formulations produces clinically demonstrable irritation.[1] Therefore we examined the pH of the formulations which you submitted for testing and it is not surprising that all three caused vaginal irritation. Note that Ortho-Creme® has a pH somewhat higher than your formulations and also appeared less irritating.

One of the problems in the development of vaginal products designed to reduce the transmission of STDs including HIV is the concomitant production of irritation of the vaginal mucosa. These cells and their secretions help produce a normal barrier to invasive pathogens. Recent studies[2] suggest that if this barrier is compromised by vaginal irritation produced by continuous use of

1. Kaminsky, M. and Willigan, D.A. (1982). *pH and the potential irritancy of douche formulations to the vaginal mucosa of the albino rabbit and rat. J. Fd. Chem. Toxic. 20, 193-196.*

2. Niruthisard, S., Roddy, R.E., and Chutivongse, S. (1992) *Use of nonoxynol-9 and reduction in rate of gonococcal and chlamydial cervical infections.* The Lancet 339: 1371-1375.

products containing nonoxynol-9, STD increases in spite of the known antimicrobial activity of this surfactant.

Notwithstanding the marked difference between vaginal pH in rabbits and women (3.5-4.2), the rabbit has been traditionally used for the assessment of irritation for products intended for vaginal use. Your *in vitro* studies are suggestive of a potential product but it will be necessary for it to less irritating. Perhaps you should consider reformulating Eosinophil and Myeloperoxidase in a less acid carrier.

I should be pleased to discuss the testing of new formulations with you.

Sincerely,

Nancy J. Alexander, Ph.D.
Chief
Contraceptive Development Branch
Center for Population Research
National Institute for Child Health
and Human Development

Roz: In addition to preparing this memo for me to sign & ~~send~~ to Donna (I could give it to her Tues. aft. at the meeting with the president), pls. call Gary Faulkner, tell him no AIDS Czar yet, that I am looking into the matter further

W 9/17

and that he should let you know when they determine ~~what~~ when they will be here in May.

CLINTON LIBRARY PHOTOCOPY

See if Kevin Thurme has any update. yet prior to my meeting this aft.

Gary w/ b in town the week of 5/17 — We'll call you then

THE WHITE HOUSE

WASHINGTON

May 6, 1993

MEMORANDUM FOR SECRETARY DONNA SHALALA

FROM: Carol H. Rasco, Assistant to the President for Domestic Policy

SUBJECT: AIDS Drug

As you will remember, I recently held a meeting in my office with individuals from Arkansas and Texas to which you sent at my invitation, Dr. Fauci and one or two others from NIH. The issue was/is the development of a drug that has promise for preventing the transmission of AIDS.

Dr. Fauci gave considerable assurances of cooperation and timeliness during the meeting. Please see the attached which has now come. Because of President Clinton's serious interest in this matter, I would appreciate any additional information you can get for me.

FYI: I have been told by Mr. Faulkner that the FDA person working with them is Dr. Walla Dempsey who can be reached at (301)443-9563.

Thank you.

MEMORANDUM OF CALL — Previous editions usable

TO: CK

☑ YOU WERE CALLED BY– ☐ YOU WERE VISITED BY–

Gary Fauekner

OF (Organization)

☐ PLEASE PHONE ▶ ☐ FTS ☐ AUTOVON

☐ WILL CALL AGAIN ☐ IS WAITING TO SEE YOU

☐ RETURNED YOUR CALL ☐ WISHES AN APPOINTMENT

MESSAGE

Per your conversation pls call:

(female) → Dr. Walla Dempsey

(301) 443-9563

FDA Team Leader

RECEIVED BY	DATE	TIME
Roz	3/19	11:30

63-110 NSN 7540-00-634-4018 **STANDARD FORM 63** (Rev. 8-81)
Prescribed by GSA
☆ U.S.G.P.O. 1992 312-070-40024 FPMR (41 CFR) 101—11.6

Call back Mon.

MEMORANDUM OF CALL — Previous editions usable

TO: CK (pl)

☑ YOU WERE CALLED BY– ☐ YOU WERE VISITED BY–

Gary Fauekner

OF (Organization)

☐ PLEASE PHONE ▶ ☐ FTS ☐ AUTOVON

(501) 375-0940

☐ WILL CALL AGAIN ☐ IS WAITING TO SEE YOU

☐ RETURNED YOUR CALL ☐ WISHES AN APPOINTMENT

MESSAGE

- Re: Aids Issue
- w/b in town next week
- As discussed, wants to meet w/ Aids Czar if announced by then

RECEIVED BY	DATE	TIME
Roz	4/19	4:30

63-110 NSN 7540-00-634-4018 **STANDARD FORM 63** (Rev. 8-81)
Prescribed by GSA
☆ U.S.G.P.O. 1992 312-070-40024 FPMR (41 CFR) 101—11.6

Stephens Enterprises, Inc.
111 Center Street, Suite 1616
Little Rock, Arkansas 72201
Tel : (501) 375-0940; Fax : (501) 375-4171

FAX-MEMO

TO: Carol Rasco
The White House
FROM: Gary Faulkner
Stephens Enterprises, Inc.
DATE: April 20, 1993

As we discussed in our meeting on March 17, 1993, one of our key concerns has been the extremely slow pace in which the NIH has moved to test AIDS related drugs such as ours. Despite Dr. Fauci's statement to the contrary, we continue to see the battle against AIDS being overwhelmed by the NIH's internal bureaucracy.

I am including with this memo a copy of a letter we have just received that is a prime example of the type of constant delays we have experienced in dealing with the NIH's Division of AIDS. For months, we have been working towards the May 13th scheduled meeting of the AIDS Clinical Drug Development (ACDDC) group. Needless to say, a tremendous amount of time and money has been expended by our small company in an effort to be prepared for our presentation at this May 13th meeting. Considering the crisis state of the AIDS problem you can understand why we were shocked and disappointed to receive this letter stating without explanation that the ACDDC meeting had been postponed until September 9, 1993. We could probably accept a month delay, but considering the seriousness of the AIDS crisis, a four month delay is unbelievable!

We are eager to discuss the status of the ACDDC review committee with the new AIDS Czar and you, whenever he or she is appointed. You had asked me to let you know when we would next be in Washington. Steve Stephens will be there next week and I could join him if you think there is a chance the AIDS Czar will be named by that time. If not, we will both be back up there sometime the first or second week of May.

Carol, we sincerely appreciate your help in trying to break up the logjam at the NIH. We know the President is committed to a fast track review process for AIDS related drugs and treatments. We look forward to meeting with the new AIDS Czar and sharing some of the experiences we have had as well as recommendations for change.

If you or Rosslyn can give me some update on the timing of our next meeting I would appreciate it. Thanks again for your assistance.

Gary Faulkner
(501) 375-0940

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

National Institutes of Health
Bethesda, Maryland 20892
Building: SOLAR
Room : 2A02
(301) 496-8213

April 16, 1993

Robert Allen, M.D., Ph.D.
ExOxEmis, Inc.
Suite 1770
One Union National Plaza
Little Rock, AR 72201

Dear Dr. Allen:

The AIDS Clinical Drug Development (ACDDC) meeting originally scheduled for May 13th has been postponed until September 9, 1993, in Bethesda, Maryland. The Division of AIDS regrets any inconvenience caused by this change.

Submitting the file for Myeloperoxidase at your earliest convenience (no later than August 12th) is recommended so that appropriate review may be given to your product. If a favorable consideration is given, I will notify you immediately to allow as much time as possible to prepare your presentation.

On behalf of the Division of AIDS and the ACDDC, thank you for your interest in AIDS research and your cooperation.

Sincerely,

Mary Anne Luzar, Ph.D.
Executive Secretary
AIDS Clinical Drug Development Committee
Division of AIDS
National Institute of Allergy and Infectious Diseases

cc: Dr. Henry Masur

Executive Secretariat

Washington, D.C. 20201

FACSIMILE

PLEASE NOTIFY OR HAND-CARRY THIS TRANSMISSION TO THE FOLLOWING PERSON AS SOON AS POSSIBLE:

Name: Carol Rasco / Rosalyn

Address: White House

Telephone:

iber of pages being transmitted (including this one) 9

FROM: Tim Harris / Glen Harelson

FAX number:

Office Number: 690-7699

for Kevin Thurm

ACDDC - related events - Interaction with ExOxEmis

Drug: Myeloperoxidase

5-29-92 - Ex. Sec of ACDDC (M.A. Luzar) spoke with Mr. Stephans about their interest in coming to ACDDC in Sept. 92.

6 -1-92 - The official ACDDC letter requesting a file of information on the product was sent to Mr. Stephens.

7-8-92 - The Exec Sec attended the company meeting here in Bethesda to review product development. It was decided that further areas of need for the product to go forward into humans included 1) animal model for prophylaxis of HSV and HIV and 2) Cell culture models for HSV and HIV. As a result of the meeting it was felt that EPO?MPO would not be ready for the ACDDC until Nov or later.

12-92 - After extensive contact with Basic Research scientists in the DAIDS, a letter from Dr. Pat Reichelderfer to Mr. Stephens was sent recommending not to procede on the present data to the Mar. 1 ACDDC meeting. M.A. Luzar confirmed by phone to company.

3 -12-93 - Dr. Tseng confirms lack of ability to test compound in animal models due to nonreceipt from company of reviesed test formulation.

5-12-93- Dr. Litterst (DAIDS) confirms that no reformulation of product has been received by DAIDS contractors.

Note: No file from the company as requested in the June 1 letter has yet been received by the ACDDC Ex Sec. Once they submit it, we can arrange for a review by conference call/meeting of the ACDDC prior to Sept (next planned meeting).

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

National Institutes of Health
Bethesda, Maryland 20892
Building: Solar
Room : 2A02
(301) 496-8213

June 1, 1992

J. T. Stephens, Jr.
Chairman and C.E.O.
ExOxEmis, Inc.
Suite 1770
One Union National Plaza
Litte Rock, AR 72201

Dear Mr. Stephens:

Thank you for your interest in AIDS treatment research. Outlined below are the additional data needed to review your purified myeloperoxidase compound pursuant to our telephone conversation on May 29th. As the Executive Secretary of the NIH AIDS Clinical Drug Development Committee (ACDDC), I am responsible for reviewing submitted information on promising agents for AIDS that require further clinical development. The Committee was established by Dr. Anthony Fauci, Director, National Institute of Allergy and Infectious Diseases (NIAID), to advise the Division of AIDS (DAIDS) regarding agents to be studied in clinical trials sponsored by the AIDS Clinical Trials Group (ACTG) and by the Community Programs for Clinical Research on AIDS (CPCRA).

The ACDDC is composed of both Government and non-Government experts. Non-Government experts include ACTG and CPCRA investigators, thus providing continuity and enabling speedier movement of drugs into clinical trials. Drugs may be submitted to the Committee by sponsors in industry, academia, and/or Government. Following a thorough review of data on each drug, the Committee assigns it a priority rating. The criteria for evaluation include a rationale, laboratory evidence of activity, safety information in animals and demonstration of *in vivo* efficacy. The DAIDS decides which drugs will enter clinical trials it sponsors, taking into account the recommendations of the Committee.

Because of the large number of suggestions proffered by individuals and organizations regarding treatment of this disease as well as the substantial time and effort required to review the relevant data, it has become necessary to limit review of suggested therapies to those which have demonstrated scientific merit. The ACDDC has established suggested guidelines for the submission of data for review in order to analyze a potential therapeutic candidate in a rational and objective scientific manner which will assure optimal use of federal funds and patient resources. We strongly encourage the inclusion of the requested data (listed below) in your submission. Material which is imprecise will be of limited value to the Committee in fairly evaluating your purified myeloperoxidase compound as a potential therapeutic approach to AIDS.

J. T. Stephens, Jr.

Please submit five (5) complete copies of the data to be reviewed (suitable for photocopying); however, if your proposal contains colored charts and/or tabs we request that you submit 80 copies. This information is for the exclusive use of the AIDS Clinical Drug Development Committee and the Division of AIDS staff, each of whom recognizes the confidential nature of the submission and his or her obligation to restrict the use of this information to the deliberations of the Committee and the Division of AIDS staff. A copy of our confidentiality policy is enclosed for your information.

Please limit the packet of information that you send us to no more than 50 - 55 pages. If any of the submitted data have been published, the specific references should be indicated where the data are presented in the body of the submission, as well as providing a separate bibliography. You are encouraged to submit reprints.

The information submitted should include a brief summary of:

1. Theoretical background

   A brief description of why your purified myeloperoxidase compound should be a) effective, b) safe, and c) feasible. For what condition is the compound proposed? What is the purported specific mechanism of action? For example, if the agent has anti-HIV activity, does it inhibit reverse transcriptase/uptake, prevent release of virus from infected cells, etc.? Similarly, if the agent is an immunomodulator, specify the effect(s) on the immune system.

2. *In vitro* documentation of efficacy

   Ideally, data from two independent laboratories should be submitted. Please provide details of how the summarized studies were performed: the specific assays used, reagents, cell lines, isolates employed, parameters used to determine a positive or negative result (eg. the background level in a laboratory doing reverse transcriptase activity assays), positive and negative controls. Please identify the laboratories where the testing was performed. If you have been unable to obtain such testing we may be able to arrange for screening of compounds for antiretroviral activity by the NIH or one of its contractors.

3. *In vivo* documentation of efficacy

   Data from animal studies, if any, indicating the safety, tolerance, and efficacy of the proposed compound in conditions possessing some similarities to AIDS. Again, in your summary of these data, please provide details that will enable us to evaluate the agent: study design, species, dose, route and duration of administration, relevant pharmacokinetic data, length and frequency of observation, measurable parameters used to define outcomes.

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4. Preclinical pharmacokinetics and toxicology

   Briefly summarize acute, subacute, and chronic toxicology studies; results of mutagenicity and teratogenicity studies; single and multiple dose pharmacokinetics in animals.

5. Results of Phase I pharmacokinetic studies

   Single and multiple dose studies; safety and tolerance. Describe patient population(s) studied and how outcome measurements were made.

6. Results of clinical trials (Phase IB or later studies)

   Summarized data should include: a) description of patient population, b) eligibility criteria, c) study design, d) objective parameters for both laboratory and clinical improvement, e) toxicity, f) concomitant therapies allowed and utilized, and g) patient status at the conclusion of the trial and at later follow-up, if done. Emphasis should be on measurable, quantitative parameters that can be summarized in tables and clearly-marked graphs. Be specific. Generalities such as "symptoms were reduced", "antiviral activity was seen", "opportunistic infections were reduced", or "lesions of Kaposi's sarcoma were reduced" are of limited value.

7. Product formulation and availability

   Data about the structure, formulation, stability-indicating assay, shelf-life, and availability should be provided to assist in determining the feasibility of conducting clinical trials with the proposed agent.

8. Cover letter delineating your overall development plan for the proposed therapy including the extent of AIDS Clinical Trials Group (ACTG) participation anticipated.

Your compliance with these guidelines will expedite the evaluation of your purified myeloperoxidase compound and will be greatly appreciated by the Committee. Information should be sent to me at the address below:

Mary Anne Luzar, Ph.D.
Executive Secretary
AIDS Clinical Drug Development Committee
Division of AIDS, NIAID
Control Data Building, Room 2A02
6003 Executive Blvd.
Rockville, MD 20852

J. T. Stephens, Jr.

Please note that future ACDDC meetings are tentatively scheduled for September 17 and December 10, 1992. We would appreciate receiving information on your agent four weeks prior to a meeting date in order for it to be considered for presentation to the committee. If a favorable consideration is given, you will be contacted by the Executive Secretary about arrangements to be made for a representative from your organization to appear before the committee for a formal presentation.

Enclosed for your information are a Committee roster and charter, as well as a set of guidelines for Biological Response Modifier Evaluation, the confidentiality statement for members and a list of ACTG and CPCRA principal investigators.

Please contact me at (301) 496-8213 if I may be of any further assistance to you.

Sincerely,

Mary Anne Luzar, Ph.D.
Executive Secretary
AIDS Clinical Drug Development Committee
Division of AIDS
National Institute of Allergy and Infectious Diseases

Encl. (5)

cc: Dr. Henry Masur