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Anti-glycoprotein D antibodies that permit adsorption but block infection by herpes simplex virus 1 prevent virion–cell fusion at the cell surface

(neutralizing antibodies/membrane fusion/glycoproteins/viral penetration)

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ABSTRACT **Certain monoclonal antibodies specific for glycoprotein D of herpes simplex virus have potent neutralizing activity but fail to block attachment of virus to cells. Here we have investigated the fate of neutralized and infectious virus after attachment to primate cells. Infectious virions fused with the cell surface such that naked nucleocapsids were detectable in the cytoplasm near or just under the plasma membrane. Neutralized virions did not fuse with the cell. They remained attached to the cell surface and could be rendered infectious by treatment with polyethylene glycol. We conclude that some anti-glycoprotein D neutralizing antibodies can inhibit the penetration of herpes simplex virus by blocking fusion of the virion envelope with the plasma membrane. These results identify a pathway of entry that initiates successful herpes simplex virus infection and a step in this pathway that is highly sensitive to neutralizing antibodies. A role for glycoprotein D in virion–cell fusion is indicated.**

Investigation of the mechanisms by which antibodies neutralize viral infectivity can provide information about route of entry of the virus into a cell as well as identify steps in the infectious process that can be blocked by antibodies. For enveloped viruses, entry leading to successful infection requires attachment to the cell and penetration of the nucleocapsid into the cytoplasm by fusion of the virion envelope with a cell membrane. In some instances, endocytosis of the virion may be a prerequisite for this membrane fusion, so that nucleocapsid penetration occurs by fusion of the virion envelope with the membrane of an endosome. The low pH of endosomes may be required to trigger the fusion activity (see ref. 1 for review).

For other enveloped viruses, nucleocapsid penetration by fusion of the virion envelope directly with the plasma membrane has been observed by EM (2–5). It is difficult to prove, however, that such events observed by EM actually lead to viral gene expression and a replicative cycle. Here we provide evidence that fusion of herpes simplex virus (HSV) with the plasma membrane of human and monkey cells is relevant to infection. We found that fusion of HSV with the cell surface (but not virus attachment) is blocked by certain neutralizing monoclonal antibodies (mAbs).

More than six membrane glycoproteins are present in the envelope of HSV, the causative agent of oral and genital herpetic lesions and, more rarely, systemic infection and encephalitis. Antibodies capable of neutralizing HSV infectivity in the absence of complement are specific for one of several glycoproteins, including gB, gD, and gH (see ref. 6 for review). Other HSV envelope glycoproteins seem not to be targets of complement-independent neutralizing antibodies.

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We have previously characterized eight mAbs specific for gD (Table 1). These mAbs fall into two groups—those that neutralize infectivity at relatively low concentrations without inhibiting virion adsorption to cells (first five listed) and those that neutralize and inhibit virion adsorption but only at relatively high concentrations (last three listed). Different anti-gD mAbs, therefore, can neutralize infectivity by at least two different mechanisms (8). Most of the anti-gD mAbs in both groups effectively block HSV-induced cell–cell fusion (Table 1), probably also by at least two different mechanisms. In this study our attention was focused on the mode of action of the potent neutralizing mAbs that block infection while permitting virion adsorption to cells.

We have investigated the fate of neutralized and infectious HSV after attachment to primate cells. The results show that the potent anti-gD neutralizing mAbs block HSV infection by preventing virion–cell fusion at the plasma membrane, that fusion at the plasma membrane is a route of entry permitting successful HSV infection, and that gD is likely to be involved in the virion–cell fusion required for infection.

MATERIALS AND METHODS

Cells and Virus. Human epidermoid carcinoma cells (HEp-2) were grown in Dulbecco's modified Eagle's minimal essential medium containing 10% fetal bovine serum (KC Biologicals, Lenexa, KS), and Vero (African green monkey) cells were grown in medium 199 supplemented with 5% fetal bovine serum. Some EM experiments were also done with primary human embryonic lung cells (HEL) maintained in basal essential medium (Eagle) supplemented with 10% fetal bovine serum. The virus strain used was a syncytial variant designated HSV-1(HFEM)syn (10). Experiments were also done using the parental strain HSV-1(HFEM), with indistinguishable results (data not shown). Purified virions were prepared from infected HEp-2 cells by centrifugation in dextran T-10 gradients as described (11).

Neutralization by mAbs. Purified mAbs were prepared by affinity chromatography on a protein A-Sepharose CL-4B column (8). The mAbs used were III-114-4, III-174-1, and III-255-2, all neutralizing antibodies specific for gD, and II-529-3, a non-neutralizing antibody that binds to virions and is specific for gC (7, 8). Purified mAb to *Salmonella* common antigen (mAb 746, Chemicon, El Segundo, CA) was also used as a control. Assays for neutralization of virus infectivity were performed (12) on HEp-2 cells or Vero cells. In some experiments, after adsorption of neutralized or control virus to Vero cells, the cells were exposed briefly to polyethylene glycol (PEG) as described previously (13), in order to

Abbreviations: HSV, herpes simplex virus; prefix g, glycoprotein (e.g., gD is glycoprotein D); mAb, monoclonal antibody; pfu, plaque-forming unit(s).

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Table 1. Activities of purified anti-gD mAbs

mAb*	Relative amounts bound to intact virions,†‡ ^{125}I cpm	Neutralization,†§ μg/ml	Inhibition of cell–cell fusion,¶ μg/ml	Inhibition of virus adsorption,†‖ μg/ml
III-174-1	750	0.06	3	>1200
III-114-4	1980	0.10	15	800
III-255-2	3460	0.10	15	>1200
II-436-1	1820	0.44	75	>1200
I-188-5	1310	2.50	15	>1200
I-99-1	1920	>6.02	15	850
I-206-7	2300	>3.98	18	380
II-886-1	3110	>6.02	76	500

*Isolation and characterization of these mAbs were described by Para *et al.* (7).

†Data from Fuller and Spear (8).

‡Radioactivity bound to purified HSV-1(HFEM)syn virions that were incubated with purified IgG (0.1 mg/ml) and ^{125}I-labeled protein A, then filtered through a polycarbonate filter that retained virions but not free protein A or antibody–protein A complexes.

§Concentration of purified IgG required for 50% reduction in titer of HSV-1(HFEM)syn (100–500 pfu input; pfu, plaque-forming units) on Vero cells. The titers of ascites fluids containing these antibodies typically range from 200,000 (reciprocal of dilution causing 50% reduction in pfu) for III-174-1 to <50 for II-886-1. The relative titers of the ascites fluids are similar for neutralization assays done on Vero and HEp-2 cells.

¶Lowest concentration of purified IgG that completely inhibited fusion of Vero cells infected with 50 pfu of HSV-1(HFEM)syn per cell. Other mAbs tested (anti-gB, anti-gC, anti-gE) were without effect. Data from Noble *et al.* (9).

‖Concentration of purified IgG required for 50% reduction in binding of radiolabeled HSV-1(HFEM)syn to HEp-2 cells.

determine whether the neutralization could be reversed. To prepare neutralized virus for EM experiments, relatively high concentrations of purified virions were incubated for 1 hr at 37°C with purified neutralizing mAb at 0.2 mg/ml, a concentration in excess (about 10-fold) of the amount required to reduce the virus titer from 10^8 plaque-forming units (pfu) per ml to <10. Similar concentrations of control mAbs, II-529-3 (specific for gC) and 746, were also used and had little effect on viral infectivity. Neutralization of the treated virions was assessed in two ways. Vero cells were exposed to dilutions of the treated virions for titration of pfu (Table 2), and HEp-2 cells were exposed to undiluted treated virions (100 pfu per cell, before neutralization) to determine whether synthesis of HSV proteins was induced, as assessed by polyacrylamide gel electrophoresis of [^{35}S]methionine-labeled cell extracts. No HSV proteins could be detected after exposure of HEp-2 cells to virions neutralized by III-114-4, III-174-1, and III-255-2, indicating that infection was blocked at an early stage. HSV protein synthesis was observed after exposure of HEp-2 cells to virions treated with the non-neutralizing anti-gC mAb II-529-3. The rate of early HSV protein synthesis was about half that observed in control cultures (data not shown).

Electron Microscopy and Quantitation. Purified HSV-1(HFEM)syn virions at a concentration of 10^8 pfu/ml were incubated with purified neutralizing or non-neutralizing IgGs at 0.2 mg/ml in a volume of 0.70 ml. After incubation for 1 hr at 37°C each mixture was diluted to a final volume of 1.0 ml and added to cells grown in 60-mm dishes at a multiplicity (before neutralization) of 200 pfu per cell or 100 pfu per cell. Adsorption was allowed for 10–60 min at either 29°C or 37°C. The dishes were placed on ice, and cells were washed with cold phosphate-buffered saline and processed for EM. In some cases when immunogold labeling was performed, the monolayers were immediately fixed with 1% paraformaldehyde and 1% glutaraldehyde in 0.1 M collidine buffer (pH 7.4). The monolayers were washed and then incubated either with antibody to glycoproteins followed by gold-labeled protein A (Janssen Pharmaceutica, Beerse, Belgium) or with anti-glycoprotein mAb directly coupled to colloidal gold as described earlier (14). All samples were fixed in 2% glutaraldehyde in 0.1 M collidine buffer, then 2% osmic acid in the same buffer, and dehydrated by a series of ethanol washes followed by three washes in propylene oxide. The samples were Epon-embedded, thin-sectioned, and photographed on a Siemens 102 electron microscope.

RESULTS

Electron Microscopy of Cells Exposed to Neutralized or Infectious Virus. Cells exposed to infectious or neutralized virions at 37°C were examined by EM at various times after the addition of virus to determine the optimal times for quantitative analysis. Thirty minutes was chosen for most experiments because, by this time, sufficient numbers of virions were bound to the cell surface, penetration of bound virions was still in progress (based on accessibility of bound virions to neutralizing antibodies), and nucleocapsid disassembly was not complete. We chose *not* to synchronize penetration by carrying out virion adsorption at low temperatures because temperature shift-up can induce endocytosis (15). Attachment and penetration at 29°C were assessed with results similar to those obtained at 37°C except that cytoplasmic nucleocapsids were more readily detectable and apparently were disassembled more slowly.

Fig. 1 (*a–f*) shows representative electron micrographs of virions in the process of fusing with HEp-2 cells at the plasma membrane. These images could be seen as early as 5 min after addition of virus and were seen only after incubation of cells with infectious virus (untreated or exposed to control mAbs). Attached or fusing virions did not appear to be associated with clathrin-coated regions or with any morphologically distinguishable feature of the plasma membrane. For cells incubated with neutralized virus, most of the virions seen were attached to the cell surface (Fig. 1*g*). For both neutralized and infectious virus samples, virions could be found in intracellular vesicles. These vesicles might contain more than one virus particle, and in some cases the virus particles looked partially degraded.

We monitored the location and numbers of intracellular and extracellular particles by EM to document differences seen for neutralized and infectious particles. Virus particles present in at least 100 cell sections of each sample were counted and categorized as to type and location (Fig. 2). The samples exposed to neutralized or infectious virus differed in numbers of cytoplasmic nucleocapsids, intact virions attached to the cell surface, and total virus particles. The absence or near-absence of cytoplasmic nucleocapsids after exposure to neutralized virions compares with 3–7 nucleocapsids per 20 cells after exposure to infectious virus. Most of the nucleocapsids detected were located near the plasma membrane. Larger numbers of virions were detected on the cell surface for neutralized virus than for infectious virus. The differences in total numbers of virus particles detected (higher for neutralized samples) probably reflect the short half-life of cytoplasmic nucleocapsids, which are no longer recognizable after disassembly during the eclipse phase of the replicative cycle. Election-dense masses that could have been viral cores were seen in cells exposed to infectious virus but were not counted. The differences described above were seen also in two experiments done with human embryonic lung (HEL) fibroblasts (data not shown). In all these experiments, no significant differences were noted in numbers of obviously damaged virions on the cell surface and numbers of virions in what appeared to be cytoplasmic vesicles (Fig.

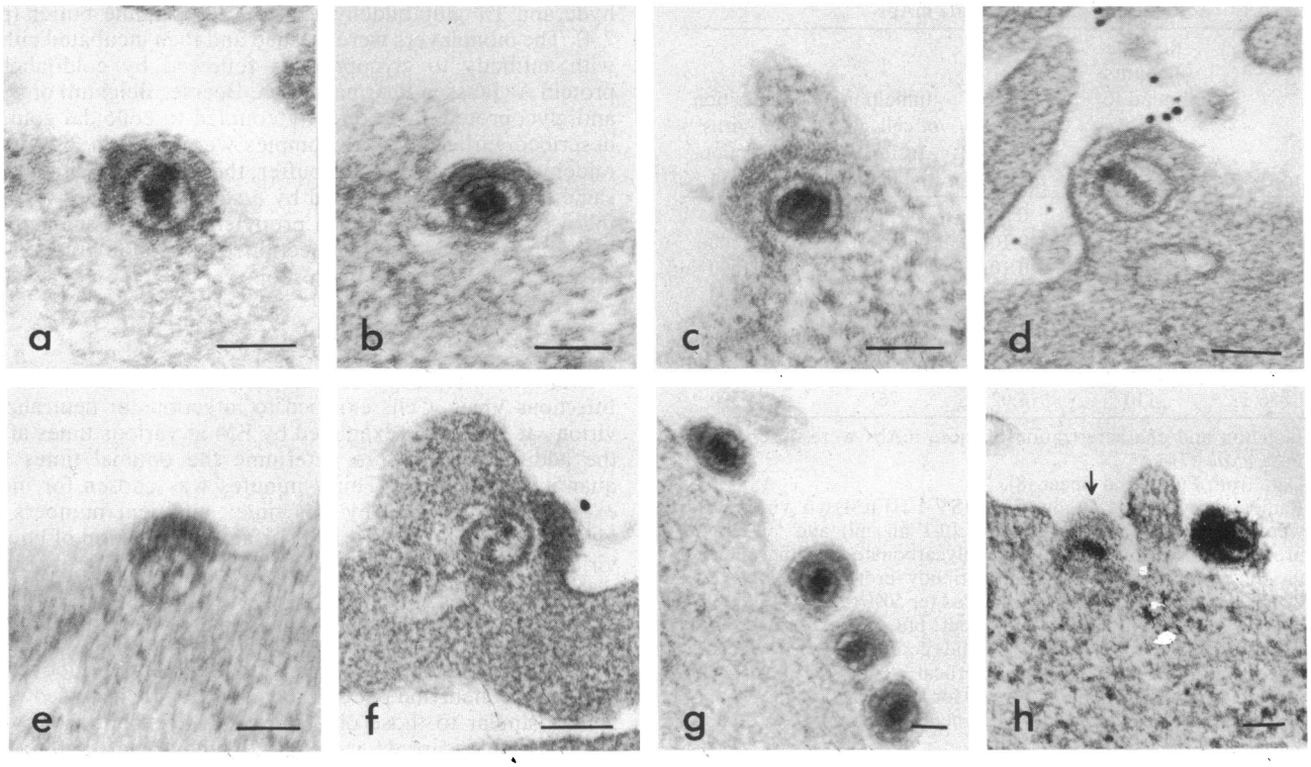

FIG. 1. Electron micrographs of cells exposed to infectious or neutralized HSV-1(HFEM)syn. After treatment with purified antibody, virus at a multiplicity (before neutralization) of 100 pfu per cell (*e*, *f*, and *h*) or 200 pfu per cell (*a–d* and *g*) was adsorbed to HEp-2 cells at 37°C for 30 min (*a–c* and *g*) or 29°C for 25 min (*d–f*) or to Vero cells at 37°C for 60 min (*h*). Antibody treatment was with anti-*Salmonella* mAb 746 (*a*), buffer only (*b*, *c*, *e*, and *f*), the anti-gC non-neutralizing mAb II-529-3 (*d*), or the anti-gD neutralizing mAb III-174-1 (*g* and *h*). In *h* the cells were treated with PEG before fixation; arrow indicates a neutralized virus particle that may have penetrated via PEG-induced fusion of the virion envelope with the cell membrane. Magnification is about ×100,000 (*a–f*) or ×50,000 (*g* and *h*). (Bars = 100 nm.)

2). The large fraction of input virions remaining on the surfaces of cells exposed to infectious virus reflects in part the fact that penetration is not complete by 30 min.

Effect of PEG Treatment on Neutralized or Infectious Virus. The quantitation presented in Fig. 2 suggests that most of the neutralized virus remained on the cell surface. This was also indicated by the results of treating cells exposed to neutralized or infectious virus with PEG, a membrane-fusing reagent that has been shown to restore infectivity of HSV mutants presumably blocked in penetration (13, 17). PEG treatment of Vero cells exposed to serial dilutions of neutralized virus substantially increased the titer, reversing the neutralization (Table 2). The titers of infectious virus were only marginally increased. Fig. 1*h* shows a virus particle (arrow) that may have been induced to penetrate by PEG-mediated fusion of the neutralized virus with a Vero cell. PEG caused no obvious differences in the overall morphology of the cells or in numbers of endocytic vesicles. (Similar experiments could not be done with HEp-2 cells because of the toxicity of PEG for these cells.)

DISCUSSION

We conclude that anti-gD mAbs can neutralize HSV infectivity by blocking penetration of virus at the cell surface, apparently by blocking fusion between the virion envelope and plasma membrane. That neutralized virus binds to cells and accumulates on the cell surface is evident from the results summarized in Fig. 2 and from the reversal of neutralization by PEG treatment (Table 2).

Entry of enveloped virus into cells can proceed by at least two routes (endocytosis or fusion at the cell surface) culminating in release of the virus nucleocapsid into the cytoplasm. Our results show that HSV-1 can enter cultured primate cells and initiate infection by fusion at the plasma membrane. This is in agreement with earlier EM observations by Morgan *et al.* (2) and Smith and de Harven (3) but disagrees with the suggestion that HSV enters cells by "viropexis" as proposed by Dales and Silverberg (18), Holmes and Watson (19), and Epstein *et al.* (20). Although we detected virions within intracellular vesicles, no differences were noted in numbers of such virions for cells exposed to neutralized or infectious virus. This virus in vesicles may be destined for destruction by lysosomes and thus unable to initiate infection.

Several other lines of evidence also support infectious entry of HSV by fusion directly at the plasma membrane. First, Para *et al.* (21) found that Fc receptors characteristic of the virion envelope could be found on the plasma membrane shortly after infection and in the absence of *de novo* viral protein synthesis. Second, DeLuca *et al.* (22) found that photosensitized HSV adsorbed to cells could be protected from light inactivation immediately after warming of cells to 37°C and proposed that this rapid protection might result from entry of the virus into the cytoplasm by fusion of the viral envelope with the plasma membrane. However, it should be noted that, in the study by DeLuca *et al.*, virus adsorption was done at 4°C and the cells then quickly warmed to 37°C. Such a temperature shift has been shown to initiate a high rate of endocytosis in some cells (15). Third, the presence of viral glycoproteins on the cell surface shortly after adsorption has been detected by flow cytofluorometry (23); adsorbed, unpenetrated virions probably did not account for the glycoprotein antigen detected because the cells were trypsinized for the analysis. Finally, although the density of labeling was not high, we detected viral antigens in the plasma membrane in the vicinity of penetrated nucleocapsids near the plasma membrane (Fig. 1 *d* and *f*).

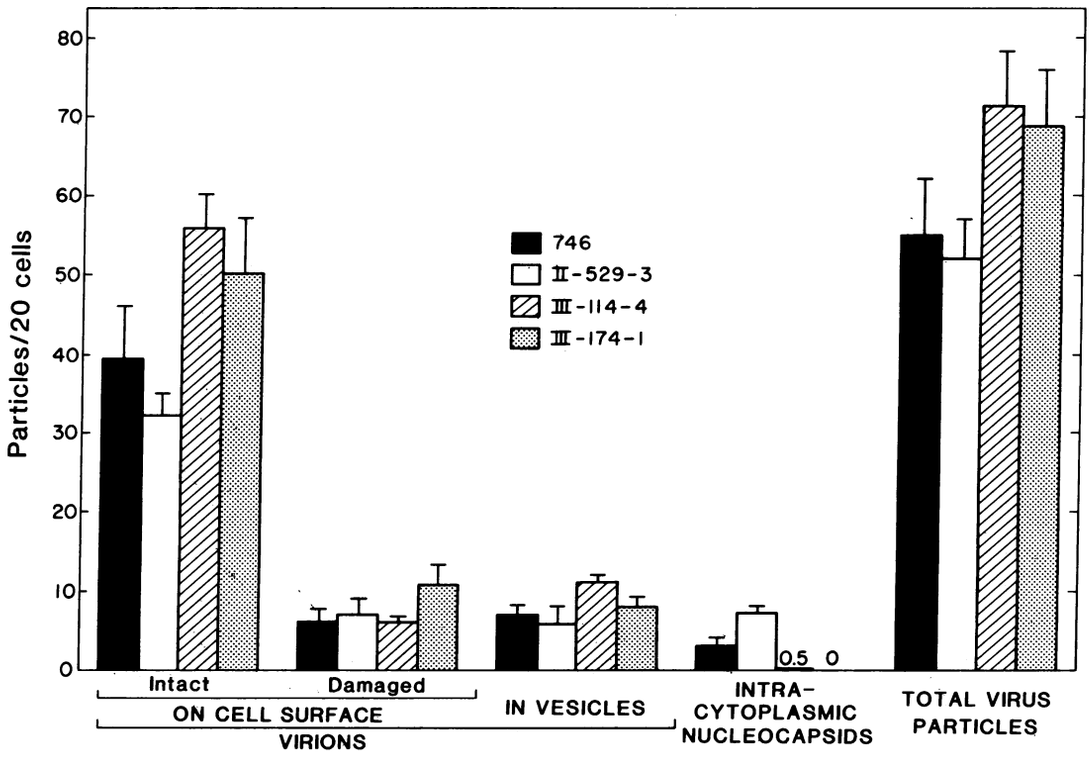

FIG. 2. Quantification and location of neutralized or infectious virus particles by EM. HEp-2 cells were exposed to neutralized or infectious virus at a multiplicity of 200 pfu per cell (before neutralization) for 30 min at 37°C. The cells were processed for EM and grids were prepared. We randomly selected from each sample 5–8 grid squares that were completely filled by thin-sectioned material. Each square contained ≈20 cell sections (range was 18–23). The total number of cells and the type, location, and number of virus particles were scored for each square. Only clearly recognizable virus particles were counted. The numbers of virus particles counted on each grid square were normalized to the number per 20 cells. The mean and standard error of the mean for normalized counts of 5–8 grid squares are presented. Data similar to that presented were also obtained using human embryonic lung (HEL) cells with an input multiplicity of 100 pfu per cell (before neutralization). III-174-1 and III-114-4, anti-gD neutralizing mAbs; II-529-3, non-neutralizing mAb that binds to gC in virions; 746, non-neutralizing mAb specific for *Salmonella* common antigen.

There is independent evidence that endocytosis is not required for HSV infectivity. Agents that block endocytosis or raise the pH of endosomes prevent infection by enveloped viruses that penetrate via endocytosis and fusion from within endosomes (1), but these agents do not interfere with initiation of infection by HSV (ref. 24; M. Wittels and P.G.S., unpublished data). Moreover, we show here that neutralized virions do not accumulate in endocytic vesicles. However, fusion from an intracellular vesicle may be used in certain cell types by some other herpesviruses. Evidence has been presented that Epstein–Barr virus enters transformed B lymphocytes by fusion with the plasma membrane but may enter normal B lymphocytes from non-clathrin-coated endosomes (4). The mechanism of successful entry of herpesviruses may be dependent on the particular virus, cell type, and other factors.

Fusogenic activity of viral envelope glycoproteins is essential for infectivity (see ref. 25 for review) and must be controlled by mechanisms, not yet understood, that allow virus–cell fusion at the appropriate time and place to initiate infection. Because HSV can infect by fusing with the plasma membrane and because HSV-induced cell–cell fusion is not dependent on low pH, it seems unlikely that low pH regulates fusogenic activity of HSV membrane glycoproteins. Interactions occurring entirely at the cell surface are probably responsible for triggering membrane fusion. At least three HSV glycoproteins (gB, gD, and gH) have been implicated in the fusion process, by the phenotype of mutations in gB (13, 17, 26) or by findings that mAbs specific for gD or gH can block cell fusion (9, 27, 29). There is no evidence to suggest that these three glycoproteins act as a single functional heteropolymeric unit. Homodimers of gB extracted from virions or infected cells are not associated with other glycoproteins (28), and gB and gD form morphologically distinct structures in the virion envelope (14).

Table 2. Effect of PEG on infectivity of neutralized and infectious HSV-1(HFEM)syn

Antibody used*	Titer, pfu/ml†	
	− PEG	+ PEG
None	2.30×10^6	3.0×10^6
III-174-1 (anti-gD)	<10	1.86×10^5
II-529-3 (anti-gC)	1.15×10^6	1.65×10^6

*Final IgG concentration of 0.24 mg/ml. Virus was incubated with antibody or buffer as described in the text, and then dilutions were prepared for plating on Vero cells. After adsorption, half of the Vero cell cultures were exposed to PEG as described (13).
†Average of duplicate counts.

Multiple interactions between the virion surface and cell surface may serve as the prelude to induction of membrane fusion. Specific interactions of one virion component with the cell may trigger conformational changes that expose fusogenic domains of other viral proteins. Thus the successful entry process may be a cooperative sequential chain of events requiring several viral products to initiate, expand, and control the location of a fusion event. Because anti-gD antibodies can inhibit HSV-induced cell–cell fusion (9, 29) as well as fusion of the virion with the plasma membrane, it seems likely that gD has some role in initiating or mediating the process of membrane fusion. How the processes of virion–cell fusion and virus-induced cell–cell fusion are

related remains to be determined. Although the same viral proteins may participate in both processes, there may be differences in mechanism to explain the fact that relative neutralizing titers of anti-gD mAbs do not necessarily correlate with relative titers in inhibition of cell–cell fusion (Table 1).

The virion–cell fusion event, whether occurring at the cell surface or after endocytosis, may be particularly sensitive to inhibition by neutralizing antibodies. For example, polyclonal neutralizing antibodies can block release of a flavivirus (West Nile virus) from endosomes, presumably by preventing virion envelope fusion with the endosome membrane, without blocking endocytosis of the virus (30). For several viruses, including a bovine herpesvirus (31), neutralizing antibodies have been shown to block a step subsequent to adsorption, although the step has not been identified. As has been previously discussed (16), virion–cell fusion is an essential step in the pathway leading to infection by enveloped viruses, one that could be exploited in the development of antiviral agents and vaccines.

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