The Dominant Linear Neutralizing Antibody-Binding Site of Glycoprotein gp86 of Human Cytomegalovirus Is Strain Specific

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Bacterial fusion proteins, constructed from overlapping fragments of the open reading frame coding for gp86 of human cytomegalovirus (HCMV) strain AD169, were used to localize antigenic regions recognized by antibodies from human convalescent sera. A major domain for binding of conformation-independent antibodies was localized on fusion protein AP86, containing amino acids 15 to 142 of gp86. Human antibodies, affinity purified on AP86, neutralized infectious virus in tissue culture. In addition, a mouse monoclonal antibody (AP86-SA4), raised against AP86, also neutralized HCMV. AP86-SA4 was reactive with viral gp86 in immunoblot assays and showed a plasma membrane staining on intact HCMV-infected fibroblasts late in infection. After exonuclease III deletions of the viral gene, the binding site of neutralizing human as well as mouse antibodies was localized between amino acid residues 34 and 43. The domain has sequence variation between laboratory strains AD169 and Towne, and binding of the antibodies was strain specific. To our knowledge, this is the first characterization of a strain-specific neutralizing epitope on HCMV.

Human cytomegalovirus (HCMV) infects the majority of the population worldwide. Fortunately, this infection usually remains clinically asymptomatic in immunocompetent individuals. In the immunologically impaired, such as organ transplant recipients or infants infected in utero, however, HCMV infection can be life threatening. Both humoral and cellular immune responses are likely involved in control of HCMV infections. Identification and immunological characterization of HCMV proteins that elicit a protective response will allow a more thorough understanding of the control of infection and eventually the design of strategies to limit disease in the infected host.

HCMV is a complex virus containing a genome of 230,000 bp with an estimated 200 open reading frames, including a coding capacity for as many as 30 glycoproteins (8). Some of the virion-associated glycoproteins are thought to play an essential role in the induction of virus-neutralizing antibody responses. During natural infection, antibodies against as many as 20 to 25 structural proteins can be detected (1, 29, 36, 39, 43). However, only two glycoproteins have consistently been shown to induce antibodies detectable by immunoblot or immunoprecipitation analysis using whole virus as antigen. We will refer to these polypeptides as gp58/116 and gp86, respectively, throughout this report, although different designations have been used by other laboratories (for a summary, see reference 31). The immunodominant gp58/116, which constitutes the gCII complex, has been characterized in some detail (6, 7, 14, 19, 24). Two nonconformational domains which are immunogenic during natural infection and result in the production of neutralizing antibodies have been identified (27, 35).

In contrast to the more thoroughly studied gp58/116 glycoprotein complex, less is known about the antigenic structure of gp86. Together with a glycosylated polypeptide of 145 kDa, gp86 constitutes the gCIII complex which is present in infected cells and virions (20). The gp86 gene is located

within the long unique region of the viral genome, and the nucleotide sequence has been established for each of the two laboratory strains, AD169 and Towne (10, 37). According to the nomenclature introduced by Chee et al. (8), the open reading frame of strain AD169 was designated HCMVUL75. Genes homologous to that encoding gp86 have been found in conserved gene blocks of other herpesviruses, including varicella-zoster virus (25), Epstein-Barr virus (4, 22), herpes simplex virus (16, 33), and herpesvirus saimiri (17), suggesting an important function in the replication cycle of the respective viruses. In herpes simplex virus, the corresponding glycoprotein gH has been shown to be essential for viral infectivity and, like its counterpart in Epstein-Barr virus, is involved in penetration of the host cell by infectious virus (11, 15, 21). The HCMV gp86 protein is capable of inducing a neutralizing immune response in humans as well as in laboratory animals, and a number of monoclonal antibodies have been isolated from these sources (3, 10, 12, 40). However, the relevant antigenic structures have not been characterized. In this report, we describe the locations and amino acid sequences of nonconformational antibody-binding sites on gp86 by means of expression of viral sequences in *Escherichia coli* and the production of neutralizing monoclonal antibodies against linear antigenic sites.

MATERIALS AND METHODS

Cells and viruses. Human foreskin fibroblast (HFF) cells were grown in minimal essential medium (Gibco BRL, Glasgow, Scotland) supplemented with 5% fetal calf serum, glutamine (100 mg/liter), and gentamicin (350 mg/liter). Propagation and gradient purification of virus strains AD169 and Towne were carried out by standard procedures.

Expression of fragments of gp86 in *E. coli*. All DNA fragments used to generate gp86 expression plasmids with the exception of the *NheI*-*HindIII* fragment were isolated from plasmid pBW10. This plasmid contains the 1,210-bp *Bam*HI fragment of HCMV AD169, which represents 91% of the gp86 sequence in pUC8. pNH86 was derived from

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plasmid pBW3, which contains the *Hind*III L fragment (11.7 kb) of AD169 in pBR322 and codes for the entire gp86 gene product. In this construct, gene expression terminates at the authentic stop codon.

The *Pvu*II-*Nhe*I fragment was cloned into the expression vector pROS (13); all others were inserted into pSEM (26). Both plasmids contain the truncated gene coding for the first 375 amino acids (aa) of *E. coli* β -galactosidase 5' to a multiple cloning site. All cloning procedures were performed by standard methods. Correct insertion of DNA fragments was monitored by establishing the nucleotide sequence at the fusion point between vector and gp86, using the chain termination method with a T7 polymerase sequencing kit (Pharmacia, Freiburg, Germany) (41).

Fusion proteins were produced in *E. coli* BMH7118 (34). The cells were grown to a density of approximately $1.0 (A_{660})$ at 37°C, and production of fusion proteins was initiated by addition of isopropylthiogalactopyranoside (IPTG; final concentration, 1 mM). Synthesis of fusion proteins was allowed for 4 to 5 h at 37°C. The cells were harvested by centrifugation (10 min, 4°C, 6,000 rpm, Sorvall GSA rotor), lysed in sodium dodecyl sulfate (SDS) gel sample buffer, and separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) (28).

To increase the levels of fusion protein, the bacterial lysates were treated with 6 M urea. Cells from an induced 100-ml culture were suspended in 4 ml of phosphate-buffered saline (PBS) containing DNase I (final concentration of 1 μ g/ml) and lysozyme (final concentration of 2.5 μ g/ml). After incubation for 30 min on ice, the lysate was sonicated for 30 s and centrifuged (20 min, 4°C, 10,000 rpm, Sorvall SS34 rotor). The sediment was washed twice in PBS, suspended in 2 ml of PBS, and added slowly to 6 M urea (4 ml) with continuous stirring on ice. After centrifugation, both the insoluble and soluble fractions were analyzed by PAGE. Enriched fusion proteins were present in the soluble fraction.

SDS-PAGE and immunoblotting. SDS-PAGE and immunoblot analysis were done as described previously (35). Murine monoclonal antibodies and human antisera were diluted in PBS containing 0.1% Tween 20. Antibody binding was detected with horseradish peroxidase-coupled anti-human immunoglobulin G (IgG) or anti-mouse IgG and either 4-chloro-1-naphthol or diaminobenzidine.

Prior to immunoblot analysis with human sera, a preincubation step was carried out to reduce background staining due to reactions with bacterial proteins. The diluted sera were incubated for 1 h on ice with a mixture of β -galactosidase (final concentration, 2 μ g/ml; Sigma, München, Germany) and a lysate of an *E. coli* culture containing the β -galactosidase portion of the fusion protein. After centrifugation (10 min, 4°C, 4,000 rpm, J6B centrifuge, JS4.2 rotor), the supernatant was saved and the preincubation step was repeated twice.

Immunoaffinity chromatography. Human antibodies specific for the amino-terminal part of glycoprotein gp86 were isolated by immunoaffinity chromatography on fusion protein AP86. AP86 was enriched as described above and dialyzed against coupling buffer (0.1 M NaHCO₃, 0.5 M NaCl [pH 8.0]). Six milligrams of protein was conjugated to 2 ml of tressyl-activated Sepharose beads (Pharmacia, Freiburg, Germany) for 3 h at room temperature as instructed by the manufacturer. After being washed with 10 ml of solution I (0.1 M sodium acetate, 0.5 M NaCl [pH 5.0]) and 10 ml of coupling buffer, the AP86 Sepharose beads were transferred to a column. Free tressyl groups were blocked

with 1 M ethanolamine (pH 9.0) for 2 h at room temperature. After a wash with 10 bed volumes of coupling buffer, antibodies were applied to the column. A concentrated IgG preparation derived from a pool of human HCMV-positive sera (kindly provided by D. Rohm, Biotest) was incubated at 56°C for 30 min to inactivate the complement factors, clarified by centrifugation (5 min, room temperature, 13,000 rpm), and adjusted to 10 mM Tris (pH 7.5). Four milliliters of this pool (IgG content of 1,200 mg, determined by a protein assay kit from Pierce, Oud-Beijerland, The Netherlands) was passed 10 times through the column. The beads were washed with 20 bed volumes each of solutions II (10 mM Tris [pH 7.5]) and III (10 mM Tris [pH 7.5], 0.5 M NaCl). Bound antibodies were eluted with 100 mM glycine (pH 2.5) and collected in fractions of 0.5 ml. The pH of the eluted antibodies was adjusted to 7.0 with 1 M Tris (pH 8.0), and their protein content was determined. Fractions containing AP86 antibodies were pooled and passed over the column a second time.

Neutralization assay. The neutralization assay has been described in detail in a previous publication (2). Briefly, infectious HCMV was mixed with diluted immune sera or murine monoclonal antibodies. Guinea pig serum at a final concentration of 5% was used as a source of complement in some assays. After a 60-min incubation at 37°C, virus-antibody mixtures were added to 96-well plates seeded previously with HFF cells. After an approximately 4-h incubation, the inoculum was removed and the monolayer was fed with fresh medium. The plates were incubated for 16 h at 37°C, fixed with absolute ethanol, and stained with a monoclonal antibody reactive with the major immediate-early protein, pp72. Antibody binding was detected by a fluorescein-conjugated anti-mouse antibody. Antibody 48, a murine monoclonal antibody that is reactive with Friend murine leukemia virus gp70, was used as a control for nonspecific reactivity. Results are expressed as percent infectivity reduction, with maximum infectivity being determined by incubation of virus with a nonreactive antibody.

Production of monoclonal antibodies. Murine monoclonal antibodies were produced as previously described (5). Briefly, β -galactosidase fusion protein AP86 was prepared as described above, purified by SDS-PAGE, emulsified in complete Freund's adjuvant, and injected intramuscularly into the hind limbs of adult BALB/c mice. Following two boosts with 50 μ g of protein emulsified in incomplete Freund's adjuvant, the draining lymph nodes were fused with the myeloma cell line Px63-Ag8 as described previously (5). Wells containing hybridoma cells were screened by immunofluorescence and by antigen binding as described previously (5).

Cell surface immunofluorescence. HFF cells were grown on 13-mm glass coverslips and infected when confluent with HCMV strain AD169 at a multiplicity of infection of 5. Five days following infection, one set of coverslips was reacted with tissue culture supernatant containing monoclonal antibody AP86-SA4 for 60 min at room temperature. After extensive washing in serum-free medium, the coverslip was fixed in freshly made 1% paraformaldehyde, permeabilized with 0.05% saponin, and reacted with monoclonal antibody. Antibody binding was detected by reacting each coverslip with fluorescein isothiocyanate-conjugated goat anti-mouse IgG. The coverslips were mounted in buffered glycerol and photographed with a Leitz diavert fluorescence microscope.

Exonuclease III-mung bean nuclease digestion. The exonuclease III-mung bean nuclease deletion system was used to shorten the gene that encodes the viral part of the fusion

protein AP86. Plasmid pAP86 was digested with *Xba*I and *Sph*I, phenol-chloroform extracted, and ethanol precipitated. *Xba*I created a 5' overlap at the end of the gp86 fragment, and *Sph*I created a 3' overlap at the end of the vector which was resistant to exonuclease III digestion. Thirty micrograms of DNA was incubated with 600 U of exonuclease III at room temperature in Exo III buffer (50 mM Tris [pH 8.0], 5 mM MgCl₂, 10 mM ZnSO₄, 10 mM β -mercaptoethanol). Every minute, an aliquot of 5 μ g was removed and directly transferred to 175 μ l of 8/7 mung bean nuclease buffer (57 mM sodium acetate [pH 5.0], 34 mM NaCl, 1.14 mM ZnSO₄) to terminate the reaction. After heating at 68°C for 15 min, the resulting single-stranded extensions were removed by incubation with mung bean nuclease (3 U/ μ g of DNA) for 30 min at 28°C, phenol-chloroform extracted, and ethanol precipitated. The shortened fragments were religated and transfected to *E. coli* BMH7118. The deletions were characterized by establishing the nucleotide sequence at the fusion point between gp86 and vector.

DNA amplification and expression of a Towne-specific gp86 motif. The Towne-specific sequence coding for the amino-terminal part of gp86 (aa 14 to 42) was amplified by using the polymerase chain reaction. DNA preparation from HCMV Towne-infected cells and amplification of the respective fragment were performed as described previously (30). Briefly, cell pellets from Towne-infected and mock-infected HFF cells were sequentially incubated with sarcosyl lysis buffer (2% sarcosyl, 50 mM Tris-HCl [pH 7.5], 10 mM EDTA) for 30 min at 56°C, RNase A for 10 min at 37°C (final concentration, 0.3 mg/ml), and proteinase K for 1 h at 37°C (final concentration, 0.7 mg/ml). DNA was purified by two extractions with phenol-chloroform, precipitated in 0.1 M NaCl and 2 volumes of ethanol, and dissolved in distilled water.

The oligonucleotide primers were prepared with a Cyclone DNA synthesizer (Biosearch, Inc., San Rafael, Calif.), using the phosphoramidite method (32). The first primer was composed of a 6-nucleotide clamp sequence, the recognition site of the restriction enzyme *Bam*HI, and the Towne-specific sequence from nucleotides 40 to 57 (5'-acggttGGATCCGTC TGTTCCTCAGCCAC-3'). The second primer represented nucleotides 108 to 126 followed by the *Hind*III recognition motif and 6 additional bases (5'-acggttAAGCTTGAGCAG TAGGTGAAACGCT-3'). The Towne-specific gp86 sequence of 87 nucleotides was amplified during 30 cycles on a Biomed Thermocycler (B. Braun, Theres, Germany). Each cycle consisted of denaturation at 95°C for 0.5 min, annealing at 45°C for 1 min, and chain elongation with *Taq* polymerase (Boehringer, Mannheim, Germany) at 72°C for 2 min.

The amplified fragment was separated on an 1.5% agarose gel and purified by using Elutip columns (Schleicher & Schuell, Dassel, Germany) according to the manufacturer's instructions. After digestion with restriction enzymes *Bam*HI and *Hind*III and an additional purification step, the fragment was cloned into the expression vector pSEM-3. Correct amplification and insertion of the fragment were examined by sequence determination according to Sanger et al. (41).

RESULTS

Recognition of linear sequences on gp86 by human antibodies. The open reading frame encoding gp86 is translated into a polypeptide of 743 aa containing six potential N-linked

glycosylation sites in HCMV strain AD169 (10). Computer predictions show that it contains a stretch of hydrophobic amino acids consistent with a signal peptide between aa 3 and 18 and a transmembrane anchor region between aa 719 and 737. The presumed cytoplasmic region of the protein is unusually short, consisting of only six residues.

To analyze antibody-binding sites that do not depend on posttranslational modifications specific for eucaryotes and/or higher-order structure, the open reading frame of gp86 was expressed in *E. coli*. Using various convenient restriction endonuclease cleavage sites within the gp86 coding sequence, we constructed a set of seven plasmids in which overlapping fragments of the protein could be expressed as fusion proteins (Fig. 1). The pSEM vector system that was used allowed for the synthesis of fusion proteins between aa 1 and 375 of β -galactosidase and the viral sequence (26). Synthesis of fusion proteins from constructs AP86 and EB86 as well as the unfused β -galactosidase could be induced to high levels in *E. coli* BMH7118 (Fig. 2A). The remaining constructs did not produce sufficient quantities to be detected on SDS-PAGE after staining with Coomassie brilliant blue. Since low levels of fusion protein could result in difficulties in the interpretation of subsequent immunological analyses of human sera, the bacterial lysates were enriched for the respective fusion proteins. Lysates from induced cultures were extracted with 6 M urea, and soluble as well as insoluble fractions were analyzed for the presence of fusion protein. With the exception of BB86, highly enriched proteins were detected in the soluble fraction (Fig. 2B). BB86, covering 570 aa of gp86, could not be induced to high levels, nor could the protein be enriched to a purity comparable to that of the other fusion proteins. In addition to the full-length product of 113 kDa, smaller proteins which most probably represent degradation products were observed after enrichment. The identity of all enriched polypeptides as β -galactosidase-gp86 fusion proteins was confirmed in immunoblot analyses using a monoclonal antibody directed against aa 1 to 375 of β -galactosidase (data not shown). The entire reading frame of gp86 was expressed excluding aa 1 to 14, which most probably represent part of the putative signal sequence and thus are not part of the mature polypeptide.

Twenty serum samples from individuals seropositive for HCMV and one seronegative control were analyzed in immunoblot assays for reactivity with the fusion proteins. With the exception of BB86, equal amounts of fusion protein, as judged by Coomassie staining after electrophoresis, were used. Because of the low solubility, the amount of BB86 had to be reduced. The serum specimens were not preselected for reactivity with viral gp86, since we found it impossible to unequivocally assess reactivity against this glycoprotein in immunoblots because most of the sera recognized a variety of proteins in this molecular weight range (for example, see Fig. 3, serum 4). Three different reaction patterns were observed (Fig. 3): (i) a strong reactivity with protein AP86 and a slightly weaker signal with EB86 (sera 1, 3, 8, 12, 14, 15, and 19), (ii) weak reaction with protein BB86 (serum 5), and (iii) no recognition of any of the fusion proteins (sera 2, 4, 6, 7, 9, 10, 11, 13, 16, 17, 18, and 20).

The negative control serum did not react with any of the bacterial or viral proteins. There was no correlation between antibody titer against HCMV and recognition of gp86 fusion proteins. This is illustrated by serum 3, which showed a low reactivity against viral proteins and a strong reaction with AP86, whereas the opposite was the case for serum 4. The lack of correlation was also found when a commercially

FIG. 1. Schematic representation of gp86-derived procaryotic expression plasmids. The open reading frame of gp86 (HCMVUL75) is depicted as an open box starting at position 110132 and extending to position 107904 (numbering according to Chee et al. [8]). (A) Recognition sites of the respective restriction enzymes and their nucleotide and amino acid positions relative to the start codon; (B) DNA fragments inserted into the expression vector pSEM.

available enzyme-linked immunosorbent assay was used for the quantitative analysis of HCMV antibodies (data not shown). In total, eight sera were considered positive for recognition of gp86 fusion proteins (Table 1). With the exception of specimen 5, which was positive for BB86 only, the sera recognized proteins AP86 and EB86. No reaction with other fusion proteins was observed. Table 1 also shows the reactivities of the sera against AD-1, a highly conserved immunodominant region of gp58 represented by Mbg58 (27).

FIG. 2. Expression of gp86-specific fusion proteins in *E. coli* BMH7118 containing either the expression plasmid pSEM (SEM) or the recombinant constructs coding for the gp86-specific sequences (AP86 to NH86; see Fig. 1) were induced by addition of IPTG. The respective cell lysates were subjected to SDS-PAGE before (A) or after (B) enrichment of the fusion proteins by treatment with 6 M urea. Proteins were stained with Coomassie brilliant blue. Molecular size standards were run in lanes M; numbers at the left represent molecular masses in kilodaltons.

Seventeen sera, including five specimens that were negative for gp86 fusion proteins, tested positive for AD-1.

The data indicate that gp86 contained a major domain for the binding of conformation-independent antibodies between aa 15 and 142. Similar observations were made by others (39a). The stronger signal of AP86 than of EB86 was consistently observed and could result from multiple antibody-binding sites on AP86 or a structural difference resulting in different binding activities (see below).

Purification of AP86-binding human antibodies and analysis of neutralization capacity. The recognition of AP86 indicated that a region between aa 15 and 142 of the gp86 molecule was capable of inducing conformation-independent antibodies. Antibodies from human sera were purified by affinity chromatography on AP86. AP86 fusion proteins were coupled to tressyl-activated Sepharose, and 4 ml of a concentrated IgG preparation derived from a pool of human sera was passed over the column. The antibody preparation showed no signs of contamination with other proteins when analyzed by SDS-PAGE. The IgG concentration was 300 mg/ml. Bound antibodies were eluted in fractions and tested in immunoblot analyses for reaction with AP86 (Fig. 4). Antibodies from fractions 2 to 6 bound to AP86, whereas a control antigen containing AD-1 of gp58 was not recognized. In immunoprecipitations with HCMV-infected HFF cells as antigens, antibodies against other viral proteins could not be detected (data not shown). A total of 19.2 µg of protein corresponding to 0.0016% of the amount of protein applied to the column, was eluted. This, however, does not represent the total amount of AP86-specific antibodies in the IgG pool, since analysis of the flowthrough still showed detectable reactivity with AP86 (data not shown). The affinity-purified AP86-specific antibodies were assayed for neutralizing activity. No complement was added to the assays, and endogenous complement was heat inactivated. The AP86-specific anti-

FIG. 3. Recognition of gp86 fusion proteins by human sera. gp86 fusion proteins (AP86 to NH86; see Fig. 1) and β -galactosidase (SEM; aa 1 to 375) were expressed in *E. coli* BMH7118, enriched by treatment with 6 M urea, separated by 10% SDS-PAGE, and transferred to nitrocellulose membranes. After blocking with PBS-0.1% Tween 20, the proteins were incubated with HCMV-positive human sera (3 to 5) at a dilution of 1:50. Anti-human IgG coupled to horseradish peroxidase and 4-chloro-1-naphthol was used as detection system. CMV, lysate from extracellular HCMV particles. Sizes of markers are indicated in kilodaltons.

bodies showed 50% neutralization of viral inoculum at a concentration of 25 ng/ml, whereas the IgG pool reached comparable levels at 60 μ g/ml. Fraction 7 from the affinity column, which had no detectable reactivity against AP86 and was used as a control, exhibited no neutralizing activity. These data indicate that antibodies binding procaryote-expressed aa 15 to 142 of gp86 could neutralize HCMV in vitro.

Definition of a linear sequence recognized by neutralizing murine monoclonal antibodies. Murine anti-gp86 antibodies were produced by immunization of mice with purified fusion proteins from two regions of the gp86 protein. Immunization with fusion protein AP86 (aa 15 to 142) induced detectable antibodies to gp86, as measured by immunoblot and immu-

nofluorescence (data not shown). In contrast, only minimal and inconsistent reactivity was found in sera from mice immunized in a similar fashion with fusion protein BB86 (aa 111 to 681). Furthermore, only sera from AP86 immunized mice exhibited virus neutralizing activity.

Murine monoclonal antibodies were produced from mice immunized with AP86 fusion proteins. Several antibody-producing hybridoma cell lines were isolated and further characterized. One monoclonal antibody, AP86-SA4, was shown to react strongly with gp86 in several different assays. Antibody AP86-SA4 reacted with HCMV-infected cells but not with varicella-zoster virus- or herpes simplex virus type 1-infected or uninfected cells. In addition, antibody AP86-SA4 reacted with the surface of HCMV-infected cells and exhibited a perinuclear staining pattern in permeabilized, infected cells 5 days after infection (Fig. 5). The protein specificity of AP86-SA4 was examined in Western immunoblots, using both viral and infected cell proteins as antigen sources. Antibody AP86-SA4 reacted with an 86-kDa protein in both virion and infected cell preparations (Fig. 6). Interestingly, the monoclonal antibody failed to react with more slowly migrating forms, suggesting that the previously described higher-molecular-weight component of the gp86 (gcIII) complex did not share antigenic reactivity with gp86 (20). Additional evidence for the gp86 specificity of antibody AP86-SA4 was obtained by demonstrating reactivity for the product of the genomic sequence encoding gp86 expressed in

TABLE 1. Differential reaction patterns of human HCMV-positive sera with gp86 fusion proteins (strain AD169) in Western blot analyses

Serum	Reactivity ^a with:							
	AP86 ^b	EB86 ^b	BB86 ^b	PN86 ^b	SN86 ^b	NB86 ^b	NH86 ^b	Mbg58 ^c
3	+++	+++	-	-	-	-	-	+++
8	+++	+++	-	-	-	-	-	+++
12	+++	+++	-	-	-	-	-	+++
15	+++	+++	-	-	-	-	-	+++
19	+++	+++	-	-	-	-	-	+++
1	+++	+++	(+)	-	-	(+)	(+)	+++
14	+++	+++	(+)	(+)	(+)	(+)	(+)	(+)
5	-	-	+++	-	-	-	-	+++
13	(+)	(+)	-	-	-	-	-	(+)
20	-	-	(+)	-	-	(+)	(+)	(+)
2	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
11	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
4	-	-	-	-	-	-	-	+++
9	-	-	-	-	-	-	-	+++
10	-	-	-	-	-	-	-	+++
16	-	-	-	-	-	-	-	+++
17	-	-	-	-	-	-	-	+++
6	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-

^a +++, positive reaction; (+), nonspecific reaction; -, negative reaction.

^b gp86-containing fusion protein (for details, see Fig. 1).

^c β -Galactosidase fusion protein containing aa 484 to 650 of gp58 of HCMV (27).

FIG. 4. Isolation of AP86-specific antibodies by affinity chromatography of pooled human IgG. Purified fusion protein AP86 was conjugated to tressyl-activated Sepharose beads. A concentrated IgG preparation derived from pooled human HCMV-positive sera was passed over the column. Bound antibodies were eluted, and the first six fractions (1 to 6) as well as the original pool of human IgG (POOL) were tested by Western blot analyses. The gp86 fusion protein AP86, a fusion protein containing the immunodominant epitope of gp58 (Mbg58), and the β -galactosidase portion of the fusion proteins (SEM) were used as antigens. Adsorption and elution of the AP86-specific antibodies were carried out as described in Materials and Methods.

FIG. 5. Immunofluorescence of AD169-infected cells with monoclonal antibody AP86-SA4. Monoclonal antibody AP86-SA4 (a and c) and a conformational antibody against gp86 (14-4b) (b and d) were tested in an immunofluorescence analysis with AD169-infected cells. Intact cells showed fluorescence of the cell surface (a and b); permeabilized cells exhibited a perinuclear staining (c and d).

recombinant vaccinia virus-infected cells (data not shown) and fusion protein AP86 (Fig. 7). Finally, in repeated experiments, antibody AP86-SA4 neutralized 80% of input virus both in the presence and in the absence of exogenous complement. The antibody did not react with HCMV strain Towne in immunofluorescence assays (data not shown).

Further characterization of antibody-binding domains on AP86. To characterize the antibody-binding site(s) on AP86 more precisely, a set of expression plasmids which would lead to the synthesis of fusion proteins with increasing deletions at the carboxy terminus was constructed. For this purpose, plasmid pAP86 was digested unidirectionally from the 3' end of the gp86 coding region by using exonuclease III. The truncated fusion proteins were analyzed for antibody binding in immunoblots.

Fusion protein from construct Exo86-35, containing residues 15 to 43, was reactive with human sera as well as with monoclonal antibody AP86-SA4. Protein Exo86-54 (aa 15 to 34) was not recognized by the antibodies. A representative analysis showing the reaction pattern of monoclonal antibody AP86-SA4 is shown in Fig. 7. Figure 8 summarizes the results and the constructs used in these experiments. It should be noted that the signal strength of Exo constructs with use of human sera was comparable to that with use of AP86, suggesting that the weaker signals obtained with EB86 were not due to multiple epitopes on AP86. The data suggest that the binding site for human as well as mouse antibodies is located between aa 34 and 43. This can be concluded from

FIG. 6. Specificity of monoclonal antibody AP86-SA4. Extracellular virus obtained by centrifugation through sorbitol density gradients was subjected to SDS-PAGE. The separated proteins were transferred to nitrocellulose membranes and incubated with tissue culture supernatant of monoclonal antibodies against gp58 (27-156), pp65 (28-19), pp28 (41-18), and antibody AP86-SA4 produced against fusion protein AP86. 125 I-labelled rabbit anti-mouse immunoglobulins were used as the detection system. Sizes are indicated at the right in kilodaltons.

the fact that in addition to the fusion proteins from the Exo constructs, the EB86 protein, starting at aa 34, was reactive.

A comparison of the amino acid sequences of viral gp86 between the laboratory strains AD169 and Towne revealed that the binding site is not completely conserved between the two strains (Fig. 8). The differences comprise an amino acid deletion of a proline (aa 36 in strain AD169) and a histidine-to-lysine change directly adjacent to the deletion. If the antibody-binding site on gp86 of strain AD169 is not restricted to the six conserved residues within the defined domain, the differences could result in strain-specific recognition of antibodies. To test this possibility, an additional expression plasmid, containing aa 14 to 42 of gp86 of the Towne strain, was constructed and analyzed in immunoblot analyses with all 20 human sera as well as antibody AP86-SA4. Again the AP86-positive human sera showed the same

FIG. 7. Characterization of the binding site of monoclonal antibody AP86-SA4. Enriched fusion proteins EB86 (aa 34 to 111) and AP86 (aa 15 to 142) as well as truncated fusion proteins Exo86-35 (aa 15 to 43) and Exo86-54 (aa 15 to 34) were analyzed in an immunoblot assay with antibody AP86-SA4. Experimental conditions were as described in the legend to Fig. 3; sequences of the fusion proteins are shown in Fig. 8. Sizes of proteins are shown at the left in kilodaltons.

15	34	43	46		
...TVYLLSHLPSQRYGADAASEAL	LDPHAFHLL	NTYGRPIRFLRENTT...	AD169		
...C...L.S...E.I...P...*	K		TOWNE		
	LDPHAFHLL	NTYGRPIRFLRENTT...	EB86	+	+
YLLSHLPSQRYGADAASEAL	LDPHAFHLL	NTYGRPIRFLRENTT...	AP86	+	+
YLLSHLPSQRYGADAASEAL	LDPHAFHLL	NTY	Exo86-79	+	+
YLLSHLPSQRYGADAASEAL	LDPHAFHLL		Exo86-35	+	+
YLLSHLPSQRYGADAASEAL			Exo86-54	-	-
VCLSHLLSSRYGAEAISEPLD*	KAFHLL		To86	-	+

FIG. 8. Linear antibody-binding site of glycoprotein gp86. The upper part shows the amino acid sequence of the amino-terminal region of gp86 from strains AD169 and Towne (bold letters). Identical amino acids are marked by dots; amino acid deletions are marked by asterisks; amino acid positions are indicated by the numbers above the sequences. The sequences below represent the viral parts of the gp86 fusion proteins. EB86, AP86, Exo86-79, Exo86-35, and Exo86-54 are AD169 derived, and To86 is Towne derived (bold letters). The antibody binding site is indicated by a box. The reaction patterns of monoclonal antibody AP86-SA4 and the AD169-specific and Towne-specific human sera are indicated at the right.

reaction pattern as did AP86-SA4 (Fig. 9). Recognition of AD169-derived fusion proteins was specific, and no cross-reaction with fusion protein To86 derived from strain Towne was observed. This result clearly demonstrates that the characterized binding site represents a strain-specific epitope. When the AP86-negative human sera were tested, one was found to react with the fusion protein To86, indicating that the corresponding sequence in strain Towne in fact is immunogenic during natural infection. The presence of Towne-specific antibodies was also detected in the original serum pool used to purify AP86-specific antibodies, whereas no such antibodies were found in the IgG fraction purified on AP86 (data not shown). This result further demonstrates the presence of strain-specific antibodies against gp86 in the large number of human sera from which the pool was derived.

DISCUSSION

We have investigated the humoral immune response against linear antigenic domains on glycoprotein gp86 of HCMV strain AD169. A major domain was identified within the amino-terminal part of the molecule and further localized to aa 34 to 43. The domain was capable of inducing strain-specific neutralizing antibodies in laboratory animals as well

as during natural infection in humans. To our knowledge, this is the first strain-specific neutralizing epitope that has been characterized for HCMV.

The methods that have been used did not allow us to determine the precise boundaries of the antibody-binding site, but this was not the primary goal of our study. The fact that the signal in the immunoblot assays with human sera was consistently lower for fusion protein EB86 than that with AP86 suggests that amino acids amino terminal of residue 34 could enhance antibody binding. Alternatively, the structure of the bacterial part of the fusion protein could influence binding of the antibodies. In this respect, the monoclonal antibody seems to be slightly different in that we did not observe a difference in binding capacity between the two fusion proteins. A detailed characterization using overlapping peptides could establish the physical properties of the epitope. Such an analysis could also establish the nature of the antibody-binding site on strain Towne. Although it seems highly likely that the site on strain Towne was located in the same area as on AD169, our analysis has not rigorously addressed this possibility.

It was surprising that the entire gp86 molecule, consisting of more than 700 aa, contained only one major conformation-independent antibody-binding site recognized by human sera. This result was different from those of similar studies of other viral glycoproteins. Glycoprotein gp58/116 of HCMV, for example, contains at least three domains spread over the entire molecule (27, 35, 42). Up to 11 continuous antibody-binding sites have been described on gp41 of human immunodeficiency virus type 1 (18, 23). However, since it turned out that the AP86 domain is strain specific, the results concerning antibody-binding sites on gp86 must be viewed in relative terms. The study has been done almost entirely by using sequences from strain AD169, and consequently we could detect only antibodies which cross-react with these antigens. The results showing that Towne-specific antibodies to the amino-terminal region exist which are completely nonreactive for the AD169-derived antigen clearly indicated that our analysis would not detect gp86-specific antibodies induced by non-AD169-like strains. Similarly, we could have missed antibodies to other regions of the molecule which were induced by non-AD169-like strains. In addition, the lack of correlation that was seen between overall titer against HCMV and recognition of AD169-derived antigens could result, at least in some cases, from strain variation. We have not found evidence for the existence of hybrid strains inducing antibodies to the AP86 domains of both AD169 and Towne. The individual sera that were tested as well as the

FIG. 9. Recognition of the Towne-specific fusion protein by monoclonal antibody AP86-SA4 and human sera. The fusion proteins were tested in immunoblot assays with monoclonal antibody AP86-SA4 and human sera 3 and 20. The first panel represents an immunoblot analysis with a monoclonal antibody against β -galactosidase (87-55/60) showing that comparable amounts of the respective fusion proteins were applied to the gel. Experimental conditions were as described in the legend to Fig. 3; sequences of the proteins are shown in Fig. 8.

affinity-purified AP86, which contained antibodies from a large number of individual donors, did not show cross-reaction between antigens.

Immunological assays using procaryote-derived fusion proteins or synthetic peptides are a widely used method to determine the antibody profiles in sera of different origins. It is generally believed that for slowly mutating viruses such as herpesviruses, the results can be extrapolated to larger populations. Our study has shown that this is not necessarily the case. Using AP86 from strain AD169, we found 35% of sera reacting with a single domain. However, only 1 in 20 sera (5%) was positive for the same domain derived from strain Towne.

At the moment, there is no information on how many different HCMV strains exist in various populations, but since HCMV is an ancient human virus, the variability should not be underestimated. Comparison of the two available sequences from strains AD169 and Towne shows additional areas within gp86 which are heterogeneous enough to give rise to strain-specific antibodies, and there are no data available which indicate that these two laboratory strains represent in fact the most widely distributed variants. In addition, the data showing that 35% of the sera that were tested contained antibodies against AD169-derived antigens cannot be taken as evidence that AD169-like strains are prevalent in our area. We have shown for gp58/116 of HCMV that antigenic domains derived from different prototype strains can exist on the same molecule (30).

The lack of B-cell epitopes on gp86 of strain AD169 in regions other than the identified domain seems to be an intrinsic property of this molecule, since mice immunized with the different fusion proteins showed an immune response comparable to that of humans. Monoclonal antibodies could be readily developed against AP86, while only a weak immune response was found against BB86, which contained the remainder of the molecule. It is worth noting that the presence of neutralizing antibody-binding sites at the very amino terminus seems to be a more general phenomenon associated with glycoproteins of herpesviruses. Examples are the glycoproteins gB and gD of herpes simplex virus as well as gp58/116 of HCMV (9, 35, 38). Whether this is a fortuitous finding or has some biological relevance remains to be determined.

A number of neutralizing monoclonal antibodies reactive with gp86 have been described (3, 10, 12, 40). These antibodies are thought to react with conformation-dependent epitopes present on a limited number of HCMV isolates (3). The antigenic structure described in this report again was represented in only a limited number of HCMV strains. In contrast to the relatively widespread conservation of two linear neutralizing antibody sites on HCMV gp58/116 (35, 42), these data suggested that gp86 induced mostly strain-specific antibodies. Although the relative contribution of various anti-envelope glycoprotein antibodies to the *in vivo* virus-neutralizing response remains undefined, if gp86 strain-specific antibodies contributed significantly to this activity, the previously infected host could be readily susceptible to reinfection and/or disease induction by a heterologous strain.

From our data, it cannot be ascertained whether the AP86-specific response constituted a minor or major fraction of the overall neutralizing response against gp86. Although it is generally believed that conformation-dependent antibodies constitute the major fraction of antibodies against proteins, it has been demonstrated in the case of gp58/116 of HCMV that a major fraction of the neutralizing response is

directed against nonconformational sites (27). Studies to determine the contribution of AP86-specific antibodies to the overall response against gp86 are in progress.

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