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Identification of a Neutralizing Epitope on Glycoprotein gp58 of Human Cytomegalovirus

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Human cytomegalovirus contains an envelope glycoprotein of 58 kilodaltons (gp58). The protein, which is derived from a glycosylated precursor molecule of 160 kilodaltons via proteolytic cleavage, is capable of inducing neutralizing antibodies. We have mapped the epitopes recognized by the neutralizing monoclonal antibody 7-17 and a second antibody (27-287) which is not neutralizing. Overlapping fragments of the carboxy-terminal part of the open reading frame coding for gp58 were expressed in *Escherichia coli* as β-galactosidase fusion proteins. The reactivities of antibodies 7-17 and 27-287 were determined by Western blot (immunoblot) analysis. Both antibodies recognized sequences between amino acids 608 and 625 of the primary gp58 translation product. The antibodies almost completely inhibited one another in a competitive binding assay with intact virus as antigen. Moreover, antibody 27-287 was able to inhibit the complement-independent neutralizing activity of antibody 7-17.

Human cytomegalovirus (HCMV) has been shown to be an important pathogen in humans. Immunocompromised individuals including organ allograft recipients, patients with acquired immunodeficiency syndrome, and human fetuses develop severe and not infrequently fatal HCMV infections (25, 28, 39). At present, no highly effective chemotherapy or widely utilized vaccine is available. A major obstacle in developing effective tools to curb the consequences of HCMV infections is the limited knowledge about the molecular biology of the virus and about the antigenic structures involved in the immune response.

The viral particle contains 35 to 45 structural proteins, most of which are capable of inducing an antibody response (20, 41). The most immunogenic polypeptides are phosphoproteins of 150, 65, and 28 kilodaltons (kDa) (20). These proteins, however, do not induce neutralizing antibodies. Only three polypeptides which are recognized by neutralizing monoclonal antibodies or monospecific sera have been identified; these are glycoproteins of 86 (9, 35), 65 (5), and 58 kDa (4, 33). More recently, a fourth glycoprotein of an estimated 47 to 52 kDa has been described (15). Of these proteins, gp58 has been characterized in the most detail (2, 4, 6, 12, 21, 30, 31, 33, 34). Within the envelope of the virus, gp58 is complexed by disulfide bonding with another protein which is highly heterogeneous in size, ranging from 95 to 130 kDa (4, 30). The 95- to 130-kDa polypeptide is antigenically unrelated to gp58 but has been shown to be structurally related to a large polyprotein precursor of gp58 (6, 21). gp58 and the diffusely migrating glycoprotein of M_r 95,000 to 130,000 are thought to be derived from a glycosylated precursor molecule of 160 kDa via proteolytic cleavage (6), with gp58 representing the carboxy-terminal portion of the processed polypeptide (24). The genomic location of the sequence encoding gp58 was identified within the *Hin*dIII F fragment of HCMV AD169 by using a procaryotic cDNA expression library and a monospecific rabbit antiserum (24). Subsequently, the nucleotide sequence revealed an open reading frame with a coding capacity for a polypeptide of 102 kDa (8). A polypeptide of similar size has been postulated as the unglycosylated precursor (31). Interestingly, the translation product of the gp58 reading frame shows homology to glycoproteins of the other human herpesviruses, namely, gpII of varicella-zoster virus (M. Mach, unpublished results), gB of herpes simplex virus, and the BALF4 reading frame product of Epstein-Barr virus (8), which has recently been shown to be a 125-kDa glycoprotein (11). The high degree of conservation which is also demonstrated by cross-reaction of antisera (1) seems to imply that these glycoproteins play an important role in the infectivity of the respective viruses. This role for gB of herpes simplex virus and gpII of varicella-zoster virus has been clearly shown (17, 22), suggesting a similar function(s) for HCMV gp58.

A more detailed analysis of gp58 is important for several reasons, the most obvious being the development of a subunit vaccine. At present, only live, attenuated virus strains are available as immunogens (32). The efficacy of these vaccines is controversial, and their use is precluded within certain populations. On the other hand, it has been demonstrated that in the case of herpes simplex virus, recombinant DNA-derived antigen preparations (3) or even synthetic peptides (10) could evoke an immune response capable of neutralizing infectious virus in vitro and in vivo. Towards a similar understanding of the immunologic activities of HCMV gp58, we have expressed large parts of gp58 in procaryotic expression systems and have used these recombinant derived HCMV proteins to identify a linear neutralizing epitope. In addition, within this same fragment of gp58 we have located a second nonneutralizing epitope which is in close proximity to the neutralizing epitope.

MATERIALS AND METHODS

Virus and cell culture. HCMV AD169 was propagated in human foreskin fibroblasts by standard procedures.

Recombinant plasmids. The fragments used to generate gp58 expression clones were excised from plasmid pBUM5801, which contains a 4.7-kilobase *Hin*dIII-*Bam*HI fragment of the *Hin*dIII F fragment of HCMV. All cloning procedures were performed by standard methods. The

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expression plasmids, except for pUU5816e, were constructed in the pEX vector system, which allows insertion of DNA fragments in all three reading frames (40). pUU5816e contains a 1.3-kilobase *Eco*RI-*Bam*HI fragment in a pUR vector (37).

Induction of fusion proteins. Fusion proteins were produced in *Escherichia coli* strain pop2136. The cells were grown to a density of 0.2 to 0.3 (A_{600}) at 30°C and induced by a quick temperature shift to 42°C. Synthesis of the fusion protein was allowed to continue for 90 min. Then the cells were harvested by centrifugation, lysed in sodium dodecyl sulfate-gel sample buffer and analyzed on 8% acrylamide gels as described elsewhere (19).

Protein gel electrophoresis and Western blot (immunoblot) analysis. Polypeptides were analyzed on polyacrylamide gels essentially as described previously (4, 24). For Western blot analysis, proteins were transferred to nitrocellulose membranes as described previously (5). Binding of antisera or monoclonal antibodies was detected by ^{125}I-protein A as described previously (5).

In vitro mutagenesis. Plasmid pUU5813 was digested with *Bgl*II, and the recessed termini were filled in with Klenow polymerase. *Kpn*I linker molecules (5′-GGGTACCC-3′) were ligated, and the DNA was transfected into *E. coli* pop2136. DNA from recombinant clones was digested with *Kpn*I, and excessive linker molecules were removed by separation on agarose gels. The DNA was religated and transfected again into *E. coli*. Recombinant clones were isolated, and the insertion of the linker molecule was confirmed by a *Kpn*I digest of the resulting plasmid. Multiple insertions of linker molecules were excluded by digestion with *Sma*I. The nucleotide sequences at the mutated site was determined by the chain termination method with the Pharmacia T7 sequencing kit (38). A 17-base-pair oligonucleotide (5′-ACTGGGCGAGGACACCG-3′) which binds 54 base pairs upstream was used as a primer.

Competitive antibody binding and neutralization assays. Competitive binding assays were carried out by using a modification of a virus-binding assay in which gradient-purified, intact virions were used as an antigen source (4). Briefly, flexible microdilution plates were coated with virions overnight at 4°C. After blocking the plates with Tris-buffered saline (0.05 M Tris plus 0.15 M NaCl, pH 7.5) containing 2% gelatin, the inhibitor antibody was added at an approximate concentration of 1 μg/ml and incubated at 37°C for 45 min. After extensive washing, ^{125}I-labeled indicator antibody was added and incubated for 30 min. Results are expressed as percent inhibition of binding of the labeled antibody.

Neutralization kinetics were determined by a modification of a previously published method (42). A known quantity of input virus (~400 PFU) was incubated with a single monoclonal antibody (1 to 3 μg/ml) or with competing antibodies at equal concentrations. Samples were removed at various times and inoculated on indicator human fibroblast monolayers. The surviving virus was calculated by dividing the number of PFU at indicated times by the infectivity of duplicate cultures containing only medium. All incubations were done in the absence of exogenous complement.

RESULTS

Expression of fragments of gp58 and recognition by various monoclonal antibodies. Since the gp58 molecule has not been characterized extensively on the protein level, two assumptions were made before regions of the molecule were expressed. First, although the transmembrane portion of the molecule has not been analyzed experimentally, a computer analysis revealed two hydrophobic domains (amino acids [aa] 711 to 748 and 752 to 772) which fit the criteria for a transmembrane region of an envelope glycoprotein. Whether these potential transmembrane sequences are arranged in a similar fashion as has been proposed for gB of herpes simplex virus, of which three transmembrane-spanning segments have been found (7), is not known. Second, the exact site at which gp58 is cleaved from the gB precursor molecule has not been determined. Digestion of gp58 with endoglycosidase F, which removes complex N-linked sugars, results in a protein of approximately 47 kDa (data not shown), suggesting that the amino acid backbone contains about 440 to 450 amino acids, provided that O-linked sugars or other modifications do not contribute significantly to the molecular weight. Assuming that the open reading frame of gp58 encodes a primary translation product of 906 aa (8) with the amino terminus of gp58 located around aa 460, it was reasoned that the extramembrane portion of the protein would extend to approximately aa 710 to 750. We therefore concentrated on expressing the region beginning with a *Pst*I site at nucleotide 1451, which corresponds to aa 484 (Fig. 1).

All constructs were made in a vector system in which the HCMV DNA was ligated into the 3′ termini of the *lacZ* gene. Expression of the gene(s) resulted in the synthesis of fusion proteins containing HCMV sequences in addition to β-galactosidase. The gp58-coding sequence was covered in two overlapping constructs. Plasmid pUU5813e contains a 900-base-pair fragment coding for aa 484 to 783, thus covering most of the portion of the protein and the entire transmembrane region. The fusion protein was stably expressed in *E. coli* and could be readily detected in Coomassie-stained polyacrylamide gels as a polypeptide of 150 kDa (Fig. 2A). Plasmid pUU5816e contained sequences between aa 685 and the end of the molecule. This hybrid protein was unstable. In addition to the full-size product of approximately 145 kDa, a polypeptide of 118 to 120 kDa accumulated in large quantities after the induction of the gene (Fig. 2A). In Western blot analyses, the fusion proteins from both constructs were recognized by a monospecific rabbit antiserum raised against viral gp58 (24). This indicates that the inserted DNA sequences are in the correct reading frame and orientation (Fig. 2B).

The reactivities of various monoclonal antibodies specific for gp58 were analyzed in Western blots. Antibodies 7-17, 27-160, 27-287, and 27-180 reacted with pUU5813e but not with pUU5816e. In control experiments, all monoclonal antibodies specifically recognized viral gp58 (4, 6). However, in this panel of antibodies only 7-17 exhibited neutralizing activity, raising the possibility that we could use these procaryotic constructs to identify the neutralizing epitope recognized by antibody 7-17. To determine the binding site precisely, various overlapping subclones of pUU5813e were constructed and tested for reactivity with antibodies 7-17 and 27-287.

Mapping of the epitope recognized by antibodies 7-17 and 27-287. Four subclones of the 900-base-pair *Pst*I region were constructed (Fig. 1). Plasmids U132e and U135e span the same region as pUU5813e. They are separated by a *Bgl*II site at nucleotide 1847 (aa 615). Plasmid U170e contains 101 aa (550 to 651), and U5005e contains 57 aa (aa 643 to 700). All constructs could be induced to synthesize stable fusion proteins which were recognized specifically by the monospecific anti-gp58 serum (Fig. 2). Monoclonal antibodies 7-17 and 27-287 were tested for their reactivities with the fusion

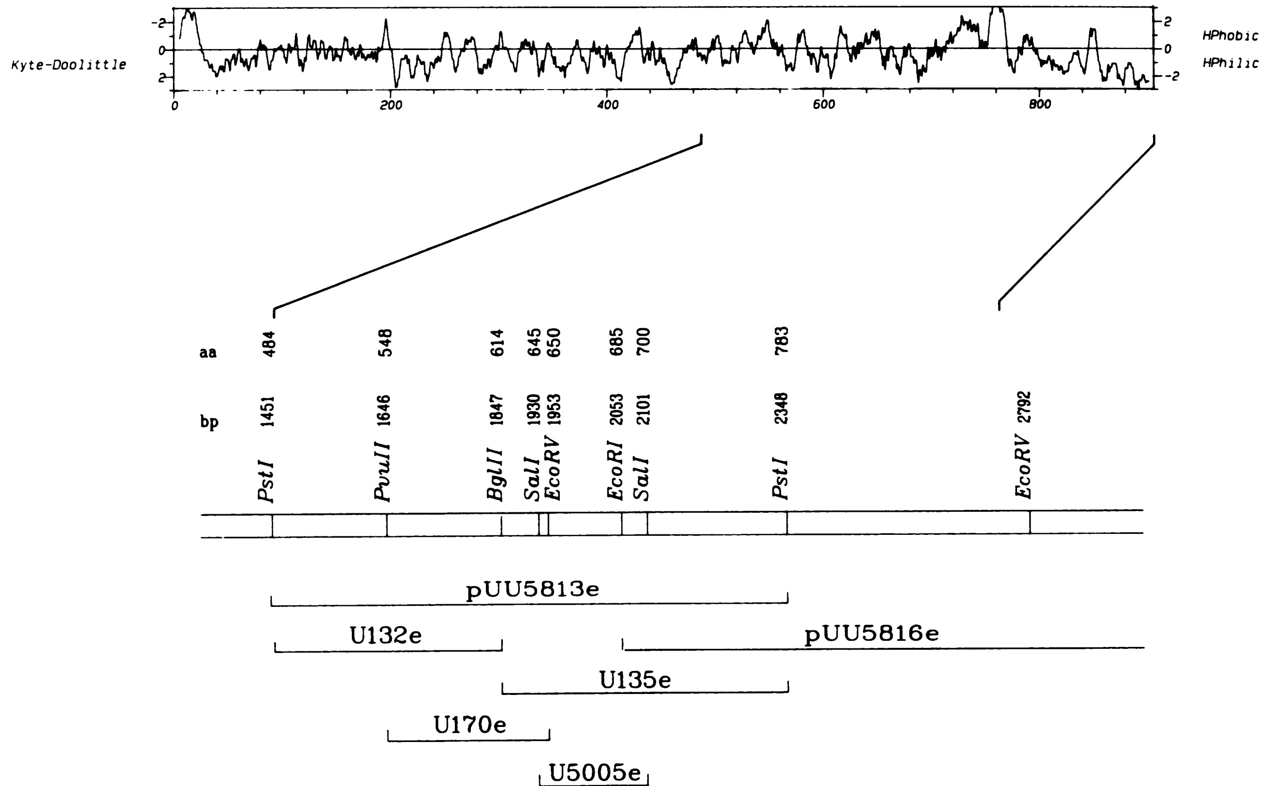

FIG. 1. Schematic representation of plasmids expressing various parts of gp58. A hydrophobicity profile as reported by Kyte and Doolittle (18) of the primary translation product is shown in the upper part. The region expressed in *E. coli* is shown, on the DNA level, at the bottom. Plasmid pUU5816e contains a 1.6-kilobase *Eco*RI-*Bam*HI fragment which includes the authentic stop codon of the gp58 reading frame. HPhobic and HPhilic, Hydrophobic and hydrophilic, respectively.

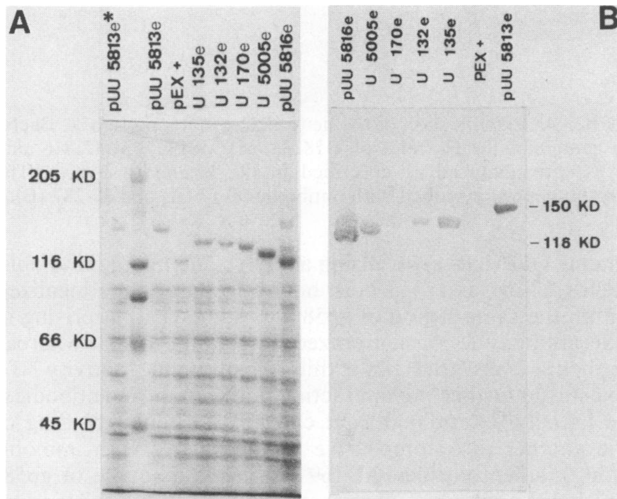

FIG. 2. Expression of various parts of gp58 as β-galactosidase fusion proteins and immunoblot analysis with a monospecific gp58 antiserum. Bacteria containing the respective plasmids were induced, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on an 8% gel, and stained with Coomassie brilliant blue (A) or transferred to nitrocellulose membranes (B). The blots were probed with a monospecific serum against viral gp58. Antibody binding was detected by protein A coupled with horseradish peroxidase and 4-chloro-1-naphthol as chromogen.

proteins. They showed identical patterns of recognition (Fig. 3). The smallest fusion protein that reacted with 7-17 was U170e. Subclones U132e and U135e, which also cover this region, did not react, indicating that the epitope recognized by 7-17 was destroyed by the separation of amino acids surrounding the *Bgl*II site at nucleotide 1847. The fusion protein synthesized from U5005e was also not recognized by the monoclonal antibodies (data not shown).

To define this finding further, the DNA sequence at the *Bgl*II site was mutagenized in vitro. Plasmid pUU5813e was cut with *Bgl*II and blunt ended by filling in. A *Kpn*I linker (5′-GGGTACCC-3′) was inserted, and the DNA was religated. The insertion of more than one *Kpn*I linker molecule was excluded by the absence of a *Sma*I site, which would be generated by a tandem insertion of more than one linker (data not shown). The addition of a single *Kpn*I linker molecule should result in an in-frame insertion of 4 aa. Figure 2A shows a comparison of protein extracts from pUU5813e and the mutagenized plasmid pUU5813e*. Both clones synthesized a fusion protein of equal size, indicating that an in-frame insertion had in fact occurred. In Western blots the respective fusion proteins were recognized by the monospecific anti-gp58 serum (data not shown). The DNA sequence of pUU5813e* revealed the insertion of the amino acids Met-Gly-Tyr-Pro (data not shown). In Western blots, monoclonal antibodies 7-17 (Fig. 4A) and 27-287 (Fig. 4B) were analyzed for the recognition of the mutated fusion protein pUU5813e*. Both antibodies did not bind to this polypeptide. The specificity of this effect is demonstrated by the included controls. As in the analysis described above,

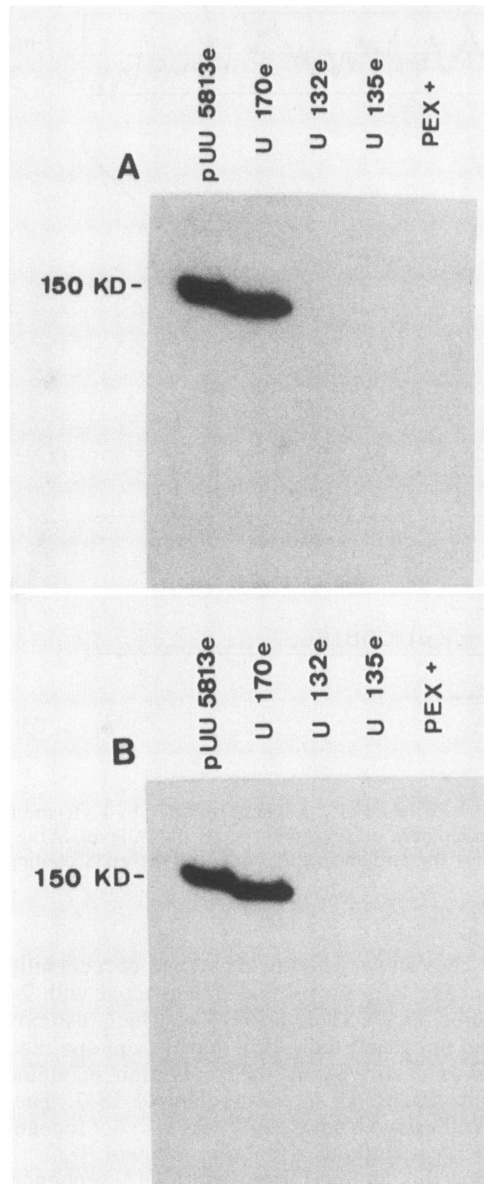

FIG. 3. Immunoblot analysis of the neutralizing epitope of gp58. Bacteria containing plasmids pUU5813e, pUU170e, p132e, p135e, and pEX-3 were induced, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. Duplicate membranes were probed with monoclonal antibodies 7-17 (A) and 27-287 (B). Antibody binding was detected by rabbit anti-mouse immunoglobulin G followed by ^{125}I-protein A and autoradiography.

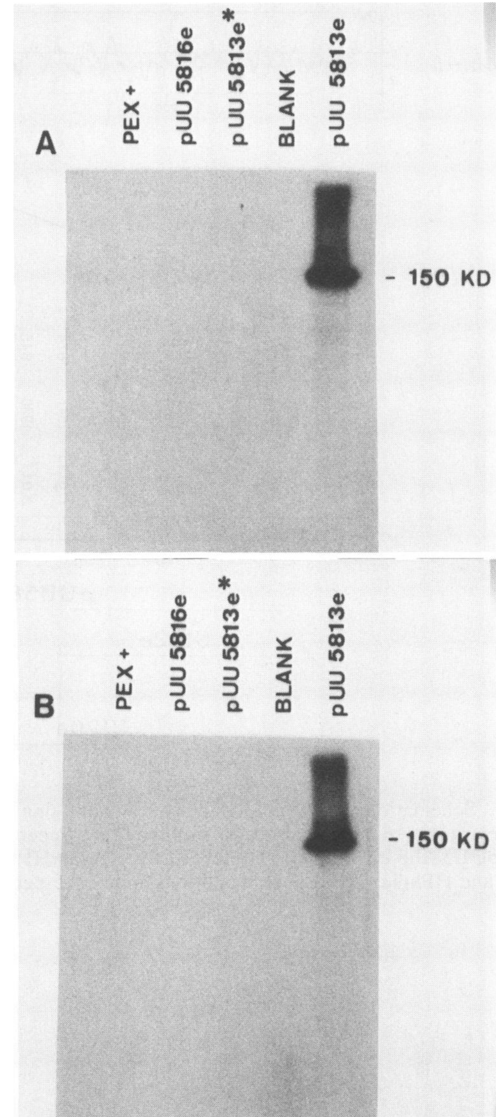

FIG. 4. Identification of the neutralizing epitope of gp58. Bacteria containing the plasmids pUU5813e, pUU5813e*, pUU5816, and pEX were analyzed as described in the legend to Fig. 3. The membranes were probed with antibodies 7-17 (A) and 27-287 (B).

(Fig. 3), both antibodies strongly recognized the fusion protein pUU5813, whereas protein extracts from the vector (pEX) and fusion proteins from a plasmid expressing carboxy-terminal sequences of gp58 (pUU5816e) did not react. This provides additional proof that the amino acid sequence surrounding the *Bgl*II site is critical for the binding of the neutralizing antibody 7-17. Surprisingly, the binding of the nonneutralizing antibody 27-287 was also dependent on the integrity of the *Bgl*II site at nucleotide 1847, suggesting close proximity of the epitopes recognized by these antibodies (Fig. 4B).

Antibody 27-287 competitively inhibits antibody 7-17 gp58-binding and virus-neutralizing activity. The finding that antibodies 27-287 and 7-17 both bound to an epitope localized within the same region of gp58 was somewhat surprising in that antibody 7-17 neutralized infectious virus, whereas antibody 27-287 did not exhibit neutralizing activity. To investigate further the interaction between these antibodies, we initially determined their capacity to inhibit binding of one another in a competitive binding assay with immobilized, gradient-purified AD169 virions as a source of gp58. Unlabeled antibody 7-17 almost completely inhibited binding of labeled antibody 27-287 to gp58, and likewise, unlabeled antibody 27-287 prevented binding of antibody 7-17 (Table 1). The specificity of this interaction was demonstrated by the use of an unrelated, anti-gp58 monoclonal antibody, 27-39, and a monoclonal antibody, 14-9, which is directed against an HCMV glycoprotein of M_r 65,000 (5). These antibodies did not inhibit binding of 7-17 or 27-287 to gp58, nor was their binding inhibited by antibody 27-287 or 7-17

TABLE 1. Competitive binding inhibition by monoclonal antibodies

Unlabeled inhibitor[a]	Inhibition of binding (%)[b] of labeled:		
	7-17	27-287	14-9
7-17	96	92	0
27-287	83	87	5
27-39	25	22	20
14-9	18	NT	100

[a] 7-17, 27-287, and 27-39 are anti-gp58, and 14-9 is anti-gp65. Wells coated with 0.1 μg of gradient-purified virus were preincubated with approximately 1 μg of antibody per ml for 45 min and washed extensively before addition of labeled antibody.

[b] Percent inhibition of binding in the presence of diluent only. Purified monoclonal antibody was radiolabeled with ^{125}I (specific activity, 1 to 10 μCi/μg), and approximately 200,000 cpm was added to virus-coated wells preincubated with unlabeled inhibitor antibody. After a 30-min incubation, the wells were extensively washed and bound radioactivity was determined.

(Table 1). Thus, in agreement with results presented in the section above, it appeared that both antibody 27-287 and 7-17 reacted with either the same or proximally located epitopes.

We then investigated the interaction between these antibodies in the complement-independent neutralization of HCMV. As was reported previously (4), antibody 7-17 neutralized HCMV whereas antibody 27-287 failed to cause a significant reduction in infectivity (Fig. 5). When equal concentrations of antibody 7-17 and 27-287 were incubated together with infectious virus, antibody 27-287 inhibited the neutralizing activity of antibody 7-17 by nearly 50% (Fig. 5). This effect on the neutralizing activity of antibody 7-17 was specific, since addition of antibody 27-287 failed to inhibit the neutralizing activity of a second, unrelated anti-gp58 antibody, 27-39 (Fig. 5). Because of the apparent proximity of the epitopes recognized by antibodies 7-17 and 27-287, these results suggest that antibodies generated in vivo after HCMV infection may also interact to reduce the overall neutralizing response. Future experiments will examine the relevance of this finding.

DISCUSSION

Glycoprotein gp58 of HCMV is clearly immunogenic, since previous studies have shown that both recombinant derived gp58 and affinity-purified protein can induce neutralizing antibodies (8, 34). In addition, because of its abundance and its homology to major glycoproteins of other herpesviruses, this protein probably plays an essential role in the life cycle of HCMV. A detailed description of the immunoreactive sequences of gp58, particularly those involved in recognition by virus-neutralizing antibodies, would be of extreme value in designing potential subunit vaccines to protect against severe HCMV disease.

We used a number of monoclonal antibodies reactive with

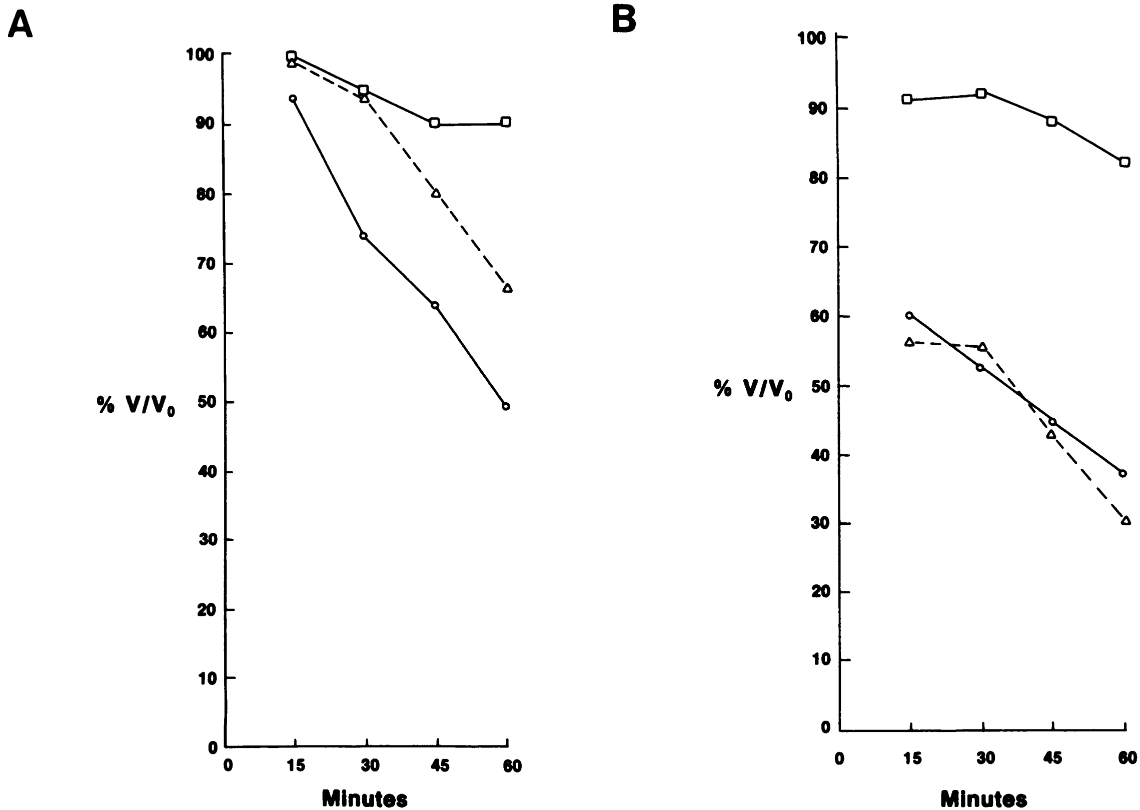

FIG. 5. Competitive-neutralization kinetics of anti-gp58 monoclonal antibodies. Neutralization kinetics assays were performed as described in Materials and Methods, and the percentage of surviving virus (% V/V_0) was plotted as a function of the length of the incubation period. The neutralizing activity of monoclonal antibody 27-287 (□) or monoclonal antibody 7-17 (○) was compared with the activity of a mixture containing equal concentrations of both antibodies (△). The neutralizing activity of monoclonal antibody 27-287 (□) or monoclonal antibody 27-39 (○) was compared with the neutralizing activity of a mixture of the two antibodies (△).

denatured HCMV glycoprotein for the epitope-mapping study of this report. Monoclonal antibodies 27-287, 27-160, and 27-180 were nonneutralizing, whereas 7-17 was neutralizing. Our strategy to localize the sequences involved in antibody recognition by these monoclonal antibodies allowed for the synthesis of gp58-β-galactosidase fusion proteins in *E. coli*. The advantage of mapping epitopes via expression in procaryotes is that once the subclones have been constructed, different antibodies can be mapped fairly easily. However, this approach leads only to the definition of the maximum limits of an epitope. The minimum length for a peptide to be recognized by a monoclonal antibody has been shown to be between 6 and 9 amino acids (13, 16, 43). This defines the maximum boundaries for the neutralizing epitopes of gp58 as between glutamic acid residues 608 and 625. The recognition pattern of nonneutralizing antibody 27-287, which seems to bind to an epitope in close proximity to 7-17, supports the hypothesis that there may be a limited number of antigenic regions but that each site may contain a substantial number of overlapping epitopes which can be distinguished by different antibodies. Interestingly, the stretch of amino acids binding to 7-17 is rather hydrophobic, and none of the computer algorithms available to us (software provided by the University of Wisconsin Genetics Computer Group) predicted the antigenic site we describe in this report. Similar observations have been made by Gnann et al. (14), who investigated synthetic peptides from the transmembrane glycoprotein of human immunodeficiency virus for their recognition by human sera. In fact, first attempts to synthesize synthetic peptides in order to define the binding site of monoclonal 7-17 more precisely have failed because of the poor solubility of the peptides (M. Mach, unpublished results). In addition to the epitope recognized by antibody 7-17, the glycoprotein gp58 contains other neutralizing epitopes. Rasmussen et al. (33) have described a monoclonal antibody which is capable of neutralizing HCMV in the presence of complement. This antibody, however, does not react with gp58 in immunoblots (34), suggesting that the epitope is probably conformational, since it is denatured by the sodium dodecyl sulfate treatment involved in immunoblotting techniques. Similarly, antibody 27-39 efficiently neutralizes HCMV; however, it also does not recognize denatured gp58. Of particular interest for the development of a subunit vaccine is the conservation of the essential neutralizing epitopes between various HCMV isolates. Although we have tested only a limited number of different clinical HCMV strains so far (25 clinical isolates), all were recognized by antibody 7-17 in immunofluorescence or Western blots, despite extensive variation in the restriction enzyme pattern of the gp58 reading frame (M. Mach, manuscript in preparation). This indicates that on the genomic level the epitope is conserved. The immune response of HCMV-seropositive individuals against this particular epitope is also of interest. Such studies are in progress.

The partial inhibition of the neutralizing activity of monoclonal antibody 7-17 by the nonneutralizing antibody 27-287 was not unexpected, because both antibodies inhibited one another in a competitive binding assay and recognized epitopes located within a small fragment of the gp58 molecule. At this time we cannot definitively assign the epitopes defined by these two different antibodies; however, their dependence on the integrity of the amino acid sequence specified by the nucleotides surrounding the unique *Bgl*II restriction site in the gp58 molecule suggests that the epitopes are proximal to one another or that secondary structure of the molecule contributes to the antibody recognition sites. Regardless of which explanation is correct, we have defined two nonconformational epitopes which can induce antibodies with different biologic activities. Other studies dealing with the interactions between antibodies against herpesvirus glycoproteins have been reported (26, 36) and recently, an extensive study of conformational epitopes present on HCMV gp58 (gC-1) has been published (23). This study also noted the competitive inhibition of several monoclonal antibodies directed against related domains of gp58 (23). In contrast to our studies, this investigation used only complement-dependent neutralizing antibodies in binding assays which used nondenatured antigens. Precise definition of the mechanism(s) of interaction between competing antibodies awaits further elucidation of the mode of neutralization by anti-HCMV antibodies.

The biologic relevance of antibody inhibition of neutralizing antibodies remains unknown. Earlier studies have suggested that nonneutralizing antibodies may inhibit complete neutralization of a number of viruses and may account for the pathogenesis of several persistent viral infections (27). Unfortunately, there is very little in vivo-derived information to support such a hypothesis. However, if neutralizing antibodies are an important component of the host response to HCMV, then even partial inhibition of this response could be detrimental to the host. Future vaccine preparations may include only immunogenic regions eliciting protective responses and not entire virion components.

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