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Glycoprotein D-Negative Pseudorabies Virus Can Spread Transneuronally via Direct Neuron-to-Neuron Transmission in Its Natural Host, the Pig, but Not after Additional Inactivation of gE or gI

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Envelope glycoprotein D (gD) is essential for entry of pseudorabies virus (PRV) into cells but is not required for the subsequent steps in virus replication. Phenotypically complemented gD mutants can infect cells and can spread, both in vitro and in mice, by direct cell-to-cell transmission. Progeny virions released by infected cells are noninfectious because they lack gD. The aim of this study was to determine the role of gD in the neuropathogenicity of PRV in its natural host, the pig. We investigated whether gD-negative PRV can spread transneuronally via synaptically linked neurons of the olfactory and trigeminal routes. High doses of a phenotypically complemented gD mutant and gD mutants that are unable to express either gI or gI plus gE were inoculated intranasally in 3- to 5-week-old pigs. Compared with the wild-type virus, the virulence of the gD mutant was reduced. However, pigs inoculated with the gD mutant still developed fever and respiratory signs. Additional inactivation of either gI or gI plus gE further decreased virulence for pigs. Immunohistochemical examination of infected pigs showed that a PRV gD mutant could replicate and spread transneuronally into the central nervous system (CNS). Compared with the wild-type virus, the gD mutant had infected fewer neurons of the CNS on day 2. Nevertheless, on day 3, the gD-negative PRV had infected more neurons and viral antigens were present in second- and third-order neurons in the olfactory bulb, brain stem, and medulla oblongata. In contrast, gD mutants which are unable to express either gI or gI plus gE infected a limited number of first-order neurons in the olfactory epithelium and in the trigeminal ganglion and did not spread transneuronally or infect the CNS. Thus, transsynaptic spread of PRV in pigs can occur independently of gD. Possible mechanisms of transsynaptic transport of PRV are discussed.

Pseudorabies virus (PRV) (Aujeszky's disease virus) is a highly neurotropic alphaherpesvirus that causes neurological disorders in domestic and wild animals (11, 38). Pigs are the natural host of the virus. Natural infection occurs mainly by the respiratory route. The primary replication sites are the nasopharyngeal mucosa, tonsils, and lungs (39, 41). From here, the virus may enter peripheral nerves and, via axonal transport, is able to invade the central nervous system (CNS) (22, 23, 27, 32), where it causes a severe encephalitis that is often fatal in young pigs (2, 7). Older pigs usually survive the infection but may develop fever and pneumonia. Infection of sensory ganglia generally results in the establishment of latency (9, 44).

The herpesvirus envelope glycoproteins mediate several steps in virus infection (29, 47). Nine envelope glycoproteins have been identified so far: gB, gC, gD, gE, gG, gH, gI, gK, and gL. The initial binding of PRV to target cells involves the interaction of gC with heparan sulfate on the cell surface. Primary attachment of the virion, which is sensitive to competition by exogenous heparin, converts to a heparin-resistent attachment (30, 45). This secondary binding appears to be specifically mediated by gD (15, 16). Attachment is followed by fusion between the virion envelope and the cytoplasmic membrane. The glycoproteins involved in the fusion process (gB, gD, gH, and probably gL) are essential for virus entry (35–37). When phenotypically complemented mutants are used to infect noncomplementing cells, gD mutants are still able to produce plaques but gB and gH mutants are not. In contrast to the analogous gD mutants of herpes simplex virus, PRV gD mutants can spread in vitro to other cells via direct cell-to-cell transmission (36, 42). Furthermore, the gD protein is essential for primary infection but not for subsequent replication and transneuronal spread in mice (1, 37).

In rodents, the invasion of the CNS by PRV and other alphaherpesviruses, for example, herpes simplex virus, occurs in an ordered fashion in which the virus replicates within synaptically linked populations of neurons (24, 48). Herpesviruses can therefore be used as self-amplifying tracers to study the connections of neuronal circuits (3). However, at present it is unclear how transsynaptic spread occurs. Although viral spread of PRV gD mutants in the CNS of mice has been demonstrated, their role and relative importance of transneuronal spread and the pathological implications in pigs are unknown. In the present study, we investigated the virulence and spread of gD mutants into and within the CNS of 3- to 5-week-old pigs. In addition, we studied the neuronal spread of gD mutants that are unable to express either gI or gI plus gE. gE and, to a lesser extent, gI confer an ability for transneuronal spread

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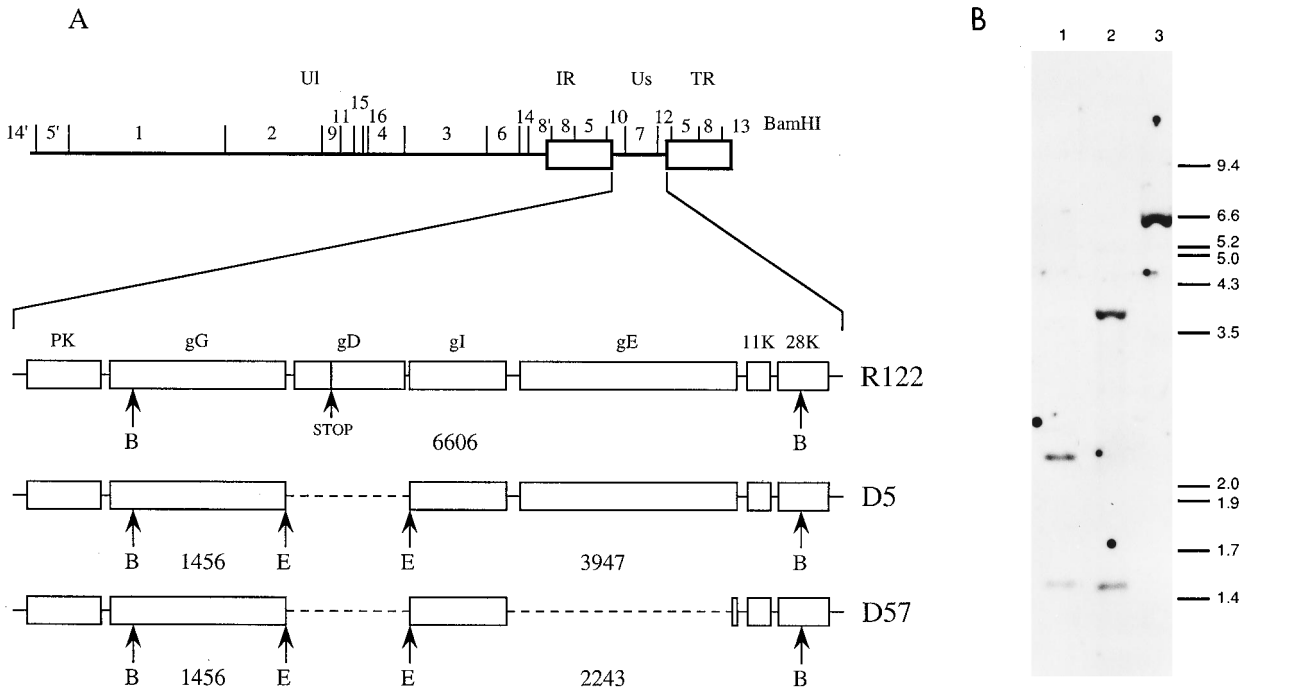

FIG. 1. (A) Physical map of the PRV genome (top line). Open rectangles represent the internal and terminal inverted repeats (IR and TR), which divide the genome into a unique long (UL) region and a unique short (Us) region. The positions of the restriction fragments generated by *Bam*HI are shown and are numbered according to size. The lower part shows the locations of the gD, gI, and gE genes and the locations of the mutations. Strain R122 (36, 37) contains a premature translation termination codon behind amino acid codon 122 in the gD gene. The positions of the gD deletion of mutant strain D5 and the gD-plus-gE deletion of mutant strain D57 are indicated by dashed lines. The positions of the *Bam*HI (B) and *Eco*RI (E) sites and the sizes of the corresponding fragments are indicated (compare with panel B). (B) Southern blot of *Bam*HI-*Eco*RI-digested viral DNA hybridized with PRV fragment *Bam*HI 7 as a probe (compare with panel A). The DNA fragments were separated on a 0.8% agarose gel and blotted onto Hybond N+ (Amersham). The *Bam*HI 7 fragment was labelled with the DIG DNA labeling kit (Boehringer Mannheim), and hybridizing fragments were visualized with the DIG luminescent detection kit as specified by the supplier (Boehringer Mannheim). Lanes: 1, D57; 2, D5; 3, NIA-3. The positions and sizes (in kilobase pairs) of marker fragments are indicated to the right.

(22, 23, 32). Furthermore, in vitro, gE is involved in cell fusion and promotes the cell-to-cell spread of the virus (reviewed in reference 13). Together, gE and gI form a noncovalently linked complex (51). Because progeny virions of phenotypically complemented gD mutants are noninfectious, the virus content of various tissues could not be determined by plaque titer determination (34). Therefore, the neuronal spread of these PRV gD mutants was studied by immunohistochemical detection of viral antigen. Our results show that transsynaptic spread of PRV in pigs can occur independently of gD. Additional inactivation of either gI or gE plus gI strongly reduced viral spread in peripheral tissues and prevented the spread of PRV to the CNS.

MATERIALS AND METHODS

Cells and viruses. The PRV strain NIA-3 (28) was used as a parent strain for the construction of the mutant strains and was used as the wild-type strain in the animal experiments. The mutant strains used in this study are illustrated in Fig. 1. The construction and properties of the PRV gD linker insertion mutant R122 have been described previously (36, 37). R122 shows normal morphogenesis and viral egress in epithelial cells of porcine nasal mucosa explants (50).

To construct the gD deletion mutants D5 and D57, unique restriction sites were created directly in front of the gD gene and immediately behind the gD gene in a plasmid that contained a 3.8-kb *Sca*I-*Dra*I fragment derived from the Us region of PRV. By means of in vitro site-directed mutagenesis (Transformer kit; Clontech) the nucleotide sequence ACTCAC (nucleotides −17 to −12 with respect to the gD gene; numbering according to reference 40 was converted to an *Spe*I site (ACTAGT) and the sequence CCTCCG (nucleotides 1210 to 1215 [40]) was converted to an *Avr*II site (CCTAGG). A 3.3-kb *Nco*I-*Pst*I fragment containing the gD gene and part of the flanking gG and gI genes was cloned in pGEM5Zf+ (Promega), yielding pBP52. The gD gene was removed by *Spe*I-*Avr*II digestion and replaced by a synthetic oligonucleotide containing the recognition sequences for *Spe*I, *Eco*RI, *Eco*RV, *Hin*dIII, and *Avr*II (in that order). The resulting plasmid was designated pBP53. To rule out the presence of mutations in the PRV sequences of pBP53, we replaced most of the PRV sequences of pBP53 with the original PRV sequences (i.e., those which had not undergone the mutagenesis procedure) by exchanging restriction fragments. Two small regions which were not replaced were verified by DNA sequencing. We did not observe any differences between the sequenced regions and the original PRV sequences, indicating that the PRV sequences which are present in pBP53 are correct.

To construct a PRV gD deletion mutant, the *Nco*I-*Pst*I fragment of pBP53 and *Eco*RI-digested DNA of the PRV gD mutant R122 were used for the cotransfection of VII/50 cells (see below). Progeny plaques were analyzed for the presence of the expected gD deletion by restriction enzyme analysis and Southern blot hybridization (36). A mutant with the desired gD deletion was plaque purified three times and designated D5 (Fig. 1).

The gD-plus-gE deletion mutant D57 was constructed as follows. Plasmid pN3HBΔgE contains the *Hin*dIII B fragment of PRV (spanning the entire Us region and a large part of the flanking inverted repeats [49]) in which a sequence spanning almost the entire gE gene (nucleotides 1183 to 2913 according to the numbering of Robbins et al. [43]) was replaced by the sequence TAGGCTA GAATTAATTCTAGCCTA. After cotransfection of VII/50 cells with (i) *Eco*RI-digested D5 DNA, (ii) the *Nco*I-*Pst*I fragment of pBP53, and (iii) a *Bgl*II-*Hin*dIII fragment derived from pN3HBΔgE, a gD-gE double-deletion mutant, designated D57, was isolated (Fig. 1).

The complementing cell line VII/50, which is able to express PRV gD, was constructed as follows. An *Sph*I fragment containing the gB promoter and the major part of the gB gene (43) was cloned in the *Sph*I site of pUC19. The gB promoter was recovered as a *Sal*I-*Ppu*MI fragment (*Ppu*MI at position −71 with respect to the ATG codon), treated with Klenow DNA polymerase to create blunt ends, and cloned in the blunted *Bam*HI site of plasmid pEVneo10 (36), yielding pEVneo2. The gD gene was recovered from plasmid pBP52 (see above) as an *Spe*I-*Avr*II fragment and cloned in the *Xba*I site (which is located downstream of the gB promoter) of pEVneo2, yielding pEVneo2gp50. After transfection of Vero cells with pEVneo2gp50, neomycin-resistant colonies were isolated and the cells were tested for the ability to complement gD mutant R122. One cell line which showed the best complementation was cloned by limiting dilution and was designated VII/50. This cell line was used for the propagation of strains D5 and D57. Since VII/50 cells contain exactly those PRV sequences

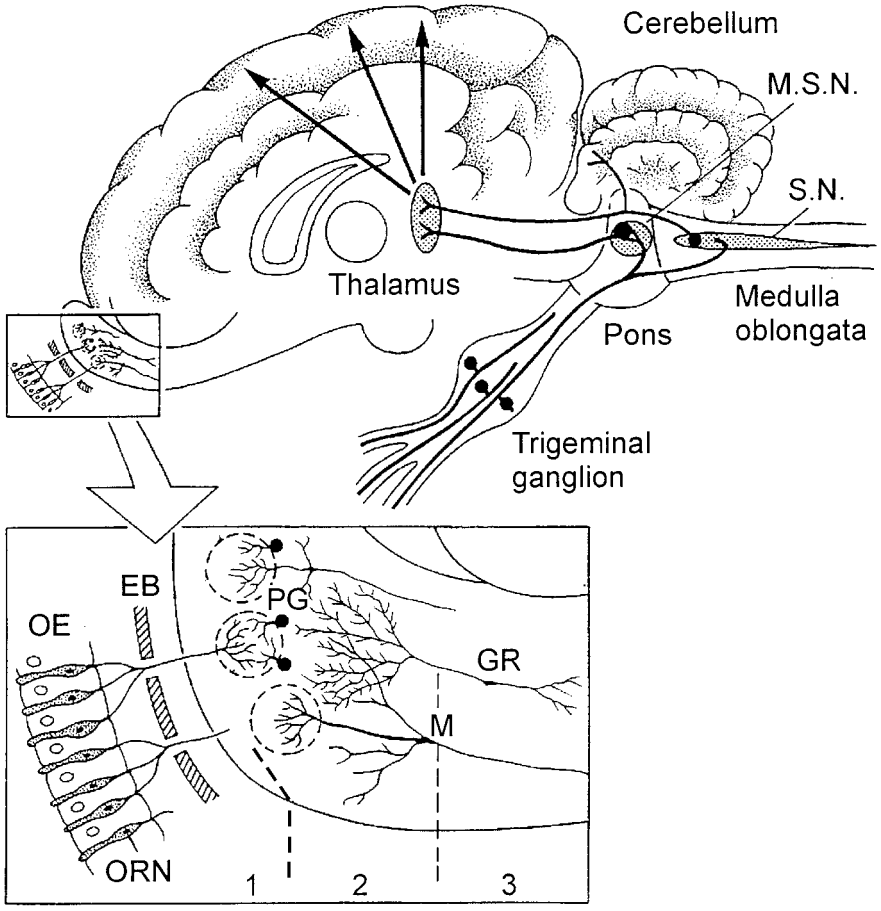

FIG. 2. Schematic diagram of the synaptically linked sensory neurons of the olfactory and the trigeminal routes by which PRV can spread transneuronally. Below the diagram is an enlargement showing details of the olfactory route. Bipolar olfactory receptor neurons (ORN) in the olfactory epithelium (OE) are the first-order neurons of the olfactory route. They send axons through the ethmoid bone (EB) into the olfactory bulb, where they end in spherical neurophils called glomeruli (dashed circles). Here the axons of the ORN synapse on the second-order neurons, i.e., the periglomerular neurons (PG) and the mitral neurons (M). Finally, the granule neurons (GR), third-order neurons, make reciprocal synapses with the mitral neurons. Pseudo-unipolar neurons in the trigeminal ganglion are the first-order neurons in the trigeminal route. They receive afferent axons from the ophthalmic nerve, the maxillary nerve, and the mandibular nerve. These trigeminal neurons send efferent axons through the trigeminal nerve that end in the brain stem and medulla oblongata. The majority of these axons synapse on second-order neurons of the main sensory nucleus (M.S.N.) of the trigeminal nerve located in the brain stem at the pons cerebri. Another part of these axons end in the spinal nucleus (S.N.) in the medulla oblongata. We focused our investigations on these main terminals of the trigeminal nerve located in the brain stem and medulla oblongata. We did not examine third-order neurons of the trigeminal route located in the cerebellum or in the thalamus and the cerebral cortex.

which are absent from strains D5 and D57, they are unable to rescue the gD deletion of strains D5 and D57 by means of homologous recombination.

Animals and experimental design. Dutch Landrace pigs 3 to 5 weeks old 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 either 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.

The pigs were sedated and placed on their backs before being inoculated as described previously (32, 41). Virus suspension (4 ml containing 10^7 PFU/ml) was instilled dropwise into the nostrils. Six pigs were infected with the wild-type strain NIA-3, and six pigs were infected with strain R122. Furthermore, nine pigs were infected with strain D5 and nine pigs were infected with strain D57. Two days after infection, three pigs from each group were killed and various tissues were examined for the presence of viral antigen. In addition, on day 3 after inoculation with NIA-3 or R122 and on days 4 and 7 after infection with D5 or D57, three pigs from each group were killed and various tissues were examined for the presence of viral antigen.

The pigs were observed twice daily for clinical signs of disease. Rectal temperatures were measured once daily at approximately the same time early in the morning. Clinical signs of Aujeszky's disease were monitored as described by Kimman et al. (19). Briefly, fever was defined as a rectal temperature above 40°C; respiratory signs were defined as nasal discharge, sneezing, coughing, and forced respiration; and 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 the sections was detected by indirect immunohistochemical staining (41) with a polyclonal rabbit serum directed against PRV. Specific binding was detected with a biotin-labelled goat anti-rabbit serum followed by streptavidin-peroxidase. Sections were counterstained with hematoxylin for 10 s. Histological examinations of tissues from inoculated pigs included selection of five sections per tissue, taken 1 mm apart. Control experiments were done to analyze gD and gB expression in nervous system tissue from R122-, D5-, and D57-infected pigs by using monoclonal antibodies directed against gD and gB (33). All of the detected viral antigen was indeed gD negative and gB positive (data not shown).

Although we also examined oropharyngeal mucosa and tonsils for the presence of viral antigen, we focused on the transsynaptic spread of PRV strains in nervous tissues of the trigeminal and olfactory routes as described previously (32). In Fig. 2, a schematic diagram of the synaptically linked neurons of the olfactory and trigeminal routes is given.

RESULTS

Phenotype of mutant strains. The virus strains that were used in this study are shown in Fig. 1. PRV strain R122 is a gD mutant that was constructed by means of linker insertion mutagenesis (35, 36). This strain contains a premature stop codon

TABLE 1. Neuronal spread of different PRV strains in pigs as detected with immunohistochemistry

Virus strain	Inactivation	Tissue (order of neurons)[a]	Presence of viral antigen[b] on day:			
			2	3	4	7
NIA-3	None	Olfactory epithelium (1)	+++	+++	ND[c]	ND[c]
		Olfactory bulb (2 and 3)	+	++		
		Trigeminal ganglion (1)	++	++		
		Brain stem, MSN (2)	++	++		
		Medulla oblongata, SN (2)	+	++		
R122[d]	gD	Olfactory epithelium (1)	++	+++	ND[c]	ND[c]
		Olfactory bulb (2 and 3)	−	+		
		Trigeminal ganglion (1)	++	++		
		Brain stem, MSN (2)	+	++		
		Medulla oblongata, SN (2)	±	++		
D5[d]	gD + gI	Olfactory epithelium (1)	±	ND	±	−
		Olfactory bulb (2 and 3)	−		−	−
D57[d]	gD + gI + gE	Trigeminal ganglion (1)	±	ND	±	−
		Brain stem, MSN (2)	−		−	−
		Medulla oblongata, SN (2)	−		−	−

[a] MSN, main sensory nucleus; SN, spinal nucleus.
[b] Symbols: −, no infected neurons detected; ± and +, scattered infection; ++, moderate infection; +++, dense infection. ND, not done.
[c] Pigs could not be tested on days 4 and 7 because they would have already died; e.g., some already had temperatures above 41.5°C.
[d] Mutant strains were phenotypically complemented for gD.

in the gD gene. Strains D5 and D57 contain deletions of the gD gene and the gD plus gE genes, respectively. Since gD is an essential gene, phenotypically complemented virus stocks were prepared on complementing VII/50 cells, which are able to express gD. It is important to note that the complementing VII/50 cells do not share homologous PRV sequences with deletion mutants D5 and D57. Therefore, these mutants are unable to pick up the gD gene by means of homologous recombination. To check our D5 and D57 virus stocks for the presence of wild-type virus, we passaged phenotypically complemented virus stocks (2×10^7 PFU) on noncomplementing Vero cells (on which only gD-positive viruses yield infectious virus) and determined the number of PFU in the progeny. We were unable to detect infectious virus among the progeny, indicating that no gD-positive virus was present in the original virus stock (data not shown). Phenotypically complemented stocks of strain R122 were prepared as described previously (35).

Although, as indicated above, strains D5 and D57 did not yield revertants on VII/50 cells, we were able to isolate revertants after passage on complementing G5 or F8 cells. The latter cell lines contain not only gD but also flanking PRV sequences (parts of gG and gI [35, 36]), which overlap with D5 and D57 sequences. We have analyzed several such revertants and have found that the genome structures and the properties of the revertants were similar to those of the wild-type strain NIA-3 (D5 revertants) and a gE deletion mutant (D57 revertants), respectively (data not shown). These findings indicate that no mutations other than the gD deletion and the gD plus gE deletion were present in strains D5 and D57, respectively.

It has been reported that the gI gene, which is located behind the gD gene (Fig. 1), is expressed by a transcript that starts at a promoter located directly before the gD gene and ends shortly after the gI gene (21). Since the putative promoter region is still present in deletion mutants D5 and D57, we expected that the gI gene would still be expressed by these strains. However, we were unable to detect expression of gI in either D5- or D57-infected cells in an immunoperoxidase monolayer assay with monoclonal antibody 3M8 (8). Since expression of gI could readily be detected in wild-type or R122-infected cells (data not shown), we conclude that strains D5 and D57 do not express detectable levels of gI. Since the sequences of the gD promoter and the gI gene were similar to those of the wild-type virus, we assume that sequences within the gD gene itself contribute to promoter activity. We are currently examining this phenomenon more closely.

Virulence of PRV wild-type NIA-3 and mutant strains R122, D5, and D57. From day 2 after inoculation, all wild-type- and R122-infected pigs had fever, most of them with temperatures above 41°C. The pigs were dull and had no appetite. On days 2 and 3, wild-type-infected pigs sneezed and scratched. One wild-type-infected pig died within 2 days and two wild-type-infected pigs died within 3 days after inoculation. The R122-infected pigs sneezed on day 2 and day 3 after inoculation, but none of the pigs died.

On day 2 after inoculation with strains D5 and D57, gD mutants that do not express either gI or gI plus gE, half of the pigs had fever. The maximum temperatures of D5- and D57-infected pigs were 40.3 and 40.6°C, respectively. Five days after inoculation, the temperatures of all pigs was normal again. Except for one animal that sneezed on day 3 after inoculation with D57, pigs inoculated with D5 or D57 developed no respiratory or neurological signs, although the pigs were dull and had less appetite shortly after inoculation. However, 3 days after inoculation with D5 and 2 days after inoculation with D57, the pigs had recovered.

Localization of viral antigen in the olfactory route. The results of the immunohistochemical investigations in tissues of the olfactory route after inoculation with the different PRV strains are summarized in Table 1. The organization of the synaptic connections in the olfactory route is shown in Fig. 2 (5). On day 2, viral antigen was detected in olfactory epithelium, stromal tissue, and axons of olfactory receptor neurons after inoculation with either wild-type strain NIA-3 or gD mutant strain R122 (Fig. 3a and b). Both viruses caused a widespread infection of the olfactory epithelium and stromal tissue, and large areas of the olfactory epithelium had sloughed off. However, the wild-type virus caused a more extensive in-

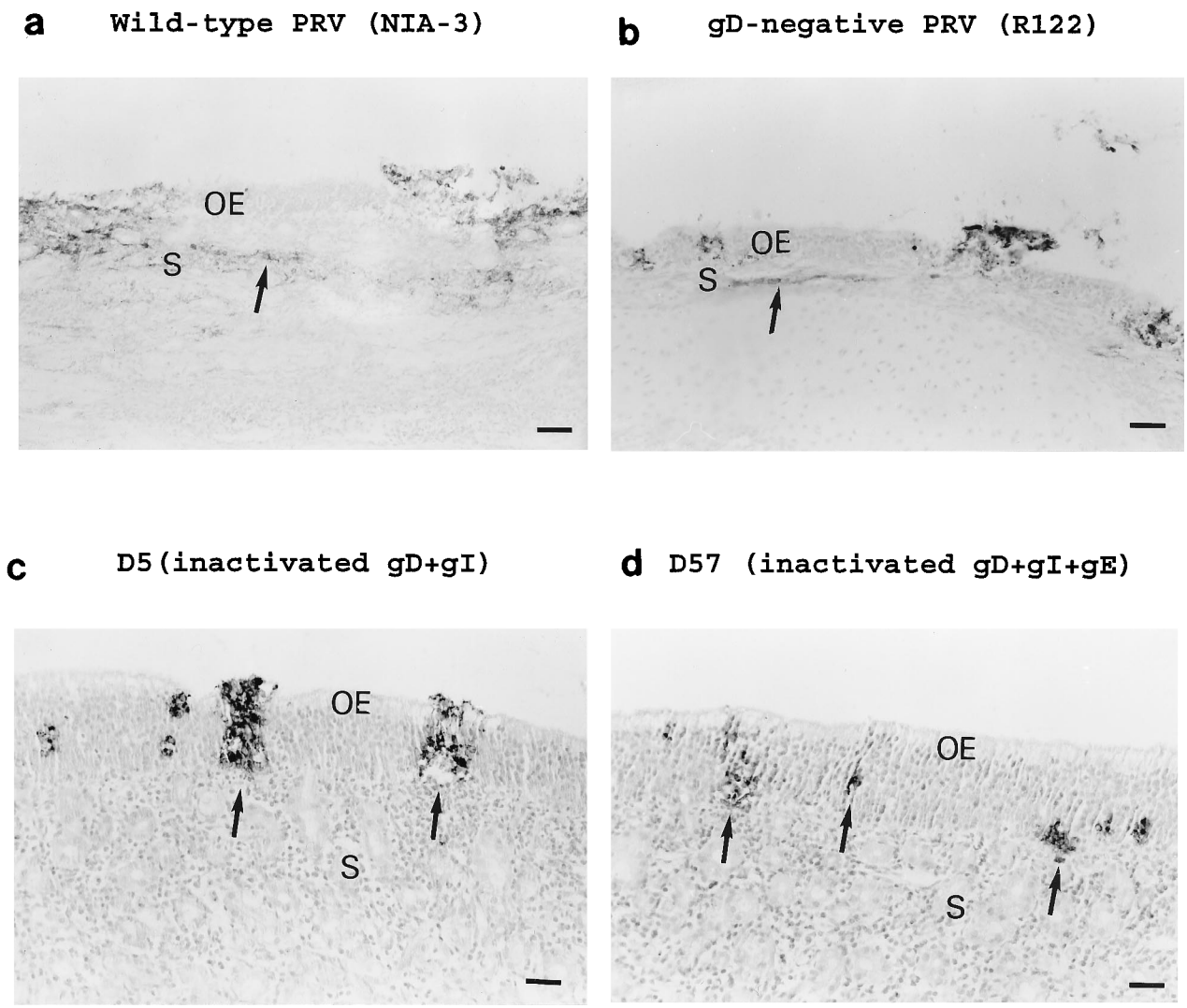

FIG. 3. Immunostains of viral antigen (dark stain) in the olfactory epithelium (OE) 2 days after infection with wild-type PRV strain NIA-3 (a), with gD-negative PRV strain R122 (b), with strain D5 (inactivated gD plus gI) (c), and with strain D57 (inactivated gD plus gI plus gE) (d). (a and b) Dense infection with wild-type PRV and R122 (gD^-). Viral antigen was also present in axons of the olfactory receptor neurons (arrows) located in the stromal (S) tissue. (c and d) Details of scattered infection with D5 (inactivated gD plus gI) and D57 (inactivated gD plus gI plus gE). Viral antigen (arrows) was present only in the olfactory epithelium. Scale bars, 50 μm (a and b) and 25 μm (c and d).

fection in these tissues than R122 did. On day 3, the R122 infection in the olfactory epithelium and stromal tissue had increased. Also on day 2, viral antigen of both virus strains was detected in olfactory nerve bundles in the ethmoid bone that lead into the olfactory bulbs (data not shown).

In contrast, double-mutant strains D5 and D57 caused very limited infections in the olfactory epithelium but no infection in stromal tissue. Two days after infection with D5 and D57, a few focal infections in the olfactory epithelium that contained less viral antigen than the infections caused by the wild-type virus or R122 were seen (Fig. 3c and d). On days 4 and 7 after inoculation with D5 or D57, no viral antigen was detected in the olfactory epithelium. Olfactory nerves bundles in the ethmoid bone that lead into the olfactory bulbs were not infected by strains D5 or D57.

Two days after inoculation with the wild-type virus, viral antigen was detected in the olfactory bulb in all three pigs (Fig. 4a). On day 2, viral antigen was detected in second-order neurons that synapse on the axons of the olfactory receptor neurons, i.e., in the periglomerular neurons, and in the mitral neurons. Viral antigen was also detected in third-order neurons of the olfactory bulb, the granule neurons. On day 3, the number of wild-type virus-infected second- and third-order olfactory neurons had increased. Widespread infections of periglomerular-, mitral, and granule neurons were seen (Fig. 4c). The transneuronal spread of strain R122 via the olfactory route was reduced compared with that of the wild-type virus. Strain R122 also infected the olfactory bulb on day 2 in two pigs. A few foci with viral antigen were detected below the glomeruli, the spherical structures where the axons of the olfactory receptor neurons end, but second- and third-order neurons of the olfactory route were not infected (Fig. 4b). Nevertheless, on day 3, R122 infected both second- and third-order neurons of the olfactory route in all pigs. Details of infected periglomerular neurons (Fig. 4d) and R122 (gD^-)-infected granule neurons (Fig. 4e) are shown. The infection of R122 in the olfactory bulb was reduced compared with that of the wild-type virus.

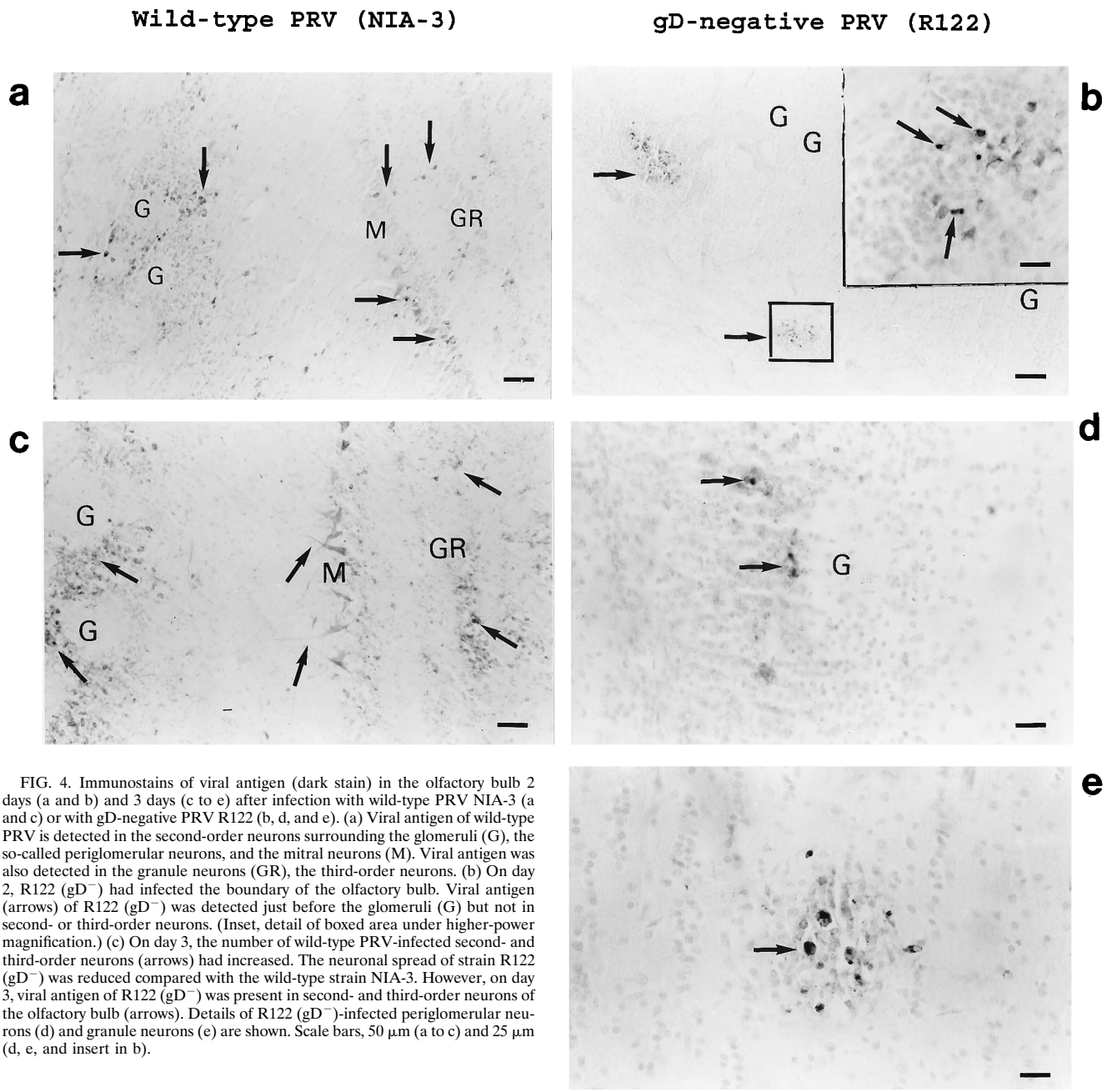

FIG. 4. Immunostains of viral antigen (dark stain) in the olfactory bulb 2 days (a and b) and 3 days (c to e) after infection with wild-type PRV NIA-3 (a and c) or with gD-negative PRV R122 (b, d, and e). (a) Viral antigen of wild-type PRV is detected in the second-order neurons surrounding the glomeruli (G), the so-called periglomerular neurons, and the mitral neurons (M). Viral antigen was also detected in the granule neurons (GR), the third-order neurons. (b) On day 2, R122 (gD^-) had infected the boundary of the olfactory bulb. Viral antigen (arrows) of R122 (gD^-) was detected just before the glomeruli (G) but not in second- or third-order neurons. (Inset, detail of boxed area under higher-power magnification.) (c) On day 3, the number of wild-type PRV-infected second- and third-order neurons (arrows) had increased. The neuronal spread of strain R122 (gD^-) was reduced compared with the wild-type strain NIA-3. However, on day 3, viral antigen of R122 (gD^-) was present in second- and third-order neurons of the olfactory bulb (arrows). Details of R122 (gD^-)-infected periglomerular neurons (d) and granule neurons (e) are shown. Scale bars, 50 μm (a to c) and 25 μm (d, e, and insert in b).

In contrast, D5 and D57 did not infect second- or third-order neurons of the olfactory bulb on day 2, 4, or 7 (Table 1).

Localization of viral antigen in the trigeminal route. The results of the immunohistochemical investigations of tissues of the trigeminal route after inoculation with the different PRV strains are also summarized in Table 1. The organization of the synaptic connections of the trigeminal route is shown in Fig. 2 (25, 26). On days 2 and 3, both the wild-type strain NIA-3 and gD mutant strain R122 infected many first-order neurons of the trigeminal ganglion in all pigs. No major differences in quantities of viral antigen in infected trigeminal neurons were observed on these days. Details of wild-type- and R122-infected trigeminal neurons and their efferent axons are shown in Fig. 5a and b.

In contrast, on days 2 and 4, double-mutant strains D5 and D57 infected a limited number of first-order neurons in the trigeminal ganglion. On day 2, in two pigs inoculated with D5 and in one pig inoculated with D57, viral antigen was detected in single infected trigeminal neurons (Fig. 5c and d). Also on day 4, in one pig inoculated with D5 and one pig inoculated with D57, single infected trigeminal neurons were detected. On day 7 after inoculation with either D5 or D57, no infected trigeminal neurons were detected. In contrast to infection with wild-type virus and R122, efferent axons of trigeminal neurons were not infected by D5 or D57 on any day (data not shown).

On day 2, in all three pigs inoculated with the wild-type virus and in two pigs inoculated with R122, viral antigen was detected in second-order neurons of the sensory nucleus of the

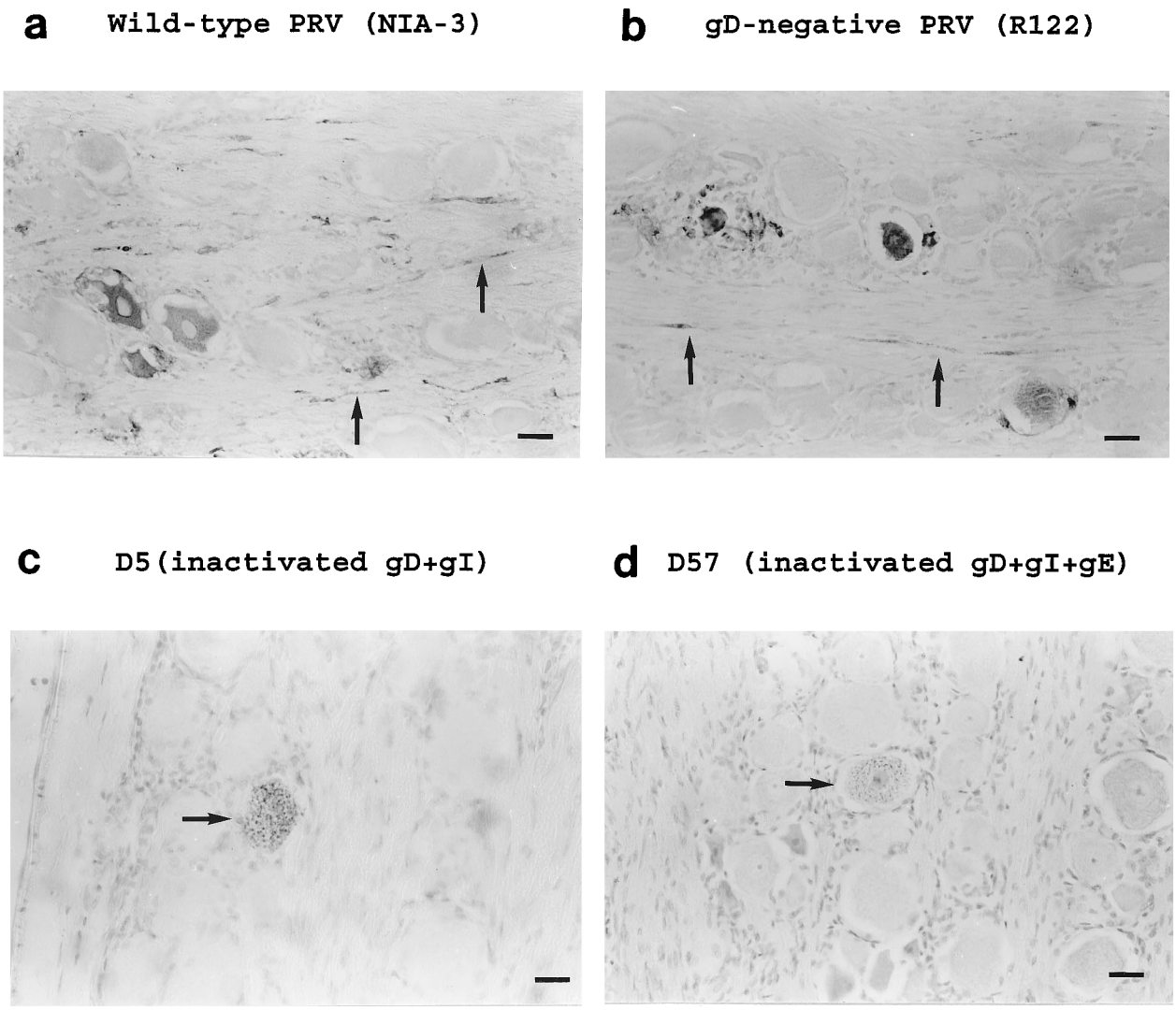

FIG. 5. Immunostains of viral antigen (dark stain) in the trigeminal ganglion 2 days after infection with wild-type PRV NIA-3 (a), gD-negative strain R122 (b), strain D5 (inactivated gD plus gI) (c), and strain D57 (inactivated gD plus gI plus gE) (d). (a and b) Details of wild-type PRV- and R122 (gD^-)-infected trigeminal neurons. Viral antigen was also present in efferent axons of trigeminal neurons (arrows). (c and d) Details of single D5 (inactivated gD plus gI)- and D57 (inactivated gD plus gI plus gE)-infected trigeminal neurons (arrows) are shown. No viral antigen of D5 (inactivated gD plus gI) and D57 (inactivated gD plus gI plus gE) was detected in efferent axons of trigeminal neurons. Scale bars, 50 μm (a and b) and 25 μm (c and d).

trigeminal nerve in the brain stem (Fig. 6a and b). The axons of the sensory neurons of the trigeminal ganglia synapse upon the soma and dendrites of neurons in these main sensory and spinal sensory nuclei. On day 2, the wild-type virus had infected more second-order neurons in the brain stem than R122 had. On day 3, the number of R122-infected neurons in the brain stem had increased and no major differences from the wild-type virus infection in the brain stem were seen (Fig. 6c and d). A similar infection pattern was observed for the second-order neurons of the spinal nucleus in the medulla oblongata. On day 2 in two pigs infected with wild-type virus and one pig infected with R122, viral antigens were detected in these neurons of the spinal nucleus. On day 2, the wild-type virus had infected more second-order neurons in the medulla oblongata than R122 had. Again, on day 3, both viruses had infected more of these neurons in all pigs (Table 1). On day 3, no major differences were seen in the immunostaining of the medulla oblongata between strain R122 and the wild-type virus.

Similar to the results obtained for the olfactory route, strains D5 and D57 did not spread transneuronally by the trigeminal route. No viral antigen was detected in the second-order neurons of the sensory nucleus of the trigeminal nerve in the brain stem or in the spinal nucleus in the medulla oblongata (Table 1).

DISCUSSION

The relative importance of gD for transneuronal spread of PRV leading to infection of the CNS and the pathological implications were investigated by using pigs, the natural host of PRV. The virulence of the phenotypically complemented gD mutants was reduced compared with that of the wild-type virus. Pigs inoculated with the wild-type virus developed both respiratory and neurological signs, and three of six pigs had died on day 3, whereas pigs inoculated with R122 developed less severe signs of disease. The immunohistochemical investigations of nervous tissues showed that the transneuronal spread of R122 into the CNS was reduced compared with that of the wild-type virus. As a consequence, R122-inoculated pigs will probably

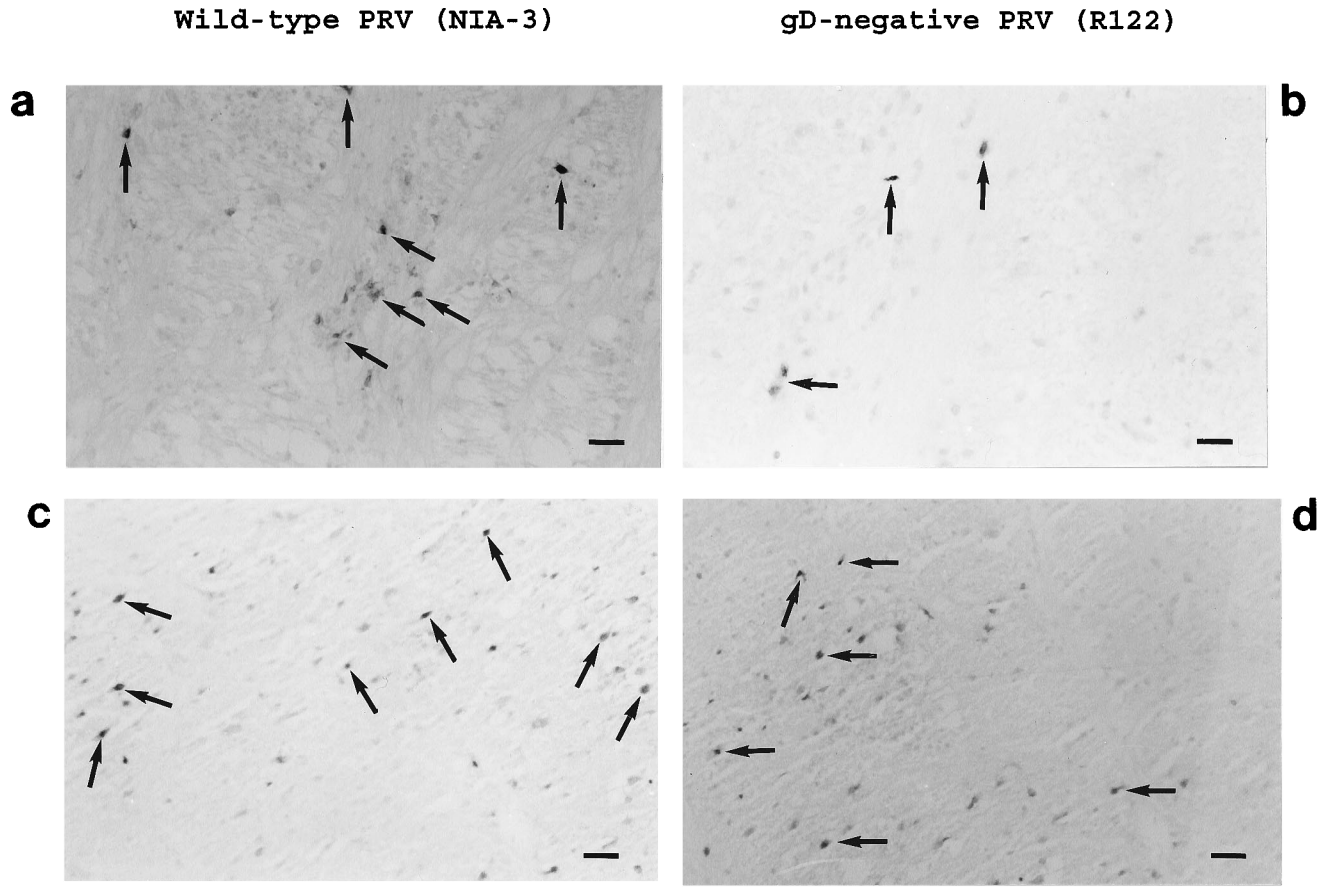

FIG. 6. Immunostains of viral antigen (dark stain) in the brain stem, 2 days (a and b) and 3 days (c and d) after infection with wild-type PRV NIA-3 (left panel) or with gD-negative PRV R122 (right panel). (a and b) Viral antigen (arrows) of wild-type PRV and R122 (gD^-) was detected in the second-order neurons of the main trigeminal nucleus of the trigeminal nerve in the brain stem. R122 (gD^-) had infected less of these neurons than the wild-type PRV had. (c and d) On day 3, the number of R122 (gD^-)-infected second-order neurons in the brain stem had increased and no major differences were observed with the wild-type PRV infection. Scale bars, 50 μm.

develop the corresponding neurological signs later than pigs infected with the wild-type virus or the emerging immune responses may prevent this. Therefore, the period of 3 days may have been too short to monitor neurological signs caused by R122 infection. However, pigs inoculated with a lower dose (10^5 PFU) of R122 did not develop neurological signs (34).

Pigs inoculated with gD mutant strains D5 and D57, which are unable to express either gI or gI plus gE, were monitored until 7 days after inoculation. With the exception of one pig that sneezed on day 3 after inoculation with strain D57, no respiratory or neurological signs were noted after inoculation with these viruses. The inability of double mutants D5 and D57 to spread efficiently in peripheral tissues and subsequently invade the CNS probably causes their lack of virulence for pigs.

The transneuronal spread of wild-type virus and its derived gD-negative strains into and within the porcine CNS via the olfactory and trigeminal routes was investigated immunohistochemically. We showed that R122 gD mutants are able to spread transneuronally and to reach second- and third-order neurons in the CNS. Compared with the wild-type virus, the spread of R122 was reduced; as a consequence, this strain infected fewer second-order neurons shortly after infection. This difference in transneuronal spread between wild-type virus and gD-negative virus was most clear in the olfactory route on day 2. Previous findings demonstrated that gD is not required for viral spread both in vitro and in mice (1, 36, 37). Our findings show that gD is also not required for transneuronal spread in pigs, its natural hosts. The exact mechanism for spread of herpesviruses through neurons and across synapses is unknown. Card et al. (4) suggested that transneuronal transfer of PRV occurs in a stepwise fashion and requires the fusion of viral envelopes with the synaptic membranes, leading to release of virions in the synaptic cleft and subsequent reinfection of postsynaptic neurons. Our finding that transneuronal transfer of PRV can occur independently of gD suggests that transneuronal transfer of PRV may occur by similar mechanisms as cell-to-cell spread of PRV in tissue culture. We speculate that the nature of the cell surface may determine whether gD is required for penetration. It has been reported that apical attachment of herpes simplex virus type 1 to polarized MDCK cells depends on gC but that basal attachment is gC independent (46). Similarly, gD may be required to allow entry of PRV only via the apical cell surface but not via the lateral or basal cell surface. Different proteoglycans present on the cell surface may be responsible for different interactions with the virus (20). For instance, heparan sulfate proteoglycan has been shown to play a major role in the gC-mediated attachment of herpes simplex virus type 1 and PRV to cells (47).

In contrast to gD, gE and gI or the gE-gI complex are required for transneuronal spread. Previous studies show that

gE-negative PRV replicates in peripheral tissues, infects first-order neurons, and spreads toward the CNS via both the olfactory and trigeminal routes. However, gE-negative PRV has a greatly reduced capacity to infect second- and third-order neurons in the porcine CNS (22, 23, 32). Enquist et al. (reference 10 and references therein) used genetic complementation to demonstrate that the effect of gE or gI on transneuronal spread occurs in some central visual projection pathways of the rat retina and occurs after entry of PRV in first-order neurons. In vitro, the gE-gI complex promotes cell-cell spread and is involved in release of the virus from cells and in cell fusion (reviewed in reference 13). Thus, the gE-gI complex may promote the release of virus from first-order neurons or the fusion of synaptically linked neurons. Finally, we cannot completely exclude the possibility that the gE-gI complex fulfills other functions in transneuronal transfer of PRV such as promoting intraneuronal transport of the virus.

In contrast to strain R122, gD double mutants D5 and D57, which lack expression of either gI or gI plus gE, did not infect second- or third-order neurons of the CNS. The lack of neuroinvasiveness by D5 and D57 is probably caused by two combined defects. Deletion of gD in D5 or D57 progeny virions prevents reinfection of other cells. In addition, lack of expression of either gI or gI plus gE will reduce both the cell-to-cell spread and the transneuronal spread of the virus. Thus, inactivation of both gD and gE plus gI will affect both pathways of viral spread in peripheral tissues and will also affect transsynaptic transport of PRV into and within the CNS.

Phenotypically complemented gD mutants are promising candidates for the development of a new generation of safer (carrier) vaccines, because progeny virions are noninfectious and prevent spread to other animals, including other species (12, 31, 34). Inactivation of gD alone might not be sufficient for development of completely nonvirulent PRV vaccines. As demonstrated, PRV can be further attenuated by inactivation of gI or gE or inactivation of viral genes involved in DNA replication (thymidine kinase, ribonucleotide reductase), or phosphorylation (US3-encoded protein kinase) (6, 18, 19). However, further attenuation will reduce the viral spread and, as a consequence, may reduce the immunogenicity (18, 34). Thus, further attenuation will increase the safety but may decrease the efficacy of PRV gD mutants as (carrier) vaccines.

We conclude that transsynaptic spread of PRV in pigs can occur independently of gD. Future research should clarify the mechanism for cell-to-cell and neuron-to-neuron transmission of PRV.

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