VIROLOGY 216, 133–145 (1996)
ARTICLE NO. 0040

Antibodies Specific for the Antigenic Domain 1 of Glycoprotein B (gpUL55)

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Received August 28, 1995; accepted November 17, 1995

Glycoprotein B (gB, gpUL55) is a major antigen for the induction of neutralizing antibodies against human cytomegalovirus, making it an attractive antigen for active and passive immunoprophylaxis. The immunodominant region on gB is the antigenic domain 1 (AD-1), a complex structure which requires a minimal linear amino acid sequence of more than 75 amino acids (aa 552–635) for antibody binding. We have analyzed the fine specificity of neutralizing and nonneutralizing AD-1-binding monoclonal antibodies. Point mutations were introduced into AD-1 and mutants were expressed as bacterial fusion proteins. The antigens were analyzed in immunoblots using a panel of 13 human and murine monoclonal antibodies. Complete loss of binding of all antibodies was observed with mutations at cysteine residues 573 and 610 as well as with a combinatorial exchange of prolines at position 577 and 613. The remaining mutations had different effects on antibody binding. Six individual recognition patterns were observed, indicating various antigenic substructures on AD-1. Changing the Fc portions of 3 murine monoclonal antibodies to human IgG1 showed that neutralization of AD-1-binding immunoglobulins is exerted by different mechanisms. Dependent on the recognized substructure within AD-1, avidity-dependent as well as Fc portion-mediated effects were observed. © 1996 Academic Press, Inc.

INTRODUCTION

Infections with human cytomegalovirus (HCMV) continue to be an important clinical problem in certain patient populations. The virus remains a major cause of morbidity and mortality in immunocompromised hosts, such as transplant recipients and patients with AIDS. Both cellular and humoral immune responses are likely to be involved in the restriction of HCMV infections. In several previous studies passive transfer of immunoglobulins has been shown to modulate serious HCMV disease by limiting virus dissemination, possibly by providing neutralizing antibodies (for review see Zaia, 1993). Also, in a recent vaccination trial, protection from reinfection was found to correlate with neutralizing antibodies (Adler *et al.*, 1995). In the closely related murine cytomegalovirus system antibodies can limit dissemination of recurrent virus and also provide protection from a lethal challenge (Jonjic *et al.*, 1994, Rapp *et al.*, 1993). However, the results from clinical studies remain controversial (for review see Zaia, 1993). A significant problem in the interpretation of these clinical trials has been the use of a number of different immunoglobulin preparations, all of which could be expected to vary in their titer of HCMV-specific antibodies (Chehimi *et al.*, 1987; Schmitz and Essuman, 1986). This problem will remain as long as immunoglobulin preparations are derived from a pool of different individual donors.

Glycoprotein B (gB) is the dominant antigen on the envelope of HCMV and nearly 100% of HCMV-infected individuals develop antibodies against this protein (Kniess *et al.*, 1991; Marshall *et al.*, 1992). Antibody preadsorption experiments with recombinant-derived gB have shown that in some human sera a considerable fraction of the neutralizing response is directed against gB (Britt *et al.*, 1990; Marshall *et al.*, 1992). Consequently gB has been proposed as a candidate for a subunit vaccine, and protein purified from virus preparations, as well as recombinant gB, has been used in first vaccination studies (Gonczol *et al.*, 1991; Pass *et al.*, personal communication). The majority of gB-specific antibodies which are developed during natural infection are directed against the antigenic domain 1 (AD-1). During natural infection and after immunization in mice, AD-1 is capable of inducing neutralizing and competing nonneutralizing antibodies in a single individual, a fact which could complicate its inclusion in a subunit vaccine or its use as target antigen for selection of neutralizing human monoclonal antibodies (Ohlin *et al.*, 1993; Utz *et al.*, 1989). In addition, AD-1 is an unusual complex structural domain. Although antibody binding takes place under denaturing conditions, a minimum of more than 75 residues of continuous primary amino acid sequence between amino acids (aa) 552 and 635 is essential for reactivity (Wagner *et al.*, 1992). Using amino acid deletions from the amino- or

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carboxyterminal boundary of AD-1, we were not able to establish substructures or to differentiate individual binding patterns for neutralizing or nonneutralizing human and mouse antibodies. Likewise attempts to define subunits of AD-1 using synthetic peptides of various length have been unsuccessful (Wagner *et al.,* 1992). These results raised the question whether AD-1 as a structure is inducing only a single type of antibody with the differences in biological activity being due to events secondary to AD-1 binding or whether AD-1 contains substructures which can induce neutralizing or nonneutralizing antibodies.

In this investigation we have further analyzed the fine specificity of AD-1-binding monoclonal antibodies. Our data indicate that AD-1 contains various substructures to which antibodies can bind. When Fc portions of AD-1-binding antibodies were changed from murine to human, a significant increase in neutralization capacity was noted for one antibody, suggesting a participation of the Fc portion in the neutralization mechanism of AD-1-specific antibodies.

TABLE 1

Characteristics of AD-1-Specific Murine and Human Monoclonal Antibodies

Monoclonal antibody	Recognition subgroup	Isotype	Virus	
			Neutralization	Avidity index[a]
27-156	A	IgG2b/κ	Yes[b]	0.88
27-78	A	ND[c]	Yes	0.88
27-83	A	ND	Yes	0.87
ITC63B	A	IgG1/λ	Yes[d]	0.86
ITC52	A	IgG1/κ	Yes	0.74
ITC39[e]	**A**	IgG1/λ	No	0.02
28-287	B	IgG2b/κ	Yes	0.87
ITC33	**B**	IgG1/λ	No	0.65
7-17	C	IgG3/κ	Yes	0.45
7-5	**C**	IgG1	No	0.06
9-3	D	IgG2a	Yes	0.14
ITC48	E	IgG1/κ	Yes	0.44
27-11	**F**	ND	No	0.41

[a] Avidity index was performed as described under Materials and Methods and calculated according to the formula (OD value sample plus 7 *M* urea/OD value sample without urea).

[b] Neutralization capacity of murine mabs established in this study according to the procedures described under Materials and Methods.

[c] Not determined.

[d] Neutralization capacity of ITC series according to Ohlin *et al.,* 1993.

[e] Nonneutralizing antibodies are shown in bold.

MATERIALS AND METHODS

Cells and viruses

Human foreskin fibroblasts were grown in minimal essential medium (Gibco BRL, Glasgow, Scotland) supplemented with 5% fetal calf serum (FCS), glutamine (100 mg/liter), and gentamycin (350 mg/liter). Propagation and gradient purification of HCMV strain AD169 was carried out by the method of Talbot and Almeida (1977).

Monoclonal antibodies and bacterial fusion proteins

Hybridoma cell lines were propagated in RPMI 1640 (Gibco BRL) supplemented with 10% FCS, glutamine, and gentamycin. Antibodies were purified from tissue culture supernatant by ammonium sulfate precipitation or on protein G–Superose columns (Pharmacia, Freiburg, Germany) (Harlow and Lane, 1988). Specifications of AD-1-binding antibodies are shown in Table 1. The Exo-proteins contained the following amino acids of gB as trpE fusion proteins: Exo 58-314 (aa 549–636), Exo 58-315 (aa 549–635), Exo 58-310 (aa 549–632), Exo 58-36 (aa 549–630), Exo 58-35 (aa 549–628), Exo 58-5A15 (aa 552–653), Exo 58-5E1 (aa 556–653), Exo 58-5E8 (aa 557–653), Exo 58-D7 (aa 561–635), and Exo 58-5E39 (aa 564–635) (Wagner *et al.,* 1992).

PCR amplification and cloning of variable light (Vl) and variable heavy (Vh) coding sequences

Total RNA was prepared from 2×10^7 cells according to Chomczynski and Sacchi (1987). Reverse transcription and PCR amplification of the mRNAs coding for variable regions of the antibodies 7-17, 27-156, and 27-287 were carried out by using the Ig-Prime kit (Novagen, Madison, WI). All primers were provided by the kit and the manufacturer's instructions were followed precisely. The mRNA coding for the variable light chain of the antibody 7-17 could not be amplified by this procedure. Therefore a G-tailing reaction of the reverse-transcribed cDNA of hybridoma 7-17 was performed as described by Saiki *et al.* (1988), followed by PCR amplification. Two 5′-primers (G-tailing 1, G-tailing 2) as well as one 3′-primer (G-tailing) were used simultaneously (Table 2). The aberrant chains secreted by the myeloma fusion partner P3X63–Ag8.653 and Vl of the antibody 7-17 were amplified by this procedure (Carroll *et al.,* 1988). The PCR products were directly cloned into the vector pT7Blue (Ig-Prime kit) and used to transform bacterial strain Nova Blue (Ig-Prime kit) by electroporation. Bacterial colonies containing the aberrant κ-light chain were identified by hybridization using a specific oligonucleotide (5′-CACATTAGGGAGCTTACA-CGT3′) for the aberrant chain. The DNA of clones which showed no signal in the colony hybridization was sequenced. All working procedures were performed according to Sambrook *et al.* (1989).

The following accession numbers were assigned: U39898 to sequence 7-17Vh of antibody 7-17, U39899 to 27-156Vh, U39900 to 27-287Vh, U39901 to 7-17Vl, U39902 to 27-156Vl, U39903 to 27-287Vl.

Chimerization of antibodies 7-17, 27-156, and 27-287

DNA from clones containing Vh or Vl sequences was PCR-amplified from the respective plasmid using the

TABLE 2

Sequences of Primers Used for G-Tailing Reaction and Cloning of Vl/Vh into the Vectors pUHWκ and pUHWγ

Primer	Sequence
5′ primer G-tailing (1)	5′-CGTAGAATTCGCTGCAACCGTGCCCCCCCCCCCC3′
5′ primer G-tailing (2)	5′-CGTAGAATTCGCTGCAACCGTG3′
3′ primer G-tailing	5′-GGAAGCTTACTGGATGGTGGGAAGATGGA3′
5′ primer 7-17 Vl	5′ GATCGTCGACATGAAGTTGCCTGTTAGGC3′
5′ primer 27-156 Vl	5′ GATCGTCGACATGGAGACAGACACACTCC3′
5′ primer 27-287 Vl	5′ GATCGTCGACATGGAGACAGACACACTCC3′
3′ primer κ	5′-GAGCGCGGCCGCACTTACGTTTTATTTCCAGCTTGG3′
5′ primer 7-17 Vh	5′ CATAGTCGACATGAAATGGAGCTGGGTC3′
5′ primer 27-156 Vh	5′ CATAGTCGACATGGAATGGACCTGGGTC3′
5′ primer 27-287 Vh	5′ CATAGTCGACATGGAATGCAGCTGGGTC3′
3′ primer γ1	5′ GAGCGCGGCCGCACTTACCTGAGGAGACGGTGAC3′

primers shown in Table 2 and inserted into the vectors pUHWκ and pUHWγ, respectively (Weissenhorn *et al.*, 1991). All 3′-primers contained a splice donor site 5′ACT-TAC3′, since plasmids pUHWκ and pUHWγ were originally constructed for the expression of genomic variable region sequences and contain the respective splice acceptor site at the 5′-end of the constant coding region. Vectors were linearized at their unique *Pvu*I sites before transfection into hybridoma cell lines SP2/O and CB-F7 (Charité, Berlin, Germany) (Grunow *et al.*, 1988). To generate stable transformants with a high frequency the electroporation conditions were optimized for each DNA preparation. In general, 5×10^6 cells were mixed with 15 μg linearized DNA in 0.8 ml HEPES buffer. Usually electroporation conditions of 960 μF, 9–11 ms, and 280–340 V produced more than 50% mortality of cells and stable transfectants could be generated. Transfected cells were propagated in 96-well round-bottom plates. Stable clones were isolated by G418 selection (1 mg/ml) added 24 hr after electroporation. Chimeric antibody-producing cells were detected by a capture ELISA. The mouse monoclonal antibody anti-human IgG1 (Dako, Bremen, Germany) was diluted to 1.5 μg/ml in 0.05 *M* carbonate buffer, pH 9.5, and used to coat microtiter plates for 16 hr at 4° in a humid chamber. Supernatant from transfected cells was added (50 μl/well) and after 2 hr of incubation at 37° a peroxidase-conjugated mouse monoclonal antibody directed against human κ chains (Dako) was used to detect correctly assembled antibodies. Cells producing intact antibody molecules were repeatedly subcloned and expanded.

Site-directed *in vitro* mutagenesis of the AD-1 coding sequences

Starting with the construct Exo 58-315 which expresses aa 549–635 of HCMV gB as trpE fusion protein (Wagner *et al.*, 1992), point mutations were introduced into AD-1 according to Dulau *et al.* (1989) using PCR amplification with degenerate primers. Mutant DNA was transfected into *Escherichia coli* C600 and DNA sequence analysis of all plasmids was performed to confirm single point mutations within AD-1.

Evaluation of antibody fine specificity

Preparation of bacterial fusion proteins, as well as immunoblot analysis, was performed as described by Wagner *et al.* (1992) with the exception that in some assays antibody binding was detected with an enhanced chemoluminescence Western blotting system (ECL; Amersham International, UK). ELISAs using AD-1 sequences as antigen were performed as described by Wagner *et al.* (1992). Neutralization assays were carried out as described by Andreoni *et al.* (1989) with slight modifications. Various amounts of virus and antibody were preincubated in 100 μl RPMI 1640 for 4 hr at 37°. Human fibroblasts (3×10^4) were added in a volume of 25 μl medium and the mixture was seeded in 1 well of a 96-well plate. After infection for 20 hr at 37°, cells were fixed with absolute ethanol and stained with a monoclonal antibody (P63-27) reactive with the major immediate-early protein of HCMV (UL123). Antibody binding was detected by a fluorescein-conjugated anti-mouse antibody. Generally, infectious doses were adjusted to 150 infected cells counted on a fluorescence microscope (Zeiss/Axioskop MC80) using a 200× magnification. To enable the comparison of murine and chimeric antibodies with respect to neutralizing capacity, two approaches were followed. The protein concentration of each antibody preparation was measured with a BCA Protein Reagent Assay (Pierce, Rockford, IL) and the relative amount of AD-1 binding antibodies was determined in an ELISA using AD-1 as antigen. To minimize the effect of potentially different avidities of anti-human and anti-mouse peroxidase-coupled antibodies, we used polyclonal immunoglobulins as a detection system. Neutralization capacity of each given antibody preparation was normalized in the AD-1 ELISA (A_{490nm}) and the corresponding protein concentration was calculated. Two murine monoclonal

antibodies, 3H82 and E6H6 (anti-β-galactosidase and anti-tryptophan synthetase), were used as a control. The standard error of the assay was in the range of 10%.

To determine antibody avidity, the standard ELISA was modified. After formation of the antibody–antigen complex, wells were treated for 4 min with PBS containing various concentrations of urea (5–7 *M*). Plates were washed three times (5 min each) with PBS–0.05% Tween 20 and anti-mouse or anti-human antiserum coupled to horseradish peroxidase was added. Alternatively, antibody–antigen complexes were treated with PBS–6 *M* urea for various times (1–7 min). The avidity index for each monoclonal antibody was calculated according to the formula (OD value sample plus urea/OD value sample without urea). Comparative analyses were performed by testing all antibodies simultaneously on the same ELISA plate since the incubation time of preformed antibody–antigen complexes with different concentrations of urea was critical for the avidity index.

RESULTS

Monoclonal antibodies define antigenic substructures within antigenic domain AD-1

AD-1 is a complex antigenic domain which has been characterized in detail in previous investigations (Wagner *et al.,* 1992; Utz *et al.,* 1989; Ohlin *et al.,* 1993). The binding of antibodies to this domain was analyzed with a panel of bacterial fusion proteins expressing sequences between residues 549 and 645 of gB. These previous studies have suggested that a linear sequence of the AD-1 antigenic site was recognized as a single epitope by AD-1-specific neutralizing and nonneutralizing monoclonal antibodies (mabs). Amino acid insertions as well as carboxy- or aminoterminal deletions abrogated mab reactivity, suggesting this region exhibited a unique conformation, which was dependent on an intact primary sequence. However, competition experiments also suggested that different antibody binding structures might exist on AD-1. In the present study an attempt was made to differentiate these specificities by mutating single amino acids in AD-1. Amino acids were selected for mutation based on computer predictions which indicated contribution of the residues to secondary structure. In particular the proline residues at positions 570, 577, and 613, as well as the serine residues at positions 586 and 587, were mutated. In addition, the cysteine residues which are present in AD-1 at positions 573 and 610 and which potentially could form disulfide bonds were altered to serine. The mutated fusion proteins were analyzed in immunoblots with eight mouse and five human AD-1-specific mabs. In each group two of the antibodies have been shown to lack virus neutralizing activity (Table 1). Using this approach it was possible to discriminate binding specificities between antibodies. As an example, the individual binding patterns that were observed for the murine monoclonal antibodies 27-156 and 7-17 are shown in Fig. 1. Qualitative differences in binding to individual fusion proteins were observed for all mabs. These differences were reproducible in repeated analyses. In all immunoblots great care was taken to apply equal amounts of fusion proteins to the analysis, and controls included the AD-1-specific murine monoclonal antibody B1B6 which was reactive with all fusion proteins (see Fig. 2). The data for all mabs are summarized in Table 3. Six different binding patterns were observed (Groups A–F, Table 3). Mutations involving cysteine residues at positions 573 or 610 or combinatorial exchange of prolines 570+577 as well as 577+613 resulted in loss of binding for all antibodies. For the reason mentioned above the weak reactivity of antibody 7-17 with lower molecular weight proteins in the proline 570+613 mutant represented nonspecific signals. The remaining mutations had different effects on antibody binding. From these data it must be concluded that a number of antigenic substructures exist on AD-1 against which antibodies are formed after natural infection or immunization. However, as in our previous analyses, we were unable to establish a correlation between specificity of a particular antibody and neutralizing capacity. Within group A, for example, one nonneutralizing antibody (ITC39) as well as five neutralizing antibodies (ITC52, ITC63B, 27-156, 27-83, 27-78) were found. Similarly, groups B and C contained neutralizing and nonneutralizing antibodies.

Avidity of AD-1-specific antibodies

In the next series of experiments we determined the avidity of AD-1-specific antibodies in order to evaluate potential correlation between antibody reactivity and neutralization capacity. An ELISA was developed in which the preformed antibody–antigen complex was treated with different concentrations of urea and residual bound antibody was expressed as function of urea concentration. The results were expressed as relative avidity index as described under Materials and Methods. When antibodies within one species were compared, avidity was not correlated to neutralizing activity (Table 1). This was illustrated for example by the lower avidity index of the neutralizing murine antibody 9-3 compared to the nonneutralizing antibody 27-11A. Likewise, the human antibody ITC33, which is nonneutralizing, had a higher avidity index than ITC48, which is neutralizing. Interestingly, within antibodies from one subgroup increasing avidity to AD-1 was correlated to neutralizing activity, suggesting that avidity might be important for neutralizing activity in those cases where similar substructures on AD-1 are recognized.

Synthesis of chimeric antibodies having human Fc and murine Fv regions and analysis of binding specificities

In attempting to establish correlations between avidity and neutralizing activity, we did not differentiate between

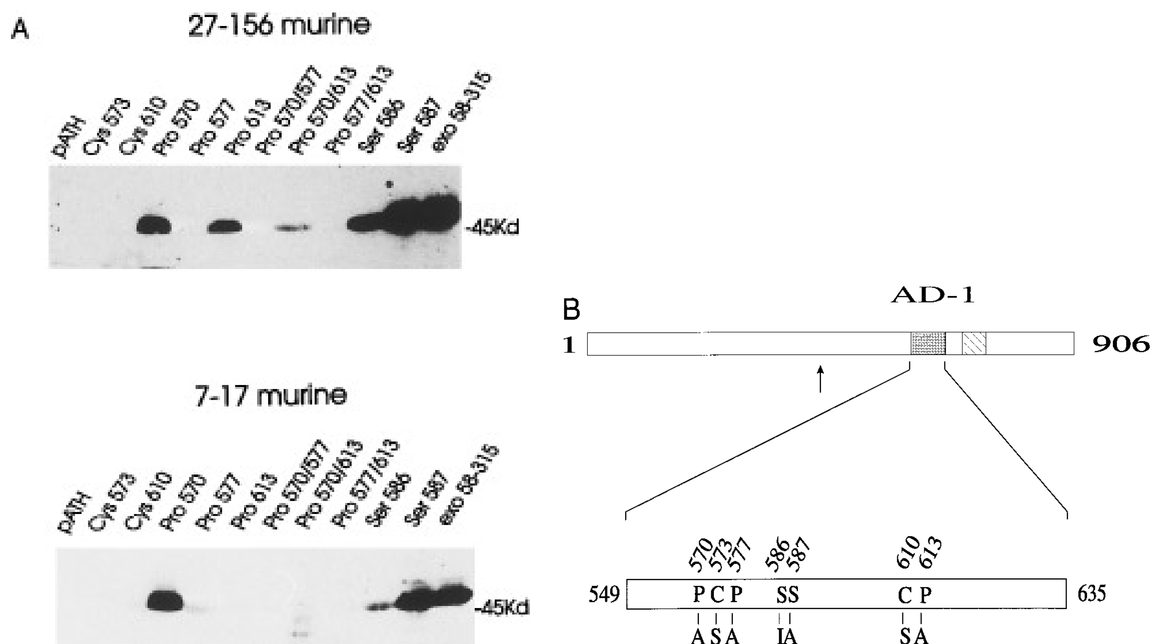

FIG. 1. (A) Reactivity of murine monoclonal antibodies for bacterial fusion proteins containing mutations in AD-1. *E. coli* lysates containing the indicated fusion proteins were separated on SDS–PAGE and transferred to nitrocellulose membranes. After blocking, individual blots were incubated with monoclonal antibodies 27-156 and 7-17, respectively. Bound antibody was detected with alkaline phosphatase-conjugated anti-mouse IgG and developed with the ECL chemoluminescence system (Amersham). The position of the molecular mass standard (in kilodaltons) is indicated. Fusion proteins were designated according to the amino acid that was changed within AD-1, i.e., Cys 573 represents an AD-1 mutant with a replacement of cysteine at position 573. Double mutants were designated accordingly. pATH and Exo 58-315 represent control antigens containing the fusion partner trpE and AD-1 (aa 549–635), respectively. (B) Schematic diagram depicting location of AD-1 relative to gB and mutations within AD-1. At the top full-length gB is shown with AD-1 indicated by the shaded box. Membrane anchor (dark box) and proteolytic cleavage site (arrow) are also indicated. The AD-1 mutants are shown on an expanded scale. Residues inside the bar represent the original AD169 gB sequence. The mutated amino acids are shown below the bar. Numbers refer to amino acid position within gB.

human and mouse monoclonal antibodies. The validity of this interpretation can be questioned, since it has been shown that the Fc portion, which is different between human and mouse IgG, has a potential influence on antibody avidity and hence on neutralization capacity (Morelock *et al.,* 1994; Cooper *et al.,* 1991; Bruggemann *et al.,* 1987; Persson *et al.,* 1988). However, the limited number of available AD-1-specific antibodies did not allow a separate evaluation of the two species. We addressed this problem by providing the human constant region γ1 to three murine monoclonal antibodies (27-156, 27-287, and 7-17), thereby generating antibodies with identical human Fc portions in groups A and B and with different Fc portions in group C. In addition, mab 7-17 was the only murine antibody with an IgG3 constant region which could mediate biological activity via cooperativity through Fc–Fc interactions (Fulpius *et al.,* 1993; Greenspan and Cooper, 1993; Cooper *et al.,* 1991). Sequence information of these antibodies would also provide information on the degree of diversity between AD-1-specific antibodies.

We generated human–mouse chimeric antibodies by first isolating mRNA from the respective hybridomas. After reverse transcription of mRNA to cDNA, coding sequences for the Vh and Vl chains were amplified by PCR and inserted into plasmids and the nucleotide sequence was determined. During the process of constructing the chimeric antibodies we noted several features of the murine antibodies 7-17, 27-156, and 27-287. The most relevant characteristics are summarized in Fig. 3 and Table 4 and included:

(i) the Vl chains originated from different Vκ gene groups and showed between 73 and 77% homology on the nucleotide level and 54 and 59% on the amino acid level (data not shown);

(ii) Vh chains had similarities between 86 and 92% (nucleotide level) and 67 and 85% (amino acid level) and could be assigned to the J558 family. However, the Vh chain of antibody 7-17 was derived from a different gene segment from that of the other two murine antibodies;

(iii) the length of the third hypervariable region of the heavy chain (CDR3), which for most antibodies is crucial for contact to the antigen, varied from 10 to 15 amino acids, indicating independent recombination events. No apparent requirement for particular amino acid residues at any position of this region was found. The third hypervariable region CDR3 is generated by Vh–Dh–Jh joining. Random nucleotide addition (N regions) and junctional flexibility makes assignment of the Dh origin problematic. The D segment of antibody 7-17 showed highest homology to the DSP 2-4 element and was flanked on both

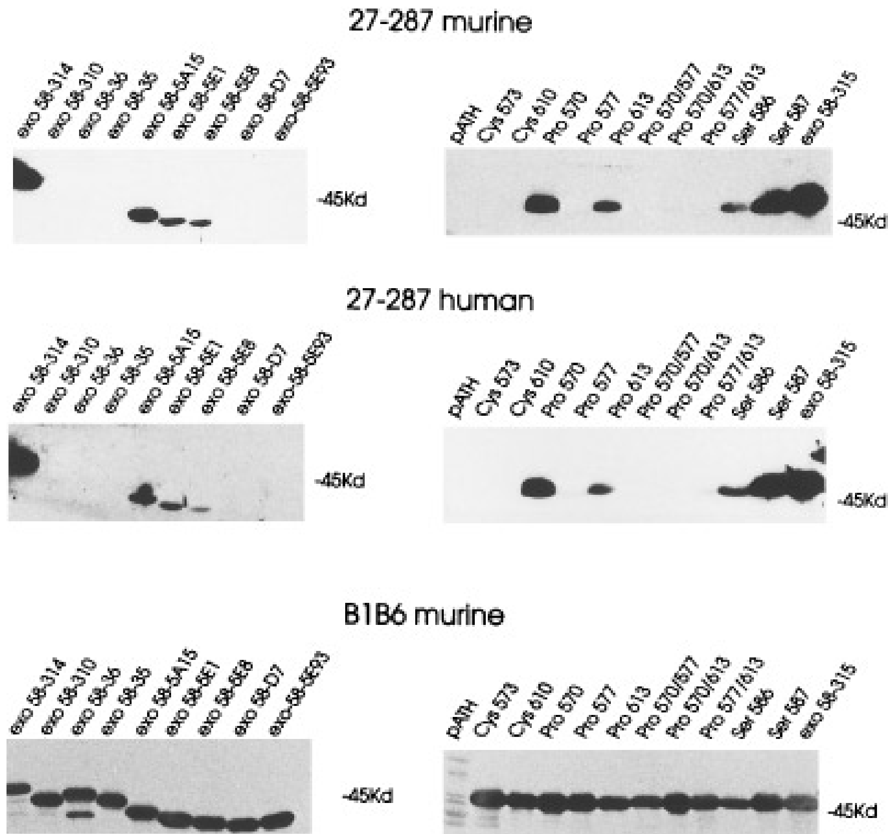

FIG. 2. Reactivity of murine (27-287 murine) and chimeric (27-287 human) monoclonal antibodies with AD-1-containing bacterial fusion proteins. Immunoblot analyses were performed as described in legend to Fig. 1. The position of the molecular mass standard (in kilodaltons) is indicated. For specification of the bacterial fusion proteins see Fig. 1A and Material and Methods. The murine monoclonal antibody B1B6, reactive with all AD-1-containing fusion proteins, was used as control.

sites by N regions. For the other two antibodies no clear homology to any of the known murine D segments could be established. However, both antibodies most probably contain D–D segment fusions which have occasionally been observed (Meek *et al.,* 1989). From these data it must be concluded that a significant degree of structural diversity exists among murine mabs that share specificity for the same antigenic determinant on gB. These findings were consistent with the antigenic specificities of the antibodies.

The chimeric antibodies were constructed by combining the Fv coding sequences of the AD-1-specific murine antibodies with the constant regions of human immunoglobulin γ1 heavy chain and the κ light chain. The resulting antibodies were designated 27-287hu, 27-156hu, and 7-17hu, respectively. To exclude the possibility that chimerization had changed the specificity of the antibodies we compared reactivity of murine and chimeric antibody pairs with a panel of fusion proteins expressing sequences from AD-1 in immunoblots. In addition to the AD-1 mutant fusion proteins a set of previously described polypeptides (the Exo-series; see Materials and Methods) was used which contained amino- and carboxyterminal deletions of AD-1 (Wagner *et al.,* 1992). Reaction patterns that were observed in repeated analyses showed no qualitative differences between murine and chimeric antibodies when fusion proteins from mutated and nonmutated AD-1 sequences were used as antigen. As an example the results obtained with murine and chimeric antibody 27-287 are shown (Fig. 2). Again, the difference in signal strength that was seen between the fusion proteins was not due to varying amounts of antigen present on the nitrocellulose filters as shown by control antibody B1B6 (Fig. 2). Reactivity of the respective mouse and chimeric antibody pairs was determined on the same nitrocellulose filter with stripping of the first antibody followed by binding of the second antibody to the same filter. There was no difference in reactivity with antibodies derived from SP2/O and CB-F7 cells (data not shown).

Analysis of binding avidity and neutralizing activity of murine and chimeric antibodies

We further characterized the chimeric antibodies by comparing their relative binding avidities to AD-1 with their respective parental murine antibodies. All three immunoglobulins showed changes in avidity after chimerization. At concentrations of 7 *M* urea the avidity index of antibody 7-17hu increased from 0.45 to 0.77 (Fig. 4B). In

TABLE 3

Recognition Patterns of Murine and Human Monoclonal Antibodies with Different AD-1 Mutants

Fusion proteins containing AD-1	Recognition patterns of AD-1-specific antibodies					
	A	B	C	D	E	F
Exo 58-315	+	+	+	+	+	+
C573 (→S)[a]	−	−	−	−	−	−
C610 (→S)	−	−	−	−	−	−
P570 (→A)	+	+	+	+	−	+
P577 (→A)	−	−	−	+	−	−
P613 (→A)	+	+	−	−	−	+
P570+P577	−	−	−	−	−	−
P570+P613	+	−	−	−	−	+
P577+P613	−	−	−	−	−	−
S586 (→I)	+	+	+	+	+	−
S587 (→A)	+	+	+	+	+	+
	27-156 27-83 27-78 **ITC39** ITC52 ITC63B	27-287 **ITC33**	7-17 **7-5**	9-3	ITC48	**27-11**[b]

[a] Amino acid exchange produced by site-directed mutagenesis.
[b] Nonneutralizing antibodies are shown in bold.

contrast, the avidity indices for antibodies 27-287hu and 27-156hu decreased from 0.87 to 0.22 and 0.88 to 0.47, respectively (Figs. 4A and 4C). Identical changes in avidity were seen when antigen–antibody complexes were treated with 6 *M* urea for various times (data not shown). The observed effect was independent of the antibody preparations as well as of the antibody concentration used in the assays (Fig. 4D). The changes in relative binding avidities of antibodies 27-287 and 27-156 compared to antibody 7-17 were unexpected. However, these antibodies now provided a test panel for the hypothesis that neutralizing activity within one AD-1-binding anti-

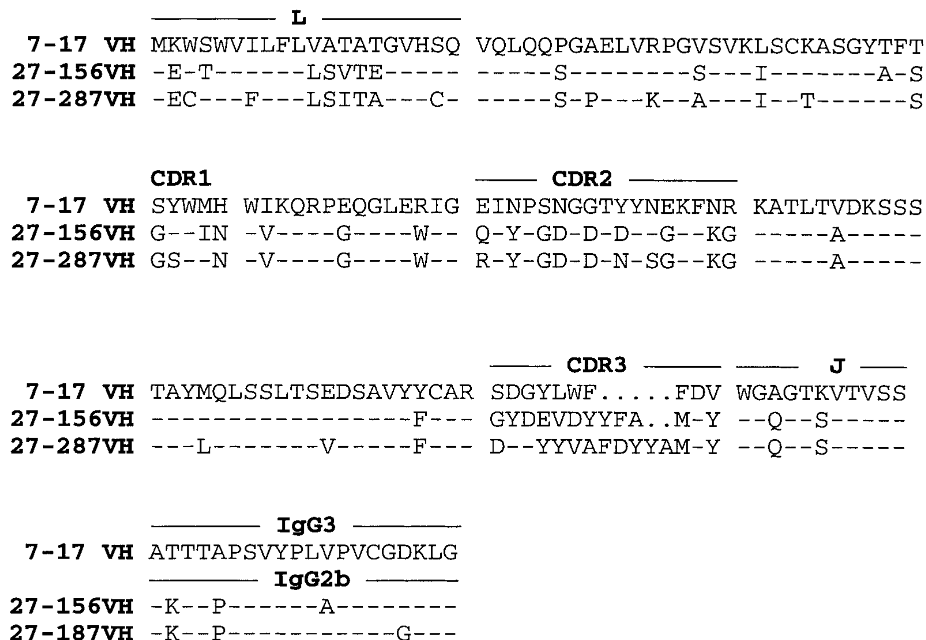

FIG. 3. Deduced amino acid sequences of the Vh chains of murine antibodies 7-17, 27-156, and 27-287. Sequences of antibodies 27-156 and 27-287 were compared to 7-17. Identity with 7-17 is indicated by dashes and differences are displayed. Gaps have been introduced to facilitate alignment of sequences and are marked by dots. Functional domains of the antibodies were identified according to Kabat *et al.* (1991) and are indicated.

TABLE 4

Characteristics of Anti-AD-1 Murine Monoclonal Antibodies

Antibody	V gene expression				
	Vl	Jl	Vh	D	Jh
7-17	V-κI/1A	J2	J558/VH-IIb	DSP2-4	J1
27-156	V-κII/V8	J2	J558/VH-I	?[a]	J4
27-287	V-κIII/V21E	J2	J558/VH-I	?	J4

[a] Antibodies 27-156 and 27-287 possibly contain D–D fusions.

body subgroup was correlated with avidity. Since neutralization is critically dependent on the actual concentration of functional antigen-binding molecules in the respective preparations, we first carried out titrations of the respective antibody preparations in ELISAs using AD-1 as antigenic substrate. This allowed us to express the neutralizing activity of an individual antibody as a function of AD-1-binding activity. This approach gave reproducible results when two different preparations of the same immunoglobulin were compared (Fig. 4D). This was not the case when comparative analyses where performed on the basis of protein concentrations (data not shown). Neutralization assays were carried out at least two times with differences between assays of less than 10%. The observed effects were independent of a number of parameters such as the cell line from which the chimeric antibodies were derived (CB-F7 or SP2/O) or the purification procedure used (untreated tissue culture supernatant, purification via protein G chromatography, or ammonium sulfate precipitation) (data not shown). No differences were seen with the chimeric antibodies 7-17hu and 27-156hu, respectively, compared to their murine counterparts. As an example, data for 7-17 and 7-17hu are shown in Fig. 5. Murine antibody 7-17 reduced input infectivity by 73% at the maximum concentration of antibody in the assay, which is comparable to previous studies (Britt, 1984). The most surprising finding was the markedly increased activity of the 27-287 chimera. The

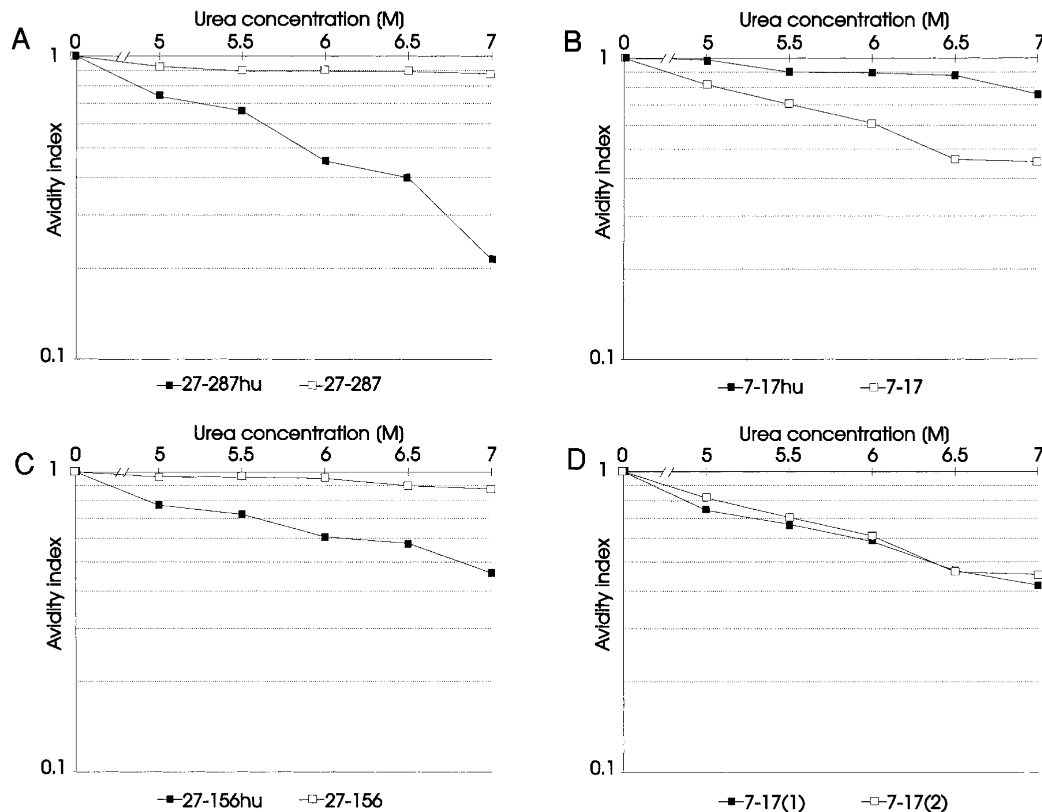

FIG. 4. Avidity determination of murine monoclonal antibodies and their chimeric counterparts. Determination of the individual avidity indices was performed as described in legend to Fig. 2. (A–C) Murine–chimeric pairs. (D) Two different preparations of antibody 7-17 at different concentrations [antibody 7-17(1), A_{490nm} = 3.579 at 0 *M* urea; antibody 7-17(2), A_{490nm} = 2.715].

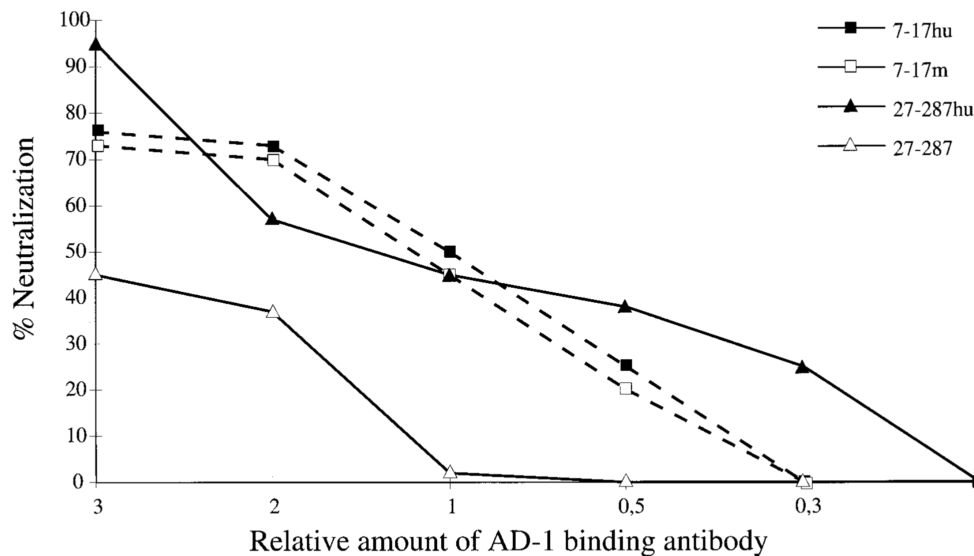

FIG. 5. Neutralization of HCMV by murine antibodies 27-287 and 7-17 and their chimeric counterparts. Neutralization analysis was performed as described under Materials and Methods. Percentage of reduction of input infectivity was plotted as a function of AD-1-binding antibody concentration. The relative concentration of AD-1-binding antibodies in individual preparations was determined in an ELISA assay using AD-1 as antigen. Absolute amount of antibody at the highest concentration corresponded to 5.2 μg/ml for 27-287hu, 28.3 μg/ml for 27-287m, 8 μg/ml for 7-17hu, and 27.3 μg/ml for 7-17m.

murine antibody 27-287 had a maximal neutralization capacity of 45%. However, the chimeric molecule almost completely reduced infectivity of virus and reached neutralization levels of 95% (Fig. 5). The difference between 27-287 and 27-287hu was also observed in assays where the number of infectious virus particles was titrated against a constant concentration of antibody (data not shown). Tissue culture supernatants of murine monoclonal antibodies reacting with β-galactosidase or tryptophan synthetase did not reduce input infectivity.

DISCUSSION

Glycoprotein B is an important antigen for the induction of neutralizing antibodies against HCMV. This makes gB an attractive antigen for strategies of active and passive immunoprophylaxis (Azuma *et al.*, 1991; Plotkin, 1991; Starr *et al.*, 1991; Aulitzky *et al.*, 1991; Werner *et al.*, 1993; Snydman, 1990). AD-1 represents the dominant antigenic structure for induction of antibodies against gB. One hundred percent of infected individuals who are seropositive for gB have antibodies against AD-1 (Kniess *et al.*, 1991). The domain has been characterized in previous work and was shown to consist of a minimal linear amino acid sequence of more than 75 amino acids between residues 552 and 635 of gB (Wagner *et al.*, 1992). Binding of different neutralizing and nonneutralizing murine and human monoclonal antibodies to this domain is competitive (Utz *et al.*, 1989; Ohlin *et al.*, 1993).

The present study was undertaken to obtain more information on the complex interaction of AD-1 with antibodies. Two major conclusions can be drawn:

(i) AD-1 contains antigenic substructures which can be defined by anti-AD-1 antibodies. Antibodies for which competition for binding to AD-1 has been shown in previous investigations did not fall into single subgroups (Wagner *et al.*, 1992; Utz *et al.*, 1989; Ohlin *et al.*, 1993).

(ii) The nature of the Fc portion of AD-1-binding antibodies contributes to virus neutralizing activity.

The fact that monoclonal antibodies exhibited individual binding specificities indicated recognition of different antigenic substructures on AD-1. Even with our limited set of AD-1 mutant fusion proteins we were able to differentiate six antibody binding patterns suggesting the existence of at least as many different antigenic substructures. This likely represents the lower estimate of the total number of antibody binding substructures on AD-1 since the mutations that were introduced most probably had a rather profound impact on AD-1 structure. This might have resulted in assignment of antibodies to one group which, upon a more refined analysis using additional mutant fusion proteins, could be differentiated with respect to AD-1 recognition. Moreover, the limited number of available AD-1-specific antibodies represented an additional restriction in our analysis.

The complete loss of antibody binding with the cysteine mutant proteins indicated that these residues are crucial for the formation of the antigenic structure AD-1. There are two possible ways in which the cysteine residues could be involved in the architecture of AD-1: (i) formation of a disulfide bond between Cys573 and Cys610 thereby creating a loop structure and (ii) formation of oligomers via disulfide bonds between different

AD-1 molecules. Oligomerization of HCMV-gB via disulfide bonds is well documented but the specific cysteine residues responsible for these interactions have not been identified (Britt and Vugler, 1992). The fact that the antibody binding form of AD-1 was observed at an apparent molecular mass equivalent to the monomers does not necessarily argue against this possibility since the oligomers could form during the blotting procedure. Formation of disulfide bonds at late stages in the analysis has to be postulated for both models since fusion proteins were reduced and denatured prior to analysis. However, our results do not rule out the possibility that formation of AD-1 is independent of the formation of disulfide bonds. Reactivity with antibodies was also abrogated by mutations other than cysteine. Whether these modifications influenced formation of AD-1 structure independent of disulfide bonds, prohibited correct formation of disulfide bonds, or altered the structure of AD-1 in the presence of correct disulfide bonds cannot be determined from our analysis. No matter what the underlying mechanism for the differential antibody recognition, it must be concluded that a variety of nonrelated antibodies having different biological activities are induced by AD-1.

The second major conclusion from our study concerns the mechanisms of neutralization exerted by AD-1-specific antibodies. Neutralization of viruses can be achieved by a variety of mechanisms which in some cases can operate simultaneously, even with monoclonal IgG (for a review see Dimmock, 1987). In most cases the exact mechanism by which an antibody is exerting its neutralizing activity is not known. This is also true for AD-1-binding antibodies. Given the individual recognition patterns of the antibodies it is conceivable that they neutralize through independent mechanisms. For antibodies of the 27-156 and 7-17 type the Fc portion of the molecule seems to be irrelevant for neutralization since exchange of the Fc portion from murine IgG2b and IgG3 to human IgG1 did not result in a significant alteration in neutralization capacity. This includes potential cooperativity mediated through murine IgG3 Fc portions (Fulpius *et al.*, 1993; Greenspan and Cooper, 1993; Cooper *et al.*, 1991). In contrast, antibody 27-287 showed a significant increase in neutralization capacity after exchange of the Fc portion. Since all neutralization experiments were carried out without complement, there are at least two possible explanations.

Steric effects mediated by the entire Fc portion

The results with the point mutations in AD-1 clearly show that the three antibodies bind to different substructures of AD-1. This probably results in a different orientation of the Fc portions in the three-dimensional space. Exchange of the murine Fc part against the human IgG1 portion could lead to steric effects which affect events such as adsorption or penetration. Combined with the original neutralization mechanism, probably mediated through the Fab part of the antibody, an increased neutralization activity could occur. Steric effects that influence neutralization of viruses have been observed in a number of different systems (Possee *et al.*, 1982; Taylor *et al.*, 1987; Taylor and Dimmock, 1985).

Segment flexibility

Segmental flexibility of IgG molecules is a well-known phenomenon and potentially facilitates bivalent antigen binding. The order of segmental flexibility has been shown to be IgG2b > IgG2a > IgG3 > IgG1 for mouse antibodies and IgG3 > IgG1 > IgG4 > IgG2 for human immunoglobulins with the range of mobility remarkably similar in both species (Dangl *et al.*, 1988; Gorman and Clark, 1990). On the other hand, glycoproteins on the surface of virions can adopt different conformations resulting in the formation of a limited number of neutralization-sensitive sites (Roben *et al.*, 1994; Taylor *et al.*, 1987; Taylor and Dimmock, 1985; Dimmock, 1987). Increased segmental flexibility of 27-287hu could therefore result in an enhanced bivalent binding to neutralization-sensitive sites. In this context it is worth mentioning that AD-1-specific IgG molecules that are produced during natural infection exclusively belong to subclass 1, which is in contrast to other glycoproteins of HCMV such as gH, where IgG1 and IgG3 can be detected (Urban *et al.*, 1994). If segmental flexibility is critical for efficient neutralization of HCMV *in vivo,* anti-AD-1 antibody responses restricted to the IgG1 subclass may provide a mechanism favoring persistence of HCMV.

Data in the literature on correlation of antibody avidity and neutralizing capacity are conflicting. While some studies did observe a positive correlation, others did not (Roben *et al.*, 1994; Beidler *et al.*, 1988). For AD-1-binding antibodies, we originally observed a correlation between avidity and neutralization capacity with immunoglobulins belonging to one recognition group. The data obtained with chimeric antibodies confirmed this correlation for immunoglobulins of groups A and C. Chimerization of murine antibody 27-287, which belongs to group B, showed different results. The avidity of the chimeric 27-287hu was drastically reduced, while its neutralizing activity was increased. It can be speculated that immunoglobulins binding to different substructures within AD-1 mediate neutralization via mechanisms which are dependent or independent of the avidity of the respective antibody. While group A-binding antibodies have a strong correlation between avidity and neutralizing capacity, group B-binding immunoglobulins do not. An alternative explanation could be that antibodies 27-287 and ITC33 do actually belong to two different recognition groups which we were not able to distinguish with the available set of mutants.

What could be the consequences of these complex

antibody–antigen interactions for the natural infection? Since AD-1 is the immunodominant domain on gB, an inefficient immune response against this domain could have implications on the course of infections. The AD-1 coding region displays a high degree of conservation between strains. Nevertheless, between one and six divergent amino acids within AD-1 have been predicted, when nucleotide sequences from clinical isolates were compared to the prototype sequence AD169 (Lehner *et al.,* 1991; Chou and Dennison, 1991; Darlington *et al.,* 1991; Chou, 1992). The virus could use these variations to evade an efficient immune response in at least two ways:

(i) Point mutations in AD-1 could abrogate binding of antibodies belonging to one or more groups. For example a clinical isolate has been described with a threonine residue instead of proline in position 570 (Roy *et al.,* 1993). According to our data, this virus would not be recognized by antibodies belonging to group E such as ITC48, which is a neutralizing human monoclonal antibody.

(ii) Point mutations could influence the proportion of neutralizing versus nonneutralizing antibodies which are induced during infection. Loss of neutralizing capacity of antibodies mediated by point mutations in immunogenic domains is well documented for a number of virus systems (Thali *et al.,* 1994; Iorio *et al.,* 1991; van-Wyke-Coelingh and Tierney, 1989; Pfaff *et al.,* 1988). Combination of point mutations in AD-1 and other important antigens such as gH might augment the effect of efficient immune evasion through minor changes in primary sequence. Antigenic variation of gH between different HCMV strains has been repeatedly observed (Simpson *et al.,* 1993; Urban *et al.,* 1992; Baboonian *et al.,* 1989; Rasmussen *et al.,* 1984).

The role of neutralizing antibodies in protective immunity against HCMV is incompletely understood. Despite accumulating data on strain-specific immune responses the contribution of group- and type-specific neutralizing antibodies is unclear. However, there is increasing evidence that strain-specific immune responses might be important in certain clinical situations. In seropositive renal transplant recipients reinfection with a genetically different donor virus is more frequently associated with severe HCMV disease (Grundy *et al.,* 1988). Virus isolates from AIDS patients exhibit a greater degree of divergence in AD-1 than strains from renal transplant recipients (Roy *et al.,* 1993; Spector *et al.,* 1984). In the face of the declining ability of AIDS patients to mount an efficient immune response against *de novo* antigens these alterations might provide a replication advantage to particular strains. Lastly, vaccination with gB derived from a single prototype virus might provide insufficient protection against infection with antigenically different strains.

ACKNOWLEDGMENTS

We thank Ulrich H. Weidle for vectors pUHWκ and pUHWγ and SP2/O cells; S. Jahn for CB-F7; M. Schwirzke for advice in generating antibody-producing cell lines; and Th. Winkler for interpretation of sequence data. This work was supported by grants from the Johannes und Frieda Marohn-Stiftung and the Deutsche Forschungsgemeinschaft (FL 91/10-3).

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