

# Antigenic domain 1 of human cytomegalovirus glycoprotein B induces a multitude of different antibodies which, when combined, results in incomplete virus neutralization

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Glycoprotein B (gB, gpUL55) is the major antigen for the induction of neutralizing antibodies against human cytomegalovirus (HCMV), making it an attractive molecule for active and passive immunoprophylaxis. The region between aa 552 and 635 of HCMV gB (termed AD-1) has been identified as the immunodominant target for the humoral immune response following natural infection. AD-1 represents a complex domain which requires a minimal continuous sequence of more than 70 aa for antibody binding. Neutralizing as well as non-neutralizing antibodies can bind to AD-1 in a competitive fashion. The fine specificity of AD-1-binding monoclonal antibodies (MAbs) and affinity-purified human polyclonal antibodies was analysed by using recombinant proteins containing single amino acid substitutions spanning the entire AD-1 domain. Our results revealed that all MAbs had individual patterns of binding to the mutant proteins indicating the presence of a considerable number of distinct antibody-binding sites on AD-1. The neutralization capacity of antibodies could not be predicted from their binding pattern to AD-1 mutant proteins. Polyclonal human antibodies purified from different convalescent sera showed identical binding patterns to the mutant proteins suggesting that the combined antibody specificities present in human sera are comparable between individuals. Neutralization capacities of polyclonal human AD-1 antibodies did not exceed 50% indicating that, during natural infection, a considerable proportion of non-neutralizing antibodies are induced and thus might provide an effective mechanism to evade complete virus neutralization.

## Introduction

Human cytomegalovirus (HCMV) represents a significant pathogen in persons with a compromised or immature immune system. In contrast, infections of immunocompetent individuals are of limited consequence in the vast majority of cases, indicating the importance of an effective immune response in the control of HCMV (Ho, 1992). However, the immunological effector functions which control HCMV infections are difficult to analyse since the virus is strictly species-specific and no experimental animal model system is available. Although cellular immunity may be responsible for recovery from HCMV infection, humoral immunity probably has an important role in protection against primary infection and limiting the severity of the disease. For example, it is well documented that preconception seroimmunity to HCMV provides sub-

stantial protection against symptomatic infection of the newborn and that high levels of neutralizing antibodies are associated with protection from reinfection (Adler *et al.*, 1995; Fowler *et al.*, 1992; Tanaka *et al.*, 1991). Moreover, in the closely related murine cytomegalovirus system, it has been demonstrated that antibodies can limit virus spread and confer protection from a lethal challenge (Jonjic *et al.*, 1994; Reddehase *et al.*, 1994; Rapp *et al.*, 1993). Collectively, these findings point to a major role of antibodies in limiting the consequences of HCMV infection.

Glycoprotein B (gB) is the dominant antigen on the envelope of HCMV. The 906 aa polypeptide of gB strain ADI69 is post-translationally modified into a 160 kDa glycosylated precursor molecule which is subsequently proteolytically cleaved into the subunits gp116 and gp58 (Britt & Vugler, 1989; Kari *et al.*, 1990; Mach *et al.*, 1986; Meyer *et al.*, 1990). The subunits remain covalently linked via disulfide bonds. Disulfide-linked gB homodimers represent the mature intracellular form as well as the virion form of gB (Britt &

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Vugler, 1992). Nearly 100% of HCMV-infected individuals develop antibodies against this protein (Kniess *et al.*, 1991; Marshall *et al.*, 1992; Schoppel *et al.*, 1997). Several studies have demonstrated that a considerable fraction of the virus-neutralizing activity found in human serum following natural infection is directed against gB (Britt *et al.*, 1990; Marshall *et al.*, 1992; Gonczol *et al.*, 1991). Consequently, gB has been proposed as the logical candidate for the development of a subunit vaccine and the first trials have been initiated (Wang *et al.*, 1996).

Three antibody-binding sites have been identified on gB: antigenic domain 1 (AD-1), located between aa 552 and 635; AD-2, between aa 50 and 77; and AD-3, between aa 783 and 906 (nomenclature for strain AD169). AD-2 is composed of two sites, residues 68–77 (site I) and 50–54 (site II). Of the three domains, AD-1 and site II of AD-2 are capable of inducing virus-neutralizing antibodies during natural infection (Meyer *et al.*, 1992; Wagner *et al.*, 1992; Kniess *et al.*, 1991). The gB protein also contains a number of additional non-linear or assembled epitopes (Lussenhop *et al.*, 1988; Kari & Gehr, 1991; Qadri *et al.*, 1992). AD-1 represents the immunodominant site on gB. In fact, nearly 100% of infected persons who are seropositive for gB have antibodies against AD-1, whereas AD-2 is recognized by 47% of serum samples from persons with a past HCMV infection (Schoppel *et al.*, 1997). AD-1 is an unusually complex structural domain consisting of more than 75 aa between residues 552 and 635 of gB. Antibody binding requires the presence of the entire AD-1 sequence. Attempts to define conventional linear epitopes within AD-1 using synthetic peptides of various length and monoclonal antibodies (MAbs) as well as human sera have been uniformly unsuccessful (Wagner *et al.*, 1992; Bonci *et al.*, 1993; Ohlin *et al.*, 1993). Point mutations within AD-1, most notably those of the two internal cysteines or prolines, eliminate the antibody-binding properties of the entire polypeptide, indicating that the domain is recognized as a single antigenic determinant. A recent study has suggested that a limited number of distinct antibody-binding sites might exist on AD-1 (Schoppel *et al.*, 1996). A remarkable feature of AD-1 is the fact that after natural infection or immunization of mice, MAbs from one individual can be isolated which show an extreme range of virus-neutralizing activity. Binding of these antibodies to AD-1 is competitive (Ohlin *et al.*, 1993; Utz *et al.*, 1989). The ratio at which the different types of antibodies are induced following natural infection is not known.

Here, using a set of AD-1 mutant proteins, we have analysed binding requirements for neutralizing and non-neutralizing MAbs as well as for polyclonal immunoglobulins purified from convalescent sera. Our data show that on the level of murine as well as human MAbs, individual binding patterns can be defined for each antibody. No common binding characteristics could be found for neutralizing or non-neutralizing antibodies. In contrast, polyclonal AD-1-specific antibodies present in human convalescent sera showed identical

binding patterns. The neutralizing capacity of polyclonal AD-1-specific antibodies did not exceed 50% of input virus indicating that, during natural infection, a balanced ratio of neutralizing and competing non-neutralizing antibodies specific for AD-1 is induced.

## Methods

■ **MAbs.** MAbs have been described previously: 89-104, 7-17, 27-287 (Wagner *et al.*, 1992; Schoppel *et al.*, 1996), 27-11, 9-3, 27-156 (Schoppel *et al.*, 1996), the ITC series (Ohlin *et al.*, 1993) and AP86-SA4 (Urban *et al.*, 1992). MAb BIB6 was raised against a prokaryotically expressed AD-1 fusion protein lacking Cys-573. It was found to bind a synthetic peptide spanning aa 600–619. Propagation of hybridomas and purification of antibodies have been described previously (Schoppel *et al.*, 1996).

■ **Neutralization assay.** Comparative neutralization assays were carried out as previously described (Schoppel *et al.*, 1996). Briefly, HCMV strain AD169 was preincubated with serial log<sub>2</sub> dilutions of antibodies in 100 µl minimal essential medium supplemented with 5% foetal calf serum, glutamine and gentamicin for 4 h at 37 °C. Human foreskin fibroblasts (1.5 × 10<sup>4</sup>) were added in a volume of 25 µl medium each and the mixtures were seeded in a 96-well plate. After infection for 20 h at 37 °C, cells were fixed with absolute ethanol and infected cells were stained with antibody P63-27, which is specific for the major immediate early protein, UL123, of HCMV. Antibody binding was detected by a Cy3-conjugated rabbit anti-mouse IgG F(ab')<sub>2</sub> fragment (Dianova). In general, the infectious dose was adjusted to produce 150 infected cells counted on a fluorescence microscope using 200-fold magnification. Neutralization was determined as 1 – (number of infected cells in the presence of antibody/number of infected cells in the absence of antibody) and expressed as percentage inhibition of infection. Results reflect mean values of three independent assays. Variations between assays were in the range 10–20%. To adjust equal concentrations of antibodies in different preparations, total IgG was measured by ELISA. In brief, polystyrene 96-well plates were coated with serial dilutions of antibodies in 50 mM carbonate buffer, pH 9.5, for 16 h at 4 °C and the relative amount of IgG antibodies was determined by using polyclonal peroxidase-conjugated rabbit anti-human IgG antibody (DAKO) as described previously (Schoppel *et al.*, 1996).

■ **Random *in vitro* mutagenesis.** Region-specific random mutagenesis was performed on ssDNA by the method described by Myers (1989) with modifications. To obtain both strands as templates for chemical mutagenesis, the AD-1 coding region was inserted into M13mp10 in both orientations. The DNA fragment was derived from the plasmid gig 58-1, which contains a 488 bp *Pst*I–*Sal*I fragment encompassing nt 1448–1935 of the gB coding region (Kniess *et al.*, 1991). ssDNAs were isolated from culture supernatants by standard procedures and treated with mutagenic chemicals (Sigma) as follows. ssDNA (40 µl 1 mg/ml) was incubated with (i) 50 µl 250 mM sodium nitrite, 10 µl 2.5 M sodium acetate, pH 4.3, (ii) 60 µl 18 M formic acid or (iii) 60 µl 12 M hydrazine at 20 °C for 10 min in separate reactions. Reactions were terminated by the addition of 30 µg carrier tRNA (Fluka) and immediate ethanol precipitation. After purification by two additional ethanol precipitations, the modified ssDNAs were used as templates for PCR amplification.

■ **DNA amplification and expression of mutagenized fragments in *E. coli*.** DNAs derived from different mutagenization reactions as well as untreated controls were amplified, cloned and analysed separately. Incorporation of incorrect nucleotides at chemically damaged bases and amplification of AD-1 coding fragments was achieved by PCR

using the primers 58mut5' and 58mut3'. 58mut5' was composed of the recognition site for the restriction enzyme *EcoRI* and the gB coding sequence from nt 1643–1659 (5' CAATGAATCCCAGCTGCGTG-ACCATC 3') and 58mut3' represented nt 1920–1936 followed by the *HindIII* recognition motif (5' GGCAAAGCTTTGTCGACGGTGGAG-ATA 3'). PCR reactions were performed as described previously (Urban *et al.*, 1992) and fragments were inserted into a *trpE*-based expression vector. All cloning procedures were performed by standard methods. Screening of mutated clones was based on binding properties of antibodies to the corresponding fusion proteins. To facilitate this, the expression vector pATH 1 (Spindler *et al.*, 1984) was modified as follows. The coding sequence for a linear epitope (AD86) was inserted downstream of the cloning site for AD-1 fragments to function as a C-terminal epitope tag in fusion proteins. Using the primers gHALT5' (5' TATAAAGCTTCCGAAGCGCTGGACCT 3') containing the *HindIII* recognition site and gHALT3' (5' CCCCATCGATTAGGTGTTGAGTAGTAG 3') containing the *Clal* restriction motif and a stop codon, the fragment was amplified by PCR. The previously described plasmid AP86 (Urban *et al.*, 1992) was used as template. This fragment was inserted into the *HindIII/Clal* sites of pATH 1. The resulting amino acid sequence for the tag was EALDPHAFHLLNT. To discriminate clones with correctly inserted AD-1 fragments from clones containing religated vector on the basis of their epitope tag, a frameshift between the restriction sites for insertion of AD-1 was created. To this end, the vector was cleaved with *BamHI*, the 5' overhangs were filled in with Klenow fragment of DNA polymerase I and the vector was religated. The modified vector was designated pATH-86-B. Mutagenized AD-1 fragments were inserted into the *EcoRI/HindIII* sites of pATH-86-B and transfected into *E. coli* C600.

■ **Immunological screening for mutants.** AD-1 fusion proteins revealing a reduction in binding by AD-1-specific MAbs were screened in colony immunoblots. Individual colonies from a master LB agar plate were transferred to five M9 agar plates containing ampicillin using sterile toothpicks and incubated for 2 h at 37 °C. To enable induction of fusion proteins directly on agar plates, 82 mm nitrocellulose membranes were soaked in indoleacrylic acid (0.1 mg/ml) and dried on 3MM paper. Membranes were placed onto the surface of the agar and plates were incubated for 4 h at 37 °C in an inverted position. For lysis of colonies, the filters were removed and transferred, colony side up, onto 3MM paper soaked with lysis buffer (1% SDS, 1 mM EDTA in 62.5 mM Tris, pH 6.8) and incubated at 80 °C for 10 min. Filters were rinsed twice in PBS. Immunological detection of fusion proteins was performed as described previously (Kropff & Mach, 1997) using AD-1-specific antibodies 7-17, 27-287, 27-11 and 9-3 as well as MAb AP86-SA4, which is specific for the epitope tag.

■ **Evaluation of antibody fine specificity by immunoblotting.** Induction of fusion protein expression in *E. coli* containing pATH-based plasmids was performed as described previously (Spindler *et al.*, 1984). SDS-PAGE of total protein lysates and immunoblotting was done by standard procedures. Nitrocellulose membranes were blocked with PBS containing 0.1% Tween 20 and for analyses of human sera, 5% powdered milk was added. Antibodies and sera were diluted in PBS containing 0.1% Tween 20. Prior to immunoblot analysis with human sera, a pre-incubation step was carried out. Diluted sera were incubated three times for 1 h with membrane strips containing total protein lysates of an induced pATH culture. The blots were incubated with antibodies or sera for 16 h at 4 °C. MAb binding to AD-1 mutants was detected after incubation with alkaline phosphatase-coupled anti-immunoglobulins by BCIP/NBT staining. For detection of bound antibodies in human sera, a peroxidase-conjugated anti-human antibody and the ECL chemoluminescence system (Amersham) were used as previously described

(Schoppel *et al.*, 1996). For characterization of HCMV-positive sera, the AD86 epitope tag was removed from fusion proteins by insertion of an oligonucleotide linker containing stop codons in both orientations at the *HindIII* site of the expression plasmids. The linker was created by annealing the oligonucleotides 5' AGCTTAGTGACCCGGAGTTATCA 3' and 5' AGCTTGATAACTCCGGTCACTA 3'.

■ **Immunoaffinity chromatography.** Human AD-1-specific antibodies were isolated from sera by immunoaffinity chromatography on AD-1 fusion protein Mbg58. Mbg58 was purified as described (Kropff *et al.*, 1993), dialysed against coupling buffer and conjugated to CNBr-activated Sepharose (Pharmacia) according to the manufacturer's instructions (2 mg/ml). Preparation of columns and the purification procedure were performed as described previously (Urban *et al.*, 1992). Each serum sample (25 ml), diluted 1:1 (v/v) with 10 mM Tris-HCl, pH 7.5, was purified over 1.5 ml antigen-coupled beads. BSA was added to eluted fractions to a final concentration of 5 mg/ml and fractions were dialysed against PBS.

■ **ELISA with selected antigenic determinants of HCMV.** Individual antigens and the assay procedure for determining antibody titres of human sera have been described previously in detail (Schoppel *et al.*, 1997).

## Results

### Fine specificity of AD-1-binding antibodies

In order to quantify neutralizing and non-neutralizing AD-1-specific antibodies in human sera, reagents had to be produced which allowed separate analysis of the respective antibody type. Fine specificity of AD-1-binding antibodies, however, cannot be analysed by use of short (10–40 aa) synthetic peptides since the entire primary amino acid sequence between residues 552 and 635 of gB is necessary for antibody binding (Wagner *et al.*, 1992; Bonci *et al.*, 1993; Ohlin *et al.*, 1993; Schoppel *et al.*, 1996). Therefore, to investigate the binding requirements of different types of antibodies for AD-1 in more detail, a number of mutant plasmids were constructed resulting in point mutations within the primary amino acid sequence. To this end, a fragment encoding aa 484–650 of gB was inserted into M13mp10 and ssDNA derived from this plasmid was subjected to random mutagenesis. Chemically treated DNA was amplified by PCR and inserted into the bacterial expression vector pATH-86-B, allowing synthesis of *trpE* fusion proteins (Spindler *et al.*, 1984). Bacterial colonies were screened (i) for the presence of fusion proteins using antibody AP86-SA4, which is specific for the epitope tag only present in fusion proteins containing full-length AD-1, and (ii) with antibodies 9-3, 7-17, 27-11 and 27-287, which are specific for AD-1. Bacterial colonies giving signals of comparable intensity with antibody AP86-SA4 but showing greatly decreased binding by one or more of the AD-1-specific antibodies were isolated and further analysed. In total, 600 colonies were screened by this method and 13 bacterial clones were further characterized. Nucleotide sequence analysis revealed that 12 of the clones had single amino acid changes within AD-1 while one clone contained a triple mutation. A truncated polypeptide containing aa 549–633 of AD-1 was

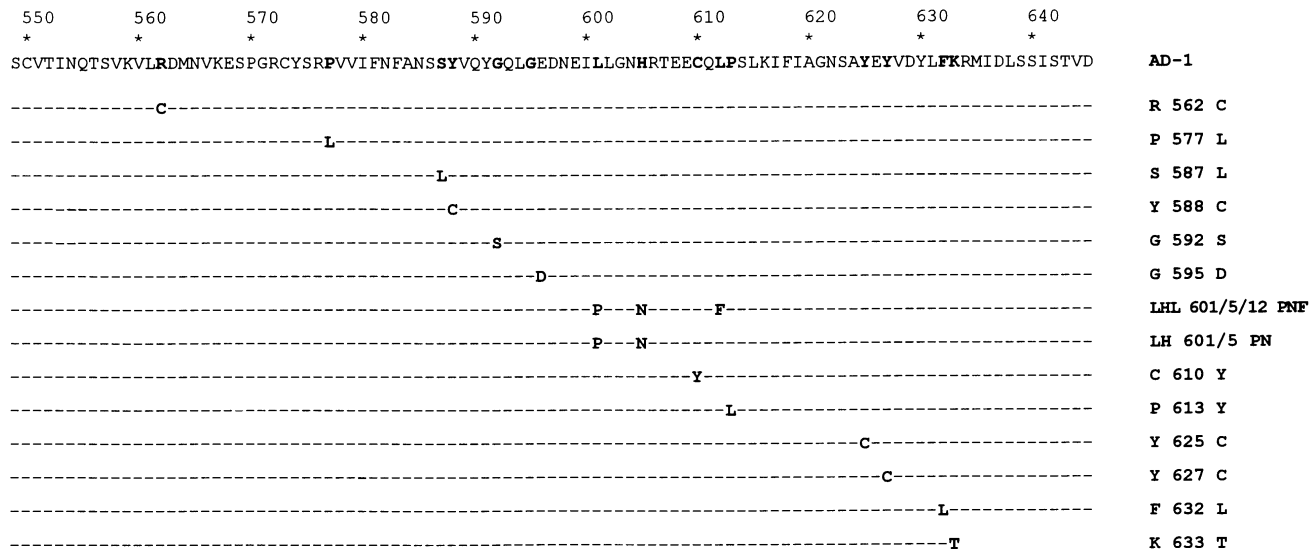


Fig. 1. Amino acid sequences of AD-1 mutant fusion proteins were generated by random mutagenesis and selected for decreased antibody binding. Dashes indicate identity to the laboratory strain AD169 and substituted amino acids are shown in bold. Clones are designated by type and position of the exchanges (i.e. in R562C, the arginine at position 562 within gB was replaced by cysteine). Protein K633T showed a C-terminal truncation as indicated.

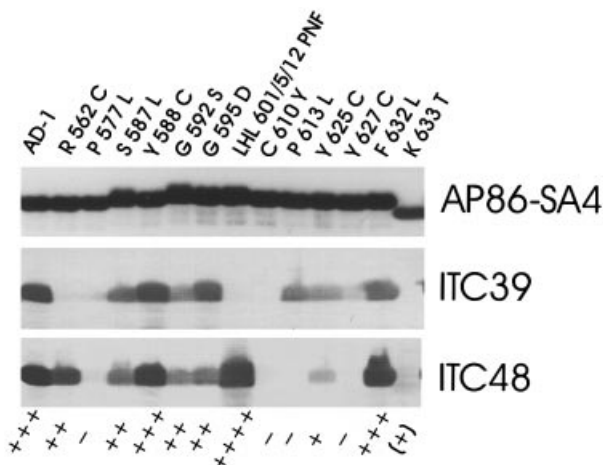


Fig. 2. Reactivity of human MAbs ITC39 and ITC48 with prokaryotically expressed fusion proteins containing mutations in AD-1. For designation of fusion proteins see Fig. 1. *E. coli* lysates were subjected to PAGE and analysed in immunoblots. Antibody binding was detected with alkaline phosphatase-conjugated secondary antibodies and BCIP/NBT staining. The murine MAb AP86-SA4, specific for the linear epitope tag, was used as a control. The classification of reactivities corresponding to Table 1 is shown for ITC48 (+++ ++, enhanced; +++ equal; ++, slightly decreased; +, decreased; (+), barely detectable; -, no reactivity in comparison to AD-1).

produced in one case. Designation of the clones included the type and position of the amino acid within gB as well as the introduced amino acid, i.e. in clone R562C, the arginine at position 562 was changed to cysteine. Amino acid exchanges were evenly distributed over AD-1 (Fig. 1). The mutated fusion proteins were analysed in immunoblots with five murine and six human AD-1-specific MAbs. Equal amounts of fusion proteins were applied to the immunoblots and controls

included the tag-specific antibody AP86-SA4, which was reactive with all antigens (Fig. 2), as well as antibody B1B6, which is specific for aa 600–619 of AD-1 (data not shown).

Qualitative as well as quantitative differences in binding to the fusion proteins were observed for all MAbs. Since they were not due to varying amounts of antigen present on the nitrocellulose filters they must be interpreted as different amounts of antibody bound to the respective fusion protein. The differences in signal intensity allowed the definition of an individual binding pattern for each antibody. Among the antibodies tested, we did not find two antibodies with the same recognition pattern for fusion proteins. As an example, the reactivities observed for the human MAbs ITC39 and ITC48 are shown in Fig. 2 and a summary of reactivities for all tested MAbs is given in Table 1. Binding of individual MAbs was influenced by mutations distributed over the entire AD-1 region, i.e. reactivity of MAb ITC48 was abolished by substitutions in residues 577, 610, 613 and 627 and, when compared to intact AD-1, diminished by several other mutations (Table 1, Fig. 2). The only amino acid substitution which resulted in a negative reaction with all tested MAbs involved C610, which is in agreement with our previous results (Schoppel *et al.*, 1996). All remaining amino acid exchanges showed positive reactions with more than one antibody. Mutational introduction of cysteine residues at positions 562, 588, 625 and 627, respectively, did not lead to an overall loss of antibody binding (Table 1).

With one exception, binding of antibodies to mutant fusion proteins was unchanged or reduced when compared to intact AD-1. For antibody ITC48, however, we observed a greatly enhanced reactivity with the recombinant protein containing the triple mutation LHL601/5/12PNF (the 'LHL' protein)

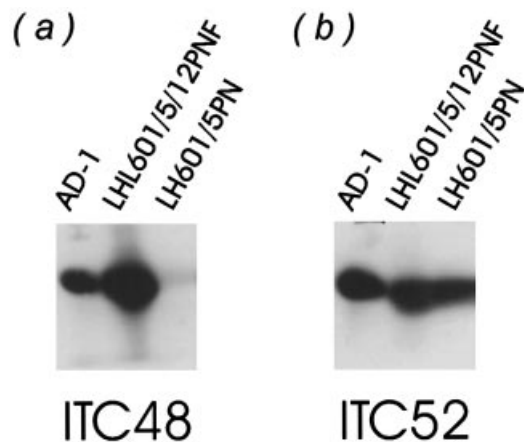
**Table 1.** Binding of MAbs and human sera to mutant AD-1 fusion proteins as determined by Western blotting

Recognition patterns of antibodies and human sera are shown. Reactivities of antibody with fusion proteins in comparison to AD-1 are given as follows: + + + +, enhanced; + + +, equal; + + slightly decreased; +, decreased; (+), barely detectable; -, no reactivity in comparison to AD-1. ND, Not determined.

AD-1 fusion protein*	Human MAb						Murine MAb					Human serum (n = 5)	
	89-104†	ITC52†	ITC48†	ITC63B†	ITC33	ITC39	7-17†	9-3†	27-287†	27-156	27-11		
AD-1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
R562C	++	++	++	++	+	-	-	++	-	+	++	++	++
P577L	-	+	-	+	(+)	-	-	-	(+)	+	-	(+)	+
S587L	++	++	++	+++	++	++	++	+	++	++	(+)	++	++
Y588C	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	-	+++	+++
G592S	(+)	++	++	++	++	+	++	+	++	+++	(+)	++	++
G595D	+++	+++	++	+++	+++	+++	++	+	+++	+++	++	+++	+++
LHL601/5/12PNF	-	+++	+++	++	-	-	-	-	+	+++	-	-/+	-/+
LH601/5PN	ND	+++	-	++	-	ND	-	-	-	+++	-	(+)	(+)
C610Y	-	-	-	-	-	-	-	-	-	-	-	-	-
P613Y	++	++	-	++	+	++	++	-	+++	+++	++	++	++
Y625C	+++	++	+	++	+	+	+	(+)	(+)	(+)	++	+++	+++
Y627C	-	-	-	-	-	(+)	-	-	+	(+)	+	(+)	(+)
F632L	+++	+++	+++	+++	+++	+++	-	+++	(+)	+++	+++	ND	ND
K633T	ND	-	(+)	-	-	-	-	+	-	-	++	ND	ND

\* For mutant fusion proteins, amino acid exchange was produced by random mutagenesis; for nomenclature see legend to Fig. 1.

† Neutralizing antibodies (Schoppel *et al.*, 1996; Wagner *et al.*, 1992; Ohlin *et al.*, 1993; Britt, 1984).



**Fig. 3.** Analysis of the influence of amino acid substitution L612F on binding of MAbs ITC48 (a) and ITC52 (b). In the triple mutant LHL601/5/12PNF, Leu-601, His-605 and Leu-612 are replaced by proline, asparagine and phenylalanine, respectively, whereas LH601/5PN lacks the substitution at position 612. Immunoblot analyses were performed using peroxidase-conjugated anti-human IgG antibody and developed by autoradiography with the ECL detection system.

(Figs 2 and 3). Interestingly, among the limited number of amino acid variations within AD-1 that can be found among different clinical isolates, the only position that is substituted in a number of strains is leucine at position 612 which is changed to phenylalanine (Lehner *et al.*, 1991; Darlington *et al.*, 1991; Chou & Dennison, 1991). To analyse the contribution of Phe-

612 to the enhanced binding of ITC48, a fusion protein was tested which contained only the LH601/5PN substitution (the 'LH' protein). As can be seen in Fig. 3(a), the reactivity of ITC48 was almost completely lost when the 'LH' protein was used as antigen, indicating an important role for Phe-612 in the enhanced binding of antibody ITC48 to the 'LHL' protein. In contrast, all other MAbs showed similar reactivity with the 'LHL' and 'LH' proteins. Fig. 3(b) shows the human MAb ITC52 as an example and Table 1 summarizes the results for all MAbs. When intact AD-1 and the 'LHL' protein were purified and analysed by ELISA using ITC48, a 200-fold stronger signal was seen with 'LHL' compared to AD-1 (data not shown).

From these data, it must be concluded that AD-1-binding antibodies all have individual binding specificities which can be differentiated using AD-1 mutant proteins. In addition, mutations in AD-1 can lead to greatly reduced or enhanced binding of antibodies. However, the results also indicated that prediction of neutralization capacity of antibodies based on their binding pattern to AD-1 mutant proteins is impossible since there are no common characteristics for either type of antibody.

#### Recognition of AD-1 mutants by human sera

Since a direct quantification of neutralizing and non-neutralizing antibodies in human sera did not seem to be possible we decided to use an indirect approach to this problem

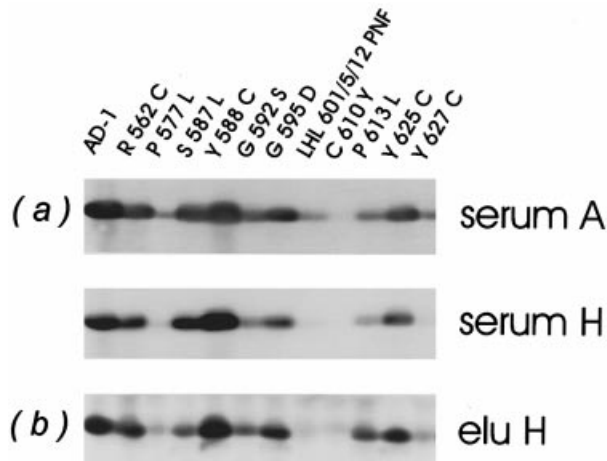


Fig. 4. Binding of AD-1-specific antibodies present in human sera (a) and a corresponding affinity-purified AD-1-specific antibody preparation (b) to mutant AD-1 fusion proteins. Immunoblot analyses were performed as described in the legend to Fig. 3.

and to determine the overall neutralization capacities of AD-1-specific antibodies purified from human sera. Five sera (I, III, A, D and H) were chosen for analysis which showed different concentrations of AD-1-specific antibodies as determined in an ELISA using recombinant AD-1 as antigen (data not shown). Firstly, reactivity of these sera was analysed in immunoblots with a restricted set of AD-1 mutant fusion proteins which did not contain the epitope tag. In contrast to MAbs, the recognition patterns of all sera were very similar and two representative sera are shown in Fig. 4(a). It can be seen, for example, that fusion protein C610Y was not recognized and the remaining mutant proteins gave signals of different intensities. The only obvious difference between sera was seen with the 'LHL' protein. Serum A did react with this protein, whereas serum H was negative. Of the remaining three sera, I was positive, whereas D and III were negative (data not shown). However, the signal intensity obtained with the 'LHL' protein, in general, was lower than that with AD-1, which is in contrast to the results obtained for ITC48.

Antibodies from the five sera were purified by affinity chromatography on recombinant protein Mbg58 containing AD-1 of gB. Mbg58 was coupled to CNBr-activated Sepharose and 25 ml 1:1 diluted serum was passed over the column. Bound antibodies were eluted and analysed for purity. To this end, a previously described ELISA was performed in which antibodies against twelve known antigenic determinants from HCMV-specific structural as well as non-structural proteins were individually analysed (Schoppel *et al.*, 1997). Whereas the sera contained considerable antibody titres against a number of different antigens such as pp150 or glycoprotein H (gH), only AD-1-specific reactivity was detectable in the affinity-purified antibody fractions (data not shown). The absence of antibodies against gH, the second major antigen capable of inducing neutralizing antibodies during natural infection, was also

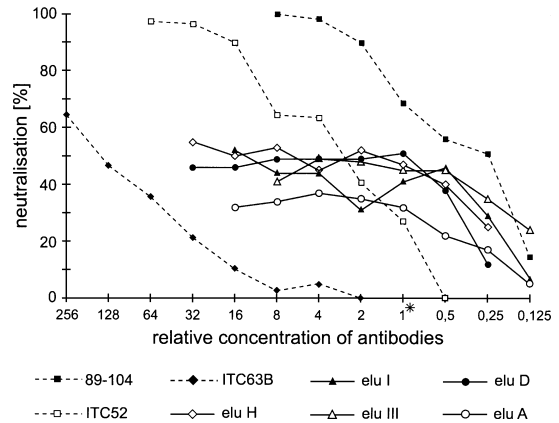


Fig. 5. Neutralizing activity of mono- and polyclonal AD-1-specific antibodies. Affinity-purified AD-1-specific fractions (elu) of five human sera (H, I, III, D and A) as well as three human neutralizing MAbs (89-104, ITC52 and ITC63B) were tested for neutralization of HCMV strain AD169 as described in Methods. Percentage neutralization is plotted as a function of relative antibody concentration. Log<sub>2</sub> titrations of antibodies are shown (abscissa) with 1\* corresponding to the IgG concentration giving an OD of 1 in an IgG-specific ELISA.

confirmed in indirect immunofluorescence analysis using insect cells infected with a HCMV gH recombinant baculovirus (Urban *et al.*, 1996). In contrast, the affinity-purified antibodies from all sera were still positive in immunofluorescence analysis with HCMV-infected fibroblasts and insect cells infected with a HCMV gB recombinant baculovirus, indicating that the purified antibodies retained their capacity to react with native antigen (data not shown). When affinity-purified antibodies were analysed in immunoblots with the AD-1 mutant fusion proteins they exhibited exactly the same recognition pattern as the corresponding sera. An example is shown in Fig. 4(b).

In summary, these data indicate that human sera contain antibody mixtures resulting in highly similar recognition patterns with respect to AD-1 mutant fusion proteins. Affinity purification produced a pure fraction of AD-1-specific polyclonal antibodies having an overall composition of individual antibody specificities identical to that of the respective serum.

#### Neutralization capacity of polyclonal AD-1-specific human antibodies

Next, we analysed the neutralizing capacity of AD-1 affinity-purified antibody fractions. Immunoglobulin concentration of the respective preparations as well as for human neutralizing MAbs 89-104, ITC52 and ITC63B was determined in an ELISA and comparable amounts were titrated for neutralization of HCMV strain AD169. As can be seen in Fig. 5, the 50% neutralization titres of the human MAbs differed by nine log<sub>2</sub> dilutions, again emphasizing the highly different neutralization capacities of AD-1-specific MAbs. In contrast, the polyclonal antibody fractions gave comparable titration curves resulting in a rather constant reduction of approximately 50% of input infectivity over an extended concentration range

(Fig. 5). The low level of neutralization exerted by the polyclonal antibody fractions was not secondary to the affinity purification since we have previously shown that affinity-purified antibodies specific for two other antigenic domains of HCMV, namely AD-2 of HCMV gB as well as AD-86 of HCMV gH, were capable of 100% neutralization of HCMV strain AD169 in an identical assay (Urban *et al.*, 1992; Meyer *et al.*, 1992). It should be noted that concentrations of AD-1-specific antibodies in these assays were comparable to those present in human sera as determined by an ELISA with recombinant AD-1 (data not shown).

## Discussion

gB is the dominant antigen for the induction of neutralizing antibodies against HCMV. Consequently, this protein has been the focus of strategies for active and passive immunoprophylaxis (Werner *et al.*, 1993; Wang *et al.*, 1996; Starr *et al.*, 1991). AD-1 represents the most highly antigenic structure for the humoral immune response against gB (Knies *et al.*, 1991; Schoppel *et al.*, 1997). Previous work has characterized AD-1 as a complex antibody-binding domain consisting of a minimal primary amino acid sequence containing residues 552–635 of gB (Wagner *et al.*, 1992). As defined by MAbs, AD-1 is capable of reacting in a competitive fashion with antibodies having highly different neutralizing capacities and the fact that the MAbs of the ITC series were all isolated from a single donor indicated that multiple binding specificities are simultaneously present in human sera (Ohlin *et al.*, 1993; Utz *et al.*, 1989). The mechanism(s) underlying these different neutralization capacities remain unexplained. The influence of different Fc portions of the respective IgG subclasses can be ruled out since, in human convalescent sera, antibodies which are reactive with AD-1 or gB are exclusively of the IgG1 subtype, which is in contrast to the immunoglobulin subtype distribution found against other glycoproteins such as gH (Urban *et al.*, 1994). Complement as a contributing factor is also unlikely, since the biological activity of AD-1-specific antibodies does not change by the addition of complement (Ohlin *et al.*, 1993; Klein *et al.*, 1999). Lastly, binding of antibodies to AD-1 is most probably not affected by glycosylation of gB since antibodies recognize deglycosylated protein equally well (Britt & Vugler, 1989, 1992). Thus, the properties of the paratope are expected to represent the decisive factor for the differences in neutralizing capacity between individual antibodies.

The major conclusion from our study is that AD-1 represents a structure which induces a multitude of antibodies during natural infection or after immunization of mice. Using a small set of AD-1 mutant fusion proteins, we have speculated in a previous analysis that MAbs could probably be assigned to a limited set of individual recognition patterns (Schoppel *et al.*, 1996). However, the present study clearly indicates that differentiation into distinct recognition subgroups is no longer possible when a larger set of mutant fusion proteins is used.

Nevertheless, on the level of MAb, binding patterns to AD-1 mutant proteins can be identified that are specific for every antibody. It should be kept in mind that our screening procedure was selective for AD-1 mutant fusion proteins which resulted in decreased antibody binding. This was dictated by the need to optimize the yield of fusion proteins having single amino acid substitutions in a 300 nt coding sequence without having to analyse a vast excess of multiple or silent mutations (Myers *et al.*, 1985). Nevertheless, an amino acid substitution was identified that resulted in drastically enhanced binding of a naturally occurring AD-1-specific antibody. It is conceivable that additional residues leading to similar effects upon mutation are present in AD-1. Also, it is expected that a number of residues within AD-1 could be changed without any effect on antibody binding. One such substitution is a Ser-557 to glycine change, which showed no influence on binding of MAbs as well as of human sera (data not shown). Similarly, it cannot be assumed that loss of antibody reactivity with a given fusion protein is secondary to the substitution of a critical contact residue. Local structural alterations within AD-1 as well as allosteric effects could equally well have contributed to the observed effects. However, epitope mapping of MAbs was not the goal of our study and, given the complexity of interactions, might require structural analysis of antigen–antibody complexes.

Human sera, on the other hand, contain a mixture of antibody specificities which results in an overall recognition pattern that is indistinguishable between individual samples. This could mean that either a single or few antibody types which are common to all infected individuals predominate. However, this seems unlikely since all AD-1-specific MAbs isolated so far show different recognition patterns. Moreover, isoelectric focusing analyses of the affinity-purified polyclonal AD-1 antibodies revealed a complex protein composition (D. Glykofrydes, unpublished observation). The more likely explanation is that during natural infection, the humoral immune response produces a mixture of many different antibody specificities against AD-1. As a consequence, only those mutations (i.e. cysteines, prolines) within AD-1 which profoundly disturb the overall conformation and thereby destroy the binding structure of the majority of the polyclonal antibodies result in altered recognition. The neutralization curves that were observed with the polyclonal antibody fractions can best be explained by the same argument. Although neutralization of viruses is a highly complex event (for review see Dimmock, 1993, 1984) and we have no information on the actual mechanism of the observed effects, the most plausible explanation is the presence of a balanced ratio of neutralizing to non-neutralizing antibodies resulting in incomplete virus neutralization.

What could be the *in vivo* relevance of our data? The importance of AD-1 for the immune response against HCMV would suggest that this area of the molecule exhibits an increased frequency of amino acid substitutions as a result of

the selective pressure of the host immune responses. However, this has not been observed. Whereas other domains of the molecule show considerable variation between isolates, AD-1 appears to be one of the most highly conserved regions of gB. Nevertheless, a few point mutations can also be found in AD-1, most of which are scattered throughout the entire region (Chou & Dennison, 1991; Lehner *et al.*, 1991; Roy *et al.*, 1993). On the level of individual antibody specificities, these changes could result in drastically diminished or improved binding to AD-1 as we have observed during our analyses. However, on the level of the combinatorial specificities which are present in human sera, these differences are balanced out with respect to recognition and neutralization. Impairing this balance will be equally difficult for the virus and the immune system. Thus, it seems as if the polyclonal antibody response against AD-1 represents yet another example of an effective immune evasion mechanism developed by this virus whereby the immune system has locked onto this highly conserved and important structural domain as a neutralization-relevant structure. The virus, on the other hand, has evolved mechanisms to evade this response via the induction of a multitude of antibodies which, when combined, achieves only incomplete virus neutralization. These properties could have important consequences for the dissemination of virus during natural infection as well as for the efficacy of a HCMV gB subunit vaccine.

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