Glycoprotein gE-negative pseudorabies virus has a reduced capability to infect second- and third-order neurons of the olfactory and trigeminal routes in the porcine central nervous system

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We investigated the spread of glycoprotein gE (gE)-negative pseudorabies virus (PRV) and its rescued ‘wild-type’ strain into and within the central nervous system (CNS) of 3- and 10-week-old pigs. This is the first study that demonstrates PRV invasion of the porcine CNS via the synaptically linked neurons of the olfactory and trigeminal routes and that demonstrates the role of gE in this invasion. After intranasal inoculation with high doses of virus, gE-negative PRV replicated less efficiently in peripheral tissues. The titres of the gE-negative virus in the oropharyngeal mucosa, olfactory epithelium, draining lymph nodes and trigeminal ganglion were approximately 100-fold lower in 3-week-old pigs and 10-fold lower in 10-week-old pigs than titres of the ‘wild-type’ virus. In contrast to the ‘wild-type’ virus, titres of the gE-negative virus were very low or undetectable in the olfactory bulb, brain stem and other tissues of the CNS. Viral antigen of rescued ‘wild-type’ PRV and of gE-negative PRV was detected immunohistochemically in the olfactory epithelium and in neurons of the trigeminal ganglion, and also in the olfactory and trigeminal axons leading towards the CNS. But, in contrast to ‘wild-type’ virus, no viral antigen of the gE-negative virus was detected in second- or third-order neurons in the olfactory bulb or in the brain stem. We conclude that gE-negative PRV can infect first-order neurons of the olfactory and trigeminal routes and is able to spread via their axons towards the CNS. Yet, gE-negative PRV has a greatly reduced capacity to infect second- or third-order neurons. Finally, we report lateral spread of ‘wild-type’ PRV in the trigeminal ganglion, i.e. nonsynaptic transport from neuron to neuron. Possible mechanisms that could explain the reduced levels of the gE-negative virus in the CNS are discussed.

Introduction

Pseudorabies virus (PRV) is a highly neurotropic alphaherpesvirus that causes Aujeszky’s disease in domestic and wild animals (Gufstafson, 1986; Pensaert & Kluge, 1989). Pigs are relatively resistant to PRV and are the natural host of the virus. The natural port of entry is the nasopharyngeal region. The virus is able to replicate in cells of the nasal and pharyngeal mucosa (Pol et al., 1989). After infection of neural cells at peripheral sites, the virus is transported to the central nervous system (CNS), where it causes a severe encephalitis that is often fatal in young pigs (Baskerville et al., 1973; Dow & McFerran, 1962). Older pigs usually survive the infection, but they may develop fever and pneumonia. Infection of the CNS generally results in the establishment of latency (Rziha et al., 1986).

In several rodent models the entry into the brain of PRV and herpes simplex virus (HSV) has been described. Invasion of the CNS of rats occurs in an ordered fashion in which the virus is transmitted through synaptically linked neurons (Strack & Loewy, 1990; Card et al., 1990). Herpesviruses have thus been used as transneuronal markers to study the neuroanatomical organization of functionally related neuronal circuits (reviewed in Kuypers & Ugolini, 1990).

Glycoprotein E (gE), previously called glycoprotein I (gI), is one of at least nine glycoproteins of PRV (Mettenleiter, 1991; Peeters et al., 1992). (In this paper the herpesvirus protein nomenclature is used as agreed during the 18th International Herpes Workshop held in July 1993 in Pittsburgh, Pa., U.S.A. Hence pseudorabies
virus (PRV) glycoproteins are named as their homologues of herpes simplex virus type 1. PRV glycoprotein gE was previously called gI, and glycoprotein gI was previously called gp63.) It is important for expression of virulence of PRV (Lomniczi et al., 1987; Mettenleiter et al., 1987a; Kimman et al., 1992; Jacobs et al., 1993a, b), but gE is not required for the virus to grow in vitro or in vivo. In vitro, gE is involved in cell fusion and promotes the cell-to-cell spread of the virus (Zsak et al., 1992). Moreover, in some cell types gE promotes the release of PRV (Mettenleiter et al., 1987b; Zsak et al., 1989; L. Jacobs and others, unpublished results). PRV glycoprotein gI, previously called glycoprotein gp63, forms along with gE a non-covalently linked complex (Zuckermann et al., 1988).

Previously, we demonstrated that 4 to 7 days after intranasal inoculation with PRV gE-negative mutants no virus could be recovered from the CNS, suggesting that the lack of gE affected the virulence of PRV by blocking the spread through or the replication of the virus in the porcine CNS (Kimman et al., 1992; Jacobs et al., 1993b). In these studies the olfactory route was not examined and no detailed histology was done. The purpose of the present work was to describe the entry of PRV into the porcine CNS by the olfactory and trigeminal routes, and to investigate possible mechanisms which reduce the capacity of gE-negative PRV to enter or to replicate in the brain. We investigated the transneuronal spread of the gE-negative virus and its rescued ‘wild-type’ at several time points, shortly after inoculation with high doses of virus. This is the first report that demonstrates the invasion by PRV of the porcine CNS via the synaptically linked neurons of both the olfactory and the trigeminal routes. We showed that the capacity of gE-negative PRV to enter or to replicate in the porcine CNS was strikingly reduced. No viral antigen was detected in the second- or third-order neurons of both olfactory and trigeminal routes. Compared to the rescued ‘wild-type’ PRV, no or only minute quantities of the gE-negative virus were isolated from various tissues of the CNS.

Methods

Cell cultures and virus titration. The swine kidney cell line SK6 (Kasza et al., 1971) was cultivated in Dulbecco’s minimal essential medium (DMEM) supplemented with 5% fetal calf serum, t-glutamine (0.3 mg/ml), and the antibiotics penicillin (90 U/ml), streptomycin (100 µg/ml) and fungizone (4.5 µg). For quantification, virus was plaque-titrated on SK6 cells. To determine the virus content of tissues, a 10% (w/v) tissue suspension was made in DMEM supplemented with 2% fetal calf serum, the antibiotics mentioned above, and the antibiotics kanamycin (100 µg/ml) and polymyxin (50 µg/ml). Virus was allowed to adsorb for 1 h at 37 °C before the cells were overlaid with 1% methylcellulose in DMEM supplemented with 5% fetal calf serum and antibiotics. About 64 h after infection, the overlay was removed and the cells were stained with a solution containing 0.1% amido black, 6% acetic acid, 0.8% sodium acetate and 8.5% glycerol. Plaques were counted and the virus titres were expressed as log_{10} plaque-forming units (p.f.u.)/g of tissue sample.

Virus strains. The NIA-3 strain of PRV (McFerran & Dow, 1975) was used as parent strain for the development of the gE mutant virus and its rescued ‘wild-type’. The construction of the virus strains has been described previously (Jacobs et al., 1993a). Briefly, a gE-negative virus strain (M301) was constructed by introducing ten nucleotides at amino acid position 124, which resulted in a frame shift. To rescue the mutation of M301, the DraI/BamHI fragment of 2400 bp containing the gE coding sequence and DNA of strain M301 were co-transfected into SK6 cells. Recombinant viruses with an intact gE gene were detected using MAb directed against gE. One rescued strain with intact gE, designated M307, was plaque purified three times and used as ‘wild-type’ PRV in the experiments.

Animals. Dutch Landrace pigs (3-week-old, experiment 1; 10-week-old, experiment 2) were obtained from the specific pathogen-free herd of the Institute for Animal Science and Health. The pigs were born from unvaccinated sows and had no antibodies against PRV before the start of the experiments. Pigs of mixed sex from different litters were randomly assigned to experimental groups. During the experiments, each group was housed in a separate isolation room and had continuous access to food.

Experimental design. In the first experiment, twelve 3-week-old pigs were inoculated intranasally with 10^7 p.f.u. of the gE-negative mutant (M301) and twelve pigs were inoculated with 10^7 p.f.u. of the rescued ‘wild-type’ virus (M307). Two 3-week-old pigs were mock-infected with virus-free medium. The virus suspension (0.5 ml) was slowly administered into each nostril during inhalation. On days 1, 2, 3, and 4 after inoculation, three pigs from each group were killed and various tissues were examined for the presence of viral antigen and virus content. The mock-infected pigs were killed on days 1 and 6 after inoculation.

In the second experiment, 10-week-old pigs were inoculated as described by Pol et al. (1989) with high doses of virus in order to infect as many first-order neurons as possible in the oropharyngeal region. Briefly, six 10-week-old pigs were sedated for at least 1 h by injection with Stresnil and Hypnomil (Jansen) before inoculation. Virus suspension (2.5 ml, 10^7 p.f.u./ml) was instilled dropwise into each nostril. The head was held in a horizontal position with the pigs positioned on their backs for at least 1 h. Three pigs were inoculated with the gE mutant (M301), and three pigs were inoculated with the rescued ‘wild-type’ PRV (M307). Pig tissues were examined on day 2 after inoculation for the presence of viral antigen and virus content.

Clinical signs of disease. The pigs were observed once a day (experiment 1) or twice a day (experiment 2) for clinical signs. Rectal temperatures were measured once daily. Clinical signs of Aujeszky’s disease were monitored as described by Kimman et al. (1992). Briefly, fever was defined as a rectal temperature above 40 °C. Respiratory signs were defined as nasal discharge, sneezing, coughing and forced respiration. Neurological signs were defined as itching, ataxia, vomiting, paralysis, tremor and convulsions.

Immunohistochemical investigations. Tissue samples for histological examination were fixed in 4% buffered formalin (pH 7.4), dehydrated with alcohol and acetone, embedded in paraffin, and cut into 6 µm sections. Viral antigen in sections was detected by indirect immunohistochemical staining (Pol et al., 1991) using a polyclonal rabbit serum directed against PRV. Specific binding was detected by a biotin labelled goat anti-rabbit serum and a streptavidin–peroxidase serum. Sections were counterstained with haematoxylin for 10 s. Histological examinations of neural tissues of all inoculated pigs included selection of
Table 1. Virus titres in tissues of 3-week-old pigs inoculated with 10^7 p.f.u. of gE-negative PRV or with ‘wild-type’ PRV

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Titre [log_{10} (p.f.u./g)]* of M301 (gE-negative PRV) at day:</th>
<th>Titre [log_{10} p.f.u./g]* of M307 (‘wild-type’ PRV) at day:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Nasal mucosa</td>
<td>2.6 (1.5)</td>
<td>3.8 (1.0)</td>
</tr>
<tr>
<td>Pharyngeal mucosa</td>
<td>3.5 (0.9)</td>
<td>3.0 (1.9)</td>
</tr>
<tr>
<td>Olfactory epithelium</td>
<td>1.2 (1.1)</td>
<td>2.4 (2.4)</td>
</tr>
<tr>
<td>Tonsil</td>
<td>4.5 (2.5)</td>
<td>4.2 (2.1)</td>
</tr>
<tr>
<td>Soft palate</td>
<td>24.1 (7.4)</td>
<td>11.1 (1.2)</td>
</tr>
<tr>
<td>Mandibular lymph node</td>
<td>0.3 (0.6)</td>
<td>2.5 (0.3)</td>
</tr>
<tr>
<td>Subparotideal lymph node</td>
<td>0.1 (1.3)</td>
<td>1.5 (0.3)</td>
</tr>
<tr>
<td>Trigeminal ganglion</td>
<td>2.2 (0.3)</td>
<td>3.1 (0.8)</td>
</tr>
<tr>
<td>Olfactory bulb</td>
<td>0.0 (0.9)</td>
<td>1.5 (0.9)</td>
</tr>
<tr>
<td>Brain stem</td>
<td>0.0 (0.9)</td>
<td>0.2 (0.3)</td>
</tr>
<tr>
<td>Medulla oblongata</td>
<td>0.0 (0.3)</td>
<td>0.3 (0.5)</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0.0 (0.3)</td>
<td>0.2 (0.3)</td>
</tr>
</tbody>
</table>

* Virus titres are the geometric mean values for three pigs, ± S.D. (expressed as log_{10}).

Results

Virulence of gE-negative and rescued ‘wild-type’ PRV

In experiments 1 and 2, both the gE-negative and the rescued ‘wild-type’ virus strains caused fever. From day 2 after inoculation with the ‘wild-type’ virus onwards all pigs had fever. On day 2 after inoculation with the gE-negative virus, half of the pigs had fever. From day 3 after inoculation with the gE-negative virus onwards all pigs had fever. The pigs were dull and had no appetite. In contrast to gE-negative PRV, the ‘wild-type’ PRV caused coughing, ataxia and paralysis. In experiment 1, all three pigs died within 4 days after inoculation with the ‘wild-type’ virus, whereas pigs survived after inoculation with gE-negative virus and developed no respiratory or neurological signs.

Virus content of tissues

In experiment 1, the virus content (geometric mean titre of three pigs) of several tissues of 3-week-old pigs was determined on days 1, 2, 3 and 4 after intranasal inoculation with 10^7 p.f.u. of the gE-negative virus (M301) or with 10^7 p.f.u. of the rescued ‘wild-type’ virus (M307) (Table 1). Compared to the ‘wild-type’ virus, the gE-negative virus replicated somewhat less efficiently at the site of peripheral infection, i.e. the oropharyngeal mucosa, olfactory epithelium, trigeminal ganglion and draining lymph nodes. The titres of the gE-negative virus in the olfactory epithelium and trigeminal ganglion were approximately 100-fold lower on average on days 2 and 3 than the titres of the ‘wild-type’ virus. Furthermore, the gE-negative virus was cleared more rapidly from these peripheral sites. The highest virus titres of the gE-negative PRV in the peripheral tissues were recovered mainly on day 3. In contrast, the virus titres of ‘wild-type’ virus in these peripheral tissues were higher on day 4 than on day 3.

The ‘wild-type’ virus replicated in the olfactory bulb, brain stem and other tissues of the CNS, and on day 4 10^9 to 10^4 p.f.u. of ‘wild-type’ virus/g of tissue sample was recovered from these tissues. In contrast, titres of the gE-negative virus were very low or undetectable in tissues of the CNS. Although on days 2 and 3 a few p.f.u. of gE-negative PRV were recovered from the olfactory bulb, brainstem and medulla oblongata, the CNS contained no detectable gE-negative PRV by day 4.

In experiment 2, pigs were inoculated with high doses of virus in order to infect as many first-order neurons as...
possible in the oropharyngeal region. The virus content (geometric mean titre of three pigs) of several tissues was determined at day 2 after inoculation. Compared to the ‘wild-type’ virus, the gE-negative virus replicated to almost similar titres at peripheral sites. The titres of the gE-negative virus were now approximately only 10-fold lower in the oropharyngeal mucosa, olfactory epithelium and trigeminal ganglion than the titres of the ‘wild-type’ virus (Table 2). In contrast, in the olfactory bulb and brainstem, tissues which contain second- and third-order neurons, the titres of gE-negative virus were respectively 5000- and 2500-fold lower than the titres of the ‘wild-type’ virus. The differences in virus replication in the tissues examined between the ‘wild-type’ virus and the gE-negative virus are indicated in Table 2 as ratios between the ‘wild-type’ virus and the gE-negative virus.

In conclusion, in this experiment the virus titres of the gE-negative virus and the ‘wild-type’ virus were almost comparable in the peripheral sites, but only very low quantities of the gE-negative virus were recovered from tissues of the CNS.

Localization of viral antigen

Sections of mucosa and nervous tissues were immunohistochemically stained to examine the spread of PRV via the olfactory and trigeminal routes in more detail, and to try to define the lack of neurotropism of gE-negative PRV. The organization of the synaptic connections of the olfactory and trigeminal routes is shown in Fig. 1 (DeVries & Baylor, 1993; Martin & Dolivo, 1983). Because the olfactory epithelium contains many epithelial cells, the cell bodies of the olfactory receptor neurons cannot be discriminated in the immuno- stains.

In experiment 1, viral antigen was detected in the olfactory epithelium of all pigs inoculated with the ‘wild-type’ virus on days 2, 3 and 4. The ‘wild-type’ virus caused small foci of infection in the olfactory epithelium. The number and size of infected areas increased with time. After inoculation with the gE-negative virus, viral antigen was detected in the olfactory epithelium in one pig on day 2, in two pigs on day 3, and in one pig on day 4. The gE-negative virus caused small foci of infection in the olfactory epithelium which contained less viral antigen than the infections caused by the ‘wild-type’ virus (data not shown).

Two days after inoculation with the ‘wild-type’ virus, viral antigen was detected in the olfactory bulb in two pigs, and in all pigs on day 3 and day 4. On day 3 viral antigen was detected in second-order neurons of the olfactory bulb that synapse on the axons of the olfactory

<table>
<thead>
<tr>
<th>Tissue</th>
<th>M301 (gE-negative PRV)</th>
<th>M307 (‘wild-type’ PRV)</th>
<th>Ratio M307/M301</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasal mucosa</td>
<td>6.2 (0.3)</td>
<td>6.7 (0.6)</td>
<td>3</td>
</tr>
<tr>
<td>Pharyngeal mucosa</td>
<td>2.8 (0.9)</td>
<td>3.5 (1.5)</td>
<td>5</td>
</tr>
<tr>
<td>Olfactory epithelium</td>
<td>6.8 (0.4)</td>
<td>8.0 (0.3)</td>
<td>16</td>
</tr>
<tr>
<td>Tonsil</td>
<td>3.8 (1.9)</td>
<td>4.2 (1.0)</td>
<td>3</td>
</tr>
<tr>
<td>Trigeminal ganglion</td>
<td>4.6 (1.8)</td>
<td>5.6 (0.1)</td>
<td>10</td>
</tr>
<tr>
<td>Olfactory bulb</td>
<td>1.3 (1.2)</td>
<td>5.0 (2.0)</td>
<td>5012</td>
</tr>
<tr>
<td>Brain stem</td>
<td>1.1 (1.2)</td>
<td>4.5 (0.4)</td>
<td>2512</td>
</tr>
<tr>
<td>Medulla oblongata</td>
<td>1.6 (0.9)</td>
<td>4.2 (1.3)</td>
<td>398</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0.6 (1.1)</td>
<td>2.1 (0.4)</td>
<td>32</td>
</tr>
</tbody>
</table>

* Virus titres are the geometric mean values for three pigs, ± S.D. (expressed as log_{10}).

Fig. 1. Schematic diagram of the transneuronal spread of PRV via (a) the synaptically linked sensory circuits of the olfactory route and (b) the trigeminal route. (a) Bipolar olfactory receptor neurons (ORN) in the olfactory epithelium send axons through the ethmoid bone (EB) into the olfactory bulb (OB), where they end in spherical neuropils called glomeruli (dashed circles). Here the axons of the ORN synapse on the second-order neurons; the periglomerular neurons (PG) and the mitral/tufted neurons (M/T). Finally, the granule neurons (GR), third-order neurons, make reciprocal synapses with the mitral/tufted neurons. (b) Pseudo-unipolar neurons in the trigeminal ganglion receive afferent axons from the ophthalmic nerve (V1), maxillary nerve (V2) and mandibular nerve (V3) and send efferent axons through the trigeminal nerve that end in the brain stem. The majority of these axons synapse on second-order neurons of the sensory nucleus of the trigeminal nerve located in the brain stem at the pons cerebri. We focused our investigations on this main terminal of the trigeminal nerve. Not shown in the diagram are other efferent axons which end in the spinal cord or in the mesencephalic nucleus of the trigeminal nerve.
Fig. 2. Immunostains of viral antigen in the olfactory bulb in pigs from experiment 1, 3 days after infection with 'wild-type' PRV or gE-negative PRV. The positions of first- (1), second- (2) and third-order (3) neurons are indicated. (a) Transneuronal spread of 'wild-type' PRV in the olfactory bulb reaches second- and third-order neurons. (b) Viral antigen (arrows) of the gE-negative PRV is transported via the axons of the olfactory receptor neurons (1) into the olfactory bulb, but the neuronal spread of gE-negative PRV stops just before the glomeruli (G). (Inset, detail of boxed area under higher power magnification.) (c) Viral antigen was detected in the periglomerular neurons after infection with 'wild-type' PRV (arrowheads). (d) No viral antigen was detected in the periglomerular neurons after infection with the gE-negative virus. Mononuclear infiltrates were seen in the glomerular layer of the olfactory bulb (arrowhead). (e) Viral antigen was detected in many mitral/tufted neurons (large arrows) and in many granule neurons (small arrows) after infection with 'wild-type' PRV. (f) No viral antigen was detected in the mitral/tufted neurons and in the granule neurons after infection with gE-negative PRV. Scale bars: (a) 50 μm; (b) 125 μm (inset 25 μm); (c to f) 25 μm.

receptor neurons (Fig. 2a), i.e. in the periglomerular neurons (Fig. 2c), and in the mitral/tufted neurons (Fig. 2e). Viral antigen was also detected in third-order neurons of the olfactory bulb, the granule neurons (Fig. 2e). Three days after inoculation with the gE-negative virus, viral antigen was detected in the olfactory bulb in
two pigs, and in one pig on day 4. At this time, the gE-negative PRV had spread via the axons of the olfactory receptor neurons into the glomerular layer of the olfactory bulb. Small amounts of viral antigen were detected below the glomeruli, the spherical structures where the axons of the olfactory receptor neurons end (Fig. 2b). Infection with the gE-negative virus in the glomerular layer was accompanied by mononuclear infiltrates. In contrast to the ‘wild-type’ virus, the gE-negative virus did not infect second- or third-order neurons of the olfactory bulb. No viral antigen was detected in the periglomerular neurons (Fig. 2d), or in the mitral/tufted neurons or in the granule neurons (Fig. 2f).
The trigeminal pathway is another main route for PRV to invade the porcine CNS after intranasal inoculation. On days 2 and 3 after inoculation with either virus, viral antigen was detected in the neurons of the trigeminal ganglion. No major differences in quantities of viral antigen were detected in the trigeminal ganglion on these days. On day 4, the amount of viral antigen in the trigeminal ganglion of two pigs infected with ‘wild-type’ virus had increased, whereas at this time after infection with the gE-negative virus, viral antigen was detected in the trigeminal ganglion of only one pig. On day 2 after inoculation with either virus, viral antigen was detected in the neurons of the trigeminal ganglion (Fig. 3a, b). Details of infected trigeminal neurons are shown in Fig. 3(c, d). Viral antigen was also seen in infiltrating macrophages. Furthermore, viral antigen was also seen in trigeminal nerves leading to the brainstem (Fig. 3e, f).

In one pig on day 2 and in two pigs on days 3 and 4 after inoculation with the ‘wild-type’ virus, viral antigen was detected in second-order neurons of the sensory nucleus of the trigeminal nerve in the brainstem. In contrast, after inoculation with the gE-negative virus, no viral antigen was detected in these second-order neurons in the brainstem (data not shown). Four days after inoculation with the ‘wild-type’ virus, viral antigen was also detected in the cerebellum (data not shown).

In experiment 2, viral antigen was detected in all pigs in the olfactory epithelium and in axons of the olfactory receptor neurons after inoculation with either virus (Fig. 4a, b). Also, viral antigen was detected in olfactory nerves directed through the ethmoid bone leading towards the olfactory bulbs (Fig. 4c, d). Both viruses caused extensive infection of the olfactory epithelium and stroma and large areas of the olfactory epithelium had sloughed off. No major differences were seen in the quantity of viral antigen in the olfactory epithelium after infection with gE-negative virus and ‘wild-type’ virus.
All three pigs inoculated with the 'wild-type' virus and two pigs inoculated with the gE-negative virus had viral antigen in the olfactory bulb. As in experiment 1, the 'wild-type' virus infected second- and third-order neurons of the olfactory bulb, whereas the gE-negative virus only infected the glomerular layer of the olfactory bulb. Again, no viral antigen was detected in the second- or third-order neurons of the olfactory bulb after inoculation with the gE-negative virus (data not shown).

As in experiment 1, 2 days after inoculation with either virus, viral antigen was seen in neurons of the trigeminal ganglion and trigeminal nerves leading to the brain stem in all pigs. No major differences in quantity of viral antigen were seen (data not shown).

Two days after inoculation with the 'wild-type' virus, viral antigen was detected in the second-order neurons of the sensory nucleus of the trigeminal nerve in the brainstem in two pigs (Fig. 5a, c). These neurons synapse on some of the efferent axons of the trigeminal neurons. Again, 2 days after inoculation with the gE-negative virus, no viral antigen was detected in these second-order neurons in the brainstem (Fig. 5b, d).

**Immunohistochemical and ultrastructural characterization of virus replication in the trigeminal ganglion**

Immunohistochemical and electron microscopical examinations were done to study the PRV infection in the trigeminal ganglion. From 2 days after inoculation with both 'wild-type' virus and the gE-negative virus in experiment 1, viral antigen was not only present in the trigeminal neurons, but also in infiltrating macrophages (Fig. 3c, d) and in satellite cells that surround these trigeminal neurons. An example of immunostaining of viral antigens in a trigeminal neuron and in surrounding satellite cells is shown, 3 days after infection with the gE-negative virus (Fig. 6).

In addition, we demonstrated by electron microscopy that PRV replicated both in trigeminal neurons and in...
Role of PRV gE in spread into CNS of pigs

Fig. 6. Immunostain of viral antigen in trigeminal ganglion in pigs from experiment 1, 3 days after infection with gE-negative virus. Viral antigen was present in the nucleus and cytoplasm of infected trigeminal neurons (arrows), and viral antigen was also clearly present in surrounding satellite cells (arrowheads). Scale bar, 20 μm.

satellite cells. Fig. 7 shows an example of both trigeminal neurons and satellite cells that produce virus particles, 3 days after infection with the ‘wild-type’ virus. The production and subsequent release of virus particles from these satellite cells probably leads to infection of other non-infected trigeminal neurons. Together, these electron microscopic and immunohistochemical data are supporting evidence that in the trigeminal ganglion of the pig, PRV may also be transferred non-synaptically by lateral spread via infected satellite cells or via infected macrophages, in addition to specific trans-synaptic transfer via synaptically linked neurons.

We could not detect, by electron microscopy, changes in virus production or virus release in trigeminal ganglia infected with the gE-negative virus that could explain the restricted spread of this mutant into the CNS.

Discussion

This is the first report that demonstrates the invasion by PRV of the porcine CNS via the synaptically linked neurons of both the olfactory and the trigeminal routes. The capacity of the gE-negative PRV to enter or to replicate in the porcine CNS was strikingly reduced compared to its rescued ‘wild-type’ PRV. In contrast to the ‘wild-type’ virus, no or only minute quantities of the gE-negative virus were isolated from various tissues of the CNS. After inoculation with the gE-negative virus, viral antigen was detected in first-order neurons, but not in second- or third-order neurons of both olfactory and trigeminal routes. Presumably, the minute quantities of gE-negative PRV in these tissues detected by virus titration were too low to be detected by the immunostaining method. Furthermore, the virulence of gE-negative PRV was greatly reduced. In experiment 1, all three pigs died 4 days after inoculation with the rescued ‘wild-type’ virus, whereas pigs inoculated with the gE-negative virus developed no respiratory or neurological signs of disease. The restricted spread or replication of gE-negative PRV through the porcine CNS probably explains its lack of virulence for pigs.

Several mechanisms may explain the reduced levels of gE-negative PRV in the CNS. First, the gE-negative virus may infect fewer first-order neurons at the peripheral site of inoculation. In experiment 1, the titres of the gE-negative virus in the oropharyngeal mucosa, draining lymph node, olfactory epithelium and trigeminal ganglion were approximately 100-fold lower than the titres of the rescued ‘wild-type’ virus (Table 1). Hence, an explanation for the reduced levels of PRV in the CNS may be that insufficient gE-negative virus gained access to the CNS thereby limiting the neuronal infection. To investigate this possibility, we performed a more intense method of infection in experiment 2, in order to infect as many first-order neurons as possible in the oropharyngeal region. In this experiment, the virus titres of the gE-negative virus and the rescued ‘wild-type’ virus were similar in the peripheral sites, but nonetheless again only minute quantities of the gE-negative virus were recovered from tissues of the CNS (Table 2). In Table 2 the ratios of recovered ‘wild-type’/gE-negative virus titres in the various tissues are shown. There is a drop in this ratio between tissues that contain second-order neurons (olfactory bulb, brainstem) and tissues that contain first-order neurons (olfactory epithelium, trigeminal ganglion). This finding makes it unlikely that defective replication of gE-negative PRV at the periphery explains the failure of gE-negative PRV to enter the CNS. Furthermore, PRV can infect the olfactory receptor neurons in the olfactory epithelium directly, without the need for replication of the virus.

Second, gE-negative virus may spread more slowly in the CNS, because cell-to-cell transmission or release of the virus from infected neurons is reduced, or both. After intranasal PRV infection in mice, direct cell-to-cell transmission appears to be an important mode of PRV
Fig. 7. Virus replication in trigeminal neurons and satellite cells in pigs from experiment 1, 3 days after inoculation with ‘wild-type’ virus. (a) Production of virus particles in the nucleus of trigeminal neuron (N) and in nuclei of surrounding satellite cells (S). The boxed areas are illustrated at high power magnification in (b) and (c). (b) Detail of virus replication in the nucleus of the infected neuron showing large aggregations of empty capsids (open arrowheads) and capsids filled with DNA (filled arrowheads). P, pores in nuclear membrane. (c) Details of virus replication in the nucleus (N) of a satellite cell showing aggregations of empty capsids (open arrowheads) and capsids filled with DNA (filled arrowheads). Mature virions were seen outside infected satellite cells (arrows). Cy, cytoplasm of infected trigeminal neuron. Scale bars: (a) 5 μm; (b, c) 500 nm.
spread (Peeters et al., 1993; Heffner et al., 1993). In vitro, gE also promotes cell fusion and as a consequence cell-to-cell transmission of the virus (Zsak et al., 1992). In addition, gE promotes the release of PRV in certain cell types (Mettenleiter et al., 1987b; Zsak et al., 1989; L. Jacobs and others, unpublished results). Dingwell et al. (1994) demonstrated that homologous herpes simplex virus glycoproteins gE and gI facilitate cell-to-cell spread in vivo and across junctions of cultured cells. Our results make it tempting to speculate that deleting gE of PRV affects cell-to-cell transmission in neurons or release from infected neurons in vivo.

Third, gE-negative PRV may be unable to efficiently cross a synapse. The virus must cross synapses to spread from the olfactory receptor neurons in the olfactory epithelium to the second- or third-order neurons of the olfactory bulb, and to spread from the trigeminal neurons to the second-order neurons in the brainstem. In contrast, the virus remains in the same neuron and spreads directly from the nerve endings in the oropharyngeal mucosa to the neurons of trigeminal ganglion without crossing a synapse. Our findings in pigs (natural host) are similar to those of Whealy et al. (1993), Card et al. (1992) and L. W. Enquist and others (personal communication) in rats. These authors found that after PRV infection in the rat anterior eye chamber, gE and gI are required for the infection of second- and third-order neurons in some circuits, but not in others. They suggested that certain subsets of retinal ganglion cells contain receptors which are not recognized by gE-negative or gI-negative PRV.

Electron microscopical and immunohistochemical examinations of infected trigeminal ganglia demonstrated that virus replication of PRV not only occurred in the trigeminal neurons, but also in surrounding satellite cells and infiltrating macrophages. These results suggest that in the trigeminal ganglion of pigs PRV can also spread from neuron to neuron via infected satellite cells and macrophages. Thus, PRV may also be transferred by lateral spread via a nonsynaptic route. These observations add another mechanism in addition to virus spread within synaptically linked neurons by a specific trans-synaptic mechanism (Strack & Loewy, 1990; Card et al., 1990, 1993). Our results are in contrast with the findings of McCracken et al. (1973) who reported that PRV-infected neurons of spinal ganglia of infected calves were almost invariably surrounded by a sheath of uninfected satellite cells.

In conclusion, gE-negative PRV can infect first-order neurons of both olfactory and trigeminal routes and is able to spread via their axons towards the CNS. gE-negative PRV, however, has a reduced capacity to infect second- or third-order neurons. Thus, this study indicates that gE confers an ability for transneuronal spread.

Future research should clarify the mechanisms by which PRV is transported through porcine neurons and across synapses, and at which level glycoprotein gE influences the neurotropism of PRV.

References


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