Antigenic Analysis of a Major Neutralization Site of Herpes Simplex Virus Glycoprotein D, Using Deletion Mutants and Monoclonal Antibody-Resistant Mutants

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Herpes simplex virus glycoprotein D is a component of the virion envelope and appears to be involved in attachment, penetration, and cell fusion. Monoclonal antibodies against this protein can be arranged in groups on the basis of a number of biological and biochemical properties. Group I antibodies are type common, have high complement-independent neutralization titers, and recognize discontinuous (conformational) epitopes; they are currently being used in several laboratories to study the functions of glycoprotein D. We have used a panel of neutralization-resistant mutants to examine the relationships between these antibodies in detail. We found that they can be divided into two subgroups, Ia and Ib, such that mutations selected with Ia antibodies have little or no effect on binding and neutralization by Ib antibodies and vice versa. In addition, Ia antibodies are able to bind deletion and truncation mutants of glycoprotein D that Ib antibodies do not recognize, suggesting that their epitopes are physically distinct. However, with one exception, Ia and Ib antibodies block each other strongly in binding assays with purified glycoprotein D, whereas antibodies from other groups have no effect. We have therefore defined the sum of the Ia and Ib epitopes as antigenic site 1.

Glycoprotein D (gD) of herpes simplex virus (HSV) plays an important role in virus infection and pathogenesis. It is present on the surface of purified virions and infected cells (7, 34, 41, 42) and, since there have been no reports of viable mutants lacking a gD gene, appears to be essential for virus replication in tissue culture. Moreover, it is a target for humoral and cellular immune responses (10, 15, 22, 31, 38, 45) and protects animals against a lethal virus challenge (1, 3, 4, 8, 11, 26, 27, 35). Studies with monoclonal antibodies (MAbs) specific for gD have indicated possible roles in adsorption (16), penetration (17, 23), and cell fusion (23, 33). A detailed knowledge of the relationships between various antibodies would be useful for correlation of particular functions with distinct sites on the molecule and for comparison of results obtained in different laboratories. We have previously arranged anti-gD MAbs in several groups on the basis of various characteristic properties (12, 14). Three of the groups (II, V, and VII) recognize denatured gD and define continuous epitopes. Antibodies in groups I, III, IV, and VI bind only to native gD; they recognize discontinuous epitopes. Antibodies in group I bind to gD from HSV type 1 (HSV-1) and HSV-2 (type common), and have high complement-independent neutralization titers (13, 16, 25, 32, 37, 39). Some of them are able to inhibit cell fusion by syncytial strains of HSV-1 (23, 33). Group III antibodies are also type common but have low neutralizing activity (6). Antibodies in groups IV and VI are type 1 specific (6); group IV antibodies

have some neutralizing activity, whereas group VI antibodies do not (6).

An alternative method of grouping MAbs involves the selection of monoclonal antibody-resistant (i.e., neutralization-resistant) *mar* mutants (18, 24), which are then tested for reactivity with a panel of antibodies. Those antibodies that give a similar pattern of reactivity with the mutants are grouped together, with each group defining a distinct antigenic site on the target protein. Antibodies within a group may each recognize a unique epitope but, as these epitopes overlap, will block each other in binding assays. In contrast, there is usually no interference between antibodies in different groups. Examples of proteins studied in this way are influenza hemagglutinin (19) and HSV-1 gB (29) and gC (30).

We are now categorizing gD MAbs by this method. It has recently been shown that a selection of 11 such antibodies recognize a minimum of four antigenic sites on gD (23). We have concentrated on group I MAbs in this study, as their biological effects indicate that they bind to a functionally important region of gD. Previously, four antibodies (HD1, DL11, 114-4, and 174-1) were assigned to group I (6, 12, 13); because of their similar properties, another three (LP2, 4S, and D2) were included in this study. We found that at least six of the seven MAbs block each other in a nitrocellulose binding assay. We then selected a number of *mar* mutants with individual antibodies and tested them for binding and neutralization with all seven antibodies. The results show that MAbs grouped by our original criteria fall into two subgroups, each recognizing a cluster of overlapping epitopes. The two subgroups (Ia and Ib) can also be distinguished by their reactivity with gD deletion mutants. The detailed antibody relationships revealed by these assays will

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TABLE 1. Properties of group I anti-gD MAbs

Antibody	Type specificity	Neutralizing activity	Inhibition of fusion ^a	Inhibition of attachment ^b	Neutralization-resistant virus	Reference(s)
HD1	Common	High	ND ^c	ND	MarD.HD1	37
LP2	Common	High	No	ND	MarD.LP2	20, 32
DL11	Common	High	ND	ND	MarDL11	6
4S	Common	High	ND	ND	MarD.4S	39
D2	Common	High	Yes	Weak	MarD2.1	23, 25
III-114-4 ^d	Common	High	Yes	Moderate	ND	16, 17, 33, 36
III-174-1 ^d	Common	High	Yes	Weak	ND	16, 17, 33, 36

^a For D2, 114-4 and 174-1, fusion was assayed by penetration (i.e., virus-cell fusion) and by syncytium formation (i.e., cell-cell fusion). For LP2, only the latter assay was used.

^b Attachment of radiolabeled virus to cell monolayers at 4°C.

^c ND, Not done.

^d Referred to as 114-4 and 174-1, respectively, throughout this article.

be of value in determining the role of gD in HSV infections, as well as providing further information about its antigenic structure.

MATERIALS AND METHODS

Cells and virus strains. CV1 cells were grown in Eagle minimal essential medium supplemented with 5% fetal calf serum. All virus stocks were grown and titers were determined with these cells. COS-1 cells were grown in Dulbecco modified minimal essential medium containing 10% fetal calf serum. Each *mar* mutant virus was selected for resistance to one neutralizing MAb. MarD.LP2 and MarD2.1 have been described previously (23, 32). MarD.HD1 and MarDL11 were selected by three successive neutralization steps, each time by using virus surviving the previous step, then plaque purified twice. MarD.4S was isolated by a similar procedure, but with two additional neutralization steps that included guinea pig complement. The relationships between parent and *mar* mutant strains of virus are as follows. MarD.4S was derived from the parent strain KOS, MarDL11 was derived from the parent strain Patton, MarD.LP2 (32) was derived from the parent strain HFEM, and MarD2.1 (23) and MarD.HD1 were derived from the parent strain KOS321. The antibody used to select each mutant is included in its name.

Antibodies. The properties and origins of the seven MAbs in group I are presented in Table 1. HD1 was kindly provided by Lenore Pereira, 4S was provided by Martin Zweig, and III-114-4 and III-174-1 were provided by Pat Spear. Throughout this paper, III-114-4 and III-174-1 will be abbreviated to 114-4 and 174-1, respectively. MAbs 1D3 and DL6 recognize continuous epitopes of gD-1 (residues 11-19 and 272-279, respectively, of the mature protein) (5, 9, 14). MAb 1C8 is specific for glycoprotein C (gC) of HSV-1 (40).

Construction of gD expression vectors and deletion mutants. The construction of vectors expressing wild-type or mutant forms of gD from the Patton strain of HSV-1 has been described previously (7a). Plasmid pRE4 expresses wild-type protein, pWW17 lacks the sequence coding for amino acids 234 to 244, and pDL24 lacks the sequence coding for amino acids 243 to 286.

Preparation of extracts of infected cells. CV1 cells in T75 tissue culture flasks were infected with parent or mutant virus at 1 PFU per cell. After incubation at 37°C for 18 h, the cells were scraped into 5 ml of phosphate-buffered saline and pelleted by low-speed centrifugation. The pellet was suspended in 10 mM Tris hydrochloride (pH 7.5)–10 mM NaCl–3 mM MgCl₂, followed by the additions of Nonidet P-40 to

1% and sodium deoxycholate to 0.5%. Nuclei were removed by low-speed centrifugation, and the supernatant (cytoplasmic extract) was frozen at –20°C.

DNA transfection and preparation of transfected-cell extracts. COS-1 cells were transfected by the calcium phosphate coprecipitation procedure (21), and cytoplasmic extracts were prepared 48 h later.

Iodination of MAbs. Iodination of antibodies HD1 and DL11 was performed by the iodobead procedure (Pierce Chemical Co.) (28).

Blocking assays. Blocking (competition) assays were performed as previously described (14). Briefly, immunoaffinity-purified gD was spotted on nitrocellulose strips in two-fold serial dilutions, from 16 to 2 ng. The strips were incubated with a protein-blocking solution to minimize non-specific binding (5) and then incubated with an unlabeled MAb. After being washed extensively, the strips were incubated with iodinated antibody (2.5×10^5 cpm), and the spots were located by autoradiography. Binding was quantitated by scanning densitometry and analyzed by linear regression to determine ¹²⁵I bound per nanogram of gD. Percentage blocking was calculated according to the following formula:

$$\text{blocking} = \left(1 - \frac{^{125}\text{I bound/nanogram in presence of test MAb}}{^{125}\text{I bound/nanogram in presence of MAb 1C8}} \right) \times 100$$

Native gel electrophoresis and Western blotting (immuno-blotting). The native polyacrylamide gel system and Western blotting procedure have been described previously (6). All gels used in this study contained 10% acrylamide–0.1% bisacrylamide (separating gel) and 5% acrylamide–0.3% bisacrylamide (stacking gel).

Antigenic analysis of mutants. Cytoplasmic extracts of infected or transfected cells were electrophoresed on native gels with no comb and transferred to nitrocellulose. The nitrocellulose sheets were incubated with a protein-blocking solution (5) and then cut into strips of equal width. These were incubated sequentially with a MAb (1 h at room temperature [RT]) and ¹²⁵I-protein A (1 h at RT), followed by autoradiography. Radioactive bands were cut out, with the autoradiograph as a guide, and counted in a gamma counter. Because nonspecific binding varied between antibodies, background counts were determined for each strip individually. Binding to each *mar* mutant was then expressed as a percentage of binding to its parent. Binding to the deletion mutants was compared with binding to wild-type gD from the vector pRE4. The relative amounts of gD in the extracts

TABLE 2. Analysis of competition between group I antibodies^a

Cold antibody	% Competition against MAb ^b :	
	HD1	DL11
HD1	100	100
LP2	100	98
DL11	99	100
4S	66	100
D2	79	88
114-4	94	99
174-1	28	41

^a Serial dilutions of gD-1 were spotted on nitrocellulose and then incubated with a cold MAb followed by an iodinated MAb. Binding of the second MAb was quantitated by scanning densitometry of the autoradiogram. Calculation of the percentage competition is described in Materials and Methods.

^b Iodinated MAbs.

were determined by probing the strips with MAbs DL6 or 1D3, which bind equally to mutant and parent forms, and the other ratios were normalized to these values.

Neutralization of *mar* mutants. Ascitic fluid of each MAb was incubated at various dilutions (from 1 in 250 to 1 in 5,000) with 10⁴ PFU of the four parent viruses. Incubation was for 2 h at RT in a total volume of 100 μ l. The virus was then titrated on CV1 cells to determine the amount of antibody required to reduce the titer to between 0 and 10 PFU. These dilutions (one for each combination of MAb and parent virus) were then tested for neutralization of the respective *mar* mutants.

RESULTS

Properties of MAbs. Mutant forms of gD were analyzed with a panel of seven MAbs, which were isolated in six different laboratories. These antibodies share various properties (Table 1), the combination of which distinguishes them from other anti-gD MAbs, and are referred to as group I.

Blocking experiments. Our grouping of anti-gD MAbs is based largely on their biological properties. To show that this is a reflection of clustered binding sites, we tested the antibodies for their blocking ability in nitrocellulose binding assays (Table 2). Serial dilutions of purified gD-1 were spotted on strips of nitrocellulose and incubated with an unlabeled MAb. After being washed to remove unbound antibody, the strips were incubated with an iodinated MAb, either HD1 or DL11. To allow for nonspecific blocking, one strip was incubated with MAb 1C8, which is specific for HSV gC-1. The maximum binding of iodinated MAb (i.e., no blocking) was therefore defined as being the amount bound in the presence of 1C8. Binding was quantitated by scanning densitometry of autoradiographs, and the percentage of blocking was determined by linear regression analysis.

Six of the seven MAbs blocked the binding of DL11 and HD1 to gD-1 by 66% or more, at dilutions ranging from 1 to 15 μ l/ml. The exception was 174-1, which at 20 μ l/ml blocked HD1 by only 28% and DL11 by only 41%. In light of subsequent results with antigenic variants of gD, we think that the lower level of blocking by 174-1 has a technical cause, such as a low concentration of antibody in the ascitic fluid. Several MAbs from other groups were also tested; none of them significantly affected the binding of DL11 or HD1 (data not shown). The results of the assay are therefore in agreement with our previous grouping. Since the group I MAbs have clustered binding sites on gD, we will refer to this region of the protein as antigenic site I.

Neutralization of *mar* mutants. Epitopes within an antigenic site may be adjacent or overlapping. To investigate the

FIG. 1. Neutralization of *mar* mutants by group I MAbs. Mutant viruses selected for resistance to individual neutralizing MAbs were tested for sensitivity to a panel of group I MAbs. In each case, 10⁴ PFU of virus was incubated with an amount of antibody sufficient to reduce the titer of the parent strain from 10⁴ PFU to between 0 and 10 PFU (i.e., 3 to 4 logs).

relationships between the seven group I epitopes, we used a panel of five *mar* mutants, each selected for resistance to neutralization by a group I MAb (Table 1). Two antibodies are likely to have overlapping epitopes if a mutation selected with one of them abolishes neutralization by the other. Therefore, we tested each mutant for resistance to neutralization by each MAb. Since the neutralization titer varies between antibodies, we first determined the dilutions of each antibody that would reduce the titers of the parent strains of virus by a factor of 10³ to 10⁴ (i.e., from 10⁴ PFU to between 0 and 10 PFU) when incubated for 2 h at RT (data not shown). The same dilutions were then tested with 10⁴ PFU of the *mar* mutants. By titrating the infectious virus remaining after incubation with antibody, we examined the ability of each *mar* mutant to escape neutralization by an amount of antibody able to neutralize its parent strain.

The results are summarized in Fig. 1, which shows the reduction in titer for each combination of MAb and mutant. A corresponding figure for the parent strains of virus would show filled black circles for each combination. By comparing the vertical patterns, subgroups of antibodies can be identified. Antibodies within a subgroup have similar, but not necessarily identical, patterns. HD1 and LP2 have the same pattern of neutralization with the five mutants, strongly suggesting that they recognize identical or overlapping epitopes on gD. 174-1, DL11, 4S, and D2 form a second group with overlapping epitopes; their reactions with MarD2.1 indicate that they recognize at least three different epitopes. The neutralization pattern obtained with 114-4 is unlike those of the other antibodies. However, its failure to neutralize MarD2.1 suggests that the 114-4 and D2 epitopes overlap, and this is confirmed by the binding experiments described in the next section.

Binding of antibodies to gD from *mar* mutants. If antigenic site I is a topographically distinct region of gD, then the mutations selected with group I MAbs should not affect recognition by antibodies in other groups. Because non-group I MAbs have low activity in our neutralization assay, we used a binding assay to test this prediction. Infected-cell extracts were electrophoresed on combless native polyacrylamide gels (to minimize the denaturation of discontinuous

FIG. 2. Binding of MAb 4S to gD on Western blots. Cytoplasmic extracts of cells infected with parent (P) or mutant (M) strains of HSV-1 were electrophoresed on nondenaturing polyacrylamide gels, transferred to nitrocellulose, and probed with one of the group I MAb followed by 125 I-protein A.

epitopes) and transferred to nitrocellulose. Strips were cut from the nitrocellulose sheet, incubated with a MAb followed by 125 I-protein A, and then autoradiographed. We found that binding of group III and VI antibodies to the mutants is not diminished relative to binding to the parents (data not shown). Therefore, antigenic sites III and VI do not appear to overlap with site I.

This Western blot binding assay provided additional information about relationships between the seven group I antibodies. Figure 2 shows autoradiographs obtained with the 4S, DL11, and D2 mutants and their respective parents. MarD.4S (panel A) showed greatly reduced binding with four of the antibodies (4S itself, D2, DL11, and 174-1), which suggests that they have overlapping epitopes. Of the three remaining antibodies, HD1 and LP2 recognized the mutant and its parent equally well and so are not affected by the mutation. In contrast, the binding of 114-4 was increased more than twofold. In the context of this assay, any alteration in binding (an increase or decrease) is indicative of overlapping epitopes, so this is additional evidence that 114-4 should be assigned to the subgroup comprising 4S, D2, DL11, and 174-1. The spectrum of activity of MarDL11 (panel B) was similar to that of MarD.4S, as in the neutralization assay, which suggests that they may have the same

FIG. 3. Binding of MAb 174-1 to gD on Western blots. Cytoplasmic extracts of cells infected with parent (P) or mutant (M) strains of HSV-1 were electrophoresed on nondenaturing polyacrylamide gels, transferred to nitrocellulose, and probed with one of the group I MAb followed by 125 I-protein A.

mutation. MarD2.1 (panel C) was somewhat different, reacting with DL11 and 4S, as well as HD1 and LP2, but not with 114-4.

The analysis of MarD.HD1 and MarD.LP2 is shown in Fig. 3. For six of the MAb, the data agree with the results from the neutralization assay; 174-1, DL11, 4S, D2, and 114-4 bound to both mutants, whereas LP2 bound to neither. However, HD1 did bind to both mutants, including the one it selected, although it neutralized neither of them. To discover whether this binding was an artifact of the nitrocellulose assay, binding of HD1 to MarD.HD1 virus was examined. MarD.HD1 (10^4 PFU) was incubated for 2 h at RT, either alone or with a 1/200 dilution of HD1, followed by 1 h at RT in the presence of DL11 at a 1/500 dilution. The virus was then plated on CV1 cells, which were examined for cytopathic effect 2 days later. Preincubation in the absence of antibody had no effect on subsequent neutralization by DL11, whereas preincubation with HD1 blocked DL11-mediated neutralization. This result confirms that HD1 binds to gD of MarD.HD1 virus and also shows that the nitrocellulose blocking assay (in which binding of iodinated DL11 was blocked by HD1) is a reflection of binding to gD in virions.

Quantitation of the Western blot binding assay, as described in Materials and Methods, enabled binding percentages to be determined for each combination of mutant or parent and MAb. Variations in the amount of gD in each extract were taken into account by normalizing the percentages to those obtained with the group II MAb DL6; this reacts with a continuous epitope downstream from the group I binding region and so binds equally to each mutant and parent. Thus, after normalization, an antibody that reacts equally with a mutant and its parent would have a value of 100%. The data, summarized in Fig. 4, together with the neutralization results (Fig. 1), support the division of group I antibodies into two subgroups, Ia (HD1 and LP2) and Ib (174-1, DL11, 4S, D2, and 114-4).

Binding to deletion mutants. We have previously described a plasmid, pRE4, containing a gD-1 gene under the transcrip-

FIG. 4. Quantitation of the binding of group Ia and Ib MAbs to mutant forms of gD. Bands shown in the autoradiograms in Fig. 2 and 3 were excised and counted in a gamma counter. Binding of a MAb to a mutant was expressed as a percentage of binding to the corresponding parent strain. Because the extracts did not contain identical amounts of gD, the percentages were normalized to those obtained with MAb DL6, which binds equally to each parent and mutant. The normalized values are represented in the figure.

tional control of a Rous sarcoma virus long terminal repeat promoter (7a). The gD protein expressed after transfection of this plasmid into COS-1 cells is antigenically indistinguishable from gD produced in HSV-1-infected cells. We found that deletion of the coding sequences for amino acids 234 to 244 or 243 to 286 of gD (plasmids pWW17 and pDL24, respectively) has a profound effect on the binding of MAb DL11 (Ib). However, binding of group III and group VI antibodies is not reduced, suggesting that the overall conformation of the protein is not affected by the deletions. We have now tested these deletion mutants with the two Ia MAbs (HD1 and LP2) and with two other Ib MAbs (4S and D2). Plasmids were transfected into COS-1 cells, and cytoplasmic extracts were prepared 48 h later. These were analyzed by the native gel-Western blot procedure (Fig. 5), as described for infected-cell extracts. MAb 1D3 was used to ensure that equal amounts of gD were run on the gels, as the epitope for DL6 is missing from the pDL24 mutant. The 1D3 epitope is at the N-terminal end of gD (residues 11 to 19) (5, 9). All five group I antibodies reacted with gD from pRE4, as expected, but only HD1 and LP2 reacted well with gD from the deletion mutants pWW17 and pDL24. Binding of DL11, 4S, and D2 to the mutants was greatly reduced or abolished. These results suggest that the site recognized by the Ia antibodies is independent of amino acids on the C-terminal side of residue 233, whereas residues between 234 and 244 and between 243 and 286 contribute to the Ib binding site.

DISCUSSION

We have previously arranged anti-gD-1 MAbs into seven groups, of which three recognize continuous epitopes (II, V, and VII) and four recognize discontinuous epitopes (I, III, IV, and VI) (12-14). The epitopes for antibodies in groups II, V, and VII have been mapped precisely by using synthetic peptides (4, 5, 9, 14). This approach is not applicable when locating discontinuous epitopes because they consist, by definition, of noncontiguous amino acids brought together by

FIG. 5. Western blot analysis of gD deletion mutants. COS-1 cells were transfected with plasmids expressing wild-type or deletion mutant forms of gD. Cytoplasmic extracts were electrophoresed on nondenaturing polyacrylamide gels, transferred to nitrocellulose, and probed with one of the group I MAbs followed by 125 I-protein A. (1) pRE4, wild type; (2) pWW17, amino acids 234 to 244 deleted; (3) pDL24, amino acids 243 to 286 deleted.

folding of the protein. To study discontinuous epitopes, a nondenaturing (native) polyacrylamide gel system was developed (6); when coupled with Western blotting, this procedure allows MAbs that recognize discontinuous epitopes to bind to gD on nitrocellulose sheets. Using this system, we found that DL11, a representative of the group I MAbs, can bind to a truncated form of gD that consists of residues 1 to 275. However, it cannot bind to a similar mutant that ends at residue 233, suggesting that its epitope includes some amino acids between 233 and 275 (6).

DL11 is one of seven anti-gD MAbs that we currently classify as belonging to group I on the basis of their similar biological and biochemical properties. To find out if the epitopes for these antibodies are clustered on gD, we examined their ability to block each other in a nitrocellulose binding assay. Apart from 174-1, all of the MAbs strongly blocked the binding of iodinated HD1 and DL11. The failure of 174-1 to cause significant blocking could be due to a low concentration of antibody in the ascitic fluid; 174-1 gave a much weaker signal with wild-type gD on Western blots than did the other MAbs, unless it was used at a higher concentration. There was little blocking with MAbs from other groups. This experiment therefore supports our belief that the antibodies within our previously defined groups have clustered epitopes.

Relationships between groups of MAbs, and therefore of the epitopes that they recognize, can be deduced from studies of antibody recognition of mutants. One type of mutant that has been especially valuable for analyses of this sort is the *mar* mutant, an antigenic variant selected for resistance to neutralization. This technique, introduced by Gerhard and Webster (18), has been used to study glycoproteins from several viruses, including gB-1 (29), gC-1 (30), and, more recently, gD-1 (23) of HSV. MAbs with similar patterns of reactivity with the mutants have overlapping epitopes and define an antigenic site. With the influenza virus hemagglutinin, for which a three-dimensional structure has been obtained (43, 44), the five antigenic sites correspond to topographically distinct regions of the protein (2).

We have analyzed HSV *mar* mutants selected with five of the seven group I MAbs, using a combination of neutralization and binding assays. We found that the antibodies can be divided into two subgroups, comprising HD1 and LP2 (group Ia) and DL11, 4S, D2, 114-4, and 174-1 (group Ib). A previous analysis of gD *mar* mutants suggested that the 4S and D2 epitopes are in different antigenic sites (23). The data from that study agree with those presented here, but their interpretation was limited by the availability of only one

group I-selected mutant. The epitopes recognized by the members of a subgroup presumably overlap, though not necessarily in every combination. We do not know if there is any overlap between the corresponding antigenic subsites, Ia and Ib. Perhaps this could be determined by the isolation and analysis of more mutants or with additional group I antibodies. However, since HD1 and LP2 (Ia) can block the binding of DL11 (Ib) and at least four members of group Ib can block binding of HD1, we feel that all seven antibodies recognize a single antigenic site, which is distinct from the sites recognized by antibodies in groups III, IV, and VI.

MarD.HD1 is a particularly interesting mutant, in that it retains the ability to bind HD1. This might be construed as an indication that the mutation is not within the HD1 epitope. Alternatively, binding of HD1 to MarD.HD1 (and to MarD.LP2) may be altered in a subtle way that abolishes neutralization.

Knowledge of the relationships between the seven MABs discussed here will help us to interpret experiments in which they are used to elucidate the functions of gD. Moreover, sequencing of the mutant genes will identify some of the amino acids that constitute antigenic site I. The finding that *mar* mutations selected in essential proteins are almost always caused by single-base-pair substitutions holds true for MarD.LP2, in which a G-to-A transition converts serine-216 to asparagine (32). However, the observation that the amino acid sequence of gD is highly conserved between isolates suggests that this approach to mapping may be limited. It suffers from the precondition that only those mutations that do not destroy the functions of gD can be isolated. There may be little scope for such viable mutations.

An alternative approach to the mapping of this major neutralization site is therefore to construct mutant genes in vitro and examine the antigenicity of the resulting proteins after expression in a transient transfection system. So far, we have examined two mutants made in this way. Both had deletions, one from amino acids 234 to 244 inclusive, the other from amino acids 243 to 286, and both retained the discontinuous epitopes recognized by MABs in groups III and VI (7a). However, of the group I antibodies, only those designated Ia in the *mar* mutant assays recognized both mutants normally, suggesting that residues downstream of 233 are involved in the Ib recognition site. This result is consistent with our finding that DL11 binds to a 1 to 275 fragment of gD but not to a fragment from amino acids 1 to 233 (6). In addition, we have found that HD1 and LP2, but none of the Ib MABs, bind to the 1 to 233 fragment on Western blots (data not shown). Subdivision of the group I MABs is therefore supported both by the *mar* mutants and the deletion mutants. However, the question of whether there is an overlap between the Ia and Ib sites has not been resolved, since both could involve amino acids upstream of 233.

The two deletion mutants described here were constructed by using convenient restriction enzyme sites in the gD gene. We now intend to make smaller deletions, using site-directed mutagenesis, to identify those residues involved in site Ib.

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