Characterization of a Discontinuous Neutralizing Epitope on Glycoprotein B of Human Cytomegalovirus

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Human cytomegalovirus (HCMV) is a ubiquitously distributed pathogen that causes severe disease in immunosuppressed patients and newborn infants infected *in utero*. The viral envelope glycoprotein B (gB) is an attractive molecule for active vaccination and passive immunoprophylaxis and therapy. Using human monoclonal antibodies (MABs), we have recently identified antigenic region 4 (AD-4) on gB as an important target for neutralizing antibodies. AD-4 is formed by a discontinuous sequence comprising amino acids 121 to 132 and 344 to 438 of gB of HCMV strain AD169. To map epitopes for human antibodies on this protein domain, we used a three-dimensional (3D) model of HCMV gB to identify surface-exposed amino acids on AD-4 and selected juxtaposed residues for alanine scans. A tyrosine (Y) at position 364 and a lysine (K) at position 379 (the YK epitope), which are immediate neighbors on the AD-4 surface, were found to be essential for binding of the human MABs. Recognition of AD-4 by sera from HCMV-infected individuals also was largely dependent on these two residues, indicating a general importance for the antibody response against AD-4. A panel of AD-4 recombinant viruses harboring mutations at the crucial antibody binding sites was generated. The viruses showed significantly reduced susceptibility to neutralization by AD-4-specific MABs or polyclonal AD-4-specific antibodies, indicating that the YK epitope is dominant for the AD-4-specific neutralizing antibody response during infection. To our knowledge, this is the first molecular identification of a functional discontinuous epitope on HCMV gB. Induction of antibodies specific for this epitope may be a desirable goal following vaccination with gB.

Human cytomegalovirus (HCMV) is a ubiquitously distributed herpesvirus. Infection in hosts with a functional immune system is usually clinically asymptomatic. In contrast, in immunocompromised hosts, such as transplant recipients or congenitally infected newborns, the virus can cause significant morbidity and mortality (1). For example, HCMV is the most common viral infection of the fetus, ranging from 0.2% to 2% in all live births, and 5 to 15% of congenitally infected children develop long-term sequelae (2).

The virus is controlled by a multilayered immune response, including innate and adaptive immune effector functions. Within the adaptive arm of the immune response, the development of antiviral antibodies contributes to effective control of HCMV infections. Antibodies directed against viral glycoproteins could mediate protection by direct virus neutralization and/or induction of immunoglobulin Fc receptor-mediated effector functions, such as antibody-dependent cytotoxicity and/or complement-mediated effects which may result in destruction of infected cells (3).

In cases of congenital HCMV infection, naturally acquired antiviral antibodies have been considered to be important components of the maternal immune response in protection, although protection is not complete (4). In recent studies, prevention of congenital HCMV infection by passive transfer of HCMV hyperimmune globulin has been reported (5–7). The protective effect of serum antibodies has also been demonstrated in animal model systems. In the mouse CMV model, complete protection from infection was achieved in animals which lack functional T and B cells by prophylactic or therapeutic administration of polyclonal antibody preparations (8).

The correlates of protection in polyspecific serotherapy are not known. An important antibody target is glycoprotein B (gB). An-

tibodies directed against gB can be detected in all infected individuals (9, 10). A major fraction of neutralizing antibodies in human sera seems to be directed against gB, and the overall *in vitro* neutralizing capacity in sera from HCMV-infected individuals correlates with anti-gB antibody titer (9, 11). Anti-gB antibodies are also effective in preventing cell-to-cell spread of the virus (12). In the guinea pig model of maternal and congenital HCMV infection, gB-specific antibodies have also been shown to protect from infection or transmission (13). Significant protection from brain pathology in murine CMV-infected mice by passive administration of a gB-specific MAb was also reported (14).

The immunogenicity of gB during infection has made gB an attractive vaccine candidate. In experimental settings, gB vaccinations have been shown to elicit protective antibody responses (13, 15). Recombinant gB also represents the most advanced vaccine in humans, partially protecting from maternal and congenital infection and reducing the duration of viremia in transplant recipients (16, 17).

gB is one of the few indispensable viral envelope glycoproteins

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that is conserved between the herpesviruses, indicating a fundamental role for the biology of these viruses. It is involved in the membrane fusion process and in cell-to-cell spread of the virus, but it is not required for attachment, assembly, or viral egress (18). The recent determination of the crystal structure of gB from herpes simplex virus 1 (HSV-1) and Epstein-Barr virus (EBV) has identified gB as a class III fusion protein (19, 20). Given the high degree of conservation, it is reasonable to assume that gB has similar functions in other herpesviruses, including HCMV. However, to enable fusion of the viral envelope and target cell membranes, gB requires interaction with additional viral proteins. In the case of HSV-1, these include the gH/gL complex, gD, and a cellular receptor (21). For HCMV, expression of gB, either alone or in combination with the gH/gL complex, has been described to be sufficient for fusion of a number of different cell types (12, 22–24). Studies using neutralizing murine monoclonal antibodies (MAbs) allowed the dissection of different regions on HSV-1 gB that are functional either in interaction with the gH/gL complex or in membrane association or fusion (25). Thus, gB-specific antibodies which are capable of neutralizing infectious virus may execute their function via different mechanisms.

There are still significant gaps in our knowledge of the antibody response against gB during natural infection with HCMV or following vaccination with recombinant gB. Five antigenic domains (AD) which induce antibodies during infection have been identified (26–28). Our recent analysis of the antibody repertoire of anti-gB antibodies as it is developed during infection has shown that >95% of anti-gB antibodies are not capable of neutralizing the virus in *in vitro* tests. Neutralizing antibodies were found to be mainly directed against AD-4 and AD-5 (28). AD-4 is a highly immunogenic structure, since >90% of HCMV-infected individuals develop antibodies against this domain (28). AD-4 represents a discontinuous protein domain formed by amino acids (aa) 121 to 132 and 344 to 438 of gB of HCMV strain AD169. The corresponding protein domain in HSV-1 gB is likely a domain involved in interaction with gH/gL (25). Thus, antibodies directed against AD-4 may target a function that is essential for the proper function of the viral fusion machinery.

In order to obtain more information on the interaction of human antibodies with AD-4, we have started to characterize antibody binding epitopes. Our data reveal that a tyrosine residue at position 364 and a lysine residue at position 379 (the YK epitope), which are juxtaposed on the surface of AD-4, are particularly important for binding of human MAbs. Importantly, AD-4-specific human polyclonal antibodies, affinity purified from pooled immunoglobulin preparations, showed requirements for binding similar to those of the MAbs, indicating a more general relevance of the YK epitope on AD-4 for the antibody response against gB. The relevance of the critical contact residues for virus neutralization by AD-4-specific antibodies was verified by generation of recombinant HCMV variants within AD-4 which were found to resist neutralization.

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney cells (Cos7), primary human foreskin fibroblasts (HFF), and human fetal lung fibroblasts (MRC-5) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Germany) supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich, Germany), glutamine (100 mg/ml), and gentami-

cin (350 mg/ml). HCMV-TB40/E and its recombinant derivatives were propagated in MRC-5 cells.

Antibodies. The following antibodies were used: gB AD-4-specific human MAb SM5-1, SM1-6, SM3-1, and SM6-5 (28); gB AD-2-specific human MAb C23 (Ti23) (29); an AD-4-specific affinity-purified human polyclonal antibody preparation (poly-AD-4) (28); HCMV immediate-early 1 protein (IE-1)-specific murine MAb p63-27 (30); murine antihemagglutinin (anti-HA) MAb (clone HA-7) from Sigma-Aldrich (Germany); and murine anti-glutathione *S*-transferase (anti-GST) MAb (clone vpg66) from BIOZOL (Germany). Secondary antibodies were purchased from Dianova (Germany) or Dako (Germany).

Expression plasmids. The expression plasmid for gB of HCMV strain AD169 has been described previously (28). The plasmids coding for AD-4 and its derivatives were constructed by inserting the appropriate DNA fragment into the vector pcUL132-sig-HA. This pcDNA3.1 (Invitrogen, Germany)-based plasmid contains the coding sequence of the HCMV gpUL132 authentic signal sequence aa 1 to 27 (28), followed by the coding sequence for the HA epitope YPYDVPDYA. To insert the appropriate AD-4 coding sequences, restriction sites EcoRI and XbaI were used, which are located immediately downstream of the HA coding sequence. The respective AD-4 coding sequence (aa 112 to 132 and aa 344 to 438 of gB strain AD169) was chemically synthesized by Life Technologies (Germany). To generate plasmids for expression of GST fusion proteins in *E. coli*, the respective nucleotide sequences were amplified by PCR from the pcDNA-based plasmids and inserted into the expression vector pGEX-6P-1 (GE Healthcare, Germany). Candidate residues for the alanine scan were identified based on a structural model of HCMV gB (28). In the first step, residues with a solvent-exposed surface area larger than 50 Å² were identified and subsequently grouped into spatially proximal pairs of residues. Initially, this procedure resulted in a total of 19 pairs of residues selected for subsequent mutagenesis.

Transient protein expression and image analysis. Cos7 cells (5×10^4 per well) grown in 24-well plates on 13-mm glass coverslips were transfected with 0.8 µg of plasmid DNA using Lipofectamine (Invitrogen, Germany). Forty-eight hours after transfection, cells were fixed and permeabilized with ice-cold methanol. Primary antibodies were then added for 90 min at 37°C. Unbound primary antibody was removed by three phosphate-buffered saline (PBS) washing steps. Binding of the primary antibody was detected with the appropriate fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Dako, Germany) (60 min at 37°C). Counterstaining of cell nuclei was done with DAPI (4',6-diamidino-2-phenylindole). Images were collected using a Zeiss Axioplan 2 fluorescence microscope fitted with a VisiTron Systems charge-coupled device camera (Puchheim, Germany). Images were processed using MetaView software and Adobe Photoshop.

Preparation of AD-4 proteins. Plasmid DNA was used to transform *E. coli* DH10B for expression of GST fusion proteins. Expression of respective fusion proteins was induced using IPTG (isopropyl-β-D-thiogalactopyranoside), and the soluble form of the proteins was purified from *E. coli* lysates. *E. coli* lysates were incubated with glutathione Sepharose 4B (GE Healthcare, Germany) for 2 h at room temperature, unbound bacterial protein was removed by washing with PBS, and proteins were eluted with 10 mM reduced L-glutathione. To remove the GST part, the fusion protein was proteolytically cleaved on the glutathione Sepharose 4B using PreScission protease (GE Healthcare) by incubation for 5 h at 4°C. The cleaved protein was eluted from glutathione Sepharose 4B. A second purification step via anion-exchange chromatography was performed. To this end, AD-4 proteins without the GST tag were dialyzed against binding buffer (20 mM Tris, pH 8.0) and anion-exchange chromatography was carried out using a HiTrapQ FF 1-ml column (GE Healthcare) on an ÄKTApurifier (GE Healthcare). Unbound proteins were removed by washing (20 mM Tris [pH 8.0]), and the proteins were eluted using a linear gradient of elution buffer (20 mM Tris, 0.6 M NaCl [pH 8.0]). The proteins were dialyzed against PBS and used in an enzyme-linked immunosorbent assay (ELISA).

ELISA. Proteins were diluted to 1 to 2.5 $\mu\text{g/ml}$ in 0.05 M sodium carbonate buffer, pH 9.6, and 50 $\mu\text{l/well}$ was used to coat polystyrene 96-well plates overnight at 4°C. All the following reactions were performed at room temperature. Reaction wells were blocked with PBS containing 2% FCS for 2 h, washed with PBS, and incubated with primary antibody for 2 h. Unbound antibody was removed by washing with PBS plus 0.1% Tween 20, and the appropriate peroxidase-conjugated secondary antibody was added for 45 min. The plate was washed 3 times (PBS plus 0.1% Tween 20), and 100 μl of tetramethylbenzidine (TMB) peroxidase substrate was added for 5 min, diluted 1:1 in peroxidase substrate solution B (KPL, USA). The reaction was stopped by adding 100 μl of 1 M phosphoric acid. The optical density at 450 nm (OD_{450}) was determined using an Emax microplate reader (Eurofins MWG Operon, Germany). In assays using GST fusion proteins as the antigen, equivalent concentrations of GST were run as control antigens and OD values were subtracted. Equivalent coating of nonfused AD-4 proteins was controlled by a murine polyclonal anti-AD-4-specific serum. Human sera were used at a dilution of 1:50.

Surface plasmon resonance (SPR) analysis. Kinetic experiments were performed at 25°C using a Biacore T100 (GE Healthcare, Germany). AD-4–GST fusion proteins were captured on the Series S Sensor Chip CM5 (GE Healthcare) using *N*-hydroxysuccinimide-*N*-ethyl-*N*-dimethylamino-propyl-carbodiimide chemistry. PBS–0.05% P20 was used as the running buffer. Approximately 300 response units (RU) of AD-4–GST and GST as reference surface antigen were captured with a contact time of 400 s and a flow rate of 10 $\mu\text{l/min}$. Different concentrations of the MABs were injected with a contact time of 90 s, a dissociation time of 600 s, and a flow rate of 30 $\mu\text{l/min}$. The sensor surface was regenerated between each binding reaction with 10 mM glycine (pH 2.0) with a contact time of 20 s and a flow rate of 30 $\mu\text{l/min}$. The kinetics data were fitted to a 1:1 binding model.

CD spectroscopy. AD-4 and its mutated derivatives were used at a concentration of 8 μM for circular-dichroism (CD) spectroscopy. They were dialyzed against 20 mM sodium phosphate buffer (pH 7.0). CD spectroscopy was performed at 20°C using a Jasco J-815 CD spectrometer (Jasco, Japan) and a cuvette with a 0.1-cm path length. Spectra were recorded from 185 to 260 nm and were corrected for the contribution of phosphate buffer. Spectra were accumulated eight times with a bandwidth of 1.0 nm and a sensitivity of 100 millidegrees (mdeg). The scan speed was 20 nm/min, the time response 1 s, and the data pitch 0.1 nm.

BAC mutagenesis and reconstitution of BAC-derived viruses. An HCMV-TB40/E-derived bacterial artificial chromosome (BAC) (TB40-BAC4) was used to generate mutant viruses using the mutagenesis protocol of Tischer et al. (31). The BAC was a kind gift of Christian Sinzger, University of Ulm, Germany (32). Briefly, recombination cassettes were amplified from plasmid pEP-kan-S using primers containing the desired mutational sequence and flanking nucleotide sequences in gB from strain TB40 at the sequence of interest (see Fig. S1 in the supplemental material). Recombination cassettes were used to transform *E. coli* GS1783 and inserted into TB40-BAC4 by homologous recombination, and clones were selected on kanamycin. To remove all non-HCMV sequences, an I-SceI digestion and a subsequent red-mediated recombination followed. Integrity of the BAC DNAs was verified by restriction enzyme digestions, and nucleotide sequencing was performed to confirm correct introduction of the respective mutations. To reconstitute virus, 2.3×10^5 MRC-5 cells/well were seeded into 6-well dishes 48 h before transfection. Cells were transfected with 5 μg of BAC DNAs together with 1 μg of pcDNAp71tag DNA (kindly provided by B. Plachter, University of Mainz, Germany) using FuGENE (Roche, Germany). Cells were supplied with fresh medium 24 h after transfection and every second day until they were transferred into 25-cm² cell culture flasks. Plaques were detected approximately 10 days posttransfection. Viral DNA was isolated from infected cells using proteinase K, and correctness of reconstituted viruses was confirmed by nucleotide sequencing analysis.

For growth curves, HFF were infected at a multiplicity of infection

(MOI) of 0.4 to 0.6. After adsorption of virus for 4 h, the inoculum was removed and cells were washed with PBS and cultured in fresh medium. Supernatants from infected cultures were harvested at the time points indicated below and stored at room temperature until use. Virus titers of the supernatants were determined by immunostaining of IE-1 as described previously (30). In brief, MRC-5 (1×10^4 per well) cells were seeded in 96-well plates. Cells were incubated with dilutions of cell culture supernatants for 4 h at 37°C. After removal of the inoculum, cells were washed with PBS and fresh medium was added. Following incubation for 24 h at 37°C, cells were fixed with cold ethanol and washed. The number of infected cells was determined by indirect immunofluorescence using MAb p63-27 as the primary antibody and Cy3-conjugated anti-mouse IgM plus IgG (Dianova, Germany) as the secondary antibody.

Virus neutralization assay. HFF (1×10^4 per well) were seeded in 96-well plates. HCMV TB40/E or recombinant viruses were preincubated with serial log₂ dilutions of antibodies for 1 h at 37°C. The mixture was added to the cells for 4 h. The inoculum was removed and the cells were washed with PBS, followed by incubation for 24 h at 37°C. Cells were fixed with ice-cold ethanol, and infected cells were determined with the IE-specific antibody p63-27. After removal of unbound antibody by washing with PBS, binding was detected with Cy3-conjugated anti-mouse IgG plus IgM antibody (Dianova, Germany). The infected cells were counted on a fluorescence microscope Axiovert 200 (Carl Zeiss MicroImaging, Germany), and percent neutralization was determined as $[1 - (\text{number of infected cells in the presence of antibody} / \text{number of infected cells in the absence of antibody})] \times 100$. Prior to performance of the neutralization assay, viral titers were adjusted to give 60 to 100 infected cells per field of vision without addition of antibody.

RESULTS

The complete AD-4 peptide sequence is essential for antibody binding. AD-4 of gB, which corresponds to structural domain II, is formed by a discontinuous sequence comprising aa 121 to 132 and aa 344 to 438 in HCMV strain AD169 (Fig. 1A). In order to express it *in toto*, a 5-aa linker sequence (IAGSG) was inserted between the two protein sections (Fig. 1B). In the first set of experiments, we tested whether the complete AD-4-encoding sequence is required for binding of human antibodies which are induced during infection. The respective gB sequences were expressed containing a signal sequence for insertion into the endoplasmic reticulum, followed by an HA tag to facilitate detection. Four human MABs (SM1-6, SM3-1, SM5-1, and SM6-5), derived from an HCMV-infected healthy donor, as well as a previously described (28) AD-4-specific affinity-purified polyclonal antibody preparation (termed poly-AD-4) were used. Binding of antibodies was analyzed in Cos7 cells after transient expression of the respective gB parts followed by indirect immunofluorescence. Both the MABs and the poly-AD-4 preparation, which has been shown to be monospecific for AD-4, were found to be completely dependent on the presence of the complete AD-4 sequence as defined above. Deletion of short amino acid stretches at either the amino or carboxy terminus of AD-4 resulted in complete loss of antibody binding. Representative data are shown in Fig. S2 in the supplemental material. We concluded from this result that AD-4 in its entirety is required for binding of antibodies and that either no or very low concentrations of antibodies which recognize subdomains or linear epitopes within AD-4 are present in sera of HCMV-infected individuals. In addition, the MABs showed only limited competition for binding to the antigen (see Fig. S2).

Identification of surface-exposed residues on AD-4 and recognition of AD-4 mutant polypeptides by human MABs. To obtain more detailed information on the critical binding residues for

FIG 1 AD-4 of gB and recognition by human antibodies. (A) Linear representation of HCMV gB and its structural domains. The regions representing individual structural domains are displayed in different colors in analogy to the HSV-1 gB structure (19). Brackets indicate disulfide bonds and numbers the beginning of the domains, respectively. (B) 3D model of the domain architecture of trimeric HCMV gB. Two monomers are shown in gray, and one monomer is colored consistently with panel A. Structural domain II, corresponding to AD-4, is enlarged. Numbers indicate the beginning and end of domain II parts that were linked by a 5-aa synthetic linker (IAGSG) for protein expression (magenta). (C) Structural models of the AD-4 mutant polypeptides. AD-4 is displayed as a ribbon diagram, and residues exchanged to alanine are shown as spheres in red.

the MABs, alanine scans of AD-4 were performed. Only residues which were predicted to be surface exposed were considered for exchange to alanine. In addition, due to the size of AD-4, we elected to mutate two neighboring, surface-exposed residues si-

multaneously to alanine. The respective amino acids within AD-4 were identified using the three-dimensional (3D) model of gB as shown in Fig. 1B, and this prediction was used to design 19 potential candidate pairs for the dialanine scan. The location of mutated

FIG 2 Recognition of AD-4 mutant polypeptides by MAbs. (A) Cos7 cells transiently expressing the indicated AD-4 polypeptides were analyzed in indirect immunofluorescence with the respective MAbs. (B) ELISA with AD-4 mutant polypeptides. AD-4–GST fusion proteins were purified from *E. coli* lysates and used in an ELISA to analyze binding of the indicated antibodies.

residues within the 3D model of AD-4 is provided in Fig. 1C. (A linear representation of the respective mutants is given in Fig. S3 in the supplemental material.) Again, the AD-4 polypeptides were transiently expressed in Cos7 cells and antibody recognition was analyzed by indirect immunofluorescence. Two patterns of recognition were observed (representative data shown in Fig. 2A). Loss of signal was seen for three MAbs (SM1-6, SM3-1, and SM6-5) with the KK_{378/79} mutant, in which lysine residues 378 and 379 were changed to alanine. The remaining mutations had no effect on MAb binding. SM5-1 retained binding to all 19 dialanine mutants (Fig. 2A and data not shown).

To confirm the results obtained by indirect immunofluorescence in an independent and more quantitative assay, seven AD-4 mutant polypeptides were expressed as GST fusion proteins in *E. coli* and the respective proteins were affinity purified. In our previous analysis of AD-4, we demonstrated that bacterially expressed wild-type (wt) AD-4 retains a native structure that is recognized by the conformation-dependent MAbs (28). The purity of fusion proteins was analyzed by SDS-PAGE and was estimated

to be >90% for all polypeptides (data not shown). The purified fusion proteins were used as antigens in ELISAs. Fusion proteins used in this series of experiments included the KK_{378/79} mutant and proteins with the structurally closely located mutations ED_{359/62}, QE_{380/81}, YL_{364/76}, and ES_{422/24}, as well as the more distantly located mutations NS_{383/85} and NT_{405/06} (Fig. 2B). In accordance with the data obtained in the indirect immunofluorescence assays, MAb binding to the ED_{359/62}, QE_{380/81}, ES_{422/24}, NS_{383/85}, and NT_{405/06} mutants was unaffected (Fig. 2B). Also, the KK_{378/79} mutant was not recognized by the MAbs with the exception of SM5-1, confirming that the dilysine motif at positions 378 and 379 is crucial for binding of MAbs SM1-6, SM3-1, and SM6-5. The analysis of YL_{364/76} revealed additional information. Whereas reactivity of SM5-1 and SM6-5 to this mutant was comparable to that to wt AD-4, MAbs SM3-1 and SM1-6 showed reduced reactivity to this peptide, indicating that tyrosine 364 and/or leucine 376 may contribute to the antibody binding site of at least some MAbs (Fig. 2B). Surface plasmon resonance analysis revealed reduced binding capacity or loss of binding of AD-4-specific MAbs (SM5-1 and SM6-5) to the KK_{378/79} and YL_{364/76} mutants

TABLE 1 Binding parameters for MAbs toward AD-4 and AD-4 mutant polypeptides^a

Antibody	AD-4			KK _{378/79}			YL _{364/76}		
	k_a (1/Ms)	k_d (1/s)	K_D (M)	k_a (1/Ms)	k_d (1/s)	K_D (M)	k_a (1/Ms)	k_d (1/s)	K_D (M)
SM5-1	2.94×10^6	1.29×10^{-4}	4.37×10^{-11}	3.13×10^4	1.84×10^{-3}	5.86×10^{-8}	4.90×10^5	1.57×10^{-2}	3.19×10^{-8}
SM6-5	4.84×10^5	5.08×10^{-4}	1.05×10^{-9}	ND	ND	ND	2.27×10^4	1.96×10^{-3}	8.63×10^{-8}

^a k_a , apparent association rate constant; k_d , apparent dissociation rate constant; K_D , apparent dissociation equilibrium; ND, no binding detectable.

FIG 3 Epitope identification AD-4-specific MABs. (A) Schematic representation of AD-4 mutant polypeptides and recognition by MABs in indirect immunofluorescence. The designation and relevant AD-4 amino acid sequence are given. Colors on the right side indicate recognition of the respective AD-4 mutant in indirect immunofluorescence following transient expression in Cos7 cells. Green, recognition; red, no recognition; yellow, significantly reduced recognition. (B) ELISA with AD-4 mutant proteins. GST fusion proteins were purified from *E. coli* lysates and used in an ELISA to analyze binding of the indicated antibodies. (C) Structural models of relevant AD-4 mutant polypeptides. AD-4 is displayed as a ribbon diagram, and residues exchanged to alanine are shown as spheres in red.

(Table 1). SM5-1 displayed reduced affinity for both the KK_{378/79} and YL_{364/76} mutants, while SM6-5 showed reduced affinity for the YL_{364/76} mutant and no binding to the KK_{378/79} mutant. So far, the data indicated that residues Y₃₆₄, L₃₇₆, K₃₇₈ and K₃₇₉ of AD-4 are involved in antibody binding but that information on critical contact residues for MAb SM5-1 was still lacking. In addition, the question remained unanswered of whether mutation of both lysine residues in the KK_{378/79} motif was critical for MAb binding or whether a single amino acid exchange would also have an effect. In order to identify contact residues for those antibodies which were critically dependent on the KK_{378/79} sequence, additional AD-4 mutants were generated (Fig. 3A). Again, antibody recognition was tested following transient expression of the respective

mutant proteins in Cos7 cells as well as in ELISA after purification of GST fusion proteins. Indirect immunofluorescence and ELISA gave congruent results. For MABs with reactivity dependent on the KK_{378/79} residues, a clear distinction was seen for mutants containing only a single mutation in K₃₇₈ or K₃₇₉. Whereas the K₃₇₈ mutant showed comparable binding for all MABs, the K₃₇₉ mutant was not recognized by SM1-6 or SM3-1 and was only weakly recognized by SM6-5 both in immunofluorescence and in ELISA (Fig. 3A and B). Recognition of SM5-1 and SM6-5 was greatly reduced or negative for the YK_{364/79}, YKK_{364/78/79} and KKQE₃₇₈₋₈₁ mutant polypeptides (Fig. 3A and B), indicating that the motif YK_{364/79} is important for binding of these two MABs (Fig. 3A and B). In summary, the binding studies using independent test for-

mat indicated that residue K₃₇₉ represents a critical binding residue for SM1-6, SM3-1, and SM6-5, while for SM5-1, residues YK_{364/79} of AD-4 were required for binding. The fact that recognition of the KKQE₃₇₈₋₈₁ mutant protein by SM5-1 was reduced compared to that of the KK_{378/79} mutant (Fig. 3B) further indicated that residues outside the YK motif probably contributed to binding of the MABs. All residues relevant for binding of the human MABs were predicted to be in close proximity on the surface of AD-4 (Fig. 3C).

Structural integrity of AD-4 mutant protein domains. The question arises of whether the reduced reactivity of MABs with the AD-4 mutant polypeptides was caused by loss of critical contact residues or incorrect structural folding of the recombinant proteins. We attempted to answer this question by purification of the respective AD-4 mutant polypeptides and analysis by CD spectroscopy. To obtain pure wt AD-4 as well as the mutant polypeptides (K₃₇₈, K₃₇₉, KK_{378/79}, QE_{380/81}, YL_{364/76}, YK_{364/79}, YKK_{364/78/79}, and KKQE₃₇₈₋₈₁ mutants), GST was proteolytically cleaved from the fusion proteins and removed by affinity chromatography on GST-Sepharose, and the AD-4 polypeptides were additionally purified via anion-exchange chromatography. This procedure resulted in AD-4 polypeptide preparations which were >95% pure (see insets in Fig. 4). When analyzed for recognition in ELISAs, the purified AD-4 polypeptides showed specificities identical to those of the GST fusion proteins used for the studies described above, indicating that the purification procedure did not alter the protein structure (data not shown).

In CD spectroscopy, all protein domains showed maximum ellipticity at approximately 189 nm and minimum ellipticity at approximately 208 nm, which is typical for structures consisting of α -helices and β -sheets and thus consistent with the predicted 3D model of AD-4. Examples are shown in Fig. 4. From these data, we concluded that the mutations that were introduced into AD-4 did not lead to significant structural alterations. Thus, loss of binding of the MABs to the mutant protein was most likely secondary to loss of critical contact residues and not structural alterations in the antigen.

Binding patterns of antibodies correlate with neutralization activity. Loss of binding of an antibody to a recombinant antigen does not necessarily have to correlate with loss of virus-neutralizing activity (33). We therefore tested the relevance of the identified binding residues for virus neutralization. To this end, nine recombinant viruses which contained the mutations listed in Fig. 5 were constructed in the genetic background of strain TB40/E using a TB40/E-derived bacmid and a markerless mutagenesis protocol (31). Replication-competent virus could be retrieved for all nine mutants, and nucleotide sequencing analysis of viral DNA confirmed the introduction of the respective mutation within AD-4.

To analyze a potential impact of the respective mutations in AD-4 on virus replication, multistep growth curves were performed following infection of fibroblasts (Fig. 6). Since it is technically challenging to compare 10 viruses simultaneously in a single assay, we performed several assays involving 3 to 5 viruses at a time so that every recombinant virus was analyzed at least twice. With the exception of RV gB-YKK_{364/78/79}, the recombinant viruses replicated comparably to BAC-derived TB40/E (RV TB40). Recombinant viruses harboring a mutation at Y₃₆₄ showed a somewhat reduced kinetics of virus production; the final titers at day 13 postinfection (p.i.), however, were only slightly lower than for RV TB40. In contrast, the virus titer in the tissue culture su-

pernatant of RV gB-YKK_{364/78/79} was reduced by approximately 3 logs at this time point.

The neutralizing capacity of the AD-4-specific MABs (SM5-1, SM1-6, SM3-1, and SM6-5) was analyzed against the mutant viruses. The AD-2-specific human MAB C23 (29), which should not be influenced by the mutations in AD-4, served as a control. The neutralization capacity of the MABs, when analyzed for RV TB40, was on par with our previous results (28). For SM5-1, SM6-5, and C23, the 50% inhibitory concentration (IC₅₀) was obtained at IgG concentrations of 0.2 to 0.4 μ g/ml, while for SM1-6 and SM3-1, IgG concentrations of 0.6 to 0.8 μ g/ml resulted in 50% neutralization (Fig. 5).

Mutation of residues K₃₇₈ and QE_{380/81} had little influence on the IC₅₀s of the SM MABs. For SM5-1 and SM6-5, there were only minor effects on the IC₅₀ for the mutations at Y₃₆₄ or YL_{364/76}, while for SM1-6 and SM3-1, the IC₅₀ was >5 μ g/ml. Only SM5-1 was capable of neutralizing the RV gB-K₃₇₉ and the RV gB-KK_{378/379} mutant viruses, albeit at concentrations which were 10- to 15-fold higher than those of the wt RV TB40 virus. For the remaining mutations, the IC₅₀ with all SM MABs was >5 μ g/ml. As expected, the neutralization capacity of the AD-2-specific MAB C23 was unaffected by any of the mutations introduced in AD-4, indicating that the differences observed in the replication kinetics of the recombinant viruses had no impact on the neutralization assay as it was performed (neutralization curves are presented in Fig. S4 in the supplemental material).

Analysis of human polyclonal antibodies against AD-4. The data so far indicated that K₃₇₉ and its neighboring amino acids are critical binding residues for the AD-4-specific MABs. These MABs were derived from a single donor, and the question arises of whether this epitope is specific for this donor or whether it is of general significance for the antibody response against AD-4. To test this, we first analyzed 23 randomly selected sera from HCMV-seropositive individuals in an ELISA using potentially relevant AD-4 mutants. OD values obtained with wt AD-4 were set to 100%, and percent recognition of the mutants was calculated (Fig. 7). The overall recognition of the serum panel was significantly reduced for the AD-4 mutant polypeptides compared to recognition of wt AD-4. When a reduction in OD value by 50% was arbitrarily chosen as a reference point, loss of antibody reactivity to the AD-4 mutant polypeptides was seen that corresponded with the binding pattern of the MABs. While for the K₃₇₈ mutant 96% of the sera showed reactivity that was higher than 50% of the wt value, this value decreased to 57% with the KKQE₃₇₈₋₈₁ mutant, indicating that within this random selection of sera, SM-like antibodies constituted a considerable fraction of the overall anti-AD-4 response. The reduction in reactivity of the entire serum panel for the AD-4 mutant polypeptides was statistically highly significant. However, it should also be noted that binding capacity of some sera for the AD-4 mutant polypeptides was largely unaffected, indicating that in these specimens, antibodies to the YK epitope did not represent the predominant reactivity. When serum reactivity was plotted individually for recognition of the AD-4 mutant polypeptides, we observed a gradual decrease in recognition of mutant polypeptides for a majority of the sera which were also relevant for binding of the SM MABs; i.e., the mutation of residue K₃₇₈ only slightly affected binding of the polyclonal antibodies, while recognition of the KKQE₃₇₈₋₈₁ mutant was severely inhibited (see Fig. S5 in the supplemental material). However, there was also a small fraction of sera that showed indi-

FIG 4 CD spectra of AD-4 mutant proteins. The indicated AD-4 mutants were first purified as GST fusion proteins. Following removal of the GST tag by proteolytic cleavage, the AD-4 part was further purified by anion-exchange chromatography and analyzed by PAGE (insets). CD spectra were recorded from 185 to 260 nm.

vidual recognition patterns with the AD-4 mutant polypeptides which differed from the rest, further indicating that in these sera, epitopes other than the YK epitope defined by the SM MAbs are relevant for the anti-AD-4 immune response (see Fig. S5). The reactivity of the poly-AD-4 preparation confirmed the results obtained with the small serum panel (Fig. 7). Recognition of the YKK_{364/78/79} and KKQE₃₇₈₋₈₁ mutants was considerably reduced compared to that of wt AD-4.

To analyze the contribution of antibodies with SM-like specificity to neutralization on total AD-4-specific neutralization ca-

capacity, we also used the poly-AD-4 preparation. As previously reported (28) and also shown in Fig. 5, poly-AD-4 showed IC₅₀s comparable to those of the SM MAbs. There was a clear effect with respect to neutralization of the AD-4 mutant viruses, although it was not as pronounced as for the human MAbs. Mutant viruses RV gB-K₃₇₉ and RV gB-YK_{364/79} showed IC₅₀s of 5 to 6 µg/ml, while for RV gB-YKK_{364/78/79} and RV gB-KKQE₃₇₈₋₈₁, the IC₅₀s were >10 µg/ml (Fig. 5).

In summary, the data obtained with polyclonal IgGs provided convincing evidence that antibodies having a specificity similar to

	SM1-6	SM3-1	SM5-1	SM6-5	poly-AD-4	C23
RV TB40	0.77	0.58	0.22	0.26	1.76	0.24
RV gB-K ₃₇₈	0.79	1.05	0.37	0.37	n. d.	0.31
RV gB-K ₃₇₉	>5	>5	2.20	>5	5.53	0.34
RV gB-KK _{378/79}	>10	>5	3.30	>10	n. d.	0.28
RV gB-QE _{380/81}	1.13	1.35	0.38	0.39	n. d.	0.27
RV gB-Y ₃₆₄	>5	>5	0.40	0.45	n. d.	0.25
RV gB-YL _{364/76}	>5	>5	0.65	0.74	n. d.	0.35
RV gB-YK _{364/79}	>5	>5	>5	>5	6.00	0.38
RV gB-YKK _{364/78/79}	>10	>5	>10	>10	>10	0.39
RV gB-KKQE ₃₇₈₋₈₁	>10	>10	>10	>10	>10	0.27

FIG 5 IC_{50} s of anti-gB antibodies for recombinant viruses harboring mutations in AD-4. HFF were infected with virus-antibody mixtures. The inoculum was removed 4 h after infection, and 20 h later, the number of infected cells was determined by immunostaining for the IE protein. The IC_{50} s of the indicated anti-gB antibodies are given in μ g/ml. Green, IC_{50} at the given concentration; red, IC_{50} not reached at the given IgG concentrations. n. d., not determined.

that of the SM MAbs make up a significant fraction of the human anti-AD-4 response.

DISCUSSION

Existing knowledge about the antibody response against gB following HCMV infection is still fragmentary. Five antigenic domains have been identified. AD-1 and AD-2, both of which induce neutralizing antibodies during infection, were described 2 decades ago. AD-1 was defined as a stretch of at least 70 residues between aa 552 and 635 of gB strain AD169 (26, 34). AD-2, located between aa 50 and 77, harbors two antibody binding sites, of which site I (aa 50 to 54) has been described as a target of neutralizing antibodies, whereas site II (aa 68 to 77) binds nonneutralizing antibodies (29). In addition, site II is different between HCMV strains (27). However, despite the fact that these antibody binding domains have been known for quite some time, detailed information on critical contact residues within these domains and information on epitope-paratope interaction are still lacking. AD-4 and AD-5 have been identified only recently (28). Studies of AD-4 have indicated that it is a highly immunogenic structure expressed on gB

FIG 7 Reactivities of human sera with AD-4 mutant polypeptides. GST fusion proteins were purified from *E. coli* lysates, the GST tag was removed, and the indicated AD-4 mutants were purified and used in an ELISA to analyze binding of sera from HCMV-infected individuals. Reactivity of the individual samples with wt AD-4 was set to 100%, and reactivity with the mutant proteins was calculated. Fifty percent reactivity is indicated by the broken line, and percent sera that showed more than 50% reactivity compared to the wild type is given. Human sera were diluted 1:50. Red symbols, reactivity of the poly-AD-4 preparation at a concentration of 1 μ g/ml. **, $P > 0.05$; ***, $P > 0.001$ (*t* test).

that induces an antibody response in >90% of HCMV-infected individuals and is thus similar to AD-1, which has been considered immunodominant since nearly 100% of HCMV-infected individuals develop antibodies against AD-1 (35). For comparison, AD-2 and AD-5 are recognized by approximately 50% of HCMV con-

FIG 6 Replication of viral mutants containing mutation in AD-4. Replication capacities of viruses containing mutations excluding residue Y₃₆₄ (A) and including mutation of residue Y₃₆₄ (B) are shown. HFF were infected with the respective recombinant virus. At the indicated time points, supernatants were harvested and virus titers were determined. Titers represent means $\pm$ SDs of 2 to 8 independent experiments. IU, infectious units.

FIG 8 The YK epitope. A tube representation of a gB trimer is shown on the left. The structural domain II representing AD-4 is shown as an accessible surface area representation and displayed in an enlarged format. Residues relevant for antibody binding of neutralizing antibodies are highlighted by different colors, and the position in gB is given.

valescent-phase sera. A more detailed characterization of the antibody target structure(s) on AD-4 could be expected to aid in our understanding of the neutralizing antibody response against gB and potentially define functional sites on gB that are essential for infection.

The YK epitope: structural considerations. According to our 3D model of HCMV gB, AD-4 is formed by a discontinuous amino acid sequence comprising aa 121 to 132 and aa 344 to 438 of gB in strain AD169 (Fig. 1). Expression of both sequence parts, joined by a 5-aa linker sequence, was prerequisite for binding of MAbs and affinity-purified polyclonal human antibodies with reactivity for AD-4. Thus, the fraction of gB-specific antibodies which bind to linear sequences within AD-4 appears to be small, a finding that is similar to the situation found for AD-1 but in contrast to AD-2, for which peptides can be used for antibody mapping (27). Based on the 3D model of gB, surface-exposed residues were identified and used for dialanine scans to search for critical binding residues of the SM MAbs. Using this approach, residues Y₃₆₄ and K₃₇₉ (the YK epitope) were identified as essential for binding of AD-4-specific MAbs, but additional residues may also be involved in antibody binding in human sera. According to our 3D model of AD-4, all critical amino acids are located directly adjacent on the surface of AD-4 (Fig. 8). Experiments using AD-4 expressed in bacteria or mammalian cells resulted in the same conclusion with respect to epitope identification, indicating that glycosylation of AD-4 is most probably not required for antibody binding. AD-4 contains 2 or 3 potential sites for the addition of N-linked glycosylation (NetNGlyc 1.0 Server [<http://www.cbs.dtu.dk/services/NetNGlyc/>]), and the peptide is glycosylated following expression in mammalian cells (N. Spindler, unpublished results). Also, the affinities of the SM antibodies toward binding of whole gB and the bacterially derived AD-4 were similar (28).

The fact that gB recognition by MAb SM5-1 required mutations in both residues of the YK epitope while for the remaining MAbs only K₃₇₉ was critical indicated that the interaction of SM5-1 with AD-4 differed from that of the other antibodies. In addition to the core residues, YK_{364/79}-neighboring residues such as QE_{380/81} most probably are involved in binding of the SM MAbs. However, the contribution of QE_{380/81} to antibody binding

was seen only when additional residues within AD-4 were mutated, indicating that by themselves they do not represent critical binding residues. It can be speculated that other residues in close proximity to the YK epitope which were not tested in our study will also participate in epitope-paratope interaction. However, the complete epitope-paratope interaction can only be resolved by X-ray crystallography of the respective antibody-antigen complexes.

The idea that residues other than YK_{364/79} contribute to binding of the SM MAbs is consistent with current concepts on epitope-paratope interaction. The interface between epitope and paratope usually covers an area of 600 to 900 Å² on both binding partners, with multiple contacts between the two surfaces (36). Despite the large epitope-paratope contact area, single residues have been identified in a number of cases as being critical for binding or function of antibodies since they contribute the majority of the binding energy in antibody-antigen interactions, and the respective residues have been termed “hot spots” of the interaction (37). An amino acid that is frequently found in such hot spots is tyrosine, which can form aromatic π interactions, hydrogen bonds, and hydrophobic interactions. Similarly, lysine can constitute an essential binding partner due to the formation of salt bridges. The YK epitope covers approximately 150 Å². However, as can be calculated from data presented in Table 1, the YK motif accounts for most of the total interaction energy. This finding strongly suggests that these two residues represent a key site of the interaction of SM5-1 with AD-4, while the remaining residues play only a moderate role in binding affinity. The smaller energetic contributions of the YK surrounding residues to the binding energy can also offer an explanation for the fact that additional residues of the epitope were not mapped in the dialanine scan, as their mutation did not significantly reduce binding.

Introducing mutations in a given molecule inevitably brings up the issue of the structural integrity of the mutant protein. The fact that the CD spectra were not altered between wt AD-4 and mutant proteins indicated that the mutations did not have a significant impact on folding of AD-4. In addition, an indirect support for structural integrity of the AD-4 mutant protein is provided by the fact that polyclonal sera still recognized these polypeptides. Since our data suggest that binding of polyclonal human antibodies to AD-4 requires the entire AD-4 sequence and/or intact conformation, recognition of structural epitopes other than the YK epitope seems to be a plausible explanation for this finding.

YK epitope binding antibodies: potential mode of action. The fact that SM MAbs block infectivity indicates that the YK epitope on AD-4 has an essential role during infection. One possible mechanism could be a block of interaction between gB and a receptor molecule. Receptor binding properties of gB have been described for a number of human herpesviruses, including HCMV (38–40). The fact that the AD-4-specific MAbs act at a postadsorption stage, as has been shown in our previous analyses (28), does not argue against this possibility, since attachment of herpesviruses to target cells is most probably dependent on the interaction of a number of different molecules on both the virus and target cell which could still allow attachment when the gB-receptor interaction is blocked (21).

Another possible mechanism which could also be operative postattachment is blocking of fusion between the viral and target cell membranes. It is the current consensus that gB represents the

fusion protein of herpesviruses (19). The herpesviral core fusion machinery is represented by gB and the gH/gL complex (21). However, in order to execute its function, gB needs accessory molecules. In the case of HSV, in most studies binding of gD to gH/gL has been shown to be required for activation of membrane fusion (41, 42). For EBV gB, gH/gL and gp42 are required for fusion of B cells, while only gB and the gH/gL complex are required for fusion of epithelial cells (43). HCMV enters host cells by different mechanisms, thereby exposing gB to different pH environments. Fusion at the plasma membrane for fibroblasts (44), endocytosis for endothelial and epithelial cells (45, 46), and macropinocytosis for dendritic cells (47) have been identified so far. The entry pathway is thought to be determined by both virus and host cell factors. While in addition to gB, the trimeric complex gH/gL/gO is required for infection of fibroblasts, the pentameric gH complex consisting of gH/gL/UL128-131 is essential for infection of endothelial and epithelial cells (48). However, the minimal fusion complex for HCMV seems to be gB/gH/gL, since cell fusion can be seen following recombinant expression of gB and gH/gL in the absence of additional proteins. Interestingly, cell-cell fusion can be observed by expression of the gB and gH/gL proteins either in *cis* or in *trans* (22). The fact that AD-4-specific antibodies block infection of different cell types with comparable potencies (28) indicates that a universal function of gB that is required for infection is targeted by the MAbs.

The transition from the fusion-inactive to the fusion-active state of gB is thought to involve conformational changes in gB. Thus, neutralizing anti-gB antibodies could block infection by at least two different mechanisms: (i) blocking of interaction of gB with gH/gL or (ii) blocking of the conformational change that is required for transition from the fusion-inactive to the fusion-active conformation. The AD-4-corresponding region in HSV-1 gB is structural domain II (Dom II), which includes functional region II (FRII) (49). Atanasiu et al. (25) reported that in bimolecular fluorescence complementation (BIMC), anti-Dom II neutralizing MAbs block fusion and the gB-gH/gL interaction. Interestingly, a conformation-dependent MAb had a higher capacity to block fusion than MAbs that recognize continuous epitopes on Dom II (see Fig. S6 in the supplemental material). It can be speculated that the AD-4-specific MAbs recognizing the YK epitope may have a similar mode of action.

Blocking of conformational changes required for gB to become a fusogen is another possible mode of action for AD-4-specific MAbs. Entry of HCMV has been reported to be accompanied by a conformational change in viral gB, as evidenced by differential susceptibility to protease (50). gB has homology to other fusion proteins such as vesicular stomatitis virus (VSV) G and gp64 of baculovirus. These so-called type III fusion proteins undergo a pronounced reversible, pH-dependent conformational change in order to become fusion active (51). The crystal structure as it was solved for gB of HSV-1 and EBV most likely represents a postfusion form of the protein. Whether herpesviral gB undergoes extensive pH-dependent structural changes similar to those of other type III fusion proteins is unknown. Crystallization under low pH has revealed only minor changes in HSV-1 gB (52). Thus, there is currently no experimental proof that a prefusion conformation of gB exists which is similar to the prefusion form of VSV G. Nevertheless, a hypothetical model of a prefusion form of EBV gB has been proposed by Backovic et al. (20). In this model, the overall conformation of domain II, which carries the AD-4 epitope in

HCMV gB, is unchanged between pre- and postfusion conformations. However, the relative domain arrangement in the EBV gB prefusion model is altered, placing the structural domain II at the tip of the molecule. An analogous model building for the HCMV gB prefusion conformation indicated that the YK epitope is still accessible to antibodies (P. Rücker, unpublished observation).

Pattern of antibody binding to recombinant AD-4 and virus neutralizing capacity. The patterns of binding of MAbs to the isolated AD-4 domain and its mutants may not be absolutely required to correlate with neutralizing activity. As noted above, the epitope-paratope interaction is complex and sterically could also include residues in gB outside AD-4, especially considering the lack of knowledge on the prefusion form of gB. Thus, to verify our findings on binding of SM antibodies to recombinant proteins in a biological assay, we constructed viral mutants and tested the neutralizing activity of the AD-4-specific MAbs. Introducing mutations in AD-4 had little influence on viral replication with the exception of the RV gB-YKK_{364/78/79} virus, which showed a 3-log-lower virus yield on day 13 after infection than did wt TB40.

However, overall the results obtained in neutralization assays using the viral mutants confirmed the data on binding to the isolated AD-4 domain. Loss of neutralization capacity was observed for those viral mutants that also showed reduced binding capacity when expressed as isolated protein domains. For example, RV gB-YL_{364/76} was neutralized by SM5-1 and SM6-5 comparably to wt TB40, while neutralization by SM3-1 and SM1-6 was lost, which reflected MAb recognition of these mutants when expressed as isolated domains. Our overall conclusion from neutralization of viral AD-4 mutants is that residues outside AD-4 most probably do not significantly contribute to the neutralization capacity of SM-like antibodies. It is important to note that the neutralization activity of MAb C23, which is directed against AD-2, was completely independent of the mutations introduced in AD-4, indicating that the differences in replication kinetics between the recombinant viruses could not account for the differences in neutralization by the AD-4-specific MAbs.

General importance of the YK-epitope for the AD-4 response. The panel of MAbs that was used in this study was isolated from a single donor, and the V gene usage of the CDR sequences was highly related (28). Thus, the question arose of whether the YK epitope is specific for this single donor or whether it is of more general relevance for the antibody response against gB during infection. We approached this question in two ways. First, we analyzed the ELISA reactivities of randomly selected sera for recognition of wt AD-4 and the AD-4 mutant polypeptides. The data clearly showed that introduction of mutations within AD-4 results in loss of binding by antibodies. When the entire serum panel was considered, a significant reduction was already observed for the K₃₇₈ polypeptide, a mutation which did not affect binding of the SM MAbs. The effect became more pronounced with the more complex mutant proteins, including the YK epitope mutant for which 30% of sera showed less than 50% reactivity. Thus, the YK epitope seems to be of general importance for the anti-AD-4 antibody response during infection. This assumption was also supported by the reduced reactivity of the poly-AD-4 preparation with the mutant proteins, which closely resembled an average of the individual serum specimens, although this preparation was most probably derived from a large number of serum donors. The residual binding activity in the majority of the individual serum samples for the mutant polypeptides as well as the fact that in

some serum reactivity against the mutants was only slightly decreased indicated the existence of additional antibody binding regions on AD-4. In fact, we have evidence for a number of additional neutralization-relevant epitopes, not structurally related to the YK epitope, on AD-4 (N. Spindler, unpublished observation). Further studies are necessary to define the epitope density on AD-4 and to evaluate the antiviral activity of the binding antibodies. Since the antibody concentrations required for 50% neutralization were comparable between MAbs and polyclonal anti-AD-4 antibodies, it can be assumed that the non-YK-specific antibodies do not compete for neutralization.

The second approach to derive information about the general importance of AD-4 for the anti-gB antibody response was the analysis of the neutralization capacity of the poly-AD-4 antibody preparation. In neutralization assays, the poly-AD-4 showed activity comparable to that of the SM MAbs. The recombinant viruses containing mutations in the YK epitope were considerably more resistant to neutralization than the wild type. The effect, however, was not as pronounced as for the SM MAbs, further supporting the assumption that additional neutralization-relevant epitopes exist on AD-4. The redundant coverage of AD-4 by the antiviral antibody response seems plausible given the potential importance of this protein domain for the function of gB in the infection process. Evasion of antibody neutralization by gB sequence variation will be impeded. It should be mentioned, however, that the AD-4 domain in general and the YK epitope in particular seem to be highly conserved between HCMV strains (see Fig. S7 in the supplemental material).

In summary, we have characterized a conformation-dependent functional epitope on AD-4 of gB that is bound by neutralizing MAbs. Y₃₆₄ and K₃₇₉ were identified as critical contact residues. Data obtained by human MAbs and monospecific human polyclonal antibody preparations support an important role for the YK epitope in the neutralizing IgG response against gB. Induction of antibodies specific for this epitope may be a desirable goal following vaccination with gB.

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