An Analysis of the Biological Properties of Monoclonal Antibodies against Glycoprotein D of Herpes Simplex Virus and Identification of Amino Acid Substitutions that Confer Resistance to Neutralization

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SUMMARY

Four monoclonal antibodies to glycoprotein D (gD) of herpes simplex virus (HSV) types 1 and 2 neutralized virus in the presence of complement but exhibited diverse activities in its absence. Amino acid substitutions that conferred resistance to neutralization by each antibody were identified by deriving the nucleotide sequence of the gD gene from resistant mutants. Each antibody selected a substitution from different parts of the molecule and mutants resistant to a single antibody always arose from the same mutation. One of the antibodies reacted with a synthetic oligopeptide corresponding to the region of the molecule in which amino acid substitution conferred resistance, but the remaining three antibodies failed to react with predicted oligopeptide targets. These antibodies may therefore react with 'discontinuous' epitopes, a view supported by the observation that two of these three antibodies competed with each other in binding assays despite the fact that substitutions conferring resistance to neutralization arose nearly 100 residues apart in the primary sequence. The four antibodies had very different biological properties. One antibody neutralized infectivity but did not inhibit cell fusion, one antibody inhibited cell fusion but did not neutralize, while a third antibody had both activities. One antibody had neither activity but enhanced the infectivity of HSV-2 in a type-specific manner. The ability of antibodies to inhibit cell fusion by syncytial virus strains correlated with an ability to prevent plaque enlargement by a non-syncytial virus strain, implying a role for gD in the intercellular spread of virus that is independent of the syncytial phenotype. We found no correlation between neutralizing activity and anti-fusion activity suggesting that, while gD is involved in cell fusion, it has at least one other function which is required for infectivity.

INTRODUCTION

Glycoprotein D (gD) of herpes simplex virus (HSV) is a component of the virus envelope that has been recognized for many years as a major target for virus neutralization (Sim & Watson, 1973; Honess & Watson, 1974; Cohen et al., 1972, 1978) and recent evidence suggests that this antigen elicits neutralizing antibody more efficiently than any other virus component (Para et al., 1985). The severity of experimental HSV infection in mice is decreased by passive immunization with monoclonal antibodies to gD and by immunization with purified gD (Chan, 1983; Lasky et al., 1984; Long et al., 1984; Simmons & Nash, 1985), and since the antibody response to gD is largely cross-reactive between HSV-1 and HSV-2, gD is considered a useful candidate as a component of future HSV vaccines.

The identification and characterization of antigenic sites on glycoprotein D has been approached by grouping anti-gD monoclonal antibodies according to their neutralizing activity, their HSV type-specificity, their ability to react with denatured and native forms of gD, and
their ability to react with truncated forms or peptidase fragments of gD. On this basis Eisenberg et al. (1982) divided monoclonal antibodies to gD into eight groups. The antigenic targets of three groups was defined with some precision by reacting monoclonal antibodies with synthetic oligopeptides whose probable involvement as antigenic sites was predicted from analyses of the primary amino acid sequence of gD (Dietzschold et al., 1984; Eisenberg et al., 1985). Thus, group VII antibodies react with a site within amino acids 11 to 19, close to the amino terminus, while groups II and V react against sites within amino acids 268 to 287 and 340 to 356 respectively, these sequences corresponding to hydrophilic regions of the polypeptide lying close to the internal and external surfaces of the membrane. Of these three groups, only group VII has significant neutralizing activity. Antibodies in the remaining groups, some of which are strongly neutralizing, fail to react with denatured gD implying that their antigenic targets are 'discontinuous', and little is known of these sites other than that they lie in the globular, glycosylated portion of the molecule.

The mechanisms by which antibodies to glycoprotein D neutralize virus infectivity are uncertain. High concentrations of monoclonal antibody have been shown to inhibit the binding of purified virions to cell monolayers, but this inhibition occurs only at concentrations orders of magnitude higher than those which efficiently neutralize virus and there is no correlation between the neutralizing activity of different antibodies and their ability to inhibit binding (Fuller & Spear, 1985). Anti-gD antibodies will also inhibit the fusion of cells infected with syncytial strains of HSV-1 (Noble et al., 1983) and this ability to suppress the syncytial phenotype shows a reasonable correlation with neutralizing activity (Para et al., 1985). These results imply a role for gD in cell fusion and in fusion of the virus envelope to the cell membrane during penetration by virus particles. Blocking of the latter process would then account for the neutralizing activity of those gD antibodies which exhibit anti-fusion activity. It remains to be seen whether fusion inhibition will continue to correlate with infectivity neutralization when larger numbers of antibodies are examined, and at present we cannot correlate the biological activities of monoclonal antibodies to gD with information about the sites against which these antibodies are directed. The aim of the work described here is to extend the antigenic analysis of Eisenberg et al. (1985) by determining the amino acid substitutions in gD that confer resistance to neutralization by monoclonal antibodies, and to investigate the biological properties of these antibodies.

METHODS

Cells and viruses. BHK-13 and Vero cells were grown in Glasgow modified Eagle’s medium supplemented with 10% tryptose phosphate broth and 10% newborn bovine serum. Stocks of HSV-1 strains HFEM and SC16 were prepared in BHK cells at 37 °C after infection at an m.o.i. of 0.01. Stocks of HSV-2 strains 333 and 25766 were similarly prepared but at 33 °C. Infectivity assays were performed in BHK cells.

Monoclonal antibodies. Antibodies LP2, LP14, AP7 and AP12 react with gD of HSV-1 and HSV-2 and were prepared as described by McLean et al. (1982). AP12 is an IgG1 while the other three are of the IgG2a subclass. LP2 and LP14 have been classified in group I and group VII respectively in the classification of Eisenberg et al. (1985; G. H. Cohen, personal communication). AP7 and AP12 have not been classified in this way.

Antibody-resistant mutants. 10⁶ p.f.u. parental virus in 1 ml tissue culture medium was incubated with ascites fluid at a final dilution of 1 in 20 for 1 h at 37 °C and then at 4 °C overnight. Neutralizations with LP2 were performed in the absence of complement while neutralizations with the other antibodies were performed in the presence of a source of complement (normal rabbit serum at a final concentration of 1 in 20), since neutralization with these antibodies was either complement-dependent or was considerably enhanced by complement. Under these conditions infectivity was reduced by 100- to 1000-fold so that several rounds of neutralization and virus growth were required to achieve a resistant population. At this stage resistant viruses were cloned by limiting dilution and individual clones were tested for resistance to neutralization.

Solid-phase enzyme linked immunoassays. Solid-phase targets were prepared by treating 96-well flat bottomed plates with 100 µl 1% glutaraldehyde in phosphate-buffered saline (PBS) for 6 h at room temperature. The glutaraldehyde solution was then removed, the plate was washed once with PBS, and 50 µl of antigen in PBS was delivered to each well. After incubation at room temperature overnight the antigen solution was removed and the plate allowed to dry. Oligopeptide antigens were used at a concentration of 50 µg/ml. HSV antigens were prepared by harvesting infected cells 8 h after infection at a m.o.i. of 10, sonicating the cell suspension and diluting with PBS to the equivalent of 10⁵ infected cells per ml.
Mutation to antibody resistance in HSV gD

The IgG fraction was purified from ascites fluid and biotinylated as described by Guesdon et al. (1979). Solid-phase targets were pretreated with 100 μl foetal bovine serum before addition of 50 μl biotinylated antibody diluted in PBS containing 0-3% gelatin and 1% foetal bovine serum (dilution buffer). After 30 min the antibody was removed and the wells were washed three times with dilution buffer. Fifty μl of streptavidin–horseradish peroxidase (Bethesda Research Laboratories) in dilution buffer was added to each well, allowed to react for 15 min and removed by washing three times with dilution buffer. Bound peroxidase was measured as described by Richman et al. (1982) by conversion of o-phenylenediamine to the coloured product. All incubations and washing were performed at room temperature.

Oligopeptides corresponding to amino acids 7 to 23 and 11 to 32 of HSV-1 gD were gifts from Dr S. Welling-Wester (Laboratory of Medical Microbiology, Groningen, The Netherlands) and Dr M. Hall (Roche Ltd., Welwyn Garden City, U.K.), respectively. Other oligopeptides were prepared by Cambridge Research Biochemicals, Cambridge, U.K.

Competition binding assays were performed using an immunofiltration assay system described by Richman et al. (1982). 10⁵ HSV-1-infected cells were trapped on the filter and reacted with dilutions of unlabelled competing antibody in 50 μl dilution buffer. After 30 min a fixed concentration of biotinylated antibody was added to each well and allowed to react for a further 30 min. The targets were then washed with dilution buffer and the bound biotinylated antibody was measured by addition of streptavidin-horseradish peroxidase and o-phenylenediamine as described above. The concentrations of biotinylated antibodies used in these assays were in slight excess over target antigen in the absence of competitor.

Neutralization assays. Four × 10⁴ p.f.u. HSV-1 were reacted with dilutions of antibody in 3 ml tissue culture medium for 1 h at room temperature. Five-hundred μl and 50 μl samples were then assayed on BHK cells.

Fusion assays. Confluent monolayers of BHK cells were infected with strain HFEM at a m.o.i. of 20, and the virus was allowed to adsorb for 1 h at 37 °C. The inoculum was then replaced with medium containing dilutions of antibody and the monolayers were fixed and stained after incubation for a further 7 h.

Plaque inhibition assays. Monolayers of Vero cells were infected with approximately 50 p.f.u. HSV-1. After adsorption for 1 h the inoculum was replaced with medium containing antibody and the monolayers were fixed and stained after 3 days. Relative plaque areas were measured by projecting the image of the monolayer onto squared paper and counting the number of 1 mm squares within the image of each plaque.

Nucleotide sequencing. The glycoprotein D coding sequences of HSV-1 and HSV-2 wild-type and mutant viruses were determined by the dideoxy chain termination method using sequences cloned into phage M13mp8 (Sanger et al., 1977, 1980; Messing & Vieira, 1982). The modifications described by Bankier & Barrell (1983) were used. The BamHI-I fragment of HSV-1 strains was cloned into pAT153 and the resulting plasmids were digested with HindIII and NruI. The 2.1 kb fragment containing the gD coding sequence was then prepared by agarose gel electrophoresis and used for sequencing. The EcoRI-N fragment or the Hind III-L fragment were cloned from type 2 viruses into pAT153. These plasmids were then used to prepare a 2-2 kb XhoI fragment which contains the gD-2 coding sequence.

Initial sequencing studies were performed by digesting DNA with restriction enzymes AklI, HaeIII or Sau3AI prior to random cloning into the Smal or BamHI sites of M13mp8. In some experiments DNA was randomly sheared by sonication and end-repaired before cloning into the M13mp8 Smal site (Deininger, 1983). The gD coding sequences of wild-type viruses were determined on both DNA strands. Mutant sequences were determined on one strand only and mutations were identified by direct comparison of sequencing gels.

RESULTS

Characterization of antigenic sites recognized by monoclonal antibodies

Mutants selected for resistance to neutralization by antibodies LP14, AP7, AP12 or LP2 were resistant to the selecting antibody only, and remained sensitive to the other three antibodies. Each of the four antibodies therefore recognizes a different type-common gD epitope. The results of competition binding assays shown in Fig. 1 indicate that the binding of LP14 or AP7 is not influenced by binding of the other three antibodies. Antibodies LP2 and AP12 compete with each other in binding assays but the competition appears to be partial, homologous competition being more efficient than heterologous competition.

We attempted to identify the binding site of each antibody in terms of the primary amino acid sequence by determining the nucleotide sequence of the gD gene from antibody-resistant mutants. Mutants resistant to antibodies LP14, AP7 and LP2 were isolated from HSV-1 strains SC16 and HFEM. A single mutant resistant to AP12 was isolated from strain SC16, and an LP2-resistant mutant was isolated from HSV-2 strain 25766. The nucleotide sequence of the gD gene from the three parental viruses and from HSV-2 strain 333 was determined. The corresponding
Fig. 1. Competition binding assays. Target antigens were reacted with dilutions of unlabelled antibodies (ascites fluids) prior to addition of a fixed concentration of biotinylated antibody (identified by an asterisk in each component of the figure). Bound biotinylated antibody was then measured by addition of avidin–horseradish peroxidase and o-phenylenediamine as described in Methods. The upper series of curves in each figure are the results obtained using infected cells as targets and the lower series show the results of identical experiments using uninfected cell targets. Competition was considered complete when the signal obtained using infected cells was indistinguishable from that obtained with uninfected cells.

Mutant sequences were determined in full with the exception of the LP14- and AP7-resistant mutants from strain HFEM. Only the 5' portion of the gene was determined from these mutants, and when this revealed identical base changes to those found in the corresponding mutants of SC16, no further sequencing was performed. Each mutant gene that was completely sequenced contained a single base change when compared to its parent. These data are summarized in Fig. 2 which shows the predicted amino acid sequences of gD from HSV-1 strain HFEM and HSV-2 strain 333. Amino acid differences found in HSV-1 strains SC16, Patton (Watson et al., 1982) and strain 17 (McGeoch et al., 1985) and HSV-2 strain 333 and strain G (Watson, 1983) are shown. A comparison of gD amino acid sequences of the HSV-1 and HSV-2 strains illustrates considerable conservation within the two types. Amino acid differences were found at three positions among three HSV-2 strains and at seven positions among four HSV-1 strains. Changes were conservative and were concentrated in the signal peptide, trans-membrane region and carboxy terminal region. It is worth noting that strain HFEM was isolated many years ago and has been subjected to many passages in vivo, in ovo and in vitro, yet differs in gD by only one amino acid from strain SC16, a recent isolate with low passage history. In addition to those differences which resulted in amino acid changes, silent nucleotide changes were found in a further three positions among the four HSV-1 strains. Thus of a total of ten positions at which nucleotide changes were found, only three were silent. We compared sequences of the HSV-1 thymidine kinase gene (which is similar in size to gD) from strains CI101 (Wagner et al., 1981), MP (McKnight, 1980), SC16 (M. Inglis, personal communication) and HFEM (U. Gompels, personal communication) and found differences at 22 positions of which 14 were silent. The frequency of silent nucleotide changes in the gD gene seems remarkably low by comparison to the thymidine kinase gene, but the numbers are too small to be significant, particularly since this comparison of the two genes involves different virus strains.
Mutation to antibody resistance in HSV gD

Mutations that confer resistance to neutralization by monoclonal antibodies are also shown in Fig. 2. Where different mutants resistant to the same antibody were examined they always arose from the same mutation and since such mutants were isolated from different virus strains they cannot be clonally related. Thus LP14 selected the transition CGC (Arg-16) → CAC (His), AP7 selected the transition CTG (Leu-25) → CCG (Pro), AP12 selected the transition ATC (I1e-129) → ACC (Thr), and LP2 selected the transition AGC (Ser-216) → AAC (Asn). Of these sites, one, the mutation to LP14 resistance, lies within one of the three regions identified as 'continuous' antigenic sites (Eisenberg et al., 1985) and amino acids substituted in antibody-resistant mutants are boxed. The residues are numbered from the amino terminal lysine of the mature protein (Eisenberg et al., 1984).

To find whether each mutation site identified a continuous epitope with which the corresponding antibody would react, oligopeptides covering each substitution site were used as solid-phase targets in enzyme immunoassays with each biotinylated monoclonal antibody. The results are given in Table 1. As expected for a group VII antibody, LP14 reacted with oligopeptides 7 to 23 and 11 to 32. Thus arginine-16, the residue substituted by histidine in LP14-resistant mutants, lies within or very close to the antibody binding site. The remaining three monoclonal antibodies failed to react with any of the synthetic peptide targets. The failure of LP2 to react with synthetic peptide 206 to 226 was expected since this antibody has been placed in group I (G. H. Cohen, personal communication) and group I antibodies do not react with denatured forms of gD (Eisenberg et al., 1982). Our results suggest that AP7 and AP12 may also be directed against discontinuous epitopes.
Table 1. Reaction of monoclonal antibodies with oligopeptide targets*

<table>
<thead>
<tr>
<th>Assay target</th>
<th>Antibody</th>
<th>AP7</th>
<th>AP12</th>
<th>LP2</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-I</td>
<td>1.09</td>
<td>0.98</td>
<td>0.56</td>
<td>0.54</td>
</tr>
<tr>
<td>HSV-2</td>
<td>0.89</td>
<td>0.01</td>
<td>0.51</td>
<td>0.84</td>
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<tr>
<td>7–29†</td>
<td>1.07</td>
<td>0.01</td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>11–32†</td>
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<td>&lt;0.00</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>17–31†</td>
<td>&lt;0.00</td>
<td>&lt;0.00</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>124–136†</td>
<td>0.01</td>
<td>0.01</td>
<td>0.03</td>
<td>0.07</td>
</tr>
<tr>
<td>206–226†</td>
<td>&lt;0.00</td>
<td>0.01</td>
<td>0.01</td>
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</tr>
</tbody>
</table>

* Each biotinylated antibody was used at a concentration of approximately 1 µg/ml. Values are absorbance at 492 nm of duplicate samples after subtraction of a value of 0.11, the absorbance given by a well containing substrate only. The absorbance values were obtained using a multiwell plate reader.

† gD oligopeptide.
Mutation to antibody resistance in HSV gD

Fig. 3. Infectivity neutralization. HSV-1 strain SC16 was incubated for 1 h at room temperature with dilutions of ascites fluids and surviving infectivity was determined. The dashed line represents 50% infectivity survival; ○, LP2; ●, LP14; ■, AP7; □, AP12; △, LP6 (a HSV-2-specific antibody directed against gC-2).

virus, monolayers of Vero cells were infected at approximately 50 p.f.u. HSV-1 SC16 (a non-syncytial strain), and antibodies were added to the medium 1 h after infection. The relative plaque areas were measured after 3 days. Vero cells, rather than BHK cells, were used in these experiments because Vero cells gave more symmetrical and better defined plaques. The results are shown in Fig. 4. All four anti-gD antibodies caused a significant decrease in plaque size at the highest concentration used (1:100 ascites fluid). The effect was much less dramatic than that observed with an anti-gH antibody, where plaque formation was undetectable at an equivalent concentration (Buckmaster et al., 1984). Antibodies LP14 and AP12 reduced plaque size more efficiently and at lower concentrations than antibodies LP2 and AP7, and the results of this assay therefore correlate with anti-fusion activity rather than with neutralizing activity. These results cannot be interpreted unambiguously because the ascites fluids used might contain factors other than antibodies that could influence virus growth or cell behaviour. Ideally these experiments should have been performed using purified immunoglobulin but we found it difficult to prepare sufficient amounts of antibody AP12. This antibody does not bind to Protein A and yields were very low following ion-exchange chromatography. As an alternative method of demonstrating that the observed effects on plaque enlargement were due specifically to antibody activity we compared the effects of each ascites fluid on plaque formation by wild-type virus and by virus mutants resistant to neutralization. A single antibody concentration was used. The results in Table 2 reinforce the conclusion that antibodies LP14 and AP12 inhibit plaque enlargement while AP7 and LP2 have little effect. Mutants resistant to neutralization by AP12 and LP14 show normal plaque formation in the presence of ascites fluid containing the relevant antibody. This confirms that the observed activity on wild-type virus is due to the specific action of antibody. The results shown were all obtained using SC16, a non-syncytial virus, but similar experiments were done with a syncytial strain, HFEM. Measurement of plaque areas proved more difficult when HFEM was used but the results were qualitatively similar; LP14 and AP12 considerably decreased plaque size while LP2 and AP7 had little effect.
Fig. 4. Plaque inhibition. HSV-1 strain SC16 was used to infect preformed monolayers of Vero cells, and after 1 h the inoculum was replaced by medium containing dilutions of ascites fluid. The plates were fixed and stained after 3 days and the relative plaque areas were measured after projection of the monolayer image. Each point is the mean of 20 determinations and the area given is that of the plaque images projected to achieve a linear magnification of approximately tenfold. ○, LP2; ■, AP7; ●, LP14; □, AP12; △, LP6.

Table 2. Effect of monoclonal antibodies on plaque formation by wild-type HSV-1 and antibody-resistant mutants*

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Virus None</th>
<th>LP6</th>
<th>LP2</th>
<th>AP7</th>
<th>LP14</th>
<th>AP12</th>
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<tbody>
<tr>
<td>SC16</td>
<td>26</td>
<td>29</td>
<td>20</td>
<td>25</td>
<td>4-6</td>
<td>4-3</td>
</tr>
<tr>
<td>LP2r</td>
<td>40</td>
<td>32</td>
<td>31</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AP7r</td>
<td>53</td>
<td>67</td>
<td>-</td>
<td>54</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>AP12r</td>
<td>37</td>
<td>40</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>47</td>
</tr>
</tbody>
</table>

* Approximately 100 p.f.u. of HSV-1 strain SC16 or antibody-resistant mutants derived from SC16 were used to infect monolayers of Vero cells. After 1 h at 37 °C the inoculum was replaced with medium containing ascites fluids at a final concentration of 1:100. After 3 days the monolayers were stained and the plaque areas measured after projection. Each value is the mean area in mm² of 30 projected plaques.

Antibody AP7 was without significant activity in any of the assays described. However we noted that in neutralization experiments with HSV-2, incubation of virus with AP7 consistently resulted in a three- to fivefold increase in infectivity titre. Similar results were never observed with HSV-1 (Fig. 3), and in the presence of complement AP7 neutralized HSV-1 and HSV-2 equally well. We do not understand this phenomenon, but it is unlikely to be due to increased infectivity from Fc receptor effects (Peiris & Porterfield, 1979) because the outcome of the experiment was not influenced by using cell types of macrophage origin and, in any case, such effects should be equally applicable to HSV type 1 and type 2. Neither does AP7 appear to stabilize HSV-2 infectivity because the virus has the same inactivation kinetics in the presence and absence of antibody (Fig. 5). It appears that AP7 is capable of 'rescuing' non-infectious virus from populations of HSV-2 particles.
Fig. 5. Enhancement of infectivity by antibody AP7. Approximately 10⁴ p.f.u./ml HSV-2 strain 25766 was incubated at room temperature with AP7 ascites fluid (■) at a final dilution of 1/100 or with normal mouse serum at the same concentration (○). Triplicate 10 µl samples were removed at various times and assayed.

Table 3. Properties of monoclonal antibodies

| Antibody | Antibody group* | Amino acid substitution conferring resistance | Reaction with oligopeptide† | Competitive binding | Neutralization‡ | Antifusion activity§ | Plaque inhibition|| |
|----------|-----------------|---------------------------------------------|-----------------------------|---------------------|-----------------|---------------------|-------------------|
| LP14     | VII             | Arg (16) → His                               | Yes (7-23)                  | None                | Yes (20%)       | > 1/7290            | ~1/7290            | ~1/2700           |
|          |                 | (11-32)                                      |                             |                     |                 | <1/7290            |                   |                  |
| AP7      | ?               | Leu (25) → Pro                               | No (11-32)                  | None                | No              | (enhances HSV-2)   | None              | <1/100            |
|          |                 | (17-31)                                      |                             |                     |                 | (<1/30)            |                   |                  |
| AP12     | ?               | Ile (129) → Thr                              | No (124-136)                | LP2                 | No              | ~1/7290            | 1/900-1/2700       |                  |
| LP2      | I               | Ser (216) → Asn                              | No (206-226)                | AP12                | Yes             | ~1/7290 (0.2%)     | None              | <1/100            |

* Antibody groups as defined by Eisenberg et al. (1982).
† Ability of antibody to bind to an oligopeptide predicted to include the binding site on the basis of the site of the amino acid substitutions conferring resistance. The relevant oligopeptides are shown in parentheses.
‡ Highest dilution that gives significant (>50%) reduction in infectivity. Number in parentheses is the infectivity remaining at high antibody concentrations.
§ Highest dilution which gave a clear reduction in cell fusion.
|| Highest dilution that gave a reduction in plaque area of 50%.

DISCUSSION

The study reported here is limited to four monoclonal antibodies directed against glycoprotein D of HSV-1 and HSV-2, and many more antibodies must be incorporated into this study before broad generalizations can be made. Nevertheless our results are consistent with previous studies of the antigenic structure of gD and give some indication of the sites involved in the different biological functions of the molecule. Table 3 summarizes the properties of the antibodies used.
There is one previous report in which a mutation site has been identified in an attempt to localize the binding site of a gD monoclonal antibody. Rawls et al. (1984) examined a variant of HSV-1 that reacted with an HSV-2-specific antibody, 17 A3, and found that asparagine-72 had been substituted by histidine, the amino acid found at this position in HSV-2 gD. It is not surprising that this substitution is unrelated to those we have identified because the antibodies used in our study were all type-common.

Eisenberg et al. (1982) have classified gD antibodies into eight groups of which three, groups II, V and VII, react with continuous antigenic sites. Antibody LP14 has been placed in group VII and reacts with synthetic peptides 7 to 23 and 11 to 32. Mutants resistant to this antibody are substituted with histidine for arginine-16 and this alteration therefore lies within or very close to the antibody binding site. Studies of the solution structure of the 7 to 23 peptide of gD show the presence of two tight turns centred on residues Pro-14, Asn-15 and Arg-18, Gly-19 (Williamson et al., 1986). Hydrogen bonding between the Arg-16 NH-group and the Asp-13 side-chain probably contributes to the stability of the first of these turns, but there is no reason to suppose that the substitution of histidine for arginine-16 would alter this arrangement.

Antibody LP2 has been placed in group I, according to the classification of Eisenberg et al. (1982), but antibodies AP7 and AP12 have not been assigned to an antibody group, and the properties of these antibodies are not entirely consistent with any of the eight groups described by Eisenberg et al. The amino acid substitutions selected by these three antibodies are not particularly informative. The hydrophilicity profile of gD shown in Fig. 6 shows that none of these substitutions lies in hydrophilic regions of the polypeptide chain, and the regions in which these substitutions occur would not be predicted as potential antigenic sites on the basis of this analysis. This may reflect nothing more than the weakness of hydropathy analysis when applied to glycosylated proteins. Secondary structure predictions based on the rules devised by Robson & Suzuki (1976) and Garnier et al. (1978) give no strong indications of secondary structure around the AP7 or AP12 substitutions and predict $\beta$-sheet around the LP2 site. The Ser-16 to Asn change selected by LP2 does not alter the $\beta$-sheet prediction. AP7, AP12 and LP2 failed to react with oligopeptides that included the residue substituted in resistant mutants. This result was expected for LP2 because group I antibodies are thought to react with a discontinuous antigenic site (Eisenberg et al., 1982). Our results suggest that AP7 and AP12 are also directed against discontinuous epitopes. We can be confident that the position of the amino acid change

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Fig. 6. Positions of amino acid substitutions that confer antibody resistance in relation to hydrophilicity. Mean hydrophilicity values were determined for each hexapeptide using the hydrophilicity values for each residue given by Hopp & Woods (1981). The position of each amino acid substitution found in antibody-resistant mutants is shown. Residues are numbered from the N-terminal lysine.
conferring resistance to LP14 lies within or very close to the antibody binding site because LP14 reacts with an oligopeptide which includes arginine-16. The same is not true for the remaining three antibodies, and it is possible that each of the amino acid substitutions conferring resistance to AP7, AP12 and LP2 occurs at some distance from the corresponding antibody binding site, but modifies the site by allosteric effects within the molecule. Mutations that confer antibody resistance by long-range effects have been observed in poliovirus (Diamond et al., 1985) and tobacco mosaic virus (Al Moudallal et al., 1982), but in these instances protein–protein interactions play an important part in determining the secondary and tertiary structure of individual subunits. It seems unlikely that mutations could act at long range (i.e. modify tertiary structure) to alter antigenic sites without interfering with the functions of a viral glycoprotein and no firm example of such a mutation exists. Where clear-cut evidence is available for the relationship between mutation site and antibody binding site in a viral glycoprotein, the evidence supports the view that the resulting amino acid substitution acts only locally and lies within the antigenic site (Knossow et al., 1984, and the mutation to LP14 resistance described in this paper).

The results of competition binding assays are surprising in that LP14 and AP7 do not compete with each other despite the fact that mutations which conferred resistance to these antibodies resulted in amino acid substitutions separated by only nine residues (positions 16 and 25 respectively). Conversely AP12 and LP2 exhibited efficient heterologous competition, but resistance was conferred by amino acid substitutions separated by 87 residues (positions 129 and 216 respectively). The simplest interpretation of the efficient competition between AP12 and LP2 in binding assays is that amino acids 129 and 216 lie close together in native gD. While this interpretation is consistent with the conclusion that both antibodies recognize discontinuous epitopes, the very different biological effects of AP12 and LP2 are difficult to reconcile with the proposal that these antibodies bind to the same region of the gD molecule, and it is equally possible that the binding of either antibody results in allosteric changes which inhibit binding of the other.

The ability of the four antibodies examined to select amino acid substitutions at very different positions in the polypeptide chain is mirrored by the very different biological activities of these antibodies. Thus LP2 efficiently neutralizes but does not inhibit cell fusion, AP12 inhibits cell fusion but does not neutralize, while LP14 has both activities. AP7 has neither activity but neutralizes in the presence of complement and protects in vivo (Simmons & Nash, 1985). In addition AP7 has the unusual property of enhancing HSV-2 infectivity and is type-specific in this respect. Fuller & Spear (1985) reported a good correlation between dilution endpoints of neutralization and anti-fusion activities among a group of gD monoclonal antibodies and suggested that the neutralizing activity of these antibodies was a consequence of their anti-fusion activity. While the neutralizing activity of antibody LP14 may result from its anti-fusion activity, the properties of antibodies LP2 and AP12 indicate that anti-fusion activity is not a prerequisite for neutralization; nor is neutralization an inevitable consequence of anti-fusion activity. The conflict between our findings and those of Fuller & Spear may result partly from the problem of ranking antibodies according to their neutralizing activity. On the basis of dilution endpoint, preparations of antibody LP14 have higher neutralizing titres than preparations of LP2 (Fig. 3), but at high concentrations LP2 reduces virus infectivity far more efficiently than LP14. We do not know why this is so but, at least in the case of the antibodies that we have examined, the neutralizing potency of an antibody cannot be adequately described by a single criterion.

Although some gD monoclonal antibodies are capable of suppressing the syncytial phenotype, mutations that affect cell fusion have not been observed in the gD coding region. Mutations of this type occur in the gB coding sequence (Manservigi et al., 1977; Honess et al., 1980; Bzik et al., 1984) and in a recently sequenced open reading frame at map position 0-74 on the HSV-1 physical map (Bond & Person, 1984; Debroy et al., 1985). It is notable that those antibodies which suppressed the syncytial phenotype were also the most effective in inhibiting the enlargement of plaques (i.e. the spread of virus from infected to uninfected cells), and that this phenomenon was observed with syncytial and non-syncytial viruses. This suppression of
plaque formation is not due to neutralization of released virus because LP2 inhibits plaque formation poorly, while AP12, which does so efficiently, is non-neutralizing. The ability of some gD antibodies to inhibit plaque enlargement is most simply explained by supposing that gD is involved in the formation of intercellular bridges that allow passage of both syncytial and non-syncytial virus from infected to uninfected cells in the presence of neutralizing antibody. The formation of such bridges might be a pre-requisite for the extensive cell fusion seen in syncytial strains, and this would explain why those antibodies which inhibit plaque enlargement also inhibit cell fusion. This reasoning implicates gD in a function responsible for intercellular virus spread, but only indirectly in the expression of the syncytial phenotype. While our results support the view that gD is involved, albeit indirectly, in cell fusion, the properties of antibody LP2 imply that gD has at least one other function that is essential for virus infectivity.

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REFERENCES


Mutation to antibody resistance in HSV gD


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