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Glycoprotein gI of Pseudorabies Virus Promotes Cell Fusion and Virus Spread via Direct Cell-to-Cell Transmission

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Mutants of pseudorabies virus defective in either glycoprotein gI or gIII are only slightly less virulent for mice and chickens than is wild-type virus, while mutants defective in both gI and gIII are avirulent. To clarify the reason for the lack of virulence of the gI$^-$ gIII$^-$ mutants, we have analyzed in some detail the interactions of these mutants with their hosts. The results obtained showed that the gI glycoprotein is an accessory protein that promotes cell fusion. This conclusion is based on the findings that in some cell types, syncytium formation is significantly reduced in mutants deficient in gI. Furthermore, despite efficient replication, gI$^-$ mutants form significantly smaller plaques on some cell types. Finally, while wild-type and gI$^-$ virus are neutralized similarly by antisera, the size of the plaques formed by gI$^-$ mutants, but not by wild-type virus, is reduced by the presence of neutralizing antibodies in the overlay. Passive immunization of mice with neutralizing antipseudorabies virus sera is also considerably more effective in protecting them against challenge with gI$^-$ mutants than in protecting them against challenge with wild-type virus. These results show that gI$^-$ mutants are deficient in their ability to form syncytia and to spread directly by cell-to-cell transmission and that these mutants spread mainly by adsorption of released virus to uninfected cells. Wild-type virus and gIII$^-$ mutants, however, spread mainly via direct cell-to-cell transmission both in vivo and in vitro. We postulate that the lack of virulence of the gIII$^-$ gI$^-$ virus is attributable to its inability to spread by either mode, the defect in gIII affecting virus spread by adsorption of released virus and the defect in gI affecting cell-to-cell spread. Although a gI$^-$ gIII$^-$ mutant replicates as well as a gIII$^-$ mutant, it will be amplified much less well. Our results with in vitro systems show that this is indeed the case.

Pseudorabies virus (PrV), a herpesvirus of pigs, causes latent as well as acute, often fatal, infection of the nervous system in pigs and acute infection in other domestic and wild animals (10). Not much is known about the viral functions that affect the virulence of either human herpesviruses such as herpes simplex virus (HSV) and varicella-zoster virus or of PrV; because of the complexity of these viruses, information concerning the genetic basis of their virulence is just beginning to emerge.

Our previous efforts to determine the functions necessary for the expression of virulence of PrV have centered around two complementary approaches: (i) marker rescue of avirulent strains, thereby attempting to identify the functions that are defective in these strains, and (ii) introduction of mutations into genes of wild-type PrV(Ka) encoding functions nonessential for growth in vitro and ascertainment of how these mutations affect both virus growth in cell culture and virulence. The main conclusions that could be drawn from these analyses were that defects in several different loci of the PrV genome may affect virulence without detectably affecting virus growth in certain cell types (14, 15) and that defects in three of the nonessential viral glycoproteins of PrV (gI, gp63, and gIII) can affect virulence (20, 21). Because these glycoproteins are present in all primary field isolates, even though the virus replicates in their absence in cell culture, it is likely that these glycoproteins play an essential role in the interaction of the virus with its animal hosts, i.e., in the pathogenesis of the virus. A detailed analysis of the role of these glycoproteins in the growth of the virus is therefore of interest.

Glycoproteins gI, gp63, and gIII are the homologs of glycoproteins gE, gI, and gC, respectively, of HSV (24, 25, 27, 28). While mutants defective in gI, gp63, or gIII are only slightly, if at all, less virulent than wild-type virus, the double mutants gI$^-$ gIII$^-$ and gp63$^-$ gIII$^-$ are almost completely avirulent for mice as well as chickens and pigs (20, 21).

Even though gIII is the main glycoprotein that mediates adsorption of the virus to cells in culture (in its absence adsorption is considerably reduced [29, 37]), mutants deficient in gIII retain levels of virulence similar to those of wild-type virus. The high level of virulence of gIII$^-$ mutants may reflect the fact that in vivo virus spread is not mediated mainly by adsorption, the main mode of virus spread being direct transmission of virus from one cell to another. Alternatively, it is known that the requirements for gIII in adsorption to different cell types vary (22) and that gIII may not be essential for adsorption of the virus to its target cells in vivo. The reason behind the observation that mutants deficient in gIII, gI, or gp63 retain essentially wild-type virus levels of virulence while the double mutants gI$^-$ gIII$^-$ and gp63$^-$ gIII$^-$ have lost most of their virulence remained obscure.

It was to clarify the reason for both the high level of virulence of the gIII$^-$ mutants and the loss of virulence of the gI$^-$ gIII$^-$ mutant that the experiments described here were designed. In these experiments, we have attempted to ascertain how a defect in either gI or gIII or in both may affect virus spread. The salient findings to emerge were that while wild-type virus can spread by direct transmission from

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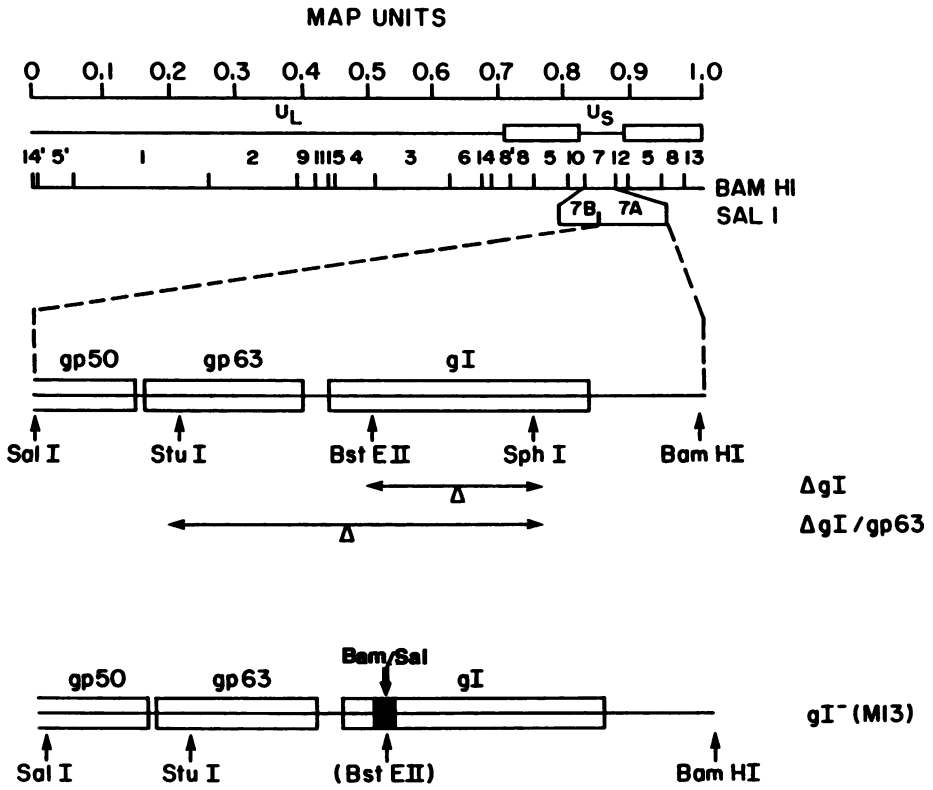

FIG. 1. Restriction map of the genome of PrV indicating the structure of the gI⁻ mutants. Lines are numbered from top to bottom: lines 1 to 3, Structure and *Bam*HI restriction map of PrV DNA; line 4, map of the *Bam*HI-*Sal*I fragment 7A; lines 5 and 6, arrows indicate the deletions introduced into the gI⁻ and gI⁻ gIII⁻ mutants; line 7, structure of the gI⁻ deletion-M13 insertion mutants. In these mutants, approximately 200 bp flanking the *Bst*EII site was removed by digestion with *Bal* 31 nuclease and a *Hae*III fragment of M13, 285 bp in size, that includes the multiple cloning site was inserted (21).

an infected cell to adjacent uninfected cells, the ability of gI⁻ mutants to do so is diminished. Virus spread can also occur after release of the virus from the infected cells, followed by its adsorption to uninfected cells. While this is the main mode of spread of the gI⁻ mutants, it is probably impaired in gIII⁻ mutants because adsorption of gIII⁻ PrV to its host cells is known to be poor (29). We postulate that interference with either one of these modes of spread (cell-to-cell transmission in gI⁻ mutants and adsorption in gIII⁻ mutants) does not greatly affect virulence because the virus can spread by either mode. Interference with both modes of spread, such as in gI⁻ gIII⁻ mutants, will drastically reduce the virulence of the virus.

MATERIALS AND METHODS

Virus mutants and cell culture. PrV(Ka) is a strain that has been carried in our laboratory for more than 30 years. The isolation and characterization of the gIII⁻, gI⁻, gI⁻ gIII⁻, and gI⁻ gp63⁻ gIII⁻ mutants of PrV(Ka) used have been described previously (18, 19, 29). The product of the gI gene forms a complex with the gp63 gene. Mutants deficient in gI, gp63, or gI and gp63 behave similarly. Thus, gI⁻ mutants, gp63⁻ mutants, and gI⁻ gp63⁻ mutants are functionally equivalent (38). The gIII mutants of the Becker strain, PrV2, PrV4, and PrV10, were obtained from Lynn Enquist (DuPont); their characteristics have been described previously (33). The gI gene was deleted from wild-type PrV(Be), from PrV2, and from PrV4, as had been done previously with the PrV(Ka) strain (19). A restriction map of the regions of the gI gene of the genomes of these mutants is illustrated in Fig. 1. Madin-Darby bovine kidney (MDBK), rabbit kidney (RK), pig kidney (PK), and pig testis cells as well as chicken embryo fibroblasts (CEFs) were cultivated in Eagle's medium containing calf serum (5%).

Antisera. Mouse PrV antiserum was obtained by injecting mice four times at intervals of 3 weeks with 10^5 PFU of PrV(Ka)TK⁻ (i.e., a mutant from which the thymidine kinase gene had been inactivated). The mice were bled 10 days after the last injection. Goat anti-gII antiserum was a generous gift from Lynn Enquist. Incubation of PrV(Ka) for 1 h with a 1:5,000 dilution of the mouse antiserum and with a 1:400 dilution of the goat anti-gII serum reduced the virus titer by approximately 50%.

Determination of LD_{50}. Tenfold dilutions of virus stocks were injected into mice intramuscularly into the right hind leg. The number of animals that died each day up to 2 weeks after inoculation (no animals died later than 7 days after inoculation) was determined, and the 50% lethal dose (LD_{50}) obtained was calculated by the Reed and Muench method (26).

Plaque assay. The virus stocks were titrated on RK, MDBK, PK, or CEF monolayers grown in 50-mm-diameter petri plates. After a 1-h adsorption period, the unadsorbed virus inoculum was removed by washing and the cells were overlaid with Methocel (1%) in Eagle's medium. In some cases, anti-PrV sera were incorporated into the overlay. At daily intervals the sizes of the plaques were measured either

by using an inverted microscope with an ocular containing a micrometer or by measuring the sizes of the plaques in fixed and stained monolayers.

Passage of virus in cell culture. Cells were infected at a low multiplicity (0.01 PFU per cell) with a mixture of wild-type virus and a mutant or with a mixture of two mutants. After a cytopathic effect had developed, fresh cultures were infected at a low multiplicity (approximately 0.01 PFU per cell) with the virus progeny. The process was repeated three times. The viral particles present in the original virus mixture used to infect the cultures, as well as those present after the third passage in cell culture, were purified, and viral DNA was isolated. The DNA was digested with the appropriate restriction enzymes, electrophoresed, transferred to nitrocellulose paper, and hybridized to nick-translated probes. The ratio of the two type virions in the populations before and after passage in cell culture was ascertained from the ratio of the restriction fragments diagnostic of each virus type.

RESULTS

Rationale for the premise that gI may promote direct cell-to-cell spread. Glycoprotein gIII is important in mediating adsorption of PrV to its host cells (29), as well as in mediating virus release (29, 33). Glycoprotein gI has also been shown to affect virus release either by itself (the absence of gI enhances release from CEFs [18]) or in conjunction with gIII (gI^- $gIII^-$ virions are released poorly from RK cells [29, 34]). During the course of experiments designed to ascertain the biological characteristics of these mutants, we also observed that despite the fact that gI^- virus replicated as well as wild-type virus and was released more effectively than was wild-type virus from CEFs, it consistently formed smaller plaques on these cell monolayers. Mutants defective in gI sometimes also formed somewhat smaller plaques than did wild-type virus on RK and MDBK cells, but the effect was not consistent, and it is possible that the physiologic state of the cells plays a role in this phenomenon. Furthermore, syncytium formation did not appear to be as extensive in cells infected with mutants deficient in gI as in cells infected with wild-type virus. These observations led us to the proposition that gI may be an accessory protein that promotes cell fusion and that consequently gI^- mutants may have a reduced ability to spread via direct cell-to-cell transmission. This assumption provides an explanation for the observation that while gI^- or $gIII^-$ mutants are as virulent as is wild-type virus, the double gI^- $gIII^-$ mutants are almost avirulent. Thus, wild-type virus would spread in vivo both (or either) by direct cell-to-cell transmission and via adsorption of virus that has been released from infected cells to uninfected cells. Since $gIII^-$ mutants are defective in adsorption (29), virus spread by adsorption to uninfected cells would proceed poorly and these mutants would spread mainly via direct cell-to-cell transmission. If, on the other hand, cell-to-cell transmission were reduced in gI^- virus-infected cells, this virus mutant would spread mainly via adsorption of released virus to uninfected cells. The gI^- $gIII^-$ mutants, being defective in both modes of virus spread, would be unable to spread efficiently and therefore would be avirulent.

The growth characteristics of the mutant viruses that affect virus spread in the animal should also, in principle, affect virus spread in at least some cell types in culture. We therefore first attempted to test the prediction that direct cell-to-cell spread of gI^- virus is impaired using cell cultures infected in vitro.

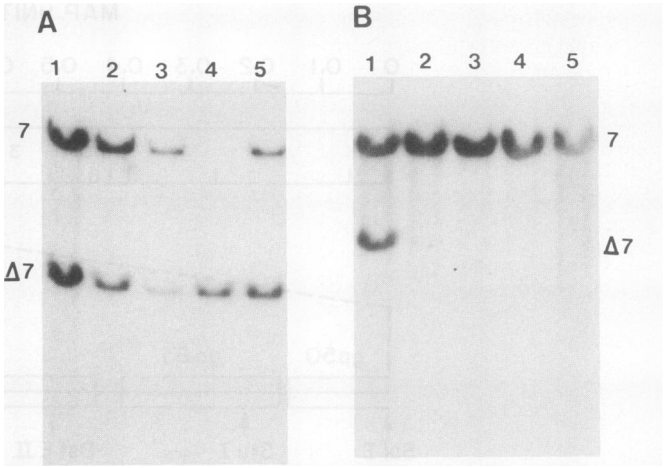

FIG. 2. Effect of passage in cell cultures of mixtures of the wild type and a gI^- $gp63^-$ mutant and of $gIII^-$ and $gIII^-$ gI^- $gp63^-$ mutants. Mixtures of equal numbers of PFUs of wild-type virus and a gI^- $gp63^-$ mutant of PrV(Ka) (A) or a $gIII^-$ mutant and a $gIII^-$ gI^- $gp63^-$ mutant of PrV(Ka) (B) were passaged three times at a low multiplicity (0.01 PFU per cell) in four different cell types. The virions in the original virus mixture, as well as after passage in cell culture, were purified, digested with *Bam*HI, electrophoresed, transferred to nitrocellulose filters, and hybridized to nick-translated *Bam*HI fragment 7. Lanes 1, original virus mixture; lanes 2, after passage in MDBK cells; lanes 3, after passage in RK cells; lanes 4, after passage in CEFs; lanes 5, after passage in PK cells.

Growth advantage or disadvantage of gI^- mutants when passaged at low multiplicity. We have previously determined the growth of gI^-, $gIII^-$, gI^- $gIII^-$, and gI^- $gp63^-$ $gIII^-$ mutants in single-step growth experiments in cell cultures (34). No difference in the growth of gI^- virus and wild-type virus was detected, nor was there any difference between the growth of $gIII^-$ and $gIII^-$ gI^- or $gIII^-$ gI^- $gp63^-$ virus. The only notable difference observed was that cells infected with the mutants defective in gIII ($gIII^-$, gI^- $gIII^-$, and gI^- $gp63^-$ $gIII^-$) yielded lower titers than did those infected with wild-type virus or $gp63^-$ or gI^- mutants. This was shown to be due to the lower specific infectivity of the $gIII^-$ mutants resulting from the poor adsorption of these mutants (29); the titers of the $gIII^-$ mutants could be significantly increased by assaying the virus under conditions which promote its adsorption (35). Of particular interest was the finding that even though no differences between the growth characteristics of these viruses in single-step growth experiments were detected, $gIII^-$ virus was virulent and gI^- $gIII^-$ virus was not (20).

Figure 2 illustrates the results of an experiment in which cells were infected at low multiplicity with a mixture of two viruses. In this type of experiment, the growth of the mutants after several cycles of replication is compared so that virus spread, as well as virus growth, affects virus amplification. A mixture of gI^- $gp63^-$ mutants and of wild-type virus and a mixture of gI^- $gp63^-$ $gIII^-$ mutant (gI and gp63 form a complex; mutants defective in gI, gp63, or both behave similarly [38]) and of a $gIII^-$ virus were grown in four different cell lines. The behavior of the gI^- $gp63^-$ mutant rather than that of the gI^- mutant is illustrated here because it is easier to distinguish the wild type from a gI^- $gp63^-$ mutant than to distinguish the wild type from a gI^-

mutant by restriction fragment analysis. However, the same experiment was also performed with mixtures of the wild type and a gI$^-$ mutant or of gIII$^-$ gI$^-$ and gIII$^-$ mutants, and identical results were obtained.

The gI$^-$ gp63$^-$ mutant replicated and spread as well as did wild-type virus in MDBK, PK, and RK cells (Fig. 2A); the ratio of the bands characteristic of gI$^-$ gp63$^-$ to those characteristic of wild-type virus did not change appreciably before and after passage in these cell cultures. As expected, the lack of gI and gp63 conferred a decided growth advantage on the virus in CEFs (Fig. 2A). This is probably because of the enhanced release of gI$^-$ gp63$^-$ mutants from these cells (18, 34), a result that confirms previously published data which showed that mutants deficient in either gI or gp63 behave in a similar manner (38). Thus, no evidence that gI may be necessary to mediate virus spread in cell culture was obtained.

When a mixture of gIII$^-$ and gIII$^-$ gI$^-$ gp63$^-$ viruses was similarly passaged, the lack of the gI gp63 genes did affect the ability of the virus to be amplified (Fig. 2B). After passage of the virus mixture in all four cell types, the band characteristic of the gI$^-$ gp63$^-$ virus had disappeared, indicating a distinct growth disadvantage for the gIII$^-$ gI$^-$ gp63$^-$ mutants relative to the gIII$^-$ mutants. Thus, the deletion of gI and gp63 confers a growth disadvantage when gIII is also defective (Fig. 2B) but does not have a similar effect when a functional gIII is present (Fig. 2A). Similar results were also obtained when the same experiment was performed using mixtures of gIII$^-$ and gIII$^-$ gI$^-$ viruses or gIII$^-$ and gIII$^-$ gp63$^-$ viruses (data not shown). When a mixture of wild-type virus and gIII$^-$ virus was passaged (data not shown), the gIII$^-$ virus had a decided growth disadvantage because gIII$^-$ virus adsorbs poorly (29).

Thus, the gI$^-$ gIII$^-$ mutant has a growth disadvantage relative to the gIII$^-$ mutant, but the gI$^-$ virus does not have a growth disadvantage relative to the wild type. The growth disadvantage of the gI$^-$ gIII$^-$ virus relative to the gIII$^-$ virus is detectable only under conditions when virus spread and recycling are required. These observations are consistent with the notion that gI is necessary for efficient cell-to-cell transmission but that gI$^-$ virions, even though deficient in cell-to-cell spread, can spread efficiently by adsorption of released virus to uninfected cells. However, gIII$^-$ gI$^-$ mutants cannot do so efficiently because the gIII glycoprotein is necessary for efficient adsorption. The fact that the gI$^-$ viruses are amplified as well as is the wild type (Fig. 2A) indicates that cell-to-cell transmission of these viruses does not contribute sufficiently to virus spread (under the conditions when spread is not limited by a Methocel overlay) to be detectable against the background of the high level of readsorption of the released virus. However, against a background of a gIII$^-$ phenotype, when adsorption is poor, the poor cell-to-cell transmission resulting from the defect in gI confers a decided growth disadvantage on the virus.

To confirm that it is a defect in adsorption due to the lack of a functional gIII that, in conjunction with a defect in the gI gp63 genes, results in the growth disadvantage of the gIII$^-$ gI$^-$ gp63$^-$ mutant, we performed a similar experiment using another mutant, PrV2, which has an internal in-frame deletion that removes 134 amino acids from its gIII gene (33, 36a). PrV2 adsorbs well to MDBK cells and to CEFs but adsorbs poorly to PK and RK cells (36). If our interpretation of the experiments described above is correct, the deletion of gI from PrV2 should confer upon the virus a growth disadvantage in PK and RK cells (to which it adsorbs poorly) but not in CEF and MDBK cells (to which it adsorbs well).

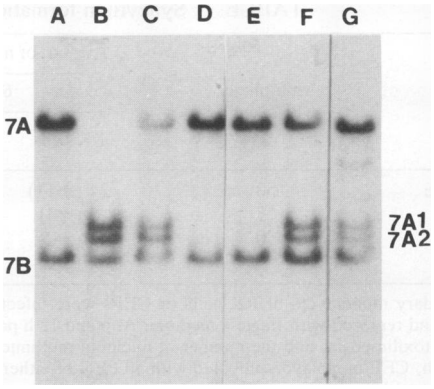

FIG. 3. Effect of passage in cell culture of mutants PrV2 and PrV2gI$^-$. PrV2 and PrV2gI$^-$ viruses were mixed to give approximately the same number of PFU and were treated as described in the legend to Fig. 2. PrV2gI$^-$ is a gI deletion-M13 insertion mutant into which the multiple cloning site of M13 has been inserted (see Fig. 1 for map); it includes a *Bam*HI site and an adjacent *Sal*I site that are not present in the wild-type gI gene. The DNA was digested with *Sal*I and *Bam*HI, electrophoresed, transferred to nitrocellulose filters, and hybridized to nick-translated *Bam*HI fragment 7. Lane A, PrV2 DNA; lane B, PrV2gI$^-$ DNA; lane C, before passage in cell culture; lane D, after passage in MDBK cells; lane E, after passage in RK cells; lane F, after passage in CEFs; lane G, after passage in PK cells.

(As expected, the deletion of the gI gene from PrV2 does not affect its adsorption to either MDBK or PK cells ([data not shown]).

Passage at a low multiplicity of a mixture of PrV2 and PrV2gI$^-$ in CEF and MDBK cells did not change significantly the ratio of the bands diagnostic of PrV2 and of PrV2gI$^-$; i.e., the two mutants were amplified similarly in these cells (Fig. 3). However, PrV2gI$^-$ had a distinct growth disadvantage in PK cells and RK cells relative to PrV2; after three cycles of growth at a low multiplicity of infection in these cells, the bands diagnostic of the PrV2gI$^-$ virus were no longer detectable. Since RK and PK cells are cell types to which PrV2 adsorbs poorly while MDBK and CEF are cell types to which it adsorbs well (36), these results are consistent with the premise that gI mutants have no significant growth disadvantage when adsorption can occur efficiently, i.e., when the gIII protein is functional. However, when adsorption is defective, gI$^-$ virus has a growth disadvantage under conditions which require recycling of the virus. These findings indicate that gI may be necessary for effective cell-to-cell spread.

Formation of multinucleated cells in cultures infected with the wild type and gI$^-$ virus. As mentioned above, differences in the cytopathic effect produced by infection of cells with gI$^-$ mutants and with wild-type virus had been observed; less-extensive syncytia appeared in cell cultures infected with gI$^-$ virus than in cultures infected with wild-type virus. Because the type of cytopathic effect observed and the sizes of the syncytia formed are often affected by the initial multiplicity of infection, we quantitated the degree of syncytium formation under conditions in which the multiplicity of infection was rigorously controlled. Table 1 summarizes the results of an experiment in which the sizes of multinucleated cells (the number of nuclei per syncytium) in cultures infected at different multiplicities of infection with gI$^-$ and wild-type virus was ascertained. In RK cells, extensive syncytia were observed by 6 h after infection with wild-type

TABLE 1. Syncytium formation in cultures infected with the wild type and with gI⁻ mutant[a]

Virus	Multiplicity (PFU/cell)	Avg no. of nuclei/multinucleated cell (± variation) at:			% of nuclei in multinucleated cells at:		
		6 h in:		12 h in RK cells[b]	6 h in:		12 h in RK cells[b]
		RK cells	CEFs		RK cells	CEFs	
Wild type	50	24 (±11)	6 (±3)	80 (±25)	87	43	100
	5	8 (±4)		63 (±24)	89		100
gI⁻	50	4 (±2)	0	10 (±5)	45	0	93
	5	2 (±1)		9 (±5)	14		83

[a] Secondary monolayers of RK cells or CEFs were infected with the indicated multiplicity of infection. After a 1-h adsorption period, the inoculum was removed and replaced with Eagle's medium. At 6 and 12 h postinfection the medium was removed, the monolayers were fixed with glutaraldehyde and stained with hematoxilin-eosin, and the number of nuclei in multinucleated cells was counted.

[b] At 12 h, CEF monolayers infected with 50 PFU of either virus exhibited many rounded cells.

virus. Multinucleated cells also appeared at that time in RK cultures infected gI⁻ virus, but they were significantly smaller. Fewer and smaller syncytia were formed in wild type-infected CEFs than in RK cells. The syncytia formed in gI⁻ virus-infected CEFs were much smaller than those formed in wild type-infected CEFs, and in the experiment summarized in Table 1 they were undetectable. Thus, differences in the size of the multinucleated cells produced by gI⁺ and gI⁻ viruses could be detected in both cell types. It appears therefore that gI promotes the formation of syncytia but that its absence does not completely preclude their formation.

It should be mentioned that the degree of reduction in syncytium formation in cells infected with gI⁻ virus compared with formation in wild-type-virus-infected cells is dependent on the physiologic state of the cells. Thus, a significant difference between gI⁻ and wild-type viruses in the size of the syncytia formed is observed in RK cultures when the experiment is performed within 7 days after explanation of the cells. This difference becomes less marked as the cells become older.

Analysis of the early interactions of gI⁻ mutants with host cells. Entry of enveloped viruses into their host cells is believed to occur by fusion of the viral and cellular membranes (for a review, see references 31 and 32). Since glycoprotein gI of PrV appears to play a role in syncytium formation, i.e., in cell fusion, it seemed possible that entry of the virus into the cells and initiation of the infectious cycle could also be affected by the lack of gI. However, as mentioned above, virus replication of gI⁻ and wild-type viruses proceeds similarly (Fig. 2A), and differences between these mutants at the level of initiation of the infectious process would therefore not be expected. Indeed, no difference in the rates of adsorption of the wild type and gI⁻ virus or of gIII⁻ and gIII⁻ gI⁻ viruses to MDBK cells (29) or to RK cells and CEFs (data not shown) was observed. Development of resistance to low pH, i.e., virus penetration, between the wild-type and gI⁻ virus after inoculation of MDBK cells (Fig. 4) or PK cells (data not shown) was also similar. Furthermore, no difference in the time of initiation of immediate-early and early protein synthesis in CEFs or RK cells infected with the wild type or with gI⁻ virus could be detected (data not shown). Thus, if gI plays an auxiliary role in the fusion of the viral and cellular membranes during virus entry into the cells, the effect is too subtle to be detected by the means we have used.

Effect of antiserum on the sizes of plaques formed by wild-type, gI⁻, gIII⁻, and gI⁻ gIII⁻ virions. To ascertain whether cell-to-cell virus spread is indeed impaired in gI⁻ virus-infected cells, we have determined the effect of the presence of neutralizing antibodies in the overlay on plaque development. The rationale for this experiment was that the presence of neutralizing antibodies should not affect the kinetics of plaque development if the main mode of virus spread is direct cell-to-cell transmission. On the other hand, if the main mode of virus spread is via adsorption of released

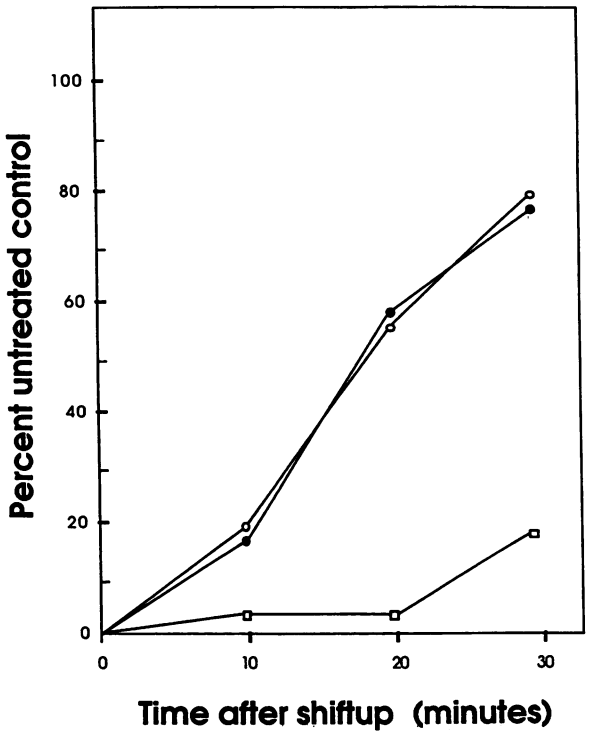

FIG. 4. Acquisition of low-pH resistance by adsorbed wild-type virus and a gI⁻ mutant. MDBK cells in 50-mm-diameter plastic dishes were preincubated for 20 min at 4°C and infected with approximately 200 PFU of virus. They were incubated for 1 h, when unadsorbed virus was removed by washing and the cells were incubated at 37°C in prewarmed Eagle's medium. At various times after temperature shift-up, the plates were washed once with phosphate-buffered saline and incubated either in phosphate-buffered saline (control plates) or with citric acid buffer (40 mM citric acid, 10 mM KCl, and 135 mM NaCl [pH 3.0]) for 2 min at room temperature. The plates were washed again and overlaid with agarose. Plaques were counted 4 days later. ●, wild-type virus; ○, gI⁻ virus; □, gIII⁻ virus.

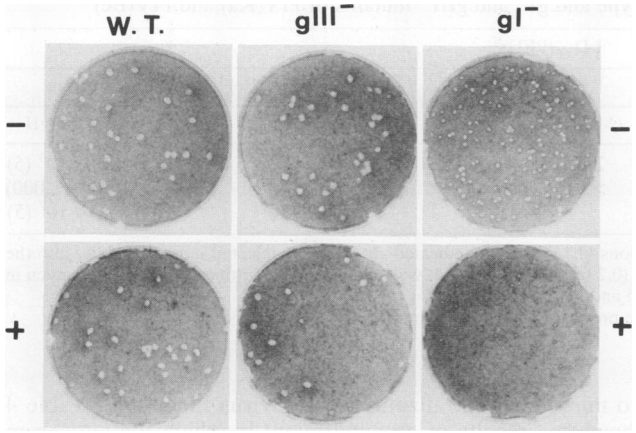

FIG. 5. Effect of antiserum on the sizes of plaques formed by the wild type (W.T.) and gI⁻ and gIII⁻ mutants on CEF cells. The viruses were assayed on CEFs and overlaid either with Methocel (−) or with Methocel containing anti-gII goat antiserum at a final dilution of 1:100 (+).

virus to adjacent cells, the presence of the serum should cause a decrease in the sizes of the plaques because of the neutralization of the released virus. The results obtained are illustrated in Fig. 5 and summarized in Table 2.

In the absence of antiserum in the overlay, the sizes of the plaques of wild-type virus and gIII⁻ virus formed on CEF monolayers were the same. The sizes of the plaques of the gI⁻ mutants were significantly smaller; the diameters of the gI⁻ plaques were approximately half those of the gI⁺ plaques. Thus, despite the fact that gI⁻ virus grows as well as wild-type virus and is released more efficiently from CEFs than is wild-type virus, (18, 34) and consequently has a significant growth advantage on these cells (Fig. 2A), it forms smaller plaques than does wild-type virus on these cells. Furthermore, while the sizes of the plaques of wild-type virus or gIII⁻ virus were not affected by the presence of antiserum, indicating that adsorption of released virus to neighboring uninfected cells (a process that would be sensitive to antisera) is not a decisive factor in the plaque development of these viruses, the sizes of the plaques produced by gI⁻ virus were reduced considerably.

In RK cells also, the presence of antiserum did not affect the sizes of the plaques formed by wild-type or gIII⁻ virus but did decrease the size of the plaques of gI⁻ virus (Table 2). This effect was, however, much less marked in RK cells than in CEFs. The lesser inhibitory effect on gI⁻ plaque size in RK cells than in CEFs was consistently observed with

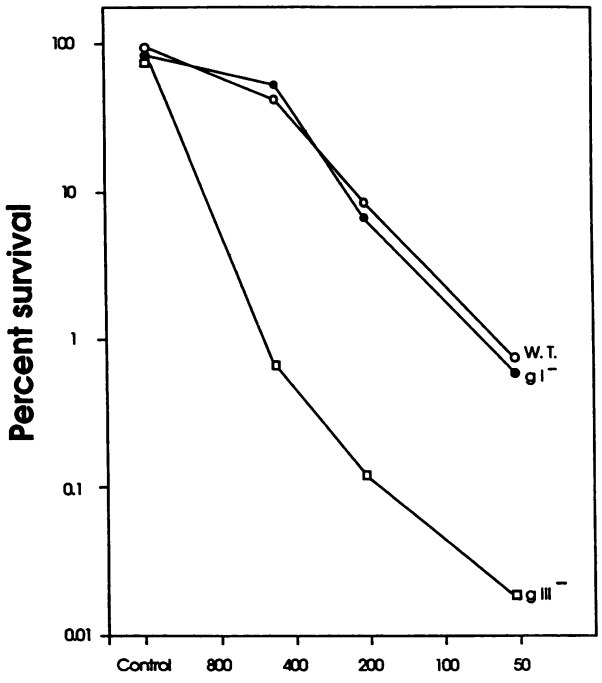

FIG. 6. Neutralization of wild-type (W.T.), gI⁻, and gIII⁻ viruses by goat anti-gII serum. Approximately 10^6 PFU of the viruses was incubated with the indicated dilutions of the antiserum for 1 h at 37°C. The titer of the viruses was determined by plaque assay of 10-fold dilutions of the viruses.

different-age RK cells and may well be related to the fact that syncytium formation in RK cells is more extensive than it is in CEF (Table 1). Thus, even though reduced in gI⁻ virus-infected RK cells, syncytium formation could still be sufficient to allow significant cell-to-cell spread in these cells.

The fact that plaque formation of gI⁻ virus was affected by the presence of the antiserum while plaque formation of the wild type was not is not due to an intrinsic greater sensitivity of the gI⁻ mutants to the antisera. Figure 6 shows that gI⁻ mutants and wild-type virus are inactivated similarly by the antisera but that, as previously observed (37), gIII⁻ virus is supersensitive to the antiserum. Thus, the ability of the antisera to reduce the plaque size of gI⁻ virus but not of gIII⁻ virus or of the wild type must be related to the mode of spread of these viruses.

TABLE 2. Effect of antiserum on the plaque sizes of the wild type and gI⁻ and gIII⁻ mutants[a]

Virus	Avg plaque diam (range [mm]) on:			
	CEFs		RK cells	
	−Antiserum	+Antiserum	−Antiserum	+Antiserum
Wild type	2.62 (±0.20)	2.57 (±0.21)	3.94 (±0.15)	3.88 (±0.25)
gI⁻	1.40 (±0.15)	0.68 (±0.13)	3.20 (±0.07)	2.60 (±0.10)
gIII⁻	2.60 (±0.21)	2.65 (±0.19)	3.97 (±0.25)	3.94 (±0.13)
gI⁻ gIII⁻	1.31 (±0.20)	0.63 (±0.09)	3.14 (±0.20)	2.37 (±0.20)

[a] The indicated mutants were plaque assayed on CEFs or on RK cells and overlaid with Methocel or with Methocel containing anti-gII antiserum (1%). The monolayers were fixed (CEFs after 72 h; RK cells after 48 h) and stained with crystal violet. The sizes of the plaques were measured in a microscope with an ocular micrometer. In each case, at least 30 randomly chosen plaques were measured.

TABLE 3. Passive protection of mice inoculated with the wild type and gI$^-$ and gIII$^-$ mutants of PrV(Ka) and PrV(Be)[a]

Virus	LD_{50} (PFU)[b]				
	PrV(Ka) with indicated serum:			PrV(Be) with indicated serum:	
	None	Anti-gII	Mouse anti-PrV	None	Anti-gII
Wild type	1×10^2	4×10^2 (4)	2×10^3 (20)	2×10^2	1×10^3 (5)
gI$^-$	1×10^2	$>10^5$ (1,000)	$>10^5$ (1,000)	1×10^2	$>10^5$ (1,000)
gIII$^-$	9×10^1	4×10^2 (4)	ND	9×10^1	5×10^2 (5)

[a] The description of the mutants is given in Materials and Methods. Tenfold dilutions (0.1 ml) of the indicated viruses were injected intramuscularly into the right hind leg of BALB/c mice (five mice per dilution). Six hours later the antiserum (0.2 ml of a 1:2 dilution) was injected intraperitoneally. The LD_{50}, given in PFU, was calculated after 8 days (no animals died thereafter) according to the Reed and Muench method (26).

[b] The numbers in parentheses indicate the increase in LD_{50} resulting from passive protection by the antisera. ND, not determined.

Passive protection of mice with PrV antiserum against challenge with wild-type, gI$^-$ and gIII$^-$ viruses. To test whether a difference in virus spread between wild-type, gI$^-$, and gIII$^-$ viruses could also be detected in infected animals, passive-protection experiments were performed. If gIII$^-$ virus spread mainly by direct transmission from cell to cell while gI$^-$ virus spread mainly by adsorption of released virus to uninfected cells, passive immunity resulting from the administration to the animals of anti-PrV neutralizing serum should provide better protection against challenge with gI$^-$ mutants than against challenge with gIII$^-$ virus. The degree of protection by the antiserum against wild-type virus would depend on its relative use of each of the two modes of spread.

These considerations are based on the premise that passive protection is mediated, at least in part, by neutralization. However, it has been previously reported that antibody-dependent cellular cytotoxicity, as well as virus neutralization, can be responsible for passive protection against virus challenge. Thus, passive protection by monoclonal antibodies of mice against challenge with wild-type PrV did not necessarily correlate with the ability of the antibodies to neutralize the virus (6, 16), and a similar situation has also been extensively documented for HSV (1, 17, 30). Because the dominant factor in passive protection could be related to the quality of the serum, we used two different antiserum types to perform these experiments, in the hope that at least one would provide protection mainly by virus neutralization. One of the antiserum types we used was raised against glycoprotein gII, and the other was raised against whole virus; both antisera had relatively high neutralizing activities.

Table 3 summarizes the LD_{50}s obtained from a representative experiment in which the mice were passively immunized with mouse anti-PrV serum or with goat anti-gII serum. To ensure the validity of the data, the animals were challenged with mutants of two different strains of PrV, PrV(Ka) and PrV(Be). In both cases, the mice challenged with wild-type or gIII$^-$ virus were provided with similar low level of protection by the sera. The mice that were challenged with the gI$^-$ virus mutants were, however, completely protected by both antisera. Since gI$^-$ mutants are not more sensitive to neutralization by the antisera than is wild-type virus (Fig. 6), these findings indicate that the gI$^-$ virus spreads mainly by adsorption of released virus to uninfected cells and is therefore accessible to the neutralizing activity of the antisera, while wild-type virus as well as the gIII$^-$ virus spread mainly via direct cell-to-cell transmission.

In the experiments summarized in Table 3, the mice were injected with the antisera 6 h after inoculation with the virus (to minimize neutralization of the virus inoculum). Table 4 shows the results of an experiment in which the antiserum was administered at different times prior to or after virus challenge. The mice inoculated with the gI$^-$ mutants were provided considerably better protection by the antiserum than were the mice inoculated with gIII$^-$ or wild-type virus if the antiserum was administered before or up to 8 h after inoculation with the virus. Delaying the administration of the antiserum until 24 h after inoculation with the virus no longer protected the animals.

DISCUSSION

The experiments described in this paper were designed to clarify the basis for the dramatically reduced virulence of mutants defective in both gI and gIII. While mutants defective in either gI or gIII have an LD_{50} that is only slightly higher than is that of the wild type, the double mutants deficient in both gI and gIII have an LD_{50} that is more than a 100-fold higher. The experiments we have performed to clarify this question yielded results which impinge not only on the understanding of the reason for the reduced virulence of gI$^-$ gIII$^-$ mutants but also on the understanding of the function of the gI glycoprotein.

Function of the gI glycoprotein. The role of glycoprotein gIII in virus adsorption has been well documented (22, 29, 38); however, the role of gI in the virus growth cycle was less clear. Thus, the growth kinetics of gI$^-$ and gI$^+$ viruses, as measured in one-step growth experiments, have been shown to be indistinguishable from each other (34), and the only function of gI that had been identified was that it is somehow involved in virus release (18, 34). We had, however, observed that even though gI$^-$ mutants replicate as well as wild-type virus and are released better than is the wild type

TABLE 4. Time course of passive protection of PrV with antiserum against gII[a]

Time (h) of administration of serum	LD_{50} (PFU)		
	Wild type	gIII$^-$ virus	gI$^-$ virus
−5	1.0×10^3	1.0×10^3	$>1.0 \times 10^5$
+8	2.0×10^2	2.0×10^2	$>1.0 \times 10^5$
+24	1.0×10^2	8.0×10^1	3.0×10^2
None	1.0×10^2	8.0×10^1	1.0×10^2

[a] The various virus mutants were injected intramuscularly with 10-fold dilutions of the virus (0.1 ml) into the right hind leg. Five mice each were used for each dilution. Anti-PrV gII goat serum (0.1 ml) was injected intraperitoneally at the indicate time. (The time of inoculation of the mice with the virus was considered time zero). The LD_{50} was calculated according to the Reed and Muench method (26).

from CEFs, they form smaller plaques on these cell monolayers (Fig. 5). Since plaque formation is determined not only by the rate of virus replication and its release and readsorption to neighboring cells (which are not negatively affected in gI$^-$ mutants) but also by cell-to-cell transmission, i.e., by the formation of intercellular bridges or channels that would allow direct cell-to-cell virus transmission, we surmised that cell bridging might be diminished in gI$^-$ virus-infected cultures. Indeed, gI$^-$ mutants cause the formation of smaller syncytia than does wild-type virus, and the gI glycoprotein thus appears to be one of the factors that promotes cell fusion. While in its absence cell fusion is not completely abrogated, it is considerably diminished.

Our results also showed that plaque formation of gI$^-$ virus (in contrast to that of wild-type virus) occurs mainly by a process in which released virus to neighboring cells is adsorbed rather than by direct cell-to-cell spread. This conclusion is based on the finding that the plaque size of gI$^-$ mutants but not of wild-type or gIII$^-$ virus was significantly reduced by the presence of antiserum in the overlay, indicating that cell-to-cell spread of the gI$^-$ mutant is impaired. From the fact that the size of the plaques formed by wild-type and gIII$^-$ viruses on CEF and RK monolayers are not detectably affected by the presence of antiserum, we can infer that plaque development of these viruses is determined mainly by direct cell-to-cell transmission. By comparing the sizes of the plaques of gI$^-$ and gI$^+$ virus that develop under an overlay containing antiserum, we can estimate that in the experiment summarized in Table 2, cell-to-cell transmission of the gI$^-$ mutants in CEFs was at least 75% less efficient than was that of wild-type virus. (Under an antiserum-containing overlay, wild-type virus produced plaques 2.57 mm in diameter and gI$^-$ virus produced plaques 0.68 mm in diameter.) This estimate is based on the assumption that the size of the gI$^-$ plaques that form on CEFs in the presence of antibody is determined by the residual cell-to-cell virus spread rather than by residual nonneutralized released virus. We favor the first possibility because syncytium formation in gI$^-$ virus-infected cultures, though diminished, is not completely inhibited.

It should be mentioned that the magnitude of the effect of antiserum on the sizes of the plaques of gI$^-$ virus as well as the reduction in syncytium formation of gI$^-$ virus relative to that of wild-type virus is dependent on the physiological state of the cells and varies somewhat between experiments. However, the effect is always observed, and only its magnitude varies.

We have also ascertained the ability of passively administered antibodies to protect animals against challenge with wild-type, gIII$^-$, or gI$^-$ virus. The antisera were administered either before or several hours after challenge with the virus. Two different serum types with relatively high neutralizing titers were used in these experiments. Both behaved similarly in that they were much more effective in protecting mice challenged with gI$^-$ virus than in protecting those challenged with wild-type virus or gIII$^-$ virus. Antibody-dependent cellular cytotoxicity as well as neutralization have previously been implicated as being responsible for passive protection by antiserum against challenge with HSV and PrV (1, 5, 16, 17, 30). Antibody-dependent cellular cytotoxicity is directed against cell surface antigens, and one would expect the anti-gII sera that were used in our experiment to have a similar level and the anti-PrV sera to have a higher level of antibody-dependent cellular cytotoxic activity against the wild type than against gI virus. However, the gI$^-$ virus-infected mice were provided considerably better passive protection than were wild type-infected mice. We are therefore led to conclude that the passive protection of the gI$^-$ virus-infected animals that was obtained was mediated mainly through neutralization of virus that had been released into extracellular fluids and subsequent inhibition of its adsorption to uninfected cells. The most likely reason mice inoculated with wild-type and gIII$^-$ viruses were less well protected than were those inoculated with gI$^-$ virus is that wild-type and gIII$^-$ viruses remained inaccessible to the serum; i.e., virus spread was in this case mainly by direct cell-to-cell transmission.

In HSV, glycoproteins gB, gD, and gH have been implicated in cell fusion (2–4, 7–9, 11, 13, 23, 31, 32). Homologs of these glycoproteins (gII, gp50, and gH) have been identified for PrV (12, 24, 25, 27, 28), and some have been implicated in cell fusion (5, 17a, 37). The results summarized here show that gI also plays a role in cell fusion. No direct role for gE of HSV (the homolog of gI of PrV [25]) in virus growth has as yet been identified. It would be interesting to ascertain whether gE performs in HSV a function similar to that of its homolog, gI, in PrV.

Reduced virulence of gI$^-$ gIII$^-$ mutants. While gI$^-$ mutants or gIII$^-$ mutants retain most of their virulence, the gI$^-$ gIII$^-$ mutants have significantly reduced levels of virulence. We propose that this is the case because these mutants are unable to spread efficiently either by adsorption of released virus (the gIII gene being defective) or by direct cell-to-cell spread (the gI gene being defective). Mutants defective in either gI or gIII alone can, however, still spread by either adsorption of released virus or by direct cell-to-cell transmission and therefore retain considerable levels of virulence.

That defects in both gIII and gI may affect virus spread was indicated by in vitro experiments. Thus, while gIII$^-$ gI$^-$ mutants and gIII$^-$ mutants grew similarly as measured in one-step growth curves, the gIII$^-$ gI$^-$ virus had a considerable growth disadvantage when the cells were infected at a low multiplicity and virus spread (in addition to virus replication) was monitored. Inactivation of gI in wild-type virus did not affect its ability to spread; the defect in gI had to be coupled to a defect in gIII. This was clearly shown by the results obtained with the mutant PrV2gI$^-$. PrV2 is a mutant with a defect in the gIII gene which affects its ability to adsorb to cells in a cell type-specific manner (36). The inactivation of the gI gene in PrV2 affected its ability to be amplified only in those cells to which PrV2 adsorbs poorly. Since gI plays a role in cell-to-cell transmission (see above), these findings are consistent with the premise that inactivation of gI affects significantly the ability of a virus to recycle only if this virus is also defective in adsorption. It was, however, surprising to find that even though gI$^-$ virus appears to spread mainly by adsorption of released virus to neighboring cells and gIII adsorbs poorly, mutants defective in gIII and gI and mutants defective in gI alone produced similar-size plaques. Since the lack of gIII should slow the process of adsorption, in principle, the gIII$^-$ gI$^-$ virus plaques should be smaller. The reason no difference in plaque sizes of gI$^-$ and gIII$^-$ gI$^-$ viruses was observed may well be that in cell culture, the cells that are adjacent to the infected cells are exposed to a large number of infectious viruses so that the less efficient adsorption of the gIII$^-$ virus does not affect detectably the dynamics of plaque formation.

The results of the passive protective experiments described in this paper showed that virus spread in mice infected with wild-type virus (as well as with gIII$^-$ mutants) occurs to a large extent by cell-to-cell transmission but that it occurs mainly by adsorption of released virus in gI$^-$ virus-infected

mice. These passive-protection experiments were performed with mice because these animals provide a convenient model system. Because the virulence profile of the mutants is similar in mice, chickens, and pigs, it is likely that the mode of spread of the viruses is similar in all three animal systems.

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