Localization of Epitopes of Herpes Simplex Virus Type 1 Glycoprotein D

ROSELYN J. EISENBERG,^{1,2*} DEBORAH LONG,^{2,3} MANUEL PONCE DE LEON,^{2,3} JAMES T. MATTHEWS,^{1,2,3†} PATRICIA G. SPEAR,⁴ MARYLOU G. GIBSON,⁴ LAURENCE A. LASKY,⁵ PHILLIP BERMAN,⁵ ELLIS GOLUB,^{2,6} AND GARY H. COHEN^{2,3}

Department of Pathobiology, School of Veterinary Medicine,¹ and Department of Microbiology,³ Department of Biochemistry,⁶ and Center for Oral Health Research,² School of Dental Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104; Department of Microbiology, The University of Chicago, Chicago, Illinois 60637⁴; and Department of Vaccine Development, Genentech, Inc., South San Francisco, California 94080⁵

Received 20 August 1984/Accepted 23 October 1984

We previously defined eight groups of monoclonal antibodies which react with distinct epitopes of herpes simplex virus glycoprotein D (gD). One of these, group VII antibody, was shown to react with a type-common continuous epitope within residues 11 to 19 of the mature glycoprotein (residues 36 to 44 of the predicted sequence of gD). In the current investigation, we have localized the sites of binding of two additional antibody groups which recognize continuous epitopes of gD. The use of truncated forms of gD as well as computer predictions of secondary structure and hydrophilicity were instrumental in locating these epitopes and choosing synthetic peptides to mimic their reactivity. Group II antibodies, which are type common, react with an epitope within residues 268 to 287 of the mature glycoprotein (residues 293 to 312 of the predicted sequence). Group V antibodies, which are gD-1 specific, react with an epitope within residues 340 to 356 of the mature protein (residues 365 to 381 of the predicted sequence). Four additional groups of monoclonal antibodies appear to react with discontinuous epitopes of gD-1, since the reactivity of these antibodies was lost when the glycoprotein was denatured by reduction and alkylation. Truncated forms of gD were used to localize these four epitopes to the first 260 amino acids of the mature protein. Competition experiments were used to assess the relative positions of binding of various pairs of monoclonal antibodies. In several cases, when one antibody was bound, there was no interference with the binding of an antibody from another group, indicating that the epitopes were distinct. However, in other cases, there was competition, indicating that these epitopes might share some common amino acids.

Glycoprotein D (gD) of herpes simplex virus (HSV) is a structural component of the virion envelope which stimulates production of high titers of virus-neutralizing activity (7, 9, 11, 15-17, 34) and is likely to play an important role in the initial stages of viral infection. It was recently shown that anti-gD antibodies can block the fusion of infected cells (39). In addition, mice immunized with gD are protected from a lethal HSV challenge (4, 30, 34, 40). Tryptic peptide analysis (2, 16) and amino-terminal sequencing (14) showed that gD of HSV type 2 (HSV-2) (gD-2) is structurally similar though not identical to gD of HSV-1 (gD-1). Recently, the genes for gD-1 and gD-2 were localized and sequenced (29, 32, 50, 51). Although the deduced amino acid sequences for the two glycoproteins were shown to be 85% homologous, little is known about the secondary or tertiary structure of these two proteins. Using monoclonal antibodies (MCAB), we previously defined eight epitopes within gD (15), some of which are type common and others of which are type specific. Our goal is to relate the structure of the protein to its biological functions. The high degree of amino acid sequence homology between gD-1 and gD-2 probably accounts for the immunological cross-reactivity of polyclonal antibodies and MCAB directed against gD (15, 41). On the other hand, the type specificity of other MCAB is undoubtedly related to differences in the structures of gD-1 and gD-2 and, consequently, in amino acid sequence.

Recently (7, 12), using synthetic peptides, the type common group VII epitope was localized to residues 11 to 19 of the mature form of gD (residues 36 to 44 of the predicted sequence [29, 50, 51]). Polyclonal sera to certain of the synthetic peptides in the region of the first 23 amino acids of gD-1 and gD-2 also exhibited type common virus-neutralizing activity (7), and immunized mice were protected from a lethal virus challenge (R. J. Eisenberg, G. H. Cohen, and B. Dietzschold, unpublished data). During these studies, we localized two additional epitopes within the first 23 amino acids of gD-2 (7, 12). The type 2 specificity depended on two amino acid differences between gD-1 and gD-2 (29, 50, 51).

The purpose of the current investigation was to continue to delineate the location and characteristics of the antigenic epitopes of gD. Three different sets of terms have been used to distinguish epitopes which are lost under denaturing conditions, such as reduction and alkylation, from those which are retained: (i) conformational versus sequential (47); (ii) discontinuous versus continuous (1); and (iii) assembled topographic versus segmental (3). We have chosen to use discontinuous and continuous as operational definitions, without any implication that this terminology is preferred over any other. We have localized the site of binding of two additional MCAB groups. As with group VII, these also recognize continuous epitopes. Groups II and V (15) bind specifically to residues 268 to 287 of gD-1 and gD-2 and residues 340 to 356 of gD-1. In addition, we carried out competition studies to map the relative positions of four discontinuous epitopes, corresponding to MCAB in groups I, III, IV, and VI (15).

* Corresponding author.

† Present address: Department of Pathology, Harvard University School of Medicine, Boston, MA 02115.

MATERIALS AND METHODS

Cells, virus, and radioactive labeling procedures. Conditions for the growth and maintenance of BHK and KB cells and for the propagation of virus have been described (8, 11). For infection, an input multiplicity of 20 PFU of HSV-1 (HF) and 10 PFU of HSV-2 (Savage) per cell was used. Procedures for labeling of HSV-infected cells with [³⁵S]methionine (specific activity, 600 Ci/mmol) (Amersham Corp.) and [2,3-³H]arginine (specific activity, 15 Ci/mmol) (Amersham) have been described previously (9, 13, 15, 16).

Preparation of polyclonal antibody and MCAb to gD. Anti-gD-1 and anti-gD-2 sera were prepared in rabbits against immunosorbant-purified preparations of gD-1 and gD-2 (17). MCAb HD-1 (group I) and MCAb 170 (group VII) were supplied by L. Pereira (15, 41). MCAb 55S and 57S (group V), 11S (group III), 41S (group IV), and 45S (group VI) were supplied by M. Zweig (48). MCAb DL6 (group II) was prepared from mice immunized intraperitoneally (34) with 6 µg of immunosorbant-purified gD-1 (17). Three days after an intravenous boost of 1 µg, the spleen was removed and the cells were fused to SP2/0 cells by the procedure of McKearn (28). Hybridomas were cloned in soft agarose (25), and ascites fluids were prepared from pristane-primed mice immunized intraperitoneally with cloned cells.

To prepare immunosorbants, immunoglobulin G (IgG) was purified from ascites fluids of MCAb from each group (15) and linked to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Inc.). The amounts coupled ranged from 5 to 12 mg of IgG per g of Sepharose. Purified immunoglobulins (50 µg) were iodinated with ¹²⁵I (Amersham) by either the chloramine-T (19) or the lactoperoxidase (36) method. For certain MCAb, the chloramine-T method inactivated the binding activity of the antibody. Group IV MCAb were inactivated by both procedures.

Synthetic peptides. Synthetic peptides to residues 1 to 23 of gD-1 (1-23[1]) and gD-2 (1-23[2]) were prepared as described previously (7). The peptides representing residues 340 to 356 of gD-1 (340-356[1]) and 268 to 287 of gD-1 (268-287[1]) were prepared by Peninsula Laboratories, Inc. Cysteine was added to the amino terminus of 340-356[1] and to the carboxy terminus of 268-287[1]. The procedures for coupling of peptides to keyhole limpet hemocyanin (KLH) were described previously (7, 33). Briefly, the maleimide group of the peptide was incorporated into KLH with M-malimido-benzol-*N*-hydroxysuccinimide ester and the M-malimidobenzol-*N*-hydroxysuccinimide ester-modified proteins were allowed to react with a 20 M excess of the peptide (22). The coupling ratio of peptide/carrier was previously determined to be 8 (7). All peptides were dissolved in 0.1 M Tris (pH 7.8)–0.15 M NaCl for assay by the immunoblot method (see below).

Preparation of antisera to the synthetic peptides. A female New Zealand White rabbit was immunized with peptide 340-356[1] coupled to KLH at three weekly intervals with a total of 2.4 mg of coupled peptide. The animal was given an intravenous booster dose of 120 µg of the coupled peptide 3 to 4 days before each bleeding. A total of five bleeds were obtained. Two female New Zealand White rabbits were immunized with peptide 268-287[1] coupled to KLH at three weekly intervals with a total of 1.4 mg of coupled peptide per rabbit. The animals were boosted intravenously once with 70 µg of coupled peptide and then three times with 500 µg of free peptide.

Preparation of native and denatured gD. gD-1 and gD-2 were each purified from cytoplasmic extracts of infected

cells by affinity chromatography, using a previously described procedure (17). For our purposes, the proteins eluted from the immunosorbant column with KSCN and dialyzed against 0.01 M Tris (pH 7.5)–0.15 M NaCl–0.1% Nonidet P-40 (TSN buffer) are designated as "native." For denaturation, purified gD-1 or gD-2 was suspended in disrupting buffer to yield a final concentration of 3% sodium dodecyl sulfate (SDS)–100 mM Tris (pH 7.0)–10% 2-mercaptoethanol–0.5% glycerol. The sample was boiled for 5 min. Iodoacetamide (0.1 M in 0.1 M Tris, pH 8.0) was added to give a final concentration of 33 mM iodoacetamide, and the mixture was incubated for 1 h at room temperature. The samples were dialyzed extensively against TSN buffer.

Preparation of truncated forms of gD. (i) **The 38K fragment of gD-1.** Preparation of the 38K fragment was by a modification of a previously described procedure (15). Briefly, a cytoplasmic extract (100 µl) of HSV-1-infected cells labeled with [³H]arginine was added to 50 µl of HD-1–IgG–Sepharose (125 µg of IgG). The immunosorbant was washed extensively with 0.01 M Tris (pH 7.5)–0.1% Nonidet P-40–0.5 M NaCl–0.1 mM phenylmethylsulfonyl fluoride (washing buffer) and then with V8 enzyme buffer (50 mM Tris, pH 8.0) and incubated with 50 µg of *Staphylococcus aureus* protease V8 in enzyme buffer at 37°C for 2 h. The immunosorbant was washed extensively with washing buffer. To test the preparation for the presence of uncleaved gD, a portion of the immunosorbant was suspended in SDS-disrupting buffer, boiled for 3 min, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). V8 proteolysis resulted in a 38K fragment (15) and no full-length gD. The 38K fragment linked to HD-1–IgG–Sepharose was then used to test the binding of other MCAb as described in Results.

(ii) **Truncated gD-1, residues 1 to 275.** The gene for gD-1 was cloned into a pBR322-simian virus 40 shuttle vector and included a DNA fragment from a *Hind*III site upstream of the gD gene to a *Hinf*I site at residue 300 of the predicted gD-1 sequence (residue 275 of the mature protein). When this plasmid is grown in Chinese hamster ovary cells, the glycoprotein is secreted (30). The glycoprotein was purified by affinity chromatography, using a gD-specific MCAb (17).

(iii) **Truncated gD-1, residues 1 to 287.** Truncated gD, residues 1 to 287, was produced and secreted by the HSV insertion mutant designated 111 (M. G. Gibson and P. G. Spear, J. Cell Biochem. Suppl. 8B, in press; Gibson and Spear, 13th Ann. UCLA Symp. 1984, abstr. no. 1337, p. 191). The virus was constructed by methods previously described (18) except that a truncated form of the gD-1 gene (extending from the *Sac*I site upstream of the gD gene to the *Nar*I site at residue 312 of the predicted sequence or residue 287 of the mature protein) was inserted into the *Bgl*II site of the thymidine kinase gene. The expressed protein contains 48 amino acids at its carboxy terminus that are translated from the noncoding strand of HSV-1 DNA at the 5' end of the thymidine kinase gene. The protein was affinity purified from the medium of HEP-2 cells infected with 111 virus by a previously described method (17), using a gD-1-specific MCAb designated II-436-1 (39).

Immunoprecipitation and SDS-PAGE. gD was immunoprecipitated from HSV-1- or HSV-2-infected cell extracts (cells infected for 6 h) prepared as previously described (26, 42), using antisera or MCAb and *S. aureus* protein A (IgG Sorb; New England Enzyme Center). SDS-PAGE was carried out in slabs of 10% acrylamide cross-linked with 0.4% *N,N'*-diallyltartardiamide (13, 49). For autoradiography, gels were dried on filter paper and placed in contact with Kodak XAR-5 film. For fluorography, the gels were treated with

Amplify (Amersham), dried on filter paper, and exposed to Kodak XAR-5 film at -70°C .

Immunoblot and neutralization assays. The immunoblot assay was done as previously described (7, 20), using antisera or MCAB and iodinated protein A (Amersham). Virus neutralization assays (50% plaque reduction method), using HSV-1 (HF) or HSV-2 (Savage), were carried out as previously described (9, 11).

Competition assays. Two competition assays were used.

(i) **Immunosorbants.** A total of 100 to 200 μl (representing 150 to 600 μg of IgG) of MCAB linked to Sepharose was incubated for 2 h at 37°C with 100 μl of a cytoplasmic extract of unlabeled HSV-1-infected cells (9, 13, 15, 16). The immunosorbant was washed 10 times with washing buffer, and the iodinated second antibody (at least 250,000 cpm) was added. The complex was washed exhaustively and counted in a gamma counter.

(ii) **Nitrocellulose.** Purified gD-1 (0.45 to 15 ng) was spotted onto nitrocellulose strips; the strips were washed as previously described (7) and incubated with unlabeled first antibody and then with iodinated second antibody (ca. 250,000 cpm). The spots were located by autoradiography, cut out, and counted in a gamma counter. The counts were subjected to linear regression analysis, and the slope of the line (counts per minute versus concentration of gD) was calculated.

RESULTS

Comparison of the predicted secondary structures of gD-1 and gD-2. Figure 1 shows a computer representation (7) of the secondary structure of gD-1 (Fig. 1A) and gD-2 (Fig. 1B), derived from the predicted amino acid sequences (29, 50, 51) and rules established by Chou and Fasman (5, 6). It should be noted that these predictive "rules" of secondary structure result in an accuracy of prediction in a three-state model (helix-sheet-turn) of approximately 50 versus 33% for chance (24). However, in the absence of any additional structural information, we have found that these predictions have heuristic value in that they focus attention on certain regions of the glycoprotein. Furthermore, we have also analyzed both glycoproteins with a second empirical analysis (Fig. 1) which assumes that hydrophilic regions of protein structure have a greater immunological potential (22). For these calculations, the first 25 amino acids of the predicted sequence were excluded from consideration, since direct N-terminal sequence analysis showed that, for both gD-1 and gD-2, lysine residue 26 of the deduced sequence was the amino terminus of the mature protein (14). In our numbering system, this lysine is residue 1. The criteria used for predicting the probability of β -turns (7) were modified to increase the likelihood of locating possible epitopes. The modified-turn criteria predict four additional β -turns in gD-1 at residues 200, 225, 255, and 298. None of these additional turns involves highly hydrophilic regions of the protein. Interestingly, a very hydrophilic region, residues 77 to 95, is not in a predicted β -turn. When the criteria were relaxed even further, this stretch of amino acids was still not predicted to be in a β -turn. The working hypothesis is that epitopes are likely to be located in regions where highly hydrophilic residues are present in β -turns (45). If, in addition, the epitope is continuous, synthetic peptides could be used to mimic the reactivity of the epitope. The program has also been expanded to indicate the positions of predicted *N*-asparagine-linked carbohydrates (shown as balloons) based on the sequence Asn-X-Thr or Asn-X-Ser (23). For gD-1 and gD-2, all three positions are glycosylated (10). Predictions for hydrophilicity use the same criteria as before (7, 22). The

homology in amino acid sequence is reflected in similarities in both secondary structure and regions of hydrophilicity in the two proteins. In at least one case, however, two differences in amino acid sequence in region 1 to 23 have been correlated with both changes in predicted secondary structure and antigenicity (7, 12, 29, 50, 51). For both gD-1 and gD-2, there are two regions in which β -turns intersect a highly hydrophilic region, i.e., residues 11 to 19 and 265 to 282. A third region in gD-1, residues 340 to 356, is hydrophilic and contains a predicted β -turn overlapping the hydrophilic region. These β -turns are present even when more stringent criteria for predicting turns are applied (7). In gD-2, however, there is no β -turn in this region, even with the modified criteria.

Reaction of MCAB in groups I to VII against native and denatured gD. To test the structural predictions, we used the immunoblot assay (7, 20) to determine which of the groups of MCAB reacted with discontinuous or continuous epitopes. Previous studies indicated that only certain MCAB groups reacted with denatured gD (32, 37; J. T. Matthews, G. H. Cohen, and R. J. Eisenberg, unpublished data). Native gD-1 (Fig. 2) reacted with polyclonal anti-gD serum and with MCAB in groups I to VII (rows 1 to 8, lane a). Native gD-2 (lane b) reacted with polyclonal anti-gD (row 1, lane b) and also reacted with MCAB in groups I, II, III, V, and VII (rows 2, 3, 4, 6, and 8, lane b). The reaction of native gD-2 with group V was unexpected, since group V failed to immunoprecipitate gD-2 from infected cell extracts (15, 48). Furthermore, denatured gD-2 reacted either weakly or not at all against group V MCAB, whereas native and denatured gD-1 reacted equally well against the same antibodies. In addition to group V, MCAB in groups II and VII recognized the denatured form of gD-1 and gD-2 (lanes c and d). Thus, three groups of MCAB, II, V, and VII, appeared to recognize continuous epitopes, and four groups of MCAB, I, III, IV, and VI, apparently reacted with discontinuous epitopes that require the native conformation of gD-1 (34).

It should be noted that under the denaturing conditions used considerable secondary and tertiary structure might remain in gD. Therefore, that the protein retained antigenic activity for antibodies in groups II, V, and VII is not proof per se that these epitopes are continuous. In the case of group VII, the proof was provided by the reactivity of a synthetic peptide mimicking residues 8 to 23 of gD-1 against group VII MCAB 170 (7). One of the goals of the present study was to obtain similar proof for the epitopes specified by MCAB in groups II and V.

Prediction of the location of the group V epitope. Several lines of evidence enabled us to localize the group V epitope. Previously, using V8 proteolysis, we found that group V MCAB reacted with a 15K fragment of gD-1 (15). Tryptic peptide analysis showed that this fragment represented the carboxy terminus of the protein (15; D. Long, G. H. Cohen, and R. J. Eisenberg, unpublished data). Further evidence indicated that the epitope was located downstream from the membrane-anchoring region (i.e., presumably after residue 339 [51]). First, group V MCAB reacted with fixed, but not with unfixed, HSV-1-infected cells (37). This suggested that the epitope is not exposed on the external face of the plasma membrane of infected cells. Second, when gD-1 was synthesized and processed in an *in vitro* system, the processed protein was partially protected from proteolysis by trypsin (37). Approximately 3,000 daltons of the protein was removed by this treatment, and the trypsin-resistant fragment could not be immunoprecipitated by group V MCAB. Furthermore, when truncated forms of the gD gene, lacking the

FIG. 1. Predicted secondary structure and hydrophilicity maps of gD-1 (A) and gD-2 (B). Secondary structures were predicted by a computer program, using the rules of Chou and Fasman for determining Pt, Pa, and Pb (5, 6). Probabilities for the occurrence of β -turns were evaluated by using modified conditions: $P_t > 7.5 \times 10^{-5}$ or $P_t > 5 \times 10^{-5}$; $P_t > P_a$ and $P_t > P_b$. Shaded circles indicate hydrophobic regions; open circles indicate hydrophilic areas. The radius of a circle over a residue is proportional to the mean hydrophilicity as calculated for that residue plus the next five residues according to the method of Hopp and Woods (22). The value is therefore distorted at the C-terminal end. The hexagonal balloons indicate predicted sites (Asn-X-Thr or Ser) of *N*-asparagine-linked glycosylation (23).

information for the transmembrane-anchoring region plus the carboxy terminus, were cloned into *Escherichia coli*, the expressed gD-like protein was not recognized by 57S antibody (R. J. Watson, J. H. Weis, J. H. Salstrom, and L. W. Enquist, J. Invest. Dermatol., in press). These results, taken together, suggested that the group V epitope was located between residues 340 and 369 of gD-1.

To localize the epitope further, we relied on the computer predictions (Fig. 1 and 3) and differences in the sequences of gD-1 and gD-2 at the carboxy terminus (Fig. 3) (29, 50, 51). We argued that the epitope is largely type 1 specific, based on the immunoprecipitation data (15), but that certain simi-

larities in sequence might account for the reactivity of gD-2 against group V MCAb in the immunoblot. Region 340 to 356 of gD-1 is highly hydrophilic and contains a predicted β -turn at residues 346 to 349 (Fig. 1 and 3). The homologous region of gD-2 is also hydrophilic, but does not contain this predicted turn. In addition, the sequences of gD-1 and gD-2 in the region 340 to 356 (Fig. 3) show similarities (e.g., residues 346 to 349 are Ala-Pro-Lys-Arg and residues 351 to 356 are Arg-Leu-Pro-His-Ileu-Arg in both proteins) and differences (e.g., residues 343 to 345 are Thr-Arg-Lys in gD-1 but are Ala-Gln-Met in gD-2). These differences appear to have a profound effect on the predicted secondary struc-

FIG. 2. Immunoblot analysis of monoclonal antibodies directed at HSV gD. The antibodies used (grouped as described in reference 15) were as follows: row 1, anti-gD-1 (rabbit 1); row 2, group I, HD-1; row 3, group II, DL6; row 4, group III, 11S; row 5, group IV, 41S; row 6, group V, 57S; row 7, group VI, 45S; row 8, group VII, 170. Antigens: lane a, immunosorbant-purified (17) (native) gD-1 (15 ng); lane b, native gD-2 (15 ng); lane c, denatured gD-1 (15 ng); lane d, denatured gD-2 (15 ng); lane e, truncated gD-1, residues 1 to 275 (30) (60 ng).

ture of the downstream residues of the two proteins. Based on this information, residues 340 to 356 of gD-1 appeared the most likely to contain the group V epitope.

Recognition of the synthetic peptide 340-356[1] by group V MCAb and by polyclonal anti-gD sera. Group V MCAb (Fig. 4, row 7) reacted with purified native gD-1 (lane a), gD-2 (lane b), and the synthetic peptide corresponding to residues 340 to 356 of gD-1 (lane e). This antibody did not react with synthetic peptides corresponding to other portions of gD-1 (lanes c and d). Two polyclonal sera prepared against gD-1 (rows 1 and 2) reacted with this peptide. In one case (row 1) the reaction was strong, and in the other case the reaction was weak (row 2). In addition, the peptide reacted weakly with one polyclonal serum prepared against purified gD-2 (row 3), but failed to react with a second anti-gD-2 serum (row 4). These results show that (i) the synthetic peptide

340-356[1] contains the group V epitope; (ii) this epitope appears to be immunogenic in the native protein since several polyclonal sera prepared against gD, including one against gD-2, reacted with the peptide; and (iii) there is limited antigenic cross-reactivity with an analogous region of gD-2.

Anti-340-356[1] serum reacted in the immunoblot assay (Fig. 5A) against native gD-1 (lane a), gD-2 (lane b), and the synthetic peptide 340-356[1] (lane h). The specificity of this serum for the carboxy terminus of gD is demonstrated by the lack of reactivity against truncated gD-1, residues 1 to 275 (lane e), and the synthetic peptides 1-23[1] and 268-287[1] (lanes f and g, respectively). The reactions against native gD-1 and gD-2 were stronger than those against the dena-

FIG. 3. Comparison of amino acid sequence and predicted secondary structures of carboxy-terminal sequences of gD-1 (residues 338 to 369) and gD-2 (residues 338 to 368). Probabilities for the occurrence of β -turns were evaluated as in the legend to Fig. 1. The single-letter code designations are as follows: A, alanine; R, arginine; D, aspartic; N, asparagine; C, cysteine; E, glutamic acid; Q, glutamine; G, glycine; H, histidine; I, isoleucine; L, leucine; K, lysine; M, methionine; F, phenylalanine; P, proline; S, serine; T, threonine; W, tryptophan; Y, tyrosine; V, valine.

FIG. 4. Immunoblot analysis of synthetic peptides which mimic portions of gD-1, using polyclonal antibodies and MCAB. The antibodies used were as follows: row 1, anti-gD-1 (rabbit 1); row 2, anti-gD-1 (rabbit 2); row 3, anti-gD-2 (rabbit 3); row 4, anti-gD-2 (rabbit 4); row 5, group VII, 170; row 6, group II, DL6; row 7, group V, 57S. Antigens: lane a, native gD-1 (15 ng); lane b, native gD-2 (15 ng); lane c, 8-23[1] (500 ng); lane d, 268-287[1] (1 μ g); lane e, 340-356[1] (100 ng).

tured forms of these proteins (lanes c and d). Anti-340-356[1] serum immunoprecipitated both gD-1 (Fig. 5B, lane c) and gD-2 (lane d) from infected cell extracts. Lanes a and b (Fig. 5B) represent negative controls in which the HSV-1 and HSV-2 extracts were tested with serum obtained from the animal before immunization with 340-356[1]. As positive controls, the same extracts were immunoprecipitated with anti-gD-1 serum (lanes e and f). It is clear that anti-340-356[1] was more reactive against gD-1 than gD-2. As with group V MCAB, the anti-340-356[1] serum failed to show any neutralizing activity against HSV-1 or HSV-2 (data not shown).

Localization of the group II epitope. Group II, represented by DL6 MCAB, reacted with the native and denatured forms of both gD-1 and gD-2 (Fig. 2, row 3), exhibited type-common membrane immunofluorescence (data not shown), and neutralized HSV-1 at 1:50 dilution and HSV-2 at 1:20, using a 50% endpoint (9, 11). The continuous epitope recognized by DL6 MCAB was distinct from those recognized by either group VII or V, since DL6 did not react with either the 8-23[1] or the 340-356[1] synthetic peptide (Fig. 4, row 6, lanes c and e). Preliminary localization of the group II epitope was accomplished by testing the reactivity of two truncated forms of gD-1, representing residues 1 to 275 and

FIG. 5. Analysis of anti-340-356[1] serum by immunoblot (A) and SDS-PAGE (B). For immunoblot analysis, the antigens were: a, native gD-1; b, native gD-2; c, denatured gD-1; d, denatured gD-2; e, truncated gD-1, residues 1 to 275; f, 8-23[1]; g, 268-287[1]; h, 340-356[1]. The concentrations of these antigens were the same as in Fig. 2. For SDS-PAGE analysis (B), extracts from HSV-1-infected cells are in lanes a, c, and e and extracts from HSV-2-infected cells are in lanes b, d, and f. The sera used were: lanes a and b, preimmunization bleed from rabbit immunized with 340-356[1]; lanes c and d, anti-340-356[1]; lanes e and f, anti-gD-1 (17).

1 to 287, against MCAB (Fig. 6). Both forms reacted with group VII antibody (row 1, lanes c to e), indicating the presence of residues 8-23 in the truncated proteins. As expected, neither form reacted with group V antibody (row 3, lanes c to e). The truncated form, 1-275[1], also failed to

FIG. 6. Immunoblot analysis of truncated forms of gD-1, using MCAB. The antibodies used were as follows: row 1, group VII, 170; row 2, group II, DL6; row 3, group V, 57S. Antigens: lane a, native gD-1 (15 ng); lane b, native gD-2 (15 ng); lane c, truncated gD-1, residues 1 to 275 (30, 60 ng); lane d, truncated gD-1, residues 1 to 287 (Gibson and Spear, in press), 100 ng of protein; lane e, residues 1 to 287, 200 ng of protein.

react with group II MCAB (row 2, lane c). However, this antibody did react with 1-287[1] (row 2, lanes d and e). These results suggest that the group II epitope is probably located between residues 275 and 287, although it could include several residues upstream. From computer predictions (Fig. 1), the region from residue 266 to approximately 282 represents a potential epitope in both gD-1 and gD-2 since this region is highly hydrophilic and contains a β -turn (45). This combination of predictions and data suggest that a peptide consisting of residues 268 to 287 would encompass the group II epitope.

Localization of the group II epitope by using synthetic peptides. To confirm the location of the group II epitope, we tested the reactivity of a peptide representing residues 268-287[1]. This peptide reacted with group II MCAB (Fig. 4, row 6, lane d) but not with antibodies in group V (row 7, lane d) or VII (row 5, lane d). Thus, the group II epitope is located between residues 268 and 287 of gD-1. In addition, the peptide reacted against three of the four polyclonal sera prepared against gD (Fig. 4, rows 1 to 3, lane d).

Peptide 268-287[1] was coupled to KLH and used to immunize two rabbits. After the initial immunization protocol and one intravenous booster dose, serum samples were assayed for anti-gD and anti-peptide antibodies by the immunoblot assay. Neither serum reacted with native or denatured gD (data not shown). The animals were then given three intravenous booster doses of the free peptide and successive serum samples were assayed again. Again, none of the sera reacted with native or denatured gD. One serum sample reacted with the synthetic peptide 268-287[1] (data not shown). None of the sera exhibited any neutralizing activity (data not shown).

Localization of discontinuous epitopes of gD-1. Previously, we developed a procedure (15) to partially fragment gD, using *S. aureus* protease V8, an enzyme which cleaves specifically at glutamic acid residues. In that procedure, metabolically labeled gD was immunoprecipitated with a MCAB plus *S. aureus*-bearing protein A and the complex was treated with the protease (15). The antibody protected that portion of gD to which it was bound, and the bound and unbound fragments were characterized by SDS-PAGE and tryptic peptide analysis. We found that group I, IV, and VI MCAB remained bound to a 38K fragment which, on the basis of N-terminal amino acid sequencing (14; D. Long, G. H. Cohen, R. Hogue-Angeletti, and R. J. Eisenberg, unpublished data), was found to contain the amino terminus of pgD (the precursor form of gD). Analysis of the tryptic glycopeptides (10) of the 38K fragment showed that glycopeptides 1 and 2 were present but that glycopeptide 3 (at position 262) was missing (data not shown). Thus the 38K fragment appears to be located between residues 1 and 262. A possible V8 cleavage site is located at glutamic acid residue 260. The V8 experiments implied that the epitopes reacting with group I, IV, VI, and VII MCAB were located within this portion of gD-1. Antibodies in groups II and III could not be localized by this technique, since no fragments remained associated with them after V8 proteolysis. However, we now know that group II is located downstream of the 38K fragment. Thus, it was also possible that the group III epitope was located downstream of the carboxy terminus of the 38K.

Figure 2 (lane e) shows that group I, III, IV, VI, and VII MCAB bound to another truncated form of gD-1 consisting of residues 1 to 275 (30). These results agree with those of V8 proteolysis (15) with the exception of group III (11S). One possibility was that the group III epitope included residues

TABLE 1. Localization of the 11S epitope on the 38K fragment

Sample ^a	¹²⁵ I bound (cpm) ^b		
	HD-1- ¹²⁵ I	11S- ¹²⁵ I	57S- ¹²⁵ I
Control	9,459	10,205	57,433
gD-1-HD-1 (no V8)	7,868	690,120	350,784
gD-1-HD-1 (+ V8)	10,898	589,136	54,826

^a The control sample consisted of HD-1-Sepharose with no gD-1 added.

^b For each assay, 100 μ l of a cytoplasmic extract of HSV-1-infected cells was added to 50 μ l of HD-1-IgG-Sepharose. The immunosorbant was washed with washing buffer and incubated with 50 μ g of *S. aureus* V8 protease in 50 mM Tris (pH 8.0) for 2 h at 37°C. The complex was washed with washing buffer and then incubated with iodinated MCAB. This complex was washed and counted in a gamma counter.

between 260 and 275. Another possibility is that binding of group III occurred within the 38K fragment, but that this binding did not protect the fragment from further proteolysis (15). To determine directly whether group III MCAB could bind to the 38K fragment, we carried out the following experiment. A group I MCAB (HD-1) immunosorbant was used to bind purified gD-1. This complex was washed extensively, and a portion was treated with *S. aureus* protease V8 and washed again. Then various iodinated MCAB were added, and the complex was washed extensively and then counted in a gamma counter. Table 1 shows that, in the HD-1 control (HD-1 as immunosorbant and iodinated probe), no significant counts bound above background in either the V8-treated or the untreated sample. We also probed the V8-treated and untreated samples with group V antibody (57S) and found that the untreated sample contained a significant number of counts and the V8-treated sample contained no counts above background. This indicated that the proteolysis was complete and that the group V epitope was not present after V8 treatment. With iodinated 11S (group III MCAB) as the probe, approximately the same number of counts bound to the V8-treated and untreated gD-1-HD-1 complexes. These results show that the group III epitope is present on the 38K fragment and, furthermore, that this epitope is distinct from the group I epitope.

Topographical relationship of epitopes located in residues 1 to 287 of gD-1. Previous experiments indicated that six epitopes of gD-1 were located within the first 287 residues of the protein, two of which were continuous and four of which were discontinuous. Two different experiments were carried out to begin to define the relative positions of these epitopes. These will be referred to as competition experiments, although we recognize that the term does not accurately describe the type of analysis being performed.

First, representative MCAB from the six groups were covalently bound to Sepharose. A preliminary experiment was carried out to determine the amount of each immunosorbant required to bind a given amount of gD-1 present in infected cell extracts. The appropriate amount of each immunosorbant was used to bind similar amounts of unlabeled gD-1. After this, a different and iodinated second antibody was added, and the complexes were washed extensively and counted in a gamma counter. The underlined values in Table 2 show that each antibody group competed against itself, since only background levels of counts bound when the same antibody was used as immunosorbant and iodinated probe. Three types of results were obtained: (i) no competition, in which a significant number of counts were bound, e.g., using group III as immunosorbant and group I as probe, or vice versa; (ii) complete competition, in which only a background number of counts were bound, e.g.,

TABLE 2. Competition analysis using MCAb linked to Sepharose 4B^a

Immuno-sorbant	Iodinated antibodies (cpm of ¹²⁵ I bound)				
	Group I (HD-1)	Group II (DL6)	Group III (11S)	Group VI (45S)	Group VII (170)
Control	<u>3,166</u>	<u>2,227</u>	<u>710</u>	<u>7,800</u>	<u>1,066</u>
I (HD-1)	<u>2,914</u>	62,135	34,158	15,806	37,909
II (DL6)	ND ^b	<u>3,175</u>	ND	ND	ND
III (11S)	30,619	80,231	<u>700</u>	2,958	10,972
IV (41S)	ND	ND	<u>578</u>	32,265	ND
VI (45S)	17,558	85,565	3,304	<u>9,819</u>	30,581
VII (170)	27,618	80,048	30,581	<u>25,435</u>	<u>1,027</u>

^a Antibodies are included in groups according to original definitions (15). For each assay, 100 to 200 µl of MCAb linked to Sepharose was incubated for 2 h with 100 µl of a cytoplasmic extract of HSV-1-infected cells (9,13,15,16). The immunosorbant was washed and the iodinated second antibody (ca. 250,000 cpm) was added. The washed complexes were counted in a gamma counter. The underlined values show that each antibody group competed against itself.

^b ND, Not done.

comparing group IV against group III; (iii) partial competition, in which some counts were bound, e.g., comparing group VI and group I. In this assay, there is no way to know what the maximal level of binding should be.

As a second approach (Table 3), different concentrations of purified gD-1 were spotted onto nitrocellulose strips which were then incubated with an excess of unlabeled antibody. The strips were washed and incubated with iodinated second antibody. The maximal level of binding (no competition or 0% in Table 3) was determined from a control in which the strip was incubated only with labeled antibody. The values underlined in Table 3 represent the percent competition which occurred when the same antibody, both unlabeled and labeled, was used to compete against itself. Theoretically, these value should approach 100%. For group II, this value was 93%; however, for groups I and III, the value was 70%. The reason for this is not understood, since, in each case, the first antibody was presumably present in excess. It may be a problem of antibody affinity or presentation of the antigen on nitrocellulose. Nevertheless, when heterologous antibody groups were compared, the results agreed with the results in Table 2. There was no competition between groups I and III or I and VII. Furthermore, there was partial competition between groups I and IV and I and VI. Group III showed partial competition with groups IV and VI. Group II MCAb showed slight competition with

group VI and possibly group I. Thus, the two kinds of experiments lead to the same conclusions and form the basis for topographical positioning of these epitopes in gD-1 (Fig. 7).

DISCUSSION

In previous studies, we defined eight antigenic epitopes of gD, based on an analysis with a panel of MCAb (15). We attempted to associate the binding of particular MCAb with different fragments of the protein and found that several of them were in a 38K fragment which encompasses the amino terminus. We further defined the position of one continuous epitope of gD, amino acid residues 11 to 19, which reacts with group VII MCAb (7, 12). Localization of that epitope was based on the V8 proteolysis studies (15) as well as the use of computer predictions of secondary structure and hydrophilicity in choosing an appropriate synthetic peptide to test the predictions. Here, our goal was to localize the precise locations of two other continuous epitopes, recognized by group II and V MCAb, and to begin to define the location of discontinuous epitopes of gD-1. Computer predictions were instrumental in helping to choose synthetic peptides to demonstrate the location of continuous epitopes. In each case, the epitope was found to be located within stretches of highly hydrophilic amino acid residues making up predicted β -turns (45).

The group V epitope was localized to residues 340 to 356 of gD-1 which is downstream from the membrane-anchoring region (50, 51). A synthetic peptide consisting of this sequence was found to bind specifically to group V MCAb. The location of this epitope is thus on the portion of gD-1 which faces the inside of the virion or infected cell and confirms predictions of its location based on other studies (15, 37; Watson et al., J. Invest. Dermatol., in press). Thus, the failure of group V MCAb to neutralize virus or to bind to the surface of HSV-1-infected cells is due to the inaccessibility of this epitope when gD is associated with membrane. Recently, Rector et al. (44) used a group V antibody (55S) (15, 48) to examine whether non-neutralizing antibodies could be protective in passive immunization studies. Their data showed that 55S was not protective. Since group V MCAb would not have reacted with intact virions or intact infected cells, their result is not surprising.

The results of the present study indicate that the group V epitope is in itself immunogenic in purified gD, since the synthetic peptide reacted with several polyclonal sera pre-

TABLE 3. Competition analysis using the immunoblot assay

Cold antibody	Group I (HD-1)		Group II (DL6)		Group III (11S)	
	Slope ^b	% Competition ^c	Slope	% Competition	Slope	% Competition
None	173	0	728	0	1,051	0
Group I (HD-1)	<u>60</u>	<u>70</u>	704	3	1,050	0
Group II (DL6)	139	20	<u>52</u>	<u>93</u>	951	10
Group III (11S)	167	3	<u>695</u>	<u>5</u>	<u>377</u>	<u>64</u>
Group IV (41S)	124	28	ND ^d		713	32
Group VI (45S)	137	21	642	20	714	32
Group VII (170)	167	3	793	0	1,050	0

^a In each assay, various concentrations of gD-1, ranging from 0.45 to 15 ng, were spotted onto nitrocellulose, incubated with cold antibody for 2 h, and then reacted with iodinated antibodies for 1 h. The spots were cut from the nitrocellulose and counted in a gamma counter. The results were plotted as counts bound versus concentration of gD.

^b Slope is given in counts per minute per nanogram of gD.

^c Percent competition was derived by the following equation: $100 - (\text{counts per minute per nanogram of gD bound with unlabeled antibody present} / \text{counts per minute per nanogram of gD bound with no unlabeled antibody})$. Values underlined represent the percent competition which occurred when the same antibody, both, unlabeled and labeled, was used to compete, against itself.

FIG. 7. Topographic map of HSV-1 gD. The positions of epitopes binding to MCAb in groups VII, II, and V are shown. Also indicated (as balloons) are the three *N*-asparagine-linked glycosylation sites. The transmembrane region is depicted as a box. The positions of discontinuous epitopes (ellipses at bottom) were derived from the competition experiments. Three of these epitopes appear to involve S-S bonds.

pared against gD, including an antiserum prepared against gD-2. More recently, we have tested additional polyclonal sera prepared in rabbits against gD-1 and gD-2 and have found that six of eight sera, including two prepared against gD-2, reacted with this peptide. In addition, antiserum prepared to the peptide reacted with gD-1 and gD-2 in immunoblot and immunoprecipitation assays, although the reaction was much stronger against gD-1. A somewhat puzzling observation was that this antiserum was more reactive against the native than the denatured forms of gD-1 and gD-2 (Fig. 5A, lanes a to d). It is possible that the conformations of peptide 340-356[1] on KLH might well be nearer to the conformation of the same segment in the native protein than to the different conformational ensemble of the denatured protein.

Another puzzling observation was the reactivity of group V MCAb against gD-2 detected by immunoblot. Previously, these antibodies were considered type 1 specific based on immunoprecipitation and immunofluorescence assays. Although most of the amino acids in the epitope specified by group V antibodies may be unique to gD-1, it is clear from examination of the sequence in this region that several amino acids in the epitope must be common to gD-1 and gD-2. The more sensitive immunoblot assay might better detect this partial overlap. Alternatively, gD-2 might assume different conformations under the conditions used in different assays (27, 38). The few type common residues in this sequence may be arranged close together in the native gD's, so that a weak type common reactivity can be seen, although the sequences otherwise differ substantially. If so, it would explain why group V MCAb appeared to be more reactive against native than against denatured gD-2 (Fig. 2, row 6, lanes b and d). Fine mapping of the group V epitope should take into account the differences in sequence between gD-1 and gD-2 in this region.

Localization of the group II epitope was accomplished first by analyzing the reactivity of truncated forms of gD-1 against DL6 MCAb. The antibody failed to react with a truncated form of gD ending at residue 275 of the mature protein (30) but did react with a form of gD ending at residue 287 (M. G. Gibson and P. G. Spear, in press). These results suggested that the epitope was between residues 275 and 287. The computer predictions (Fig. 1) showed that gD-1 and gD-2 each contained a region with hydrophilic residues within a β -turn. Interestingly, this region is rich in acidic

residues (both aspartic and glutamic acids) and proline, but lacks the basic amino acids, such as lysine, that are often associated with epitopes (31). In choosing a synthetic peptide for confirmation of the location, we assumed that the epitope could include some residues upstream of 275. Peptide 268-287[1] reacted with DL6 MCAb and with three polyclonal sera prepared against gD. This peptide also reacted with several other polyclonal anti-gD rabbit sera, a total of four to gD-1 and two to gD-2. However, when rabbits were immunized with this peptide coupled to KLH, there was no antibody response. After several injections with the free peptide, one of the sera exhibited reactivity against the peptide but not against gD. In this case, it is possible that none of the conformations of the peptide coupled to KLH corresponded to the structure of the peptide as it is found in native or denatured gD.

The synthetic peptide approach we took in these studies is not yet likely to be fruitful in localizing discontinuous epitopes (1, 45). This is because these epitopes depend on a certain tertiary structure of gD, which in part involves disulfide bonds. The position of these bonds is not yet known, but such information should aid in localization. However, we do know that four discontinuous epitopes of gD-1 are located within the first 260 amino acids of the protein (Fig. 7), since antibodies in groups I, III, IV, and VI reacted with truncated gD-1 (1 to 275) as well as with the 38K fragment (presumably residues 1 to 260) generated by V8 proteolysis. Six of the seven cysteine residues of gD-1 are located within residues 66 to 202. It is quite possible that disulfide bonds formed by these six cysteines play a role in the structure of discontinuous epitopes. Cysteine at residue 333 of the mature protein is within the transmembrane region of gD-1 and is not involved in formation of these epitopes. Thus, our data predict that this cysteine is probably not involved in intramolecular disulfide bonds in gD-1. However, it may be involved in intermolecular disulfides, perhaps in formation of the gD-1 dimer (17, 18). In this regard, it is interesting to note that gD-2, which lacks this cysteine, does not form dimers (17, 18). In preliminary experiments, we have found that the group III epitope is destroyed by boiling gD-1 in SDS in the absence of mercaptoethanol, whereas destruction of groups I, IV, and VI required reduction and alkylation (M. Ponce de Leon, G. H. Cohen, and R. J. Eisenberg, unpublished data).

Competition experiments were carried out to determine the relative orientation of the four discontinuous epitopes. In some cases, the binding of one antibody to gD-1 had no effect (no competition) on the binding of another. These results are further evidence that the MCAb groupings are valid and that there are distinct discontinuous epitopes on gD-1. In other cases, there was competition. This indicated that (i) some amino acids in these epitopes are shared; or (ii) the epitopes were so close that there was steric hindrance in the binding of a second antibody; or (iii) binding of one antibody altered the conformation of gD so that binding of the second antibody was affected (35). Previously, we speculated that binding of group III MCAb altered the conformation of gD-1, making the molecule more susceptible to protease V8 cleavage (15). This explanation is still consistent with the present results.

On the basis of these studies, as well as the studies of three continuous epitopes, we have constructed a two-part topographic map for gD-1 (Fig. 7). First, we have depicted the protein essentially as a linear molecule with the positions of the three continuous epitopes indicated. The discontinuous epitopes have been depicted in a separate drawing as

ellipses located downstream from group VII, each of which includes amino acids prior to residue 260. The discontinuous epitopes corresponding to MCAb which exhibited competition are shown as overlapping. Thus, group III overlaps groups IV and VI and group I also overlaps IV and VI. Groups I and III do not overlap at all. Preliminary experiments indicate that antibodies in all of the MCAb groups except possibly group IV are able to immunoprecipitate the gD-like protein produced by tunicamycin-treated, HSV-1-infected cells (42; Matthews et al., unpublished data). Three of the epitopes are depicted as involving disulfide bonds (S-S in Fig. 7), although we do not know how many cysteines are disulfide bonded in gD or how many are involved in determining the structure of any one epitope.

Further localization of discontinuous epitopes will require other approaches, including a more complete understanding of the contribution of disulfide bonds to the structure of gD. One possible approach will be to analyze the amino acid changes associated with mutants which exhibit an altered pattern of reactivity with MCAb. Such mutants would include those which are no longer neutralized by antibody, such as the "mar" mutants (21). Another approach would be to examine the amino acid changes found in natural isolates of HSV which exhibit an anomalous pattern of reactivity with MCAb (41, 43). This approach was recently exploited (43) to explain the reactivity of an HSV-1 strain with a gD-2-specific MCAb called 17 β A3 (2). Analysis of the DNA sequence of the gD gene of the isolate revealed a change which altered the codon for asparagine (residue 72 of the mature protein) present in the prototype HSV-1 strain to histidine, normally present in the HSV-2 strain. Grouping of 17 β A3 has not yet been accomplished. However, it might be in group VIII (15). Our uncertainty about the grouping of 17 β A3 illustrates the need for a common classification of gD-specific MCAb. We are now in the process of grouping a number of additional gD-specific MCAb from several laboratories to overcome this difficulty.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grants DE-02623 from the National Institute of Dental Research, AI-18289 from the National Institute of Allergy and Infectious Diseases, and CA-21776 from the National Cancer Institute. A portion of this work was supported by a grant to G.H.C. and R.J.E. from the American Cyanamid Co. M.G.G. is a fellow of the Leukemia Society of America, and J.T.M. was a predoctoral trainee supported by Public Health Service grant NS-07180 from the National Institute of Neurological and Communicative Disorders and Stroke.

We thank B. Hampar, M. Zweig, and L. Pereira for monoclonal antibodies, Wesley Wilcox for carefully reading the manuscript, and Madeline Cohen, Valerie Rinaldt, and Michael Nobel for excellent technical assistance.

LITERATURE CITED

- Atassi, M. Z. 1978. Precise determination of the entire antigenic structure of lysozyme. Molecular features of protein antigenic structures and potential of 'surface-stimulation' synthesis—a powerful new concept for protein binding sites. *Immunochimistry* 15:909–936.
- Balachandran, N., D. Harnish, W. E. Rawls, and S. Bacchetti. 1982. Glycoproteins of herpes simplex virus type 2 as defined by monoclonal antibodies. *J. Virol.* 44:344–355.
- Benjamin, D. C., M. A. Berzofsky, 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.
- Chan, W. 1983. Protective immunization of mice with specific HSV-1 glycoproteins. *Immunology* 49:343–352.
- Chou, P. Y., and G. D. Fasman. 1974. Conformational parameters for amino acids in helical β -sheet and random coil regions. *Biochemistry* 13:211–222.
- Chou, P. Y., and G. D. Fasman. 1974. Prediction of protein conformation. *Biochemistry* 13:222–245.
- 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 production of neutralizing antibody. *J. Virol.* 49:102–108.
- 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.
- 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.
- Cohen, G. H., D. Long, J. T. Matthews, M. May, and R. Eisenberg. 1983. Glycopeptides of the type-common glycoprotein gD of herpes simplex virus types 1 and 2. *J. Virol.* 46:679–689.
- 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.
- 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.
- 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. *J. Virol.* 31:608–620.
- Eisenberg, R. J., D. Long, R. Hogue-Angeletti, and G. H. Cohen. 1984. Amino-terminal sequence of glycoprotein D of herpes simplex virus types 1 and 2. *J. Virol.* 49:265–268.
- Eisenberg, R. J., D. Long, L. Pereira, B. Hampar, M. Zweig, and G. H. Cohen. 1982. Effect of monoclonal antibody on limited proteolysis of native glycoprotein gD of herpes simplex virus type 1. *J. Virol.* 41:478–488.
- 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.
- Eisenberg, R. J., M. Ponce de Leon, L. Pereira, D. Long, and G. H. Cohen. 1982. Purification of glycoprotein gD of herpes simplex virus types 1 and 2 by use of monoclonal antibody. *J. Virol.* 41:1099–1104.
- Gibson, M. G., and P. G. Spear. 1983. Insertion mutants of herpes simplex virus have a duplication of the glycoprotein D gene and express two different forms of glycoprotein D. *J. Virol.* 48:396–404.
- Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of ^{131}I -labeled human growth hormone of high specific radioactivity. *Biochem. J.* 89:114–123.
- Hebrink, 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.
- 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.
- Hubbard, S. D., and R. J. Ivatt. 1981. Synthesis and processing of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* 50:555–583.
- Kabsch, W., and C. Sander. 1983. How good are predictions of protein secondary structure? *FEBS Lett.* 155:179–182.
- Kennett, R. H. 1980. Cloning of hybridomas. Cloning in semi-solid agarose, p. 372–373. *In* R. H. Kennett, T. J. McKearn, and

- K. Bechtol (ed.), Monoclonal antibodies. Hybridomas: a new dimension in biological analyses. Plenum Press, New York.
26. Kessler, S. W. 1975. Rapid isolation of antigens from cells with a staphylococcus protein A antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. *J. Immunol.* **115**:1617-1624.
 27. Kuusmanen, E., B. Bang, M. Hurme, and R. F. Pettersson. 1984. Uukuniemi virus maturation: immunofluorescence microscopy with monoclonal glycoprotein-specific antibodies. *J. Virol.* **51**: 137-146.
 28. 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 Press, New York.
 29. 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.
 30. 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.
 31. Leach, S. J. 1983. How antigenic are antigenic peptides? *Biopolymers* **22**:425-440.
 32. Lee, G. T.-Y., M. F. Para, and P. G. Spear. 1982. Location of the structural genes for glycoproteins gD and for other polypeptides in the S component of herpes simplex virus type 1 DNA. *J. Virol.* **43**:41-49.
 33. Liu, F. T., M. Zinnecker, T. Hamaoka, and D. H. Katz. 1979. New procedures for preparation and isolation of conjugates of proteins and a synthetic copolymer of D-amino acids and immunochemical characterization of such conjugates. *Biochemistry* **18**:690-697.
 34. 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.* **37**:761-764.
 35. Lubeck, M., and W. Gerhard. 1982. Conformational changes at topologically distinct antigenic sites on the influenza A/PR/8/34 virus HA molecule are induced by the binding of monoclonal antibodies. *Virology* **118**:1-7.
 36. Marchalonis, J. J. 1969. An enzymic method for the trace iodination of immunoglobulins and other proteins. *Biochem. J.* **113**:299-305.
 37. 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.
 38. Molday, R. S., and D. MacKenzie. 1983. Monoclonal antibodies to rhodopsin: characterization, cross-reactivity and application as structural probes. *Biochemistry* **22**:653-660.
 39. 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.
 40. 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 virus surface antigen and the herpes simplex virus glycoprotein D. *Proc. Natl. Acad. Sci. U.S.A.* **81**:193-197.
 41. 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.
 42. Pizer, L. I., G. H. Cohen, and R. J. Eisenberg. 1980. Effect of tunicamycin on herpes simplex virus glycoproteins and infectious virus production. *J. Virol.* **34**:142-153.
 43. 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.
 44. 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.
 45. Rose, G. D. 1978. Prediction of chain turns in globular proteins on a hydrophobic basis. *Nature (London)* **272**:586-590.
 46. Ruyechan, W. T., L. S. Morse, D. M. Knipe, and B. Roizman. 1979. Molecular genetics of herpes simplex virus. II. Mapping of the major viral glycoproteins and of the genetic loci specifying the social behavior of infected cells. *J. Virol.* **29**:677-697.
 47. Sela, M., B. Schechter, I. Schechter, and A. Borek. 1967. Antibodies to sequential and conformational determinants. *Cold Spring Harbor Symp. Quant. Biol.* **32**:537-545.
 48. 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.
 49. 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.
 50. Watson, R. J. 1983. DNA sequence of the herpes simplex virus type 2 glycoprotein D gene. *Gene* **26**:307-312.
 51. Watson, R. J., J. H. Weis, J. S. Salstrom, and L. W. Enquist. 1982. Herpes simplex type 1 glycoprotein D gene: nucleotide sequence and expression in *Escherichia coli*. *Science* **218**: 381-383.