

Contribution of single genes within the unique short region of Aujeszky's disease virus (suid herpesvirus type 1) to virulence, pathogenesis and immunogenicity

T. G. Kimman,^{1*} N. de Wind,² N. Oei-Lie,¹ J. M. A. Pol,¹ A. J. M. Berns² and A. L. J. Gielkens¹

¹Central Veterinary Institute, Department of Virology, P.O. Box 365, 8200 AJ Lelystad and ²The Netherlands Cancer Institute, Division of Molecular Genetics, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

Pigs (3 and 10 weeks old) were infected intranasally with Aujeszky's disease virus (ADV) mutants that functionally lacked one of the non-essential genes in the unique short region of the genome (except the gene encoding the 11K protein). Virus excretion in oropharyngeal fluid and disease symptoms were monitored. Some pigs were killed to study pathogenesis, whereas others were challenged with virulent ADV 8 weeks after the primary infection. Mutants lacking protein kinase, or glycoproteins gp63 or gI showed reduced virulence, but mutants lacking gX or the 28K protein showed normal virulence. Glycoprotein gI appears to affect the tissue tropism of ADV in pigs, presumably by

facilitating the spread of the virus through the central nervous system. In this study, there was no correlation between virulence and virus multiplication in either cultured cells or in the oropharynx *in vivo*. All mutants induced neutralizing antibody and complete or partial protection against challenge infection. Complete protection was obtained by inoculation with the gI and gX mutants, whereas incomplete protection was obtained using gp63 and protein kinase mutants. Complete clinical and virological protection was associated with the absence of secondary antibody responses in the serum.

Introduction

Aujeszky's disease virus (ADV; synonyms pseudorabies virus and suid herpesvirus type 1) causes an important disease of swine. Young pigs may die of severe meningoencephalitis; older pigs usually survive the infection, but may develop fever and pneumonia, and may grow more slowly. Several countries try to minimize the spread of the virus by vaccination alone or in combination with slaughter of infected pigs. Conventional attenuated vaccine strains, such as Bartha and Norden, have deletions in the gI, gp63, 11K and 28K genes in the unique short (U_S) region of the genome (Petrovskis *et al.*, 1986*a*). Also, genetically engineered virus strains have been developed with deletions in the gI, gIII, gX and thymidine kinase genes (Kit *et al.*, 1987; Marchioli *et al.*, 1987; Moormann *et al.*, 1990). Existing vaccines prevent the development of clinical signs upon infection and they may reduce, but usually do not prevent, virus excretion and the establishment of latency after infection (van Oirschot *et al.*, 1990).

The genome of ADV is a linear dsDNA molecule of 150 kbp and consists of a (U_S) region flanked by inverted repeats and a unique long (U_L) region (Ben-Porat *et al.*,

1979). It encodes at least 50 proteins. The U_S region is the best characterized part of the genome and has been sequenced completely. It contains the genes encoding a protein kinase (PK), glycoproteins gX, gp50, gp63 and gI, and 11K and 28K proteins (Petrovskis *et al.*, 1986*b, c*, 1987; Rea *et al.*, 1985; van Zijl *et al.*, 1990). Comparison with herpes simplex virus type 1 (HSV-1) has revealed similarity between ADV gp50 and HSV-1 gD, ADV gp63 and HSV-1 gI, ADV gI and HSV-1 gE, ADV 11K and HSV-1 US9, and ADV PK and HSV-1 US3 (Petrovskis *et al.*, 1986*b, c*; Petrovskis & Post, 1987; van Zijl *et al.*, 1990). Only gp50 is essential for virus replication in cell culture (de Wind *et al.*, 1990), probably playing a role in penetration of the virus into cells (B. Peeters, unpublished results). The other genes may play a role in determining the outcome of infection, in transmission of the virus, or in other processes of infection. GX seems to have no, or only a minor, role in virulence; a gX deletion mutant is fully virulent for mice (Thomsen *et al.*, 1987). Virus mutants lacking gI show reduced virus release from infected cells *in vitro* and reduced virulence *in vivo* (Mettenleiter *et al.*, 1987; Zuckermann *et al.*, 1988). However, the mechanism by which gI affects virulence is not known. The contribu-

tion of the gp63, 11K and 28K genes to the reduced virulence of the Bartha and Norden strains is not known (Petrovskis *et al.*, 1986a).

Similarly the contribution of each of these proteins to the development of complete or partial immunity is unknown. Single gene products may be involved in determining the immunogenicity of the virus, either because they are important target antigens for antibody or T cells, or because they are important for virus replication. Both gp50 and gIII have been identified as targets for neutralizing antibodies (Ben-Porat *et al.*, 1986; Eloit *et al.*, 1988; Marchioli *et al.*, 1988; Zuckermann *et al.*, 1990), and gIII is also a target for cytotoxic T lymphocytes (Zuckermann *et al.*, 1990). However, the relative importance of each of these responses is unknown.

By linker insertion mutagenesis, ADV mutants with a single inactivated gene in the U_S region have been constructed (de Wind *et al.*, 1990). The results of this study indicated that gX and 28K do not contribute to the virulence of ADV for pigs, in contrast to PK, gp63 and gI. GI probably contributes to virulence by facilitating the spread of the virus through the central nervous system (CNS). Pigs immunized intranasally with PK, gX, gp63 and gI mutants are all protected considerably or completely against a challenge with virulent virus; the gI and gX mutants induce complete protection against the challenge with virulent virus, indicating that these genes may be dispensable for the induction of complete immunity.

Methods

Cells, viruses and medium. Virus was isolated, grown and titrated by plaque assay on monolayers of secondary porcine kidney cells in Earle's MEM (EMEM) supplemented with 2% foetal bovine serum and antibodies in a humidified CO₂ incubator at 37 °C. The porcine kidney cell lines PK15 (de Leeuw & van Oirschot, 1985) and SK6 (Kasza *et al.*, 1971) were used in transfection experiments and for plaque purification of mutant virus strains.

The NIA-3 strain of ADV, originally isolated from a field case of Aujeszky's disease in Northern Ireland (McFerran & Dow, 1975), was used as the parent strain for the development of mutant viruses and as the challenge virus in animal experiments. Mutant viruses were generated as described previously by insertion mutagenesis using an oligonucleotide with translational stop codons in all reading frames, and also containing an *EcoRI* site (de Wind *et al.*, 1990). The positions of the linker insertion sites are shown in Fig. 1. From all the insertion mutants used in this study, the sequences flanking the oligonucleotide insertion sites were determined; all the mutants contained a correctly inserted translation stop codon and no alterations were found in ADV sequences flanking the oligonucleotides. Mutants M101, M102 and M104 have a premature translation termination codon to the 5' side of the genes encoding gI, gp63 and gX respectively. These mutants have been previously called 522, 149 and 247 (de Wind *et al.*, 1990). Mutants M110 and M118 (previously 549) have a premature translation termination codon at the same position to the 5' side of the gene

encoding PK. M118 was constructed because *BamHI* fragment 6 of M110 appeared to be approximately 100 bp smaller than that of the parent strain, NIA-3. A slight variation in the size of the *BamHI* 6 region is frequently observed among field isolates of ADV. Mutant M113 (previously 351–357) has a deletion of approximately 80% of the gene encoding 28K. This deletion was generated between two *EcoRI* linker insertion sites located 5' of and in the 3' part of the gene (de Wind *et al.*, 1990). Using protein-specific polyclonal and monoclonal antibodies, absence of expression of the respective proteins was confirmed for M101, M113 and M118 by Western blotting (de Wind *et al.*, 1990; van Zijl *et al.*, 1990), and for M102 and M104 using fixed infected cell monolayers (data not shown). Mutant M120 was generated from the PK mutant M118 by marker rescue. For this purpose, a purified, cloned *BamHI* 10 fragment from the parental NIA-3 strain was cotransfected with viral DNA from strain M118. Of the resulting plaques, 5% contained the wild-type *BamHI* 10 fragment. M209 was generated by cotransfection into cells of four purified overlapping DNA fragments, together comprising the entire wild-type viral genome, as described previously (van Zijl *et al.*, 1988). M209 has virulence similar to that of NIA-3 and was used as a positive control in the animal experiments (van Zijl *et al.*, 1988). Mutant virus strains were plaque-purified three times on SK6 cells.

Animals. Dutch Landrace pigs were from the specific pathogen-free herd of the Central Veterinary Institute; the pigs were born to unvaccinated sows and before the start of the experiments had no antibodies directed against ADV. Pigs of different litters were randomly assigned to experimental groups. During the experiments, each group was housed in a separate isolation room. Three-week-old pigs had access to food *ad libitum* during the whole day, whereas 10-week-old and older pigs were given food during two daily periods of 20 min each.

Collection of samples. Oropharyngeal fluid (OPF) samples were collected using swabs once daily from 1 day before to 10 days after primary infection and challenge, using five pigs per group. Swabs were extracted with 4 ml of EMEM supplemented with 4% foetal calf serum. The weight of the fluid collected was measured after centrifugation of the swab in a special container. Blood samples were collected once a week.

A number of organs were collected for virological and pathological examination from pigs that died or were killed.

Virological and serological examinations. Virus multiplication after primary infection and challenge was determined by titration of OPF samples and organ suspensions on secondary porcine kidney cells. From tissues collected at autopsy, a 10% (w/v) suspension was made in tissue culture medium. Plaques were confirmed as being caused by ADV by complement-independent neutralization with a specific ADV antiserum. Virus titres were expressed as log₁₀ p.f.u./g of sample.

Sera were tested for neutralizing antibody against the NIA-3 strain using secondary porcine kidney cells and a serum/virus incubation period of 24 h at 37 °C (Bitsch & Eskildsen, 1976). Titres are expressed as log₁₀ of the reciprocal of the highest serum dilution inhibiting the viral c.p.e. in 50% of the cell cultures. Antibodies directed against gI were detected in a blocking ELISA as described (van Oirschot *et al.*, 1988).

Clinical observations. Clinical observations were made as described (de Leeuw & van Oirschot, 1985) with some modifications. The pigs were observed for clinical signs twice daily and rectal temperatures were taken daily; the animals were weighed once a week. During the 3 weeks after primary infection and challenge, they were weighed three times a week. For each pig, the numbers of days of growth arrest, fever, respiratory and neurological signs, and virus shedding were determined. Pigs that died or were killed were not included. The growth arrest period was defined as the number of days needed to regain the

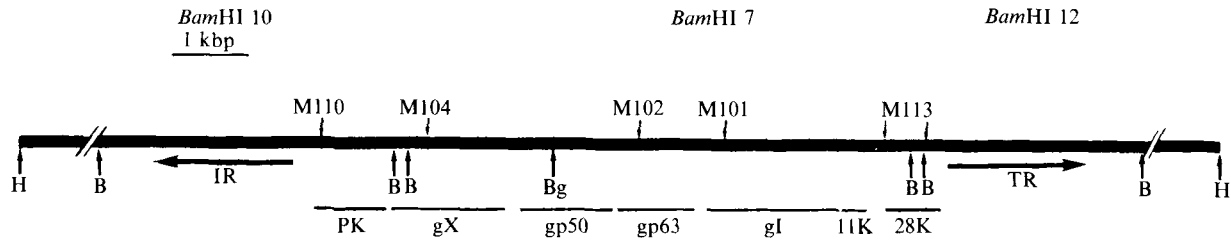


Fig. 1. Physical map of the U_S region of ADV NIA-3 enclosed by inverted repeats (IR and TR) (de Wind *et al.*, 1990; van Zijl *et al.*, 1988). Cleavage sites for *Bam*HI (B), *Hind*III (H) and *Bgl*II (Bg) are indicated. Oligonucleotide insertion sites in virus mutants are indicated by vertical arrows. In mutants M110 and M118 (not shown) the PK gene encodes a truncated protein of 44 amino acids (aa) or 98 aa, depending on the translational start site (van Zijl *et al.*, 1990). M110 has an additional deletion of approximately 100 bp in the variable *Bam*HI 6 region. In mutant M104, the gX gene encodes a truncated protein of 147 aa; in mutant M102 the gp63 gene encodes a protein of 141 aa, and in M101 the gI gene encodes a truncated protein of 105 aa. M113 has a deletion starting 16 bp upstream of the ATG start codon of the 28K gene extending to aa 209.

animal's weight on the day of challenge. Fever was defined as a rectal temperature >40 °C. Respiratory signs were nasal discharge, sneezing, coughing and forced respiration. Neurological signs were defined as itching, ataxia, paralysis, tremor and convulsions.

Pathological examinations. Tissues for histological examination were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 5 µm, and stained with haematoxylin and eosin. Viral antigen in tissues was detected by an indirect immunoperoxidase test using a specific polyclonal rabbit antiserum against ADV.

Experimental design. Table 1 shows the experimental design. In experiments 1 and 2, groups of 10-week-old pigs were infected intranasally with 10⁵ p.f.u. mutant virus or mock-infected with tissue culture medium. Virus suspension (0.5 ml) was slowly administered into each nostril during inspiration. Four days post-inoculation (p.i.), two pigs from each group were killed and examined for the presence of virus and lesions in tissues. Eight weeks after the first infection, the remaining pigs were challenged intranasally with 10⁵ p.f.u. of the seventh cell culture passage of the virulent ADV strain NIA-3. This challenge inoculum contains between 100 and 1000 median infective doses for pigs (de Leeuw & van Oirschot, 1985).

In experiments 3 and 4, groups of 3-week-old pigs were infected intranasally with 10⁵ p.f.u. of mutant virus. In experiment 4, a number of pigs were killed on days 4 and 7 p.i., and examined postmortem as above. Pigs that died spontaneously were also autopsied.

Results

Virulence of mutant strains in 10-week-old pigs

Pigs (10 weeks old) infected with M209 (ADV generated from four overlapping viral DNA fragments) developed clinical signs typical of Aujeszky's disease (fever, loss of appetite, weight loss and nasal discharge) from 2 days p.i. onwards. Some pigs developed neurological signs, such as tremors, ataxia and paralysis, and two pigs eventually died in both experiments.

Mutants M104 (gX⁻) and M113 (28K⁻) evoked similar clinical signs, including neurological signs and mortality, as M209. Thus, virulence was not decreased through inactivation of the genes encoding the gX or 28K

Table 1. *Experimental design*

Group (primary infection)	Age†	Number* of pigs	Challenge‡	Number of pigs challenged
Experiment 1§				
Controls	10	8	NIA-3	6
M102 (gp63)	10	9	NIA-3	7
M110 (PK)	10	9	NIA-3	7
M209	10	8	NIA-3	3
Experiment 2§				
Controls	10	8	NIA-3	6
M101 (gI)	10	9	NIA-3	7
M104 (gX)	10	9	NIA-3	4
M113 (28K)	10	9	ND	
M209	10	8	NIA-3	4
Experiment 3§				
M118 (PK)	3	5	ND	
M120	3	5	ND	
M209	3	5	ND	
Experiment 4§				
M101 (gI)	3	13	ND	
M209	3	5	ND	

* Number of pigs at the start of the experiment.

† Age of the pigs at the start of the experiment is given in weeks.

‡ Challenge infection was done 8 weeks after the primary infection.

§ In experiments 1 and 2, two pigs from each group were killed on day 4 p.i. In experiment 4, four M101- and two M209-inoculated pigs were killed on day 4 p.i.; on day 7 p.i., four M101- and three M209-inoculated pigs were killed.

|| ND, Not done.

proteins. Mortality was even higher and occurred sooner after inoculation with M113 than with M209.

Mutants M101 (gI⁻), M102 (gp63⁻) and M110 (PK⁻) had reduced virulence. Fever was lower and of shorter duration in pigs inoculated with these strains than in pigs inoculated with M209. Also, periods of growth arrest were shorter or absent. Some pigs inoculated with M102 or M110 showed some dullness, fever or reduced appetite. The group inoculated with M101 had the least

Table 2. Clinical signs and virus content of OPF from 10-week-old pigs inoculated intranasally with 10^5 p.f.u. of ADV mutant

Group (primary infection)	Mortality	MTD†	Mean \pm s.d. (days)*					Daily mean \pm s.d. virus excretion (log ₁₀ p.f.u./g OPF)
			Growth arrest	Fever	Respiratory signs	Neurological signs	Virus excretion	
Experiment 1								
Controls	0/6		0	0	0	0	0	0
M102 (gp63)	0/7		0.3 \pm 0.8 ¹	4.3 \pm 1.4 ⁴	0	0	6.2 \pm 1.6 ⁴	1.5 \pm 0.3 ⁴
M110 (PK)	0/7		1.0 \pm 2.6 ¹	5.3 \pm 0.7 ³	0.1 \pm 0.4	0	8.6 \pm 1.8	2.4 \pm 0.8 ²
M209	2/6‡	8.0	10.3 \pm 7.1	6.8 \pm 0.5	0.5 \pm 0.8	0.8 \pm 1.5	9.7 \pm 0.6	3.6 \pm 0.5
Experiment 2								
Controls	0/6		0	0	0	0	0	0
M101 (gI)	0/7		0	3.6 \pm 2.0 ³	0	0	9.8 \pm 1.1	3.1 \pm 0.5
M104 (gX)	3/7	9.3	10.0 \pm 1.2	4.3 \pm 1.3 ¹	1.0 \pm 0	1.3 \pm 1.0	7.0 \pm 1.0	2.5 \pm 0.2
M113 (28K)	5/7	7.4	11.0§	4.5 \pm 0.7	0.5 \pm 0.7	2.0§	11.0§	4.2 \pm 0.6
M209	2/6	10.0	11.3 \pm 2.1	6.5 \pm 0.6	1.3 \pm 0.5	1.3 \pm 0.9	8.7 \pm 2.6	3.0 \pm 0.6

* Statistical evaluation was done by Student's *t*-test in comparison with M209-inoculated pigs. The level of significance is indicated as ¹ $P < 0.05$; ² $P < 0.02$; ³ $P < 0.01$; ⁴ $P < 0.001$.

† MTD, Mean time to death (days after infection).

‡ One pig developed chronic signs (wasting) and was killed before challenge.

§ Excluding one pig that developed chronic neurological signs (ataxia).

symptoms, showing only fever, their appetite remaining normal and gaining weight at a rate similar to the uninoculated controls. Table 2 summarizes the clinical and virological findings.

Small areas of pneumonia were observed 4 days p.i. in pigs infected with M101, M113 and M209, and in pigs that died. Immunohistological examination revealed catarrhal bronchopneumonia in these areas, sometimes accompanied by viral antigen in alveolar airspaces. In the CNS, lesions were minimal or absent in pigs that were killed, but typical ADV lesions and antigen were seen in the CNS of pigs that died.

Virus content in OPFs and tissues of 10-week-old pigs

Pigs inoculated with M102 and M110 had lower virus titres in OPF than M209-inoculated pigs, whereas pigs inoculated with M101, M104 or M113 had similar or higher virus titres in OPF samples than M209-inoculated pigs (Table 2). There appeared to be no clear correlation between virus replication in the oropharynx and virulence.

Virus was recovered from a large number of tissues (CNS, respiratory tract, draining lymphoid tissues, spleen, liver, kidneys and adrenal glands) of inoculated pigs killed 4 days p.i., or of those which died spontaneously. Remarkably, in the pigs infected with M101 no virus was recovered from the CNS.

Virulence of mutant strains in 3-week-old pigs

To confirm and extend the observations on the contribution of gI and PK to the virulence of ADV, we did two

experiments on 3-week-old pigs. The reported susceptibility of pigs to ADV decreases with age. As M110 appeared to have a small deletion of approximately 100 bp in the variable *Bam*HI 6 region of the genome (such variation is also seen among plaque and field isolates of ADV), we used another PK mutant (M118) with the linker insertion at the same position in the PK gene as that in M110, and a *Bam*HI fragment pattern identical to that of the parent strain NIA-3. We also inoculated pigs with M120, which was generated from M118 by marker rescue.

In experiment 3, severe and typical signs of Aujeszky's disease were observed in M209-inoculated pigs, and four of five of these pigs died. The pigs had high fever from 2 days p.i. Some of the pigs vomited or sneezed, and they all showed neurological signs before they died. The surviving pig had fever between days 1 and 7 p.i. and also had convulsions, but recovered from 8 days p.i. Similar signs were observed in pigs inoculated with rescue mutant M120; of five M120-inoculated pigs, three died. The two pigs that recovered had fever between days 2 and 7 p.i., and they showed all the typical signs of Aujeszky's disease before they recovered. In contrast, pigs inoculated with PK mutant M118 remained healthy. These pigs had fever for 2 to 4 days (mean \pm s.d.: 2.8 \pm 0.8), but their appetite remained good and they did not lose weight.

Experiment 4 confirmed that gI is a marker of virulence. Pigs (3 weeks old) inoculated with 10^5 p.f.u. of mutant M101 showed only very mild signs. Of the five pigs that were not used for postmortem studies, three had fever for 1 or 2 days. M209-inoculated pigs developed severe and typical signs of Aujeszky's disease before they died or were killed.

Table 3. Virus content (p.f.u./g) in tissues of 3-week-old pigs that were inoculated intranasally with 10⁵ p.f.u. of ADV mutant

	Pigs inoculated with												
	M101								M209				
	Day 4 p.i.				Day 7 p.i.				Day 4 p.i.		Day 7 p.i.		
	1*	2	3	4	5	6	7	8	9	10	11	12	13
Nasal mucosa	3.9	3.0	-†	3.5	-	-	5.4	-	5.4	5.8	4.9	5.6	6.0
Pharyngeal mucosa	3.3	1.9	3.7	6.0	3.1	-	4.1	1.6	4.6	4.6	5.0	6.0	6.0
Tonsil	3.8	4.3	6.4	5.6	4.8	2.1	5.9	3.1	6.0	4.7	6.0	6.0	6.0
Subparotidial lymph node	5.3	-	3.1	3.9	-	-	-	-	6.0	3.2	4.1	5.6	5.5
Mandibular lymph node	5.4	3.5	3.4	4.2	-	-	1.6	-	6.0	4.1	4.9	6.0	5.9
Retropharyngeal lymph node	-	4.0	2.6	3.6	-	-	-	-	6.1	-	2.3	4.8	5.1
Lung	-	6.0	6.0	6.0	4.9	-	-	-	6.0	6.0	6.0	6.0	6.0
Liver	-	-	-	-	-	-	-	-	-	-	-	4.0	3.5
Kidney	-	-	-	-	-	-	-	-	2.3	-	4.2	3.1	4.1
Adrenal gland	2.4	-	-	-	-	-	-	-	5.8	-	5.9	5.9	6.0
Spleen	2.5	-	-	1.6	-	-	-	3.8	4.4	-	4.2	6.0	4.4
Trigeminal ganglion	3.8	3.4	2.1	2.7	-	-	-	-	5.4	4.8	4.7	6.0	5.9
Bulbus olfactorius	-	-	-	-	-	-	-	-	5.2	5.4	5.5	5.6	6.0
Pons cerebri	-	-	-	-	-	-	-	-	3.4	2.4	3.6	3.9	5.1
Brain stem	-	-	-	-	-	-	-	-	3.5	2.7	3.2	4.3	4.8
Medulla oblongata	-	-	-	-	-	-	-	-	4.2	2.5	3.2	4.3	4.6
Cerebellum	-	-	-	-	-	-	-	-	3.1	3.4	2.5	5.4	6.0

* Numbers refer to an individual pig.

† No virus recovered.

At autopsy on days 4 and 7 p.i., small areas of pneumonia were found in the ventral parts of the lungs of both M101- and M209-inoculated pigs; immunohistological examination revealed a catarrhal bronchopneumonia, and degeneration and inflammation of the nasal mucosa. Encephalitis, characterized by lymphocytic infiltrates, perivascular cuffing and the presence of viral antigen, was widespread in M209-inoculated pigs, but nearly absent in M101-inoculated pigs. Small lymphocytic infiltrates were observed locally in the brain stem and medulla oblongata, in only one of eight M101-inoculated pigs, but viral antigen could not be demonstrated in these areas.

Virus content of OPF and tissues of 3-week-old pigs

Pigs (3 weeks old) inoculated with PK mutant M118 excreted virus in OPFs between days 1 and 9 p.i.; the mean duration of virus excretion was 8 ± 1 days (mean \pm S.D.) and mean daily virus excretion (days 1 to 10 p.i.) was $\log_{10} 2.7 \pm 0.6$ p.f.u. M120- and M209-inoculated pigs that survived excreted virus in approximately the same amounts and for the same periods of time. M101-inoculated pigs excreted virus in OPFs from days 1 to 11 p.i.; the mean duration of virus excretion was 9.2 ± 1.3 days and the mean daily virus excretion (days 1 to 10 p.i.) was $\log_{10} 2.7 \pm 0.6$ p.f.u.

In the M120- and M209-inoculated pigs that died, virus was identified in the lungs, tonsils, nasal and

pharyngeal mucosa, kidney, trigeminal ganglion, bulbus olfactorius, brain stem, pons, cerebellum and medulla oblongata. The virus contents of the organs of M101- and M209-inoculated pigs (experiment 4) is given in Table 3. M101 was detected in the trigeminal ganglia on day 4, but not on day 7 p.i., and this strain was not detected in other parts of the CNS. However, M101 was recovered from most peripheral tissues, albeit at somewhat lower titres than M209. The findings suggest that gI mutants of ADV can enter neural cells in the nasal or pharyngeal mucosa, that they can be transported to the CNS by axonal transport and replicate locally in the trigeminal ganglion, but that they do not easily spread further into the CNS.

Immunogenicity of mutant strains

All mutant viruses induced a neutralizing antibody response that increased sharply until day 17 p.i. and then more slowly until the day of challenge (Fig. 2). However, the deletion and insertion mutants induced neutralizing antibody titres that were approximately one- to 1.5-fold lower than those induced by M209. All mutants, except the gI mutant M101, induced an antibody response to gI.

After challenge with the virulent strain NIA-3, a further increase in neutralizing antibody titre was seen in pigs immunized with M102 and M110, but not in pigs immunized with M101, M104 or M209. The lack of a further increase in neutralizing antibody titre correlated

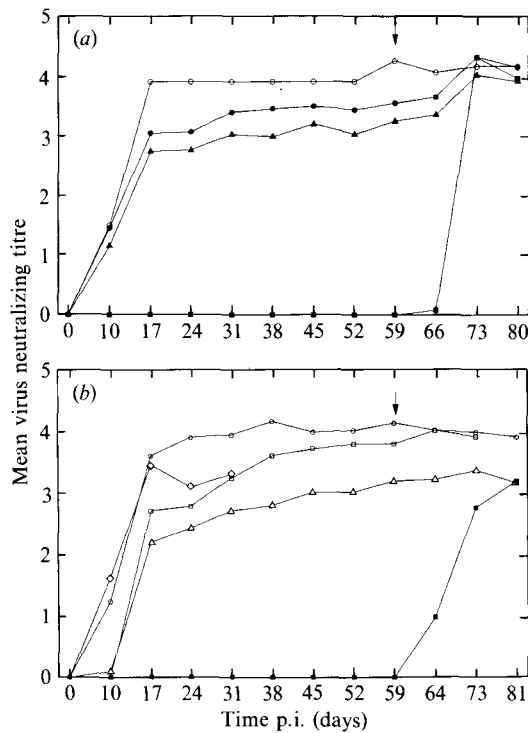


Fig. 2. Mean virus neutralizing antibody titres in sera of 10-week-old pigs infected intranasally with 10^5 p.f.u. of ADV mutant M102 (gp63) (●), M110 (PK) (▲) (experiment 1; a), M101 (gI) (△), M104 (gX) (□), M113 (28K protein) (◇) (experiment 2; b) or M209 (○) (experiments 1 and 2; a and b), or an uninfected control (■), and subsequently challenged 59 days after the primary infection (arrow) with 10^5 p.f.u. of virulent ADV strain NIA-3. The number of infected and challenged pigs is given in Table 1.

with a lack of virus excretion after challenge (see below) and apparently indicates complete immunity to challenge. M101-immunized pigs also failed to mount a gI antibody response after challenge.

Pigs immunized with M101, M102, M104, M110 or M209 did not show clinical signs after challenge. In contrast, pigs from the control groups exhibited severe and typical clinical signs (loss of appetite, growth retardation and fever), but did not die. A few of these pigs showed neurological signs.

No virus was detected in OPF samples from pigs immunized with M101, M104 (experiment 2) or M209 (experiments 1 and 2) after challenge. Reduced virus titres were found in pigs immunized with M102 or M110 (experiment 1). However, the results from the control groups indicate that the challenge infections did not have the same results in both experiments. Nevertheless, the high titres of neutralizing antibodies induced by M102 and M110 could not completely prevent virus replication and excretion. Table 4 summarizes clinical and virological findings after challenge.

Discussion

Major steps in the pathogenesis of Aujeszky's disease in pigs are virus multiplication in the oropharyngeal and nasal mucosa, uptake and transport of the virus by axons, and multiplication and spread of the virus in the CNS. In the CNS, the virus may cause neuronal necrosis and inflammation. The virus may also disseminate by

Table 4. Clinical signs and virus content of OPF from 18-week-old pigs after intranasal challenge infection with 10^5 p.f.u. of ADV strain NIA-3

Group (primary infection)	Mortality	Mean \pm s.d. (days)*					Daily mean \pm s.d. virus excretion (\log_{10} p.f.u./g OPF)
		Growth arrest	Fever	Respiratory signs	Neurological signs	Virus excretion	
Experiment 1							
Controls	0/6	17.3 \pm 2.3	5.3 \pm 0.8	0.3 \pm 0.8	0.5 \pm 0.8	10.0 \pm 0	4.4 \pm 0.2
M102 (gp63)	0/7	0	0.1 \pm 0.4 ⁴	0	0	4.0 \pm 2 ⁴	1.1 \pm 0.5 ⁴
M110 (PK)	0/7	0	0.1 \pm 0.4 ⁴	0.1 \pm 0.4	0	4.0 \pm 1.6 ⁴	1.3 \pm 0.5 ⁴
M209	0/3	0	0	0	0	0	0
Experiment 2							
Controls	0/6	16.3 \pm 1.5	5.8 \pm 1.2	1.2 \pm 0.4	0.7 \pm 1.0	5.6 \pm 0.5	2.2 \pm 0.4
M101 (gI)	0/7	1.0 \pm 2.6 ⁴	0	0	0	0	0
M104 (gX)	0/4	0	0	0	0	0	0
M209	0/4	0	0	0	0	0	0

* Statistical evaluation was done by Student's *t*-test in comparison with the non-vaccinated control pigs. The level of significance is indicated as ¹ $P < 0.05$; ² $P < 0.02$; ³ $P < 0.01$; ⁴ $P < 0.001$.

viraemia, but this route seems less important (Wittmann *et al.*, 1980). Virulence of ADV is multigenically controlled (Lomniczi *et al.*, 1984, 1987*a, b*), and the role of single genes in virulence and their functional activity are not well established. The results of this study indicate that PK, gp63 and gI determine the virulence of ADV for pigs, whereas 28K and gX appear to have no significant role. In other hosts, similar findings have been reported for gI and gX mutants of ADV, and for PK mutants of HSV (Thomsen *et al.*, 1987; Meignier *et al.*, 1988; Mettenleiter *et al.*, 1987, 1988*a, b*). The 28K and gX proteins are apparently dispensable for virus multiplication in cells *in vitro*, for virus multiplication in the oropharynx, for spread into and multiplication in the CNS, and for the expression of virulence.

Glycoprotein gX is made as a cell-associated precursor that is subsequently processed, presumably by proteolysis, and secreted extracellularly (Bennet *et al.*, 1986). The gene encoding gX has no homologue in the U_S region of HSV-1, but part of gX is homologous to glycoprotein gG of HSV-2 (McGeoch *et al.*, 1987; van Zijl *et al.*, 1990). No functions have been ascribed to either gG or gX.

Part of the 28K protein of ADV is homologous to the 32K protein encoded by the US2 gene of HSV-1 (McGeoch *et al.*, 1985; van Zijl *et al.*, 1990). No function has been ascribed to these proteins, or can be predicted from the sequence. Van Zijl *et al.* (1990) have demonstrated the expression of the 28K protein in cells infected with wild-type virus.

PK activity associated with ADV was demonstrated some years ago (for a review see Leader & Purves, 1988), but the PK gene has been identified, sequenced and its homology to the PK-encoding US3 gene of HSV-1 established only recently (Purves *et al.*, 1987; van Zijl *et al.*, 1990; Zhang *et al.*, 1990). The mechanism by which PK affects virulence is unknown. Although purified PK can phosphorylate a major viral protein *in vitro* which is also phosphorylated *in vivo* (Zhang *et al.*, 1990), the physiological substrate and function of PK are unknown. An HSV-1 mutant lacking US3 can still establish latency in mice (Meignier *et al.*, 1988). Because the PK mutant of ADV can be isolated from peripheral tissues, OPF and the CNS, PK apparently does not affect tissue tropism. It might influence virulence by enhancing virus multiplication in different cells. Both *in vivo* (OPF samples, this study) and *in vitro* (SK6 cells; de Wind *et al.*, 1990), PK-deficient mutants replicate less well than the parental strain. Interestingly, in nasal mucosa explant cultures, the PK mutant has an altered morphogenesis; budding through the outer nuclear membrane is delayed resulting in accumulations of enveloped virus in the perinuclear space (J.M.A. Poi, unpublished results). These findings suggest that a viral or cellular phosphoprotein has a role in budding of the virus at the nuclear membrane.

The significance of gI or the non-covalent gI-gp63 complex for expression of virulence of ADV has been well documented (Mettenleiter *et al.*, 1987, 1988*b*) and is confirmed in this study. Mutants lacking either gI or gp63 have some biological characteristics in common, such as growth advantage in primary chicken fibroblasts, decreased virus release from certain cells (a function shared with glycoprotein gIII; Schreurs *et al.*, 1988; Zsak *et al.*, 1989) and a decreased virulence for 1-day-old chickens after intracerebral inoculation (Zsak *et al.*, 1989; Zuckermann *et al.*, 1988). We found that inactivation of gI or gp63 alone strongly reduced virulence for pigs. Mettenleiter *et al.* (1987, 1988*a*), however, have reported that deletion of either gI, gp63 or gIII alone has only a slight effect on virulence for 1-day-old chickens, but deletion of gIII in combination with either gp63 or gI completely abolishes virulence in that model. In contrast to the homologous gE-gI complex of HSV-1 (Bell *et al.*, 1990; Dubin *et al.*, 1990), the gI-gp63 complex of ADV does not appear to have immunoglobulin Fc receptor activity (Zuckermann *et al.*, 1988). The significance of the gE-gI complex and Fc receptor activity in the expression of HSV-1 virulence is unknown. Although an Fc receptor could decrease the effects of antibody on infected cells, its reported absence from ADV and ADV-infected cells suggests that an Fc receptor is not of major importance for the expression of virulence of herpesviruses. Our results suggest that gI of ADV affects tissue tropism. The gI mutant could be recovered from the trigeminal ganglia but not from other parts of the CNS after intranasal application in 3-week-old pigs, and thus we hypothesize that gI facilitates the spread from neuron to neuron.

ADV mutants specifically lacking a single functional gene also allow the role of individual proteins in the induction of immunity to be established. Glycoproteins gIII and gp50 have been identified as prime targets for neutralizing antibodies, in contrast to gI, gX and gp63 (Ben-Porat *et al.*, 1986; Eloit *et al.*, 1988; Marchioli *et al.*, 1988; Zuckermann *et al.*, 1990). This study confirms that high levels of neutralizing antibody (induced by immunization with gp63 and PK mutants) could not completely prevent virus excretion after challenge with virulent ADV. However, pigs previously immunized with ADV or with the gI or gX mutants were completely protected against the challenge, as evidenced by the lack of clinical signs, the lack of virus excretion in OPF samples and the lack of a secondary neutralizing antibody response in the serum.

We thank the animal technicians for excellently taking care of the animals. Drs J. G. van Bakkum, R. J. M. Moormann and C. Terpstra were helpful with their comments on the manuscript. This work was partly supported by the Netherlands Technology Foundation, Duphar (a member of the Solvay Animal Health Group) and Intervet (subsidiary of AKZO).

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(Received 17 June 1991; Accepted 8 October 1991)