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Localization of Discontinuous Epitopes of Herpes Simplex Virus Glycoprotein D: Use of a Nondenaturing ("Native" Gel) System of Polyacrylamide Gel Electrophoresis Coupled with Western Blotting

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Previously, a panel of monoclonal antibodies (MCAb) was used to define specific epitopes of herpes simplex virus glycoprotein D (gD) (R. J. Eisenberg et al., J. Virol. 53:634–644, 1985). Three groups of antibodies recognized continuous epitopes; group VII reacted with residues 11 to 19 of the mature protein (residues 36 to 44 of the predicted sequence), group II reacted with residues 272 to 279, and group V reacted with residues 340 to 356. Four additional antibody groups recognized discontinuous epitopes of gD, since their reactivity was lost when the glycoprotein was denatured by reduction and alkylation. Our goal in this study was to localize more precisely the discontinuous epitopes of gD. Using a nondenaturing system of polyacrylamide gel electrophoresis ("native" gel electrophoresis) coupled to Western blotting, we analyzed the antigenic activity of truncated forms of gD. These fragments were generated either by recombinant DNA methods or by cleavage of purified native gD-1 (gD obtained from herpes simplex virus type 1) and gD-2 (gD obtained from herpes simplex virus type 2) with *Staphylococcus aureus* protease V8. Antibodies in groups III, IV, and VI recognized three truncated forms of gD-1 produced by recombinant DNA methods, residues 1 to 287, 1 to 275, and 1 to 233. Antibodies in group I recognized the two larger forms but did not react with the gD-1 fragment of residues 1 to 233. On the basis of these and previous results, we concluded that a portion of epitope I was located within residues 233 to 259 and that epitopes III, IV, and VI were upstream of residue 233. Antibodies to continuous epitopes identified protease V8 fragments of gD-1 and gD-2 that contained portions of either the amino or carboxy regions of the proteins. None of the V8 fragments, including a 34K polypeptide containing residues 227 to 369, reacted with group I antibodies. This result indicated that a second portion of epitope I was located upstream of residue 227. Two amino-terminal fragments of gD-1, 33K and 30K, reacted with group III, IV, and VI antibodies. A 33K fragment of gD-2 reacted with group III antibodies. Based on their size and reactivity with endo-β-*N*-acetylglycosaminidase F, we hypothesized that the 33K and 30K molecules represented residues 1 to 226 and 1 to 182 of gD-1, respectively. These results suggest that epitopes III, IV, and VI are located within the first 182 residues of gD. The association of group I monoclonal antibodies with several important biological properties, including virus neutralization, protection by passive immunization, and inhibition of cell-to-cell fusion, implicates residues 233 to 259 of gD in these functions. The association of group VI antibodies with virus adsorption implicates gD residues upstream of 182 in this function.

Glycoprotein D (gD) is a virion envelope component of herpes simplex virus (HSV) types 1 (HSV-1) and 2 (HSV-2) which stimulates the production of high titers of virus-neutralizing activity and is likely to play an important role in the initial stages of viral infection. gD has been shown to protect animals from HSV challenge (3, 8, 9, 16, 21, 39, 41, 47) and is a subunit vaccine candidate. Studies have shown that gD is structurally and antigenically similar, although not identical, in the two serotypes of HSV. These and other properties of gD have recently been reviewed (54). The function(s) of this glycoprotein has been inferred mostly from studies with anti-gD antibodies. For example, certain monoclonal antibodies (MCAb) directed at gD blocked virus adsorption (30), whereas others interfered with the fusion of infected cells (46). These data suggest that gD participates in at least two different steps in infection and that different parts of the protein appear to be involved in these activities.

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We examined a panel of gD MCAb and arranged them into groups, presumably recognizing distinct epitopes of HSV-1 gD (gD-1) and HSV-2 gD (gD-2) (22, 24, 25). Three continuous epitopes (1) (epitopes not destroyed by reduction and alkylation) were localized to residues 11 to 19 (group VII) (9, 10, 18), 268 to 287 (group II) (25), and 340 to 356 (group V) (16). Group I, III, IV, and VI MCAb recognized discontinuous epitopes (1) of gD-1 (epitopes destroyed by reduction and alkylation). Blocking studies showed that the four discontinuous epitopes were different from each other (25). This type of analysis was also used to group MCAb from other laboratories. We found that MCAb which had been reported to exhibit the highest titers of neutralizing and fusion-blocking activity (46) were in type-common group I, whereas MCAb with the highest virus adsorption-blocking activity (30) were in the HSV-1-specific group VI (R. J. Eisenberg, V. Rinaldt, M. Nobel, and G. H. Cohen, unpublished data). Thus, two functions of gD in viral infection are associated with two discontinuous epitopes.

The purpose of the current investigation was to localize more precisely the discontinuous epitopes of gD. Our approach was to analyze the antigenic activity of truncated forms of gD generated either by recombinant methods or by proteolysis. Previously, we showed that the four discontinuous epitopes were located within residues 1 to 275 of gD-1 (25). In those studies, the protein or fragments were spotted onto nitrocellulose and tested for reactivity in the antigen spot test (9, 10, 18, 22, 25, 33). However, that system did not permit visualization of a reactive fragment within a mixture; moreover, we could not use Western blots (6, 55) of standard sodium dodecyl sulfate (SDS)-polyacrylamide gels to visualize the fragments, since the discontinuous epitopes were destroyed by the denaturing conditions.

To circumvent these problems, we adapted a nondenaturing system of polyacrylamide gel electrophoresis (PAGE) ("native" gel electrophoresis) coupled to Western blotting (6) for use with each of the MCAb groups. Since many MCAb are directed against discontinuous epitopes (2, 5), the native gel-Western blotting system should have general applicability. This technique allowed us to visualize the reaction of group I, III, IV, and VI MCAb with native gD and fragments of the molecule. Fragments were generated in two different ways. First, three truncated forms of gD-1 (residues 1 to 287, 1 to 275, and 1 to 233) were produced and secreted by Chinese hamster ovary cells transformed with shuttle vectors containing portions of the gD-1 gene (39). Second, *Staphylococcus aureus* protease V8 was used to generate fragments of gD-1 or gD-2 under conditions of partial digestion. These results, together with those of previous studies (24), showed that a portion of epitope I is located between residues 233 and 259 of gD-1. We speculate that another portion of epitope I is located further upstream. Evidence is also presented to show that the other three discontinuous epitopes are located within the first 182 residues of gD.

MATERIALS AND METHODS

Cells and virus. Conditions for the growth and maintenance of BHK cells (14, 26) and for the propagation of virus have been described previously (11, 12, 14). The NS strain of HSV-1 was kindly provided by H. Friedman (29). The SAVAGE strain of HSV-2 was used as a source of gD-2 (11).

Preparation of polyclonal antibody and MCAb to gD. Polyclonal anti-gD-1 serum was prepared in rabbits (12) against an immunosorbent-purified preparation of gD-1 (27). MCAb DL2 (group VI) and DL11 (group I) were prepared from mice immunized intraperitoneally (41) with 6 μg of immunosorbent-purified gD-1 and gD-2 (27), respectively. Three days after an intravenous boost of 1 μg, the spleens were removed, and the cells were fused to SP2/0 cells (44). Hybridomas were cloned in soft agarose (36), and ascites fluids were prepared from Pristane-primed mice immunized intraperitoneally with cloned cells. The preparation and characteristics of MCAb DL6 (group II) were described previously (25). MCAb 11S (group III) (24, 52), MCAb 41S (group IV) (24, 52), and anti-358-369 serum were supplied by M. Zweig. The latter was prepared against a synthetic peptide which mimicked the amino acid sequence of gD-1 at residues 358 to 369. MCAb 1D3 (group VII) (24) was supplied by H. Friedman (29). Antibodies were grouped by their ability to react with native or denatured gD-1 and gD-2 or both, blocking studies (previously termed competition analysis [25]), neutralizing activity, and reactions with synthetic peptides, as in previous studies (9, 10, 18, 22, 24, 25, 52). Imunoglobulins were purified from sera and ascites fluids by previously described methods (45).

Preparation of native gD and denatured gD. gD-1 and gD-2 were each purified from cytoplasmic extracts of infected cells by affinity chromatography on a column of MCAb DL6 (group II) immunoglobulin G (25) linked to Sepharose by a previously described procedure (6). Denaturation by boiling of gD-1 and gD-2 in the presence of SDS and mercaptoethanol, followed by alkylation with iodoacetamide, was carried out as previously described (25, 43).

Preparation of truncated forms of gD. The gene for gD-1 was cloned into a pBR322-Simian virus 40 shuttle vector (39; P. W. Berman and W. Nunes, submitted for publication) and included a DNA fragment extending from a *Hin*dIII site upstream of the gD-1 gene (38, 40, 57) to one of three restriction sites within the gene: (i) an *Acc*I site at residue 258 of the predicted sequence (residue 233 of the mature protein); (ii) a *Hin*fI site at residue 300 of the predicted sequence (residue 275 of the mature protein); or (iii) a *Nar*I site at residue 312 of the predicted sequence (residue 287 of the mature protein). When these plasmids are grown in Chinese hamster ovary cells, glycoproteins are secreted (39; Berman and Nunes, submitted); the glycoproteins used in this study were concentrated from the medium and, in the case of the form containing residues 1 to 275, purified by affinity chromatography (27).

V8 proteolysis of gD. Purified gD-1 and gD-2 (2-μg amounts) were each suspended in 50 mM Tris hydrochloride buffer (pH 8.0) and digested with 20 μg of *S. aureus* protease V8 (20) (Miles Laboratories, Inc.) for 1 h at 37°C. The reaction was stopped by freezing the sample at −70°C, and samples were maintained at this temperature until electrophoretic analysis.

Endo F treatment of gD. Purified gD-1 (2 μg) was suspended in 0.1 M sodium phosphate buffer (pH 6.0) and digested with 0.5 U of endo-β-*N*-acetylglycosaminidase F (endo F) (New England Nuclear Corp.) (28) at 37°C for 4 h. A duplicate sample of gD-1 was incubated in endo F buffer without enzyme. At the end of the digestion period, each sample was adjusted to a final concentration of 50 mM Tris hydrochloride (pH 8.0). A 10:1 ratio of *S. aureus* V8 protease was added, and the samples were incubated for 1 h at 37°C. The reaction was stopped as described above.

Electrophoresis under nondenaturing conditions (native gels). All of the experiments presented here used a Hoefer minigel system for vertical electrophoresis. The resolving gel consisted of either 10 or 12.5% acrylamide cross-linked with either 0.1 or 0.125% bisacrylamide, respectively, in 0.425 M Tris hydrochloride buffer (pH 9.18) containing 0.1% SDS. The gel was polymerized with ammonium persulfate and *N,N,N',N'*-tetramethylethylenediamine (TEMED). The stacking gel consisted of 3.2% acrylamide, 0.2% bisacrylamide, and 0.1% SDS in 0.054 M Tris-sulfate (pH 6.1). Samples were diluted into the sample buffer (0.054 M Tris, 0.1% SDS, 10% glycerol, 0.001% bromophenol blue), loaded into the wells, and then overlaid with running buffer (0.1% SDS in 0.04 M Tris-borate buffer [pH 8.65]). Electrophoresis was carried out for 45 min at 150 V. Following electrophoresis, the proteins were electrophoretically transferred to nitrocellulose (type BA-85, 0.45 μm; Schleicher & Schuell, Inc.) by using a Hoefer Transphor Electrophoresis Unit at 150 V for 30 min. The blotting buffer consisted of 0.02 M Tris base, 0.150 M glycine, and 20% methanol (pH 8.3). Following transfer, the nitrocellulose was washed with a protein-blocking solution (10) and then incubated with antibody, followed by iodinated protein A (Amersham Corp.) as pre-

TABLE 1. Properties of continuous and discontinuous epitopes of HSV gD

Epitope	Reaction of MCAb with [a]: gD-1	gD-2	Position in gD (amino acids)	Biological properties[b]
Continuous				
VII	+	+	11–19	Neutralization, protection
II	+	+	272–279	Neutralization, protection
V	+	+/−	340–356	Unknown
Discontinuous				
I	+	+	216, 233–259, and upstream	Neutralization, protection, fusion
III	+	+	1–182	Neutralization
IV	+	−	1–182	Neutralization
VI	+	−	1–182	Virus adsorption, protection

[a] Reactivity was based on the results of immunoblot analysis with either the antigen spot test (10, 25, 33) or Western blots of native gels (this paper). In immunoprecipitation (24), group I, II, III, IV, VI, and VII MCAb gave essentially the same results as those indicated; however, group V MCAb showed no cross-reactivity against any strain of HSV-2 gD (24,52).

[b] Neutralization properties were reported in references 24, 30, 46, 48, and 52. The ability of a particular epitope to participate in protection is based on the results of experiments with MCAb for the passive immunization of mice, followed by a live HSV-1 or HSV-2 challenge via the footpad route (19; R. Dix, personal communication for groups II and VI). By this criterion, epitope V does not participate in protection (51). The participation of epitope VII in protection was also shown by using synthetic peptides which mimic residues 1 to 23 of gD-1 or gD-2 as vaccines against a lethal HSV-2 challenge in mice (9, 21). To assess the gD epitopes involved in fusion (46) and adsorption (20), we grouped antibodies kindly supplied by P. G. Spear by reactivity against gD-1 and gD-2, as well as by blocking studies (22, 25; Eisenberg et al. unpublished data). The groupings were as follows: antibodies III-114-4 and II-174-1, shown to be best at inhibiting fusion (46), were in group I; antibody I-99-1 was in group III; antibody II-436-1 was in group IV; and antibodies I-206-7 and II-886-1, shown to be best at inhibiting adsorption (30), were in group VI.

viously described (10). The blots were air dried and then exposed to Kodak XAR-5 film with a Cronex Lightning-Plus screen (Du Pont Co.) at −70°C.

General comments on the native gel system. There are a number of methods by which electrophoretic separation and blotting of proteins are carried out in such a way as to retain much native conformation or to restore it (4, 17, 31). The method we have chosen uses a low concentration of SDS, so that proteins will still be separated by apparent molecular weight. This concentration of SDS might have some denaturing effect. However, the native gel system worked well with MCAb that recognized either continuous or discontinuous epitopes of gD. In addition, we found that the method could also detect discontinuous epitopes of other HSV glycoproteins (R. J. Eisenberg and G. H. Cohen, unpublished data). However, in a few cases, e.g., MCAb DL2 (group VI), the antigen-antibody reaction was more difficult to detect and might have been negatively affected by the electrophoretic conditions. Attempts to alter the electrophoretic conditions, e.g., to further lower the SDS concentration in the gel or running buffer, had an adverse effect on the resolving power of the gel system. Therefore, in cases in which the antigen-antibody reaction was particularly sensitive to SDS, we increased the antigen concentration 5- to 20-fold, until a reaction was detected (see Figure legends).

We also compared the separation of polypeptides in both native and denaturing gels. The denaturing gels were used to determine the apparent molecular weights of the gD fragments against the SDS-denatured protein standards. When these calculated values for the fragments were assigned to the equivalent polypeptides in the native gels, the migration rates of the polypeptides in the native gel system were found to be inversely proportional to the log of molecular weight over a wide range.

RESULTS

Use of native gels to visualize native and denatured gD. A summary of the grouping of gD epitopes is shown in Table 1. Continuous epitopes (those recognized by group II, V, and VII MCAb) were localized precisely by fragmentation studies (24), SDS-PAGE (43, 58), immunofluorescence (43), and immunoblot analysis (9, 10, 18, 22, 25). However, discontinuous epitopes (those recognized by group I, III, IV, and VI MCAb) were previously localized to a large fragment of gD (amino acids 1 to 275). To localize these epitopes more precisely, we used a native gel system for electrophoresis coupled to Western blotting to examine the reactivity of gD fragments.

(i) Reactivity of group VII and II MCAb against gD. To test the resolving power of the gel and blotting system, we first examined the reactivity of gD-1 and gD-2 against antibodies which recognize continuous epitopes. Native and denatured gD-1 and gD-2 were electrophoresed in a native gel, blotted onto nitrocellulose, and reacted with group VII (Fig. 1A) and II (Fig. 1B) MCAb. Epitope VII was localized to residues 11 to 19 (10, 18). Epitope II was originally localized to residues 268 to 287 (25) and more recently was further localized to residues 272 to 279 (Conrad Heilman, unpublished data). Both native and denatured gD-1 and gD-2 migrated in the gel in a manner similar to that seen in standard SDS-PAGE (inversely according to size) and reacted with both MCAb. The precursor, product, and dimer forms of gD-1 were easily distinguished (13, 23, 27, 32, 53). In the denatured gD-2 preparation, two breakdown products were observed, one of

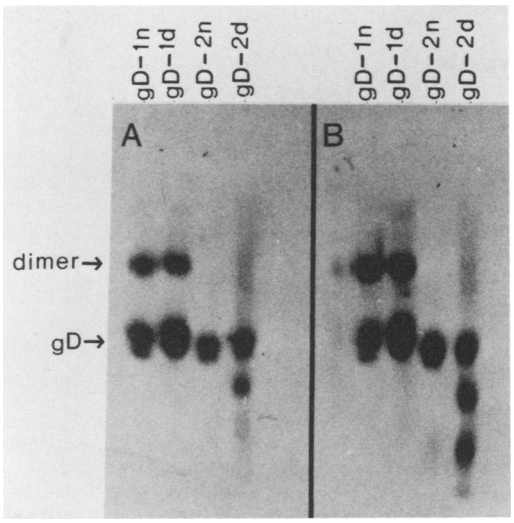

FIG. 1. Reactivity of group VII and II MCAb against native and denatured gD-1 and gD-2. Immunosorbant-purified native (n) or denatured (d) gD-1 and gD-2 (25 ng) were electrophoresed in a native gel (10% acrylamide cross-linked with bisacrylamide) and transferred to nitrocellulose. (A) Reaction with group VII MCAb (residues 11 to 19); (B) reaction with group II MCAb (residues 272 to 279).

which reacted with group VII and both of which reacted with group II. This result suggests that the larger of the two breakdown products extended at least from residues 11 to 279 and that the smaller one lacked residues 11 to 19 but contained residues 272 to 279.

(ii) Reactivity of MCAb which recognize discontinuous epitopes of gD (groups I, III, IV, and VI). Native and denatured gD-1 and gD-2 were electrophoresed in a native gel, transferred to nitrocellulose, and tested for binding of MCAb which recognize discontinuous epitopes. Group I (Fig. 2A) and III (Fig. 2B) MCAb recognized the native forms of gD-1 and gD-2 but not the denatured forms. The HSV-1-specific group IV (Fig. 2C) and VI (Fig. 2D) MCAb recognized only native gD-1. Again, each of the antibodies reacted with precursor, product, and dimer forms of gD. In Fig. 2C, group IV appears to be less reactive against the dimer. In this case, a different preparation of purified gD-1 which contained less dimer was used. The amount of dimeric gD-1 varied in each preparation, for reasons which are not understood. These results demonstrate that the native gel system can be used to visualize both continuous and discontinuous epitopes of gD.

Reactivity of MCAb against truncated forms of gD-1. Mammalian cell lines which synthesize three different truncated forms of gD-1 (amino acids 1 to 233, 1 to 275, and 1 to 287) were constructed (39; Berman and Nunes, submitted). The truncated proteins each lacked the carboxy terminus, the transmembrane anchor region (38, 43, 56, 57), and various numbers of upstream residues.

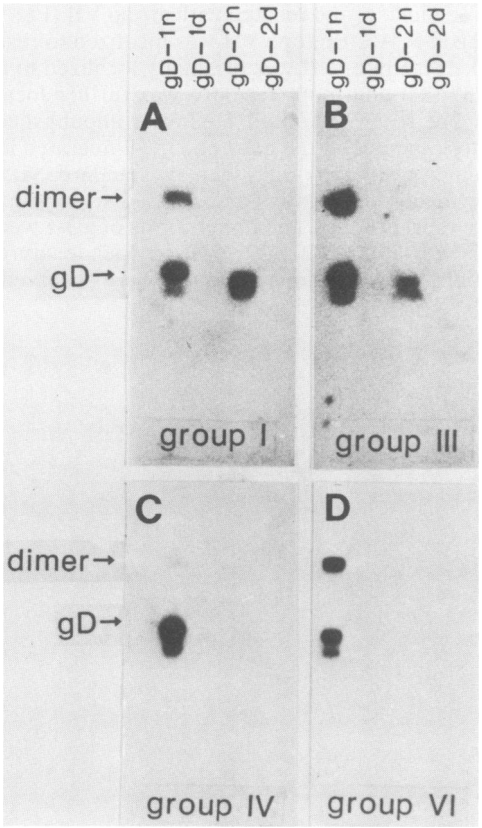

FIG. 2. Reactivity of group I, III, IV, and VI MCAb against native and denatured gD-1 and gD-2. Native (n) or denatured (d) gD-1 and gD-2 were electrophoresed in a 10% native gel and transferred to nitrocellulose. (A) 25 ng of gD-1n, 50 ng of gD-1d, 25 ng of gD-2n, and 50 ng of gD-2d; (B) 25 ng of gD-1n, 50 ng of gD-1d, 50 ng of gD-2n, and 100 ng of gD-2d; (C) 150 ng of gD-1n, 150 ng of gD-1d, 300 ng of gD-2n, and 300 ng of gD-2d; (D) 150 ng of gD-1n, 150 ng of gD-1d, 300 ng of gD-2n, and 300 ng of gD-2d.

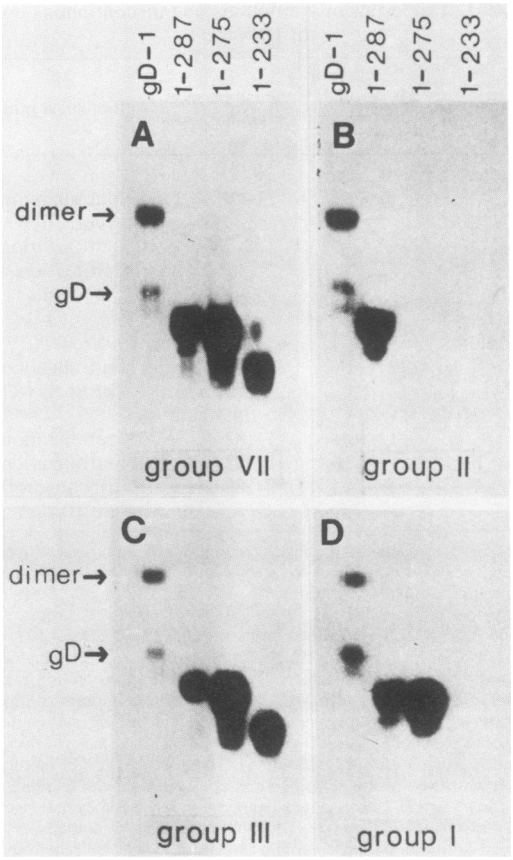

FIG. 3. Reactivity of MCAb against truncated forms of gD-1. Truncated forms of gD-1 were electrophoresed in a 10% native gel and transferred to nitrocellulose. The amounts of protein used in each case were as follows: gD-1, 5 ng; residues 1 to 287, 25 ng; residues 1 to 275, 50 ng; and residues 1 to 233, 50 ng.

(i) Reactions against MCAb which recognize continuous epitopes. Each of the truncated gD-1 forms reacted with group VII MCAb (Fig. 3A), confirming that they all contained residues near the amino terminus of gD-1 (residues 11 to 19). Among the truncated forms, only that containing residues 1 to 287 reacted with group II MCAb (Fig. 3B). These results confirm and extend the results of our previous studies (25) which showed that group II MCAb (residues 272 to 279) did not react with gD-1 residues 1 to 275, although a portion of the epitope is contained in this molecule. None of the truncated gD-1 forms reacted with group V MCAb (24, 52), which recognize residues 340 to 356 (25) (data not shown).

(ii) Reactions against MCAb which recognize discontinuous epitopes. Each of the truncated gD-1 forms reacted with group III MCAb (Fig. 3C), showing that this discontinuous epitope was entirely contained within residues 1 to 233 of gD-1. Similar results were obtained for group IV and VI MCAb (data not shown). In contrast, group I MCAb reacted with the truncated forms containing residues 1 to 287 and 1 to 275 but not with the truncated form containing residues 1 to 233 (Fig. 3D). This result indicates that a portion or all of epitope I is located between residues 233 and 275.

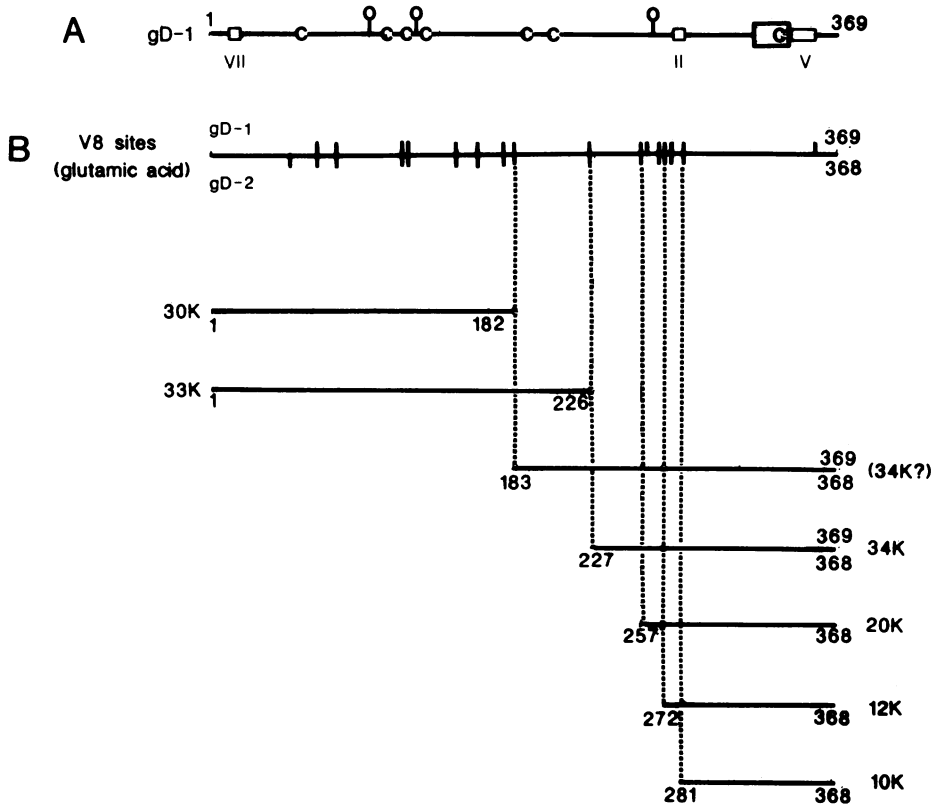

FIG. 4. Diagrammatic representation of the sequence of gD-1 and potential V8 sites in gD-1 and gD-2. In the line drawing of gD-1 in panel A (38, 57), the positions of cysteine residues are denoted with a "C," N-asparagine-linked oligosaccharides (13, 38, 57) are denoted by circles, and the transmembrane anchor (43) is indicated as a heavy rectangular box. Epitopes VII (amino acids 11 to 19), II (amino acids 272 to 279), and V (amino acids 340 to 356) are denoted by boxes. These features are identical in gD-2, except that the cysteine at residue 332 is replaced by a serine (38, 56). The line drawing in panel B indicates the positions of the potential V8 sites (glutamic acid residues [20]) in gD-1 and gD-2. Also shown are the V8 fragments of gD-1 and gD-2, with probable residue numbers and apparent molecular weights (in thousands [K]) derived from the experiments in Fig. 5 and 6.

V8 proteolysis of gD-1 and gD-2. (i) Potential cleavage sites. The purpose of the proteolysis experiments was to generate antigenically active fragments of gD-1 and gD-2 which would be missing portions of either the amino- or carboxy-terminal areas. *S. aureus* protease V8 specifically cleaves proteins on the carboxyl side of glutamic acid residues (20). Figure 4 shows the potential protease V8 cleavage sites of gD-1 and gD-2 based on their deduced amino acid sequences (38, 56, 57; W. Wilcox, G. H. Cohen, and R. J. Eisenberg, unpublished results for HSV-2 strain 333 gD). Fourteen of these sites are identical in the two proteins; however, gD-1 contains two additional sites (residues 259 and 357) not present in gD-2, and gD-2 contains one additional site (residue 45) not present in gD-1. The sizes of the potential V8 fragments vary from 3 to 44 amino acids. However, a partial digest was able to yield a number of larger fragments, some of which are indicated in Fig. 4.

Previously (24, 25), we treated gD-1 bound to a specific MCAb with protease V8 and examined the sizes and tryptic peptide profiles of the fragments remaining bound to the antibody. We identified a 38K fragment of gD-1 containing epitopes I, III, IV, and VI and probably representing residues 1 to 259. Further cleavage products included a fragment containing epitope VII and another fragment containing epitope V.

In this study, we treated purified gD-1 and gD-2 with protease V8 in the absence of antibody and under conditions of partial proteolysis. The fragments were separated by electrophoresis in a 12.5% native gel and transferred to nitrocellulose.

(ii) Identification of gD residues contained in the antigenically active fragments. To identify and position the polypeptides in the digests, we used both polyclonal antibodies, and MCAb that recognized specific continuous epitopes of gD. We first examined the digestion products with polyclonal antisera to gD-1 (Fig. 5A) and gD-2 (data not shown). The results indicate that gD-1 and gD-2 were each cleaved into a number of faster-migrating antigenically reactive polypeptides which varied in apparent molecular weight over a wide range.

Group VII MCAb (Fig. 5B) were used to identify fragments containing residues 11 to 19 (termed amino-terminal fragments), group II MCAb (Fig. 5C) were used to identify fragments containing residues 272 to 279, and anti-358-369 serum (Fig. 5D) was used to identify fragments containing the carboxy-terminal residues of gD (termed carboxy-terminal fragments). These results, together with the apparent molecular weights of the reactive fragments, were used to deduce the compositions of V8 fragments shown in Fig. 4.

Two V8 fragments of gD-1, 33K and 30K, and one fragment of gD-2, 33K, reacted with group VII MCAb (residues 11 to 19) (Fig. 5B). These fragments of gD-1 migrated more rapidly than the truncated form containing residues 1 to 233 (see Fig. 6F). From these results, we

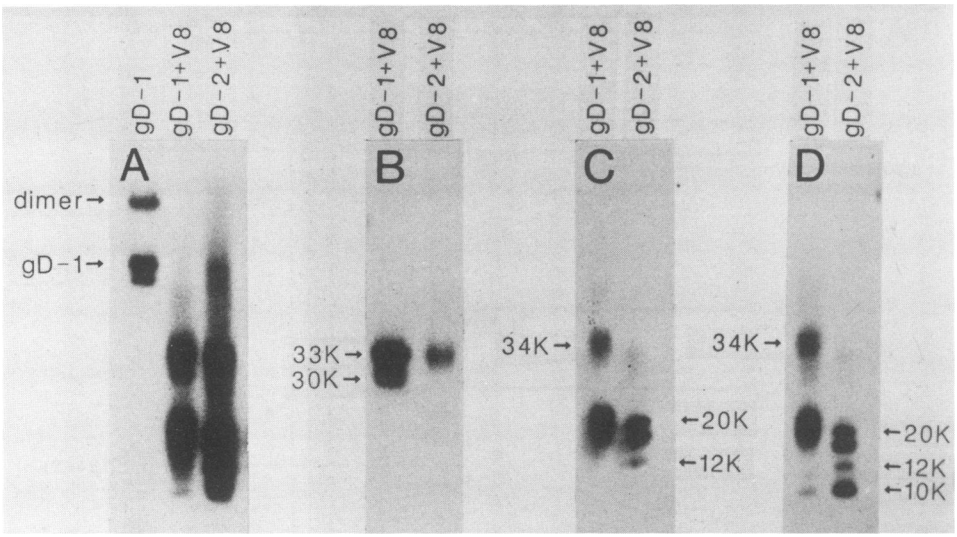

FIG. 5. V8 proteolysis of gD-1 and gD-2: orientation of fragments. Purified gD-1 and gD-2 were each digested with *S. aureus* protease V8, electrophoresed in a 12.5% native gel, and transferred to nitrocellulose. The antibodies were as follows: A, polyclonal anti-gD-1 antibody (1, 6, 16); B, group VII MCAb; C, group II MCAb; and D, anti-358-369 serum.

deduced that the 33K fragment was most likely generated by cleavage of gD-1 or gD-2 at residue 226 or further upstream. The 30K fragment derived from gD-1 could either be a precursor (e.g., less glycosylated) form of the 33K fragment (13, 23) or a further V8 cleavage product, such as residues 1 to 182 (see below).

Group II MCAb (residues 272 to 279) reacted with a 34K fragment of gD-1 (Fig. 5C) as well as with lower-molecular-weight polypeptides. These antibodies, when reacted with the gD-2 digest, recognized a 34K fragment (trace amounts) and three well-resolved lower-molecular-weight fragments. These results suggested that several overlapping gD fragments had been generated. Since epitope II is in the middle of the molecule, the fragments could contain different amino or carboxy termini. However, none of the fragments comigrated with those reacting against group VII MCAb (residues 11 to 19).

The V8 digests were analyzed further with group V MCAb (data not shown) and with anti-358-369 serum (Fig. 5D). For gD-1, both antibodies produced patterns similar to those shown for gD-1 digested by anti-358-369 serum (Fig. 5D). However, group V MCAb reacted poorly against gD-2 (25) and could not be used to identify V8 fragments derived from this molecule. In contrast, anti-358-369 serum was cross-reactive (Fig. 5D). The results clearly show that all of the V8 fragments of gD-1 and gD-2 which were recognized by group II MCAb (Fig. 5C) were also recognized by anti-358-369 serum (Fig. 5D). In addition, anti-358-369 serum reacted against a 10K polypeptide of gD-1 and gD-2 which was not recognized by group II MCAb. These results indicate that all of the V8 fragments containing epitope II also contained the carboxy terminus of gD. In addition, the V8 digest contained a 10K carboxy-terminal fragment which lacked epitope II.

The smallest fragment of gD-2 that could have reacted with anti-358-369 serum but not with group II MCAb would consist of residues 281 to 368 (87 amino acids, approximately 10K, Fig. 4), although it could have extended upstream to residue 275 and still be lacking epitope II. A 12K carboxy-terminal fragment of gD-2 (Fig. 5D) contained epitope II and therefore included at least residues 272 to 368 (Fig. 4). The 16K and 20K carboxy-terminal fragments would contain additional upstream residues (indicated for 20K as residues 257 to 368 in Fig. 4); the 20K fragment would also appear larger if it contained the N-asparagine-linked oligosaccharide on residue 262 (13). Based on its reactivity and apparent molecular weight, the 34K fragment (Fig. 5C and D) appeared to contain at least residues 227 to 369 (Fig. 4) and could conceivably extend further upstream, possibly to residue 183.

(iii) Reaction of group I, III, IV, and VI MCAb with fragments in the V8 digests. Group I MCAb did not react with any of the fragments present in either V8 digest (Fig. 6A). Assuming that the 34K fragment includes at least residues 227 to 369, it should also contain that portion of epitope I localized to residues 233 to 275 (Fig. 3D). This result suggests that another portion of epitope I might be located upstream of residue 227. Alternatively, the tertiary structure of the 34K fragment and possibly epitope I could have been lost because of V8 proteolysis.

Group III MCAb reacted with the 33K and 30K fragments of gD-1 (Fig. 6B and C) and the 33K fragment of gD-2, which contained epitope VII (Fig. 5B and 6F). This result suggests that epitope III is located at least within the first 226 residues of gD-1 and gD-2. Group IV (Fig. 6D) and VI (Fig. 6E) MCAb also reacted with the 33K and 30K fragments of gD-1, suggesting that these epitopes are also located within the same portion of the protein as epitope III.

Endo F digestion of V8 fragments. One question raised in the previous experiment was whether the 33K and 30K fragments represented two different glycosylated forms of gD-1; alternatively, the 30K fragment could contain fewer amino acids than the 33K fragment. To answer this question, we used the enzyme endo F, which removes both high-mannose (present on the precursor) and complex (present on the product) N-asparagine-linked oligosaccharides from glycoproteins (28). If the 33K and 30K fragments were precursor-product, digestion with endo F would convert both to the same-sized polypeptide. Alternatively, if the 33K and 30K fragments contained different numbers of amino acids, we would detect two fragments.

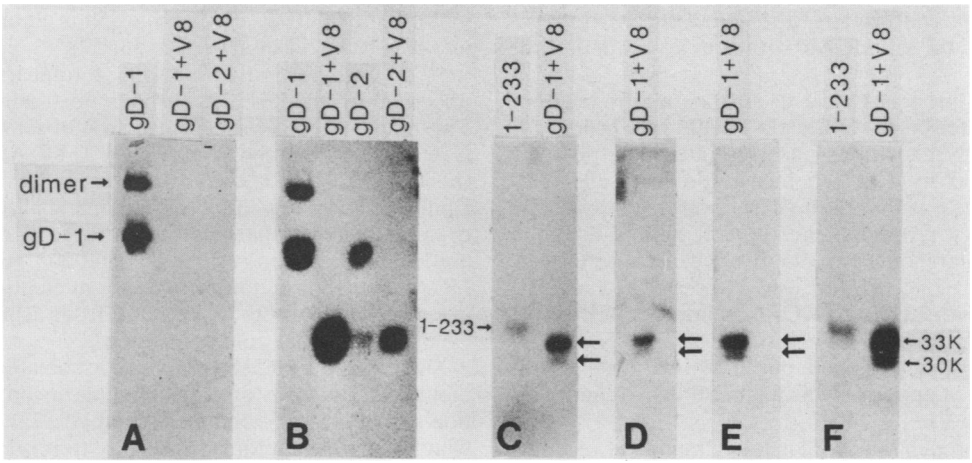

FIG. 6. V8 proteolysis of gD-1 and gD-2: identification of fragments retaining discontinuous epitopes. Purified gD-1 and gD-2 were each digested with *S. aureus* protease V8, electrophoresed in a 12.5% native gel, and transferred to nitrocellulose. The truncated form (residues 1 to 233) (5 ng) was electrophoresed as a marker in panels C and F. The antibodies were as follows: A, group I; B, group III; C, group III; D, group IV; E, group VI; and F, group VII.

gD-1 was first treated with the enzyme endo F for 4 h and subsequently with protease V8 for 1 h. A control sample was incubated in endo F buffer (no enzyme) and then treated with protease V8. The fragments were separated by electrophoresis, transferred to nitrocellulose, and probed with group III MCAb (Fig. 7). The control sample (with V8, without endo F) contained 33K and 30K fragments which reacted with group III MCAb. In contrast, the sample which had first been treated with endo F and then with V8 contained 26K and 22K fragments, both of which reacted with group III MCAb. These polypeptides had the expected sizes of unglycosylated forms of the 33K (residues 1 to 226) and 30K (residues 1 to 182) fragments.

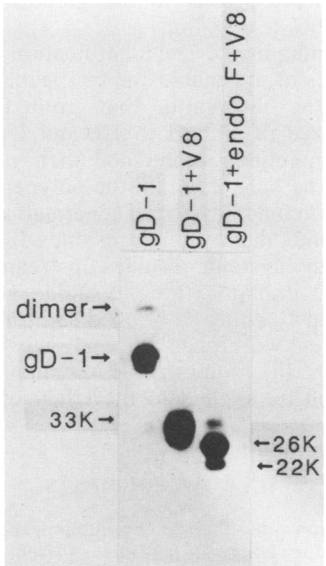

FIG. 7. Endo F and protease V8 treatment of gD-1. Purified gD-1 was incubated at 37°C with endo F for 4 h and then at 37°C with protease V8 for 1 h. Control samples (gD-1 or gD-1 + V8) were incubated under the same conditions in the appropriate enzyme buffers but without one or both enzymes. The samples were electrophoresed in a 12.5% native gel, transferred to nitrocellulose, and probed with group III MCAb.

On the basis of the results in Fig. 6 and 7, we conclude that discontinuous epitopes III, IV, and VI are at least within residues 1 to 182 of gD-1.

DISCUSSION

Previously, we defined antigenic epitopes of gD based on an analysis with a panel of MCAb (10, 24, 25). Three epitopes were continuous and were located at residues 11 to 19 (group VII) (10, 18), 272 to 279 (group II) (25; Heilman, unpublished data), and 340 to 356 (group V) (25, 43, 58). Four epitopes were discontinuous and were localized to residues 1 to 275 of gD-1 (24, 25). Our long-term goal is to define discontinuous epitopes with the same precision achieved for the continuous epitopes (9, 10, 18, 22, 25). On the basis of blocking studies (25), a topographic map of gD-1 was constructed that indicated that epitopes I and III (both type common) were distinct from each other and that epitopes IV and VI (type 1 specific) were also distinct from each other. In addition, some epitopes appeared to partially overlap. In particular, the binding of group II MCAb partially blocked the binding of group I MCAb but had no effect on the binding of group III MCAb. Therefore, epitope I was depicted in the map as being closer to epitope II, and epitope III was depicted further upstream.

In the present study, we developed methods to localize more precisely the positions of discontinuous epitopes of gD. For other HSV glycoproteins, the mapping of discontinuous epitopes has relied on the availability of mutants (7, 34, 36, 37, 42) but has not been as precise as in cases in which the tertiary structure of the protein is known (2, 15, 59). For gD, a paucity of mutants is available for study. No temperature-sensitive mutants have thus far been identified. Several MCAb-resistant mutants have been isolated (37) and, in some cases, sequenced (A. Minson, personal communication). One natural variant with an altered pattern of reactivity with MCAb has been sequenced (50). Here, our strategy was to examine the antigenic activity of gD fragments that retained their native conformation. A nondenaturing system of PAGE (native gel elctrophoresis) coupled with Western blotting (6, 55) was adapted for use with the MCAb groups. This method appears to have general applicability for the study of MCAb which recognize discontinuous epitopes (2,

5). The technique has allowed us to visualize the reactions of group I, III, IV, and VI MCAb with native gD and with fragments of gD.

Two different approaches were used to obtain fragments. First, three truncated forms of the gD-1 gene were produced by recombinant DNA methods, inserted into a shuttle vector, and expressed by Chinese hamster ovary cells (39; Berman and Nunes, submitted). The expressed proteins lacked the carboxy terminus, the transmembrane anchor, and various numbers of upstream residues. The shortest form, residues 1 to 233, reacted wih group III, IV, and VI MCAb but not with group I MCAb. Thus, a portion of epitope I appeared to be within residues 233 to 275. This finding confirmed and extended our previous speculation (25) that a portion of epitope I was further downstream than epitope III, IV, or VI.

Our second approach was to generate fragments of both the amino and carboxy portions of gD-1 and gD-2 with *S. aureus* protease V8. We identified the fragments by using group II and VII MCAb as well as an antiserum to a synthetic peptide which mimics the gD carboxy terminus (provided by M. Zweig), anti-358-369 serum. We found that none of the fragments reacted with group I MCAb, not even a 34K fragment tentatively identified as residues 227 to 369. This result indicated that another portion of epitope I was located upstream of residue 227. Alternatively, the 34K carboxy-terminal fragment could have contained all the amino acids of epitope I but might have lost the proper tertiary structure for reacting with group I MCAb. The other three discontinuous epitopes were located within two amino-terminal V8 polypeptides, 33K and 30K, tentatively identified as residues 1 to 226 and 1 to 182. The 30K fragment could have contained fewer amino acids, since there were glutamic acid residues just upstream of residue 182. Further experiments will be necessary to determine the precise compositions of these polypeptides.

In the present experiments, the major amino-terminal V8 fragment represents residues 1 to 226. Previously, the major amino-terminal V8 fragment was deduced to represent residues 1 to 259 (24). We speculate that the difference depends on the experimental conditions used to generate the fragments. In the original experiments (24), gD was first bound to group I MCAb and then treated with V8. In the present study, the purified native protein was directly exposed to the protease. We speculate that the bound antibody protected or masked residue 226 from V8 cleavage.

Epitope I. Our results indicate that a portion of epitope I is located downstream of residue 233. Our previous studies with V8 indicated that epitope I was located on a 38K fragment which lacked one N-asparagine-linked glycosylation site (13, 24; D. Long, G. H. Cohen, and R. J. Eisenberg, unpublished results). Based on the deduced amino acid sequence of gD-1 (38, 57) and tryptic peptide analysis (24), the 38K fragment most probably contains residues 1 to 259. Thus, we speculate that one portion of epitope I is located between amino acids 233 and 259.

Since group I MCAb show equivalent reactivity against gD-1 and gD-2, our working hypothesis is that the residues which comprise epitope I are identical in the two glycoproteins. In this regard, MCAb DL11 (group I) was developed with spleen cells of mice immunized with gD-2 (25), whereas other group I MCAb, such as HD-1 (49) and 4S (52), were prepared against HSV-1. There are three positions in which gD-1 and gD-2 differ in amino acid sequence within the region containing residues 233 and 259. Residue 233 is valine in gD-1 and leucine in gD-2; residue 246 is alanine in gD-1 and proline in gD-2; and residue 259 is glutamic acid in gD-1 and aspartic acid in gD-2 (38, 56, 57).

A second portion of epitope I appears to be located upstream of residue 227, perhaps upstream of residue 183. This speculation is based on several observations. (i) There is a lack of reactivity of group I MCAb with the 34K (residues 227 to 369) carboxy-terminal V8 fragment. (ii) Epitope I is destroyed by reduction and alkylation; all of the cysteine residues that might contribute to disulfide bonding are located between residues 66 and 202. (iii) Three group I MCAb-resistant mutants contain a nucleotide change in the gD gene which alters residue 216 from asparagine to serine (Minson, personal communication).

Since group I MCAb have been associated with important biological functions of gD, including fusion (46), neutralization (24, 30, 49, 52), and protection (19, 22), the amino acids which comprise epitope I are likely to represent biologically important portions of gD.

Epitope III. Previous experiments suggested that epitopes I and III are structurally distinct (25). We have confirmed this conclusion, since a portion of epitope I is between residues 233 and 259, but epitope III appears to be located upstream of residue 182. In contrast to group I MCAb, the binding of group III MCAb was always greater with gD-1 than with an equimolar amount of gD-2 (Fig. 2B and 6B). This difference suggests that epitope III is not identical in structure in gD-1 and gD-2. It is possible that the amino acids which comprise epitope III differ in the two glycoproteins; alternatively, the amino acids could be identical, but their tertiary structure might be different.

Epitopes IV and VI. These type 1-specific epitopes are also located within residues 1 to 182. Epitope VI appears to be associated with virus adsorption (30), based on the following observations. (i) Blocking studies indicated that MCAb DL2 (group VI) is in the same group as MCAb I-206-7 and II-886-1 (obtained from P. Spear) (Eisenberg et al., unpublished data). (ii) The latter antibodies inhibited the adsorption of HSV-1 to uninfected cells to the greatest extent of the anti-gD antibodies tested (30).

Disulfide bonding in gD. The identification of antigenically active fragments of gD enables us to speculate about disulfide bonding. The observation that group III, IV, and VI MCAb recognized residues 1 to 226 and 1 to 182 suggests that these polypeptides maintained their normal disulfide bond pattern. The only way that the polypeptide containing residues 1 to 182 could have been generated without disrupting disulfide bonds is if cysteine residues 189 and 202 were not bonded to any cysteine residues upstream of residue 182 (Fig. 4). If gD contains three disulfide bonds, cysteine residues 202 and 189 may be bonded to each other.

Future studies will focus on an analysis of disulfide bonding in gD. In addition, the techniques of in vitro mutagenesis will be applied to the study of discontinuous epitopes of gD.

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LITERATURE CITED

1. **Atassi, M. Z.** 1978. Precise determination of the entire antigenic structure of lysozyme. Molecular features of protein antigenic structures and potential of 'surface-simulation' synthesis—a powerful new concept for protein binding sites. Immunochemistry **15**:909–936.
2. **Benjamin, D. C., J. A. Berzofsky, I. J. East, F. R. N. Gurd, C. Hannum, S. J. Leach, E. Margoliash, J. G. Michael, A. Miller, E. M. Prager, M. Reichlin, E. E. Sercarz, S. J. Smith-Gill, P. E. Todd, and A. C. Wilson.** 1984. The antigenic structure of proteins: a reappraisal. Annu. Rev. Immunol. **2**:67–101.
3. **Berman, P. W., T. Gregory, D. Crase, and L. A. Lasky.** 1985. Protection from genital herpes simplex virus type 2 infection by vaccination with cloned type 1 glycoprotein D. Science **227**:1490–1492.
4. **Bers, G., and D. Garfin.** 1985. Protein and nucleic acid blotting and immunobiochemical detection. Biotechniques **3**:276–288.
5. **Braun, D. K., L. Pereira, B. Norrild, and B. Roizman.** 1983. Application of denatured, electrophoretically separated, and immobilized lysates of herpes simplex virus-infected cells for detection of monoclonal antibodies and for studies of the properties of viral proteins. J. Virol. **46**:103–112.
6. **Burnette, W. N.** 1981. "Western blotting." Electrophoretic transfer of proteins from SDS-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**:195–203.
7. **Bzik, D. J., B. A. Fox, N. A. DeLuca, and S. Person.** 1984. Nucleotide sequence of a region of the herpes simplex virus type I gB glycoprotein gene: mutations affecting rate of virus entry and cell fusion. Virology **137**:185–190.
8. **Chan, W.** 1983. Protective immunization of mice with specific HSV-1 glycoproteins. Immunology **49**:343–352.
9. **Cohen, G. H., B. Dietzschold, D. Long, M. Ponce de Leon, E. Golub, and R. J. Eisenberg.** 1985. Localization and synthesis of an antigenic determinant of herpes simplex virus glycoprotein D that stimulates neutralizing antibody and protects mice from lethal virus challenge, p. 24–30. *In* S. E. Mergenhagen and B. Rosan (ed.), Molecular basis of oral microbial adhesion. American Society for Microbiology, Washington, D.C.
10. **Cohen, G. H., B. Dietzschold, M. Ponce de Leon, D. Long, E. Golub, A. Varrichio, L. Pereira, and R. J. Eisenberg.** 1984. Localization and synthesis of an antigenic determinant of herpes simplex virus glycoprotein D that stimulates the production of neutralizing antibody. J. Virol. **49**:102–108.
11. **Cohen, G. H., M. N. Factor, and M. Ponce de Leon.** 1974. Inhibition of herpes simplex virus type 2 replication by thymidine. J. Virol. **14**:20–25.
12. **Cohen, G. H., M. Katze, C. Hydrean-Stern, and R. J. Eisenberg.** 1978. Type-common CP-1 antigen of herpes simplex virus is associated with a 59,000-molecular-weight envelope glycoprotein. J. Virol. **47**:172–181.
13. **Cohen, G. H., D. Long, J. T. Matthews, M. May, and R. J. Eisenberg.** 1983. Glycopeptides of the type-common glycoprotein gD of herpes simplex virus types 1 and 2. J. Virol. **46**:679–689.
14. **Cohen, G. H., M. Ponce de Leon, and C. Nichols.** 1972. Isolation of a herpes simplex virus-specific antigenic fraction which stimulates the production of neutralizing antibody. J. Virol. **10**:1021–1030.
15. **Colman, P. M., J. N. Varghese, and W. G. Laver.** 1983. Structure of the catalytic and antigenic sites in influenza virus neuraminidase. Nature (London) **303**:41–44.
16. **Cremer, K. J., M. Macket, C. Wohlenberg, A. L. Notkins, and B. Moss.** 1985. Vaccinia virus recombinant expressing herpes simplex virus type 1 glycoprotein D prevents latent herpes in mice. Science **228**:737–740.
17. **Daniel, T. O., W. J. Schneider, J. L. Goldstein, and M. S. Brown.** 1983. Visualization of lipoprotein receptors by ligand binding. J. Biol. Chem. **258**:4606–4611.
18. **Dietzschold, B., R. J. Eisenberg, M. Ponce de Leon, E. Golub, F. Hudecz, A. Varrichio, and G. H. Cohen.** 1984. Fine structure analysis of type-specific and type-common antigenic sites of herpes simplex virus glycoprotein D. J. Virol. **52**:431–435.
19. **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.
20. **Drapeau, G. R., Y. Boily, and J. Houmard.** 1972. Purification and properties of an extracellular protease of *Staphylococcus aureus*. J. Biol. Chem. **247**:6720–6726.
21. **Eisenberg, R. J., C. P. Cerini, C. J. Heilman, A. D. Joseph, B. Dietzschold, E. Golub, D. Long, M. Ponce de Leon, and G. H. Cohen.** 1985. Synthetic glycoprotein D-related peptides protect mice against herpes simplex virus challenge. J. Virol. **56**:1014–1017.
22. **Eisenberg, R. J., and G. H. Cohen.** 1986. Identification and analysis of biologically active sites of herpes simplex virus glycoprotein D, p. 74–84. *In* R. L. Crowell and K. Lonberg-Holm (ed.), Virus attachment and entry into cells. American Society for Microbiology, Washington, D.C.
23. **Eisenberg, R. J., C. Hydrean-Stern, and G. H. Cohen.** 1979. Structural analysis of precursor and product forms of type-common envelope glycoprotein D (CP-1 antigen) of herpes simplex virus type 1. J. Virol. **31**:608–620.
24. **Eisenberg, R. J., D. Long, L. Pereira, B. Hampar, M. Zweig, and G. H. Cohen.** 1982. Effect of monoclonal antibodies on limited proteolysis of native glycoprotein gD of herpes simplex virus type 1. J. Virol. **41**:478–488.
25. **Eisenberg, R. J., D. Long, M. Ponce de Leon. J. T. Matthews, P. G. Spear, M. G. Gibson, L. A. Lasky, P. Perman, E. Golub, and G. H. Cohen.** 1985. Localization of epitopes of herpes simplex virus type 1 glycoprotein D. J. Virol. **53**:634–644.
26. **Eisenberg, R. J., M. Ponce de Leon, and G. H. Cohen.** 1980. Comparative structural analysis of glycoprotein gD of herpes simplex virus types 1 and 2. J. Virol. **35**:428–435.
27. **Eisenberg, R. J., M. Ponce de Leon, L. Pereira, D. Long, and G. H. Cohen.** 1982. Purification of glycoprotein gD of herpes simplex virus type 1 and 2 by use of monoclonal antibody. J. Virol. **41**:1099–1104.
28. **Elder J. H., and S. Alexander.** 1982. Endo-β-N-acetylglucosaminidase F: endoglycosidase from *Flavobacterium meningosepticum* that cleaves both high-mannose and complex glycoproteins. Proc. Natl. Acad. Sci. USA **79**:4540–4544.
29. **Friedman, H. M., G. H. Cohen, R. J. Eisenberg, C. A. Seidel, and D. B. Cines.** 1984. Glycoprotein C of HSV-1 functions as a C3b receptor on infected endothelial cells. Nature (London) **309**:633–635.
30. **Fuller, O., and P. G. Spear.** 1985. Specificities of monoclonal and polyclonal antibodies that inhibit adsorption of herpes simplex virus to cells and lack of inhibition by potent neutralizing antibodies. J. Virol. **55**:475–482.
31. **Gershoni, J. M., and G. E. Palade.** 1983. Protein blotting: principles and applications. Anal. Biochem. **131**:1–15.
32. **Gibson, M. G., and P. G. Spear.** 1983. Insertion mutants of herpes simlex virus have a duplication of the glycoprotein D gene and express two different forms of glycoprotein D. J. Virol. **48**:396–404.
33. **Herbrink, P., F. J. van Bussel, and S. O. Warnaar.** 1982. The antigen spot test (AST): a highly sensitive assay for the detection of antibodies. J. Immunol. Methods **48**:293–298.
34. **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.
35. **Kennett, R. H.** 1980. Cloning of hybridomas. Cloning in semi-solid agarose, p. 372–373. *In* R. H. Kennett, T. J. McKearn, and K. B. Bechtol (ed.), Monoclonal antibodies. Hybridomas: a new dimension in biological analysis. Plenum Publishing Corp., New York.
36. **Kousoulas, K. G., P. E. Pellett, L. Pereira, and B. Roizman.** 1984. Mutations affecting conformation or sequence of neutralizing epitopes identified by reactivity of viable plaques segregated from *syn* and *ts* domains of HSV-1(F) gB gene. Virology

135:379–394.

37. **Kumel, G., H. C. Kaerner, M. Levine, C. H. Schroder, and J. Glorioso.** 1985. Passive immune protection by herpes simplex virus-specific monoclonal antibodies and monoclonal antibody-resistant mutants altered in pathogenicity. J. Virol. **56**:930–937.
38. **Lasky, L. A., and D. Dowbenko.** 1984. DNA sequence analysis of the type-common glycoprotein D genes of herpes simplex virus types 1 and 2. DNA **3**:23–29.
39. **Lasky, L. A., D. Dowbenko, C. C. Simonsen, and P. W. Berman.** 1984. Protection of mice from lethal herpes simplex virus infection by vaccination with a secreted form of cloned glycoprotein D. Biotechnology **2**:527–532.
40. **Lee, G. T.-Y., M. F. Para, and P. G. Spear.** 1982. Location of the structural genes for glycoprotein gD and for other polypeptides in the S component of herpes simplex virus type 1 DNA. J. Virol. **43**:41–49.
41. **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.
42. **Marlin, S. D., T. C. Holland, M. Levine, and J. Glorioso.** 1985. Epitopes of herpes simplex virus type 1 glycoprotein gC are clustered in two distinct antigenic sites. J. Virol. **53**:128–136.
43. **Matthews, J. T., G. H. Cohen, and R. J. Eisenberg.** 1983. Synthesis and processing of glycoprotein D of herpes simplex virus types 1 and 2 in an in vitro system. J. Virol. **48**:521–533.
44. **McKearn, T. J.** 1980. Fusion of cells in an adherent monolayer, p. 368–369. *In* R. H. Kennett, T. J. McKearn, and K. B. Bechtol (ed.), Monoclonal antibodies. Hybridomas: a new dimension in biological analysis. Plenum Publishing Corp., New York.
45. **Montgomery, P. C., K. J. Dorrington, and J. H. Rockey.** 1969. Equine anti-hapten antibody. The molecular weights of the subunits of equine immunoglobulins. Biochemistry **8**:1247–1258.
46. **Noble, A. G., G. T.-Y. Lee, R. Sprague, M. L. Parish, and P. G. Spear.** 1983. Anti-gD monoclonal antibodies inhibit cell fusion induced by herpes simplex virus type 1. Virology **129**:218–224.
47. **Paoletti, E., B. R. Lipinskas, C. Samsonoff, S. Mercer, and D. Panicali.** 1984. Construction of live vaccines using genetically engineered poxviruses: biological activity of vaccinia virus recombinants expressing the hepatitis B virus surface antigen and the herpes simplex virus glycoprotein D. Proc. Natl. Acad. Sci. USA **81**:193–197.
48. **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.
49. **Pereira, L., T. Klassen, and J. R. Baringer.** 1980. Type-common and type-specific monoclonal antibody to herpes simplex virus type 1. Infect. Immun. **29**:724–732.
50. **Rawls, W. E., N. Balachandran, G. Sisson, and R. J. Watson.** 1984. Localization of a type-specific antigenic site on herpes simplex virus type 2 glycoprotein D. J. Virol. **51**:263–265.
51. **Rector, J. T., R. N. Lausch, and J. E. Oakes.** 1984. Identification of infected cell-specific monoclonal antibodies and their role in host resistance to ocular herpes simplex virus type 1 infection. J. Gen. Virol. **65**:657–661.
52. **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.
53. **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.
54. **Spear, P. G.** 1985. Antigenic structure of herpes simplex viruses, p. 425–446. *In* M. H. V. van Regenmortel and A. R. Neurath (ed.), Immunochemistry of viruses. The basis for serodiagnosis and vaccines. Elsevier Science Publishers B.V., Amsterdam.
55. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
56. **Watson, R. J.** 1983. DNA sequence of the herpes simplex virus type 2 glycoprotein D gene. Gene **26**:307–312.
57. **R. J. Watson, J. H. Weis, J. S. Salstrom, and L. W. Enquist.** 1982. Herpes simplex virus type I glycoprotein D gene: nucleotide sequence and expression in *Escherichia coli*. Science **218**:381–383.
58. **Watson, R. J., J. H. Weis, J. S. Salstrom, and L. W. Enquist.** 1985. Bacterial synthesis of herpes simplex virus type 1 and 2 glycoprotein D antigens. J. Invest. Dermatol. **83**(Suppl.): 102S–111S.
59. **Wiley, D. C., I. A. Wilson, and J. J. Skehel.** 1981. Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. Nature (London) **289**:373–378.