Role of envelope glycoproteins gl, gp63 and gIII in the invasion and spread of Aujeszky’s disease virus in the olfactory nervous pathway of the pig

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One-week-old pigs were infected intranasally with the Ka strain of Aujeszky’s disease virus (ADV) or with mutants that were lacking the non-essential envelope glycoproteins gl, gp63 or gIII. The invasion and spread of these strains in the olfactory nervous pathway were examined by assessing virus levels and by localizing viral antigens in the olfactory mucosa representing the first neuronal level, in the olfactory bulb representing the second neuronal level and in the lateral olfactory gyrus, the rostral perforated substance and the piriform lobe, all representing the third neuronal level. The Ka parental strain invaded and spread up to the third neuronal level. The extent of invasion and spread of the gII- mutant were similar to that of the parental strain. The gp63- mutant replicated normally in the olfactory mucosa, but its spread to all other levels was limited as compared with that of the parental strain. The gl- mutant showed a defect in infection at all neuronal levels. These results indicate that, of the non-essential envelope glycoproteins, gl plays the major role in neural invasion and spread of ADV in its natural host. The pattern of invasion and spread of these mutants in the olfactory pathway of pigs was similar to that previously observed in the trigeminal pathway. The type of nervous pathway therefore appears not to influence the neuropathogenesis of ADV or mutants deleted in non-essential envelope glycoproteins in the pig.

Introduction

Aujeszky’s disease virus (ADV) or suid herpesvirus 1, also designated pseudorabies virus, is an alphaherpesvirus that causes severe nervous disease in several domestic and wild animals. The pig is the natural host of the virus. Newborn pigs are most sensitive to virus invasion and to the development of neurological disorders (Kluge et al., 1992). Following oronasal uptake and replication, ADV invades the central nervous system (CNS) via the trigeminal, glossopharyngeal and/or olfactory nerves (Baskerville et al., 1973; McFerran & Dow, 1965; Sabo et al., 1968, 1969; Wittmann et al., 1980).

ADV envelope glycoproteins gl, gII, gIII, gp50, gp63 and gH are known to be involved in virus–host cell interactions such as adsorption, penetration, release and cell fusion (for reviews see Mettenleiter, 1991; Wittmann & Rziha, 1989). Glycoproteins gl, gIII and gp63 are dispensable for replication of the virus in cell culture and are designated ‘non-essential’ (Mettenleiter, 1991). Nevertheless, these non-essential envelope glycoproteins of ADV play a role in the neuropathogenesis of ADV in the natural host because their absence reduces or completely abolishes the development of neurological symptoms in 3- and 10-week-old pigs (Kimman et al., 1992; Mettenleiter et al., 1987b, 1989). Studies on the exact role of these non-essential envelope glycoproteins in neural invasion and spread of ADV in the pig have shown that a gl- mutant can reach peripheral neural tissues such as the trigeminal ganglion but not the CNS, i.e. brain stem or olfactory bulb (Jacobs et al., 1993; Kimman et al., 1992). The authors hypothesized that gl is required for the uptake of the virus into or spread through the CNS. In another study (Kritas et al., 1994), in which invasion and spread of mutants deleted in non-essential envelope glycoproteins were followed in the trigeminal nervous pathway, all mutants reached the CNS. However the spread of the gp63- mutant and particularly that of the gl- mutant were impaired. It was suggested that both gl and gp63 play a role in the viral spread in the trigeminal pathway, but that the role of gl is more important.
The olfactory bulb, which is part of the olfactory nervous pathway, is an important replication site for ADV in the CNS of pigs after its entrance through the olfactory region of the nasal mucosa (McFerran & Dow, 1965; Sabo et al., 1969; Wittmann et al., 1980). Replication of ADV occurs in the cells of the olfactory mucosa, in glial cells and neurons of the olfactory bulb (Narita et al., 1991; Pol et al., 1989; Sabo et al., 1969). However, the olfactory nervous pathway, as compared with other sensory nervous pathways, exhibits several peculiarities. First, the olfactory receptor cells which represent the first neuronal level of the olfactory pathway are the only known neurons that can be replaced in postnatal life (reviewed in Cormac, 1987). In addition, the olfactory receptor cells provide a direct connection between the brain and the external environment, a feature lacking in other neural pathways where extraneural tissues or synapses interrupt the route towards the CNS. Finally, the termini of the olfactory nervous pathway in the cerebral cortex, also known as the allocortex, have a different structure and phylogenetic development as compared with the termini of the other sensory neural pathways in the neocortex. These characteristics support the view that the olfactory nervous pathway is phylogenetically the oldest and most primitive (reviewed in Brodal, 1969). It is, therefore, possible that the cells of the olfactory pathway exhibit different features to those in other neural pathways and, as a consequence, the pattern of invasion and spread of ADV and of its mutants may be different. Although it has been suggested that the entry of ADV into the CNS via the olfactory route may be of a direct nature (Sabo et al., 1969), the pattern of spread of the virulent ADV via the olfactory pathway to the deeper tissues of the CNS is still unknown.

In this paper, the role of different non-essential envelope glycoproteins of ADV in neuroinvasion and neural spread in the olfactory pathway of pigs was investigated.

**Methods**

*Structure of the olfactory pathway.* A schematic diagram of the olfactory nervous pathway is presented in Fig. 1 and is based on information from several sources (Brodal, 1969; Jenkins, 1978;
The first neuronal level of this pathway consists of the olfactory receptor cells, which are located amongst the non-neuronal cells of the olfactory epithelium. The dendrites of the olfactory receptor cells extend on the olfactory bulb. The axons of the olfactory receptor cells or mentioned so far represent the second neuronal level of the olfactory pathway. The most important are the mitral and brush neurons. The axons of both the mitral and brush neurons leave the olfactory bulb and terminate in other structures of the brain amongst which are the LOG, RPS and PL. The LOG, RPS and PL each have a three-layer structure with an outer molecular layer (axons of the second order neurons), two layers contain neurons that represent the third neuronal level of the olfactory pathway (not shown in Fig. 1).

**Virus strains.** The wild-type Ka strain and its genetically engineered derivatives Ka gI-, Ka gp63- and Ka gII- were used. The Ka parental strain is of uncertain origin (Kaplan & Vatter, 1959) and is virulent in newborn pigs.

The construction of the Ka gI-, Ka gp63- and Ka gII- mutants is described in detail elsewhere (Mettenleiter et al., 1987a, 1988). Briefly, for the construction of the gI mutant, the DNA was cleaved with BstElI and Sphi in order to delete a large part of gI. For the construction of the gp63- mutant, a large part of the gp63 gene was deleted after cleavage with Stul and NcoI. The gII- mutant was constructed by deleting a 1.45 kbp XhoI-XhoI fragment that includes most of the regulatory and coding sequences of the gII gene. All the strains were passaged twice in swine testicle (ST) cells before use.

**Pig inoculation and collection of samples.** One-week-old pigs that did not have antibodies against ADV were inoculated with the ADV strains. Four pigs were used for the examination of each strain apart from when three pigs were used for the study of the Ka gII- mutant. Inoculations were performed intranasally by means of a catheter which was inserted into the nostrils until it reached the ethmoidal labyrinth while the pig was laid on its back. A total of 1 ml virus suspension containing 10^7 TCID50 was slowly administered, 0.5 ml being given in each nostril. The pig was kept in the same position for 5 min to ensure contact of the inoculum with the olfactory epithelium. The pigs were observed three to four times daily for the appearance of CNS symptoms. Pigs were killed at 2 and 5 days post-inoculation (p.i.) as indicated in Table 1. One pig inoculated with the Ka strain and another inoculated with the Ka gII- mutant were killed at 4 days p.i. at a moribund stage.

The following samples were collected from the left side of the body and processed for virus quantification: olfactory mucosa, olfactory bulb, LOG, RPS and PL. From several pigs, the same samples (except RPS) were collected from the right side of the body for immunohistochemical examination.

**Virus quantification.** Suspensions (20%) were made from tissues in PBS. The suspensions were centrifuged at 3000 r.p.m. for 20 min at 4°C. Subsequently, 0.1 ml of supernatant was inoculated on each of four tubes of confluent ST cells. The cell cultures were observed for 5 days for c.p.e. To measure virus levels 10-fold dilutions of the original suspensions were titrated on the cells and the endpoints were calculated by the Reed-Muench method.

**Immunohistochemical (IHC) examination.** For the IHC tissue samples were fixed in 50% alcohol for 5 days and, subsequently, embedded in paraffin. From every tissue serial sections of 6 μm were cut at equally distributed sites so that a tissue depth of 1 cm had been covered. The sections then had the paraffin extracted before being incubated in 0.5% H2O2 in methanol for 20 min to block endogenous peroxidase activity, washed in Tris-buffered saline (TBS) pH 7.6, and subsequently incubated for 30 min at 37°C with goat normal serum covered. The sections then had the paraffin extracted before being incubated in 0.5% H2O2 in methanol for 20 min to block endogenous peroxidase activity, washed in Tris-buffered saline (TBS) pH 7.6, and subsequently incubated for 30 min at 37°C with goat normal serum covered. The sections then had the paraffin extracted before being incubated in 0.5% H2O2 in methanol for 20 min to block endogenous peroxidase activity, washed in Tris-buffered saline (TBS) pH 7.6, and subsequently incubated for 30 min at 37°C with goat normal serum covered.

The sections were counterstained with haematoxylin, dehydrated through alcohol, cleared in xylene, mounted in DPX (Fluka) and examined by microscopy.

The primary antibody was produced as follows. One 10-week-old pig that did not have antibodies against ADV was infected with the ADV strains. Four pigs were used for the examination of each strain apart from when three pigs were used for the study of the Ka gII- mutant. Inoculations were performed intranasally by means of a catheter which was inserted into the nostrils until it reached the ethmoidal labyrinth while the pig was laid on its back. A total of 1 ml of the virus suspension containing 10^7 TCID50 was slowly administered, 0.5 ml being given in each nostril. The pig was kept in the same position for 5 min to ensure contact of the inoculum with the olfactory epithelium. The pigs were observed three to four times daily for the appearance of CNS symptoms. Pigs were killed at 2 and 5 days post-inoculation (p.i.) as indicated in Table 1. One pig inoculated with the Ka strain and another inoculated with the Ka gII- mutant were killed at 4 days p.i. at a moribund stage.

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**Results**

**Clinical signs observed with different ADV strains**

The pigs inoculated with the parental Ka strain showed anorexia, somnolence, poor balanced stepping, vertigo and vomiting around 2-3 days p.i. Six to 12 h later, the pigs developed typical neurological signs such as sitting on the hind legs (‘dog sitting’), seeking support from the
levels. The Ka gIII- mutant reached the third neuronal level in only one pig. There was only a slight difference in titres compared with the parental strain in the olfactory mucosa but titres with the gI- mutant were markedly lower at the second and third neuronal levels.

**Antigen localization and quantification by IHC**

(i) Olfactory mucosa (non-neuronal cells and first neuronal level)

Results concerning the degree and site of infection in the olfactory mucosa are presented in Table 2. At 2 days after inoculation with the Ka parental strain, small foci of infection containing both non-neuronal and olfactory receptor cells were observed in the epithelium. Desquamated epithelial cells were observed on the surface of the epithelium. In the lamina propria, several small foci of infection, which were often continuous with the foci in the epithelium, were found. Nerve bundles in the lamina propria were also infected. The Ka gIII- mutant infected the olfactory mucosa to an even higher degree than the parental strain. The pig inoculated with the Ka gp63- mutant and one pig inoculated with the Ka gI- mutant had an infection pattern similar to that of the parental strain, whereas infection was less extensive in the other gI- inoculated pig.

At 5 days p.i., approximately 50% of the epithelium had been destroyed after infection with the Ka parental strain. The foci of infected cells in the remaining epithelium and in the lamina propria were mainly large, sometimes extending to the bone or to the cartilage. Many infected nerve bundles were observed (see Table 2). The Ka gIII- and Ka gp63- mutants replicated to the same extent as the parental strain but fewer foci of infected cells were observed after infection with the Ka gI- strain.

(ii) Olfactory bulb (transition from the first to the second neuronal level)

At 2 days p.i., the Ka strain had formed more than 200 medium sized (0.25 to 0.5 mm²) foci of infection in the filament layer. Many of these foci extended through the entire diameter of this layer (Fig. 2a). Glomeruli were also infected. A similar degree of infection was found with the gIII- mutant whereas infection with the gp63- mutant was less extensive. However, the pattern of infection with both mutants was similar to that of the

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**Table 2. Degree of infection in the olfactory mucosa with the Ka strain of ADV and its deletion mutants in neonatal pigs**

<table>
<thead>
<tr>
<th>Pig no.</th>
<th>Strain inoculated</th>
<th>Killed (day p.i.)</th>
<th>S1</th>
<th>L</th>
<th>S</th>
<th>L</th>
<th>No. of infected nerve bundles†</th>
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<td>14</td>
<td>3</td>
<td>15</td>
<td>5</td>
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* Evaluation was performed in 10 sections.
† (-) not infected, (+) less than 10 nerve bundles infected, (+ +) 10 to 30 nerve bundles infected, (+ + +) 30 to 60 nerve bundles infected, (+ + + +) 60 to 100 nerve bundles infected, (+ + + + +) more than 100 nerve bundles infected.
‡ Small foci < 0.5 mm, large foci > 0.5 mm.
Mutants of ADV in the pig nervous system

Fig. 2. Photomicrograph of the filaments (f) and glomeruli (g) of the olfactory bulb stained by the immunoperoxidase method. At 2 days p.i., the Ka parental strain (a) had infected the filament layer through its entire diameter reaching up to the glomeruli, in contrast to the Ka gI\(^{-}\) mutant (b) which had infected only the superficial areas of the filament layer. Bars represent 80 \(\mu\)m.

### Table 3. Degree and localization of infection with the Ka strain of ADV and its deletion mutants in tissues of the olfactory pathway

<table>
<thead>
<tr>
<th>Pig no.</th>
<th>Strain of ADV</th>
<th>Killed (day p.i.)</th>
<th>Brush (2) No. of cells</th>
<th>Mitral (2) No. of cells</th>
<th>Inner granular (2) No. of foci</th>
<th>LOG Pyramidal (3) No. of cells</th>
<th>PL Pyramidal (3) No. of cells</th>
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<td>6</td>
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* Evaluation was performed in 15, 10 and five sections of olfactory bulb, LOG and PL respectively.
+ Small < 0.25 mm\(^2\), medium 0.25 to 0.5 mm\(^2\) and large > 0.5 mm\(^2\).
† NT, Not tested.

Parental strain. The Ka gI\(^{-}\) mutant had formed less than 20 small foci (< 0.25 mm\(^2\)) of infection in the filament layer and, in contrast to the other strains, these foci were found only in the superficial areas of this layer (Fig. 2b). Glomeruli were not infected by this mutant.

At 5 days p.i., the Ka strain had formed large foci of infection in the filament and glomerular layers. Infection with the Ka gIII\(^{-}\) and the Ka gp63\(^{-}\) mutants was similar and less extensive, respectively. The Ka gI\(^{-}\) mutant formed only five small foci in the filament layer and infected one glomerulus in each pig.

(iii) Olfactory bulb (second neuronal level)

The degree and localization of the infection at the second neuronal level in the olfactory bulb are presented in Table 3. At 2 days p.i., the Ka strain had infected several brush, mitral and inner granular neurons mostly
occurring within single cells. The Ka gIII− and the gp63− mutants also infected all the layers of the olfactory bulb to a larger and lesser extent than the Ka strain, respectively. However, the pattern of infection with the Ka gIII− mutant in the internal granular layer was different in so far as viral antigen was mainly located in

foci of cells. The Ka gl− mutant did not infect cells of the second neuronal level.

At 5 days p.i., the Ka strain had infected numerous brush, mitral and inner granular neurons in a focal manner (Fig. 3a). Compared with the parental strain, the Ka gIII− mutant had infected the second neuronal level to a higher degree whereas infection with the Ka gp63− mutant (Fig. 3b) was markedly lower since 50-fold fewer
brush-mitral neurons were infected and fewer foci were formed in the internal granular layer. The form of infection with both the gIII− and the gp63− mutants was however similar to that of the parental strain. The Ka gI− mutant did not infect brush-mitral cells, whereas a single small focus was observed in the internal granular layer in each pig (Fig. 3c).

(iv) LOG and PL (third neuronal level)

The degree and localization of infection occurring in the LOG and PL are presented in Table 3. At 2 days p.i., a few scattered pyramidal neurons of the LOG were infected with only the Ka and the Ka gIII− strains. At 5 days p.i., the Ka parental strain and the Ka gIII− mutant had infected large groups or foci of pyramidal neurons in the LOG and PL (Fig. 4a and b). Several scattered neurons were also infected in the polymorph layer of the LOG and PL but the molecular layer was rarely infected. Compared with the parental strain, the severity of infection with the Ka gp63− mutant was much lower, despite the similar pattern (Fig. 4c). Viral antigens were not observed with pigs infected with the Ka gI− mutant either in the LOG or in PL.

Discussion

In a previous study, the invasion and spread of the Ka strain and its non-essential envelope glycoproteins deletion mutants were examined in the trigeminal pathway of neonatal pigs after nasal inoculation (Kritas et al., 1994). In that study, it was shown that gI plays an important role in the neural invasion and spread of ADV whereas gp63 is less important than gI, and gIII does not influence the viral neuropathogenesis.

The olfactory pathway is another important nervous pathway in the pathogenesis of ADV since the virus has frequently been isolated from the olfactory bulb. As previously mentioned, this pathway exhibits several primitive characteristics compared to other neural pathways so that the cells of the olfactory pathway may behave differently with regard to the neuropathogenesis of ADV. Therefore, the invasion and spread of ADV and mutants deleted in particular non-essential envelope glycoproteins was followed in this pathway. The results obtained with the parental Ka strain in the olfactory mucosa are in agreement with those of previous studies (Narita et al., 1991; Pol et al., 1989; Sabo et al., 1969). All types of mucosal cells were infected. In addition, it was shown that all the types of neurons that constitute the second neuronal level (brush, mitral and inner granular neurons of the olfactory bulb) or the third neuronal level (pyramidal and polymorph neurons of the LOG and PL) were infected to a high degree. The pattern of viral spread at the early stages of the infection was mainly characterized by infection of individual neurons whereas neighbouring cells were found not to have viral antigens. This indicates that the main transport of ADV in the olfactory pathway occurs from neuron to neuron and that the virus subsequently spreads to the surrounding cells. In the trigeminal pathway, the Ka strain had also infected all types of neurons (Kritas et al., 1994). The only difference was that virus titres in the neuronal levels of the trigeminal pathway were somewhat lower (0.5 to 2 log10 TCID50), compared to the respective neuronal levels of the olfactory pathway. This difference may be attributed to the direct accessibility of the CNS via the olfactory nerves.

As with the Ka parental strain, the titres of the mutants in the olfactory pathway were somewhat higher than those observed in the trigeminal nervous pathway (Kritas et al., 1994). These higher titres may also be explained by the direct accessibility of the CNS via the olfactory nerves.

As shown by IHC, the Ka gIII− mutant infected all types of neuronal cells in the olfactory nervous pathway to almost the same degree as the parental strain. However, the titres of the Ka gIII− mutant were lower at all neuronal levels compared to those of the parental strain. It is known that gIII− mutants when inoculated in cell culture show a defect in adsorption and hence are detected at lower titres as compared to other strains (Mettenleiter et al., 1988; Schreurs et al., 1988; Whealy et al., 1988). Therefore, the lower titres observed with the gIII− mutant are probably not due to lower virus production in the neural tissues, but reflect the less efficient infection in cell cultures. In the trigeminal nervous pathway, the Ka gIII− mutant had also shown a spread essentially similar to that of the parental strain (Kritas et al., 1994). We conclude that absence of gIII does not appear significantly to alter neuroinvasion and neural spread of ADV in its natural host.

Kimman et al. (1992) and Jacobs et al. (1993) were not able to isolate a gI− mutant from the olfactory bulb of 3-week-old pigs. The authors hypothesized that the gI− mutant either failed to recognize specific receptors on the olfactory receptor cells or that the spread of the mutant from neuron to neuron was blocked. In the present study, it was found that the Ka gI− mutant infected and replicated in the olfactory receptor cells and that, at late stages of infection, it was able to spread from the olfactory receptor cells to the inner granular neurons. The finding that, at the early stages of infection, the Ka gI− mutant, in contrast to the other strains, had infected only superficial areas of the filament layer and had yet to reach the synapses between the first and second neuronal level indicates that a delay in spread of the Ka gI− mutant occurs at the first neuronal level. A possible explanation for this delay could be that after replication in the nerve
bodies of the olfactory receptor cells, virus is not transported efficiently towards the olfactory bulb. Neuronal transport of herpesviruses has been analysed in detail in mice, rats or human neuronal cultures infected with herpes simplex virus type 1 (HSV-1). The axonal transport of HSV-1 occurs in two directions: transport of capsids towards the nerve cell body, the so-called retrograde transport, and transport of newly formed enveloped particles away from the nerve cell body, the so-called anterograde transport (Lycke et al., 1984, 1988). HSV-1 appears to travel retrogradely in the nerves in a non-infectious form and, after replication in the nerve cell bodies, virus is anterogradely transported in an infectious form (Klein & DeStefano, 1983). The in vivo sequence of events during the neuronal transport of ADV can be easily followed in the maxillary nerve which connects the nasal mucosa with the trigeminal ganglion since the neuronal extensions in this nerve are long and clearly distinct from the nerve cell bodies of the trigeminal ganglion. It was observed that although, at 20 h after nasal inoculation, the Ka strain was not isolated from the peripheral parts of the maxillary nerve, it was isolated from the trigeminal ganglion and from the part of the maxillary nerve close to the ganglion. At 2 and 5 days p.i., the Ka strain was isolated not only from the trigeminal ganglion but also from all parts of the maxillary nerve. The Ka gl mutant was isolated from the trigeminal ganglion at both 2 and 5 days p.i. but, in contrast to the parental strain, not from the maxillary nerve (S. K. Kritas & M. B. Pensaert, unpublished). These findings indicate that both the parental and the gl strains can be efficiently transported in a non-infectious form (capsids) towards the trigeminal ganglion and that both are able to replicate in the nerve cell bodies. However, only the infectious newly formed enveloped particles of the parental strain appear to be efficiently transported in an anterograde manner towards the periphery. This defect in anterograde transport may also be responsible for the delay in spread of the Ka gl mutant along the olfactory pathway. There, the nerve cell bodies of the olfactory receptor cells are located in the mucosa so that locally produced ADV can only reach the olfactory bulb by anterograde transport through the axons of these cells (or olfactory nerves or filaments). Once this transport is impaired, neuroinvasion is reduced. Whealy et al. (1993) also proposed that gl of ADV may play a role in the anterograde axonal transport in the visual system of the rat.

As demonstrated by both virus titration and IHC, the Ka gp63- mutant exhibited a marked defect in invasion and spread in the tissues of the olfactory pathway located in the brain although its impairment was not nearly as dramatic as that of the Ka gl- mutant. The nature of this defect was not clear from the present results and so is now under further investigation. In the maxillary nerve, however, anterograde transport of the Ka gp63- mutant was found to be impaired, but to a lesser degree than that of the Ka gl- mutant (S. Kritas & M. Pensaert, unpublished).

Previous studies have shown that gl- and gp63- mutants exhibit similar characteristics in virus replication in cell cultures, virulence in chickens and neural spread in rats (Whealy et al., 1993; Zuckermann et al., 1988). This indicated that gl and gp63 exert similar functions and it has subsequently been shown that gl and gp63 form a physical glycoprotein complex (Whealy et al., 1993; Zuckermann et al., 1988). This complex was suggested to represent the functional entity. However, the different phenotypes of the Ka gl- and gp63- mutants observed in this present study indicates that gl has functions in addition to those of gp63 or the gl/gp63 complex. We conclude that gp63 plays an important role in the invasion and spread of ADV in the olfactory pathway of the pig but that this role is not as important as that of gl.

Based on their capacity to invade and spread in the nervous system, the behaviour of ADV strains devoid of non-essential envelope glycoproteins was compatible in the olfactory and trigeminal nervous pathways. The Ka gIII- mutant exhibited a spread similar to that of the parental strain, whereas the Ka gp63- mutant and particularly the Ka gl- mutant were defective in both neural invasion and spread. It appears, therefore, that differences in these two pathways do not affect the pattern of spread of the virus and its mutants deleted in non-essential envelope glycoproteins. In mice, a gl- mutant was able to invade and spread in the trigeminal but not in the olfactory pathway (Kovfics & Mettenleiter, 1991). These findings suggest that viral neuro-pathogenesis in animal species that are not the natural host may be different and that extrapolation of results from non-porcine to porcine hosts after ADV infection may be misleading.

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References


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