Distribution of Linear Antigenic Sites on Glycoprotein gp55 of Human Cytomegalovirus

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Human convalescent serum and bacterial fusion proteins constructed from overlapping open reading frames of the nucleotide sequence encoding the human cytomegalovirus gp55 component of the major envelope glycoprotein complex, gp55-116 (gB), were used to localize antigenic regions recognized by human antibodies. All donor serum analyzed contained antibody reactivity for an antigenic site(s) located between amino acids (AA) 589 and 645, a region containing a previously defined linear site recognized by neutralizing monoclonal antibodies (U. Utz, B. Britt, L. Vugler, and M. Mach, *J. Virol.* 63:1995–2001, 1989). Furthermore, in-frame insertion of two different synthetic oligonucleotides encoding four amino acids into the sequence at nucleotide 1847 (AA 616) eliminated antibody recognition of the fusion protein. A second antibody binding site was located within the carboxyl terminus of the protein (AA 703 through 906). A competitive binding inhibition assay in which monoclonal antibodies were used to inhibit human antibody reactivity with recombinant gp55-116 (gB) suggested that the majority of human anti-gp55-116 (gB) antibodies were directed against a single antigenic region located between AA 589 and 645. Furthermore, inoculation of mice with fusion proteins containing this antigenic site led to a boostable antibody response. These results indicated that the antigenic site(s) located between AA 589 and 645 was an immunodominant antibody recognition site on gp55 and likely the whole gp55-116 (gB) molecule. The enhanced immunogenicity of this region in vivo may account for its immunodominance.

Human cytomegalovirus (HCMV), the largest and most complex member of the family of human herpesviruses, is a significant cause of morbidity and mortality in immunocompromised hosts, including infants infected in utero (2, 32). Both cellular and humoral immune responses are thought to play important roles in limiting disease associated with HCMV infection (31, 33). Although little is known about virus-encoded targets of HCMV-specific cellular immune responses, considerable effort has been directed toward defining virus-specified proteins that are recognized by antibodies present in human sera. Previous studies utilizing immunoprecipitation of HCMV-infected cell proteins followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) have suggested that at least 20 electrophoretically unique proteins are recognized by human sera (1, 24, 26, 43). Additional studies with immunoblot techniques have revealed that human serum is reactive with some 15 distinct virus-encoded proteins (20, 27). Interestingly, of the five well-defined envelope glycoproteins of HCMV, only the major envelope glycoprotein complex, gp55-116 (gp58 or gB), consistently induces detectable antibody responses as measured in conventional immunoblot or immune precipitation-type assays (12, 24). This finding is in contrast to antibody responses after infection with other herpesviruses, such as herpes simplex virus and varicella-zoster virus, which usually include significant responses against a number of the envelope glycoproteins of these viruses (4, 23). The reason for the apparent singularity of the anti-envelope antibody responses after HCMV infection is not known; however, likely explanations include the relative abundance of gp55-116 and/or the immunogenicity of this

glycoprotein complex as compared with those of other envelope components of HCMV.

The HCMV gp55-116 (gB) complex represents the most abundant component of the envelope of the virus. Analysis of the gene encoding this protein has indicated homology with the glycoprotein B (gB) of herpes simplex virus (16). The HCMV gp55-116 (gB) gene is translated as a 100-kDa polypeptide and, after glycosylation and transport into the Golgi apparatus, undergoes endoproteolytic cleavage into a disulfide-linked complex consisting of a heavily glycosylated, broadly migrating protein of estimated mass of 116 kDa (gp116) and a less heavily modified 55-kDa protein (gp55) (11). gp116 represents the amino-terminal component and gp55 represents the carboxyl-terminal polypeptide of the complex (28a). To date, studies have not shown antigenic cross-reactivity between these two components.

Purified HCMV gp55-116 and a recombinant-derived gp55-116 can induce both complement-dependent and complement-independent neutralizing antibodies (14, 16, 21, 34). More recent studies have indicated that the majority of neutralizing activity of human convalescent serum is directed against this single envelope glycoprotein complex (13). This latter finding, together with the relatively abundant expression of the gp55-116 on the surface of HCMV-infected cells, suggested that the gp55-116 complex might be a target of human antibody responses that are important in the clearance of infectious virus and virus-infected cells. In this report we have extended our studies on antibody binding sites on the gp55-116 molecule by expressing fragments of the gp55 component as bacterial fusion proteins and assaying human antibody responses against specific regions of this molecule. With studies utilizing antigenic site-specific murine monoclonal antibodies as competitive inhibitors of human anti-gp55-116 antibody binding, we have defined a single immunodominant region on gp55. Because this region

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is also the target of virus neutralizing antibodies, this domain may be of importance in host protective antibody responses.

MATERIALS AND METHODS

Cells and viruses. Human fibroblast (HF) cells were propagated as described previously (13). HCMV strain AD169 was utilized for all experiments. The recombinant vaccinia virus containing the entire coding sequence of AD169 gp55-116 (gB), VVPED-8, has been described previously (13).

Virus binding and virus neutralization assays. Binding assays utilizing gradient purified AD169 virions as an antigen source have been described previously (7). Neutralizing activity was determined by using a microneutralization assay (3).

Competitive binding inhibition assay. SF-8 lepidopteran insect cells were infected at a multiplicity of infection of 10 to 20 with either wild-type *Autographa californica* nuclear polyhedrosis virus or a recombinant *A. californica* nuclear polyhedrosis virus containing the gene encoding HCMV gp55-116 (gB) under control of the polyhedron promoter (40). Nonionic detergent-solubilized lysates of infected cells were used as an antigen source. After overnight absorption of the lysates to polyvinyl microtiter plates in 0.2 M carbonate buffer (pH 9.0), nonspecific binding sites on the plates were blocked with 2% gelatin in Tris-buffered saline (pH 7.5). Tissue culture supernatants containing either monoclonal antibody 27-180 (8) or control monoclonal antibody p63-27 (3) were then added to the wells and incubated for 60 min at 37°C. Antibody 27-180 is an immunoglobulin G (IgG) antibody that is reactive with antigenic domain 1 of gp55, whereas antibody p63-27 is an IgG antibody against the major immediate-early protein (3). Antibody 27-180 was selected from a large panel of IgG anti-gp55-116 monoclonal antibodies by comparing the capacity of individual antibodies to inhibit the gp55-116 binding activity of a reference immune human serum. After an extensive wash with phosphate-buffered saline containing 2% gelatin and 0.05% Tween 20, 50 µl of human serum at the indicated dilution was added and incubated at 37°C for 60 min. After another wash, human antibody binding was detected by the addition of ¹²⁵I-labeled goat anti-human IgG (Tago, Burlingame, Calif.). Binding inhibition was calculated as shown in Table 1. In some experiments, mouse antiserum raised against bacterial fusion proteins was used instead of antibody 27-180.

Production of antiserum against gel purified bacterial fusion proteins. *Escherichia coli* containing plasmids encoding regions of HCMV gp55 were induced as described (15, 37, 39). Partially purified proteins were subjected to SDS-PAGE, and proteins were visualized by Coomassie blue staining as described previously (39). The purified protein was eluted and recovered as reported in an earlier publication (9). Protein concentration was quantitated with a commercial kit (Pierce Chemicals, Rockford, Ill.). Proteins were emulsified in complete Freund adjuvant and injected subcutaneously, followed by boosting by subcutaneous injection of purified protein in incomplete Freund adjuvant. Animals were bled and serum was analyzed as described above.

Western immunoblotting and SDS-PAGE. Western immunoblotting and SDS-PAGE were carried out essentially as described previously (39). Antibody binding was detected either with ¹²⁵I-labeled protein A or with horseradish peroxidase-conjugated anti-human IgG followed by β-4-chloronaphthol as a chromogen (39).

Recombinant plasmids. All cloning procedures were per-

formed by standard methods. The DNA fragments used to generate gp58 expression plasmids were isolated from plasmid pBUM5801, which contains a 4.7-kb *HindIII-BamHI* fragment of the *HindIII*F fragment of HCMV AD169. The fragments were inserted in a suitable pATH vector, allowing for the synthesis of tryptophane synthetase-gp58 fusion protein (37). Plasmid p5815 was constructed by inserting a 1.2-kb *PstI-BamHI* fragment containing the authentic stop codon of the gp58 reading frame of pBUM58010 into pSEM. Plasmid pSEM is a derivative of pBD-2 (15) that allows the fusion of DNA fragments to a truncated β-galactosidase gene in all three reading frames. The vector was kindly provided by Stefan Knapp, Behringwerke, Marburg, Federal Republic of Germany. Correct insertion of DNA fragments was monitored by establishing the nucleotide sequence at the fusion point between the vector and gp58.

Induction of fusion proteins and Western blot analysis. Gig fusion proteins were produced in *E. coli* C600 exactly as described previously (37). Induction of fusion protein p5815 was initiated by the addition of isopropylthiogalactopyranoside (1 mM final concentration) to bacterial cultures when cell density was approximately 1.0. Synthesis of fusion proteins was allowed for 3 to 4 h. Cells were harvested by centrifugation and lysed in SDS-gel sample buffer, and protein extracts were analyzed by PAGE as described previously (39).

In vitro mutagenesis. In vitro mutagenesis of the *Bg/II* site was performed as described previously (39). The DNA linker molecules consisted of octamers containing the sequences 5'-GCCATGGC-3' (*NcoI* linker) and 5'-GGGTA CCC-3' (*KpnI* linker). The nucleotide sequence at the mutated site was determined by the chain termination method (39). A T7 polymerase kit (Pharmacia, Freiburg, Federal Republic of Germany) was used, and the manufacturer's instructions were followed precisely. A 17-nucleotide oligomer (5'-GGTGGAGATACTGCTGA-3') binding 70 bp upstream on the complementary strand was used as a primer. The resulting amino acid sequences for gig 58-2N and gig 58-2K are as follows (letters in boldface type indicate the inserted amino acids): ₆₁₃PSLKIAMAIFIAG (gig 58-2N), ₆₁₃PSLKIGYPIFIAG (gig 58-2K).

RESULTS

Human antibody recognition of a linear sequence on gp55. The sequence encoding gp55-116 is translated into a 906 amino-acid (AA) polyprotein in HCMV strain AD169 and a 907-AA polyprotein in the Towne strain (36). The glycosylated Towne polyprotein is proteolytically cleaved at the sequence motif RTKR/STD₄₆₃ as reported by Spaete et al. (35). In HCMV strain AD169, the homologous sequence RTRR/STS₄₆₃ presumably serves as the cleavage signal. The carboxyl-terminal component of the cleaved protein (gp55) consists of a 446-AA protein with a predicted mass of 49.3 kDa. This predicted size is in close agreement with an experimentally derived estimate of the mass of nonglycosylated gp55 (11). Using several convenient restriction endonuclease cleavage sites, we constructed a series of expression plasmids in which overlapping open reading frames of the sequence encoding gp55 were expressed as procaryotic fusion proteins (Fig. 1). Expression of the HCMV sequences was accomplished by using either pATH vectors or pSEM in which the HCMV polypeptides were fused with tryptophan synthetase or truncated β-galactosidase, respectively (15, 37). With the exception of gig 58-4, all constructs could be induced to synthesize high levels of stable fusion proteins in

FIG. 1. Restriction fragments utilized for construction of bacterial fusion proteins. Amino acid and nucleotide numbers are listed above restriction endonuclease cleavage sites, and a Kyte and Doolittle hydrophobicity-hydrophilicity plot is shown at the top.

E. coli (Fig. 2). The region between AA 700 and 780 of gp55 was not stably expressed in a variety of procaryotic expression systems, possibly because of the hydrophobicity of this presumed transmembrane region of the molecule (39). In

total, approximately 75% of the entire open reading frame of gp55 was expressed, including all but 24 AA of the ectodomain of the molecule (Fig. 1).

Seventeen serum specimens from individuals seropositive for HCMV and three seronegative control specimens were tested in immunoblot assays for reactivity with the fusion proteins. Although positive serum specimens were randomly chosen, all were screened for reactivity with gp55 before use in these experiments. The reactivities of four representative sera are presented, although all sera gave identical results. A strong reaction was obtained with constructs gig 58-1 and 58-2, whereas fusion proteins from gig 58-3 and 58-7 were not recognized by seropositive sera (Fig. 3). Control negative sera did not react with the fusion proteins (Fig. 3). The finding that immune sera recognized fusion proteins from gig 58-1 and 58-2 but not gig 58-3 and 58-7 indicated that the antibody binding site was located between AA 589 and 645 (Fig. 1). When this stretch of amino acids was disrupted at position 616 (gig 58-8 and 58-9), antibody binding was abrogated (Fig. 3). This finding was consistent with our previous studies, which have shown that a neutralizing murine monoclonal antibody reacted with a linear determinant surrounding AA 616 (39). Furthermore, several neutralizing and non-neutralizing monoclonal antibodies specific for the binding site surrounding AA 616 exhibited a reactivity for the gig constructs that was identical to that of seropositive human sera (data not shown).

An additional antibody binding site was localized to the carboxyl terminus of the molecule. *E. coli* containing plasmid 58-15 synthesized a β -galactosidase fusion protein containing AA 783 through 906 of gp55, which extends from 10 AA 3' of the transmembrane region to the authentic stop codon (Fig. 1). Human sera reacted strongly with this fusion protein but not with truncated β -galactosidase, suggesting that antibody binding site(s) were also present on this region

FIG. 2. Expression of HCMV fusion proteins in *E. coli*. *E. coli* containing either the PATH expression plasmid or recombinant PATH plasmid consisting of restriction endonuclease fragments of the gene encoding gp55 (Fig. 1) were induced and solubilized as described in Materials and Methods. Total cell lysates were resolved by SDS-PAGE, and fusion proteins were identified by staining with Coomassie blue. Molecular size standards (Std) are listed in the right margin.

FIG. 3. Human antiserum reactivity for HCMV bacterial fusion proteins. Fusion proteins were electrophoretically separated as described in the legend to Fig. 2 and transferred to nitrocellulose membranes. After blocking with skim milk, individual strips were incubated with serum diluted 1:200 from an individual without serologic reactivity to HCMV (1) and three patients with serologic evidence of past infection with HCMV (4, 6, 9). After the membranes were washed, they were reacted with peroxidase-conjugated goat anti-human IgG and developed with β -chloronaphthol and H_2O_2 .

of the molecule (Fig. 4). Because all of the serum specimens used in this study exhibited different titers against gp55, the specificity of recognition of both antigenic sites was independent of the overall antibody level against gp55 in the serum.

Definition of a linear antibody recognition site dependent on integrity of the sequence surrounding AA 616. The recognition pattern of the ectodomain of gp55 by human anti-gp55-116 antibodies was unexpected; it appeared that only a single region induced antibody responses within this stretch of over 200 AA. As noted previously, this immunogenic region was located in the same position as the previously defined linear antigenic site recognized by neutralizing murine monoclonal antibodies (39). To further define the importance of the amino acid sequence surrounding position 616, plasmid 58-2 was mutagenized by insertion of either a *KpnI* (58-2K) or *NcoI* (58-2N) linker into the *BglII* site at nucleotide 1847. Insertion of these linkers created an in-frame, 4-AA insertion with the additional amino acid sequence IAMA (*NcoI* linker) or IGYF (*KpnI* linker) between AA 616 and 617 of the original sequence. Fusion proteins from 58-2N (58-2K) and 58-2 were indistinguishable by SDS-PAGE (Fig. 2); however, only the fusion protein encoded by 58-2 was recognized by human sera (Fig. 5). Identical results were obtained with 58-2K (data not shown). This result indicated that the amino acid sequence surrounding AA 616 were essential for human antibody recognition of gp55 and that alteration of this sequence by insertion of two different amino acid

FIG. 4. Reactivity of human sera for an antigenic site in the carboxyl terminus of gp55. *E. coli* containing plasmid 5815 (Fig. 1, AA 783 through 906) or control plasmid sera were induced with isopropylthiogalactopyranoside, and the fusion proteins were separated by SDS-PAGE as described in Materials and Methods. After transfer to nitrocellulose, the membrane was cut into strips and reacted with serum diluted 1:200 from a seronegative individual (1) or four patients with serologic reactivity for HCMV (3, 4, 6, 10). Antibody binding was detected as described in the legend to Fig. 3.

sequences eliminated antibody recognition of this molecule. This linear region, representing a conserved antibody binding site on gp55, was initially designated linear epitope 1 (LEp-1 [10]). Because the exact amino acid sequence of this epitope has yet to be defined and preliminary evidence suggested that additional unique antibody binding sites were located immediately adjacent to the site initially termed LEp-1, we have altered our original nomenclature to a more broadly descriptive term of antigenic domain 1 (AD-1).

Immunodominance of AD-1. The results presented above suggested that a limited number of linear antibody recognition sites were present on the gp55 component of the gp55-116 complex. In addition to the previously defined region AD-1 surrounding AA 616, the reactivity of human antibodies with the fusion protein encoded by the 58-15

FIG. 5. Reactivity of human sera for mutagenized HCMV fusion protein. Plasmid gp 58-2 (Fig. 1) was mutagenized by insertion of an *NcoI* linker into the *BglII* site (nucleotide 1847) with the resulting 4-AA (IAMA) in-frame insertion as described in Materials and Methods. *E. coli* containing either the mutated gp 58-2N or parent gp 58-2 expressed the nearly equivalent-sized fusion protein after induction and SDS-PAGE analysis of the cell lysate (Fig. 1). Antibody reactivity for representative seropositive patients (6, 9) was determined as described in the legend to Fig. 3. Reactivity was detected only for the parent plasmid fusion protein 58-2.

TABLE 1. Binding inhibition of human anti-gp55-116 (gB) antibodies by monoclonal antibody 27-180^a

Human serum	% Binding inhibition ^b					
	27-180			27-39		
	1:100	1:200	1:400	1:100	1:200	1:400
1	61	84	84	0	25	25
2	59	71	81	12	18	23
3	65	76	83	7	12	21
4	53	57	67	19	26	28
5	65	67	75	23	33	33
6	68	65	79	8	18	29
7	35	65	67	0	6	12
8	56	48	36	NT ^c	NT	NT
9	57	59	58	NT	NT	NT
10	66	57	37	NT	NT	NT
11	59	60	60	NT	NT	NT
12	44	51	68	NT	NT	NT
13	90	99	100	NT	NT	NT
14	43	68	84	NT	NT	NT

^a Recombinant gp55-116-coated microtiter wells were initially reacted with either monoclonal antibody 27-180 (8) or 27-39 (39), followed by the addition of human convalescent serum at a dilution of 1:100, 1:200, or 1:400. Human anti-gp55-116 IgG binding was determined by the addition of ¹²⁵I-labeled goat anti-human IgG.

^b Binding inhibition was calculated for monoclonal antibodies (MAb) 27-180 and 27-39 as follows: % binding inhibition = 100 (cpm of control MAb - cpm of exp Ab)/(cpm of control MAb); the control was an IgG anti-HCMV immediate early protein antibody (3), and the experimental antibodies (exp Ab) were 27-180 and 27-39.

^c NT, Specimens not tested.

construct indicated that additional antibody binding sites were present on the carboxyl terminus of gp55 (Fig. 4). Thus far our analysis of human anti-gp55-116 antibody reactivity had not addressed the relative dominance of AD-1 as compared with those of other linear antibody binding sites present on the carboxyl terminus of gp55 as well as the amino-terminal component of gp55-116 complex, gp116 (11). We obtained some measure of the dominance of AD-1 as well as an estimate of other antibody binding sites on the gp55-116 complex by the use of a competitive binding inhibition assay in which human antibody binding to gp55-116 was inhibited by murine monoclonal antibodies. After screening a large panel of gp55-116-specific monoclonal antibodies, we found several that significantly inhibited (>50%) human antibody binding to gp55-116. A single antibody, 27-180 (8), which is specific for the AD-1 was selected for further study because of its consistent inhibition of human antibody binding. As a control, a gp55-116-specific, non-AD-1 binding antibody, 27-39, was used (39). Among 14 different human sera specimens tested, all but three showed gp55-116 binding that was significantly inhibited (>50%) by the AD-1-specific monoclonal antibody (Table 1). This inhibition was observed at a relatively low dilution (1:100) of human serum and in most cases was still observable at 1:50 dilutions (Table 1; data not shown). Binding inhibition by the non-AD-1-reactive monoclonal antibody rarely exceeded 25% (Table 1).

The importance of previously defined linear antibody binding sites on the carboxyl terminus of gp55 was next investigated. For these experiments, we utilized antisera produced in mice by injection of SDS-PAGE-purified fusion proteins from *E. coli* transformed with plasmid 58-15 (AA 783 through 906). As controls we also used antisera produced in mice by injection of fusion proteins from *E. coli*

TABLE 2. Inhibition of human anti-gp55-116 antibody binding by monoclonal antibodies and immune mouse sera^a

Human serum	% Binding inhibition				
	27-180	58-3 ^b	58-7 ^b	58-15 ^b	27-39
1	70	7	12	2	1
2	52	4	5	3	0
3	77	6	5	0	7
4	51	4	12	8	5
5	65	8	13	4	1
6	64	NT ^c	18	17	NT
7	68	NT	11	0	NT
8	65	NT	18	12	NT

^a Recombinant HCMV gp55-116 was applied to microtiter plates and either monoclonal antibody (27-180, 27-39) or immune mouse serum (58-3, 58-7, 58-15) diluted 1:100 was added. After 1 h of incubation, the monoclonal antibody or mouse serum was removed, the plate was washed, and human serum diluted to 1:200 was added. Binding inhibition was calculated as described in the legend to Fig. 1.

^b Immune mouse serum was prepared by injection of purified *E. coli*-derived fusion proteins into mice as described in Materials and Methods. Mice were immunized with fusion protein from plasmids 58-3 (AA 645 through 700), 58-7 (AA 484 through 588), and 58-15 (AA 783 through 906). Pooled sera from animals in each group exhibited significant binding to purified gp55-116 in a solid-phase binding assay with the following binding indexes: 58-3, 2.6; 58-7, 1.8; 58-15, 30 (control serum from unimmunized animals is defined as 1.0). The binding index for these sera was determined in a solid-phase radioimmunoassay with the index calculated as follows: [cpm bound (experimental)]/[cpm bound (control)] (7).

^c NT, Specimens not tested.

containing either gig 58-7 (AA 484 through 588) or gig 58-3 (AA 645 through 700). Although the fusion protein encoded by 58-15 were clearly the most immunogenic, all fusion proteins induced virus and gp55-116 binding antibodies and antisera with weak but detectable neutralizing activity (data not shown). When these antisera were used in the binding inhibition assay, none significantly inhibited human antibody binding to gp55-116 compared with the AD-1 specific monoclonal antibody, 27-180 (Table 2). These findings confirmed the previous results (Table 1) and suggested that the AD-1 was an immunodominant antibody recognition site on gp55. Furthermore, because these assays were done with the intact gp55-116 complex, it was likely that the immunodominance of AD-1 extended to include the entire gp55-116 envelope glycoprotein complex.

Immunogenicity of AD-1. The apparent immunodominance of AD-1 after naturally acquired HCMV in humans was also observed after immunization of mice with purified virions. Immunization of mice with either native or SDS-denatured HCMV gp55-116 resulted in an unexpectedly high frequency of AD-1-specific monoclonal antibodies (data not shown). We investigated the relative immunogenicity of the AD-1 in vivo by immunizing mice with β -galactosidase fusion proteins containing either AD-1 (plasmid 5813) or the same open reading frame of the gp55 sequence mutated by the insertion of a *KpnI* linker at position 1847 in the plasmid 5813* (39). This insertion resulted in a 4-AA in-frame mutation as described above and ablated AD-1-specific antibody binding (39). As a control, a β -galactosidase fusion protein containing the carboxyl terminus of gp55 was also used as an immunogen (sequence 5816; AA 684 through 906) (39). Mice were inoculated initially with approximately 4 μ g of fusion protein followed by three booster injections. Because of the small amount of protein initially injected, minimal gp55-116-specific antibody binding activity was detected after this immunization schedule (Table 3). At 4 months after the final

TABLE 3. Immunogenicity of antigenic domain 1

Mouse ^a	Virus binding index ^b		β -Gal binding index ^c	Neutralizing activity ^d	
	Pre	Post		Pre	Post
5813-1	1.0	11.0	8.3	26	66
5813-2	0.6	4.5	3.2	0	51
5813-3	0.9	5.4	14.1	39	66
5813-4	0.7	4.3	3.6	5	68
5813*1	0.9	1.2	14.7		39
5813*2	0.6	1.2	24.4	44	46
5813*3	0.8	1.1	8.5	41	34
5816-1	0.6	0.9	3.5	11	42
5816-2	0.7	0.8	1.0	28	33
5816-3	0.6	1.4	1.2	28	52
5816-4	0.7	0.8	8.9	15	32

^a Adult BALB/c mice were immunized with approximately 4 μ g of purified β -galactosidase fusion proteins in complete Freund adjuvant from *E. coli* containing the indicated plasmid. Animals were boosted at 2, 6, and 12 weeks after the initial immunization with 2 μ g of the respective fusion protein emulsified in incomplete Freund adjuvant.

^b The binding index was determined in a solid-phase binding assay utilizing gradient purified AD169 virions as an antigen source (7). Antibody binding was detected by sequential addition of serum at a dilution of 1:100, rabbit anti-mouse IgG, and then ¹²⁵I-labeled protein A. The results are expressed as the ratio of counts per minute in experimental serum to that in control serum obtained from nonimmunized littermates. Values represent binding indexes before (Pre) and after (Post) boosting with 10⁷ PFU of a vaccinia virus-HCMV gp55-116 recombinant virus with the final bleed taken 6 days after virus inoculation.

^c Anti- β -galactosidase (β -Gal) binding activity of postboost sera diluted 1:100 was measured in a solid-phase binding assay with purified β -galactosidase as an antigen source.

^d Neutralizing activity was determined as described previously (3) and is represented as the percentage reduction in infectivity at a serum dilution of 1:100 in the presence of complement. Control sera from unimmunized mice and mice immunized with a recombinant gp55-116 had neutralizing activities of 15 and 79%, respectively. The mean neutralizing activities after boosting in groups 5813, 5813*, and 5816 were 63, 40, and 40%, respectively. Values represent activities before (Pre) and after (Post) boosting.

injection, all animals were given 10⁷ PFU of a vaccinia virus-HCMV gp55-116 recombinant virus. Six days later, the animals were sacrificed and their sera were analyzed. Only animals initially inoculated with the construct expressing AD-1 exhibited a boostable virus binding antibody response (Table 3). Although individual animals in each group produced low levels of neutralizing antibodies, as a group only animals initially immunized with AD-1 containing fusion protein generated a neutralizing antibody response that reduced input infectivity by greater than 50% (Table 3). Comparable levels of anti- β -galactosidase binding antibody were found in all three groups, suggesting that the initial immunization schedule was similar in all groups (Table 3). These results provided additional evidence for the immunodominance of AD-1 and suggested that its immunogenicity may account for its immunodominance in mice and possibly in humans.

DISCUSSION

In this study we have investigated the distribution of linear antibody recognition sites on the gp55 component of the HCMV gp55-116 (gB) complex. Approximately 75% of entire coding sequence of gp55 was examined, including all but 24 AA (AA 463 through 484) of the ectodomain of the molecule, a hydrophilic region which could potentially represent an antigenic domain. Thus, a reasonably complete set of potential linear epitopes was studied. Only two regions of the molecule, AA 589 through 645 (AS-1) and AA 783

through 906, were recognized by antibodies in convalescent human serum. Because a significant number of random human serum specimens from two different patient populations was utilized in this study, it was unlikely that this restricted response was secondary to genetically determined unresponsiveness in this population to other regions of the molecule. The explanation for the restricted recognition of this molecule is unknown and was unexpected given the significant immunogenicity of this molecule in vivo (4, 12, 20, 24, 26, 27). Previous reports have also noted that antibody recognition of viral proteins was restricted to discrete domains and have ascribed this restricted response to the host immunological repertoire and not to antigenic silence of other regions of the protein molecules (22). Indeed, other regions of gp55 were antigenic as evidenced by the production of antibodies after immunization of mice with fusion proteins from plasmids 58-3 (AA 645 through 700), 58-7 (AA 484 through 588), and 58-9 (AA 616 through 645) (data not shown). Furthermore, immunization of rabbits with SDS-PAGE-purified gp55 resulted in production a heterologous serum reactivity with linear domains scattered throughout the protein (39). However, it appeared that the antigenicity of these regions was not expressed in the presence of the apparently dominant region, AD-1, when animals were immunized with native gp55. Additional evidence of the restricted nature of the host antibody response to gp55 was obtained by priming mice with three different fusion proteins representing AA 484 through 783, AA 684 through 906, and AA 484 through 783* in which the AD-1 was altered by insertional mutagenesis. Only the fusion protein containing the AD-1 efficiently primed mice, leading to a boostable antibody response (Table 3). This experimental finding was consistent with previous observations demonstrating an unexpectedly high frequency of AD-1-specific monoclonal antibodies derived from mice immunized with intact HCMV virions (data not shown). Although it was clear that human antibody responses could be directed against a second domain defined by AA 684 through 906, the immunogenicity of this region was limited and appeared to represent a minor response as compared with the response to AD-1 (Table 3). Thus, it appeared that AD-1 represented a dominant antibody recognition site on the gp55. Findings consistent with our results have recently been reported for glycoprotein I of pseudorabies virus and bovine herpesvirus 1. These reports indicated that serum from experimentally infected animals contained antibodies reactive with only a limited number of regions present on denatured proteins (18, 19, 25). Furthermore, monoclonal antibodies against these linear domains exhibited neutralizing activity, suggesting that these antibody binding sites were also expressed on the native protein. In contrast to these findings, a recent report has shown that human antibody reactivity against the major capsid protein of another herpesvirus, Epstein-Barr virus, was directed against multiple epitopes scattered throughout the protein (30). The explanation for these different results is unknown, but the modes of presentation to the immune system could be expected to differ significantly for an internal, intracellular antigen such as the capsid protein of Epstein-Barr virus and the cell surface envelope glycoproteins described above. Other structural features of the gpI of pseudorabies virus and bovine herpesvirus type 1 and the gp55-116 (gB) complex of HCMV including glycosylation could limit the number of antigenic sites on native molecules.

The finding that mice initially primed with either fusion protein 5816 (AA 684 through 906) or 5813* (AA 484 through

783) in which AD-1 was mutated by insertion of 4 AA at position 616 could produce low levels of neutralizing activity after boosting with the vaccinia virus–HCMV gp55-116 recombinant virus was unexpected (Table 3). Initially we interpreted this result as evidence of an early primary antibody response after immunization with the recombinant virus. However, individual mice produced low levels of neutralizing antibodies after the initial immunization protocol (Table 3). In addition, we subsequently noted that animals injected with fusion proteins containing AA 484 through 588, 645 through 700, and 780 through 907 all produced antisera with detectable levels of neutralizing activity (data not shown). This result suggested that additional, nondominant neutralizing epitopes may be expressed on the molecule and that antibodies against these regions in the native protein were generated in significantly lesser amounts than were antibodies against the more immunogenic AD-1 region. This hypothesis, however, raised several questions about the topology of gp55-116 in the virion membrane. Initial analysis of the nucleotide sequence of gp55-116 (gB) suggested that the molecule has only one membrane-spanning region and that AA 780 through 907 were internal to the ectodomain of gp55 (16). Thus, the finding that antisera directed against AA 708 through 907 had detectable neutralizing activity raised the possibility that there are additional membrane-spanning regions in gp55. Current studies are in progress to resolve the discrepancy between these experimental observations and the predicted structure of the molecule.

Because the fragments of gp55 were expressed as bacterial fusion proteins, our investigation of the distribution of antibody recognition sites on gp55 was limited to linear sequences without appreciable secondary, tertiary, and quaternary structure. Therefore our results provide only a minimal estimate of the number of antibody binding sites, because we have ignored nonlinear or conformation-dependent epitopes that could be expressed on the native molecule. Previous studies have indicated that a number of conformation-dependent antibody binding sites exist on the gp55-116 (gB) molecule (5, 28). Interestingly, of the more well-defined epitopes, at least two were localized to the gp55 component of the complex (5). In general, conformation-dependent epitopes are thought to be of more biological significance than linear or non-conformation-dependent epitopes because the former class of antibody binding sites more consistently induces higher affinity antibodies against native protein with measurable biological activity such as virus neutralization. Initially it appeared that our results were in conflict with this hypothesis in that AD-1, which has also been shown to be the target of virus-neutralizing antibodies, was a presumed linear epitope contained within a linear sequence of approximately 57 AA. This estimate was derived by demonstrating antibody reactivity for fusion protein 58-2 (AA 548 through 645) and the lack of reactivity for 58-3 (AA 645 through 700) and 58-7 (AA 484 through 588) as well as the inability of antisera against fusion proteins 58-3 or 58-7 to inhibit antibody binding to gp55 (Fig. 3; Table 2). However, we have been unable to demonstrate antibody binding to synthetic peptides ranging in size from 10 to 21 AA with AA 616 as the midpoint (data not shown). Furthermore, several experiments utilizing fusion proteins from deletions of plasmid 58-2 (AA 548 through 645) suggested that the minimal size of AD-1 was considerably longer than 20 AA (data not shown). Thus it appeared that AD-1 was larger than the minimal 6- to 10-AA sequence required for antibody binding (38). Currently we favor the hypothesis

that AD-1 consists of at least 30 AA that can assume some higher-order structure more closely resembling the conformation of the antibody recognition site expressed by the native molecule. Similar observations have been reported in other viruses, including human immunodeficiency virus, foot-and-mouth disease virus, and poliovirus, in which linear neutralizing epitopes were localized to 26, 19, and 11 AA, respectively (6, 17, 29). Interestingly, shorter peptides from this region of poliovirus could prime mice, but primary neutralizing antibody induction required an amino acid sequence of at least 11 AA (17). Whether a similar phenomenon will occur after priming of mice with shorter sequences from the region encompassing AD-1 is currently under study.

It was of interest that AD-1 appeared to be the dominant antibody binding site for the entire gp55-116 (gB) complex, in both its native and denatured forms (Table 1). The capacity of a single anti-AD-1-specific monoclonal antibody to significantly inhibit human antibody binding to gp55-116 (gB) indicated that the majority of antibodies were directed at AD-1, or at least to regions in close proximity to this site. This binding inhibition was reproducible and detectable at even low serum dilutions. In addition, the failure of mouse antisera reactive with adjacent regions of gp55 to inhibit binding to gp55 provided additional evidence for the specificity of binding inhibition exhibited by the anti-AD-1-specific monoclonal antibody. Because a panel of conformation-specific monoclonal antibodies reacted with the immobilized antigen, it was likely that the recombinant gp55-116 used as the antigen source retained a significant amount of native structure (data not shown). Furthermore, absorption experiments with the immobilized antigen suggested that all detectable anti-gp55-116 antibodies in human serum could be absorbed by this solid-phase antigen (data not shown). Thus, it was unlikely that the inhibition assay described in this report measured only human antibody reactivity against linear epitopes, although we cannot definitively discount the possibility that additional antigenic sites were expressed only by mammal-derived gp55-116. It should also be noted that inhibition assays such as this do not adequately measure binding of low-affinity antibodies with potential biological significance, nor can these assays be utilized to definitively predict the location of antibody recognition sites. This would be especially true if conformationally dependent antibody binding sites were assembled utilizing regions of gp55 in close proximity to AD-1. However, even with these limitations in mind, these results together with the qualitative findings with fragments of gp55 expressed as bacterial fusion proteins suggested that AD-1 was a dominant antibody recognition site on the gp55-116 (gB) complex.

Although several studies have demonstrated that passively acquired anti-HCMV antibodies can modify HCMV infection in allograft recipients, premature infants, and fetuses, the specificities of these protective antibodies remain undetermined (2, 31, 41, 42). Virus neutralization remains the only well-studied biological activity of anti-HCMV antibodies. The presence of this class of antibodies correlated qualitatively with seropositivity; however, there is no reported quantitative correlation between antibody level and clinical outcome. Regardless of these limited data, it would seem likely that HCMV neutralizing antibodies are active *in vivo*, since these antibodies react with the surfaces of both infectious virions and infected cells and could be expected to mediate clearance of virus and virus-infected cells. Recently we showed that the gp55-116 (gB) complex was the major target of virus-neutralizing activity in human serum (13).

Together with the findings presented in this report, these results suggested that the AD-1 of HCMV gp55-116 (gB) might play an important role in the generation of protective antibody responses.

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