Antigenic Variants of Herpes Simplex Virus Selected with Glycoprotein-Specific Monoclonal Antibodies

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Monoclonal antibodies specific for herpes simplex virus type 1 (HSV-1) glycoproteins were used to demonstrate that HSV undergoes mutagen-induced and spontaneous antigenic variation. Hybridomas were produced by polyethylene glycol-mediated fusion of P3-X63-Ag8.653 myeloma cells with spleen cells from BALB/c mice infected with HSV-1 (strain KOS). Hybrid clones were screened for production of HSV-specific neutralizing antibody. The glycoprotein specificities of the antibodies were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of immunoprecipitates of radiolabeled infected-cell extracts. Seven hybridomas producing antibodies specific for gC, one for gB, and one for gD were characterized. All antibodies neutralized HSV-1 but not HSV-2. Two antibodies, one specific for gB and one specific for gC, were used to select viral variants resistant to neutralization by monoclonal antibody plus complement. Selections were made from untreated and bromodeoxyuridine- and nitrosoguanidine-mutagenized stocks of a plaque-purified isolate of strain KOS. After neutralization with monoclonal antibody plus complement, surviving virus was plaque purified by plating at limiting dilution and tested for resistance to neutralization with the selecting antibody. The frequency of neutralization-resistant antigenic variants selected with monoclonal antibody ranged from 4×10^{-4} in nonmutagenized stocks to 1×10^{-2} in mutagenized stocks. Four gC and four gB antigenic variants were isolated. Two variants resistant to neutralization by gC-specific antibodies failed to express gC, accounting for their resistant phenotype. The two other gC antigenic variants and the four gB variants expressed antigenically altered glycoproteins and were designated monoclonal-antibody-resistant, *mar*, mutants. The two *mar* C mutants were tested for resistance to neutralization with a panel of seven gC-specific monoclonal antibodies. The resulting patterns of resistance provided evidence for at least two antigenic sites on glycoprotein gC.

Glycoproteins expressed on the surfaces of many enveloped viruses have been shown to be essential for attachment of the virions to host cells and subsequent penetration into the cytoplasm (6, 11). Because of their role in replication and accessibility to antibody, they are the principal target antigens involved in virus neutralization (16, 17, 47, 50). Virus-neutralizing monoclonal antibodies have proven to be especially useful in defining the antigenic structure of viral glycoproteins (5, 34, 51, 53). Such antibodies can be used to select viral antigenic variants that are resistant to neutralization by the selecting antibody. These variants are likely to express viral glycoproteins with alterations in the site recognized by the selecting antibody. A monoclonal antibody interacts with a precise configuration of amino acids on the antigen surface, referred to as an epitope (19, 53). Epitopes frequently occur in clusters in discrete regions of

the antigen rather than in a dispersed fashion over the antigen's surface (1, 12, 26, 52). These clusters of overlapping epitopes are defined as antigenic sites and may be limited in number on a given antigen. Antigenic sites can be identified by the patterns of reactivity that result when a panel of monoclonal antibodies is tested against a set of independently isolated antigenic variants. Antibodies with similar or identical reactivity patterns with the set of variants can be grouped together to operationally define an antigenic site. Monoclonal antibody groups with distinct reactivity patterns can be used to enumerate nonoverlapping antigenic sites.

Herpes simplex virus (HSV) encodes at least four antigenically distinct glycoproteins, designated gC, gB, gE, and gD (4, 18, 46). The glycoproteins are incorporated into virion envelopes and the surface membranes of infected cells (4, 17, 18, 46). During active infection,

glycoproteins gC, gB, and gD are the principal immunogens and induce neutralizing and cytolytic antibodies and cell-mediated immune responses (16, 25, 32, 47). Infections with the two viral serotypes, HSV types 1 and 2 (HSV-1 and HSV-2), induce antibodies that are functionally either type specific or cross-reactive. Neutralizing antibodies specific for gC react with type-specific determinants, while antibodies against gB and gD are largely cross-reactive (9, 31, 33, 40, 47, 48). Aside from these observations, little is known about the precise antigenic structure of the individual glycoproteins or the role that these antigens play in the immunobiology of the virus. Such information is essential to understanding the immune response to both primary and recurrent HSV infections.

An analysis of the number of antigenic sites in the HSV glycoproteins may become particularly relevant if these antigens undergo antigenic variation. If the number of antigenic sites is small, then relatively few mutations in key sites in the glycoprotein structural genes could substantially alter the overall antigenicity of the virus. This phenomenon has proven relevant to the immunobiology of other viruses (14, 23, 27, 37, 50, 51) and could bear directly on the pathogenicity and epidemiology of HSV as well. For example, antigenic variation in influenza viruses, which is caused by point mutation and genetic reassortment, has been implicated as a major factor contributing to the ineffectiveness of the immune system in controlling infections in the human population (22, 37). Indeed, Laver et al. (23) were able to select a variant of influenza virus that had undergone sequential mutations in three distinct antigenic sites in the hemagglutinin molecule. The net effect of these mutations was that the variant had significantly reduced reactivity with hyperimmune sera prepared against the parental virus.

We report here on the use of monoclonal antibodies specific for the HSV-1 glycoproteins gC and gB to select neutralization-resistant virus variants. Antigenic variation in single antigenic sites of these glycoproteins occurred with high frequency. The antigenic variants of gC were analyzed for resistance to neutralization with a panel of monoclonal antibodies to estimate the number of different antigenic sites on glycoprotein gC. In a preliminary analysis of the resistance patterns of gC antigenic variants, we found that there are at least two antigenic sites on this glycoprotein.

MATERIALS AND METHODS

Virus strains. Wild-type HSV-1 (strain KOS), HSV-2 (strain 333), and virus mutants were grown by infection at low multiplicity in Vero cells at 37°C, and titers were determined by plaque assay on Vero cells

as described previously (16). A plaque-purified isolate of HSV-1 (strain KOS), designated KOS 321, was used in the production of hybridomas and the isolation of monoclonal-antibody-resistant mutants. The syncytial mutant *syn* LD 70 was isolated in this laboratory as described previously (43).

Cell culture and media. Human embryonic lung (HEL) and African green monkey kidney (Vero) cells were grown and maintained in Eagle minimal essential medium (MEM) (GIBCO Laboratories, Grand Island, N.Y.) supplemented with nonessential amino acids, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (MEMH), and 10% fetal calf serum (FCS) (GIBCO Laboratories) (MEMH-10). The BALB/c myeloma cell line, P3-X63-Ag8.653, does not express immunoglobulin heavy or light chains (20). This cell line was kindly provided by J. L. Claffin. The myeloma cells and the hybrids derived by myeloma-spleen cell fusion were grown in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with 10% FCS, 10% newborn calf serum, essential and nonessential amino acids, 2 µg of human transferrin and bovine insulin per ml (Sigma Chemical Co., St. Louis, Mo.), 5×10^{-5} M 2-mercaptoethanol, 20 mM HEPES, and 100 U of penicillin and 100 µg of streptomycin per ml (supplemented DMEM). All cloning of hybrids was performed in supplemented DMEM containing 20% conditioned medium from an actively growing culture of P3-X63-Ag8.653 myeloma cells (cloning medium).

Immunization and fusion. BALB/c mice (8 to 10 weeks old) (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were infected intraperitoneally with 2×10^7 PFU of HSV-1 strain KOS 321 on day 1 and boosted intraperitoneally on day 13 with 1×10^8 PFU of UV-inactivated virus. Virus was inactivated by exposing 2×10^8 PFU/ml, diluted in Dulbecco phosphate-buffered saline (PBS), to a UV light source (Westinghouse Sterilamp, type WL-15) at a distance of 10 cm for 20 min. This treatment reduced the titer to less than 100 PFU/ml. Three days after boosting, three mice were killed, and their spleens were removed as a source of immune lymphocytes.

Fusion of spleen cells and myeloma cells was performed by a modification of the method of Claffin and Williams (8). Spleen cells and myeloma cells were mixed at a ratio of 10:1, pelleted, and fused by the addition of 0.2 ml of 50% polyethylene glycol (PEG 6000; J. T. Baker Chemical Co., Phillipsburg, N.J.) in MEMH at 37°C. The mixture was then stirred gently and incubated at 37°C in a water bath. At 8 min after the addition of polyethylene glycol, 5 ml of MEMH was added slowly. The tube was then occasionally swirled over a period of 5 min, followed by centrifugation at $250 \times g$ for 5 min. The supernatant was aspirated, and the cell pellet was gently overlaid with 5 ml of cloning medium. After 10 min, the cell pellet was resuspended by gentle pipetting and added to 35 ml of cloning medium containing 13.6 µg of hypoxanthine, 0.1 µg of aminopterin, and 3.8 µg of thymidine per ml plus 10^7 normal spleen cells. The cells were dispensed into 96-well tissue culture plates (100 µl per well) and incubated at 37°C and fed every 4 days. Supernatants from growing colonies were screened for antibody activity in virus neutralization assays (see below). The cells from neutralization-positive wells were then transferred into cloning medium plus hypoxanthine

and thymidine in upright 25-cm² plastic tissue culture flasks and later subcloned by limiting dilution in cloning medium in 96-well plates. Subclones were re-screened for production of neutralizing antibody and routinely passaged in supplemented DMEM in upright 25-cm² tissue culture flasks.

Production of monoclonal antibody stocks. Stocks of monoclonal antibodies were prepared from either spent tissue culture medium or ascites fluids from mice injected intraperitoneally with hybridoma cells. Antibody from 1 liter of spent tissue culture medium was purified by affinity chromatography on a 1.5-g protein A-Sepharose CL-4B column (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) according to the procedure of Oi and Herzenberg (35). High-titer ascites fluids were produced by intraperitoneal injection of approximately 5×10^6 hybridoma cells into BALB/c mice primed 2 weeks previously with 0.5 ml of pristane (2,6,10,14-tetramethylpentadecane; Aldrich Chemical Co., Milwaukee, Wis.). Antibodies from clarified ascites fluids were precipitated with 40% saturated ammonium sulfate, redissolved in distilled water, dialyzed extensively against PBS, and used without further purification.

Immunoglobulin class was determined by Ouchterlony immunodiffusion in agarose using rabbit antisera to specific mouse immunoglobulins (Litton Bionetics, Kensington, Md.) (36).

Virus neutralization. Supernatants from growing hybrid clones were screened for neutralizing antibody in 96-well trays. Each well received 50 μ l of culture supernatant and 50 μ l of virus (2×10^3 PFU) diluted in MEMH plus 5% FCS (MEMH-5). Complement-enhanced neutralization was determined by the addition of 20% normal rabbit serum (NRS) as a source of complement to the virus preparation. After incubation for 3 h at 37°C, each well received 50 μ l of MEMH-10 containing 3×10^4 Vero cells, and the plates were incubated for 48 h at 37°C. The plates were rinsed once with PBS, and the cells were fixed and stained with 1% crystal violet in 50% ethanol. Wells in which no virus was neutralized exhibited 100% cytopathic effect (CPE). Wells in which greater than 50% of the cells were protected from CPE were scored as positive for neutralization of virus.

Titers were determined for monoclonal antibody stocks for neutralizing activity against HSV-1 (KOS 321), HSV-2 (333), and the mutant *syn* LD 70 by the assay described above. Serial twofold dilutions of monoclonal antibody stocks were used in place of culture supernatants. The antibody titer was expressed as the highest dilution which protected greater than 50% of the cells from CPE.

Wild-type HSV-1 and antigenic variants were tested for sensitivity to neutralization with monoclonal antibodies in 50% plaque reduction assays in a modification of the method of Rawls et al. (41). The assays were performed by adding 50 μ l of serially diluted ascites fluid containing monoclonal antibody to 50 μ l of virus suspended in MEMH-5-20% NRS in microtiter trays. As a control, MEMH-5 was substituted for the antibody. The assays were incubated for 3 h at 37°C. One-half of each mixture was then transferred to Vero cell monolayers grown in microtiter trays, and the virus was allowed to adsorb for 1 h. The monolayers were overlaid with 0.5% methylcellulose in MEM-2% FCS, and the plates were incubated at 37°C. After

development of plaques, the monolayers were washed once with PBS and stained with crystal violet. In control assays, approximately 40 plaques were produced. Antibody titers were expressed as the reciprocal of the last dilution of ascites fluid that reduced the plaque count by at least 50% relative to unneutralized controls.

Radiolabeling of cells. HEL cells (10^7) were infected with HSV-1 (KOS 321) or antigenic variants at a multiplicity of infection of 10. After adsorption of the virus for 1 h, the monolayer was overlaid with MEMH-5 and incubated at 37°C. At 4 h postinfection, the monolayer was washed once with PBS, and the cells were radiolabeled at 37°C with either 10 μ Ci of [³⁵S]methionine per ml (940 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) in methionine-free MEM-2% FCS or 1 μ Ci of [¹⁴C]galactose per ml (284 mCi/mmol; New England Nuclear Corp., Boston, Mass.) in MEM-2% FCS. At 9 h postinfection, the cell monolayers were washed three times with PBS, and the cells were harvested by scraping. The cells were pelleted by centrifugation and solubilized for 15 min in 1 ml of cold extraction buffer (1% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride in PBS, pH 8.0). Extracts were clarified by centrifugation at $15,000 \times g$ for 15 min.

Immunoprecipitation. Radiolabeled extracts of infected cells (100 μ l) were incubated with 20 μ l of purified monoclonal antibodies or ascites fluids overnight at 4°C. Antigen-antibody complexes were harvested by the addition of 50 μ l of 40% (vol/vol) protein A-Sepharose CL-4B beads (Pharmacia) in extraction buffer, followed by incubation at 4°C for 1 h. Immunoprecipitates produced with monoclonal antibodies that did not bind to protein A were harvested using protein A-Sepharose CL-4B presensitized with rabbit anti-mouse immunoglobulin (Cappel Laboratories, Cochranville, Pa.). After binding of antigen-antibody complexes to the beads, the beads were pelleted, and the supernatant was discarded. The beads were washed three times with extraction buffer and resuspended in 50 μ l of 2 $\times$ electrophoresis sample solution (2% sodium dodecyl sulfate [SDS], 5% 2-mercaptoethanol, 10 mM Tris, 10% sucrose, 2 mg of bromophenol blue per ml [pH 7.0]). Before SDS-polyacrylamide gel electrophoresis, the samples were heated to 100°C for 2 min, and the beads were removed by centrifugation.

Polyacrylamide gel electrophoresis. Immunoprecipitates and extracts of radiolabeled cells were solubilized in electrophoresis sample solution and subjected to SDS-polyacrylamide slab gel electrophoresis in 8.5% acrylamide (Bio-Rad Laboratories, Richmond, Calif.) cross-linked with 0.47% *N,N'*-diallyltartardiamide (Bio-Rad Laboratories) as previously described (16). Gels containing separated radiolabeled proteins were fixed and treated with 1.0 M sodium salicylate in 0.1 M Tris to provide fluorographic enhancement. Fluorographs of dried gels were made on Kodak XR-5 film exposed at -70°C. In each experiment, radiolabeled protein standards (Pharmacia) were electrophoresed in parallel for estimation of molecular weights of viral proteins.

Isoelectric focusing. Isoelectric focusing of monoclonal antibodies was performed on polyacrylamide gels by a modification of the method of Claflin and Cubberley (7). Gels were composed of 5% acrylamide, 0.15% *N,N'*-methylenebisacrylamide, 2.5% carrier ampho-

lytes (1.1% Pharmalytes [pH 4 to 6.5] plus 1.4% Pharmalytes [pH 6.5 to 9.0]; Pharmacia), 6.5% glycerol, and 5 M urea, polymerized with a combination of riboflavin and *N,N,N',N'*-tetramethylethylenediamine. Vertical gels 1.5 mm thick were cast on silanized glass supports prepared by soaking glass plates for 18 h in a solution of 2.5% 3-methacryloxypropyltrimethoxysilane in 0.1 M acetic acid–50% ethanol (pH 4.0), followed by extensive washing with distilled water. Samples were mixed with an equal volume of 4% carrier ampholytes. Electrophoresis of 20- μ l samples was carried out at a constant current of 2.0 mA for 18 h. Electrode buffers consisted of 0.1 M phosphoric acid and 0.2 M ethylenediamine. Gels were fixed in 20% trichloroacetic acid and stained with 0.125% Coomassie brilliant blue R-250 in 45% ethanol–10% glacial acetic acid. Destaining was performed with 45% ethanol–10% glacial acetic acid for 1 h followed with 30% ethanol–5% glacial acetic acid overnight.

Mutagenesis. Mutagenized stocks of KOS 321 were prepared with 5-bromo-2'-deoxyuridine (BUdR) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG). Vero cell monolayers were infected at a multiplicity of infection of 5 and incubated at 37°C. BUdR (2.5 μ g/ml in MEMH-5) was added after adsorption of the virus and remained in the culture medium until the virus was harvested. NG (10 μ g/ml) was added at 4 h postinfection and removed 1 h later by washing the monolayers with PBS. The monolayers were then overlaid with MEMH-5. At 24 h postinfection, the cells were scraped into the medium and frozen and thawed once, and the cellular debris was pelleted by centrifugation. Titers of the supernatants were determined on Vero cells. Mutagenized virus was then grown in Vero cells at a multiplicity of infection of 0.01 for 24 h to prevent phenotypic mixing of mutant and wild-type glycoproteins within the same virion envelope.

Isolation of antigenic variants. Variants were selected on the basis of resistance to neutralization with monoclonal antibodies. Mutagenized or nonmutagenized virus (2×10^5 to 4×10^5 PFU in 0.4 ml of MEMH-5) was incubated with 0.5 ml of purified monoclonal antibody plus 0.1 ml of NRS as a source of complement at 37°C for 3 h. The reaction mixture was then diluted with 8.5 ml of MEMH-5 and frozen, and titers were subsequently determined. Control neutralizations contained all reagents except monoclonal antibody.

The virus surviving neutralization was isolated by plating at limiting dilution on Vero cells grown in 96-well trays. After 4 to 5 days of incubation at 37°C, the trays were examined and frozen and thawed once. Trays in which less than 10% of the wells exhibited CPE were chosen, and virus isolates were picked from wells with generalized CPE. The virus isolates from these wells were then screened for resistance to neutralization. A 5- μ l amount of virus was added to duplicate wells of a 96-well tray containing 100 μ l of MEMH-5. Each well received 50 μ l of the selecting monoclonal antibody plus 20% NRS or MEMH-5 plus NRS alone. After incubation at 37°C for 3 h, 3×10^4 Vero cells in 50 μ l of MEMH-5 were added to each well. The trays were incubated at 37°C for 48 h when generalized CPE was observed in control wells. Virus isolates were tentatively identified as resistant to neutralization when CPE in wells containing monoclonal antibody was judged to be nearly as extensive as

duplicate wells containing no antibody. Neutralization-resistant isolates were plaque purified three times by plating at limiting dilution on Vero cells in 96-well trays and rescreened for resistance to neutralization. Stocks of mutant variants were grown on Vero cells.

RESULTS

Hybridoma cultures were initially screened for neutralizing antibody activity against plaque-purified wild-type HSV-1 (KOS 321). Hybrids positive for virus neutralization were expanded, subcloned, and retested for neutralization. Stocks of monoclonal antibody from stable hybrids were prepared from ascites fluids and also purified from tissue culture supernatants. The monoclonal antibodies were then characterized for antigenic specificity, monoclonality, immunoglobulin subclass, and virus-neutralizing activity.

Antigen specificity of the monoclonal antibodies. The specificity of the monoclonal antibodies was determined by immunoprecipitation of [35 S]methionine-labeled HSV-1 viral antigens from infected-cell extracts. The immunoprecipitates were electrophoresed in SDS-polyacrylamide gels, and viral antigens were identified on the basis of apparent molecular weight. A single major band with an apparent molecular weight typical for one of the HSV-1 glycoproteins was seen in each immunoprecipitation. A fluorogram of viral antigens immunoprecipitated with six gC-specific, one gB-specific, and one gD-specific monoclonal antibody is shown in Fig. 1. The monoclonal antibodies reactive with gC (lanes 1, 2, 4, 5, 6, and 7), gB (lane 3), and gD (lane 8) immunoprecipitated major bands with apparent molecular weights of 130,000, 120,000, and 58,000, respectively. No monoclonals specific for gE were produced. Similar results were obtained with immunoprecipitates of [14 C]galactose-labeled cell extracts, confirming the glycoprotein nature of the precipitated viral antigens (data not shown). None of the antibodies immunoprecipitated radiolabeled proteins from mock-infected cells.

The following nomenclature was adopted for designating monoclonal antibodies: a letter designation indicating the glycoprotein specificity followed by a number indicating independently derived antibodies. For example, antibody C1 and antibody C2 are independently derived monoclonal antibodies specific for glycoprotein gC. The monoclonal antibodies used in this study are listed in Table 1. It should be pointed out that approximately 30 other monoclonal antibodies were produced which neutralized virus but did not immunoprecipitate radiolabeled viral proteins. Detergent solubilization of glycoproteins may alter their conformation such that some antibodies which recognize the native mol-

FIG. 1. Immunoprecipitation of [35 S]methionine-labeled glycoproteins from extracts of KOS 321-infected HEL cells. Radiolabeled extracts were immunoprecipitated with monoclonal antibodies C2 (lane 1), C1 (lane 2), B1 (lane 3), C3 (lane 4), C5 (lane 5), C4 (lane 6), C6 (lane 7), and D1 (lane 8) and electrophoresed on an 8.5% SDS-polyacrylamide gel and fluorographed. The numbers on the right indicate molecular weights ($\times 10^3$). Relative positions of the glycoproteins are indicated on the left. The multiple bands seen in lanes 1 and 6 represent lower-molecular-weight precursors of glycoprotein gC.

ecule cannot combine with the soluble antigen. Alternatively, these antibodies may recognize hybrid epitopes created by the interaction of more than one glycoprotein molecule on the virion envelope. Solubilization of the complex of glycoproteins would destroy such hybrid determinants and prevent immunoprecipitation.

Isoelectric focusing and immunoglobulin subclass. The monoclonality of each antibody was determined by isoelectric focusing of purified antibodies. As shown in the stained gel (Fig. 2), each antibody exhibited a set of bands characteristic of a homogeneous antibody (2). Similar results were obtained when diluted ascites fluids were electrofocused. The multiple banding pattern was a consequence of charge heterogeneity in the monoclonal antibody population. Antibody B1 and antibody D1 produced different isoelectric focusing banding patterns and represent two different clonotypes. The isoelectric focusing patterns of antibodies C3 and C7 were different from each other and from all other anti-gC antibodies. Antibodies C6 and C5 produced similar banding patterns and are of the same immunoglobulin subclass (Table 1). These two antibodies may represent sister clones isolated from the same fusion. Antibodies C4, C2, and C1 appeared to be similar and also may represent sister clones, but they had a banding pattern different from antibodies C6, C5, C3, and C7.

TABLE 1. Characteristics of monoclonal antibodies

Mono-clonal anti-body	Immuno-globulin type	Neutralization titer			
		HSV-1		syn LD 70	HSV-2
		+ ^a	- ^b	+	+
C1	IgG2	6,400	<100	<100	<100
C2	IgG2	6,400	<50	<50	<50
C3	IgG2	6,400	<50	<50	<50
C4	IgG2	6,400	<50	<50	<50
C5	IgG3	1,280	<10	<10	<10
C6	IgG3	12,800	<50	<50	<50
C7	IgG3	640	<40	<10	<40
B1	IgG3	1,280	<10	2,560	<100
D1	IgG1	102,400	12,800	204,800	<50

^a +, Indicates that the neutralization mixture contained NRS as a complement source at a final concentration of 10%.

^b -, Indicates that no NRS was present.

These findings indicate that at least four different clonotypes of anti-gC antibodies are represented.

Immunoglobulin subclass was determined by Ouchterlony immunodiffusion. Of the nine hybrids tested, one produced antibody of the immunoglobulin G1 (IgG1) subclass, four of the IgG2 subclass, and four of the IgG3 subclass (Table 1).

Virus-neutralizing activity of the monoclonal

FIG. 2. Isoelectric focusing of monoclonal antibodies on a 5% polyacrylamide gel, pH 4.0 to 9.0. The procedure used was as described in the text. Monoclonal antibody designations are given at the top. The anode (+) and cathode (-) are indicated.

antibodies. Monoclonal ascites fluids were tested for neutralizing activity against HSV-1 (KOS 321), the KOS mutant *syn* LD 70, and HSV-2 (333). As described below, *syn* LD 70 does not express gC (see Fig. 3). This mutant was useful in confirming the specificity of monoclonal antibodies specific for gC since these antibodies neutralized wild-type virus but not *syn* LD 70. All of the antibodies exhibited significant neutralization titers against HSV-1 in the presence of complement, but all failed to neutralize HSV-2 (Table 1). Hyperimmune antisera monospecific for HSV-1 gC and the monoclonal anti-gC antibodies produced to date are type specific and fail to neutralize HSV-2 (10, 38-40, 44, 45). In contrast, both type-specific and cross-reactive monoclonal antibodies reactive with gB and gD have been produced (38, 44). Our results with gC-specific monoclonal antibodies are consistent with these findings and the hypothesis that gC contains only type-specific antigenic determinants. As expected, all of the monoclo-

nal antibodies specific for gC failed to neutralize *syn* LD 70. Monoclonal antibodies specific for gB and gD, however, neutralized this mutant to an extent equal to or greater than wild-type HSV-1. The gC- and gB-specific antibodies required complement for neutralization, while only the gD-specific monoclonal antibody, D1, had neutralizing activity in the absence of complement. Showalter et al. (44) have reported the production of both complement-dependent and -independent gD-specific monoclonal antibodies.

Mutagenesis and antigenic variant selection. Monoclonal antibodies B1 and C5 were used to select virus variants resistant to neutralization by these antibodies. Mutants were selected from unmutagenized and mutagenized virus stocks prepared from plaque-purified HSV-1 (KOS 321). Production of virus in mutagenized cultures was reduced by over 99%. Approximately 1 to 3% of the viruses survived neutralization (Table 2). Surviving virus was plaque purified by

FIG. 3. Comparison of $[^{35}\text{S}]$ methionine- and $[^{14}\text{C}]$ galactose-labeled polypeptides from wild-type- and mutant-infected HEL cells. Clarified extracts of $[^{35}\text{S}]$ methionine- or $[^{14}\text{C}]$ galactose-labeled wild-type- and mutant-infected HEL cells were electrophoresed in an 8.5% SDS-polyacrylamide gel and fluorographed. The positions of the viral glycoproteins are indicated on the right and molecular weight standards on the left ($\times 10^3$).

TABLE 2. Isolation of neutralization-resistant antigenic variants of HSV-1 (KOS 321)

Selection	Mutagen	% Virus surviving mutagenesis ^a	Selecting monoclonal antibody	% Virus surviving neutralization ^b	Frequency of resistant variants ^c	Neutralization-resistant variant designation
1	None	100	C5	1.2	4×10^{-4} to 8×10^{-4}	C5.1 C5.2 C5.3
2	2.5 μ g of BUdR per ml	0.55	C5	1.0	4×10^{-4} to 8×10^{-4}	C5.4
3	2.5 μ g of BUdR per ml	0.55	B1	1.1	1×10^{-3}	B1.1 B1.2
4	10.0 μ g of NG per ml	0.13	B1	3.1	1×10^{-2}	B1.3 B1.4

^a Equals the titer of virus from the mutagenized stock divided by the titer of the control stock. It provides a measure of the effectiveness of the mutagenesis procedure.

^b Percent virus surviving neutralization was calculated by dividing the titer of the residual virus after neutralization by the titer of a control neutralization containing all reagents except monoclonal antibody. Virus titers were determined by plaque assays on Vero cell monolayers.

^c Frequency of resistant variants equals the fraction of virus surviving after selection multiplied by the fraction of virus isolates with the neutralization-resistant phenotypes.

limiting dilution, and isolates were screened for resistance to neutralization by the selecting monoclonal antibody. Resistant isolates were plaque purified twice and retested for resistance to neutralization.

The frequencies of isolation of variants resistant to neutralization by antibodies C5 and B1 are shown in Table 2. The frequency of variants resistant to antibody C5 was approximately 4×10^{-4} to 8×10^{-4} in both the unmutagenized and mutagenized virus stocks, while the frequency of variants resistant to antibody B1 was 1×10^{-3} in the BUdR-mutagenized stock and 1×10^{-2} in the NG-mutagenized stock. We were not successful in isolating variants resistant to B1 from unmutagenized virus, and therefore, we estimated the frequency of such variants to be less than 10^{-4} . Using antibody C5, three resistant isolates were selected from an unmutagenized virus stock and one from a BUdR-treated stock. Selecting with antibody B1, two resistant isolates were obtained from the same BUdR-treated stock and two from an NG-treated stock.

Analysis of viral proteins and glycoproteins. Since the neutralization-resistant phenotype could result from the absence of the relevant glycoprotein antigen, the proteins and glycoproteins synthesized by the variants were examined. Figure 3 shows a fluorogram of an SDS-polyacrylamide gel of [³⁵S]methionine-labeled proteins and [¹⁴C]galactose-labeled glycoproteins synthesized by cells infected with wild-type, mutant *syn* LD 70, and antigenic variant viruses. *syn* LD 70 does not express gC (Fig. 3). This glycoprotein is not essential for growth of HSV-1 in tissue culture (18, 21, 28). The absence

of gC in the protein profile of *syn* LD 70 was a useful marker for identifying gC in gels. Virus isolates C5.2 and C5.3, selected with the antibody C5, had glycoprotein profiles similar to *syn* LD 70 and thus did not synthesize gC. As a consequence, these variants were not useful in the analysis of the antigenic structure of glycoprotein gC.

Virus isolate C5.4, also selected with antibody C5, synthesized gC, but in somewhat reduced amounts compared with the wild type (Fig. 3). Virus isolates B1.1, B1.2, and B1.4, which were selected with antibody B1, were similar to wild-type virus in the expression of all glycoproteins. Glycoprotein expression by isolates B1.3 (selected with B1) and C5.1 (selected with C5) was also similar to wild-type virus (data not shown). Since all of these viruses expressed the relevant glycoprotein and were also resistant to neutralization by the selecting monoclonal antibodies, we designated them monoclonal-antibody-resistant or *mar* mutants. The *mar* mutant designation includes the name of the selecting antibody and the mutant number designation. For example, *mar* C5.4 in the fourth *mar* mutant selected with antibody C5.

Quantitative neutralization of *mar* mutants with monoclonal antibodies. Different monoclonal antibodies specific for the same glycoprotein may recognize different antigenic sites. Therefore, a *mar* mutant selected by resistance to neutralization by one antibody may or may not be resistant to another antibody specific for the same antigen. The panel of monoclonal antibodies was tested against each of the *mar* mutants in 50% plaque reduction titration assays. A mutant

TABLE 3. Reactivity patterns of hybridoma antibodies with neutralization-resistant variants

<i>mar</i> mutant	Difference in neutralization titer ^a of monoclonal antibodies							
	C1	C2	C4	C5	C6	C3	C7	B1
KOS 321 (wild type)	0	0	0	0	0	0	0	0
<i>mar</i> C5.4	-8 ^b	-5 ^b	-6 ^b	-5 ^b	-6 ^b	-2	0	+2
<i>mar</i> C5.1	-8 ^b	-5 ^b	-6 ^b	-8 ^b	-6 ^b	-1	0	+4
<i>mar</i> B1.1			+2		0		0	-9 ^b
<i>mar</i> B1.2			+4		+1		0	-9 ^b
<i>mar</i> B1.3			+2		+2		0	-9 ^b
<i>mar</i> B1.4			+3		+2		+1	-9 ^b

^a The numbers indicate the differences between the neutralization titers of each antibody when tested with mutant and wild-type virus, expressed as the number of twofold dilutions. A negative number indicates that the mutant was less sensitive to the antibody than wild-type virus, while a positive number indicates that it was more sensitive.

^b The mutants were resistant to these antibodies. The criterion for resistance was that the titer of the antibody, when tested in neutralization assays with the mutant, be at least five twofold dilutions less than the titer with wild-type virus.

virus was considered to be resistant to neutralization by a monoclonal antibody if the titer of that antibody, as determined in virus neutralization assays with the mutant, was five or more twofold dilutions lower than the antibody titer obtained in assays with wild-type virus. Table 3 shows that the antibodies C1, C2, C4, C5, and C6 were not able to neutralize the mutants *mar* C5.4 and *mar* C5.1, while antibodies C3 and C7 did neutralize these mutants. This indicated that antibodies C3 and C7 recognized an antigenic site(s) different from the site(s) recognized by the other gC-specific antibodies. As expected, the antibody B1 did not neutralize mutants *mar* B1.1, *mar* B1.2, *mar* B1.3, and *mar* B1.4, since they had been selected with this antibody. It is noteworthy that most of the mutants were more sensitive than the wild type to neutralization by irrelevant antibodies by as much as four twofold dilutions. For example, *mar* B1.2 was four twofold dilutions more sensitive than the wild type when tested with antibody C4. The reason for this is not known.

Immunoprecipitation of *mar* mutant glycoproteins. Experiments were conducted to determine whether the mutant glycoproteins were precipitable with monoclonal antibodies. Viral proteins radiolabeled with [³⁵S]methionine were immunoprecipitated from Nonidet P-40 extracts of wild-type- or *mar* mutant-infected cells. As shown in Table 4, two antibodies, C5 and C6, which did not neutralize *mar* C5.4 or *mar* C5.1, did precipitate the gC produced by these mutants. This is noteworthy since antibody C5 was

used to select these mutants. In addition, antibodies C1 and C4, which also did not neutralize *mar* C5.4 or *mar* C5.1, weakly precipitated the gC produced by these mutants. Antibody C3, which neutralized these mutants, also efficiently precipitated their gC glycoproteins. Antibody B1 did not precipitate gB from extracts of cells infected with mutants selected with this antibody (data not shown). The failure of this antibody to precipitate gB from these mutants correlated with the resistance of these mutants to neutralization by these antibodies.

DISCUSSION

Monoclonal antibodies specific for the HSV-1 glycoproteins were used to select virus variants which were resistant to neutralization by the selecting antibody. Characterization of these variants with a panel of monoclonal antibodies has shown that it is feasible to use this approach to identify and enumerate antigenic sites on the HSV-1 glycoproteins. This type of analysis has been used successfully to identify the antigenic sites on the hemagglutinin and neuraminidase glycoproteins of influenza virus (15, 24, 26, 49) and the envelope glycoprotein of rabies virus (51). The basis of this procedure is that a mutation affecting an epitope recognized by one monoclonal antibody may also affect the structure of other epitopes in the same antigenic site. Thus, the mutant may be resistant to monoclonal antibodies other than the one with which it was selected. Those epitopes affected by the mutation are operationally defined to reside in the same antigenic site. Members of a panel of monoclonal antibodies directed against a single antigen may give different reactivity patterns when tested against a set of independently isolated antigenic variants. On the basis of these patterns, the antibodies can be grouped to define separate antigenic sites. Since these definitions are operational, the procedure does not provide

TABLE 4. Immunoprecipitation of HSV-1 glycoprotein gC from wild-type- and *mar* mutant-infected cell extracts by monoclonal antibodies

<i>mar</i> mutant	Immunoprecipitation ^a with monoclonal antibodies				
	C1	C3	C4	C5	C6
KOS 321 (wild type)	+	+	+	+	+
<i>mar</i> C5.4	±	+	±	+	+
<i>mar</i> C5.1	±	+	±	+	+

^a + Indicates that the antibody immunoprecipitated the *mar* mutant gC as efficiently as wild-type gC; ± indicates that the antibody precipitated *mar* mutant gC less efficiently than wild-type gC.

any direct information about the physical structure of the molecule. Furthermore, it cannot be said that a mutation affecting an epitope has occurred in the epitope itself, since the conformation of an epitope may be affected by a mutation separate from it. In addition, it is possible that a single mutation can affect more than one antigenic site through changes in the conformation of the protein. An extensive analysis using a number of variants and monoclonal antibodies should distinguish between mutations that affect single or multiple antigenic sites.

When we tested our panel of gC-specific monoclonal antibodies against *mar* C5.4 and *mar* C5.1 in virus neutralization assays, the antibodies could be arranged into two groups according to their ability to neutralize these mutants. Antibodies in group 1, C1, C2, C4, C5, and C6, failed to neutralize the mutants, while antibodies in group 2, C3 and C7, did neutralize them. Following the principles outlined above, this indicated the presence of at least two antigenic sites on gC. One of these sites, recognized by group 1 antibodies, is altered in *mar* C5.4 and *mar* C5.1. At least one other antigenic site was recognized by the antibodies in group 2. The identification of two antigenic sites on gC is based on a limited number of monoclonal antibodies and *mar* mutants. An analysis using larger panels of monoclonal antibodies and *mar* mutants will serve to confirm these findings and may identify additional antigenic sites.

It might be expected that an antibody which does not neutralize a *mar* mutant would not immunoprecipitate the relevant glycoprotein from extracts of cells infected with that mutant. This was the case with mutants selected with the antibody B1. However, some antibodies with greatly reduced capacities to neutralize *mar* C5.4 and *mar* C5.1 did immunoprecipitate the glycoprotein gC synthesized by these mutants. These antibodies included antibody C5, which was used to select these mutants. The mutations may have reduced the affinity of these antibodies for their epitopes sufficiently to affect neutralization but not enough to prevent precipitation. The different capabilities of the group 1 antibodies to precipitate *mar* C5.4 and *mar* C5.1 gC may be an indication that the antigenic site defined by these antibodies is a complex one, which might be subdivided by analysis of additional mutants selected with group 1 antibodies. The isolation of such mutants is in progress.

The mutation in *mar* B1.1 has been physically mapped by marker rescue using cloned wild-type DNA fragments. This mutation mapped to a 2.8-kilobase sequence of HSV-1 (KOS) DNA, located between coordinates 0.350 and 0.368. The temperature-sensitive mutant HSV-1 (HFEM) *ts* B5, which does not produce mature,

functional gB at the nonpermissive temperature, also mapped to the same DNA sequence (T. C. Holland, R. M. Sandri-Goldin, S. D. Marlin, M. Levine, and J. C. Glorioso, submitted for publication). These coordinates agree with the previously published map location of the gB gene (42). The mutation in *mar* C5.1 has been mapped to *Eco*RI fragment I (coordinates 0.633 to 0.721) (F. Homa and T. C. Holland, unpublished results), in agreement with the published location of the gC gene (42). These data provide important evidence that the *mar* mutations are, in fact, in the glycoprotein structural genes.

Another strategy used to analyze the antigenic structure of HSV glycoproteins has been to compare the reactivity of monoclonal antibodies with different strains of virus (13, 38, 44). The patterns of reactivity demonstrated the existence of numerous epitopes on these glycoproteins. However, epitopes operationally defined by the reactivity patterns of monoclonal antibodies with different virus strains cannot be grouped to define antigenic sites consisting of overlapping epitopes. Due to the genetic heterogeneity among strains, it should be assumed that these strains differ at more than one antigenic site. If the virus strains compared by reactivity with a panel of monoclonal antibodies differ at more than one site, it is impossible to attribute differences in antibody reactivity patterns to differences in single antigenic sites. In contrast, a mutant selected from a single virus strain with a monoclonal antibody should differ from the wild-type virus in a single antigenic site. The isolation of mutants with two or more mutations affecting different antigenic sites should be rare, since selective pressure is only applied to a single site. The differences between analyses of viral strains and mutants selected by monoclonal antibodies have been demonstrated by studies on the influenza virus hemagglutinin. In an analysis of several virus strains with monoclonal antibodies, 50 antibody reactivity patterns were identified. However, the analysis of antibody-selected variants revealed only four antigenic sites each of which was composed of multiple epitopes (53).

The frequencies with which antigenic variation occurs in an individual antigenic site can provide information about the structure or biological importance of that site. If the determinant site is in a domain of the glycoprotein that is critical to its structure or function, it may not be possible to isolate antigenic variants of that site, since the function of the glycoprotein would be lost or impaired. Antigenic sites which are located in functional sites on the protein may be highly conserved. In our study, the frequency of spontaneous mutants resistant to antibody C5 was approximately 4×10^{-4} to 8×10^{-4} . This

represents a substantial number of variants in a cloned preparation of wild-type virus.

Some of the antigenic variants selected with antibody C5 did not express gC, which accounted for their resistance to neutralization. However, most isolates of HSV-1 do express gC, and this protein may confer some as yet unknown advantage to the virus in infection (42). As a consequence, antigenic variation by the loss of expression of this glycoprotein may not occur in vivo. No spontaneous mutants resistant to antibody B1 were found. After mutagenesis, we were able to isolate mutants at frequencies of 10^{-3} and 10^{-2} , depending on the mutagen used. The ability to isolate these mutants demonstrated that variation can occur in the antigenic site recognized by this antibody without affecting the function of the glycoprotein.

Although a number of antigenically distinct strains of HSV exist within the human population, it is not known whether rapid antigenic variation occurs during the course of infection with this virus. The fact that recrudescence is common despite high titers of virus-neutralizing serum antibodies and immune effector cells (3, 29, 30) suggests that this virus also has some mechanism for evading the immune response. Furthermore, during repeated cycles of recurrent infections the virus is exposed to immunological selective pressure. This may result in significant alterations in the antigenic structure of the virus. Thus, antigenic variation could contribute to outbreaks of recurrent infection and the transmission of the virus to new hosts. Further studies on the antigenic structure of the HSV glycoproteins designed to identify critical antigenic sites and mechanisms of variation are essential to understanding the interaction of the virus with the immune system.

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