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Genetic Analysis of Type-Specific Antigenic Determinants of Herpes Simplex Virus Glycoprotein C

KAREN E. DOLTER,[1]† WILLIAM F. GOINS,[2] MYRON LEVINE,[3] AND JOSEPH C. GLORIOSO[2]*

Department of Microbiology and Immunology[1] and Department of Human Genetics,[3] The University of Michigan Medical School, Ann Arbor, Michigan 48109, and Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261[2]

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Herpes simplex virus type 1 (HSV-1) glycoprotein C (gC-1) elicits a largely serotype-specific immune response directed against previously described determinants designated antigenic sites I and II. To more precisely define these two immunodominant antigenic regions of gC-1 and to determine whether the homologous HSV-2 glycoprotein (gC-2) has similarly situated antigenic determinants, viral recombinants containing gC chimeric genes which join site I and site II of the two serotypes were constructed. The antigenic structure of the hybrid proteins encoded by these chimeric genes was studied by using gC-1- and gC-2-specific monoclonal antibodies (MAbs) in radioimmunoprecipitation, neutralization, and flow cytometry assays. The results of these analyses showed that the reactivity patterns of the MAbs were consistent among the three assays, and on this basis, they could be categorized as recognizing type-specific epitopes within the C-terminal or N-terminal half of gC-1 or gC-2. All MAbs were able to bind to only one or the other of the two hybrid proteins, demonstrating that gC-2, like gC-1, contains at least two antigenic sites located in the two halves of the molecule and that the structures of the antigenic sites in both molecules are independent and rely on limited type-specific regions of the molecule to maintain epitope structure. To fine map amino acid residues which are recognized by site I type-specific MAbs, point mutations were introduced into site I of the gC-1 or gC-2 gene, which resulted in recombinant mutant glycoproteins containing one or several residues from the heterotypic serotype in an otherwise homotypic site I background. The recognition patterns of the MAbs for these mutant molecules demonstrated that (i) single amino acids are responsible for the type-specific nature of individual epitopes and (ii) epitopes are localized to regions of the molecule which contain both shared and unshared amino acids. Taken together, the data described herein established the existence of at least two distinct and structurally independent antigenic sites in gC-1 and gC-2 and identified subtle amino acid sequence differences which contribute to type specificity in antigenic site I of gC.

Glycoprotein C (gC) is one of numerous glycoproteins found on the surface of herpes simplex virus (HSV)-infected cells and within the viral envelope. Although this glycoprotein is not essential for virus production in cell culture (36), gC serves multiple accessory functions, including the ability to bind the C3b component of complement (8, 10, 31) and to function in virus attachment to cells upon infection via heparan sulfate moieties on the cell surface membrane (18, 28). Glycoprotein C is also a major viral antigen which elicits a strong humoral and cellular immune response during infection and plays an important role in the induction of herpetic eye disease in animal models of herpesvirus keratitis (4, 17, 48).

Although many antibodies against HSV antigens react with both serotypes (HSV-1 and HSV-2), antibodies to gC are predominantly serotype specific (1, 6, 26, 33, 34, 41), although cross-reactive antibodies do exist, particularly in polyclonal antisera (8, 51). Therefore, this antigen appears to play an important role in defining type specificity of the virus. The type-specific antibody response to gC correlates with the 69% amino acid homology between gC-1 and gC-2 (5, 45), compared with the much greater homology (85%) found among other highly immunogenic HSV glycoproteins (2, 46, 47). The ability of gC-1 and gC-2 to elicit type-specific antibody responses could be due to antibody recognition of (i) structurally distinct and noncolinear regions of the two molecules or (ii) regions that are similar and colinear but contain key amino acid differences which contribute to epitope structure and determine antibody specificity.

Our laboratory previously reported on mutations which alter the antigenic structure of gC-1, resulting in a loss of recognition by some antibodies but not others (20, 30, 50). These mutations were uncovered by using a panel of monoclonal antibodies (MAbs) to select a series of neutralization escape variants referred to as MAb-resistant (*mar*) mutants (20). DNA sequencing and protein analyses demonstrated that the majority of mutant proteins contained single amino acid substitutions (19, 24, 50). Some mutants, however, lacked gC in the virus envelope as a result of the presence of chain-terminating mutations and were therefore designated gC^- rather than *mar* mutants (19, 24). Analysis of the recognition patterns of MAbs with the *mar* mutants in neutralization assays revealed nine unique reactivity patterns. These functionally defined epitopes could be segregated into two distinct antigenic sites, each composed of multiple overlapping epitopes. Antibody binding competition assays confirmed the existence of two physically separate antigenic regions of the molecule, designated antigenic sites I and II (19, 24, 30). Immunoprecipitation studies using chain-terminating mutants that express truncated gC molecules of various lengths provided evidence that these sites represent the physical antigenic determinants of gC which directly interact with the specific MAbs. Site II maintained

* Corresponding author.
† Present address: Department of Immunology and Microbiology, Wayne State University School of Medicine, Detroit, MI 48201.

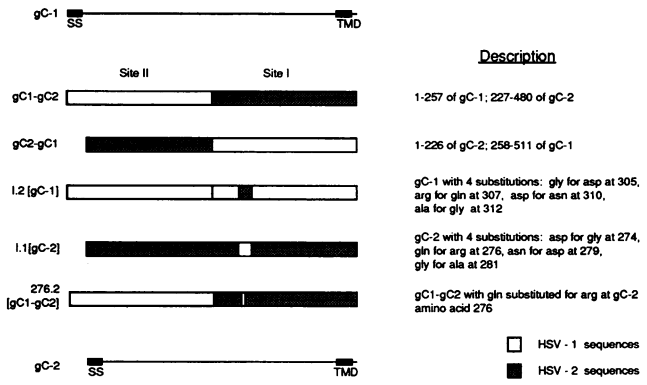

FIG. 1. Diagram of intertypic recombinant proteins. The serotype composition of each gC mutant is shown. Diagrams of gC-1 and gC-2 with their amino-terminal signal sequences (SS) and carboxy-terminal transmembrane domains (TMD) are included. Site II is currently defined as lying between residues 129 and 247, and site I is found between residues 307 and 373 (50).

its antigenic structure in the absence of the carboxy-terminal half of the molecule and therefore resides in the amino-terminal portion of gC-1. DNA sequencing of the *mar* mutant gC genes identified mutations in codons for amino acids between residues 307 and 373 for site I mutants and between 129 and 247 for site II mutants (50). These results and the later finding that a *mar* mutation in site I resulted in epitope seroconversion (25), that is, the gC-1 *mar* mutant had acquired the ability to react with a gC-2-specific MAb, suggested the possibility that both gC molecules contain two similar antigenic sites. Moreover, the sites might be localized to colinear regions of the two molecules wherein subtle amino acid sequence differences create unique but overlapping type-specific epitopes. This idea was supported by a previous example of a type-specific epitope in glycoprotein D containing a single type-specific amino acid reported by Rawls et al. (35).

This report confirms that the two dominant antigenic regions of gC-1 are distinct and that gC-2 also contains two similar antigenic regions. A more extensive genetic analysis of site I has further demonstrated that type-specific antibodies recognize individual amino acids in an environment of extensive homology between the two serotypes. It is concluded that at least for site I, amino acid sequence rather than conformational differences account for the unique and distinct antigenic character of gC-1 and gC-2.

MATERIALS AND METHODS

Plasmids. Plasmids were propagated in the DH5α strain of *Escherichia coli* and were isolated by lysis with sodium dodecyl sulfate (14). Bacteria were transformed by standard methods (3). Transformants were analyzed by restriction enzyme digestions of DNA from quick plasmid preparations (minipreps) adapted from the boiling method (22). The gC-1 gene was subcloned as a 3.4-kb *Pst*I-*Hin*dIII fragment from pGC (23). The gC-2 gene was subcloned as a 4.8-kb *Aat*II fragment from pTYL302 (which contains the *Bam*HI A fragment from strain 333 of HSV-2) (52). Each of these fragments was subcloned into pBS+ (Stratagene) to create pgC1PH and pgC2Aat, respectively.

Site-directed mutagenesis was performed by the method of Kunkel et al. (27). Mutant plasmids were detected by restriction enzyme digestion of miniprep DNA (22) followed by electrophoresis on 1 to 2% agarose gels. Some mutations caused changes in the sizes of small restriction fragments which were readily detectable on 5% polyacrylamide gels. When mutants were present at low frequencies, they were screened by colony hybridization (16) with the appropriate mutant oligonucleotide probe labeled with ^{32}P, using T4 polynucleotide kinase (29). Tetramethylammonium chloride was used for the filter washes to eliminate the effects of GC content on hybridization (49). The point mutations were confirmed by dideoxy DNA sequencing (38) of the mutant plasmids. Schematic diagrams of the proteins encoded by the mutant genes are shown in Fig. 1.

Construction of gC heterotype chimeric genes. pgC1K and pgC2K were derived from pgC1PH and pgC2Aat, respectively, by using site-directed mutagenesis to introduce a *Kpn*I site. This site is located at codon 257 in gC-1 and codon 226 in gC-2. The mutations were designed to cause no change in amino acid sequence for either molecule. This restriction site was introduced in order to divide the two gC genes into two halves, corresponding to the antigenic sites previously defined in gC-1. The chimeric plasmid pgC1-gC2 was constructed by inserting the *Ssp*I-*Kpn*I fragment of pgC2K into the *Eco*RV-*Kpn*I sites of pgC1K. Plasmid chimera pgC2-gC1 was produced by inserting the *Sal*I-*Kpn*I fragment of pgC2K into the *Xho*I-*Kpn*I sites of pgC1K. pGC::gC2-gC1 was created by insertion of the *Pst*I-*Hin*dIII fragment of pgC2-gC1 into pGC (23) to provide 1.3 kb of upstream sequence in order to facilitate recombination of the chimeric gC2-gC1 construct into the HSV-1 genome. Diagrams of the proteins which these chimeric genes encode are shown in Fig. 1.

MAbs. MAbs used were described previously (25, 30). III188-4 was kindly provided by P. Spear (33), and MP1, MP2, and MP5 were the generous gifts of G. Cohen (40).

Cells and viruses. Vero and HEL cells, HSV-1 (strain KOS), and HSV-2 (strain 333) were propagated as described previously (20). Viral DNA was isolated from lysates of infected cells or virions (37).

Construction and isolation of recombinant viruses. Recombination of mutated genes into the viral genome was accomplished by cotransfection of Vero cells with viral and plasmid DNA by the calcium phosphate technique (15). Recombinant viruses were isolated in a number of ways. When possible, transfection progeny were neutralized with antibody specific for gC of the parental virus (C11 or C2-5) to provide a growth advantage to recombinants. This was feasible because our previous results (25) demonstrated that MAb C11 binding requires glutamine at amino acid 307 of gC-1 and MAb C2-5 binding requires arginine at amino acid 276 of gC-2. Antibody was used at a dilution of 1:50 or 1:100 in the presence of 10% rabbit complement. Enrichment for the recombinant viruses was based on resistance to neutralization in three successive rounds of antibody treatment and subsequent virus propagation. Hybridization of radiolabeled probes to DNA from virus isolates on dot blot filters (29) was used when the mutated gene was introduced into a gC^- virus. Immunoreactive black plaque assays (21) were used when reactivity with a particular MAb could be predicted. A recombinant expected to react with a MAb which does not react with the parental virus could be identified by production of black plaques in this assay. Introduction of the chimeric genes into the virus genome resulted in larger gC-specific restriction fragments which could be detected in Southern blots (44). Substitution of sequences also resulted in unique patterns of restriction enzyme digestion. For the point mutations, loss or gain of some restriction enzyme

sites was also detected by Southern blotting. Radioactive probes for dot blots and Southern blots were created by labeling gel-purified restriction fragments, using the Random Primed DNA Labeling Kit (Boehringer Mannheim) (9).

Radiolabeling of viral proteins, immunoprecipitation, and polyacrylamide gel electrophoresis. HEL cells were infected at a multiplicity of infection of 10 and labeled with 40 μCi of [^{35}S]cysteine per ml in Cys-free minimum essential medium at 5 to 7 h postinfection. At 16 to 24 h postinfection, cell extracts were prepared as described previously (20). Immunoprecipitation (20) was carried out for 4 to 18 h at 4°C with MAbs (5 μl of ascites fluid for gC-1-specific MAbs and 10 μl for gC-2-specific MAbs). Immunoprecipitates were bound to protein A-Sepharose CL-4B (Sigma Chemical Co.) by shaking for 1 h at 4°C, washed five times in lysis buffer, and resuspended in 2× electrophoresis sample solution (20). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of samples followed by fluorography was performed as described earlier (20).

Virus neutralization assays. Neutralization assays (20) were performed with five 10-fold dilutions of individual MAbs in the presence of 10% rabbit complement. Viruses were incubated in this solution at 37°C for 2 to 3 hours. The surviving virus was quantitated by infection of Vero cell monolayers in 24-well trays overlaid with 0.5% methylcellulose containing 2% newborn calf serum and subjected to crystal violet staining once plaques formed. Neutralization was performed in triplicate and was considered positive when a reduction in virus titer of at least 50% was observed for all three trials.

Flow cytometry. Flow cytometry was performed with a Coulter Epics C 985 (25). Vero cell monolayers were infected at a multiplicity of infection of 10. Cells were harvested at 10 to 12 h postinfection by scraping and divided into aliquots of approximately 10^6 cells, which were kept on ice throughout the procedure. The cells were washed between each step with Hanks' balanced salt solution (pH 7.5) (GIBCO Laboratories) with 3% fetal calf serum and 0.04% sodium azide. MAbs were bound to cells by incubation for 30 min with 100 μl of ascites fluid diluted 1:100. An isotype-matched irrelevant antibody was used as a negative control. This mouse anti-rat antibody did not bind Vero cells, regardless of whether or not they were infected. One microgram of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Sigma Chemical Co.) was added to the cells for 20 min. Finally, the cells were fixed with 1% formaldehyde before analysis of membrane fluorescence.

RESULTS

Isolation and characterization of viruses expressing chimeric gC molecules. Previous results with gC-1 truncation mutants demonstrated that the N-terminal antigenic site, site II, retained antibody binding when separated from the C-terminal site I (30). One goal of these studies was to determine whether site I would also remain antigenically active when separated from site II of gC-1, which would demonstrate that both sites are structurally independent. If the sites are indeed independent, then the epitope structure of site I would not depend on the presence of the N-terminal portion of the molecule and vice versa. The experimental approach was to produce and study two chimeric gC gene products in which the N-terminal half of gC-1 was joined in frame to the C-terminal half of gC-2 (gC1-gC2) and the N-terminal half of gC-2 was joined in frame to the C-terminal half of gC-1 (gC2-gC1). Besides a difference in length of 31 amino acids and some sequence divergence near the N terminus, gC-1 and gC-2 are similar in sequence and hydrophobicity profiles (5), implying similar secondary structure. On the basis of this similarity, it was predicted that the chimeric molecules would be stable and processed in the cell in a manner suitable for membrane insertion and incorporation into viral envelopes. Moreover, these chimeric gene products could also be used to determine whether gC-2-reactive MAbs would independently recognize either the N-terminal or C-terminal portion of gC-2, thus providing evidence for the presence of at least two similar antigenic sites in the gC-2 molecule. The chimeric gC genes were constructed as described in Materials and Methods and introduced into the HSV-1 genome at the gC locus by homologous recombination.

Radioimmunoprecipitation of labeled proteins from infected cells was performed to test the reactivity of 26 MAbs with the hybrid proteins, representative examples of which are shown in Fig. 2. Because gC-1 is larger than gC-2 at the N terminus (511 amino acids versus 480 amino acids), the gC1-gC2 hybrid protein was expected to have an apparent molecular mass the same as that of gC-1 (130 kDa). For the same reason, the gC2-gC1 hybrid protein was expected to have an electrophoretic mobility comparable to that of gC-2 (85 kDa). However, it was observed that the gC1-gC2 product migrated slightly faster than gC-1 and gC2-gC1 migrated slightly slower than gC-2 (Fig. 2). Since the C-terminal portions of gC-1 and gC-2 contain the same number of amino acids, it could be surmised that differences in O-linked glycosylation between the two molecules are most likely responsible for the apparent size differences.

Initial experiments sought to evaluate the reactivity patterns of the gC-specific MAbs with the hybrid proteins. If either antigenic site is dependent on the other to maintain epitope integrity, then some antibodies might not be expected to bind to the hybrid protein as a result of disruption of the site by the presence of heterotypic residues. The results show, however, that each MAb was able to bind to one of the two hybrid proteins (Fig. 2). All 20 of the gC-1-reactive MAbs bound to the hybrid containing the appropriate antigenic site of gC-1. C13, a gC-1-specific MAb which binds to antigenic site II (the N-terminal site), immunoprecipitated gC from cells infected with KOS or gC1-gC2, the recombinant containing gC-1 sequence at the N-terminal site. C11 is a gC-1-specific MAb which binds to antigenic site I (the C-terminal site). This MAb bound to gC produced by cells infected with KOS or gC2-gC1, the recombinant containing gC-1 sequence at the C-terminal site.

The results with the gC-2-specific MAbs are consistent with the concept of two independent antigenic sites and showed that binding of the gC-2-specific MAbs could be mapped to one or the other of the two sites. The gC-2-specific MAb C2-3 bound gC from HSV-2 strain 333 and from gC2-gC1, the recombinant containing type 2 sequence at the N-terminal site (Fig. 2), indicating that C2-3 binds to antigenic site II. C2-5, a gC-2-specific MAb, bound gC from cells infected with 333 and gC1-gC2, the recombinant containing type 2 sequence at the C-terminal site (Fig. 2). This result is consistent with our previous finding that C2-5 binds to the C-terminal portion of gC-2 (25). In addition, MAb MP5 was mapped to site II of gC-2, and MAbs MP1, MP2, and III188-4 were mapped to site I of gC-2 (data not shown).

These radioimmunoprecipitation assays utilize soluble proteins extracted by detergent treatment of infected cells and thus do not provide evidence for the preservation of the antigenic sites of the protein when anchored in the mem-

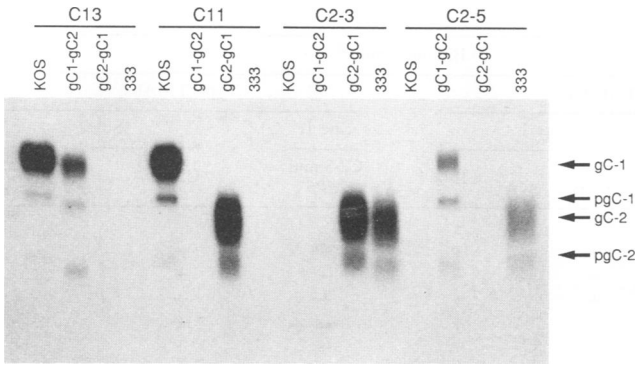

FIG. 2. Immunoprecipitation of chimeric gC molecules from [^{35}S]cysteine-labeled cell extracts. Extracts from labeled cells infected with the virus indicated above each lane were precipitated with the MAbs marked at the top. Radiolabeling of proteins, immunoprecipitation, electrophoresis, and fluorography were carried out as described in Materials and Methods. Arrows indicate positions to which precursor and mature gC molecules migrate. KOS is the wild-type HSV-1 strain, and 333 is the wild-type HSV-2 strain.

branes of either virions or infected cells. Therefore, two additional methods were used to analyze whether the hybrid proteins were inserted into virion or plasma membranes and whether the antigenic sites on these molecules were maintained in that context.

Flow cytometry was used with four representative MAbs to test antibody binding to wild-type and hybrid proteins exposed on the surface of infected cells (Fig. 3). As expected, gC-1-specific MAbs bound to KOS-infected cells, whereas gC-2-specific MAbs did not; gC-2-specific MAbs bound 333-infected cells, and gC-1-specific MAbs did not. C13, a gC-1-reactive MAb which binds site II, and C2-5, a gC-2-reactive MAb which binds site I, both bound to cells infected with the gC1-gC2 virus, whereas MAbs C11 and C2-3 did not. C11, a gC-1-specific MAb which binds site I, and C2-3, a gC-2-specific MAb which binds site II, both bound cells infected with the gC2-gC1 virus, whereas MAbs C13 and C2-5 did not. These results are consistent with those of radioimmunoprecipitation using infected cell extracts and demonstrate that the antigenic structure of gC on the surface of infected cells correlates with the structure of gC extracted from infected cells.

Neutralization assays were carried out with four representative MAbs to evaluate the ability of the antibodies to bind the hybrid proteins present in virion envelopes (data not shown). In each case, the neutralization patterns for individual MAbs were consistent with the immunoprecipitation and flow cytometry data. This result confirmed that the antigenic sites of gC-1 and gC-2 are independent from one another when present in hybrid molecules in both virion and cellular membranes.

The results of these studies with chimeric gC molecules (summarized in Table 1) provide information on the overall antigenic structure of gC-1 and gC-2. First, the data confirm that antigenic sites I and II of gC-1 are independent of one another; that is, distal type-specific residues are not essential for maintenance of each antigenic site. For each chimera, antibody binding was maintained when each site was segregated from the remaining sequences of the homologous serotype molecule. Second, similar to gC-1, gC-2 has two sites that are independent for antibody recognition; in other

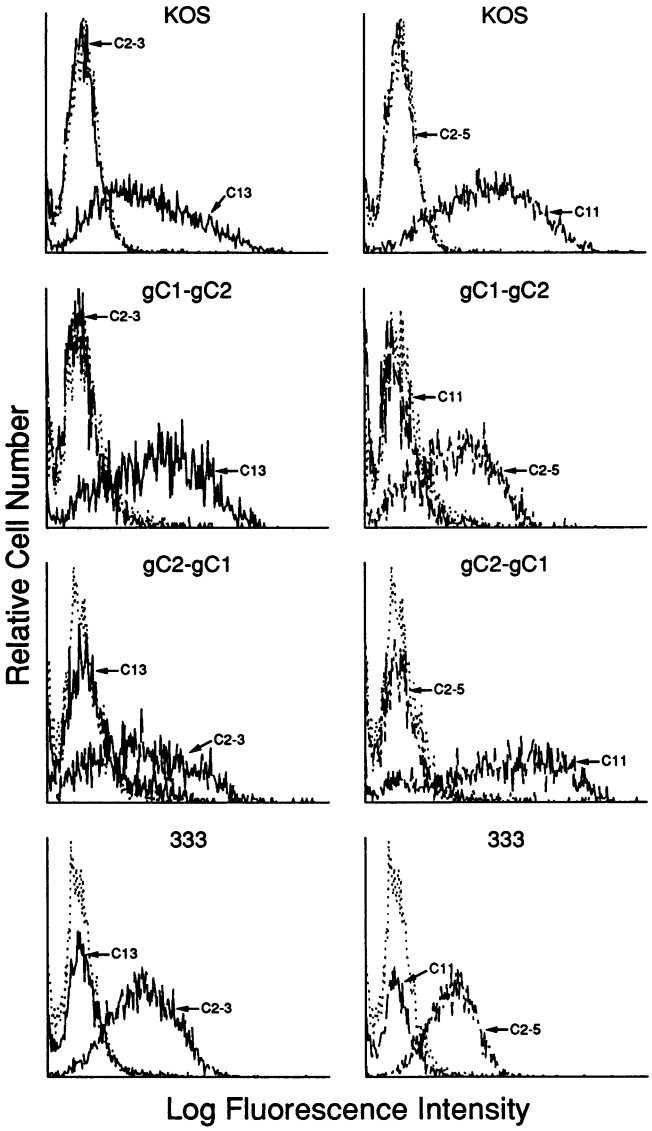

FIG. 3. Surface antigen expression of chimeric gC molecules in infected cells. Flow cytometry was carried out as described in Materials and Methods. Each panel shows the results of one virus and three antibodies. ····, negative control antibody.

words, type 2 antibodies recognize one or the other site even when the sites are segregated. Finally, the recombinant viruses could be tested with uncharacterized gC-specific MAbs to assign binding to either site I or site II.

Analysis of point mutations in antigenic site I. Previous studies in our laboratory identified a gC-2-specific MAb able to bind a *mar* mutant form of gC-1 which contained a single amino acid substitution (25). This example of seroconversion suggested that type-specific epitopes could be investigated further by selecting particular amino acids for replacement by site-directed mutagenesis, thus bypassing the laborious process of isolating and characterizing many spontaneous mutants to find an example of a seroconverted epitope. Amino acids which differ between the two serotypes but are surrounded by many identical residues are good candidates for contributing to type-specific antigenic determinants provided that they reside in hydrophilic regions in which previ-

TABLE 1. Summary of results

Virus	Site II type	Site I change[a]	Binding ability[b]					
			Type 1 MAbs			Type 2 MAbs		
			Site II	Site I		Site II	Site I	
			C13 and 13 others	C11 and 4 others	C15	C2-3 and MP5	C2-5	MP2 and 2 others
gC⁻44	1		+	±	±		−	−
KOS	1		+	+	+	−	−	−
gC1-gC2	1		+	−	−	−	+	+
gC2-gC1	2		−	+	+	+	−	−
*mar*C 11.1[c]	1	1→2	+	−	+	−	+	−
I.2[gC-1]	1	1→2	+	−	−	−	+	−
276.2[gC1-gC2]	1	2→1	+	+	−	−	−	+
I.1[gC-2]	2	2→1	−	+	+	+	−	+
333	2		−	−	−	+	+	+

[a] Direction of the amino acid change in each point mutant, type 1 to type 2 or type 2 to type 1. MAbs from each column were also used to test the viruses by neutralization assays and flow cytometry and produced the same results as those observed for immunoprecipitation.
[b] + and −, ability and inability, respectively, of MAbs to bind gC in immunoprecipitation, neutralization, and flow cytometry assays; ±, minimal MAb binding.
[c] Described in references 25, 30, and 50.

ously mapped *mar* mutations lie. It should be possible, therefore, to use a genetic approach to further characterize antigenic areas of the molecules and identify residues which play a crucial role in the formation of type-specific epitopes.

Figure 4 shows the amino acid sequence of antigenic site I of gC-1 and gC-2 and the locations of *mar* mutations affecting the binding of gC-1-specific MAbs. The substitution of amino acids at the locations of *mar* mutations in gC-1 is expected to eliminate binding of the respective gC-1-reactive MAbs. By exchanging these amino acids for those found in gC-2 at these positions, it might be possible to introduce gC-2 epitopes into the gC-1 molecule. Likewise, the reciprocal changes in gC-2 might introduce the appropriate gC-1 epitopes that were mapped to this site. To test these predictions, site-directed mutagenesis was used to introduce one or four codon changes into antigenic site I of gC-1 and gC-2 (solid rectangles in Fig. 4). The mutations were introduced at codons for amino acids which differ between the serotypes and were selected because they either represented *mar* mutations or were near these mutations.

Previous results showed that the gC-2-reactive MAb C2-5 was able to recognize and bind to the *mar*C 11.1 and *mar*C 14.1 mutant proteins (25). This mutation resulted in the substitution of an arginine for a glutamine residue at position 307. Since arginine is present in gC-2 at the corresponding position 276 whereas the remainder of the *mar*C 11.1/14.1 gC molecule contains the unchanged gC-1 sequence, it became clear that arginine was the critical gC-2 residue recognized by MAb C2-5. This finding indicated that substitution of the arginine residue with glutamine would render a gC-2 mutant resistant to neutralization by C2-5. It was predicted, therefore, that this substitution in the gC-2 background might also result in seroconversion allowing for recognition of gC-2 by C11, a gC-1-reactive antibody. This mutation was engineered into the gC-2 coding sequence at codon 276 by site-directed mutagenesis and then introduced by marker transfer into the HSV-1 recombinant virus carrying the gC1-gC2 chimeric gene. The resultant cotransfection progeny were then neutralized with MAb C2-5, which enriched for recombinant progeny carrying the mutant gene with a single point mutation in site I of gC-2. Presence of the point mutation was verified by Southern blotting. Immunoreactive black plaque assays on neutralization escape progeny revealed that C2-5 failed to bind to developing plaques, whereas MAb C11 produced black plaques (data not shown). This finding demonstrated that like the arginine-for-glutamine substitution in gC-1, the substitution of glutamine for arginine in gC-2 resulted in seroconversion of this epitope. It is concluded from these data that the type-specific epitopes recognized by MAbs C11 and C2-5 rely on this critical residue at positions 276 (gC-2) and 307 (gC-1). Despite the fact that these residues are at different amino acid positions in the molecules, they are at the same position when the two molecules are aligned for best-fit homology.

The hybrid gC1-gC2 protein with an arginine-to-glutamine substitution at the gC-2 amino acid position 276 was referred to as 276.2. 276.2[gC1-gC2] was tested for its ability to react with 26 MAbs to gC in radioimmunoprecipitation assays (examples are shown in Fig. 5). The patterns of binding of site II antibodies were the same as for wild-type gC-1 (and

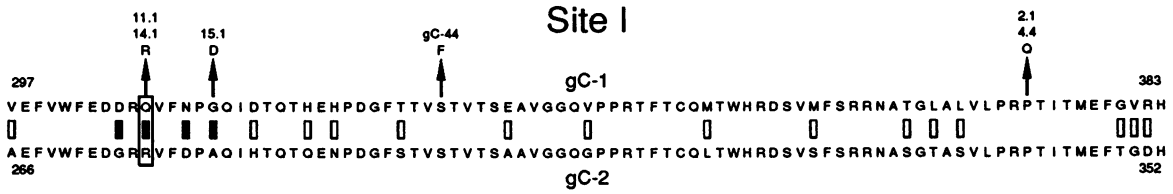

FIG. 4. Mutations in antigenic site I. The amino acid sequences of gC-1 (top) and gC-2 (bottom) in the region of site I are listed, with numbers for the end residues indicated. *mar* mutations are labeled above the sequences and denote the altered residues; rectangles mark positions where the gC-1 and gC-2 amino acids are different. The box shows the position of the *mar*C 11.1/14.1 mutation and of the single mutation introduced in 276.2[gC1-gC2]. The four solid rectangles designate the residues altered in I.2[gC-1] and I.1[gC-2].

gC1-gC2); that is, antibodies to gC-1 site II immunoprecipitated the protein and antibodies to site II of gC-2 did not. Antibodies to site I, however, showed different patterns of binding to the mutant relative to the parental protein. The results with *mar*C 11.1/14.1 indicate that the arginine residue present in these mutants is necessary for binding of MAb C2-5 (25, 50). Consistent with this observation, C2-5 was unable to immunoprecipitate the 276.2[gC1-gC2] mutant protein, which lacks the arginine residue. In addition, this mutant protein acquired the ability to be immunoprecipitated by some gC-1-specific MAbs. C11 and C14, the antibodies used to select *mar*C 11.1 and *mar*C 14.1, respectively, were able to bind 276.2[gC1-gC2], which contains, in addition to residue 276, gC-2 sequences in the remainder of site I. This result provided strong support for the conclusion that not only was the glutamine residue necessary for C11 and C14 binding, but it was sufficient for conferring type 1 specificity to this epitope. The gC-1 site I-specific MAbs C1, C2, and C4 were also able to immunoprecipitate this mutant protein (data not shown). In contrast, C15, another gC-1-specific MAb, was not able to bind site I of the 276.2[gC1-gC2] mutant protein, showing that the mutation introduced only one or a few gC-1 epitopes into the gC-2 antigenic site, confirming the presence of at least two overlapping epitopes in site I (30). In contrast to C2-5, the other gC-2-reactive MAbs directed against C-terminal determinants (MP1, MP2, and III188-4) were able to bind this mutant protein, suggesting either that these antibodies bound a different site or that other epitopes at the same antigenic site were not affected by this single substitution. These results were also confirmed by neutralization assays and flow cytometry using four site I antibodies (data not shown). The data observed when the 276.2[gC1-gC2] mutant was tested with site I MAbs are reciprocal to the results observed with the *mar*C 11.1 mutant, thus showing that the epitopes affected in gC-1 and gC-2 are homologous and differ only in one charged residue (summarized in Table 1).

To further investigate the epitope structure of site I, four point mutations (including the single base change in 276.2[gC1-gC2] and *mar*C 11.1) were introduced into site I of both the wild-type gC-1 and gC-2 genes such that each resulting protein contained four amino acids normally found in the other serotype (Fig. 4). The I.1[gC-2] plasmid, containing the gC-2 gene with four type 1 amino acids introduced into site I, was cotransfected with 333 viral DNA into Vero cells, and the progeny were neutralized with MAb C2-5. Southern blots confirmed two of the four mutations. MAb C11 reacted in immunoreactive plaque assays with the recombinant, in contrast to C2-5, confirming results obtained with the 276.2[gC1-gC2] recombinant virus, which shares a single mutation with I.1[gC-2] at amino acid 276. The I.2[gC-1] plasmid, containing the gC-1 gene with four type 2 amino acids introduced into site I, was cotransfected with KOS viral DNA, and then the progeny were neutralized with MAb C11. Three of the four mutations were verified by Southern blotting. Again as expected, C2-5 reacted in the immunoreactive plaque assay with the recombinants whereas C11 did not (data not shown).

Radioimmunoprecipitation was initially used to evaluate the loss and gain of antibody reactivities by the mutant molecules (Fig. 5). All site II antibodies bound to their respective molecules, as expected since the antigenic sites are independent of one another. As with 276.2[gC1-gC2], the I.1[gC-2] protein was bound by MAbs C1, C2, C4, C11, and C14 and was not bound by MAb C2-5. Conversely, the I.2[gC-1] protein was not immunoprecipitated by C1, C2,

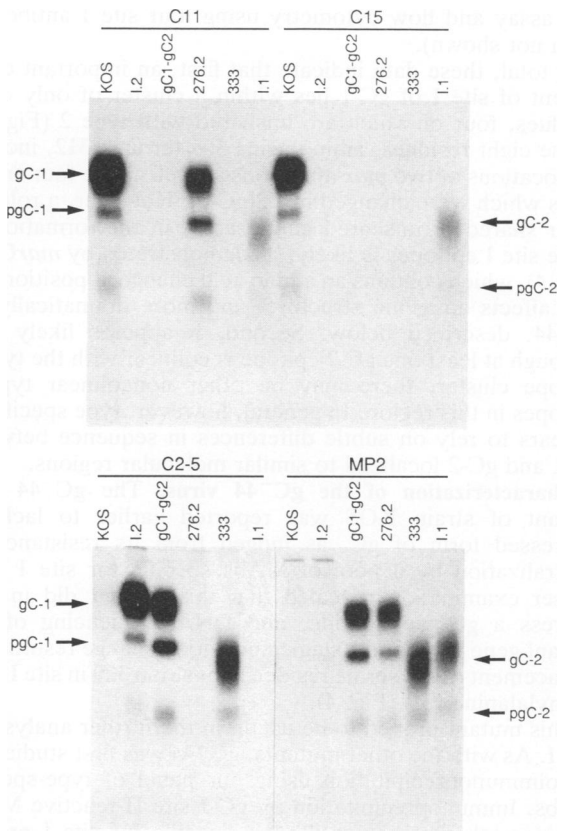

FIG. 5. Immunoprecipitation of gC molecules with mutations in antigenic site I. The MAbs indicated at the top were used to immunoprecipitate gC from extracts of labeled cells infected with the virus indicated above each lane. The MAbs listed for the upper panel are HSV-1 specific, whereas those for the lower panel are HSV-2 specific. The gC molecules expressed by each virus are as follows: KOS, wild-type gC-1; I.2, gC-1 with Gly for Asp at 305, Arg for Gln at 307, Asp for Asn at 310, and Ala for Gly at 312; gC1-gC2, amino acids 1 to 257 of gC-1 and 227 to 480 of gC-2; 276.2, Gln substituted for Arg at 276 in gC-2 of gC1-gC2; 333, wild-type gC-2; and I.1, gC-2 with Asp for Gly at 274, Gln for Arg at 276, Asn for Asp at 279, and Gly for Ala at 281.

C4, C11, or C14 but was immunoprecipitated by C2-5. This finding is consistent with the results from the analysis of *mar*C 11.1/14.1 and 276.2[gC1-gC2]; that is, the presence of the type 1 residue (glutamine) is coincident with binding of some type 1-specific MAbs (C1, C2, C4, C11, and C14). Likewise, the presence of the type 2 residue (arginine) is coincident with binding of a type 2-specific MAb (C2-5). However, binding of three type 2 site I-specific MAbs (MP1, MP2, and III188-4) was not affected by these amino acid changes, again suggesting that they bind to another area of the carboxy-terminal half of gC-2. In addition to these changes, gC-1-specific MAb C15 bound the I.1[gC-2] protein and not the I.2[gC-1] protein, indicating that at least one of the mutated residues is necessary for binding of C15. A likely candidate is the glycine residue at position 312 of gC-1 because C15 selected a *mar* mutant with a mutation in this codon (Fig. 4). This single amino acid may be sufficient for type specificity of the C15 epitope. However, the importance of the other three amino acids cannot be ruled out. These immunoprecipitation results were confirmed by neutraliza-

tion assay and flow cytometry using four site I antibodies (data not shown).

In total, these data indicate that first, an important component of site I of gC-1 lies within a cluster of only eight residues, four of which are unshared with type 2 (Fig. 4). These eight residues, amino acids 305 through 312, include the locations of two *mar* mutations as well as the four amino acids which were changed in I.2[gC-1]. However, a role for other shared or unshared amino acids in the formation of these site I epitopes is likely, as demonstrated by *mar*C 2.1 (Fig. 4), which contains an amino acid change at position 373 that affects antigenic structure, and more dramatically by gC$^-$44, described below. Second, it appears likely that although at least one gC-2 epitope is colinear with the type 1 epitope cluster, there may be other noncolinear type 2 epitopes in this region. In general, however, type specificity appears to rely on subtle differences in sequence between gC-1 and gC-2 localized to similar molecular regions.

Characterization of the gC$^-$44 virus. The gC$^-$44 *mar* mutant of strain KOS was reported earlier to lack an expressed form of gC, as judged from its resistance to neutralization by a pool of MAbs specific for site I (19). Closer examination revealed that this mutant did in fact express a gC polypeptide, and DNA sequencing of the mutant gene identified a single nucleotide change resulting in replacement of the serine residue at position 329 in site I with phenylalanine (24) (Fig. 4).

This mutant proved to be useful in the further analysis of site I. As with the other mutants, gC$^-$44 was first studied by radioimmunoprecipitation using our panel of type-specific MAbs. Immunoprecipitation by gC-1 site II-reactive MAbs was normal, whereas antibodies specific for site I precipitated a small amount of protein (Fig. 6). The reduced level of immunoprecipitation was most apparent with the mature form of the protein; the precursor form displayed levels comparable to those of the wild type. This finding suggested that complete glycosylation is important for maintaining the antigenic structure, either because of the effect of glycosylation on conformation or because of direct involvement of carbohydrate moieties in antibody binding.

As shown in Fig. 6, the site II-specific antibody C13 immunoprecipitated the gC$^-$44 protein as efficiently as did the wild-type protein. In contrast, C1, C11, C14, and C15, all site I-reactive antibodies, immunoprecipitated the gC$^-$44 protein poorly compared with wild-type gC-1. Immunoprecipitation of the gC$^-$44 protein by C1 and C14 was especially inefficient and was detectable only after very long exposure of film to the gels. None of the gC-2-reactive MAbs bound this mutant protein, as expected since the mutated residue is conserved in the two serotypes and therefore is unlikely to contribute to type specificity. These results were confirmed by neutralization assay and flow cytometry using a site II-specific antibody control and the four site I-specific antibodies mentioned above (data not shown). Neutralization of gC$^-$44 with site I-reactive MAbs was minimal, confirming our earlier results (19). Only MAb C11 caused a very slight reduction in the number of plaques. Consistent with this observation, binding of site I-specific MAbs to gC$^-$44-infected cells as measured by flow cytometry was limited, but C11 showed the greatest amount of binding. MAb C1 binding to gC$^-$44-infected cells was minimal and often undetectable. These results suggest that the antigenic structure of site I has been disrupted by disturbance of the conformational integrity of the site. This interpretation is consistent with the failure of six overlapping *mar* peptides representing the site I region to react with the appropriate

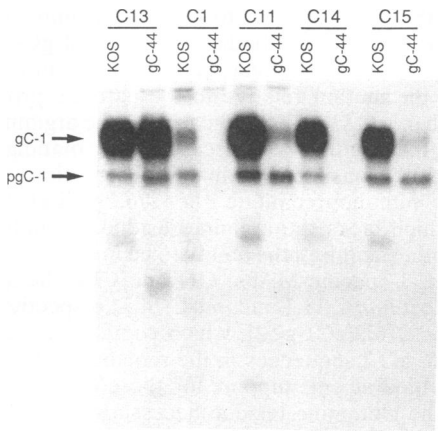

FIG. 6. Immunoprecipitation of the gC$^-$44 mutant gC molecule. The MAbs indicated at the top were used to immunoprecipitate gC from extracts of labeled cells infected with the virus indicated above each lane. C13 binds to site II, and the other antibodies bind to site I. gC$^-$44 expresses a gC-1 molecule with Phe substituted for Ser at position 329.

MAbs in binding assays (13). The fact that disruption of site I occurred without affecting site II supports the inference that site II is independent of site I.

DISCUSSION

This study was undertaken to compare the antigenic components of glycoprotein C encoded by HSV-1 and HSV-2. The interest in these molecules stems from the fact that they play important roles in the immunobiology of infection and possess the interesting property of eliciting primarily type-specific responses during infection, in contrast to most other HSV glycoproteins (1, 6, 7, 12, 26, 33, 34, 39, 41). Why the antigenic differences between gC-1 and gC-2 are distinct is unknown but may be due to certain functional characteristics of these molecules. The C3b binding and virus attachment properties differ for gC-1 and gC-2 (10, 28), which may contribute to differences in their antigenic properties. There also appears to exist limited overlap between antigenic and structural features of gC which play a role in its binding activity for the C3b component of complement (11) and perhaps other features such as attachment to cell membranes during the early stages of infection. This limited overlap between antigenic domains and functional domains may allow further divergence in antigenic structure between the two molecules.

In this study, we attempted to use extensive information on the antigenic structure of gC-1 as a starting point to extend our epitope analyses to gC-2. In particular, a major emphasis was placed on determining whether similar molecular structures of both proteins contain dominant epitope clusters. If so, we wished to investigate whether the largely type-specific antibody response to these antigenic sites is determined by single amino acid differences in largely conserved domains or whether the antigenic sites in these regions are rather dissimilar and unrelated. The study was also prompted by our earlier finding that a single mutation in a type 1-specific epitope converted it to one recognized by a type 2-specific antibody (25), raising the possibility that the antigenic sites in these molecules are colinear and probably similar.

Two types of intertypic recombinant gC genes were con-

structed. First, chimeric genes containing an antigenic site from each serotype were created. Second, individual point mutations were introduced into the C-terminal antigenic site (site I) of gC-1 and gC-2 in order to substitute specific amino acid residues from the heterotype molecule into the otherwise homotypic C-terminal half. The ability of site-specific antibodies to recognize these few residues would not only pinpoint the binding site but also provide an experimental method to precisely identify critical residues which impart type specificity to site I. The altered genes were introduced into the HSV genome in order to analyze these proteins when expressed by the virus in their native environment.

The antigenic structure of the recombinant gC molecules was evaluated by using three MAb binding assays. Immunoprecipitation tested binding of antibodies to gC extracted by detergent from infected cells, while flow cytometry tested the binding of MAbs to the recombinant molecules on the surface of infected cells. Neutralization assays tested MAb recognition of the recombinant proteins present within the virions. The results of these analyses are summarized in Table 1. It is important to note that throughout these studies, all three assays produced consistent results.

Studies of hybrid proteins revealed that antigenic sites I and II of both gC-1 and gC-2 are structurally independent of one another in that they retain their site-specific antigenicity when physically joined to part of the heterotypic molecule. The gC1-gC2 protein was bound by anti-HSV-1 MAbs to site II (N-terminal) and anti-HSV-2 MAbs to the C-terminal half of gC-2, while the gC2-gC1 protein was bound by HSV-2-specific antibodies to the N-terminal half of gC-2 and HSV-1-specific antibodies to site I (Table 1). The results also demonstrated the existence of at least one antigenic site in each of the two halves of gC-2, as previously shown for gC-1.

Results with point mutants confirmed the independence of the antigenic sites and provided a more detailed understanding of the structure of site I. By introducing specific point mutations in site I, it was possible to eliminate binding of some site I-reactive MAbs and to acquire binding of other site I-reactive MAbs without affecting the binding of site II-reactive MAbs. Analysis of mutant gC-1 molecules containing gC-2 amino acids and mutant gC-2 molecules containing the respective gC-1 amino acids yielded reciprocal results; that is, gC-2-specific antibodies recognized the substituted gC-2 residues in the otherwise gC-1 sequence background and vice versa. For example, the C2-5 MAb which bound to gC encoded by the I.2[gC-1] virus, a variant of HSV-1 with gC-2 amino acids at residues 305, 307, 310, and 312, did not bind to the I.1[gC-2] protein. This protein is expressed by a variant of HSV-2 with gC-1 amino acids at residues 274, 276, 279, and 281, the positions homologous to those of I.2[gC-1]. The gC-1-specific MAbs which bound to the I.1[gC-2] protein did not bind gC expressed by the I.2[gC-1] virus (Table 1). These results provide an example of sequence arrangements which are responsible for the serotype specificity of gC. The MAbs bound to areas of the molecule which are highly homologous between the two serotypes, meaning that type specificity in this case is not due to antibody recognition of divergent areas of the molecules. This finding rules out an early hypothesis to explain type specificity (5) based on recognition of nonhomologous areas of the molecules and suggests that the secondary structure of gC-1 and gC-2 may be similar because it was possible to effect serotype conversion of individual epitopes by introducing point mutations. If the MAbs were binding to areas of the molecules with limited homology and different secondary structure, single point mutations would not be expected to introduce epitopes specific to the heterotypic serotype. Overall, the results demonstrate that type specificity is due to the presence of different individual amino acids in a common structural framework, consistent with the hypothesis that antibodies to gC recognize areas possessing substantial homology between the molecules. These individual amino acids could be directly involved in binding as a result of their hydrophilic nature and accessibility on the antigen surface or, alternatively could be responsible for limited conformational differences which affect the spatial configuration of residues shared by the two molecules, thereby creating unique epitopes.

Results with type 2-specific antibodies suggest that site I of gC-2 may be composed of two or more subsites. gC-2 site I encoded by the 276.2[gC1-gC2] and I.1[gC-2] viruses lost the ability to bind MAb C2-5, while gC-1 from *mar*C 11.1 and I.2[gC-1] gained the ability to be recognized by this MAb (Table 1). However, binding of three other type 2-specific MAbs to site I was not altered by the residue substitutions. This finding indicates that the epitopes to which these three MAbs bind do not include the four amino acids which were changed in gC-2 or that these changes do not affect the conformation of the epitopes recognized by these MAbs. It is possible that the epitopes to which these three MAbs bind do indeed overlap the C2-5 epitope, but there is no evidence for this. Seidel-Dugan et al. (40) examined serotype 2-reactive MAbs and found that MP1 (group III) and III188-4 (group V), two of the site I antibodies, do not compete for binding, suggesting that site I of gC-2 consists of more than one antigenic site. Because of the high degree of homology between gC-1 and gC-2, gC-1 may also contain more than one antigenic site in the region identified as site I. Further site-specific mutations within site I could prove useful for delineating the specific residues involved in this other potential antigenic site.

Point mutations may result in structural changes without complete loss of antibody binding, as observed with the $gC^{-}44$ mutant. This mutant contains a point mutation in site I which reduced the level of binding of all site I-specific antibodies without affecting binding of site II-specific antibodies (Table 1). The reduction in binding was most significant for the mature, fully glycosylated form of the protein, suggesting that the mutation affects the ability of the protein containing complex-type oligosaccharides to fold into its wild-type conformation. Examples of HSV epitopes disrupted by altered glycosylation have been observed for glycoprotein D (43). In this case, elimination of one site of N-linked glycosylation resulted in the loss of some but not all epitopes within an antigenic site, demonstrating that altered glycosylation can have a localized effect without affecting other parts of the molecule. Investigation into the glycosylation of gC has indicated that some epitopes are dependent, some are independent, and some are masked by oligosaccharides (32, 42). Site II-reactive MAbs bound to the $gC^{-}44$ mutant glycoprotein normally, which confirms the independence of site II from site I. The observation that site I-specific MAbs bound poorly, if at all, to gC from $gC^{-}44$ showed that a single point mutation disrupted several epitopes and possibly the entire antigenic site (site I). This disruption of structure suggests that the site I epitopes are conformationally dependent and may consist of individual amino acids displayed on the surface of the structural framework. In addition, the possibility that a single mutation may disrupt an entire antigenic site suggests a mechanism for antigenic shift in which a strain of virus suddenly

shows a drastic change in its immunological profile. The findings in this report showed that the gC$^-$44 virus, which was previously believed to be deficient in gC, does in fact express and retain a gC molecule in its envelope. On the basis of these results, we propose that this mutant virus be renamed gC44 to reflect the presence of gC. The significant homology between gC-1 and gC-2 and the fact that the amino acid at the position of the gC44 mutation is shared between the two molecules suggest that this residue is also important to the antigenic structure of site I in gC-2. This possibility could be investigated by testing a gC-2 molecule containing the same substitution found in gC44 with site I-specific MAbs in order to further examine the importance of this residue.

Comparison of the gC-1 and gC-2 sequences in the vicinity of the *mar* mutations (see Fig. 4 for site I sequences) illustrates that the epitopes contain some critical amino acids which are different in the two serotypes and are therefore good candidates for determinants of type specificity. On the other hand, some *mar* mutations are found at residues which are identical in the two molecules, demonstrating that shared amino acids are also antigenic or contribute to the overall structure of the epitope. One of three *mar* mutations in site I and six of seven *mar* mutations in site II (50) are found at residues which are identical in gC-1 and gC-2, indicating that type-common residues are important to antigenic structure. Because they are interspersed with type-specific amino acids, shared amino acids can apparently contribute to epitopes which are serotype specific. Site I of gC-1 is currently defined as spanning amino acids 307 through 373 (50). The primary amino acid sequence within this region is 79% homologous with the gC-2 sequence. The 6 amino acids from the *mar*C 11.1 mutation to the *mar*C 15.1 mutation are 50% homologous, while 11 amino acids surrounding the *mar*C 2.1 mutation are identical to the respective gC-2 residues, showing that there is no correlation between nonhomology and importance to antigenic structure. In the case of *mar*C 2.1, an effect on antigenic structure of the mutation is not surprising since the altered residue is proline, an amino acid important to the secondary structure of proteins.

T-cell responses to gC, like humoral responses, tend to be type specific (7, 12, 39). The gC variants created in this study may therefore be useful for examination of T-cell determinants. The utility of the point mutants would depend on the overlap of antibody epitopes and T-cell determinants, however.

The studies described here show that (i) gC-2 contains at least two antigenic sites in locations similar to those found in gC-1, (ii) antigenic sites I and II of gC-1 and gC-2 are independent of one another, (iii) some epitopes of site I in gC-1 and gC-2 are colinear, (iv) the type specificity of antigenic site I is due to subtle amino acid differences between the two serotypes, and (v) epitopes of site I in gC-1 are conformationally dependent. In addition, the results imply that alteration of a few critical amino acids should allow the virus to evade the immune system of the host.

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