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Neutralizing Antibodies Specific for Glycoprotein H of Herpes Simplex Virus Permit Viral Attachment to Cells but Prevent Penetration

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Monoclonal antibodies specific for gH of herpes simplex virus were shown previously to neutralize viral infectivity. Results presented here demonstrate that these antibodies (at least three of them) block viral penetration without inhibiting adsorption of virus to cells. Penetration of herpes simplex virus is by fusion of the virion envelope with the plasma membrane of a susceptible cell. Electron microscopy of thin sections of cells exposed to virus revealed that neutralized virus bound to the cell surface but did not fuse with the plasma membrane. Quantitation of virus adsorption by measuring the binding of purified radiolabeled virus to cells revealed that the anti-gH antibodies had little or no effect on adsorption. Monitoring cell and viral protein synthesis after exposure of cells to infectious and neutralized virus gave results consistent with the electron microscopic finding that the anti-gH antibodies blocked viral penetration. On the basis of the results presented here and other information published elsewhere, it is suggested that gH is one of three glycoproteins essential for penetration of herpes simplex virus into cells.

Glycoprotein H (gH) of herpes simplex virus (HSV) is one of three envelope glycoproteins (gB, gD, and gH) shown to elicit the production of complement-independent neutralizing antibodies (3, 9, 15, 24, 25, 44, 46, 50) and to be indispensable for virion infectivity (5, 14, 31, 32, 49). HSV virions also contain at least four other envelope glycoproteins. Virions devoid of these latter glycoproteins can be infectious for cultured cells (23, 26, 33, 42), although specific infectivity may be reduced.

The two serotypes of HSV (HSV-1 and HSV-2) encode related forms of gH that are antigenically cross-reactive but not identical (50). Genes homologous to that encoding HSV-1 gH (20, 36) have been found in the genomes of other herpesviruses, including varicella-zoster virus (36), Epstein-Barr virus (36), cytomegalovirus (11), and herpesvirus saimiri (21). The detection of a gH homolog in every herpesvirus for which sufficient sequence information is available, coupled with comparisons and alignments of amino acid sequences (21), indicate that the gH family is the second most highly conserved glycoprotein family encoded by herpesviruses, second only to the gB family. It is not yet clear whether the gH homologs specified by other herpesviruses are essential for virion infectivity. It is suggestive, however, that monoclonal antibodies (MAbs) specific for the gH homologs of varicella-zoster virus (12, 17, 22, 29, 40), cytomegalovirus (11, 47), and Epstein-Barr virus (53) have complement-independent neutralizing activity.

The mechanism by which at least some anti-gD and anti-gB MAbs neutralize HSV infectivity is by blocking viral penetration into cells, not by blocking adsorption of virus to cells (19, 24, 25). We showed by quantitative electron microscopy that anti-gD MAbs can prevent the fusion between the virion envelope and plasma membrane that is required for penetration of HSV into a cell (19). Recently, by measuring dequenching of a virion-associated fluorescent probe to quantitate penetration, it was shown that a MAb specific for Epstein-Barr virus gp85, the gH homolog, also neutralized infectivity by blocking viral penetration (37).

Here we have investigated how anti-gH MAbs neutralize HSV-1 infectivity. The results indicate that, similar to anti-gD MAbs, the anti-gH MAbs block fusion of the virion envelope with the plasma membrane without having any significant effect on the number of virus particles that adsorb to cells. These findings, coupled with other information about gB, gD, and gH, indicate that gH is required for viral penetration into cells.

MATERIALS AND METHODS

Cells and viruses. Human epidermoid carcinoma cells (HEp-2) were grown in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with 10% fetal bovine serum (FBS) (Hyclone). African green monkey kidney (Vero) cells were grown in medium 199 with Hanks salts supplemented with 5% FBS. HSV-1(HFEM)syn was isolated in our laboratory from HSV-1(HFEM) as previously described (2). HSV-1(14-012) was obtained from F. Rapp (Hershey Medical Center, Hershey, Pa.). Purified virions of HSV-1(HFEM)syn were prepared from lysates of infected HEp-2 cells by centrifugation through dextran T-10 (Pharmacia) gradients as previously described (8). Infectious titers of virus preparations were determined by plaque assays on HEp-2 and Vero cells.

Antibodies. Table 1 provides pertinent information about the MAbs used in this study. Cell lines producing MAbs 52S and 53S were obtained from the American Type Culture Collection, Rockville, Md. Ascitic fluids containing MAbs

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52S and 53S as well as MAbs III-114, III-174, and III-195 were prepared as described previously (44). Ascitic fluids containing MAb LP11 were generously provided by Anthony Minson (Cambridge University, Cambridge, United Kingdom). Immunoglobulins were purified from ascitic fluids by affinity chromatography on columns of protein A-Sepharose CL-4B (Pharmacia) as described previously (18). Purified immunoglobulin G was concentrated by using Centricon-30 concentrators (Amicon Corp.) and dialyzed against 0.01 M sodium phosphate buffer (pH 7.2) before use. Protein concentrations were determined by the Bio-Rad protein assay, using immunoglobulin as the protein standard.

Plaque assays for monitoring neutralization of HSV infectivity. Neutralization of HSV-1 by purified MAbs was assayed by using either diluted or concentrated stocks of virus. For neutralization of diluted virus, purified preparations of HSV-1(HFEM)syn or crude stocks of HSV-1(14-012) were diluted in phosphate-buffered saline (PBS; 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 140 mM NaCl, 2.5 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$), supplemented with 1% FBS and glucose (PBS-GC), to yield final concentrations of approximately 200 PFU/ml when mixed with various concentrations of purified MAb. The reaction mixtures were incubated for 1 h at 37°C and then plated undiluted and in duplicate on monolayers of HEp-2 cells in 25-cm^2 flasks for titration of residual infectivity. The flasks were incubated for 2 h at 37°C, the inocula were removed, and the flasks were overlaid with medium 199 containing 1% FBS and pooled human γ-globulin or overlaid with medium containing agarose. The flasks were incubated at 37°C for 36 to 48 h. The cells were fixed with methanol and stained with Giemsa stain. Plaques were counted, and the percentage of neutralization was calculated for each sample {percent neutralization or percent inhibition = 100 × [1 − (PFU in samples incubated with MAb)/PFU in control samples]}.

For neutralization of concentrated virus, purified virions of HSV-1(HFEM)syn at approximately 10^8 PFU/ml were incubated with 0.4 or 0.2 mg/ml of purified antibody or without MAb for 1 h at 37°C. The samples were serially diluted with PBS-GC for titration of residual infectivity. Each dilution was plated in duplicate onto monolayers of HEp-2 cells, and the PFUs remaining were quantitated in a plaque assay as described above for diluted virus.

In experiments designed to determine whether polyethylene glycol (PEG) could reverse the neutralization of viral infectivity, Vero cells, instead of HEp-2 cells, were exposed to dilutions of neutralized or infectious virus as described above. After virus adsorption and removal of the virus inoculum and unadsorbed virus with washing, the cells were briefly treated with PEG (PEG 8000; Sigma Chemical Co.) as described previously (49).

Quantitation of virus adsorption to cells in the presence and absence of MAbs. Purified radiolabeled virions were prepared from HEp-2 cells that had been infected with HSV-1(HFEM)syn at 10 PFU per cell and incubated for 24 h with medium 199 containing 1% FBS, 1/10 the usual concentration of leucine, and [^{3}H]leucine at 20 μCi/ml. The purified radiolabeled virions were mixed with various concentrations of purified MAbs, incubated for 1 h at 37°C, chilled, and then plated in triplicate on HEp-2 cell monolayers in 96-well plates to quantitate virus adsorption as previously described (18). The average values obtained for counts per minute bound to cells in the presence of MAb were calculated and expressed as the percentage of counts per minute bound in the absence of MAb.

Electron microscopy and quantitation of virus particles. Mixtures of purified HSV-1(HFEM)syn virions (final concentration of about 10^8 PFU/ml) and purified MAbs (final concentration of 0.4 mg/ml or no MAb) were prepared in Ca^{2+}- and Mg^{2+}-free PBS and incubated for 1 h at 37°C. HEp-2 cells, detached by the use of EDTA and washed, were then suspended in the virus-MAb mixtures at 3 × 10^6 cells per ml and incubated for 20 or 40 min at 34°C. After incubation, the cells were chilled, washed, fixed in 2% glutaraldehyde, and then processed for preparation of thin sections of the cells and electron microscopy as previously described (19). The sections were examined and photographed with a Siemens 102 electron microscope. Virions and nucleocapsids in each cell section were counted and categorized by their location within or on the cell. At least 20 cell sections were examined for each sample. The samples were coded and examined by different individuals, and the code was broken only after the counts had been completed.

TABLE 1. Properties of MAbs used in this study

MAb	Immunoglobulin G subclass	Virus strain used for immunization	Antigen recognized[a]	Neutralizing activity[b]		Reference(s)
				HSV-1	HSV-2	
52S	2a	HSV-1(14-012)	gH-1	+	−	50
53S	2a	HSV-1(14-012)	gH-1/2	+	−	50
LP11	2a	HSV-1(SC16)	gH-1	+	−	3,20
III-114	2b	HSV-2(G)	gD-1/2	+	+	44
III-174	2a	HSV-2(G)	gD-1/2	+	+	44
III-195	3	HSV-2(G)	gG-2	NT	−	44

[a] Based on the results of immunoprecipitation tests. Three of the antibodies are type specific by this test, precipitating only the HSV-1 form of gH (gH-1) or the HSV-2 form of gG (gG-2), whereas three of the antibodies are type common, precipitating both the HSV-1 and HSV-2 forms of gH or gD (gH-1/2 or gD-1/2). Although 53S was type common by immunoprecipitation, it was type specific for neutralization (50).

[b] +, Greater than 50% plaque reduction; −, less than 50% plaque reduction observed at dilutions of ascitic fluids greater than 1:25 or 1:50; NT, not tested.

Induction of early viral protein synthesis by infectious and neutralized HSV-1(HFEM)syn. Mixtures of purified virions (final concentration of 2 × 10^6 PFU/ml) and purified MAbs (final concentrations ranging from 0 to 300 μg/ml) were prepared in PBS-GC and incubated for 1 h at 37°C. Samples (0.25 ml) of the mixtures were plated undiluted on HEp-2 cell monolayers in 24-well dishes (40 PFU per cell before neutralization), and the virus was allowed to attach for 30 min at 37°C. The inocula were removed, and the cells were incubated for 4 h in Dulbecco modified Eagle medium containing 10% FBS. At 4.5 h after the addition of virus, the cells were pulse-labeled for 15 min with [^{35}S]methionine at 20 μCi/ml of methionine-free medium. The dishes were placed on ice, and the cells were washed and solubilized in sodium dodecyl sulfate-containing buffer for electrophoresis on an 8.5% polyacrylamide gel cross-linked with *N,N'*-diallyltartardiamide. The gels were processed for autoradiography with Amplify (Amersham), dried, and exposed to Cronex film.

RESULTS

Neutralization of HSV-1 by purified anti-gH or anti-gD MAbs. The anti-gD and anti-gH MAbs used here have previously been shown to neutralize HSV-1 infectivity (Table 1). We wished to determine how much purified MAb was required to neutralize two of the virus strains used as immunogens for eliciting the MAbs. Dilute suspensions of virus were incubated with various concentrations of the purified MAbs and then plated directly on HEp-2 cells for

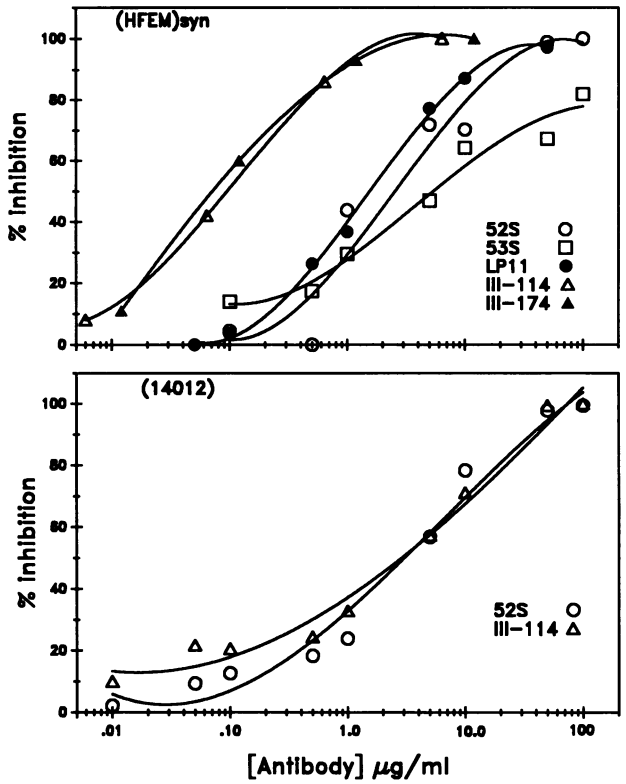

FIG. 1. Neutralization of HSV-1 by purified anti-gH or anti-gD MAbs. Purified preparations of HSV-1(HFEM)syn or crude stocks of HSV-1(14-012), diluted to approximately 200 PFU/ml, were incubated with various concentrations of purified MAb for 1 h at 37°C. The reaction mixtures were plated undiluted onto HEp-2 cells for titration of residual PFUs. The numbers of plaques counted in the absence of MAb ranged from 200 to 600 per flask. The results are presented as percent inhibition of plaque formation calculated as described in Materials and Methods. The anti-gD MAbs, III-114 and III-174, were induced in response to HSV-1(HFEM)syn antigen whereas 52S and 53S were induced in response to HSV-1(14-012) and LP11 was induced in response to HSV-1(SC16).

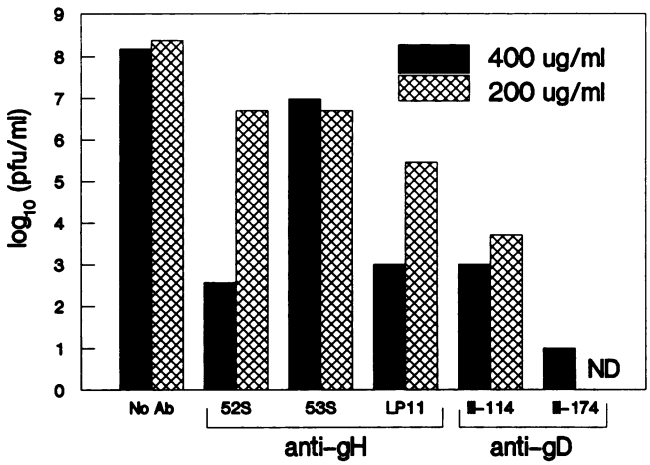

FIG. 2. Effects of the anti-gH and anti-gD MAbs on infectivity of purified, concentrated HSV-1(HFEM)syn virions. Samples of purified virions at 10^8 PFU/ml were incubated without MAb (No Ab) or with two different concentrations (200 or 400 μg/ml) of the purified MAbs indicated for 1 h at 37°C. The samples were then serially diluted for titration of residual PFUs on HEp-2 cells. ND, Not done.

quantitation of residual PFUs. Figure 1 shows that the anti-gD MAbs induced against HSV-1(HFEM)syn neutralized this virus strain better than did the anti-gH MAbs induced against other virus strains (with approximately a 10-fold difference in MAb concentration required to neutralize 50% of input virus). On the other hand, representative anti-gD (III-114) and anti-gH (52S) MAbs neutralized HSV-1(14-012) equally well. MAb 52S was induced against this strain HSV-1(14-012). Among the anti-gH MAbs, LP11 and 52S had the most potent neutralizing activity, whereas 53S failed to give complete neutralization at the highest concentrations tested.

Because some of our planned experiments required the exposure of cells to high concentrations of neutralized virus, we assessed the effects of the MAbs on the infectivity of concentrated virus under conditions approximating those to be used in these experiments. Samples of purified HSV-1(HFEM)syn at about 10^8 PFU/ml were incubated with the purified MAbs to be tested at two to four times the highest MAb concentration used in Fig. 1. The mixtures were then diluted with buffer and plated on HEp-2 cells to determine the titers of residual PFUs. At 0.4 mg/ml, all the anti-gD and anti-gH MAbs, except 53S, reduced the titer of the concentrated virus stock to about 10^{-5} of the original value (Fig. 2). Consistent with the results shown in Fig. 1, 53S reduced the titer to 10^{-1} of the original value. The MAb concentration used for all subsequent experiments was 0.4 mg/ml, except when noted otherwise.

In several instances, it has been shown that HSV-1 can bind to cells but fails to penetrate (as a result of the absence of gB or gD in the virion or of neutralization by anti-gD MAbs) and that treatment of the virus-cell complexes with PEG can induce penetration of at least part of the bound virus (4, 19, 31, 32, 49). An experiment was done to assess whether PEG could partially reverse the neutralization by anti-gH MAbs, as it does for anti-gD MAbs (19). Samples of purified HSV-1(HFEM)syn virions were incubated with an anti-gD or anti-gH MAb at 0.4 mg/ml and then diluted for plating on Vero cells (PEG is too toxic to HEp-2 cells to permit their use in this kind of experiment). After virus adsorption and washing of the cells to remove unbound virus, some of the monolayers were exposed briefly to PEG as described before (49), and replicate monolayers were left untreated. Figure 3 shows the numbers of PFUs that were scored after further incubation to allow plaque development. In two experiments, PEG significantly increased the number of PFUs detected in the neutralized samples but had no effect on the numbers detected in the control samples incubated without neutralizing MAb. There seems to be an upper limit to the level of reversal of neutralization that can be obtained, at about 1% of control values. The increased titers obtained by PEG treatment after neutralization are quite impressive, however, particularly for the anti-gD MAb. These results indicate that at least some of the virions neutralized by anti-gD or anti-gH MAbs can bind to cells and that the block to their infectivity can be overcome by PEG treatment.

The adsorption of HSV-1(HFEM)syn to HEp-2 cells was not inhibited by anti-gH MAbs. Purified radiolabeled virions were incubated at 37°C with various concentrations of each of the purified anti-gH MAbs. The mixtures were then chilled and plated on HEp-2 cells to quantitate binding of the labeled virus to the cells as previously described (18). Similar to the results obtained with neutralizing anti-gD

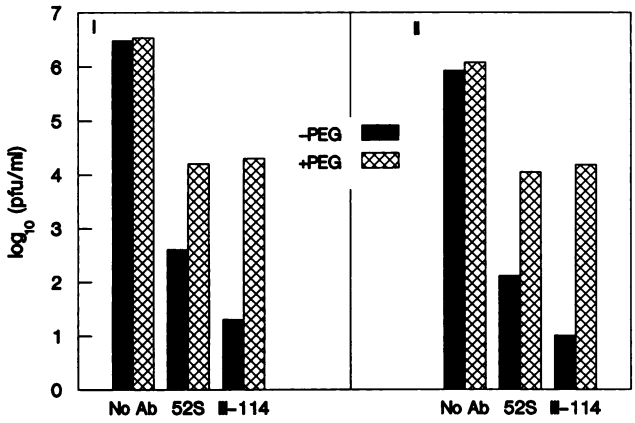

FIG. 3. Effect of PEG on the infectivity of cell-bound neutralized HSV-1(HFEM)syn. In two independent experiments, purified virions at 4×10^6 PFU/ml were incubated without MAb (No Ab) or with purified MAb (0.4 mg/ml) for 1 h at 37°C. The samples were then serially diluted for titration of residual infectivity on Vero cells. One set of replicate cultures was exposed briefly to PEG after virus adsorption, whereas the other set was untreated. The anti-gH MAb is 52S, and the anti-gD MAb is III-114.

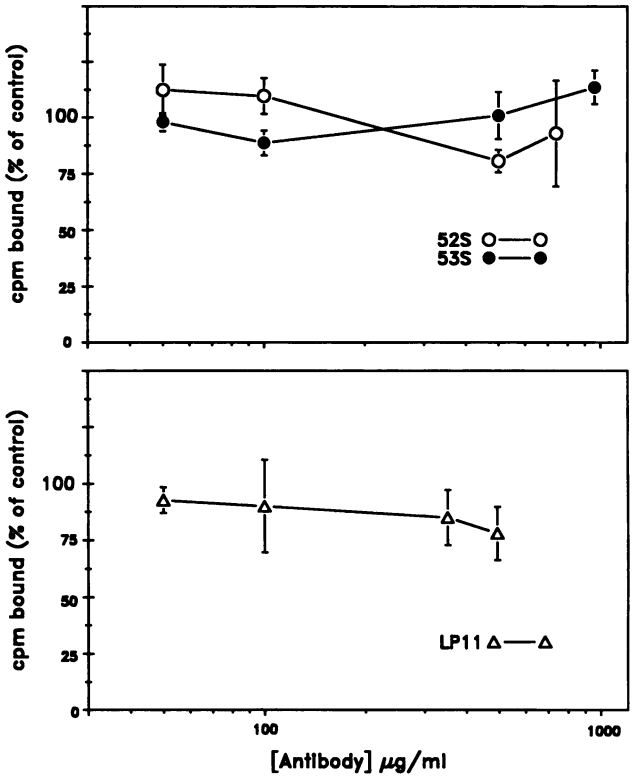

FIG. 4. Effects of the anti-gH MAbs on adsorption of HSV-1(HFEM)syn virions to HEp-2 cells. Purified radiolabeled virions were incubated with various concentrations of the purified MAbs indicated for 1 h at 37°C, chilled, and then plated undiluted on HEp-2 cells in 96-well plates (3×10^7 PFU/ml and 50 PFU per cell before neutralization). After exposure of the cells to the virus for 1 h at 4°C, the cells were washed and cell-bound radioactivity was quantitated. Averages were obtained, from the counts of triplicate wells, for the number of virus bound per well and expressed as the percentage of the number bound in the absence of neutralizing antibodies. Approximately 10% of the input radioactivity (10,000 cpm per well) remained cell-bound in the control samples. Each error bar represents the standard deviation of the mean.

MAbs (18), the anti-gH MAbs, at concentrations sufficient to reduce infectivity to 10^{-5} of control values, had little, if any, effect on virus adsorption (Fig. 4).

Quantitation of virus particles by electron microscopy in sections of cells exposed to infectious and neutralized virus. To determine whether penetration was blocked for virus neutralized by anti-gH MAbs, we compared by electron microscopy the fate of virus neutralized by anti-gH MAbs or by an anti-gD MAb with the fate of infectious virus after adsorption to cells. Suspended HEp-2 cells were exposed to infectious HSV-1(HFEM)syn or to neutralized virus for 40 min at 34°C to allow attachment and penetration. The cells were then processed immediately for sectioning and transmission electron microscopy. The number and location of virus particles (virions and nucleocapsids) were determined in at least 20 randomly chosen cell sections per sample (Fig. 5).

Consistent with the results presented in the preceding section, there was little or no significant difference in the numbers of virus particles detected by electron microscopy in thin sections of the cells exposed to infectious virus and to neutralized virus (see the legend to Fig. 5). In interpreting the results presented in Fig. 5, the following considerations are relevant. For the virus preparations that were either untreated or exposed to the nonneutralizing MAb III-195, the particle/PFU ratios were in the range of 10 to 100. Neutralization by the anti-gH MAb 53S reduced the concentration of PFUs by a factor of about 10 (Fig. 2) and therefore increased the particle/PFU ratio by a factor of 10. The anti-gH MAb 52S and the anti-gD MAb III-114 had more potent neutralizing activity (Fig. 2) and increased the particle/PFU ratios by factors of about 10^5.

The most important point of Fig. 5 is that significant numbers of nucleocapsids were detected in the cytoplasm of cells exposed to infectious, nonneutralized virus (6% of total virus particles observed in each sample were cytoplasmic nucleocapsids) but not in the cytoplasm of cells exposed to neutralized virus (except for one nucleocapsid [0.3% of total particles] in the sample prepared with the weakest anti-gH MAb, 53S). It can be concluded therefore that the anti-gH MAbs, as well as the anti-gD MAb, neutralized infectivity by blocking nucleocapsid penetration into the cytoplasm, rather than by blocking some postpenetration step such as dissociation of the nucleocapsid from envelope remnants or uncoating of the nucleocapsid.

The majority of virus adsorbed to the cell surface, in both infectious and neutralized samples, was still on the cell surface within 40 min of adding virus to the cells (Fig. 5), not inconsistent with particle/PFU ratios greater than 10. Possibly, much of this virus even in infectious samples was not able to penetrate the cell as a result of factors such as intrinsic defects in or deterioration of some virions, adsorption of some virions to "dead-end" receptors with abortion of the process leading to entry or competition for limiting numbers of cell surface components needed for steps after adsorption but before membrane fusion.

Interestingly, only a small fraction of input virus could be detected in intracytoplasmic vesicles at 40 min, with no apparent difference between samples prepared with infectious virus and with neutralized virus. It seems likely that endocytosis plays little or no role in HSV entry into cells based on (i) inability of agents that perturb endocytosis or raise the pH of endosomes to inhibit HSV entry (51); (ii) inability of neutralizing MAbs that block penetration to

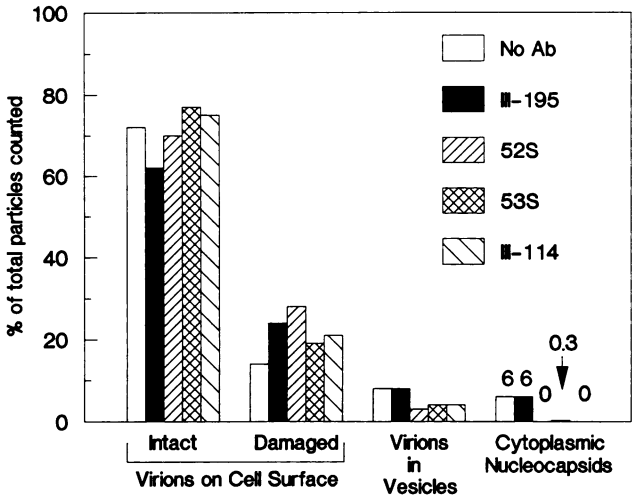

FIG. 5. Quantitation by electron microscopy of adsorbed and penetrated virus particles, after exposure of HEp-2 cells to infectious or neutralized HSV-1(HFEM)syn. Purified virions at 8.7×10^7 PFU/ml were incubated for 1 h at 37°C with the purified MAbs indicated at 0.4 mg/ml (or in the absence of MAb [No Ab]). The mixtures were then incubated with suspended HEp-2 cells at 50 PFU per cell (titer before neutralization) for 40 min at 34°C. The cells were chilled, washed, and processed for analysis of thin sections by electron microscopy. Particles were counted in at least 20 cell sections for each sample, and the location (attached to cell surface, in vesicles, or in cytoplasm) and type of particle (virions [obviously damaged or relatively intact] or nucleocapsids) were categorized. The total number of virus particles counted ranged from 291 to 477 for all samples (380 particles in 22 cell sections for the control without MAb; 328 particles in 39 sections for the nonneutralizing MAb III-195; 286 particles in 38 sections for 52S; 291 particles in 20 sections for 53S; and 477 particles in 32 sections for III-114). The twofold range in numbers of particles per section did not correlate with extent of neutralization and is probably not statistically significant. The numbers of nucleocapsids detected in the cytoplasm were 22 and 25 for the infectious samples (no MAb and the anti-gG-2 [gG-2 being HSV-2 form of gG] MAb III-195, respectively) and were 0, 1, and 0 for the neutralized samples (52S, 53S, and III-114, respectively). The calculated percentages are given on the graph for the nucleocapsid counts.

influence accumulation of virions in endosomes (19; also this study); and (iii) inability to detect virion-cell fusion at any site except the plasma membrane, at which site such events are readily detected as shown elsewhere (19) and in Fig. 6.

The electron microscopic results obtained with the anti-gH MAbs are similar to those obtained with anti-gD MAbs, as reported here and elsewhere (19), and indicate that the anti-gH MAbs, similar to the anti-gD MAbs, block the virion-cell fusion required for viral penetration.

Where virion-cell fusion appeared just to have been initiated in samples prepared with infectious virus, we sometimes observed a condensation of electron-dense material just under the envelope and opposite the point of contact with the plasma membrane. This condensation of electron-dense material appeared to persist until after the nucleocapsid had been released into the cytoplasm free of the envelope. In a previous study, we noticed this condensation phenomenon when penetration occurred at 29°C but not at 37°C (19). In the electron microscopic experiments reported here, the temperature of penetration was 34°C. Possibly the rate at which envelope components (those located under the lipid bilayer as well as those embedded in the lipid bilayer) diffuse away from the fusion site is temperature dependent. At appropriate temperatures, monitoring the location, movement, or catabolism of these envelope components might define useful markers of sequential steps in completion of the fusion process and release of the nucleocapsid from the membrane. Further exploration of this phenomenon is required.

Effects of the MAbs on induction of early viral protein synthesis. To test whether the anti-gH MAbs blocked viral penetration by a different approach, we exposed HEp-2 cells to infectious and neutralized HSV-1(HFEM)syn and pulse-labeled with [^{35}S]methionine at 4.5 h after the addition of virus. Cell lysates were then prepared for analysis of labeled proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to determine whether there was induction of viral protein synthesis and inhibition of cell protein synthesis. Immediate inhibition of cell protein synthesis is mediated by a virion component delivered to the cell during penetration (16), providing a way to monitor penetration.

Virus neutralized by the anti-gD MAbs and by two of the anti-gH MAbs failed to induce the synthesis of viral proteins (Fig. 7, solid arrowheads) and also failed to inhibit the synthesis of cell proteins (open arrowhead). Within the limits of detection in this assay, these antibodies effectively blocked viral penetration and the induction of viral protein synthesis, even at relatively high virion concentrations and over the entire range of MAb concentrations tested (3 to 300 μg/ml). The anti-gH MAb (53S) with weakest neutralizing activity (Fig. 1 and 2) was only partially effective at inhibiting viral penetration; the degree of inhibition appeared to be dependent on the MAb concentration used. The control MAb III-195 had partial inhibitory effects only at the highest concentration tested, which probably reflects a weak neutralizing activity at this dose.

DISCUSSION

The results presented here show that the neutralizing activity of three MAbs specific for gH resulted from ability of the antibodies to block HSV penetration into cells. The antibodies had no significant effect on virus adsorption to cells. HSV penetration results from fusion between the envelope of a virion adsorbed to the cell surface and the plasma membrane (19, 41). Electron microscopy revealed that this fusion was blocked by the anti-gH MAbs and that nucleocapsids were not released into the cytoplasm. Consequently, events associated with viral penetration, such as the inhibition of cell protein synthesis and induction of viral protein synthesis, were not detected. The results we obtained with anti-gH neutralizing MAbs were similar to results we obtained with anti-gD neutralizing MAbs, as reported here and elsewhere (18, 19).

Evidence summarized below suggests that entry of HSV into cells requires a cascade of multiple interactions between several virion glycoproteins and several cell surface components. Moreover, it seems likely that gB, gD, and gH are all essential for interactions that occur subsequent to the initial attachment of virus to cells.

The initial attachment of HSV to cells is binding of the virion to heparan sulfate moieties of cell surface proteoglycans (54). The viral glycoprotein(s) required for this initial attachment includes neither gB nor gD, because virions devoid of either glycoprotein can adsorb to cells normally (4, 31). Virions devoid of gH have not been tested for their ability to adsorb to cells. It seems likely that gH-negative mutants will also prove to be adsorption competent and penetration defective. The findings that neutralizing MAbs

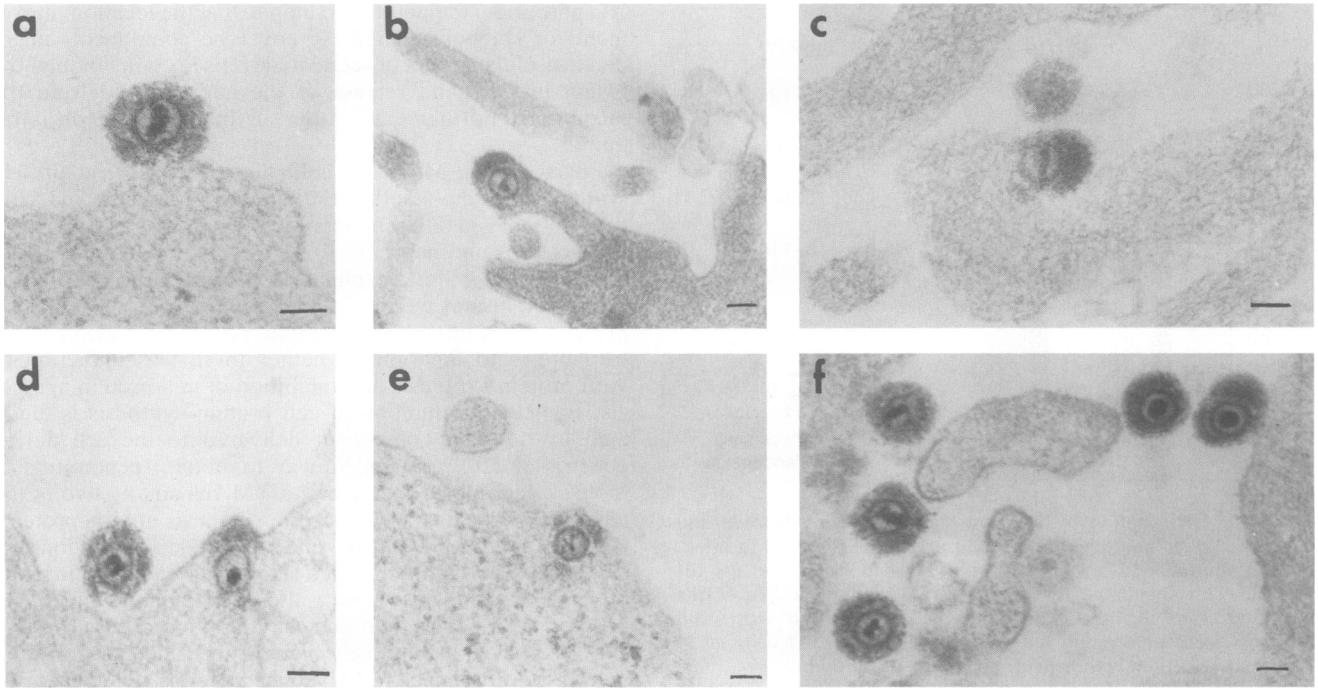

FIG. 6. Electron micrographs showing sections of HEp-2 cells exposed to infectious (a through e) or neutralized (f) HSV-1(HFEM)syn virions. Purified virions were incubated with the neutralizing anti-gH MAb 52S (f), with the nonneutralizing anti-gG-2 MAb III-195 (a, c, d, and e), or without antibody (b). They were then incubated with suspended HEp-2 cells for 20 (a, b, d, and e) or 40 min (c and f) and immediately prepared for electron microscopy (all procedures done as described in the legend to Fig. 5). The images presented in panels a through e could be seen only in samples prepared with infectious virus and were not observed when neutralized virus was used. Bars, 100 nm.

specific for gB (24), gD (19, 25), and gH (this study) can block penetration without blocking adsorption are consistent with roles for these proteins in steps subsequent to initial adsorption.

Although the initial binding of HSV to cells requires the presence of heparan sulfate on the cell surface, stable attachment may require subsequent interactions of the virion glycoproteins with other cell surface receptors. Three lines of evidence suggest that gD has its own receptor. First, prior exposure of cells to UV-inactivated virions containing gD, but not those devoid of gD, can saturate sites required for infection with infectious virus (27). These sites appear to be less numerous than those required for the initial attachment of virus to cells. Second, transformed cells expressing gD are resistant of infection with HSV (virus can adsorb to the cells but fails to penetrate), presumably because the cell-associated gD saturates or sequesters some cell component needed for interaction with gD in virions (6, 28). Third, some anti-gD MAbs can reduce the rate or extent of HSV adsorption (18, 25), despite the fact that gD is not required for initial attachment.

Glycoproteins related to gD are encoded only by HSV-1 and HSV-2, among the human herpesviruses, and by closely related animal viruses. Therefore, the cell receptors recognized by gD would be required only by these herpesviruses. These receptors must be ubiquitous and highly conserved, however, to account for the broad host range of HSV.

It is not clear whether gB and gH interact with specific cell surface receptors. Glycoproteins related to gB and gH have been detected in all herpesviruses examined to date. The gB homologs are highly conserved with respect to amino acid sequence (10, 13, 30, 39, 45, 48). Although the gH homologs are not so similar in amino acid sequence (11, 21, 36), most, if not all, induce the production of neutralizing antibodies as mentioned above. Such antibodies can act by blocking penetration, for Epstein-Barr virus at least (37), as well as for HSV. It seems likely that members of both the gB and gH families are responsible for interactions leading to viral entry and that some aspects of their functions are common to most or all herpesviruses.

Whatever their interactions with other viral or cell components, the HSV forms of gB, gD, and gH all appear to be required for events leading to membrane fusion. HSV mutants that fail to produce gB or gD (or that produce aberrant gB) also fail to induce cell fusion (4, 31, 35) and produce virions that are defective for penetration (4, 31, 32, 49). In addition, not only are MAbs specific for each of the three glycoproteins capable of blocking viral penetration, but MAbs specific for gD and gH can also block virus-induced cell fusion (20, 38, 43). Recent publications have reported that expression of gB or gD alone may be sufficient to induce cell fusion (1, 7). This may be true only for particular cell types and may be a kind of fusion that is triggered by other factors acting in concert with the viral glycoprotein. We have studied cell lines expressing gB (34) or gD (28) and have been unable to detect cell fusion that could be attributed to expression of the glycoprotein in question (unpublished observations). Cells expressing gD can be more susceptible to PEG-induced cell fusion, however (A. O. Fuller, R. M. Johnson, and P. G. Spear, manuscript in preparation).

These three glycoproteins required for membrane fusion do not function as a single multi-subunit structure. Electron microscopy of virions labeled with antibody-gold probes revealed that gB and gD, at least, form two morphologically

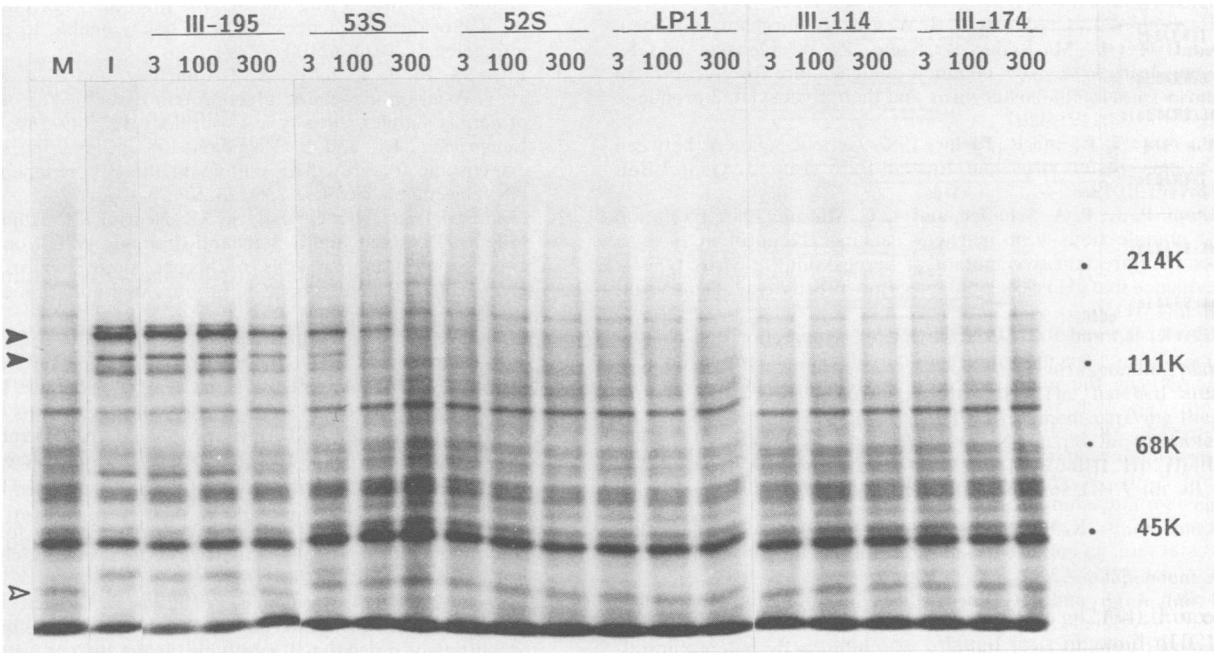

FIG. 7. Effects of the MAbs on initiation of gene expression by HSV-1(HFEM)syn, as judged by the induction of early viral protein synthesis. Purified virions at 2×10^6 PFU/ml were incubated for 1 h at 37°C with purified MAbs at the concentrations (in micrograms per milliliter) indicated. The mixtures were plated on HEp-2 cells (30-min adsorption period at 37°C) at 40 PFU per cell (titer before neutralization). The cells were pulse-labeled with [^{35}S]methionine for 15 min at 4.5 h after the addition of virus. The cells were then lysed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis. III-195 is a nonneutralizing (or very weakly neutralizing) MAb reactive with gG-2; the remainder are neutralizing MAbs (53S, 52S, and LP11 reactive with gH and III-114 and III-174 reactive with gD). M, Mock infected; I, infected with virus in the absence of MAb. The molecular weights of marker polypeptides are given (in thousands [K]) on the right margin. Arrowheads mark the positions of viral proteins made after infection (solid arrowheads) and of cell proteins whose synthesis is markedly inhibited by viral penetration (open arrowhead).

distinct spikes in the virion envelope (52). The physical arrangement of these glycoproteins in the virion envelope, therefore, could permit the multiple interactions with cell surface components proposed.

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