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Specificities of Monoclonal and Polyclonal Antibodies That Inhibit Adsorption of Herpes Simplex Virus to Cells and Lack of Inhibition by Potent Neutralizing Antibodies

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Polyclonal and monoclonal antibodies to individual herpes simplex virus (HSV) glycoproteins were tested for ability to inhibit adsorption of radiolabeled HSV type 1 (HSV-1) strain HFEMsyn [HSV-1(HFEM)syn] to HEp-2 cell monolayers. Polyclonal rabbit antibodies specific for glycoprotein D (gD) or gC and three monoclonal mouse antibodies specific for gD-1 or gC-1 most effectively inhibited HSV-1 adsorption. Antibodies of other specificities had less or no inhibitory activity despite demonstrable binding of the antibodies to virions. Nonimmune rabbit immunoglobulin G and Fc fragments partially inhibited adsorption when used at relatively high concentrations. These results suggest involvement of gD, gC, and perhaps gE (the Fc-binding glycoprotein) in adsorption. The monoclonal anti-gD antibodies that were most effective at inhibiting HSV-1 adsorption had only weak neutralizing activity. The most potent anti-gD neutralizing antibodies had little effect on adsorption at concentrations significantly higher than those required for neutralization. This suggests that, although some anti-gD antibodies can neutralize virus by blocking adsorption, a more important mechanism of neutralization by anti-gD antibodies may be interference with a step subsequent to adsorption, possibly penetration.

The viral structure that mediates attachment of herpes simplex virus (HSV) to cells for initiation of the infectious cycle has not yet been defined. Certain properties of the viral attachment component (VAC) may be inferred from the following. First, HSV has an extremely broad host range with respect to cell type and species. Consequently, the VAC must bind to a ubiquitous constituent of cell surfaces, or perhaps HSV has multiple VACs. Second, the two HSV serotypes (HSV type 1 [HSV-1] and HSV-2) apparently bind to different receptors on cell surfaces (1, 39, 40), suggesting differences in the structures of the HSV-1 and HSV-2 VACs. Finally, the polyanion heparin can block the adsorption of HSV to cells (12, 21, 37, 38).

Possible constituents of the VAC include the envelope glycoproteins designated glycoprotein B (gB), gC, gD, gE, gG, and gH (4; see references 34 and 35 for review). For all these glycoproteins except gG, antigenically related but not identical forms have been detected in both HSV-1 and HSV-2. The HSV-1 homolog of the HSV-2 glycoprotein designated gG or 92K (20, 30) has not yet been detected.

Although the physiological roles of these glycoproteins have not been adequately defined, certain activities have been associated with individual species. Experiments done with temperature-sensitive mutants altered in gB (19, 31) showed that this glycoprotein is essential for infectivity and indicated a possible role for gB in penetration. Artificial liposomes prepared with HSV envelope glycoproteins bind to cells more efficiently when gB is present than when it is absent, indicating a role for gB in adsorption as well (17). Potent neutralizing antibodies are induced in response to gD (7, 9, 15, 26, 28, 32), and anti-gD antibodies can block HSV-induced cell fusion (22). gC binds to the C3b component of complement (10). Both gC and the binding activity associated with it appear to be dispensable in cell culture based on the viability of gC-negative mutants (1, 11, 13, 14, 43). gE has Fc-binding activity (2, 24, 25). No activities have yet been associated with gG or gH.

In one approach to characterizing the HSV VAC, we tested monoclonal and polyclonal antibodies to individual glycoproteins for ability to inhibit adsorption of HSV-1 to cells. We show that certain antibodies to gD and gC can inhibit virus adsorption, as can high concentrations of Fc domains of immunoglobulin G (IgG). We also compared this inhibitory activity of the monoclonal antibodies with neutralizing activity. The results suggest that gD, gC, and perhaps gE are directly or indirectly involved in HSV adsorption and that some neutralizing antibodies can block HSV infectivity at a step subsequent to adsorption.

MATERIALS AND METHODS

Cells and viruses. Human epidermoid carcinoma (HEp-2) and African green monkey (Vero) cells were grown in monolayer cultures in Dulbecco modified Eagle minimal essential medium supplemented with 10% fetal bovine serum. HEp-2 cells were used for producing virus stocks and for adsorption assays. Vero cells were used for determining virus titers and for neutralization assays. The virus strain used was HSV-1(HFEM)syn, a syncytial mutant isolated by R. Baucke (2) from HSV-1(HFEM) obtained from A. Buchan (University of Birmingham, United Kingdom).

Preparation of purified labeled virus. The procedure for purification of virions was as described by Spear and Roizman (36) and modified by Cassai et al. (6). Briefly, HEp-2 cells grown to confluency were infected with HSV-1 (HFEM)syn at a multiplicity of infection of 3 PFU per cell. The infected cells were overlaid with medium 199 supplemented with 1% fetal calf serum (medium 199V), and at 4 to 5 h after infection the medium was changed to 199V containing 20% of the normal concentration of leucine and [^{3}H]leucine at 20 μCi/ml (Amersham Corp.). The cells were incubated at 34°C for 36 to 48 h, at which time the infected cells could be easily detached. Virus was harvested and

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purified from infected cells by centrifugation through dextran gradients (Dextran T-10; Pharmacia, Inc.). The titers of fractions containing purified virus were determined on Vero cells and radiolabel monitored by liquid scintillation counting. Portions of labeled virus were stored at −80°C until used.

Preparation and purification of monoclonal antibodies. Hybridomas secreting antibodies capable of binding to HSV virions were isolated, and ascites fluids containing these antibodies were characterized as described elsewhere (26). IgG was purified from mouse ascites fluids by affinity chromatography on protein A-Sepharose CL-4B (Pharmacia). IgG bound to protein A was eluted with 1.0 M acetic acid or 0.1 M glycine (pH 3.0), dialyzed against 0.01 M sodium phosphate buffer (pH 7.2), and concentrated by vacuum centrifugation (Savant Instruments, Inc.). Protein concentrations were determined by a Bio-Rad protein assay with immunoglobulin as the standard protein. Purified antibody preparations were centrifuged before use to remove aggregates and contained no protease activity detectable by a Bio-Rad protease detection assay with casein as the substrate.

Preparation and purification of polyclonal antibodies. All but one of the anti-HSV antisera used were previously described and characterized (Table 1). An antiserum reactive with gD was prepared by injecting rabbits with a secreted truncated form of gD-1 (gD-1^t) purified by affinity chromatography (9) from the medium of cells infected with an HSV mutant designated 111. This mutant contains a deleted form of the gD-1 gene inserted into its *tk* gene and expresses gD-1^t from this gene; in gD-1^t the 82 amino acids normally found at the carboxy terminus of gD-1 are replaced with 48 amino acids of unrelated sequence (M. G. Gibson and P. G. Spear, manuscript in preparation). The antigen (100 μg per rabbit) in 0.01 M sodium phosphate buffer (pH 7.4) was emulsified in complete Freund adjuvant and inoculated subcutaneously at multiple sites on female New Zealand White rabbits. The rabbits received boosters at 2-week intervals of 75 μg of protein in incomplete Freund adjuvant and were bled 8 weeks after the first inoculation. The antiserum immunoprecipitated purified gD-1^t and also gD-1 from extracts of HSV-1(HFEM)syn virions prepared as previously described (42).

TABLE 1. Rabbit antibodies used and binding of protein A-reactive IgG to HSV-1(HFEM)syn virons

Antibody[a]	Procedures for purification of immunogen	^{125}I-protein A bound to virion-antibody complexes (cpm)[b]
Anti-HSV-1 (R#23)	Unfractionated proteins extracted from purified HSV-1(F) virions (33)	1,600
Anti-HSV-2 (R#22)	Unfractionated proteins extracted from purified HSV-2(G) virions (33)	ND[c]
Anti-gB-1 (R#67)	Gradient centrifugation and preparative SDS-PAGE (16)	890
Anti-gC-2 (R#71)	Immunoprecipitation and preparative SDS-PAGE (42)	1,020
Anti-gD-1[d]	Affinity chromatography (9)	1,290
Anti-gE-1 (R#S24)	Affinity chromatography and preparative SDS-PAGE (24)	3,260
Anti-gD-1^t (R#72)[e]	Affinity chromatography of soluble secreted gD-1^t (see text)	ND

[a] Detergents used for extraction of immunogens were Nonidet P-40 plus sodium deoxycholate, unless otherwise indicated. The code in parentheses is the number assigned to each rabbit for the antisera produced in our laboratory.

[b] Radioactivity bound to purified virions (10^7 PFU) that had been incubated with purified IgG at a concentration of 0.1 mg/ml. Background counts (counts per minute in samples containing only IgG plus protein A or only protein A plus virus) were subtracted.

[c] ND, Not determined.

[d] A gift from Roselyn Eisenberg and Gary Cohen.

[e] No detergent was used for immunogen extraction.

For most of the antisera, IgG was purified by protein A-Sepharose CL-4B affinity chromatography as described above. For the antisera to gB-1 and gD-1, IgG was purified by chromatography on DEAE Affi-Gel Blue (Bio-Rad) (3). Bound IgG, along with transferrin, was eluted from the column with a 20 to 50 mM NaCl gradient, and the IgG was concentrated by ammonium sulfate precipitation followed by dialysis against 0.01 M sodium citrate buffer (pH 7.0). Protein concentrations were determined by a Bio-Rad protein assay.

Preparation of Fab and Fc fragments of IgG. Fab and Fc fragments of rabbit IgG were prepared by a modification of the method of Porter (29). Briefly, protein A-purified IgG at a concentration of 10 mg/ml was digested with a 1:200 ratio of papain (Worthington Diagnostics) to IgG in 0.1 M sodium phosphate buffer (pH 6.5). Papain was activated with cysteine and EDTA as specified by Worthington. The digestion mixture was incubated at 37°C for 5 h, and digestion was ended by raising the pH to 8.0, followed by freezing. The extent of digestion was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the final digest was judged free of intact IgG by Coomassie blue staining of the gel. Fab and Fc fragments were isolated by chromatography on protein A-Sepharose CL-4B. Fab fragments were collected from the column flowthrough, the column was extensively washed, and the Fc fragments were eluted with 0.1 M glycine (pH 3.0). The samples were dialyzed against 0.01 M sodium phosphate buffer (pH 7.0) and concentrated by centrifugation through Centricon 30 concentrators (Amicon Corp.). Only a single band could be detected by SDS-PAGE on 10% gels in the final preparations. The protein concentration was determined by a Bio-Rad protein assay.

Relative amount of antibody bound to intact virions. Antibody diluted with phosphate-buffered saline (10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl, 0.5 M $MgCl_2 \cdot 6H_2O$, 1 mM $CaCl_2$ [pH 7.4]) (PBS) containing 1% inactivated calf serum and 0.1% glucose (PBS-GC) was incubated with virus (10^7 PFU) in a total volume of 40 μl for 1 h at 37°C. Excess ^{125}I-protein A (30 mCi/mg; Amersham Corp.) diluted in PBS-GC to 5.0×10^5 cpm/ml was added to each sample, bringing the volume to 115 μl, and incubated on ice for an additional hour. Virus-IgG-protein A complexes were separated from free IgG-protein A complexes or free protein A by vacuum filtration through a polycarbonate membrane (0.08-μm pore size; Nuclepore Corp.) which had been prewashed three times with ovalbumin (3 mg/ml in PBS). The filters were washed four times with buffer, dried, and quantitated for ^{125}I counts per minute. Control experiments showed that the ^{125}I-protein A was specifically retained by the filters only under conditions such that virion-IgG-protein A complexes could form. The polycarbonate

filters retained 60 to 70% of the counts per minute in ^{3}H-labeled virus which had not been exposed to IgG or protein A. Complexes of IgG and ^{125}I-protein A formed in the absence of virus passed through the filter; cpm in these samples (420 to 540) were not significantly higher than those in controls of ^{125}I-protein A alone or virus plus ^{125}I-protein A (350 to 440). After incubation of virions with nonimmune IgG, near-background levels of ^{125}I-protein A were retained by the filters (500 to 620 cpm), whereas incubation of virions with HSV-specific IgG resulted in significantly greater retention of the ^{125}I-protein A (1,230 to 1,270 cpm).

Adsorption Assay. Virus adsorption was routinely quantitated by using radiolabeled virus. HEp-2 cells growing as confluent monolayers in 96-well microtiter plates were pretreated by being washed once on ice with ovalbumin in PBS-GC (2 mg/ml), followed by a 15-min incubation of cells with ovalbumin in PBS-GC (5 mg/ml) at 4°C. Radiolabeled purified virus (3×10^7 PFU) was incubated for 1 h at 25°C with antibody or reagent diluted to a final volume of 120 μl with PBS-GC. The antibody-virus mixture was divided and added in triplicate to pretreated monolayers in the 96-well plates. The plates were incubated and gently agitated at 4°C for 2 h to allow adsorption of virus to cells. The supernatant was removed to GF/A microfiber filters (Whatman, Inc.) to quantitate unbound radioactivity, and the wells were washed at least three times with cold PBS-GC. Cells and bound virus were removed from the wells by solubilization in PBS containing 1% Triton X-100 and 1% SDS. The counts per minute in bound and unbound samples were quantitated by liquid scintillation counting. Total recovery of radioactivity ranged from 90 to 100% of input. The percentage of recovered virus bound to cells was calculated from the average of triplicate samples. For most analyses, the deviation of each value from the average was no more than 10%. The percent bound and relative percent inhibition were calculated as follows: % bound = counts per minute bound/(counts per minute unbound + counts per minute bound); % inhibition = 100 × [1 − (% bound in presence of inhibitor/% bound in control)].

RESULTS

Antibodies used and relative amounts bound to HSV-1 virions. Polyclonal and monoclonal antibodies directed against different HSV glycoproteins were tested in this study for ability to block virus adsorption. The polyclonal antibodies were produced by immunization with purified preparations of individual glycoproteins (or virion extracts) and shown to precipitate selectively the glycoprotein (or glycoproteins) used for immunization as described in the papers cited in Table 1 and above. The monoclonal antibodies used (Table 2) were selected for ability to bind to HSV-1(HFEM)syn or HSV-2(G) virions and are characterized elsewhere with respect to IgG subclass, neutralizing activity, and ability to immunoprecipitate solubilized HSV glycoproteins (26).

We compared the relative efficiency of binding of these monoclonal and polyclonal antibodies to intact virions under conditions identical to those used for the adsorption experiments described below. ^{125}I-protein A was the probe used to quantitate the relative amount of IgG bound to the virions. Experiments with five monoclonal antibodies showed that an IgG concentration of 0.1 mg/ml did not saturate available IgG binding sites on virions (10^7 PFU) for most of the antibodies. Close to maximal amounts of II-105-1 IgG bound at 0.1 mg/ml, while higher concentrations were required to achieve maximal binding of antibodies I-206-7, III-255-2, II-694-6, and III-188-4 (data not shown). Tables 1 and 2 show the amounts of radioactive protein A bound to a constant number of virions (10^7 PFU) after incubation with the various HSV-specific IgG preparations at concentrations of 0.1 mg/ml.

TABLE 2. Properties of the monoclonal antibodies used and binding of the antibodies to HSV-1(HFEM)syn virons

Monoclonal antibody[a]	IgG subclass	Immunoprecipitates		^{125}I-protein A bound to virion-antibody complexes (cpm)[b]
		Type 1	Type 2	
Anti-gD				
III-255-2	2a	+	+	3,460
II-886-1	2a	+	−	3,110
1-206-7	2a	+	−	2,300
III-114-4	2b	+	+	1,980
I-99-1	2a	+	+	1,920
II-436-1	2a	+	−	1,820
I-188-5	2a	+	+	1,310
III-174-1	2a	+	+	750
Anti-gB				
I-59-2	3	+	+	4,900
I-252-4	2a	+	−	1,840
II-694-6	2a	+	+	1,250
II-105-1	2a	+	+	670
Anti-gE (III-252-4)	3a	+	−[c]	1,720
Anti-gC				
II-529-3	ND[d]	+	−	2,900
III-188-4	2a	−	+	2,070
II-474-1	ND	+	−	1,670
III-596-1	2a	−	+	1,500
II-73-3	3	+	−	910
II-512-3	ND	+	−	ND

[a] Production of these antibodies and their characterization with respect to IgG subclass and immunoprecipitating activity were described elsewhere (26).
[b] See Table 1, footnote *b*.
[c] Despite the fact that this antibody was induced in response to HSV-2(G), we were not able to detect a reaction with any HSV-2 antigen by immunoprecipitation.
[d] ND, Not determined.

These data verify that all the polyclonal and monoclonal antibodies used in this study bind to intact HSV-1 virions. For monoclonal antibodies, the amount bound to intact virions at a single concentration of IgG did not correlate with IgG subclass or with glycoprotein specificity as determined by immunoprecipitation. The variation in amounts of different monoclonal antibodies bound to the same glycoprotein may reflect differences in the proportions of active HSV-specific antibody present in each IgG preparation, differences in accessibility on virions of their respective antigenic determinants, or differences in avidities of the antibodies.

Note that the anti-gC-2 monoclonal antibodies, III-596-1, III-188-4, and polyclonal anti-gC-2, which do not recognize an HSV-1 glycoprotein by immunoprecipitation (42), did interact with intact HSV-1 virions (Table 2). These results indicate that antigenic determinants shared by gC-1 and gC-2 in intact virions may be lost on solubilization of the glycoproteins.

Assay to measure virus adsorption. Binding of purified radiolabeled virus to HEp-2 cells was at 4°C to quantitate virus adsorption under conditions that prevented uptake. It was shown by trichloroacetic acid precipitation that radio-

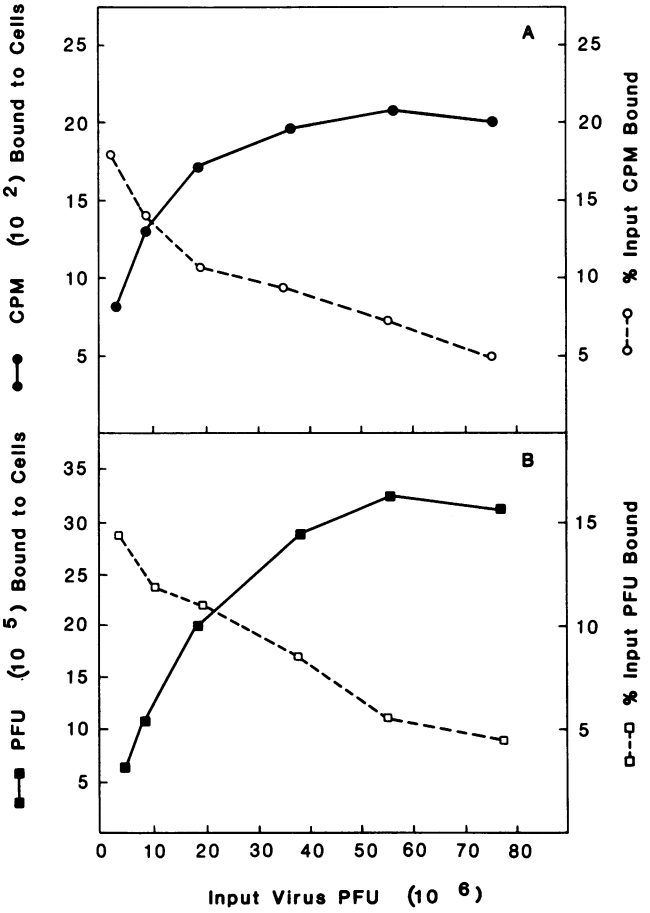

FIG. 1. Amount of HSV-1(HFEM)syn bound to HEp-2 cells as a function of input virus. The number of PFUs indicated in a total volume of 40 μl was added to monolayers in replicate wells of a microtiter tray. After 2 h at 4°C, unbound virus was removed, the cells were washed, and bound virus was quantitated by counting radioactivity of cell lysates prepared with detergent (●) or by determining the titers of infectivity in cell lysates prepared by sonication in PBS (■). Each point is the average of triplicate analyses. The percentages of input counts per minute or input PFU bound are also shown.

activity measured in purified virus samples represented radiolabel incorporated into macromolecules. Nonspecific interactions of virus with the surface of the microtiter plate and with cells were minimized by pretreatment of monolayers with ovalbumin. Pretreatment of wells without cells reduced the amount of radiolabeled virus bound to the surface of wells from 7.5% without pretreatment to less than 1.3% of input counts per minute.

That virus adsorption was to specific sites limited in number is indicated by the apparent saturability of sites for virus on HEp-2 cell monolayers (Fig. 1). The amount of virus bound to cells as a function of increasing input virus was determined by monitoring bound infectivity (PFU) or bound radioactivity. The similarity of the binding curves for infectivity and radiolabel indicated that binding of radiolabel was representative of binding of infectious particles. HEp-2 or Vero cell monolayers could be saturated with an input of about 6×10^7 PFU for different purified HSV-1(HFEM)syn preparations (the particle-to-PFU ratios in these preparations were not determined). Increasing the amount of input virus did not result in greater amounts of label or infectivity

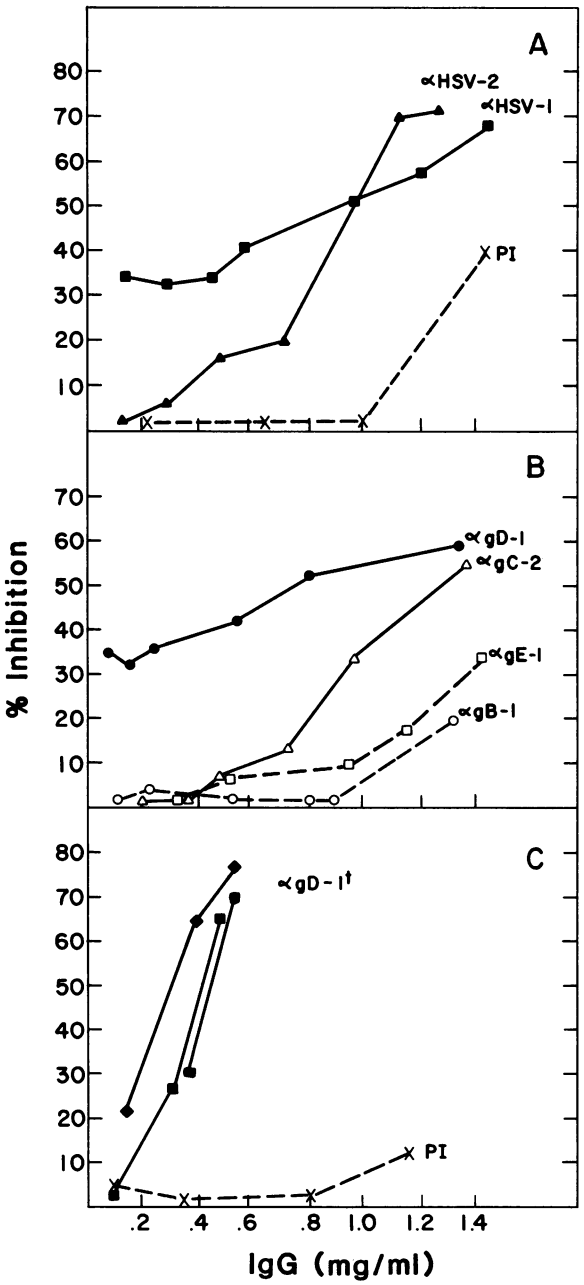

FIG. 2. Inhibition of HSV-1(HFEM)syn adsorption to HEp-2 cells by IgG purified from the rabbit antisera indicated in Table 1 or from nonimmune rabbit sera (PI). Purified radiolabeled virions were incubated for 1 h at 25°C with IgG at the final concentrations indicated. Samples (50 μl) of each reaction mixture were then added in triplicate to HEp-2 cell monolayers in the wells of microtiter plates. After adsorption for 2 h at 4°C, the unbound virus was removed, the cells were washed, and cell lysates were prepared to determine radioactivity in the cell-bound fractions. The percentage of inhibition of adsorption was calculated as described in the text. Nonimmune rabbit serum used for panel A was obtained before immunization from the rabbit that produced anti-gC-2. The nonimmune rabbit serum used for panel C was obtained before immunization from the rabbits that produced anti-gD-1^t. Three separate experiments were performed with anti-gD-1^t, as shown in panel C (■).

bound under the specific conditions described above. The fraction of input virus bound to cells could be increased by increasing the temperature for adsorption so that uptake was no longer prevented.

Experiments to determine the effects of antibodies or control reagents on virus adsorption were done with near-saturable levels (10^7 PFU) of input virus. When the effects of nonimmune goat IgG (0.33 mg/ml), ovalbumin (0.66 mg/ml), or heparin on virus adsorption were evaluated, only heparin (0.03 and 0.33 mg/ml) blocked the binding of HSV-1(HFEM)syn to HEp-2 cells (as much as 92 and 95% inhibition, respectively). Heparin was used as a control for inhibition in all experiments.

Effects of polyclonal antibodies on virus adsorption. Polyclonal antibodies to individual HSV glycoproteins or to HSV-1 or HSV-2 virion envelope extracts were evaluated for their ability to block adsorption of radiolabeled virus. Virus which had been preincubated with antibody was added in triplicate to HEp-2 monolayers and allowed to adsorb at 4°C. The percent bound in samples containing inhibitors (antibody or heparin) was compared with the percent bound in control samples containing no inhibitor. The dose-dependent effects of the polyclonal antibodies are shown in Fig. 2. Antibodies purified from antiserum to HSV-1 envelope extracts were effective at inhibiting virus adsorption when used at relatively low concentrations (0.1 to 0.5 mg/ml). Antibodies to HSV-2 extract were also effective at inhibiting adsorption of HSV-1(HFEM)syn at concentrations of 0.4 mg/ml or

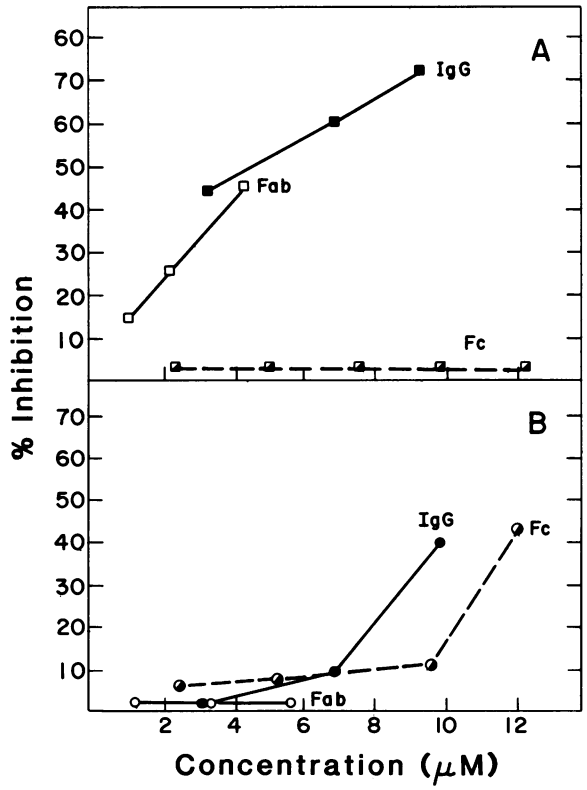

FIG. 3. Inhibition of HSV-1(HFEM)syn adsorption to HEp-2 cells by various concentrations of purified IgG and Fab and Fc fragments from rabbit anti-HSV-1 serum (A) and nonimmune rabbit serum (B). The experiments were performed as described in the legend to Fig. 2. Concentrations of IgG and fragments were calculated assuming molecular weights of 150,000 for IgG, 45,000 for Fc fragments, and 50,000 for Fab fragments.

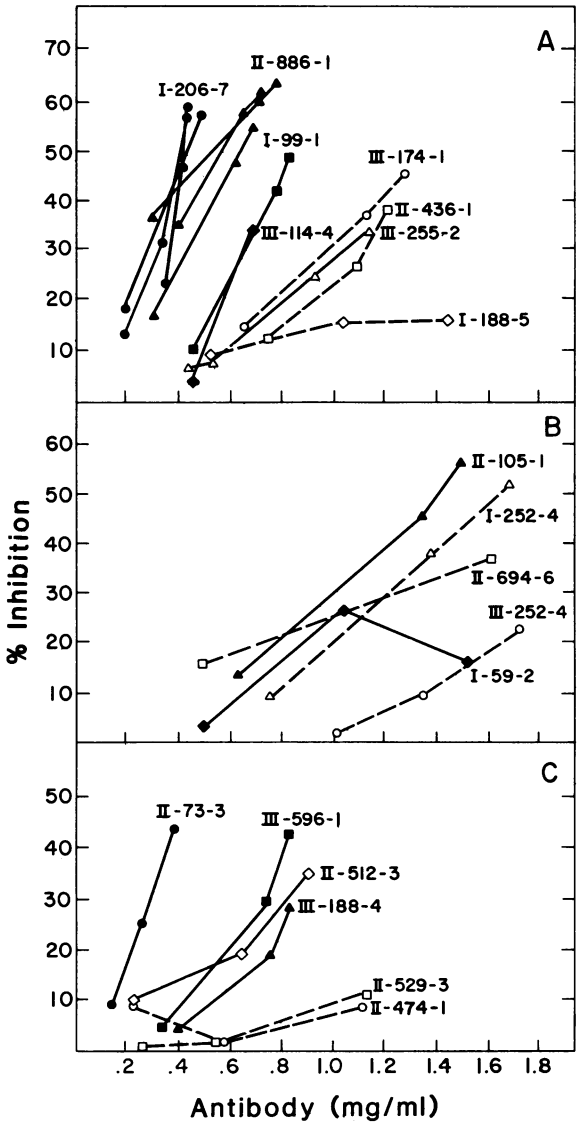

FIG. 4. Inhibition of HSV-1(HFEM)syn adsorption to HEp-2 cells by various concentrations of IgG purified from ascites fluids containing monoclonal antibodies specific for gD (A), gB and gE (B), and gC (C). The experiments were done as described in the legend to Fig. 2. Results from multiple experiments are shown for I-206-7 (●) and II-886-1 (▲).

higher (Fig. 2A), indicating the presence of cross-reactive antibodies. Among the antibodies to individual HSV glycoproteins (Fig. 2B and C), two preparations of anti-gD-1 antibodies had equal or greater activity than the anti-HSV-1 antibodies at blocking virus adsorption. Anti-gC-2 antibodies had an effect only when used at intermediate concentrations, and anti-gB-1 and anti-gE-1 antibodies were no more effective than purified IgG from nonimmune serum (Fig. 2).

At high concentrations (>1.0 mg/ml), all the IgG preparations including the IgG from nonimmune rabbit serum had an inhibitory effect on HSV adsorption. Because gE in virions has Fc receptor activity (2, 23, 24), these results raised the possibility that IgG might prevent virus adsorption by binding to virus via the Fc region. We investigated this possibility by comparing activities on a molar basis of IgG and Fab and Fc fragments from a nonimmune serum and from an anti-

TABLE 3. Neutralizing activities of purified anti-gD monoclonal antibodies

Antibody	Concn (ng/ml) required for 50% PFU reduction[a]
III-174-1	63
III-114-4	95
III-255-2	100
II-436-9	440
I-188-5	2,500
I-99-1	>6,020[b]
I-206-7	>3,980
II-886-1	>6,020

[a] Purified virions were incubated with at least four different concentrations of IgG (in the absence of complement) to permit calculation by extrapolation of the concentration required for 50% reduction in PFU.

[b] At a concentration of 6,020 ng/ml, 20% reduction in PFU was observed.

HSV-1 antiserum (Fig. 3). The inhibitory activity of HSV-specific IgG at relatively low molar concentrations (Fig. 3A) correlated well with some detectable activity for comparable molar amounts of Fab fragments from the same HSV-specific IgG. In contrast, there was no activity at this concentration for Fab fragments from nonimmune IgG (Fig. 3B). The inhibitory activity seen only at high concentrations for IgG from nonimmune serum correlated with some detectable activity of the purified Fc fragments from this IgG. The lack of activity of Fc fragments from HSV-specific IgG was unexpected. It may be due either to loss of activity in this particular preparation of Fc fragments or to variability of Fc-binding activity among different rabbits or different IgG preparations. We favor the first possibility because the purified Fc fragments from nonimmune IgG (Fig. 3B) had less activity on a molar basis than the intact IgG from which they were made.

These results support the possibility that inhibition of HSV-1(HFEM)syn adsorption by IgG at high concentrations might be mediated via the Fc domain of IgG. However, this inhibitory activity seems to require high concentrations of Fc. At relatively low concentrations, both anti-gD-1 and anti-gC-2 polyclonal IgG inhibited HSV-1(HFEM)syn adsorption (Fig. 2) much better than did nonimmune IgG, indicating a contribution of antigen-specific reactivity. The blocking activities of the polyclonal antibodies did not correlate with amounts of IgG bound to virions at 0.1 mg/ml (Table 1), indicating that differential activity depends on specificity of the antibodies.

Effect of monoclonal antibodies on virus adsorption. To further assess the ability of antibodies to one or another of the HSV glycoproteins to block virus adsorption, we tested monoclonal antibodies to HSV-1 glycoproteins (Table 2) for ability to inhibit adsorption of radiolabeled HSV-1(HFEM)syn to HEp-2 cell monolayers. The 18 monoclonal antibodies to gD, gB, gE, or gC were grouped into three categories based on the concentration dependence of their inhibitory activities (Fig. 4). Three monoclonal antibodies, two specific for gD-1 (I-206-7 and II-886-1) and one specific for gC-1 (II-73-3), significantly inhibited virus adsorption at relatively low antibody concentrations (<0.4 mg/ml). Two other anti-gD (I-99-1 and III-114-4) and three other anti-gC (III-596-1, III-188-4, and II-512-3) monoclonal antibodies were effective at blocking adsorption when used at intermediate concentrations (0.5 to 1.0 mg/ml). The other monoclonal antibodies tested inhibited adsorption only when used at high concentrations (>1.0 mg/ml). The most effective blockers of virus adsorption were not necessarily those that exhibited most efficient binding to intact virus (Table 2). For example, III-255-2, an anti-gD antibody which binds efficiently to virions, was not a very effective inhibitor of virus adsorption. In contrast, II-73-3, an anti-gC antibody which binds in relatively low amounts at 0.1 mg/ml, was an effective inhibitor of adsorption.

Comparison of inhibitory activities of anti-gD monoclonal antibodies. Monoclonal antibodies used in these experiments were assayed for neutralizing titers in ascites fluids as described elsewhere (26). Monoclonal antibodies with the highest complement-independent neutralizing titers had specificity for gD. To verify that the relative titers of the ascites fluids reflected differences in neutralizing potency rather than concentration of antibody, we determined neutralizing activities of the purified IgGs in a 50% plaque reduction assay (Table 3). The antibodies which were most effective, III-114-4, III-174-1, and III-255-2, neutralized 50% of the PFU at concentrations of ≤100 ng/ml. We ranked the anti-gD antibodies with respect to three different activities—ability to block virus adsorption and to neutralize viral infectivity based on the results presented here and ability to block virus-induced cell fusion based on results presented by Noble et al. (22) (Table 4). Note that the antibodies which best inhibit adsorption had poor neutralizing activity whereas the most potent neutralizing antibodies were the most effective at inhibition of HSV-induced cell fusion.

DISCUSSION

Our findings that some antibodies to gD and gC and high concentrations of Fc domains can inhibit HSV adsorption raise the possibility that gD, gC, and possibly gE play a role in HSV adsorption. The monoclonal antibodies that inhibited adsorption were not those with the most potent neutralizing activity. Analysis of the different activities of anti-gD monoclonal antibodies suggests that, although neutralization can be mediated by inhibition of adsorption, a more important mechanism is interference with a step subsequent to adsorption, possibly membrane fusion leading to penetration.

Evidence for involvement of gD and gC in adsorption is that polyclonal antibodies to gD and gC and certain anti-gD and anti-gC monoclonal antibodies can each independently inhibit virus attachment, while less inhibitory activity was observed with antibodies specific for other glycoproteins.

TABLE 4. Ranking of the anti-gD monoclonal antibodies for three different activities[a]

Inhibition of binding	Inhibition of fusion[b]	Neutralization without complement
I-206-7	III-174-1	III-174-1
II-886-1	III-114-4	III-114-4
I-99-1	III-255-2	III-255-2
III-114-4	I-99-1	II-436-9
II-436-9	I-188-5	I-188-5
III-174-1	I-206-7	I-99-1
III-255-2	II-436-9	I-206-7
I-188-5	II-886-1	II-886-1

[a] Monoclonal antibodies were ranked from most to least effective (top to bottom).

[b] From data presented by Noble et al. (22).

Concentrations of antibody required were relatively high, perhaps because complete saturation of specific antigen determinants is required. No correlation was found between the amount of antibody bound to virus at one antibody concentration and blocking activity, indicating that the site recognized is more important than the absolute amount of antibody bound. It is of interest that the monoclonal antibodies with the best activity in inhibiting adsorption, I-206-7, II-886-1, and II-73-3, are type specific, at least by immunoprecipitation. This is consistent with reports that HSV-1 and HSV-2 bind to different receptors, implying differences in the structures of their VACs (1, 39, 40).

Nonimmune rabbit IgG and Fc fragments thereof can also inhibit virus adsorption, possibly by binding to the Fc-binding glycoprotein, gE. However, very high concentrations are required, perhaps because the affinity of gE for Fc domains may be lower than that of antibody for viral antigen. It is difficult to assess the contribution of Fc binding to the effects of monoclonal antibodies on adsorption. For reasons documented elsewhere (18, 27; P. Parham, *in* D. Weir, C. Blackwell, L. Herzenberg, and L. Herzenberg, ed., *Handbook of Experimental Immunology*, in press), it was difficult to obtain isolated fragments from most of the monoclonal antibodies used here. Unless these antibodies (mostly of the 2a subclass) differed markedly in affinity for the Fc receptor, there must have been a large contribution of antigen-specific binding to the inhibition of adsorption observed with I-206-7, II-886-1, and II-73-3.

The results presented here do not permit identification of the HSV glycoprotein or glycoproteins that make direct contact with the cell surface to initiate infection. Our results do have certain implications as to the nature of the viral structure or structures that mediate adsorption. Either the VAC is a single structure composed of several HSV glycoproteins or multiple interactions of individual glycoproteins with the cell surface must occur, simultaneously or sequentially, to mediate stable attachment.

Assuming that the VAC is a single structure composed of several glycoproteins, gD and gC must both be components of this structure. Although gC seems not to be essential for infectivity on cultured cells (5, 11, 13, 14, 43), if present it may be a component of the VAC and serve as a target of blocking antibodies. Results obtained with Fc fragments suggest that gE is also a component of the VAC. Lack of inhibitory effects of the anti-gE antibodies does not rule out this possibility, nor can we exclude the possibility that other HSV glycoproteins are also a part of the VAC. Existence of a single multicomponent VAC implies interactions which have not yet been detected among the HSV glycoproteins.

Assuming that several HSV glycoproteins interact individually with the cell surface to mediate adsorption, these interactions could occur simultaneously or sequentially to trigger an event leading to penetration. Antibodies to any of the glycoproteins involved could block adsorption, provided that each interaction was essential for infectivity. In connection with this hypothesis, binding to cells of lipid vesicles containing HSV glycoproteins was blocked by anti-gB antibodies (17) that had little effect in this study on the attachment of virus to cells. Possibly, stable interaction of the liposomes to cells was mediated primarily by interaction of gB with a cell surface component. In virions, a similar interaction may occur but only after or in conjunction with stable attachment of virus to cells mediated by other viral glycoproteins.

Comparison of the activities of anti-gD monoclonal antibodies (Table 4) in three functional assays shows that anti-gD antibodies can neutralize infectivity by at least two different mechanisms. Weakly neutralizing antibodies such as I-206-7 and II-886-1 may act by blocking adsorption. The most potent neutralizing antibodies, however, prevent infection at doses that have little if any effect on virus adsorption. Although adsorption of neutralized virus may differ qualitatively from that of nonneutralized virus, some anti-gD antibodies probably block infectivity at a step subsequent to adsorption. The most potent anti-gD neutralizing antibodies are also the most potent inhibitors of cell fusion (Table 4). Because fusion between the virion envelope and a cell membrane is likely to be required for virus penetration, we speculate that these antibodies neutralize infectivity by blocking membrane fusion required for penetration. Studies done with two other enveloped viruses, murine hepatitis virus (8) and respiratory syncytial virus (41), also showed that potent neutralizing antibodies could block virus-induced cell fusion. In these studies effects of the neutralizing antibodies on virus adsorption were not assessed.

That the anti-gD antibodies which most effectively block fusion and inhibit adsorption differ supports a multifunctional role for gD in both adsorption and penetration. Determination of the regions of gD recognized by these different antibodies will facilitate investigation of this multifunctional role.

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