

## Neutralizing Monoclonal Antibodies Specific for Herpes Simplex Virus Glycoprotein D Inhibit Virus Penetration

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Received 7 May 1987/Accepted 13 July 1987

**Nine monoclonal antibodies specific for glycoprotein D (gD) of herpes simplex virus type 1 were selected for their ability to neutralize virus in the presence of complement. Four of these antibodies exhibited significant neutralization titers in the absence of complement, suggesting that their epitope specificities are localized to site(s) which contribute to the role of gD in virus infectivity. Each of these antibodies was shown to effectively neutralize virus after virion adsorption to cell surfaces, indicating that neutralization did not involve inhibition of virus attachment. Although some of the monoclonal antibodies partially inhibited adsorption of radiolabeled virions, this effect was only observed at concentrations much higher than that required to neutralize virus and did not correlate with complement-independent virus-neutralizing activity. All of the monoclonal antibodies slowed the rate at which virus entered cells, further suggesting that antibody binding of gD inhibits virus penetration. Experiments were carried out to determine the number of different epitopes recognized by the panel of monoclonal antibodies and to identify epitopes involved in complement-independent virus neutralization. Monoclonal antibody-resistant (*mar*) mutants were selected by escape from neutralization with individual gD-specific monoclonal antibodies. The reactivity patterns of the mutants and antibodies were then used to construct an operational antigenic map for gD. This analysis identified a minimum of six epitopes on gD that could be grouped into four antigenic sites. Antibodies recognizing four distinct epitopes contained in three antigenic sites were found to neutralize virus in a complement-independent fashion. Moreover, *mar* mutations in these sites did not affect the processing of gD, rate of virus penetration, or the ability of the virus to replicate at high temperature (39°C). Taken together, these results (i) confirm that gD is a major target antigen for neutralizing antibody, (ii) indicate that the mechanism of neutralization can involve inhibition of virus penetration of the cell surface membrane, and (iii) strongly suggest that gD plays a direct role in the virus entry process.**

The envelope of herpes simplex virus (HSV) contains at least six virus-encoded glycoproteins which are exposed on the virion surface (2, 43, 44). To more fully understand the function of these glycoproteins in HSV infection and immunity, we have carried out studies to define their antigenic structure and to determine the extent to which antigenic domains contribute to the process of virus infectivity. The experimental strategy has been to select antigenic variants, referred to as monoclonal antibody-resistant (*mar*) mutants, by using virus-neutralizing glycoprotein-specific monoclonal antibodies and to search for concomitant alterations in other aspects of the biology of these glycoprotein molecules. For example, we have recently reported that *mar* mutations in glycoprotein B (gB) interfere with both its processing and function in virus penetration at high temperature (28) and that *mar* mutations in gC affect the C3b-binding activity of this molecule (17). These data, in combination with information on the location of *mar* mutations, should prove useful in identifying regions of the glycoproteins which contribute to their biological properties.

A similar approach has now been applied to the characterization of the antigenic structure and biology of HSV type 1 (HSV-1) gD. gD is one of the more abundant and easily detected HSV glycoproteins. This molecule has received

considerable attention because it is thought to play a role in virus attachment to cells (18) and is a primary target for neutralizing antibodies (7, 9, 22, 37, 41). Although gD has been purified (16) and well characterized physically (8, 14, 30), and the genes for gD of both serotypes have been sequenced (31, 48, 49), little is known concerning its precise function in the virus replicative cycle.

In this report, we used gD-specific monoclonal antibodies and *mar* mutants to define epitopes which contribute to the antigenic structure of HSV-1 gD. An analysis of the reactivity patterns of *mar* mutants and antibodies revealed the presence of at least six distinct gD epitopes. Attempts were made to correlate epitope specificity with the ability of these antibodies to interfere with virus infection. In particular, evidence is provided to show that gD-specific monoclonal antibodies recognizing four separate epitopes neutralize virus by preventing penetration of the plasma membrane in a complement-independent manner. Moreover, most gD-specific antibodies were able to slow the rate of virus penetration, further indicating that they affect this process. In agreement with the findings of Fuller and Spear (18), some gD-specific monoclonal antibodies inhibited virus attachment to various degrees, but only at concentrations in excess of that required for neutralization. As shown previously by others (32, 34), many of these antibodies also interfered with plaque development and syncytia formation, although no

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correlation with epitope specificity or complement-independent neutralization titer was observed. However, the consistent finding that gD-specific antibodies can interfere with these events indicates that gD participates in several biological activities and may contain more than one functionally active domain.

## MATERIALS AND METHODS

**Cells and virus strains.** Human embryonic lung (HEL) and Vero cells were maintained in Eagle minimum essential medium (MEM; GIBCO Laboratories, Grand Island, N.Y.) supplemented with nonessential amino acids, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, and 10% fetal calf serum (FCS; GIBCO). Wild-type HSV and antigenic variants were grown and titered at 37°C at low multiplicity on Vero cells. A plaque-purified isolate of wild-type HSV-1 (strain KOS), designated KOS-321, was the parent strain from which all *mar* mutants were isolated (19). A plaque-purified isolate of HSV-2 strain 186 (186.111) was used to identify antibodies reactive with type-common epitopes. Purified virions for virus adsorption experiments were grown from HSV-1 strain F. The syncytial mutant *tsB5* was derived from the HFEM strain of HSV-1 and carries a temperature-sensitive mutation in the gB coding region (11). *synLD70* is a KOS derivative which possesses a chain-terminating mutation in the structural gene for gC and consequently has a gC<sup>-</sup> phenotype (19, 39). Temperature-dependent defects in virus replication were assayed by growing virus stocks at 34 and 39°C and titering the resulting progeny at 34°C as previously described (28).

**Production of hybridomas.** The BALB/c myeloma line P3-X63-Ag8.653 and the myeloma-spleen cell hybridomas were grown in supplemented Dulbecco modified Eagle medium (GIBCO) containing 20% FCS (Sterile Systems, Inc., Logan, Utah) as described previously (19). The procedure for immunization and production of hybridoma cell lines secreting HSV-specific monoclonal antibodies has been described in detail elsewhere (19). Briefly, BALB/c mice were infected intraperitoneally with  $2 \times 10^7$  PFU of live KOS-321 and boosted 13 days later with  $10^8$  PFU of UV-inactivated virus. Three days after boosting, spleens were removed and disrupted, and cells were fused to P3-X63-Ag8.653 myeloma cells with polyethylene glycol 6000 (Eastman Kodak Co., Rochester, N.Y.). Hybridoma cell lines secreting HSV-neutralizing antibody were identified by using culture supernatants in 50% plaque-reduction microneutralization assays (19).

High titer antibody preparations were produced as mouse ascites fluid (19). Antibodies were concentrated by ammonium sulfate precipitation, suspended in phosphate-buffered saline (PBS), and designated as the D series. Immunoglobulin G (IgG) concentrations were determined by using a radial immunodiffusion assay kit (Miles Scientific, Naperville, Ill.). Assays involving the use of monoclonal antibodies to alter the rate of virus penetration, interfere with syncytia formation, or inhibit plaque development employed antibody preparations normalized to 2 mg/ml. The hybridoma cell line CC406, which secretes IgG that does not react with HSV determinants, was used as a negative control antibody in virus adsorption and rate of penetration experiments (1). Antibody isotypes were determined with an enzyme immunoassay with subclass-specific antisera (MonoAb-ID EIA Kit; Zymed Laboratories, San Francisco, Calif.). The antibodies were analyzed by isoelectric focusing as described previously (29) to identify unique clonotypes.

The monoclonal antibodies 1S, 4S, and 50S were kindly provided by Martin Zweig (National Cancer Institute, Frederick, Md.). These antibodies were produced from mice immunized with the HSV-1 strain 14012 and have been characterized by Showalter et al. (42).

**Virus neutralization assays.** Wild-type virus and antigenic variants were tested for sensitivity to neutralization by HSV-specific monoclonal antibodies in 50% plaque reduction microneutralization assays described by Holland et al. (19). Virus neutralization titers were expressed as the reciprocal of the highest antibody dilution that neutralized 50% of input virus (30 PFU per well in a 96-well tray). Neutralization titers were determined in both the presence and absence of 10% normal rabbit serum as a complement source.

**Isolation of antigenic variants.** Variants of KOS-321 that were resistant to neutralization by HSV-specific antibodies were isolated as described previously (19). Virus surviving antibody neutralization in the presence of 10% rabbit serum was plaque purified three times and retested in the 50% plaque-reduction assay described above. If the titer of the selecting antibody was a minimum of 32-fold lower against the mutant than against KOS-321, the mutant was given the monoclonal antibody resistant, or *mar*, designation. Multiple *mar* mutations were introduced into a single virus by sequentially selecting antigenic alterations with different antibodies or by selecting multiple mutants produced from mixed infections of different single *mar* mutants as described earlier (28).

**Radiolabeling, immunoprecipitation, and electrophoresis.** HEL cells were infected at high multiplicity (10 PFU/cell) and labeled with [<sup>35</sup>S]methionine (New England Nuclear Corp., Boston, Mass.) (40 μCi/ml) from 4 to 18 h postinfection at either 34 or 39°C. Nonidet P-40 extracts of infected cells were precipitated with a pool of monoclonal antibodies (D1, D2, D3, D4, and D5). Precipitates were recovered with protein A-Sepharose beads (Sigma Chemical Co., St. Louis, Mo.), electrophoresed in 10% polyacrylamide slab gels, and fluorographed as previously described (19).

**Antibody-mediated alteration of plaque size.** Vero cells in 96-well trays were infected with 25 to 30 PFU of wild-type virus for 4 h at 37°C in MEM-5% FCS. Wells were washed with PBS-MgCl<sub>2</sub> and overlaid with diluted antibody and 0.5% methylcellulose. The monolayers were stained after 3 days with crystal violet, and plaque size was scored by using a dissecting microscope. Plaque reduction is reported as the highest dilution of a 2-mg/ml antibody stock that reduced the average plaque size by 50%.

**Antibody-mediated inhibition of syncytia formation.** Vero cells were grown to confluency in 24-well trays (Costar, Cambridge, Mass.). Monolayers were infected with KOS-321 or the syncytial mutant *tsB5* or *synLD70* at a multiplicity of 20 PFU per cell (37°C). After 2 h, 5 μl of monoclonal antibody was added to each inoculum, and the total volume of the inoculum was increased to 500 μl (final antibody concentration of 20 μg/ml). Viruses were tested against each of the nine gD-specific monoclonal antibodies (D1 through D9). Cells infected with syncytium-forming or wild-type virus without antibody served as controls for maximum and minimum polykaryocyte formation, respectively. After incubation for 24 h at 37°C, monolayers were stained with crystal violet and examined microscopically for inhibition of polykaryocyte formation. The degree of inhibition was on the following plus-minus scale: (++++) 1, (++++) 2 to 10, (++) 10 to 25, (+) 25 to 50, and (-) 50 or more nuclei per cell.

**Postadsorption virus neutralization.** Wild-type virus was

diluted to 1,000 PFU/ml in MEM-5% FCS and aliquoted in 0.5-ml volumes. Antibody dilution was such that approximately 50% of the input virus would be neutralized. One set of virus samples was mixed with one member of the gD-specific monoclonal antibody panel for 2 h at 4°C and then added to Vero cell monolayers in six-well trays at 4°C for 2 h. Then the monolayers were washed twice with PBS-MgCl<sub>2</sub> and incubated for additional 2 h at 4°C under MEM. The second set of virus samples was added directly to the individual monolayers for 2 h at 4°C, unadsorbed virus was removed by washing the cells, and bound virus was incubated with the individual monoclonal antibodies for an additional 2 h at 4°C. The third set of virus samples was plated for 2 h at 4°C, washed, and incubated an additional 2 h at 4°C without antibody treatment. In all three conditions, the monolayers were again washed twice with PBS-MgCl<sub>2</sub> and overlaid with methylcellulose. The monolayers were stained, and plaques were counted after 3 days of incubation at 37°C. Neutralization before and after plating was compared with that of the untreated control for each sample. Percentages reported are the averages of two separate experiments. Values differed less than 10%.

**Assays for rate of virus penetration.** The rate of virus penetration was measured by inactivation of extracellular virus with a low-pH citrate buffer by a modification of the procedure of Huang and Wagner (23). Confluent Vero cell monolayers in six-well trays (Costar) were overlaid with 0.5 ml (400 PFU) of virus inoculum. The plates were then incubated at 4°C with gentle rocking for 2 h, washed twice with PBS-MgCl<sub>2</sub>, overlaid with 0.5 ml of MEM-5% FCS, and shifted to 37°C to allow virus penetration to proceed. At various times after temperature shift, each well was treated with 1 ml of citrate buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3.0) for 1 min. Then the monolayers were washed twice with PBS-MgCl<sub>2</sub> and overlaid with methylcellulose. The plaques were visualized by crystal violet staining of the monolayer and counted at 3 days. The fraction of intracellular virus at a given time is that percent of viral PFU surviving citrate treatment, where 100% is the number of plaques formed on a monolayer not treated with citrate buffer. Antibody-restricted virus entry into cells was performed identically, except that wild-type virus was incubated with each monoclonal antibody (final concentration of 20 µg/ml) for 3 h at 37°C before the addition of virus to the monolayer.

**Purification of radiolabeled virions and inhibition of virus adsorption by anti-gD monoclonal antibodies.** Purified virions were isolated by the procedure of Spear and Roizman (45) as modified by Cassai et al. (5), except that (i) R-970 cells were infected with 5 PFU of HSV-1 strain F per cell, and (ii) infected cells were labeled with 50 µCi [<sup>35</sup>S]methionine per ml from 4 to 20 h postinfection. For adsorption assays, Vero cells were plated in 24-well trays and grown to confluency. Before plating of virus, each well was coated with 1% bovine serum albumin solution in PBS-MgCl<sub>2</sub>. Radiolabeled virus was diluted to 10<sup>4</sup> PFU/ml in the bovine serum albumin solution in 0.25-ml samples (2,500 PFU) and treated with various concentrations (0.04, 0.1, 0.25, 0.5, and 1.0 mg of IgG per ml) of different monoclonal antibodies for 2 h at 37°C. Samples were added to the bovine serum albumin-coated wells for 2 h at 4°C with rocking. The inoculum was recovered, and the wells were washed with PBS-MgCl<sub>2</sub>. The inoculum and washes were combined with 4.5 ml of aqueous counting scintillation solution (Amersham Corp., Arlington Heights, Ill.) and represented the unbound virion fraction. Monolayers were solubilized with 0.25 ml of lysis solution

TABLE 1. Characteristics of gD-specific monoclonal antibodies

Monoclonal antibody	Immunoglobulin isotype	Neutralization titer		
		HSV-1 (KOS-321)		HSV-2 (186.111)
		+ <sup>a</sup>	- <sup>b</sup>	+
D1	IgG1	102,400	12,800	<50
D2	IgG3	81,920	20,480	25,600
D3	IgG2a	64,000	64	20,480
D4	IgG2b	10,240	<5	10,240
D5	IgG3	640	<10	<50
D6	IgG2a	512	<2	<10
D7	IgG2a	4,096	<16	2,560
D8	IgG2a	5,120	320	2,560
D9	IgG2b	81,920	<20	<20

<sup>a</sup> +, Neutralization mixture contained normal rabbit serum as a complement source at a final concentration of 10%.

<sup>b</sup> -, No normal rabbit serum was present.

containing 1% Nonidet P-40, 20 mM Tris hydrochloride, and 150 mM NaCl (pH 8.5). The lysates were removed, and the wells were washed again with 0.25 ml of the lysis solution. Lysates and washes were transferred to 4.5 ml of aqueous counting scintillation solution and represented the bound virion fraction. Samples were allowed to equilibrate for 24 h before counting. Each sample was run in duplicate, and the mean values were plotted. Total recovery averaged between 90 and 95% of input counts, and duplicate counts per minute for most samples varied less than 10%.

## RESULTS

**Characteristics of monoclonal antibodies specific for HSV-1 gD.** Mice were immunized with infectious wild-type HSV-1 (KOS-321), and spleen cell-myeloma fusions were performed as previously described (19). Antibodies were identified on the basis of virus-neutralizing ability in the presence of 10% normal rabbit serum (19). Nine neutralizing antibodies representing seven independent fusions were found to be specific for gD by immunoprecipitation of radiolabeled proteins from KOS-infected cells (data not shown). The antibodies shown in Table 1 had a wide range of complement-dependent neutralization titers against HSV-1, whereas D1, D2, D3, and D8 had substantial titers in the absence of complement. Antibodies D2, D3, D4, D7, and D8 all had virus neutralization titers against HSV-2 strain 186.111, indicating that about half of the antibodies recognized gD epitopes shared by both serotype strains. The antibodies were analyzed by isoelectric focusing (29), and all possessed unique banding patterns indicative of different clonotypes (data not shown).

**Monoclonal antibodies specific for gD inhibit plaque development and syncytium formation.** Antibodies to gD have been previously reported to alter plaque size (32) and inhibit formation of polykaryocytes by syncytium-forming mutants (32, 34). These findings have been interpreted as suggesting that gD plays a role in membrane-related events involved in virus spread. Accordingly, our gD-specific antibodies were each tested for their ability to affect plaque development by the prototype strain (KOS-321) and membrane fusion induced by the syncytial mutants *tsB5* and *synLD70*, strains recognized by all of our gD-specific monoclonal antibodies (data not shown). The highest dilution of each antibody sufficient to reduce the average plaque size by 50% after 3 days of growth in the presence of the antibody is shown in Table 2. Six of nine antibodies reduced the size of wild-type

TABLE 2. Antibody effects on plaque formation and cell-cell fusion

Antibody	50% reduction titer <sup>a</sup>	Inhibition of syncytia <sup>b</sup>
D1	160	++++
D2	80	++++
D3	160	-
D4	<10	++
D5	<10	+
D6	<10	-
D7	80	++++
D8	40	++++
D9	40	++++

<sup>a</sup> Dilution of a 2 mg/ml antibody stock that reduced plaque diameter by at least 50%.

<sup>b</sup> Syncytia induced by the mutant *tsB5* (Materials and Methods), shown as the relative amount of inhibition of polykaryocyte formation: -, cell fusion of 50 or more nuclei per polykaryocyte; + + + +, no fusion, similar to wild-type infected cells. The final antibody dilution was 20 µg/ml.

plaques at dilutions ranging from 1/40 to 1/160. No antibodies were able to enhance the development of wild-type virus plaques at any concentration tested. Antibodies were scored on the degree of inhibition of fusion from complete inhibition (++++; similar to the wild-type virus infected cell control) to no inhibition (-; syncytial mutant-infected cell control). The relative ability of these antibodies to inhibit cell fusion on monolayers infected with either mutant at high multiplicity (20 PFU/cell) varied widely and did not correlate completely with complement-independent virus neutralizing titers (Table 1). For example, D1 had a very high complement-independent virus-neutralizing titer and blocked syncytia, whereas D7 blocked syncytia equally well but neutralized virus only in the presence of complement. The effects were indistinguishable on both syncytial mutants. With the exception of D3, all antibodies capable of inhibiting plaque formation also prevented syncytium formation.

**Neutralization of wild-type virus by gD-specific monoclonal antibodies after virus adsorption to cell surfaces.** It has been demonstrated that antibodies to gD can interfere with virus attachment to the cell surface membrane (18). However, this phenomenon required antibody concentrations far in excess of that required for neutralization and was carried out by antibodies with little virus-neutralizing activity. These observations suggest that inhibition of virus attachment is not the principal means by which gD-specific antibodies neutralize virus in the absence of complement. To further explore the mechanism of neutralization, complement-independent neutralizing antibodies were compared for their ability to neutralize virus before and after virus adsorption to host cells. If the mechanism of neutralization primarily involves inhibition of virus attachment, adsorbed virions should be resistant to neutralization. Alternatively, if the mechanism involves the blocking of some step in infection subsequent to adsorption, it should be possible for these antibodies to neutralize cell surface-bound virions.

Antibody was added to virus inoculum for 2 h before or after virus adsorption at 4°C. Antibody was diluted such that about 50% of input virus would be neutralized as determined by microneutralization assays (Table 1). Table 3 shows the percent neutralization achieved when the treatment was given before or after adsorption of virus on cell monolayers. In each case, the antibody was able to efficiently neutralize virus after adsorption. Regardless of when the antibody treatment was given, the levels of neutralization were remarkably similar. In addition, a single antibody specific for

gB that could neutralize virus in the absence of complement was tested. This antibody exhibited a similar ability to neutralize virus after adsorption (data not shown). This finding is consistent with the mechanism of virus neutralization involving blocking of some step after virus attachment, as previously suggested (18).

**Anti-gD antibodies inhibit the rate of virus penetration.** If antibodies directed against gD block virus infection at some step after virus attachment, inhibition of virus penetration is a likely possibility. Accordingly, the gD-specific antibodies were tested for their ability to alter the rate of virus penetration by using an acid-restricted penetration assay. The assay involved allowing antibody-treated virus particles to attach to the cell monolayers at 4°C, followed by a shift to 37°C, at which time virus begins to penetrate cell membranes. By inactivating the extracellular virus at different times after shift with a low-pH citric acid buffer, the kinetics of penetration can be determined. For antibodies that neutralized virus without complement (e.g., D2), the rate of penetration was determined on the fraction of virus that survived neutralization. In these experiments, penetration is operationally defined as the loss in sensitivity to citrate inactivation. Thus, this assay cannot distinguish between virus that has fused at the cell membrane and entered the cytoplasm and virus that may have been taken up in vesicles but has yet to cross the membrane boundary into the cytoplasm.

Figure 1 shows the kinetics of penetration for virus treated with the indicated antibodies. As expected, the complement-independent neutralizing antibodies (e.g., D1) greatly inhibited virus penetration. Those antibodies that required complement to efficiently neutralize virus (e.g., D9) also retarded the rate of virus penetration, but generally to a much lesser extent. Antibodies specific for gC representing the nine epitopes defined by Marlin et al. (29) were also tested, and none had any effect on virus penetration (data not shown). Moreover, antibody CC406, which is not reactive with HSV determinants, showed no ability to alter the kinetics of virus penetration (data not shown). These data strongly suggest that antibodies to gD exert their neutralizing effect by preventing nucleocapsid penetration of the cell surface.

**Inhibition of adsorption by gD-specific monoclonal antibodies.** The ability of some gD-specific monoclonal antibodies to inhibit virus adsorption has been previously reported by Fuller and Spear (18). Attachment to cells is assayed by measuring the number of counts from radiolabeled, purified virions that are irreversibly bound to monolayers at 4°C. In our experiments, inhibition of virus binding to cells was measured after the addition of various concentrations of the gD-specific antibodies. Concentrations ranging from 0.04 to 1.0 mg of IgG per ml were tested, since nonspecific inhibition was previously reported at approximately 1 mg/ml for preim-

TABLE 3. Neutralization by gD-specific monoclonal antibody treatment of virus before or after adsorption

Antibody	% Neutralization <sup>a</sup>		Neutralization ratio <sup>b</sup>
	Preadsorption	Postadsorption	
D1	66.8	66.6	1.00
D2	96.7	86.5	0.89
D3	46.7	31.7	0.68
D8	38.2	38.2	1.00

<sup>a</sup> Percent neutralization when virus was treated with the 50% endpoint dilution of antibody before or after adsorption to cells.

<sup>b</sup> Ratio of neutralization after virus adsorption to neutralization before virus adsorption.

mune rabbit serum (18). The fraction of input counts for each sample of virus that specifically bound to cells was then compared with the average amount bound when no antibody treatment was given.

Figure 2 shows the percent inhibition for each antibody. The negative control, antibody CC406, was unable to inhibit virus adsorption at any concentration tested. The gD-specific antibodies fell into two activity groups: moderate and negligible inhibitory effects. The ability of some antibodies to moderately inhibit virus attachment to cell membranes did not correlate with other antibody activities such as complement-independent neutralization, blocking of polykaryocyte formation, inhibition of plaque development, or alteration in the rate of virus penetration. No significant inhibition of adsorption (<5%) was seen at concentrations of antibody higher than that used for virus neutralizations (Table 3) or inhibition of virus penetration (Fig. 1). These data indicate that the neutralizing capability of these gD-specific monoclonal antibodies is not the result of inhibition of virus adsorption and further support the conclusion that neutralization occurs by preventing virus penetration.

**Development of an operational antigenic map of gD.** To associate particular antiviral activities with specific antibody recognition sites on the gD molecule, experiments were carried out to group the antibodies according to the gD epitope which they recognized. This was accomplished first by using different gD-reactive monoclonal antibodies to

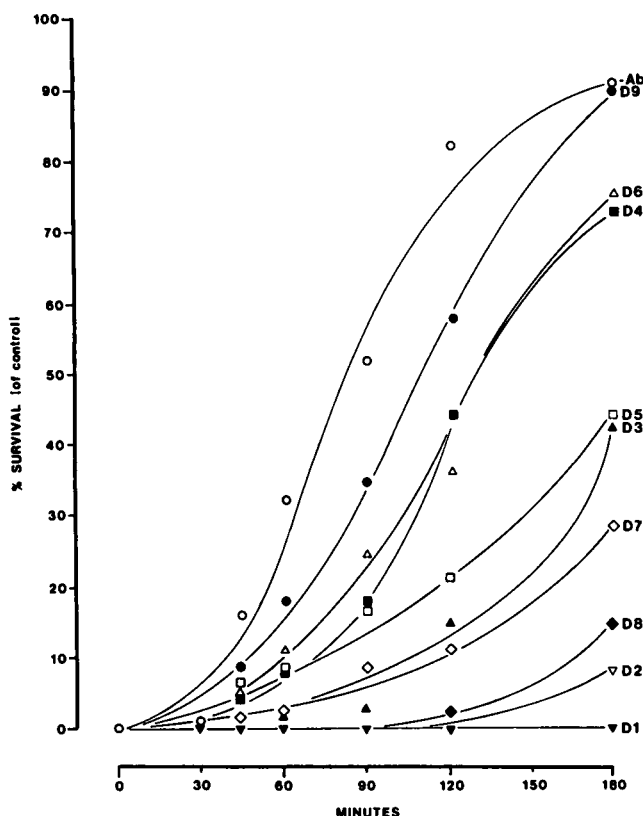


FIG. 1. Effect of gD-specific antibodies on KOS-321 entry kinetics. Virus samples were treated with each antibody (20  $\mu$ g/ml) for 3 h at 37°C and added to Vero cell monolayers. After adsorption for 2 h at 4°C, cultures were shifted to 37°C and washed with citric acid buffer (pH 3.0). The times shown are times after shift to 37°C. Survivor virus plaques were scored after 3 days at 37°C.

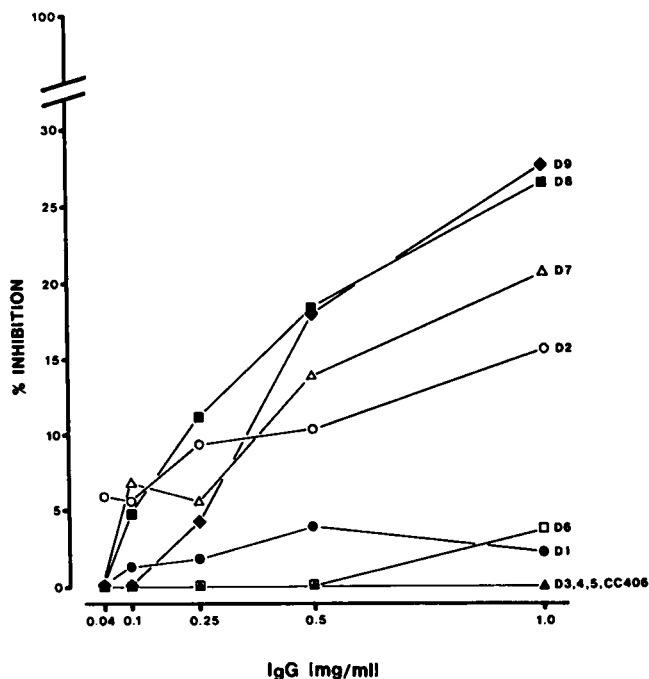


FIG. 2. Effect of gD-specific antibodies on KOS-321 adsorption to Vero cells. [ $^{35}$ S]methionine-labeled virus was treated with different concentrations of antibody at 37°C for 2 h. Virus was then allowed to adsorb to cell surfaces for 2 h at 4°C. Bound and unbound virus was determined by liquid scintillation counting. The results are reported as percent inhibition of adsorption when compared with the untreated KOS-321 control.

select a panel of monoclonal antibody-resistant (*mar*) mutants and second by grouping the selecting antibodies according to similarities and differences in the ability to neutralize each member of the *mar* D mutant panel.

Naturally occurring mutants were selected from a wild-type virus population of KOS-321 after several rounds of neutralization with individual monoclonal antibodies in the presence of rabbit complement. Surviving virus was plaque purified and screened for resistance to neutralization by using a 50% plaque reduction assay (19). The *mar* phenotype was assigned to mutants that showed at least a 32-fold reduction in 50% endpoint titer using the selecting antibody (19). However, all mutants described in this study were completely resistant to neutralization. Mutants were screened for the production of gD by immunoprecipitation with a pool of gD-specific monoclonal antibodies. All mutants were shown to express a single protein species with a mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis indistinguishable from that of the fully glycosylated form of gD expressed by wild-type virus (data not shown).

As we have described previously with studies of gC (29) and gB (28), antibodies and *mar* mutants were useful in performing an operational antigenic analysis of gD. The genetic change(s) sustained by each *mar* mutant that confer resistance to neutralization with the selecting antibody should also confer resistance to other antibodies with similar epitope specificities. In this way, the mutants and antibodies with similar reactivity patterns can be operationally grouped together to define epitopes on a protein antigen. Unique vertical patterns of neutralization resistance defined separate epitopes and are represented by the prototype antibodies

GROUP	MAR MUTANT	MONOCLONAL ANTIBODY GROUPS											
		II				I	IX			X			
		D1	D3	1S	50S	D2	D4	D7	D5	D8	D9	4S	
KOS 321													
2	D1.3	●											
	D1.2	●	●	●	●								
	D1.1	●	●	●	●								
	D3.2	●	●	●	●								
	D3.1		●	●									
1	D2.1					●							
	D2.2					●							
9	D4.1						●	●					
	D4.2						●	●					
	D4.3						●	●					

FIG. 3. Antigenic sites on HSV-1 gD. The individual gD-specific monoclonal antibodies were tested for neutralization of wild-type virus (KOS-321) and the *mar* mutants in 50% plaque reduction neutralization assays. The viruses were designated as resistant (●) or sensitive (□) to neutralization with each monoclonal antibody. The criterion for resistance was that the titer of the antibody, when tested against the mutant, was at least 32-fold less than the titer against wild-type virus.

D1, D3, 50S, D2, D4, and D5 (Fig. 3). Antibodies with similar reactivities were used to define four groups of overlapping epitopes representing single antigenic sites. For example, antibodies D1, D3, and 50S showed similar but nonidentical patterns (epitopes) and have been grouped together accordingly to define antigenic site II. These antibodies likely recognize three closely related overlapping determinants. No mutants have been selected with antibodies representing site X. Each of these antibodies may recognize a unique epitope within gD. Until mutants have been selected, it can only be said that these antibodies neutralize all existing mutants, thereby defining at least one additional epitope. Of the three gD-specific antibodies raised against strain 14012 (1S, 50S, 4S) (42), at least two are specific for antigenic sites recognized by antibodies to KOS-321, thus indicating that the two strains are capable of inducing antibodies with similar specificities.

Once antibody specificities were determined, an attempt was made to establish relationships between the effects the antibodies had on virus replication (syncytia formation, rate of penetration, etc.) and the epitope which they recognized. Antibodies recognizing each of the four antigenic sites inhibited plaque formation and syncytium formation, neutralized an HSV-2 strain (186.111), and inhibited the rate of virus penetration. In addition, virus neutralization in the absence of complement was a characteristic of antibodies that recognized at least three antigenic sites. Therefore, more than one antigenic site appears to exist in this molecule, which contributes to virus infectivity.

**Isolation and characterization of antigenic variants with multiple epitope changes.** As previously reported, antigenic variation in gB has been shown to alter the pattern of glycosylation of gB and its ability to function in initiating virus infection at high temperature (28). However, the functional defect appeared only when multiple epitope alterations were introduced into the gB gene. Although no

temperature-sensitive mutants in gD have been found, *mar* D mutants were potentially defective in some aspect of virus replication under conditions of elevated temperature. To explore this possibility, multiple epitope changes were introduced into gD employing either recombination of two different single *mar* D mutants or by serial antibody selection. Virus mutants resistant to multiple antibodies were plaque purified and screened for resistance with the entire panel of gD-specific antibodies. The mutants exhibited the expected multiple *mar* D phenotypes (Fig. 4).

The ability of these mutants to replicate at high temperature was assayed as described previously (28). None of the gD mutants shown in Fig. 3 and 4 was found to be temperature sensitive for production of infectious virus (data not shown). Since some single gB *mar* mutants showed a partial reduction in gB processing at 39°C without being temperature sensitive for virus replication (28), all *mar* D mutants were tested for a similar defect. [<sup>35</sup>S]methionine-radiolabeled extracts of infections carried out at 34 or 39°C were immunoprecipitated with a pool of gD-specific monoclonal antibodies. The precipitates from the mutant infections were electrophoresed in parallel with those of wild-type virus infections. No apparent change in the mobility or amount of mature gD was seen (data not shown).

Single *mar* mutants of gB have been shown to be altered in the rate of virus penetration (S. Highlander, unpublished data), raising the possibility that *mar* D mutants may be similarly affected. Accordingly, the single and multiple *mar* D mutants were screened for alterations in rate of penetration by using the same acid-restricted penetration assay described above. None of the mutants, however, showed any increase or decrease in rate of penetration when compared with that of KOS-321, the wild-type strain from which the mutants were isolated (Fig. 3). Thus, the gD *mar* mutations do not appear to alter glycoprotein structure(s) involved in virus replication.

DISCUSSION

Virus attachment and penetration into the host cell represent the initial steps in the infectious process. Information on the molecules which contribute to these events is accumulating for a number of different virus-host systems (24, 27, 35), although very little is known concerning the molecular mechanisms by which herpes simplex virus gains access to the cell cytoplasm. Early electron microscopic data suggested that HSV particles enter the cell by fusion of the viral envelope with the host cell surface membrane (33), although

GROUP	MAR MUTANT	ANTIBODY GROUPS								
		II		I	IX			X		
		D1	D3	D2	D4	D7	D5	D8	D9	
KOS 321										
2 & 9	D1/4.4	●	●		●	●				
1 & 9	D4/2.2			●	●	●				
1 & 2	D1/2.2	●		●						

FIG. 4. Neutralization patterns of mutants with multiple epitope changes in gD. Mutants were tested for sensitivity to neutralization with the gD-specific antibodies in 50% plaque reduction neutralization assays (17). The mutants were designated as resistant (●) or sensitive (□) to neutralization as described in the legend to Fig. 3.

evidence for other mechanisms such as uptake in vesicles has been reported (10, 21). More recently, experiments involving treatment of cells with inhibitors of endosomal activity such as chloroquine (M. Wittels and P. G. Spear, 11th Annual Herpesvirus Workshops, Leeds, United Kingdom, abstr. no. 324, 1986) and  $\text{NH}_4\text{Cl}$  (20) suggest that virus penetration does not occur by entry across endosomal membranes. In addition, virion envelope components have been detected in plasma membranes after virus penetration (36), and liposomes containing HSV glycoproteins have been shown to fuse directly with cell surface membranes (25). Together, these data are consistent with a mechanism for virus penetration involving envelope fusion with the plasma membrane.

Thus far, only two glycoproteins of HSV-1 have been shown to be essential envelope components (2, 26, 40), which play a role in either initiating infection (gB) or mediating virus release (gH). Conditional lethal mutations in the gB gene which result in single amino acid substitutions have been shown to result in the production of defective gB at high temperature (3). Mutant virions are released from infected cells and attach to, but are unable to penetrate, host cell surface membranes. This block in the replicative cycle can be overcome by artificially inducing fusion of the virus envelope with the cell surface membrane by using polyethylene glycol, indicating that gB plays an important role in virus penetration (26, 40). In further support of a role for gB in virus entry, complement-independent neutralizing antibodies reactive with gB block virus penetration, and some antigenic variants altered in gB penetrate the cell at a reduced rate (S. Highlander, unpublished data). One rate of entry mutation has been sequenced (4) and maps to a region of the gB gene where mutations affecting antigenic structure recently have been identified (S. Highlander, unpublished data). Although no conditional lethal mutants exist in the gD gene, extensive attempts to introduce Tn5 insertion mutants of gD into the virus have failed, suggesting that this gene is essential to virus replication (50). In keeping with the predicted essential nature, antibodies to gD have also been shown to inhibit virus replication, including neutralization of virus in the absence of complement (14, 37), inhibition of virus adsorption (18), alteration of plaque development (32), and prevention of cell-cell fusion caused by syncytium-forming mutants (32, 34).

The purpose of this study was to determine the mechanism by which gD-specific antibodies neutralize HSV in the absence of complement as a means of gaining insight into the possible role of this envelope component in virus infectivity. In addition, attempts were made to correlate virus neutralization and other antiviral activities displayed by these antibodies with distinct epitopes or antigenic sites. First, several antibodies inhibited plaque development and polykaryocyte formation by syncytial mutants. With the exception of one antibody, D3, all antibodies that interfered with plaque development were effective at preventing polykaryocyte formation. Whether these two phenomena are related is still unclear, but both involve events associated with the presence of gD in the infected cell membrane. Second, efficient virus neutralization by some gD-specific antibodies in the absence of complement was achieved after virus had been adsorbed to the host cell membrane. This indicated that the mechanism of neutralization involved prevention of virus penetration rather than adsorption. In addition, most antibodies specific for gD had an inhibitory effect on the rate at which virus penetrated cell membranes. Third, inhibition of virus adsorption did not correlate with virus neutralization

titers and was observed only at IgG concentrations much higher than that required for neutralization. Again, these data strongly suggest that the antibodies exert their neutralizing effect at the level of virus penetration. It should be noted that antibodies in our panel which did inhibit virus adsorption were not exclusively type-specific neutralizers. These findings are in contrast to those reported by Fuller and Spear (18), who suggested that inhibition of attachment by type-specific neutralizing antibodies was compatible with the existence of serotype-specific receptors for virus adsorption (46, 47). Together our data strongly suggest that gD, like gB, plays a role in virus penetration, and raise the possibility that these two molecules collaborate in the entry process.

Studies were conducted to define and catalog epitopes recognized by our different monoclonal antibodies. The approach was to first select a panel of *mar* mutants in gD on the basis of the ability to escape neutralization by different anti-gD antibodies and then analyze the reactivity patterns of these mutants and antibodies to distinguish unique epitopes on this antigen. This *mar* mutant analysis has defined a minimum of six distinct epitopes that can be grouped into four antigenic sites (I, II, IX, and X). As discussed below, our antigenic sites were numbered to conform to the Eisenberg et al. classification scheme for antibody groups specific for gD (14). Thus, sites I and II are compatible with the previously established antibody groups I and II, whereas sites IX and X do not correspond to any existing group. Sites I and IX appear to be represented by single epitopes, whereas site X may or may not consist of multiple epitopes. Site II is comprised of antibodies with at least three different epitope specificities. These antibodies are believed to recognize closely related determinants but are sufficiently unique that an alteration that confers resistance to one of these antibodies does not necessarily confer resistance to all antibodies in this site.

Once the antibodies were arranged in groups according to the epitope specificity, it was anticipated that the antiviral characteristics associated with particular antibodies could be assigned to distinct antigenic domains. The results showed, however, that antibody activities such as complement-independent virus neutralization were not limited to a single antigenic site. Rather, three distinct sites were identified as containing these neutralizing epitopes. Moreover, these determinants did not correlate with epitopes involved in inhibition of plaque development or syncytium formation. It is now apparent that a more complete antigenic analysis is required to align epitopes with specific functional domains of gD and that more precise information on the role of gD in membrane fusion events is required. An in-depth analysis may reveal that, although these epitopes are distinct, they do indeed lie within a single physical domain of gD. Alternatively, gD may have several functional domains, each playing a unique role in the infectious process.

The analysis of the antigenic structure of gD reported in the literature is quite extensive (6, 13–15, 32, 38). Based on the work of our laboratory and others, gD appears to be a complex antigen, containing a variety of different determinants (14, 32), which depend on both primary and secondary structure for their integrity (6, 15). Eisenberg et al. (14) have classified antibodies into groups I through VIII on the basis of various properties such as serotype specificity, complement-dependent and complement-independent neutralization, and specific immunoprecipitation of V8 protease polypeptide fragments of gD. Subsequently, synthetic peptides and production of truncated gD polypeptides from cell lines carrying mutant forms of the gD gene have ex-



tended this analysis (13, 15). V. Isola, G. Cohen, and R. Eisenberg (personal communication) have attempted to fit our monoclonal antibodies into this classification scheme. Antibody D1 was assigned to group VI based on type-specific neutralization and competitive binding studies. Antibody D2 is a member of group I based on type-common reactivity, immunoprecipitation, and competition with group I antibodies. Antibody D3 shows partial reactivity with gD2 as determined by radioimmunoprecipitation and low complement-independent neutralization titer, suggesting that it is a group III antibody. However, this antibody fails to compete with other group III antibodies and therefore does not appear to fall into any of the existing groups. In another report on gD-specific monoclonal antibodies, Minson et al. (32) were able to place only two out of four gD-specific antibodies into the groups defined by Eisenberg et al. (14), indicating that a complete definition of the antigenic structure of gD is still lacking.

Some monoclonal antibody-resistant mutants in gD have been sequenced, and the nucleotide changes conferring resistance to neutralization have been identified. In three of four cases, Minson et al. (32) showed that antibodies failed to react with synthetic peptides corresponding to wild-type sequences surrounding the sequenced mutation. This suggests that discontinuous epitopes play an important role in the antigenic character of gD. Clearly, sequencing of our *mar* D mutants will help to further define the epitope encoding regions of this molecule.

A large number of gD-specific monoclonal antibodies have now been produced by different laboratories (14, 19, 32, 37). The relationships between these antibodies have not been well established, since widely different criteria have been employed to distinguish antibody specificities. Experiments are currently underway in collaboration with R. Eisenberg and G. Cohen to further catalog the epitopes of gD by comparing the reactivities of these antibodies with a wide range of assays. In addition, immunoprecipitation of truncated gD polypeptides in a transient expression system (P. Weber, unpublished data) will help to determine the physical location of linear epitopes on this antigen. These data will be compared with information on the location of *mar* mutations which confer resistance to antibody neutralization. Ultimately, it is hoped that precise molecular studies of the antigenic structure and biologic activities associated with gD can be brought together into a coherent picture. As a consequence, specific domains involved in immunologic recognition and virus penetration may be identified and characterized.

#### ACKNOWLEDGMENTS

This work was supported by Public Health Service grants RR00200, GM34534, and AI18228 from the National Institutes of Health. S.L.H. was supported by a Horace Rackham Predoctoral Fellowship from the University of Michigan. S.L.H. and S.L.S. received support from the University of Michigan Graduate Program in Cellular and Molecular Biology (Public Health Service Training Grant 2T32GM07315 from the National Institute of Health). P.J.G. was supported by a F. G. Novy Fellowship from the Department of Microbiology and Immunology at the University of Michigan. D.C.J. is a scholar of the National Cancer Institute of Canada.

We thank Roselyn Eisenberg and Gary Cohen for helpful comments on the preparation of this manuscript. We also thank Elizabeth Smiley for excellent technical work with monoclonal antibodies, Joyce Borland for purification of radiolabeled virus, and Judy Worley for patience and expert preparation of the manuscript.

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