Epitopes of Herpes Simplex Virus Type 1 Glycoprotein gC Are Clustered in Two Distinct Antigenic Sites

STEVEN D. MARLIN,¹ THOMAS C. HOLLAND,^{2,3} MYRON LEVINE,² AND JOSEPH C. GLORIOSO^{3,4*}

Graduate Program in Cellular and Molecular Biology,¹ Department of Human Genetics,² Unit for Laboratory Animal Medicine,³ and Department of Microbiology and Immunology,⁴ University of Michigan Medical School, Ann Arbor, Michigan 48109

Received 18 June 1984/Accepted 2 September 1984

Epitopes of herpes simplex virus type 1 (HSV-1) strain KOS glycoprotein gC were identified by using a panel of gC-specific, virus-neutralizing monoclonal antibodies and a series of antigenic variants selected for resistance to neutralization with individual members of the antibody panel. Variants that were resistant to neutralization and expressed an antigenically altered form of gC were designated monoclonal antibody-resistant (*mar*) mutants. *mar* mutants were isolated at frequencies of 10^{-3} to 10^{-5} , depending on the antibody used for selection. The epitopes on gC were operationally grouped into antigenic sites by evaluating the patterns of neutralization observed when a panel of 22 antibodies was tested against 22 *mar* mutants. A minimum of nine epitopes was identified by this process. Three epitopes were assigned to one antigenic site (I), and six were clustered in a second complex site (II) composed of three distinct subsites, IIa, IIb, and IIc. The two antigenic sites were shown to reside in physically distinct domains of the glycoprotein, by radioimmunoprecipitation of truncated forms of gC. These polypeptides lacked portions of the carboxy terminus and ranged in size from approximately one-half that of the wild-type molecule to nearly full size. Antibodies recognizing epitopes in site II immunoprecipitated the entire series of truncated polypeptides and thereby demonstrated that site II resided in the N-terminal half of gC. Antibodies reactive with site I, however, did not immunoprecipitate fragments smaller than at least two-thirds the size of the wild-type polypeptide, suggesting that site I was located in the C-terminal portion. Sites I and II were also shown to be spatially separate on the gC polypeptide by competition enzyme-linked immunosorbent assay with monoclonal antibodies representative of different site I and site II epitopes.

Herpes simplex virus type 1 (HSV-1) is currently known to encode at least four glycoproteins, designated gC, gB, gE, and gD in order of decreasing apparent molecular weight (3, 35). The glycoproteins are exposed both on viral envelopes and on the surfaces of infected cells and thereby represent potentially important target antigens for either virus-neutralizing antibody or immune effector mechanisms which destroy infected cells (12, 13, 15, 31, 35). Antibodies against gC, gB, and gD initiate both complement-dependent and cell-mediated lysis of HSV-infected cells (2, 10, 11, 19, 27, 29). Monoclonal antibodies reactive with these same viral antigens have been reported to neutralize HSV in vitro and protect animals against lethal virus challenge in vivo (2, 7, 16, 29, 34). Immunization with purified gC or gD also protects against lethal challenge (6, 23, 32). The HSV glycoproteins are the major targets of virus-specific cytotoxic T cells (5, 22), and adoptively transferred, virus-reactive cytotoxic T cell clones can provide immune protection (33). Furthermore, human natural killer cell recognition of HSV-infected targets has recently been linked to the viral glycoproteins (4). Although all of these studies have provided strong evidence that the viral glycoproteins are the principal antigens involved in protective immune responses and are likely to be involved in natural resistance to infection, little is known about the antigenic structure of most of these molecules, information that is essential to understanding the immunology of HSV infection at a molecular level.

Because of their exquisite specificity, virus-specific monoclonal antibodies have proven to be especially useful in defining the antigenic structure of viral surface components

(39). A monoclonal antibody interacts with a precise configuration of amino acids on the antigen surface, referred to as an epitope (18). The amino acids delineating an epitope may be contiguous or noncontiguous, since sequentially distant amino acids can be brought into close proximity in the tertiary structure of the antigen. An antigenic site is a limited region of the antigen molecule composed of a single epitope or a cluster of distinct, but overlapping, epitopes (1, 39). Viral antigens often contain a limited number of complex antigenic sites composed of clusters of overlapping epitopes (21, 26, 39).

The availability of large panels of HSV glycoprotein-specific monoclonal antibodies has now made it possible to identify antigenic sites on the viral glycoproteins and to catalog the epitopes which compose individual sites. Our laboratory has used virus-neutralizing monoclonal antibodies to select antigenic variants of HSV referred to as monoclonal antibody resistant (*mar*) mutants (16). *mar* mutants may lose reactivity with antibodies that recognize one antigenic site, although they may remain reactive with antibodies which define other antigenic sites. Thus, by analyzing the patterns of resistance of *mar* mutants to neutralization with panels of monoclonal antibodies, we have been able to operationally define and enumerate discrete antigenic sites and their corresponding epitopes on the HSV glycoproteins.

In this report, we describe studies of the antigenic structure of HSV-1 glycoprotein C. By analyzing the reactivity patterns of *mar* mutants with a panel of gC-specific monoclonal antibodies, we identified nine epitopes on this antigen and showed them to be organized into two antigenic sites. The two antigenic sites were assigned to two distinct regions

* Corresponding author.

of gC by examining the ability of individual monoclonal antibodies to immunoprecipitate polypeptide fragments of this antigen. Finally, the two antigenic sites were shown to be topographically separated on gC by monoclonal antibody competition studies.

MATERIALS AND METHODS

Virus strains. Wild-type HSV-1 (strain KOS), HSV-2 (strain 186), and virus mutants were grown by infection at low multiplicity in African green monkey kidney (Vero) cells at 37°C, and titers were determined as described previously (12). A plaque-purified isolate of HSV-1 (strain KOS), designated KOS 321, was used as antigen in the production of hybridomas and as the parent virus strain for the isolation of antigenic variants. The syncytial mutant *synLD70* was isolated and characterized in our laboratory as described previously (16, 30). Mutants gC⁻3, gC⁻8, and gC⁻49 were isolated by resistance to neutralization with gC-specific monoclonal antibodies, and cells infected with these mutants secreted truncated forms of normally membrane-bound gC into the culture medium. These mutants have been characterized by Holland et al. (15a).

Cell culture and media. Human embryonic lung (HEL) and Vero cells were grown and maintained in Eagle minimum essential medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with nonessential amino acids, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), and 10% fetal calf serum (GIBCO Laboratories). The BALB/c myeloma cell line P3-X63-Ag8.653 and the myeloma-spleen cell hybridomas were grown in supplemented Dulbecco modified Eagle medium (GIBCO Laboratories) containing 20% fetal calf serum (Sterile Systems, Inc., Logan, Utah), as described previously (16).

Immunization and production of hybridomas. The procedures used for the production of hybridomas secreting neutralizing monoclonal antibodies specific for HSV-1 have been described in detail elsewhere (16). Briefly, BALB/c mice were infected intraperitoneally with 2×10^7 PFU of live KOS 321 on day 1 and were given booster injections on day 13 with 1×10^8 PFU of UV-inactivated virus. On day 16, immune spleen cells and P3-X63-Ag8.653 myeloma cells were fused by using polyethylene glycol. After selection in HAT medium (13.6 µg of hypoxanthine, 0.1 µg of aminopterin, and 3.8 µg of thymidine per ml), the surviving hybrid colonies were screened for the production of virus-neutralizing antibody, and positive colonies were subcloned by limiting dilution.

The monoclonal antibodies 5S, 17S, 18S, 19S, 26S, 27S, 19S, and 31S were kindly provided by Martin Zweig, National Cancer Institute, Frederick, Md. These antibodies were produced from mice immunized with HSV-1 (strain 14012) and have been characterized by Showalter et al. (34).

Production of monoclonal antibody stocks. High-titer ascites fluid preparations containing monoclonal antibodies were produced by intraperitoneal injection of ca. 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., Inc., Milwaukee, Wis.). Antibodies from clarified ascites fluid were concentrated by ammonium sulfate precipitation and characterized for monoclonality by isoelectric focusing (IEF) as previously described (16). Monoclonal antibody isotypes were determined by enzyme immunoassay (EIA) with subclass-specific antisera by using a MonoAb-ID EIA Kit (Zymed Laboratories, San Francisco, Calif.).

Virus neutralization assay. Wild-type HSV-1 and antigenic variants were tested for reactivity with monoclonal antibodies in 50% plaque reduction microneutralization assays as reported previously (16). Neutralization titers of ascites fluid stocks were expressed as the reciprocals of the highest dilutions which reduced standardized virus inputs (20 to 30 PFU/well) by at least 50% relative to unneutralized controls.

Radiolabeling, immunoprecipitation, and electrophoresis. HEL cells were infected with wild-type KOS 321 or antigenic variants at a multiplicity of 10 and radiolabeled with 40 µCi of [³⁵S]methionine or 2 µCi of [¹⁴C]glucosamine per ml from 4 to 18 h postinfection as previously described (16). Nonidet P-40 extracts of radiolabeled infected cells were immunoprecipitated with monoclonal antibodies, electrophoresed on 8.5% polyacrylamide slab gels cross-linked with diallyltartardiamide, and fluorographed as detailed previously (16).

To analyze gC-related peptides secreted into the medium, HEL cells were infected and radiolabeled with [³⁵S]methionine as described above. At 18 h postinfection, the medium was removed from the infected cell monolayer and cleared by centrifugation at $15,000 \times g$ for 15 min. Of the cleared, radiolabeled culture medium, 0.5 ml was then immunoprecipitated with monoclonal antibody.

Isolation of antigenic variants. Antigenic variants resistant to neutralization by single monoclonal antibodies were isolated essentially as described previously (16). Briefly, 10^7 PFU of wild-type HSV-1 (KOS 321) was neutralized in 1 ml of Eagle minimum essential medium-HEPES-10% fetal calf serum containing 10% normal rabbit serum as a source of complement and a 1:100 dilution of monoclonal antibody. The virus surviving neutralization was passaged at low multiplicity on Vero cells, and the progeny virus was subjected to a second round of neutralization with monoclonal antibody. Isolates of the virus surviving neutralization were plaque purified three times and tested for resistance to neutralization in the 50% plaque reduction assay described above.

Virus isolates resistant to neutralization were then characterized for expression of viral proteins and glycoproteins by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of [³⁵S]methionine- and [¹⁴C]glucosamine-labeled, Nonidet P-40-solubilized extracts of infected HEL cells as described previously (16). Antigenic variants were subsequently tested for reactivity with a panel of monoclonal antibodies in 50% plaque reduction neutralization assays as described above.

Some antigenic variants were isolated from stocks of KOS 321 mutagenized with 5-bromo-2-deoxyuridine as reported earlier (16). Briefly, virus was grown in Vero cells at a multiplicity of infection of 5 for 24 h in the presence of 2.5 µg of 5-bromo-2-deoxyuridine per ml. The mutagenized virus was then passaged on Vero cells at a multiplicity of infection of 0.01 for 24 h to eliminate phenotypic mixing of mutant and wild-type glycoproteins in the same virion envelope.

EIA. HEL cells were mock infected or infected at a multiplicity of 10 with KOS 321 and plated in 96-well trays at 3×10^4 cells per well in 50 µl of Eagle minimum essential medium-HEPES-5% fetal calf serum. After 18 h of incubation at 37°C, the cell monolayers were washed once with phosphate-buffered saline, fixed for 10 min at room temperature with 0.25% glutaraldehyde in phosphate-buffered saline, and washed four times with 0.1 M carbonate-buffered saline, pH 9.4 (CBS). Serial fourfold dilutions of monoclonal antibody diluted in CBS plus 3% bovine serum albumin, pH 9.4 (EIA buffer), were added to each well (50 µl). The plates

were then incubated at 37°C for 1 h and washed 3 times with CBS. Each well then received 50 µl of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (Cappel Laboratories, Cochranville, Pa.) diluted 1:500 in EIA buffer. After incubation at 37°C for 1 h, the wells were washed three times with CBS, and 50 µl of substrate [2 mM 2,2-azino-di-(3-ethylbenzthiazoline sulfonic acid) in 0.1 M sodium acetate, 0.05 M NaH₂PO₄, and 250 mM H₂O₂] was added. After incubation at room temperature for 15 min, the reaction was stopped by the addition of 50 µl of 5% SDS to each well. Absorbances at 405 nm were then determined using a Titertek Multiscan (Flow Laboratories, Inc., McLean, Va.). Titers of antibodies were expressed as EIA units equal to the greatest dilution of antibody producing at least half-maximum absorbance.

Biotin labeling of monoclonal antibodies. A modification of the method of Guesdon et al. (14) was used to conjugate biotin to the gC-specific antibodies C10, C13, C14, and C16 for use in competitive binding assays. Ascites fluid preparations of monoclonal antibodies were precipitated with 50% saturated ammonium sulfate. The precipitate was washed twice with 50% saturated ammonium sulfate and dissolved in distilled water. The redissolved antibody was dialyzed against 0.1 M NaHCO₃ (pH 9.0) for 4 h at 4°C and adjusted to a protein concentration of 4.5 to 5.0 mg/ml, based on absorbance at 280 nm. Antibody (1 ml) was then mixed with 200 µl of a solution of 1.7 mg of biotinyl-*N*-hydroxysuccinimide dissolved in *N,N*-dimethylformamide. After incubation at room temperature for 4 h, the biotinylated antibodies were dialyzed overnight against three changes of phosphate-buffered saline, sampled, and stored at -70°C.

Competitive binding assays. Antibody competition assays were done by a modification of the EIA described above with biotin-conjugated antibodies and horseradish peroxidase-conjugated avidin. Biotinylated antibodies were first titrated for binding to fixed, infected HEL cells as described above, except that binding was detected by the addition of 50 µl of horseradish peroxidase-conjugated avidin-D (20 µg/ml in EIA buffer) (Vector Laboratories, Inc., Burlingame, Calif.), in place of horseradish peroxidase-conjugated goat anti-mouse IgG. A minimum saturating concentration of biotinylated antibody was then used in the competitive assays. The conjugated antibodies had no detectable reactivity with uninfected cells at the concentrations used. Biotinylated antibody and serially diluted unlabeled antibody or EIA buffer alone (50 µl each) was added to triplicate wells and allowed to bind for 1 h at 37°C. The plates were washed three times with CBS, and 50 µl of horseradish peroxidase-conjugated avidin was added to each well. After 1 h of incubation at 37°C, the plates were washed, substrate was added, and the reactions were quantitated as described above. The percent competition was calculated by the formula $100 \times (A - B)/A$, where A is the absorbance in the absence of competing antibody and B is the absorbance in the presence of antibody. In all assays, the homologous unlabeled antibody was tested as a control for positive competition. In addition, the binding of each competing antibody in the absence of biotinylated antibody was assayed in parallel by using horseradish peroxidase-conjugated goat anti-mouse IgG as described above.

RESULTS

Characteristics of monoclonal antibodies. Hybridomas producing virus-neutralizing monoclonal antibodies were derived from BALB/c mice immunized by infection with a plaque-purified isolate of wild-type HSV-1 (KOS 321) as

TABLE 1. Characteristics of gC-specific monoclonal antibodies

Monoclonal antibody	Immunoglobulin isotype	Neutralization titer ^a for virus (strain):			
		HSV-1 (KOS 321)		synLD70	HSV-2 (186.111)
		+	-	+	+
C1	IgG2a	20,480	<80	<80	<80
C2	IgG2a	5,120	<40	<40	<40
C3	IgG2a	5,120	<40	<40	<40
C4	IgG2a	5,120	<40	<40	<40
C7	IgG3	640	<10	<10	<10
C8	IgG2a	20,480	<10	<10	<10
C9	IgG2a	20,480	<10	<10	<10
C10	IgG2a	20,480	<10	<10	<10
C11	IgG2a	40,960	<10	<10	<10
C13	IgG2a	327,680	<320	NT	<320
C14	IgG2b	81,920	<160	NT	<160
C15	IgG2a	1,280	<5	NT	<5
C16	IgG2a	10,240	<10	NT	<10
C17	IgG2a	5,120	<10	NT	<10

^a +, Neutralization mixture contained normal rabbit serum as a complement source at a final concentration of 10%; -, no normal rabbit serum was present; NT, not tested. Titer is the 50% endpoint titration by quantitative virus neutralization assay.

reported earlier (16). Four independent hybridoma fusions were performed. The characteristics of 14 monoclonal antibodies used in the selection and analysis of antigenic variants are shown in Table 1. The antibodies were determined to be specific for gC by immunoprecipitation of [³⁵S]methionine-labeled HSV-1 viral antigens from Nonidet P-40 extracts of infected cells. The immunoprecipitates were electrophoresed in SDS-polyacrylamide gels in parallel with immunoprecipitates produced with known gC-specific monoclonal antibodies (16; data not shown). All of the antibodies tested had high complement-dependent neutralization titers against HSV-1 but failed to neutralize HSV-2. As previously described (16), synLD70 does not express gC on the surface of infected cells or on virion envelopes and was useful in confirming the reactivity of gC-specific monoclonal antibodies; all gC-specific antibodies failed to neutralize this mutant.

Selection of antigenic variants. The gC-specific monoclonal antibodies were used to select *mar* mutants that expressed gC in infected cells but were resistant to neutralization with at least one gC-specific monoclonal antibody (16). Plaque-purified wild-type HSV-1 (KOS 321) was neutralized with single monoclonal antibodies in the presence of complement. The virus surviving neutralization was passaged at low multiplicity and subjected to a second round of neutralization, and the surviving virus was plaque purified. These isolates were tested for resistance to neutralization in 50% endpoint plaque reduction assays. An isolate was considered to be a *mar* mutant if the titer of the antibody, when tested against the isolate, was at least 32-fold less than the titer of the antibody when tested with wild-type virus. In practice, most of the *mar* mutants were not neutralized to any detectable extent. Isolates resistant to neutralization by this criterion were analyzed by radioimmunoprecipitation and SDS-polyacrylamide electrophoresis to ensure that the viral glycoproteins were expressed (data not shown).

From 13 independent selection experiments, 22 *mar* mutants were isolated from 5-bromo-2-deoxyuridine-mutagenized or nonmutagenized virus stocks by using 12 gC-specific antibodies. The frequencies of mutant isolation ranged from 2×10^{-5} to 1×10^{-3} , depending on the selecting antibody, and are similar to frequencies we previously

G R O U P	mar MUTANT	MONOCLONAL ANTIBODY GROUPS																							
		I					IIa		IIb			IIc													
		C1	C2	C4	C11	C14	C15	C16	C3	C8	C9	C17	C13	C7	C10	5S	17S	18S	19S	26S	27S	29S	31S		
KOS 321																									
1	C2.1	●	●	●																					
	C4.4	●	●	●																					
	C4.3	●	●	●	●	●	●																		
	C11.1	●	●	●	●	●	●																		
	C11.2	●	●	●	●	●	●																		
	C14.1	●	●	●	●	●	●																		
	C15.4	●	●	●	●	●	●																		
C15.1	●	●	●	●	●	●																			
2a	C3.1							●	●																
	C3.2							●	●																
	C16.1							●	●																
	C16.2							●	●																
2b	C9.1								●	●	●	●													
	C9.6								●	●	●	●													
	C17.2								●	●	●	●													
	C17.3								●	●	●	●													
	C13.1											●													
2c	C7.1							●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●		
	C7.2							●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●		
	C10.1							●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●		
	C10.3							●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●		
	C13.2							●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●		

FIG. 1. Antigenic sites on HSV-1 gC. The individual gC-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.

reported (16). The *mar* mutants were designated by the selecting antibody. For example, *mar* C2.1 was selected with antibody C2. In addition to *mar* mutants, we also isolated mutants which failed to express detectable gC on virion envelopes or on the surface membranes of infected cells and were therefore referred to as gC⁻ mutants. These mutants were isolated at an average frequency of 2×10^{-4} . The gC⁻ mutants exhibited a variety of phenotypes and have been described in detail elsewhere (15a, 19a).

Construction of an operational antigenic map of gC. The *mar* mutants should have sustained mutations that affect antigenic sites in a way that reduces the ability of antibody to bind to and neutralize the variant virus. By selecting *mar* mutants with different monoclonal antibodies, it is possible to introduce into the wild-type parental background mutations that alter different epitopes. Different epitope alterations can be distinguished by analyzing the reactivity of *mar* mutants with monoclonal antibodies. These patterns of reactivity can then be used to identify epitopes on the glycoproteins and group them into operationally defined antigenic sites (37-39).

Accordingly, we tested the panel of gC-specific antibodies for neutralization of the *mar* mutants, and the resulting patterns of resistance were used to construct an operational map of the antigenic structure of gC (Fig. 1). In this analysis, each monoclonal antibody with a distinct vertical pattern of reactivity with the *mar* mutants defined a different epitope on gC. That is, a unique pattern identified an antibody which recognized a distinct amino acid configuration on the gC molecule. Based on this criterion, nine different epitopes were identified by the prototype antibodies C1, C11, C15,

C16, C3, C8, C17, C13, and C7. Antibodies with distinct but similar reactivity patterns with the *mar* mutants were grouped together and were considered to define overlapping and operationally linked epitopes in the same antigenic site. Conversely, antibodies or groups of antibodies with mutually exclusive patterns of reactivity defined different antigenic sites. By examining the patterns of overlapping epitopes, the nine epitopes on gC could be arranged into four groups that defined two antigenic sites.

Antigenic site I was defined by antibodies in group I. The *mar* mutants selected with those antibodies (mutant group I) were resistant to neutralization with antibodies in group I and were sensitive to all other antibodies. The group I antibodies produced three distinct patterns of reactivity, indicating that antigenic site I was composed of at least three nonidentical, but overlapping, epitopes. Thus, a mutation in this antigenic site might affect only one epitope, (e.g., *mar* C15.1) or all epitopes defined by antibodies in this group (e.g., *mar* C4.3). In some instances, mutants selected with the same antibody differed in their reactivity with the panel of antibodies. For example, *mar* C4.4 and *mar* C4.3 were both selected with antibody C4 but differed considerably in reactivity with the group I antibodies. It also should be pointed out that mutants with the same patterns of reactivity are not necessarily identical since different mutations could create the same phenotypic alterations in antigenic structure.

Antigenic site II is a complex site defined by antibodies in groups IIa, IIb, and IIc. Although the patterns of reactivity of the antibodies in these three groups overlapped to varying degrees, they were sufficiently different to warrant subdivision. Mutants selected with antibodies in group IIa or IIb were resistant only to antibodies in those respective groups. However, mutants in group 2c (selected with antibodies in group IIc) were generally resistant to neutralization with antibodies of all three groups. By the principles outlined above, we interpreted these patterns as indicating the presence of one complex antigenic site. This site would be composed of a minimum of six epitopes as determined by the number of different patterns of antibody reactivity. Antibody groups IIa, IIb, and IIc would operationally define overlapping but distinguishable subregions within this antigenic site.

Analysis of the gC-specific monoclonal antibodies by IEF. The gC-specific monoclonal antibodies used in the analysis of *mar* mutants were analyzed by IEF (Fig. 2). Antibodies with identical IEF profiles are likely to be of the same clone type. The results showed that of the 14 antibodies produced against HSV-1 (KOS), at least 10 distinct clone types were represented. Of the six antibodies in group I (C1, C2, C4, C11, C14, and C15), three had similar banding patterns (C1, C2, and C4) and were indistinguishable by their reactivities with the *mar* mutants (Fig. 1). These antibodies were isolated from the same hybridoma fusion experiment and probably represent sister isolates. The other antibodies in this group, C11, C14, and C15, were unique in their banding characteristics (Fig. 2), and their patterns of neutralization of *mar* mutants were different from those of the other group I antibodies (Fig. 1). Three of the four antibodies in group IIb (C8, C9, and C17) had banding patterns similar to each other and likely represented the same clone type, but they were different from the remaining antibody in that group, C13. Thus, two sets of multiple antibodies with similar focusing profiles and identical patterns of reactivity with the *mar* mutants were isolated from the same hybridoma fusion. Finally, the four antibodies in groups IIa (C3

FIG. 2. IEF of gC-specific monoclonal antibodies. Monoclonal antibodies were electrophoresed on a 5% polyacrylamide gel (pH 4.0 to 9.0) at a constant current of 2 mA for 18 h. Electrophoretically separated proteins were visualized by staining with Coomassie brilliant blue. The anode (+) and cathode (-) are indicated.

and C16) and IIc (C7 and C10) had unique banding patterns and represented distinct clonotypes.

The IEF patterns also distinguished between antibodies that appeared to be identical in their reactivity with *mar* mutants. For example, antibodies C7 and C10 had identical reactivity patterns with the *mar* mutants but were clearly different in their isoelectric points. These results indicate that different antibodies can be reactive with the same operationally defined antigenic site and, in turn, that the same *mar* mutation can affect the binding of two different antibodies.

Reactivity of monoclonal antibodies with truncated peptides. The analysis of reactivity patterns of *mar* mutants and monoclonal antibodies provided a means of operationally defining antigenic sites. To identify regions of the gC molecule which were associated with those antigenic sites, we used a unique class of virus mutants which produce truncated polypeptide fragments of gC. These mutants, designated gC⁻3, gC⁻49, and gC⁻8, were a subset of antigenic variants selected by resistance to neutralization with monoclonal antibody C11. These variants did not express gC on virions or on the surface membranes of infected cells but produced soluble, truncated forms of the normally membrane-bound glycoprotein which were secreted into the culture medium (15a). In addition to these mutants, the mutant *syn*LD70, originally isolated on the basis of its plaque morphology and described as failing to express gC (16), was also found to secrete a truncated gC polypeptide.

The secreted products were immunoprecipitable with a pool of gC-specific monoclonal antibodies and ranged in molecular weight from 130,000 (gC⁻3) to 101,000 (*syn*LD70) (Fig. 3). Almost no detectable gC could be immunoprecipitated from the medium of cells infected with wild-type virus. The nonglycosylated precursor forms of these secreted polypeptides were identified by pulse radiolabeling in the presence of tunicamycin, and molecular weights were estimated to be 73,000 (gC⁻3), 59,000 (gC⁻49), 54,000 (gC⁻8), and 48,000 (*syn*LD70).

To locate antigenic sites I and II on the gC polypeptide, we tested representatives of the four groups of antibodies for immunoprecipitation of [³⁵S]methionine-labeled, truncated polypeptides of gC which were secreted into the medium of cells infected with mutants gC⁻3, gC⁻49, gC⁻8, and *syn*LD70 (Table 2). The antibody groups defining sites I and II differed in their reactivity with these polypeptides. Antibodies from groups IIa, IIb, and IIc immunoprecipitated the proteins secreted by all of the mutants, localizing antigenic site II to the *syn*LD70 polypeptide, the smallest tested. Antibodies of group I precipitated the gC⁻3 polypeptide but did not precipitate the *syn*LD70 or gC⁻8 polypeptides. In addition, antibody C2 did not immunoprecipitate the product of mutant gC⁻49, distinguishing it from the other group I antibody.

FIG. 3. Secretion of truncated forms of gC from cells infected with gC⁻ mutants. HEL cells were infected at a multiplicity of infection of 10 and radiolabeled with [³⁵S]methionine from 4 to 18 h postinfection. A pool of gC-specific monoclonal antibodies was used to immunoprecipitate gC from the culture medium or from detergent lysates of infected cells. The immunoprecipitated proteins were electrophoresed on a 7% SDS-polyacrylamide gel and visualized by fluorography. The locations of molecular weight standards are indicated on the left.

TABLE 2. Immunoprecipitation of truncated gC peptides with monoclonal antibodies

Virus	Mol wt (10 ³) of gC product ^a when:		Monoclonal antibody ^b in group:									
			I				IIa		IIb		IIc	
	Tunicamycin precursor	Mature	C2	C11	C14	C15	C3	C16	C13	C17	C7	C10
KOS	73	130	+	+	+	+	+	+	+	+	+	+
gC-3	73	130	+	+	+	+	+	+	+	+	+	+
gC-49	59	112	-	+	+	+	+	+	+	+	+	+
gC-8	54	109	-	-	-	-	+	+	+	+	+	+
synLD70	48	101	-	-	-	-	+	+	+	+	+	+

^a Glycoprotein gC-related peptides were identified by immunoprecipitation of lysates of [³⁵S]methionine-labeled infected HEL cells or of tissue culture medium with a pool of gC-specific monoclonal antibodies (15a). Nonglycosylated precursors were identified after a 30-min pulse with [³⁵S]methionine in the presence of the glycosylation inhibitor tunicamycin.

^b +, A radiolabeled peptide with the expected molecular weight was immunoprecipitated with the indicated monoclonal antibody; -, no radiolabeled band was observed after prolonged autoradiography.

ies. These data confirm the grouping of antibodies based on neutralization of *mar* mutants and show that antigenic sites I and II are located in different parts of the gC molecule.

Topographical analysis of antigenic sites. Antigenic sites I and II appeared to be distinct sites based on the analysis of *mar* mutants and the reactivities of monoclonal antibodies with polypeptide fragments of gC. These data suggested that sites I and II represent limited antigenic regions on the gC molecule that are spatially separate from one another. To determine the topographical relationships of the two sites, we performed competitive binding studies with representatives of the different groups of antibodies. In principle, if monoclonal antibodies define spatially separate epitopes on the same glycoprotein molecule, the binding of one antibody should not inhibit the binding of the other. Conversely, antibodies reactive with identical or topographically overlapping epitopes should be mutually competitive. Based on these considerations, competitive binding assays with monoclonal antibodies have been used to construct topographical maps of antigenic sites on a number of other viral proteins (20, 24, 25).

To determine if sites I and II were topographically separate, we tested five unlabeled antibodies representing the two antigenic sites for inhibition of binding of the four labeled monoclonal antibodies C14 (group I), C16 (group IIa), C13 (group IIb), and C10 (group IIc). Strong competition was always seen with homologous competitors (Fig. 4). The group I antibodies C11 and C14 were able to compete for binding of the labeled group I antibody C14 (Fig. 4A). However, these antibodies failed to inhibit the binding of group II antibodies C16, C13, and C10 (Fig. 4B, C, and D, respectively). Antibodies of groups IIa, IIb, and IIc (C16, C13, and C10) did not inhibit the binding of the group I antibody C14, although the three group II antibodies were mutually competitive (Fig. 4B, C, and D). In these experiments, lack of competition was not due to the inability of the competing antibody to bind since the competing antibodies were always titrated in parallel and were shown to bind strongly at the concentration used (data not shown). Reciprocal noncompetition between antibodies of groups I and II strongly indicates that antigenic sites I and II are spatially separate on the surface of the gC molecule and may represent distinct antigenic domains.

DISCUSSION

In this report, we describe experiments which provide detailed information on the antigenic structure of HSV-1 glycoprotein C. We used two approaches to identify epi-

topes on gC and catalog them into antigenic sites. (i) Monoclonal antibodies that were gC specific were used to select neutralization-resistant virus mutants with demonstrable antigenic changes in gC. The comparative analysis of these mutants with a panel of monoclonal antibodies allowed us to operationally identify epitopes on gC and group them into antigenic sites. (ii) The relative locations of these antigenic sites were identified by determining the physical

FIG. 4. Competitive binding assays of gC-specific antibodies. Biotinylated antibodies C14, C16, C13, and C10 were reacted in a competitive binding EIA with representative antibodies from each of the four reactivity groups. Unlabeled competitors were mixed with the labeled antibodies and coincubated in wells containing glutaraldehyde-fixed, KOS-infected HEL cells. All assays were done in triplicate. The percent competition was determined from the absorbance in the presence of competitor compared with that in the absence of competitor, as described in the text.

binding sites of the monoclonal antibodies on the gC molecule. By these two approaches, we have identified a total of nine epitopes on gC, which are organized into two distinct antigenic sites.

The definition of antigenic sites from the analysis of *mar* mutants was based on the principle that mutations affecting one epitope should not affect epitopes in a separate antigenic site but may affect overlapping epitopes in the same site. Thus, a *mar* mutant selected with a single monoclonal antibody could be altered in one epitope or several. For example, the two *mar* mutants selected with antibody C13 had dramatically different neutralization phenotypes; *mar* C13.1 was resistant to neutralization with only 1 antibody, whereas *mar* C13.2 was resistant to 14 antibodies defining at least four different epitopes in the same antigenic site (Fig. 1). Conversely, *mar* C4.3 and *mar* C11.1 were selected with antibodies directed at different epitopes, yet they were indistinguishable by neutralization tests. By analyzing these types of patterns of overlapping reactivity, we identified nine epitopes on gC and assigned them to two antigenic sites. Three epitopes were assigned to antigenic site I, and six were clustered into site II, a complex site composed of subsites IIa, IIb, and IIc.

Identification of the three subsites was based on the phenotypes of *mar* mutants selected with the three subgroups of antibodies. In general, *mar* mutants selected with group IIa antibodies or IIb antibodies were resistant to neutralization only with antibodies of those respective groups and might be considered to define two distinct antigenic sites. However, two observations argue against this interpretation. (i) *mar* C13.2 was selected with a group IIb antibody and was resistant to neutralization by antibodies in both groups IIb and IIc. (ii) All four mutants selected with group IIc antibodies were resistant to groups IIa, IIb, and IIc. These results are best interpreted as defining a single complex antigenic site. The alternative explanation is that mutations in the group IIc mutants affect three distinct antigenic sites by introducing conformational changes in the tertiary structure of gC that alter sites IIa and IIb. However, it is unlikely that all the mutants selected with group IIc antibodies would have mutations of this nature. It is more probable that the three subgroups of antibodies either define overlapping but distinct subregions of the same site or represent mutations that affect the same antigenic site in three generally different ways.

Of the 16 monoclonal antibodies that define antigenic site II, 8 were produced from four independent hybridoma fusions against the KOS strain of HSV-1. The remaining eight antibodies (17S to 31S) were produced independently by Showalter et al. (34) against strain 14012. Antigenic site II may therefore be a major, immunodominant site since it is recognized by a number of monoclonal antibodies produced in independent fusions and in independent laboratories.

The analysis of *mar* mutants operationally defines antigenic sites and does not provide direct information about the physical structure of the molecule. In addition, the resolution of the operational antigenic map is dependent on the number of antibodies and mutants analyzed, and the definitions of antigenic sites are subject to changes as more are isolated and tested. The antigenic map is also based on an arbitrary criterion of resistance to neutralization; the levels of overlap between epitopes might change if the *mar* mutants were selected and characterized with a more stringent or relaxed definition of resistance. However, the operational definitions of the two antigenic sites on gC are supported by the results of two sets of experiments that assayed the

physical locations of the binding sites of the gC-specific antibodies, namely, the immunoprecipitation studies of the truncated gC peptides and the competitive binding assays.

Antibodies defining sites I and II could be distinguished by the immunoprecipitation of the truncated products of the gC⁻ secretor mutants. Group II antibodies immunoprecipitated the *syn*LD70 peptide, whereas group I antibodies did not (Table 2). Based on studies of the nonglycosylated precursor (15a) and nucleotide sequencing data (F. Homa, M. Levine, and J. Glorioso, unpublished data), the *syn*LD70 polypeptide represents the N-terminal 248 amino acids of the wild-type protein (excluding the signal sequence) and thus localizes antigenic site II to the N-terminal 51% of the protein sequence. From the amino acid sequence predicted by Frink et al. (9), this polypeptide would contain one of two major hydrophilic regions of the molecule as determined by the method of Hopp and Woods (17). Strongly hydrophilic regions have been shown to be predictive of antigenic sites (17).

Of the four monoclonal antibodies defining site I, three immunoprecipitated the gC⁻49 polypeptide, but all four were unreactive with the gC⁻8 and *syn*LD70 polypeptides. These results show that antigenic site I is located within the gC⁻49 polypeptide. By subtraction, these data further suggest that site I is located in the sequences unique to the gC⁻49 polypeptide, which are not contained in the gC⁻8 polypeptide. An alternative interpretation, that the missing sequences of gC⁻8 do not code for site I but indirectly affect the site, cannot be excluded. Nucleotide sequencing of *mar* mutants in this site, combined with biochemical analysis of the mutant gene products, should help to distinguish between these possibilities and precisely identify the amino acid sequences contributing to the antigenic site. At a minimum, however, it can be stated that these C-terminal sequences are required to maintain the immunological reactivity of site I.

The competitive binding experiments also agree with the analysis of *mar* mutants and truncated gC peptides. Lack of reciprocal competition between antibodies of groups I and II strongly indicates that antigenic sites I and II are spatially separate on the surface of the gC molecule and represent distinct antigenic domains. The reciprocal competition of antibodies in groups IIa, IIb, and IIc implies that these groups define topographically overlapping epitopes within the same antigenic site. However, competition between antibodies can be due to several mechanisms other than binding to structurally overlapping epitopes. These include steric hindrance between antibodies recognizing nonoverlapping epitopes in close proximity and allosteric changes induced by the binding of antibody (24). Competition studies, therefore, tend to overestimate the degree of overlap between epitopes. Nevertheless, the results of the competition studies taken together with the analyses of *mar* mutants and radioimmunoprecipitation of peptide fragments may indicate that these three groups define a single, complex antigenic site on the gC molecule.

We were able to isolate *mar* mutants with changes in the antigenic sites on gC at relatively high frequencies. It is reasonable to ask, then, if similar antigenic variation occurs in human infections. Pereira et al. (28) addressed this question in part by testing 130 clinical isolates of HSV-1 and HSV-2 with a panel of glycoprotein-specific antibodies. All of the HSV-1 isolates were reactive with two gC-specific antibodies tested. Interestingly, 11% of the HSV-2 isolates also reacted, a surprising finding since gC-specific antibodies are predominantly type specific. Antigenic variation in gB,

gD, and gE was shown by the failure of isolates to react with antibodies specific for these glycoproteins (up to 15, 12, and 3% for gB, gD, and gE, respectively). The lack of reactivity could not be attributed to failure to express the glycoproteins, and antibodies specific for the same glycoprotein varied in reactivity with the isolates. Thus, the antigenic changes in the unreactive isolates are similar to those in *mar* mutants. Since the absence of demonstrable variation in gC was based on analysis with only two gC-specific antibodies, a further study of clinical isolates with a more extensive panel of antibodies may detect antigenic variation in gC. Furthermore, the use of antibodies with defined epitope and antigenic-site specificity would determine if variation is more common in some epitopes than others. Although the study by Pereira et al. demonstrated that there are antigenic differences in the glycoproteins expressed by clinical isolates, it is unknown if this variation has a significant impact on the immunobiology of HSV infections. At a minimum, however, the variation in the clinical isolates and the *mar* mutants stresses the requirement for the use of multiple monoclonal antibodies reactive with epitopes of known stability in diagnosis, treatment, or epidemiological studies.

Until recently, HSV-1 gC (gC-1) was thought to be type specific and to lack antigenic cross-reactivity with an equivalent glycoprotein (gC-2) in HSV-2. For example, purified gC-1 and gC-2 each induce immune responses which apparently do not cross-react with the heterologous serotype (6, 8, 32, 36). However, it has been recently demonstrated with cross-reactive monoclonal antibodies and polyclonal antisera specific for gC that the two glycoproteins do share limited antigenic homology (28, 40–42). It is of interest whether these cross-reactive antibodies identify epitopes located within the two antigenic sites identified in our study or within new antigenic sites. It also remains to be determined whether type-specific and cross-reactive antibodies generally define different antigenic regions on gC. It is possible that type-specific and cross-reactive antibodies do not identify distinct antigenic sites on gC but rather reflect different epitopes within the same antigenic site. For example, a mutation in a region originally common to the two serotypes could have created apparent type specificity by affecting binding of some antibodies in an antigenic site but not binding of others. An analogous effect has been seen in our study in which one *mar* mutant becomes resistant to some, but not all, antibodies that define one antigenic site. A definitive analysis of the antigenic structure of gC may require a comparison of the nucleotide sequences of gC-1 and gC-2, along with analyses of *mar* mutations affecting antigenic sites.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grants AI-17900, AI-13228, and RR 00200 from the National Institutes of Health. S.D.M. was supported by a Horace Rackham Predoctoral Fellowship from the University of Michigan and by training grant T32-GM07315. We are pleased to acknowledge support for T.C.H. by the Dental Research Institute Associate Program, from the Office of the Vice President for Research of the University of Michigan, and by Public Health Service grant DE02731 from the National Institute of Dental Research.

We thank Fred Homa for allowing use of his unpublished data. We thank Elizabeth Smiley for her excellent technical assistance.

LITERATURE CITED

- Atassi, M. Z., and J. A. Smith. 1978. A proposal for the nomenclature of antigenic sites in peptides and proteins. *Immunochemistry* 15:609–610.
- Balachandran, N., S. Bacchetti, and W. E. Rawls. 1982. Protection against lethal challenge of BALB/c mice by passive transfer of monoclonal antibodies to five glycoproteins of herpes simplex virus type 2. *Infect. Immun.* 37:1132–1137.
- Baucke, R. B., and P. G. Spear. 1979. Membrane proteins specified by herpes simplex viruses. V. Identification of an Fc-binding glycoprotein. *J. Virol.* 32:779–789.
- Bishop, G. A., J. C. Glorioso, and S. A. Schwartz. 1983. Relationship between expression of herpes simplex virus glycoproteins and susceptibility of target cells to human natural killer activity. *J. Exp. Med.* 157:1544–1561.
- Carter, V. C., P. A. Schaffer, and S. S. Tevethia. 1981. The involvement of herpes simplex virus type 1 glycoproteins in cell-mediated immunity. *J. Immunol.* 126:1655–1665.
- Chan, W. L. 1983. Protective immunization of mice with specific HSV-1 glycoproteins. *Immunology* 49:343–352.
- Dix, R. D., L. Pereira, and J. R. Baringer. 1981. Use of monoclonal antibody directed against herpes simplex virus glycoproteins to protect mice against acute virus-induced neurological disease. *Infect. Immun.* 34:192–199.
- Eberle, R., and R. J. Courtney. 1980. Preparation and characterization of specific antisera to individual glycoprotein antigens comprising the major glycoprotein region of herpes simplex virus type 1. *J. Virol.* 35:902–917.
- Frink, R. J., R. Eisenberg, G. Cohen, and E. K. Wagner. 1983. Detailed analysis of the portion of the herpes simplex virus type 1 genome encoding glycoprotein C. *J. Virol.* 45:634–647.
- Glorioso, J., C. H. Schröder, G. Kumel, M. Szczesiul, and M. Levine. 1984. Immunogenicity of herpes simplex virus glycoproteins gC and gB and their role in protective immunity. *J. Virol.* 50:805–812.
- Glorioso, J., M. S. Szczesiul, S. D. Marlin, and M. Levine. 1983. Inhibition of glycosylation of herpes simplex virus glycoproteins: immunogenicity and antigenicity of partially glycosylated glycoproteins. *Virology* 126:1–18.
- Glorioso, J. C., M. Levine, T. C. Holland, and M. S. Szczesiul. 1980. Mutant analysis of herpes simplex virus-induced cell surface antigens: resistance to complement-mediated immune cytolysis. *J. Virol.* 35:672–681.
- Glorioso, J. C., and J. W. Smith. 1977. Immune interaction with cells infected with herpes simplex virus: antibodies to radioiodinated surface antigens. *J. Immunol.* 118:114–121.
- Guesdon, J., T. Ternynck, and S. Avrameas. 1979. The use of avidin-biotin interaction in immunoenzymatic techniques. *J. Histochem. Cytochem.* 27:1131–1139.
- Heine, J. W., R. W. Honess, E. Cassai, and B. Roizman. 1974. Proteins specified by herpes simplex virus. XII. The virion polypeptides of type 1 strains. *J. Virol.* 14:640–651.
- Holland, T. C., F. L. Homa, S. D. Marlin, M. Levine, and J. Glorioso. 1984. Herpes simplex virus type 1 glycoprotein C-negative mutants exhibit multiple phenotypes, including secretion of truncated glycoproteins. *J. Virol.* 52:566–574.
- Holland, T. C., S. D. Marlin, M. Levine, and J. Glorioso. 1983. Antigenic variants of herpes simplex virus selected with glycoprotein-specific monoclonal antibodies. *J. Virol.* 45:672–682.
- Hopp, T. P., and K. R. Woods. 1981. Prediction of protein antigenic determinants from amino acid sequences. *Proc. Natl. Acad. Sci. U.S.A.* 78:3824–3828.
- Jerne, N. K. 1960. Immunological speculations. *Annu. Rev. Microbiol.* 14:341–358.
- Kapoor, A. K., A. A. Nash, P. Wildy, J. Phelan, C. S. McLean, and H. J. Field. 1982. Pathogenesis of herpes simplex virus in congenitally athymic mice: the relative roles of cell-mediated and humoral immunity. *J. Gen. Virol.* 60:225–233.
- Kikuchi, G. E., J. E. Coligan, T. C. Holland, M. Levine, J. C. Glorioso, and R. Nairn. 1984. Biochemical characterization of peptides from herpes simplex virus glycoprotein gC: loss of CNBr fragments from the carboxy terminus of truncated, secreted gC molecules. *J. Virol.* 52:806–815.
- Kimura-Kuroda, J., and K. Yasui. 1983. Topographical analysis of antigenic determinants on envelope glycoprotein V3 (E) of

- Japanese encephalitis virus, using monoclonal antibodies. *J. Virol.* **45**:124–132.
21. Lafon, M., T. J. Wiktor, and R. I. MacFarlan. 1983. Antigenic sites on the CVS rabies virus glycoprotein: analysis with monoclonal antibodies. *J. Gen. Virol.* **64**:843–854.
 22. Lawman, M. J. P., R. J. Courtney, R. Eberle, P. A. Schaffer, M. K. O'Hara, and B. T. Rouse. 1980. Cell-mediated immunity to herpes simplex virus: specificity of cytotoxic T cells. *Infect. Immun.* **30**:451–461.
 23. Long, D., T. J. Madara, M. Ponce de Leon, G. H. Cohen, P. C. Montgomery, and R. J. Eisenberg. 1984. Glycoprotein D protects mice against lethal challenge with herpes simplex virus types 1 and 2. *Infect. Immun.* **43**:761–764.
 24. Lubeck, M. D., and W. Gerhard. 1981. Topological mapping of antigenic sites on the influenza A/PR/8/34 virus hemagglutinin using monoclonal antibodies. *Virology* **113**:64–72.
 25. Massey, R. J., and G. Schochetman. 1981. Topographical analysis of viral epitopes using monoclonal antibodies: mechanisms of virus neutralization. *Virology* **115**:20–23.
 26. Minor, P. D., G. C. Schild, J. Bootman, D. M. A. Evans, M. Fuguson, P. Reeve, M. Spitz, G. Stanway, A. J. Cann, R. Hauptmann, L. D. Clarke, R. C. Mountford, and J. W. Almond. 1983. Location and primary structure of a major site for poliovirus neutralization. *Nature (London)* **301**:674–679.
 27. Norrild, B., S. L. Shore, and A. J. Nahmias. 1979. Herpes simplex virus glycoproteins: participation of individual herpes simplex virus type 1 glycoprotein antigens in immunocytolysis and their correlation with previously identified glycopolypeptides. *J. Virol.* **32**:741–748.
 28. Pereira, L., D. V. Dondero, D. Gallo, V. Devlin, and J. D. Woodie. 1982. Serological analysis of herpes simplex virus types 1 and 2 with monoclonal antibodies. *Infect. Immun.* **35**:363–367.
 29. Rector, J. T., R. N. Lausch, and J. E. Oakes. 1982. Use of monoclonal antibodies for analysis of antibody-dependent immunity to ocular herpes simplex virus type 1 infection. *Infect. Immun.* **38**:168–174.
 30. Sandri-Goldin, R. M., M. Levine, and J. C. Glorioso. 1981. Method for induction of mutations in physically defined regions of the herpes simplex virus genome. *J. Virol.* **38**:41–49.
 31. Sarmiento, M., M. Haffey, and P. G. Spear. 1979. Membrane proteins specified by herpes simplex viruses. III. Role of glycoprotein VP7(B₂) in virion infectivity. *J. Virol.* **29**:1149–1158.
 32. Schrier, R. D., L. I. Pizer, and J. W. Moorhead. 1983. Type-specific delayed hypersensitivity and protective immunity induced by isolated herpes simplex virus glycoproteins. *J. Immunol.* **130**:1413–1420.
 33. Sethi, K. K., Y. Omata, and E. E. Schneweis. 1983. Protection of mice from fatal herpes simplex virus type 1 infection by adoptive transfer of cloned virus-specific and H-2-restricted cytotoxic T lymphocytes. *J. Gen. Virol.* **64**:443–447.
 34. Showalter, S. D., M. Zweig, and B. Hampar. 1981. Monoclonal antibodies to herpes simplex virus type 1 proteins, including the immediate-early protein ICP 4. *Infect. Immun.* **34**:684–692.
 35. Spear, P. G. 1976. Membrane proteins specified by herpes simplex viruses. I. Identification of four glycoprotein precursors and their products in type 1-infected cells. *J. Virol.* **17**:991–1008.
 36. Vestergaard, B. F., and B. Norrild. 1978. Crossed immunoelectrophoresis of a herpes simplex virus type 1-specific antigen: immunological and biochemical characterization. *J. Infect. Dis.* **138**:639–643.
 37. Webster, R. G., and W. G. Laver. 1980. Determination of the number of nonoverlapping antigenic areas on Hong Kong (H3N2) influenza virus hemagglutinin with monoclonal antibodies and the selection of variants with potential epidemiological significance. *Virology* **104**:139–148.
 38. Wiktor, T. J., and H. Koprowski. 1980. Antigenic variants of rabies virus. *J. Exp. Med.* **152**:99–112.
 39. Yewdell, J. W., and W. Gerhard. 1981. Antigenic characterization of viruses by monoclonal antibodies. *Annu. Rev. Microbiol.* **35**:185–206.
 40. Zezulak, K. M., and P. G. Spear. 1983. Characterization of a herpes simplex virus type 2 75,000-molecular-weight glycoprotein antigenically related to herpes simplex virus type 1 glycoprotein C. *J. Virol.* **47**:553–562.
 41. Zweig, M., C. J. Heilman, Jr., S. V. Bladen, S. D. Showalter, and B. Hampar. 1983. Detection in antisera of antibodies that cross-react with herpes simplex virus type 1 glycoprotein gC. *Infect. Immun.* **41**:482–487.
 42. Zweig, M., S. D. Showalter, S. V. Bladen, C. J. Heilman, Jr., and B. Hampar. 1983. Herpes simplex virus type 2 glycoprotein gF and type 1 glycoprotein gC have related antigenic determinants. *J. Virol.* **47**:185–192.