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Involvement of Membrane-Bound Viral Glycoproteins in Adhesion of Pseudorabies Virus-Infected Cells

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Cell-associated spread of pseudorabies virus (PrV) plays an important role in the pathogenesis of the disease. Besides the already known direct cell-to-cell spread of the virus in monolayers, adhesion and subsequent fusion of suspended PrV infected cells to monolayers of uninfected cells are thought to occur. To study the adhesion of PrV-infected cells, an in vitro model was developed in SK-6 cells. Specific adhesion of PrV-infected cells to an uninfected monolayer started 5 h after infection of the cells and reached a maximum 6 h later. A correlation was found between the surface expression of PrV glycoproteins on the infected cells and the adhesion of these cells. PrV hyperimmune serum completely inhibited binding of the infected cells. To investigate which PrV envelope glycoproteins were responsible for the cell adhesion, the infected cells were incubated with antisera against glycoproteins gII, gIII, and gp50. Antiserum against either gII or gIII inhibited cell adhesion, and antisera against gII and gIII together had a cooperative effect. Antiserum against gp50 had no effect on binding when used alone but enhanced the inhibition induced by gII and gIII antisera. Heparin and neomycin inhibited adhesion, showing that the receptor for adhesion was a heparinlike substance. SK-6 cells infected with a gIII deletion mutant of PrV exhibited a much lower adhesion. This binding was heparin and neomycin independent and was not blocked by anti-gII serum. Nevertheless, it was completely inhibited with PrV hyperimmune serum and with anti-gp50 serum. This finding demonstrates that the ligand for adhesion of gIII$^-$-infected cells is glycoprotein gp50. These results strongly suggest that the mechanism for adhesion of a PrV-infected cell to an uninfected monolayer is similar to the mechanism of adsorption and penetration of a PrV virion to a host cell.

Pseudorabies virus (PrV), a member of the *Alphaherpesvirinae*, is an important pathogen in swine. The disease is characterized by nervous signs, respiratory disorders, and reproductive failures, such as abortions, mummifications, and stillbirths. Abortion is generally the result of intraplacental and intrafetal replication of PrV. Infected mononuclear cells are detected in the bloodstream of both seronegative and vaccinated sows after PrV inoculation (32), and they are thought to transport the PrV all over the body. Nauwynck and Pensaert were able to reproduce abortion in vaccinated sows by injecting in vitro-infected autologous blood mononuclear cells into the arteria uterina (15). The hypothesis may be formulated that PrV-infected mononuclear cells which are circulating in the bloodstream adhere to the vascular endothelium, after which PrV reaches the fetal tissues by means of direct cell-to-cell-spread.

Much research has been done on the direct cell-to-cell spread of PrV (16, 17, 22, 33) and herpes simplex virus type 1 (HSV-1) (2–4, 6, 8, 11). Nevertheless, cell-associated spread of PrV may not only involve direct cell-to-cell spread of the virus in cell monolayers but could also occur through attachment and subsequent fusion of infected cells in suspension with monolayers of uninfected cells. To investigate the attachment of PrV-infected cells, an in vitro model was developed. By analogy to plaque assays in which direct cell-to-cell spread is investigated in a continuous cell line, a cell adhesion assay was developed with SK-6 cells to study binding of PrV-infected cells to uninfected cells.

At least seven glycoproteins, designated gI (12, 21), gII (13, 24), gIII (25), gp50 (19, 31), gp63 (21), gX (23), and gH (18), are synthesized in cells infected with PrV. HSV-1 glycoproteins show structural and functional homology with the PrV glycoproteins. The HSV-1 homologs are gE, gB, gC, gD, gI, gG, and gH, respectively (7, 29). PrV glycoproteins gI, gIII, gp63, and gX have been shown to be dispensable for viral growth in tissue culture, whereas gII, gp50, and gH are essential for virus replication (1, 12, 20, 26, 31). The functions of gII, gIII, gp50, and gH have been studied extensively; the results have shown that initial virus adsorption to a host cell is mediated by gIII and that gII, gp50, and gH are involved in the subsequent penetration of the virions into the host cells (5, 14, 16, 17, 22, 28, 34). In cell-to-cell spread, glycoproteins gI, gII, and gH play an important role (16, 17, 22, 33); gp50 (17, 22) and gIII (33) are not required for cell-to-cell spread. The cellular receptor for binding of PrV and HSV-1 virions was identified as heparan sulfate, since heparin and neomycin inhibit binding of the virions (10, 14, 27). PrV mutants that lack gIII exhibit a much lower and weaker adsorption to host cells than do wild-type PrV virions. Nevertheless, a gIII-independent adsorption exists, mediated by a different receptor than is wild-type virus adsorption (14, 28, 34).

The adhesion experiments presented in this report were designed to determine the involvement of different PrV glycoproteins in the adhesion of PrV-infected SK-6 cells to uninfected SK-6 cells, and the nature of the adhesion receptor is identified.

MATERIALS AND METHODS

Viruses. The origin of PrV strain 89V87 [PrV (89V87)] was described earlier (15). PrV strain Kaplan [PrV (Ka)] (28) and the PrV (Ka) gIII deletion mutant (PrV gIII$^-$) (28) were kindly provided by T. Mettenleiter.

Antibodies. The porcine PrV hyperimmune serum was prepared in this laboratory. Polyclonal sheep antisera against PrV glycoproteins gII, gIII, and gp50 were kindly

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provided by A. Brun (30), and rabbit anti-gH peptide serum 1193 was kindly provided by T. Mettenleiter (9). All the sera used were mixed with 1 volume of Alsever's solution and further diluted with sodium phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS). As a control serum, we used a PrV-negative serum taken from the same animal species as that from which the positive serum was taken. In certain experiments, 1:1 mixtures of anti-gII and anti-gIII sera, anti-gII and anti-gp50 sera, and anti-gIII and anti-gp50 sera were used.

Infection and [^{3}H]thymidine labeling of cells. Monolayers of swine kidney (SK-6) cells were trypsinized and inoculated with PrV (89V87), PrV (Ka), or PrV gIII$^-$ (multiplicity of infection, 30 PFU per cell). After 1 h of incubation, the cells were labeled with 2.5 μCi of [^{3}H]thymidine per 10^6 cells. The cells were further incubated in a siliconized bottle on a rocking platform at 37°C in medium containing equal amounts of RPMI and minimal essential medium based on Earle's buffered salt solution, supplemented with 10% fetal calf serum, glutamine (0.6 mg/ml), and the antibiotics penicillin (100 U/ml), streptomycin (0.1 mg/ml), and kanamycin (0.1 mg/ml).

Cell adhesion assay. Confluent monolayers of uninfected SK-6 cells were obtained 3 days after the cells were seeded in 96-well plates (target monolayers). Immediately before the assay was started, PrV-infected SK-6 cells and uninfected SK-6 cells (effector cells) were washed three times, counted, and adjusted to 10^6 cells per ml with PBS. The target monolayers were washed with PBS, and 100 μl of effector cell suspension was added per well and centrifuged for 30 s at 50 × *g*. The effector cells were allowed to adhere to the target monolayer at 22°C for 1 or 20 min, depending on the experiment. After incubation of the effector cells to the monolayer, nonadherent cells were washed away by shaking the microtiter plate for 20 s on a Titertek shaker (Flow Laboratories) at maximum speed, after which the liquid was removed from the wells. One hundred microliters of PBS was added to the wells, the microtiter plate was shaken again for 20 s at maximum speed, and finally the liquid was removed from the wells. The adherent cells and the underlying monolayer were lysed in 0.2 ml of 1% Triton X-100. The lysates were then dissolved in Ready Safe (Beckman), and the radioactivity was counted by liquid scintillation counting. The percent specific adhesion of the effector cells to the target monolayer was calculated as follows: [(cpm of adherent infected effector cells/cpm of total infected effector cells) − (cpm of adherent uninfected effector cells/cpm of total uninfected effector cells)] × 100.

For certain experiments, the effector cells were preincubated with hyperimmune serum against PrV, anti-gII serum, anti-gIII serum, anti-gp50 serum, or anti-gH peptide serum for 30 min at 4°C prior to inoculation of the cells to the target monolayer. In other experiments, heparin (Rhône Poulenc) or neomycin (Bufa) (diluted with PBS) was added to the effector cell suspensions 20 min (37°C) prior to inoculation of the cells on the target monolayer. The percent inhibition of adhesion was calculated as follows: {[(specific adhesion of control serum) − (specific adhesion of positive serum)] × 100}/(specific adhesion of control serum). All assays were performed in duplicate and repeated independently at least one time.

Flow cytofluorographic analysis. Monolayers of SK-6 cells were trypsinized and infected with PrV (89V87), PrV (Ka), or PrV gIII$^-$ as described above. At 3, 9, 11, or 13 h after infection, the cells were centrifuged, suspended in fluorescein isothiocyanate-conjugated pig PrV hyperimmune serum, and incubated on ice for 60 min. Subsequently, the cells were washed two times with PBS containing 0.1% NaN_3 and fixed for 1 min in 1% formaldehyde. The cells were washed again two times, resuspended in PBS containing 0.1% NaN_3, and analyzed on a FACScan (Becton Dickinson). Sizing gates were set to include all nucleated cells. At least 10^4 cells were analyzed for each sample.

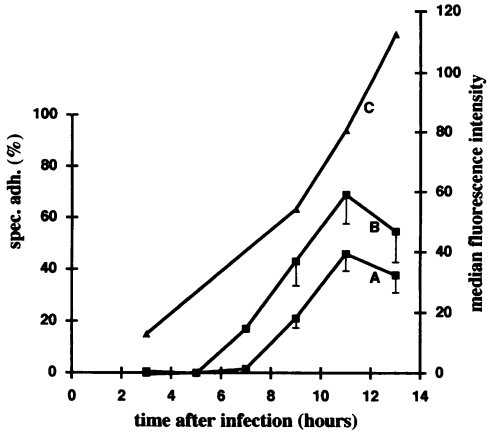

FIG. 1. Specific adhesion of [^{3}H]thymidine-labeled PrV (89V87)-infected effector SK-6 cells to an uninfected target monolayer of SK-6 cells at 1 min (curve A) or 20 min (curve B) of incubation of the effector cells on the target monolayer. Data are means minus standard deviations of at least two independent experiments each performed in duplicate. Curve C shows the median fluorescence intensity of PrV (89V87)-infected SK-6 cells at various times after infection.

RESULTS

Adhesion of PrV (89V87)-infected cells to an uninfected monolayer. In Fig. 1, specific adhesion of infected cells as a function of the infection time is shown. Specific adhesion started approximately 5 h after infection of the effector cells and reached a maximum 6 h later. Incubation of the infected effector cells for 1 min on the target monolayer resulted in a lower specific adhesion than did incubation for 20 min. Eleven hours after infection of the effector cells and after 20 min of incubation on the target monolayer, 70% of the infected cells adhered to the uninfected cell monolayer. With these experimental conditions, the cell adhesion inhibition experiments were performed. Uninfected effector cells bound only to a small extent (1 to 3%) to the target monolayer.

Expression of PrV (89V87) glycoproteins on infected SK-6 cells. Figure 1, curve C, shows the median of the fluorescence intensity for PrV (89V87)-infected cells 3, 9, 11, and 13 h after infection. Strong expression of PrV glycoproteins was noticed from 9 to 13 h after infection. A correlation was observed between the specific adhesion and the median fluorescence intensity of the infected cells 3, 9, and 11 h after infection.

Effects of hyperimmune serum against PrV and polyclonal antibodies against PrV glycoproteins gII, gIII, gp50, and gH on the adhesion of PrV (89V87)-infected SK-6 cells. Incubation of the infected effector cells with PrV hyperimmune serum for 30 min at 4°C prior to the inoculation of the cells to the target monolayer strongly inhibited the binding of infected SK-6 cells, and the inhibition was dependent on the serum concentration. A comparable inhibitory effect was

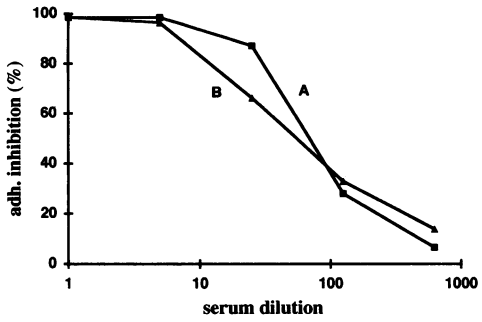

FIG. 2. Adhesion inhibition of [^{3}H]thymidine-labeled PrV (89V87)-infected SK-6 cells at various dilutions of PrV hyperimmune serum after incubation for 30 min at 4°C (curve A) or for 1 min at 37°C (curve B).

noticed when the infected cells were incubated for 1 min at 37°C with PrV hyperimmune serum (Fig. 2).

Undiluted antisera against either gII or gIII completely inhibited the binding of the infected SK-6 cells. Upon dilution of the sera, a 1:1 mixture of anti-gII and anti-gIII sera induced a higher adhesion inhibition than did anti-gII serum or anti-gIII serum. Anti-gp50 serum had no inhibitory effect when used alone but enhanced the inhibition induced by gII and gIII antisera. The anti-gH peptide serum had no effect on binding of the SK-6 cells (Fig. 3).

Effects of heparin and neomycin on the adhesion of PrV (89V87)-infected cells. Figure 4 demonstrates that concentrations of heparin higher than 0.08 μg/ml had an inhibitory effect on the adhesion of the cells and that a concentration of 10 μg/ml blocked the adhesion completely. Neomycin had a dual effect. At concentrations from 0.008 to 0.2 mM, the adhesion was enhanced, and at concentrations of 1 mM and higher, strong adhesion inhibition occurred.

Adhesion of SK-6 cells infected with PrV (Ka) and PrV gIII$^-$. More than 50% of SK-6 cells infected for 11 h with PrV (Ka) bound on the target monolayer. This binding was inhibited completely by a 1:4 dilution of PrV hyperimmune serum, a 1:4 dilution of anti-gII serum, a 1:4 dilution of anti-gIII serum, 50 μg of heparin per ml, and 5 mM neomycin. No inhibitory effect was noticed with a 1:4 dilution of anti-gp50 serum (Fig. 5).

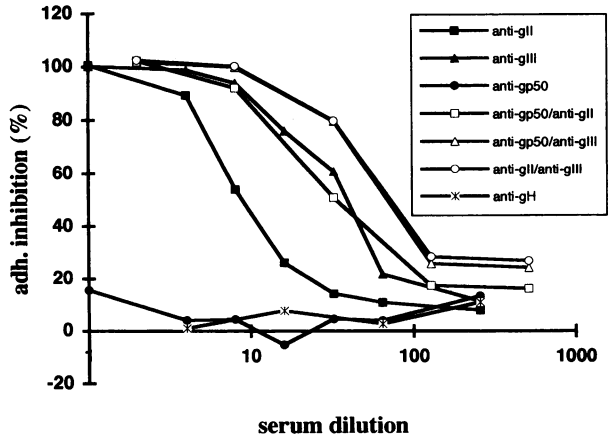

FIG. 3. Adhesion inhibition of [^{3}H]thymidine-labeled PrV (89V87)-infected SK-6 cells at various dilutions of sera against different PrV glycoproteins.

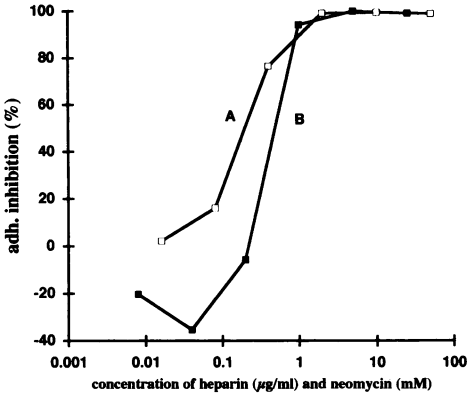

FIG. 4. Adhesion inhibition of [^{3}H]thymidine-labeled PrV (89V87)-infected SK-6 cells at various final concentrations of heparin (curve A) and neomycin (curve B).

The expression of PrV glycoproteins on the cells infected with the PrV gIII$^-$ mutant 13 h after infection was comparable to the expression of PrV glycoproteins on the cells infected with wild-type PrV (Ka) 11 h after infection, as determined by flow cytometry (data not shown). Because the expression of PrV glycoproteins on PrV gIII$^-$-infected cells was slower than the expression of glycoproteins on cells infected with wild-type PrV (Ka), the adhesion assay performed with PrV gIII$^-$-infected cells was started 13 h after infection of the cells. Five to six percent of the PrV gIII$^-$-infected effector cells bound to the target monolayer. This adhesion was inhibited completely by a 1:4 dilution of PrV hyperimmune serum and a 1:4 dilution of anti-gp50 serum. A 1:4 dilution of anti-gIII serum, which was used as a control, and a 1:4 dilution of anti-gII serum had no effect on adhesion of the cells, nor did heparin at 50 μg/ml and neomycin at 5 mM (Fig. 6).

DISCUSSION

In this report, adhesion of PrV-infected SK-6 effector cells to an uninfected SK-6 target monolayer is described. The

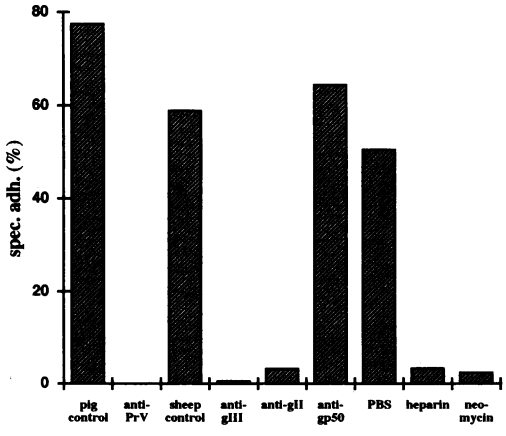

FIG. 5. Adhesion of [^{3}H]thymidine-labeled wild-type PrV (Ka)-infected SK-6 cells after incubation with PrV hyperimmune serum, sera against PrV glycoproteins, heparin, and neomycin.

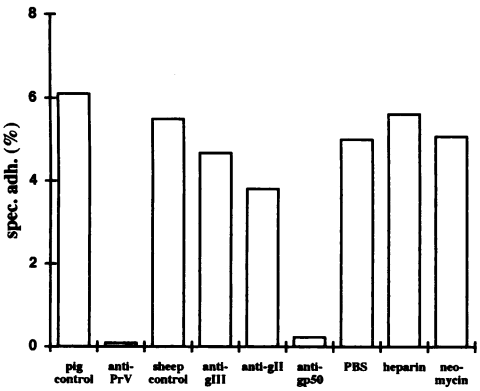

FIG. 6. Adhesion of [^{3}H]thymidine-labeled mutant PrV gIII$^-$-infected SK-6 cells after incubation with PrV hyperimmune serum, sera against PrV glycoproteins, heparin, and neomycin.

binding of the PrV (89V87)-infected cells is mediated by the cell membrane-bound viral glycoproteins expressed on the infected effector cells. This was indicated by the good correlation found between the expression of PrV glycoproteins on PrV (89V87)-infected cells and adhesion of the infected cells (Fig. 1) and clearly proven by the ability to inhibit completely the adhesion with PrV hyperimmune serum (Fig. 2).

The adhesion inhibition of wild-type PrV-infected cells induced by antisera against gII and gIII (Fig. 3) shows that the ligands for the interaction of the infected cells to the monolayer are gII and gIII. A stronger adhesion inhibition occurred when antisera against gII and gIII were used together. This finding indicates that glycoproteins gII and gIII interact with the monolayer in a cooperative way. The finding that anti-gp50 did not inhibit the adhesion when used alone suggests that gp50 is not involved in the initial step of binding of the cells. Nevertheless, when antiserum either against gII or against gIII was combined with the antiserum against gp50, a stronger adhesion inhibition was noticed. A possible explanation for that observation is that although gp50 is not responsible for the initial binding of the infected cells, it could play a role in a further stage of binding or at the start of cell fusion. The observation that both heparin and neomycin inhibited the adhesion of PrV (89V87)-infected cells to the target monolayer (Fig. 4) suggests that the receptor for binding of PrV-infected cells is a heparinlike substance.

In this study, it is shown that PrV glycoproteins gII, gIII, and gp50 are involved in the binding of infected cells and that the receptor for binding is a heparinlike substance. Glycoprotein gIII is responsible for the stable adsorption (14, 28, 34), and gII and gp50 are involved in the penetration (5, 17, 22) of the extracellular PrV virus particle to the host cell. The cellular receptor for adsorption of a PrV particle is heparan sulfate (10, 14, 27). Taken together, these data demonstrate that the adsorption and penetration of a virus particle and the binding of an infected cell to an uninfected cell are mediated by similar mechanisms. It would be interesting to investigate whether the amounts of antibodies needed to inhibit on one hand adsorption and penetration of PrV virions and on the other hand cell adhesion are also similar.

From our results and from what is known of virion adsorption and penetration, the following model can be proposed. The infected cells bind via gII and gIII on a heparan sulfate receptor present on the uninfected cells. In the process of binding, gII and gIII act together. After this initial binding, cell fusion may occur. As gp50 is involved in the penetration of a PrV particle to a host cell, we speculate that cell membrane-bound gp50 plays a role in the fusion of infected cells to uninfected cells.

The results of the adhesion inhibition experiments of SK-6 cells infected with wild-type PrV (Ka) (Fig. 5) and wild-type PrV (89V87) show that the same mechanism of adhesion occurred with cells infected with both wild-type PrV strains. It is likely that other wild-type PrV strains induce the same cell adhesion. Not only different PrV strains but also different cell types were investigated for cell adhesion. Comparable cell adhesion of PrV (89V87)-infected cells to an uninfected monolayer was also observed for Vero cells, PK-15 cells, and ST cells (data not shown). One can conclude that the adhesion mechanism described here is a general way for wild-type PrV-infected cells to bind target cells. To test the first step of our hypothesis concerning the pathogenesis of PrV-induced abortion, research will be done to determine whether the adhesion of PrV-infected mononuclear cells to an endothelial monolayer is mediated by the same mechanism as described above.

Cells infected with the PrV gIII$^-$ mutant strain showed a much lower adhesion than did the wild-type-infected cells; this observation supports the finding that gIII is essential for adhesion of PrV-infected cells. Nevertheless, some binding of PrV gIII$^-$ mutant-infected cells, approximately 10 times lower than the adhesion of wild-type-infected cells, was observed (Fig. 6). These results indicate that the mechanism for adhesion of gIII$^-$ mutant-infected cells is similar to the adsorption of gIII$^-$ virus particles. Adsorption of a PrV gIII$^-$ mutant virus particle to a cell is much slower and weaker than that of a wild-type virus particle (28, 34), and the adsorption of a gIII$^-$ virus particle is heparin independent (14). Here we show evidence that the ligand for adhesion of gIII$^-$ mutant-infected cells is gp50 and that the receptor is not a heparinlike substance.

The main conclusions that can be drawn from the results in this report are as follows. (i) Wild-type PrV-infected SK-6 cells adhere strongly to an uninfected monolayer of SK-6 cells. (ii) The mechanism of binding of wild-type PrV-infected SK-6 cells is very similar to the adsorption and penetration of wild-type PrV virions to an uninfected cell: PrV glycoproteins gII and gIII are the ligands for binding, and the receptor is a heparinlike substance. (iii) PrV gIII$^-$ mutant-infected cells adhere much more weakly to the target cells in a heparin-independent fashion, with a mechanism similar to the adsorption and penetration of PrV gIII$^-$ mutant virions to a cell. Glycoprotein gp50 acts as the ligand for adhesion of the PrV gIII$^-$-infected cells.

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