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Clonal repertoire diversification of a neutralizing cytomegalovirus glycoprotein B-specific antibody results in variants with diverse anti-viral properties

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Abstract

Cytomegalovirus induces a chronic infection that in normal individuals is controlled by the immune system. In the case of humoral immunity, epitopes, in particular antigenic domain-1, in glycoprotein B have proven to be important for the induction of virus-neutralizing activity. Such antibodies can exert potent virus-neutralizing activity but can also block neutralizing antibodies from binding. Furthermore, these antibodies differ in their fine recognition of antigenic domain-1 as determined by epitope mapping. By using combinatorial library and phage display technologies we have now isolated a large array of clonally related antibody fragments to understand the origin of this diversity. This procedure allowed us to demonstrate that much of the diversity in functional activity (virus neutralization) and epitope recognition can arise from a single parental molecule through somatic mutation processes. We have thus demonstrated that the clonal diversification of a single antigen-specific clone can account for much of the diversity in antibody anti-viral activity. These findings have implications on the development of a gB-based subunit vaccine, as an effective vaccine preparation need not only to recruit appropriate clones into the immune response but also to evolve them properly so as to maintain an appropriate biological function.

Keywords: Antibody; Cytomegalovirus; Phage display; Repertoire; Virus neutralization

1. Introduction

Cytomegalovirus (CMV) is a ubiquitous virus widely spread around the world. Despite its usually low rate of disease induction, its high frequency in many populations makes it a problematic cause of disease, in particular as the sequellae that may develop in affected individuals are quite severe. As the immune system generally seems to control virus infection, much interest has focused on immunological factors that may contribute to protection against disease. In fact, development of a vaccine against CMV has been given the highest priority by the Institute of Medicine (Institute of Medicine, 2001a,b) and several efforts are ongoing to develop such a product. Although much of the limiting and protecting efficacy resides in the T lymphocyte response, antibodies appear to confer protection or to improve prognosis, e.g. in relation to congenital infection (Chatterjee et al., 2001; Fowler et al., 1992; Plotkin, 2002; Snydman et al., 1995; Zaia, 1993). Antibodies are proteins produced by the immune system with a powerful ability to mediate biological functions, including destruction of infectious agents and toxins. In order to provide protection against e.g. infectious agents, antibodies have to bind to their target with appropriate binding properties involving reaction rate kinetics, affinity and fine specificity.

Abbreviations: AD, antigenic domain; BSA, bovine serum albumin; CDR, complementarity determining region; CMV, cytomegalovirus; gB, glycoprotein B; H, heavy; L, light; PBS, phosphate buffered saline; PCR, polymerase chain reaction; scFv, single chain antibody fragment

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Many antibodies that develop in response to an infection, such as in the case of an HIV infection, seem to recognize improperly folded antigens. Such responses will not protect against disease and may serve as a mode whereby the infectious agent even tries to reduce the pressure exerted by humoral immunity on its survival (Parren et al., 1997). CMV employs a multitude of strategies to reduce immune surveillance to a level manageable by the virus (Michelson, 1999) and certain features of the antibody response it induces may also contribute to escape from antibody-mediated surveillance of infection. CMV neutralizing humoral immunity is directed to a variety of antigens found on the viral membrane, including glycoprotein B (gB), which has also been the target for vaccine efforts (Pass et al., 1999). This membrane protein is involved in the infection process both at the level of attachment and membrane fusion (Compton, 2004). Natural infection or immunization induces antibodies against several epitopes on gB among which antigenic domain-1 (AD-1) is the dominant and perhaps the principal virus-neutralizing antibody binding site on gB (Kniess et al., 1991). This epitope, which is largely conformational in nature and depends on the formation of proper disulphide bonds, is located in the C-terminal fragment of gB (Speckner et al., 2000). AD-1 has been shown to be recognized by sets of human as well murine monoclonal antibodies displaying different fine-specificities and widely different capabilities to neutralize infectious virus *in vitro* (Schoppel et al., 1996; Speckner et al., 1999). In fact, even though certain individual antibody clones targeting AD-1 are able to completely block virus infectivity, polyclonal IgG preparations, purified from human sera, with specificity for AD-1 are only able to partly prevent infection (Speckner et al., 1999). This finding suggests that polyclonal preparations contain different types of AD-1-specifc antibodies that either (1) have no effect on virus infection, or (2) mediate or (3) prevent virus neutralization. Little is known though about the origin of the populations with different functional activities. The humoral immune response recognizing this structure is complex in its makeup and different mechanisms are likely to contribute to the diverse recognition of AD-1. Human antibodies recognizing AD-1, although demonstrating a predominance of antibodies derived from the small IGHV5 germline gene subgroup (Ohlin et al., 1994), originate from different antibody-encoding germline genes. In addition, junctional and immunoglobulin D gene-encoded diversity creating the critical third heavy-chain hypervariable loop of the antigen-binding site of antibodies is likely to contribute to the diverse recognition of AD-1. Finally, somatic hypermutation also contributes to the diversity of antibodies targeting many proteinaceous antigens, including AD-1. Thus, the antibody repertoire targeting AD-1 is quite diverse, not only with respect to anti-viral activity, but also in sequence.

In this study we have investigated how a set of clonally related antibodies are able to mediate anti-viral activity and we demonstrate how the somatic hypermutation process may contribute to functional diversity of an AD-1-specific antibody clonotype. By using one high affinity human antibody, ITC52 (Ohlin et al., 1993) originating from the IGHV5 gene subgroup that is a major contributor to AD-1 specific human antibody immunity (Ohlin et al., 1994) and *in vitro* evolution technology, we have been able to isolate clonally related antibody fragments against AD-1. We have also characterized how these related antibody specificities behave with respect to antigen recognition and mediation of virus neutralization. We demonstrate that diversity with respect to recognition of AD-1 and anti-viral activity can be contained within a single set of clonally related anti-AD-1 antibodies. Thus, the exclusive induction of highly protective antibodies via immunization using gB as antigen might be difficult to achieve.

2. Materials and methods

2.1. Antibody library

To create molecular diversity in an antibody targeting AD-1, we made use of a previously existing high affinity human antibody, ITC52, that had been isolated from a CMV-seropositive individual (Ohlin et al., 1993). This antibody is encoded for by somatically hypermutated germline genes IGHV5–51*01 (DP-73) and IGKV3–20*01 (A27). It thus represents the major clonotype, as defined by its use of IGHV5–51, which is frequently found associated with the AD-1 specificity (Ohlin et al., 1994). A variant of this specificity (GenBank accession number AY736147) in a single chain antibody fragment (scFv) format was constructed upon cloning of the corresponding gene in a phagemid vector. Diversity was subsequently incorporated into this gene in two steps, first by incorporating variability in heavy (H) chain complementarity determining region (CDR) 2 and light (L) chain CDR1 and subsequently by adding diversity in CDRL3. To identify natural sequence diversity as it may develop *in vivo*, it was amplified from antibody-encoding cDNA. This template was synthesized by the use of immunoglobulin constant region-specific primers from RNA that had been obtained from pokeweed mitogen-stimulated human peripheral blood lymphocytes. Primers (Table 1) used in the polymerase chain reaction (PCR)-based amplification process were designed so as to ensure amplification of CDRH2 from genes originating from members of the IGHV5 gene subgroup. Similarly, primers were designed so as to ensure amplification of CDRL1 and to some extent CDRL3 derived from members of the IGKV3 gene subgroup. This process ensured that the amplified sequence diversity introduced into the library was essentially similar to that expected not only to be tolerated by, but also to be introduced during somatic evolution of an antibody like ITC52 with a genetic origin in IGHV5-51 and IGKV3-20 germline genes. These amplified sequences were mixed with PCR-derived products encoding the other parts of the ITC52 gene. The different gene products carried short overlapping sequences that allowed for the successful assembly of these gene fragments into intact genes by a process of overlap extension PCR/CDR shuffling (Jirholt et al., 1998). The resulting products were cleaved with *Sfi*I and *Not*I (New England BioLabs, Beverly, MA) and ligated into a variant of the pFAB5c.His (Ørum et al., 1993) phagemid vector encoding a truncated version of M13 protein 3 covering only its C-terminal domain. Ligated vector was electroporated into *Escherichia*

Table 1
Primer combinations and templates used to prepare fragments for development of antibody libraries

Construct	Product	Template	5′-primer	3′-primer[a]
1	Vector → FRH2	Phagemid vector carrying ITC52 scFv insert	GGCCGATTCATTAATGCAGC	ATCTGGCGCACCCAAGCGATCCA
	CDRH2	cDNA prepared from peripheral blood lymphocytes	GGATCGCTTGGGTGCGCCAGATG	GATGGACTTGTCGGCTGAGATGGWGAC
	FRH3 → FRL1	Phagemid vector carrying ITC52 scFv insert	CTCAGCCGACAAGTCCATCA	GGCTCTTTCCCCTGGAGAC
	CDRL1	cDNA prepared from peripheral blood lymphocytes	CTCCAGGGGAAAGAGCCACCC	GGCCAGGTTTCTGCTGGTACCA
2	FRL2 → Vector	Phagemid vector carrying ITC52 scFv insert	GGTACCAGCAGAAACCTGGCCAG	GCCTTTAGCGTCAGACTGTAG
	Vector → FRL3	Construct 1	GGCCGATTCATTAATGCAGC	AATACACTGCAGAATCTTCAGG
	CDRL3	cDNA prepared from peripheral blood lymphocytes	GGAGCCTGAAGATTCTGCAGTGTATTACTG	GATTTCCACCTTGGTCCCTTGGCCGAA
	FRL4 → Vector	Phagemid vector carrying ITC52 scFv insert	CGGCCAAGGGACCAAGGTGGAA	GCCTTTAGCGTCAGACTGTAG

[a] The degeneracy (A, T) allowed for in one position seen in the 3′-primer intended for amplification of CDRH2 permits the amplification of sequences carrying codons for both the germline-encoded threonine and the serine found in ITC52 in position 77 of the heavy chain.

coli Top10F′ using conventional electroporation methodology (Lantto et al., 2001). ScFv-displaying phages were produced after induction of gene expression by the addition of 0.1–1 mM isopropyl β-D-1-thiogalactopyranoside. Phages were harvested and concentrated in the presence of 0.5 M NaCl and 4% PEG 6000.

2.2. Antigen-specific selection and protein production

Phages carrying scFv specifically recognizing AD-1 were selected with three different forms of recombinant protein encoding the epitope. These were: (1) AD-1 (amino acids 552–635 of gB from strain AD169) carrying a hexahistidine tag (AD-1-His_6) (Speckner et al., 2000), (2) NRD1-BCCP (a fusion of AD-1 (amino acids 541–640) to the biotin carboxyl carrier protein that is biotinylated in *E. coli* (Ohlin et al., 1997)), and (3) soluble gB produced in CHO cells (Spaete, 1991) kindly provided by Aventis Pasteur (Marcy L'Etoile, France). For phage selection, AD-1-His_6 was coated onto immunotubes (NUNC A/S, Roskilde, Denmark) and NRD1-BCCP was bound to strepatavidin-coated paramagnetic Dynabeads (Dynal A/S, Oslo, Norway). After washing, phages diluted in phosphate buffered saline (PBS) containing 3% bovine serum albumin (BSA) and 0.05% Tween 20 were added. After incubation for 1 h, non-bound phages were removed by washing and specific phages were eluted with 0.1 M HCl. After neutralization with Tris, eluted phages were allowed to infect *E. coli* Top10F′. The selection was repeated once more. Similarly, selection of specific antibody fragments was also attempted in three rounds on immunotubes coated with soluble gB (Aventis Pasteur) using an approach otherwise identical to the one described above. After the final selection, M13 gene III was removed from plasmid pools by cleavage with *Eag*I and subsequent re-ligation, and the vector was again transformed into *E. coli*. Individual colonies were picked and their produced scFv were assessed for reactivity towards AD-1 by ELISA using the anti-FLAG M2 antibody (Sigma–Aldrich, St. Louis, MO, USA) that recognizes the FLAG sequence found at the C-terminus of each scFv. Horseradish peroxidase-labelled rabbit anti-mouse immunoglobulin (DAKO A/S, Glostrup, Denmark) was used for detection. The coefficient of variation for these assays is typically below 10%. Soluble scFv was produced into culture medium and used as such or purified by immobilized metal affinity chromatography and gel filtration, essentially as described (Jirholt et al., 2002), to obtain a monovalent antibody fragment preparation.

2.3. Gene sequencing and analysis

Genes encoding scFv were sequenced directly from plasmids or from PCR products derived from such plasmids either in-house using the Big Dye Terminator kit (PE Biosystems, Warrington, UK) or at an external sequencing centre (MWG-Biotech, Ebersberg, Germany). The antibody-encoding gene nomenclature and numbering of residues are in agreement with the IMGT definitions (http://imgt.cines.fr) (Lefranc, 2003). Replacement/silent mutation ratios were calculate by dividing

the number codons that had undergone an amino acid substitution or deletion with the number of altered codons that had not changed the amino acid they encoded.

2.4. Evaluation of antibody fine specificity by immunoblotting

SDS-PAGE of total protein lysates of bacteria producing AD-1 and mutated versions thereof (Fig. 1) and immunoblotting were done by standard procedures. Nitrocellulose membranes were blocked with PBS containing 0.1% Tween 20. Filters were incubated with undiluted scFv-containing supernatants for 2 h at room temperature. ScFv binding to AD-1 and AD-1 mutants was detected after incubation with the M2 anti-FLAG monoclonal antibody and alkaline phosphatase-coupled anti-immunoglobulins by BCIP/NBT staining. Monoclonal antibody Ap86-SA4, which is specific for the epitope tag of the AD-1 mutants, was used as an application control. Thus, the presence of equal amounts of the different recombinant proteins bound to the nitrocellulose filters could be verified.

2.5. Biacore analysis

Recombinant soluble gB was immobilized onto CM5 sensorchips (Biacore AB, Uppsala, Sweden) via its free amino groups. Antibody fragments were injected at different concentrations and dissociation rate kinetic was determined using the BIA Evaluation 3.0 software (Biacore AB) as previously described (Lantto et al., 2003).

2.6. Neutralization assay

Comparative neutralization assays were carried out as previously described by Schoppel et al. (1996) with slight modifications. Serial dilutions of scFv-containing supernatants as well as purified preparations in minimal essential medium supplemented with 5% fetal calf serum, glutamine and gentamicin were pre-incubated for 1 h at 37 °C in 96-well microtitre plates with anti-FLAG M2 monoclonal antibody to crosslink scFv via their FLAG-tag. CMV strain AD169 was added afterwards for incubation for another 3 h at 37 °C. Human foreskin fibroblasts (1.0×10^4) were added in a volume of 25 μl medium each. 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, IE-1, of CMV. Antibody binding was detected by a Cy-3 conjugated rabbit anti-mouse IgG $(Fab')_2$ fragment (Dianova, Hamburg, Germany). Generally, infectious doses were adjusted to 150 infected cells as counted with 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 represent mean values of three independent assays. Variations between assays were in the range of two dilution steps.

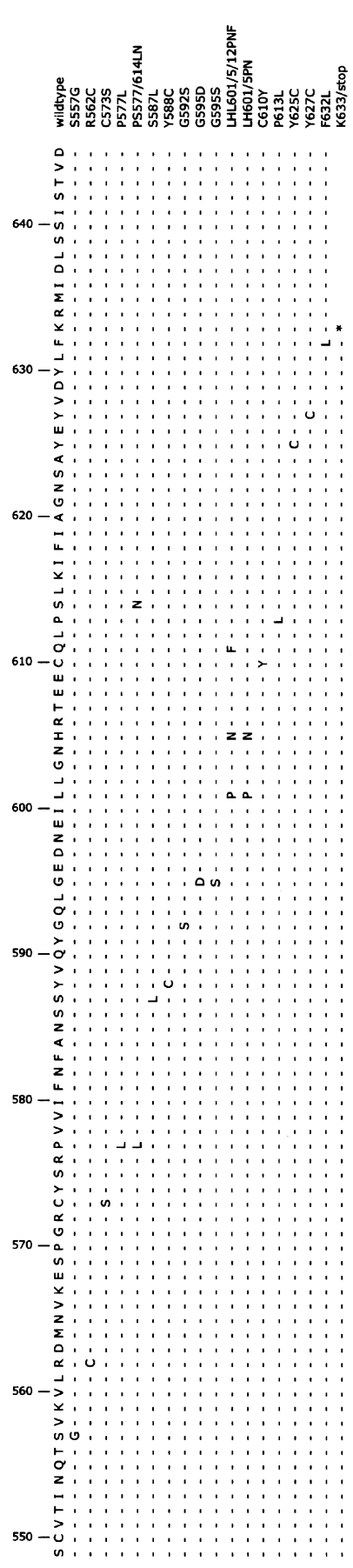

Fig. 1. Amino acid sequences of AD-1 mutant fusion proteins that were generated by random mutagenesis (Speckner et al., 1999). Dashes indicate identity to CMV strain AD169 and substituted amino acids are shown in bold. Clones are designated by type and position of the exchanges (i.e. in R562C, the arginine at position 562 within gB was replaced by cysteine). Protein K633T has a C-terminal truncation as indicated.

3. Results

3.1. Antibody library

To address the matter of whether or not a single clonotype recognizing AD-1 can evolve to generate the different recognition patterns and anti-viral profiles observed in polyclonal antibody populations, we developed a single chain antibody fragment (scFv) library based on a known AD-1 specific antibody, ITC52 (Ohlin et al., 1994), with diversity introduced in three of the hypervariable loops that are considered to be critical for antigen recognition, namely H chain CDR2, and L chains CDR1 and CDR3. Although ITC52 evidently has undergone hypermutation (Ohlin et al., 1994), all of these three particular CDR are largely unmutated. Sequencing of genes encoding heavy chain-encoding genes from this library identified germline or slightly mutated versions of this sequence as it is found in the IGHV5–51 gene. This thus represented a set of heavy chain genes originating from the antibody hypermutation process *in vivo*, as it could be expected to occur in the clonotype represented by ITC52. Sequencing of the light chain, similarly identified CDRL1 and CDRL3 mainly originating from genes belonging to the IGKV3 gene subgroup in mutated and non-mutated forms. CDRL1 encoded by different members of this gene subgroup are similar in sequence, but may differ by one codon in length. This difference has been demonstrated to translate into two different canonical loop structures (Al-Lazikan et al., 1997). We had thus succeeded in developing a library carrying sequence diversity that may arise not only from the process of point mutation but also through codon deletion. This mode of antibody hypermutation has previously (Ohlin and Borrebaeck, 1998; Wilson et al., 1998) been demonstrated to occur *in vivo*. The introduced sequence diversity thereby offered us an opportunity to gain insight into whether or not two different modes of sequence evolution, i.e. point mutation and codon deletion, may have a role in the creation of diversity of antigen recognition.

3.2. Selected diversity

Selection of phages displaying a functional AD-1-specific scFv was primarily performed on two different recombinant antigens representing the complete antibody binding site, namely AD-1-His_6 and NRD1-BCCP produced in *E. coli*. An analysis of the sequences of a large set of clones selected on these two antigens demonstrated that specifically binding clones were diverse in the varied hypervariable loops, in particular in CDRL1 and CDRL3 (GenBank accession numbers AY736148–AY736176) (Fig. 2). Little diversity was found in CDRH2 of selected clones. Additional diversity was, however, also found in other parts of the sequence, probably as a consequence of random errors occurring in the primers or by the use of a non-proofreading polymerase in the PCR amplification process. Importantly, however, amino acid substitutions were approximately 20 times more common in CDR specifically targeted by diversification than in other parts of the sequence that were only amplified through the PCR process. This fact demonstrated that the diversity largely originated from the process of antibody evolution as it occurs *in vivo* and not primarily from the PCR amplification process. We thus had access to a large set of related AD-1 specific antibody fragments that also may have originated through an evolution process as it occurs *in vivo*, as the diversity in evolved CDR had been sampled from antibody-encoding genes in human B cells. Apart from single point mutations, other major sequence differences were seen in the light chain. In particular, length variation of hypervariable loops as it would occur following insertion/deletion of codons was observed in several selected clones, demonstrating that this mode of diversification was compatible not only with proper protein folding, but also with antigen recognition. Indeed, there was no general evidence for a selective preference for the longer canonical loop in CDRL1 used by the original antibody ITC52 following selection with soluble AD-1-His_6 or NRD1-BCCP (13/21 and 17/29 of randomly picked clones harbored the shorter loop before and after selection, respectively). The fact that selection under very stringent conditions on intact recombinant gB strongly favored selection of variants carrying the longer version of CDRL1 (data not shown) demonstrated that CDRL1 is indeed involved in antigen-binding. Nevertheless, functional sequence space of this clonotype could be expanded by codon deletion in this hypervariable loop.

A further analysis of the sequence data demonstrated features of the diversification and selection processes. Indeed, a substantial selection pressure appeared to have been in operation during the selection process as the unselected library carried more diversity in CDRL1 and CDRL3 in comparison to the selected sequences (Fig. 3). However, a large fraction of the nucleotide changes, in particular in CDRL1, still resulted in amino acid replacement or deletion, as evidenced by codon replacement-to-silent mutation ratio in this CDR of 6.7. Thus, although a selective pressure operated against too much sequence diversification of these hypervariable loops, many sequence variants carrying sequence diversity mimicking that found in B cells as they occur *in vivo* could be identified by this combination of library design and selection technology.

3.3. Specificity of selected clones

After selecting sequence variants of ITC52, we now had access to a set of variants representing an antibody clonotype as it may have evolved *in vivo*. To test whether or not sequence diversity within the clonotype translated into differences in fine specificity, a set of scFv selected on soluble AD-1-His_6 (clones I2 1–16) and NRD1-BCCP (clones S2 1–16) were analyzed by western blotting for their recognition of a set of previously described different recombinant mutants of AD-1 (Fig. 1) (Speckner et al., 1999). As expected, all of the scFv retained their specificity towards wild type AD-1. Binding of the scFv to the mutant AD-1 proteins was either unaffected (e.g. mutants F632L and Y588C), reduced (e.g. mutants P613L or LH601/5PN or even completely inhibited (e.g. C610Y and C573Y) in comparison to intact AD-1 (exemplified in Table 2). With respect to some mutant AD-1 proteins, recognition by the individual scFv was clearly different. For example, whereas S2-6 showed recognition of most of the AD-1 mutant proteins comparable to wild

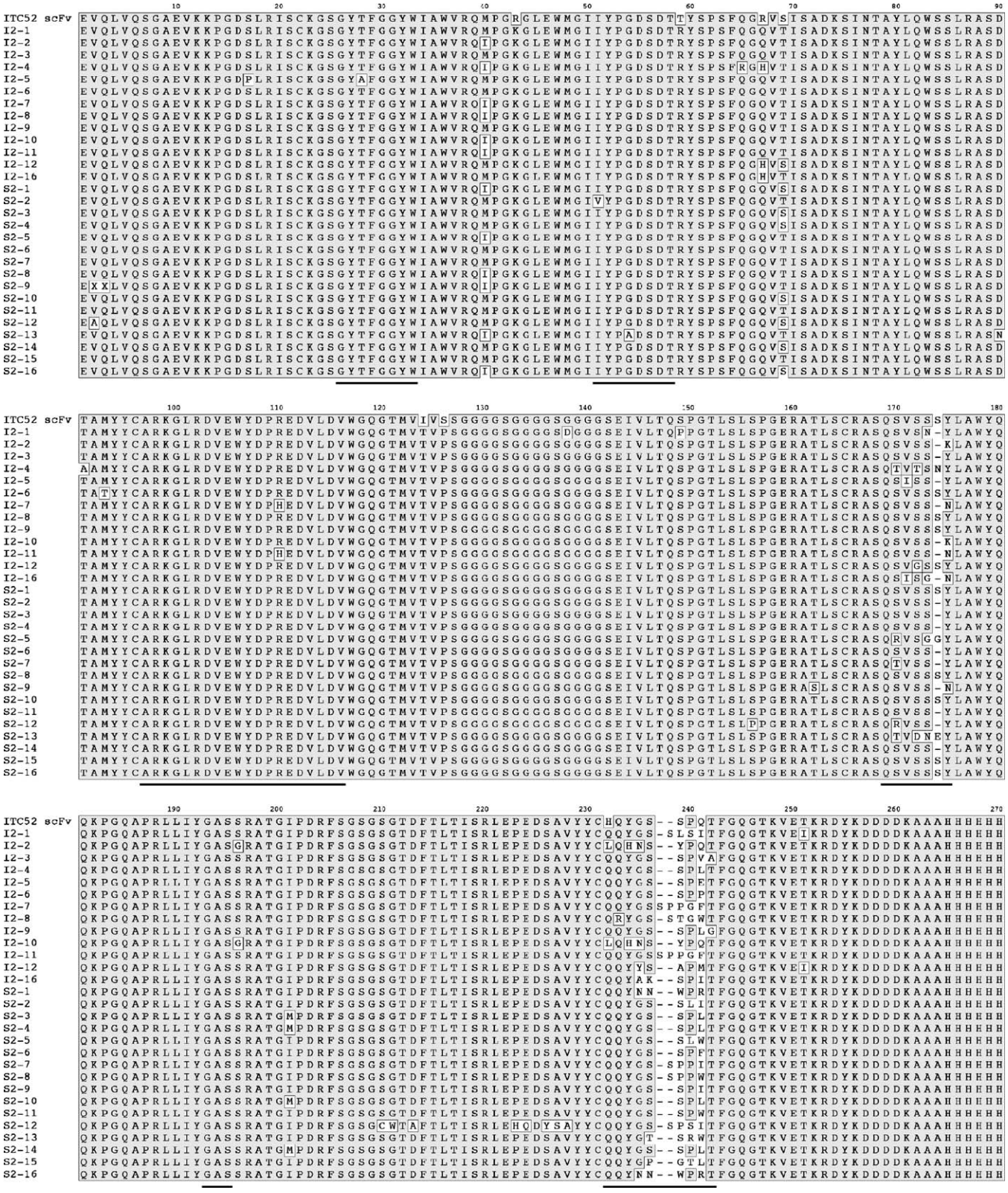

Fig. 2. Alignment of the original ITC52 scFv and 29 selected scFv variants evolved from this sequence. CDR, as defined by the IMGT nomenclature (Lefranc, 2003), are underlined. The H chain variable domain, linker, L chain variable domain, and tag sequences are represented by positions 1–127, 128–142, 143–253 and 254–270, respectively, in this linear representation of the sequences. Missing residues are located to the peaks of CDRL1 (residue 174, corresponding to IMGT residue 32) and CDRL3 (residues 237–238). Substitutions in framework region positions 40 and 69 (corresponding to residues 45 and 77 as defined by the IMGT nomenclature) were introduced into the library by the primers used for library construction.

Table 2
Examples of binding patterns of clonally related scFv to mutant AD-1 fusion proteins as determined by immunoblotting

AD-1 fusion protein*	ITC52L3 scFv selection with AD-1-His_6			ITC52L3 scFv selection with NRD-1 BCCP			ITC52scFv
	I2-1	I2-2	I2-7	S2-3	S2-6	S2-8	
AD-1	+++[a]	+++	+++	+++	+++	+++	+++
PS577LN	++	(+)	(+)	(+)	++	+	+
Y627C	+	+	–	(+)	+	(+)	+
Y625C	++	+	+	++	++	+	++
F632L	+++	+++	+++	+++	+++	+++	+++
P577L	+	(+)	(+)	+	+	+	+
S587L	++	++	++	++	++	++	++
K633Stop	–	–	–	–	–	–	–
R562C	++	++	++	++	++	+	++
Y588C	+++	+++	+++	+++	+++	+++	+++
G592S	+++	++	++	++	++	++	++
P613L	++	+	(+)	+	+	+	+
LH601/5PN	+	++	(+)	+	+	++	+
G595D	++	+++	++	++	+++	+++	++
S557G	++	+++	+++	+++	++	+++	+++
C610Y	–	–	–	–	–	–	–
G595B	+++	+++	++	++	+++	+++	+++
LHL601/5/12PNF	+++	(+)	(+)	+	++	++	++
C573S	–	–	–	–	–	–	–
pATH	–	–	–	–	–	–	–

[a] Recognition patterns of scFv are shown. Reactivities of scFv with fusion proteins in comparison to AD-1 are given as follows: +++, equal; ++, slightly decreased; +, decreased; (+), barely detectable in comparison to AD-1; –, no reactivity. *For mutant fusion proteins, amino acid exchange was produced by random mutagenesis; for nomenclature see legend to Fig. 1.

type ITC52 (data not shown), S2-3 was more severely affected in its binding capacity (Fig. 4). Differences in reactivity towards AD-1 mutant proteins were not associated with differences in loop length in CDRL1 described above, as both of the selected canonical loop lengths were associated with good recognition of certain mutants. In conclusion, a single antibody clonotype, here represented by the original clone ITC52, recognizing AD-1 can develop into several different fine-specificities, such as those observed after immunization of humans and mice (Schoppel et al., 1996; Speckner et al., 1999), and a polyclonal response does not seem to necessarily be required to obtain this diversification of specificity.

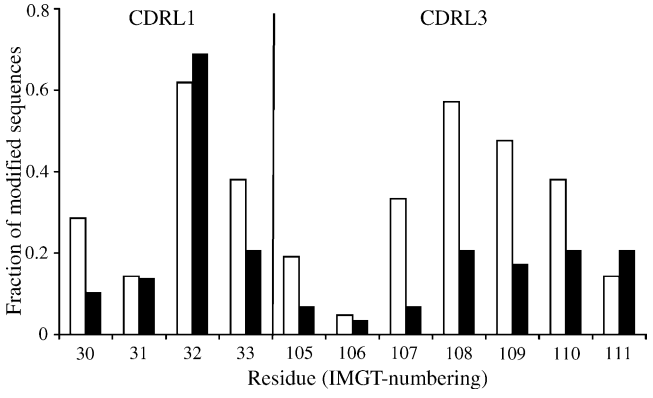

Fig. 3. Frequency of differences in selected residues in CDRL1 and CDRL3 in comparison to the germline sequence IGKV3–20 from which ITC52 light chain originates, as assessed among randomly picked unselected sequences ($n = 21$; open bars) and among selected sequences ($n = 29$; closed bars). ITC52 itself carries only one difference from the germline gene-encoded sequence in these parts of the sequence (Q105 → H) (Ohlin et al., 1994).

3.4. Biological activity

Amino acid substitutions occurring in a set of related clones through the processes of somatic hypermutation may result in loss of biological activity if it affects affinity or fine specificity. Indeed, the dissociation rate constant has been shown to directly relate to the ability of antibodies targeting another epitope on gB, AD-2, to mediate virus neutralization (Lantto et al., 2003). There was, however, no evidence that selected mutations greatly affected the dissociation rate of gB-scFv complexes (Table 3). However, it could not be excluded that sequence variation resulted in a tendency for selected clones to vary in their neutralizing capacity, for instance as a consequence of preferential binding to denatured as opposed to properly folded antigen. Therefore, we tested the ability of various evolved scFv specifically recognizing the AD-1 epitope on CMV gB to neutralize infectious virus. Initial studies demonstrated that monovalent antibody fragments in general were unable to neutralize virus

Table 3
Analysis of the dissociation rate constants of the complex between gB and ITC52 scFv and five selected scFv variants

Clone	k_{diss} (s^{-1})[a]
S2-8	3×10^{-4}
S2-9	3×10^{-4}
S2-10	2×10^{-4}
S2-13	3×10^{-4}
S2-14	1×10^{-4}
ITC52	0.5×10^{-4}

[a] The results are the average of two to three experiments with a coefficient of variation of <20%.

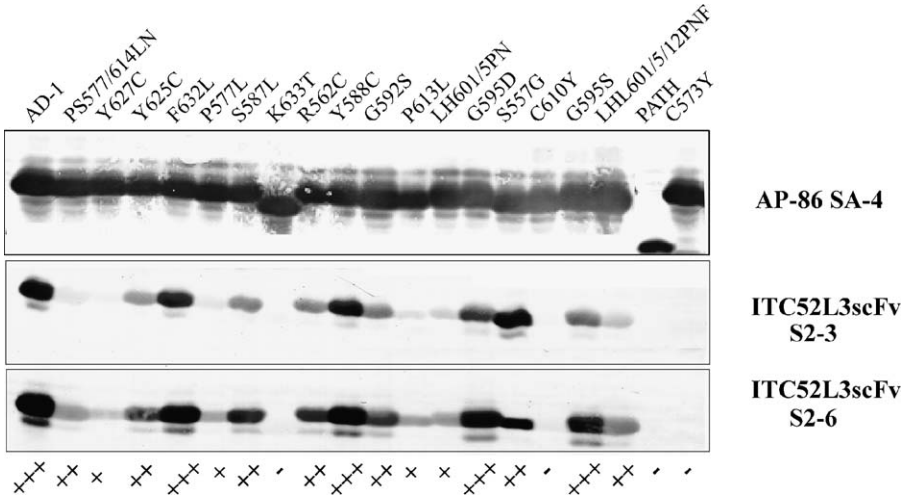

Fig. 4. Reactivity of scFv S2-3 and S2-6 with prokaryotically expressed fusion proteins (Fig. 1). *E. coli* lysates were subjected to SDS-PAGE and analyzed in immunoblots, where the disulphide-linked epitope reforms under the prevailing oxidating conditions (Speckner et al., 1999, 2000). The murine monoclonal antibody AP86-SA4, specific for the linear epitope tag present on all molecules, was used as a control. The classification of reactivities corresponding to Table 2 is shown for S2-6.

infection, as were monovalent antibody fragments targeting AD-2 on the same antigen (Lantto et al., 2002). It was only upon dimerization of these molecules using a divalent antibody recognizing the FLAG-sequence present at the C-terminus of each scFv that specific virus neutralization was achieved (data not shown). Further studies of virus neutralization were thus conducted on antibody fragments dimerized by the anti-FLAG antibody.

Since neutralization is critically dependent on the actual concentration of functional antigen-binding molecules in the respective preparations, we first carried out titrations of the samples in an ELISA using recombinant gB as antigenic substrate. In total 31 scFv in crude format were then tested for virus neutralization and the activity was correlated with the antigen binding capacity (Fig. 5). It was found that there was no strict correlation between antigen-binding capacity and anti-viral activity, as the slope of the regression line did not differ from zero. Furthermore, there were few obvious correlations between the number of mutations in the sequence and recognition of AD-1 and bioactivity. The substitution of tyrosine in codon 33 (as numbered in germline gene sequence IGKV3-20) in CDRL1, a frequent antigen-contacting residue (MacCallum et al., 1996) correlated ($p < 0.05$) to a low level of signal in antigen-specific binding assays, but not with the level of neutralizing activity (data not shown). There is thus no simple correlation between sequence and biological activity. Overall, the 50% virus neutralization capacity of the entire scFv population extended over a range of six dilution steps. Detailed studies of the neutralizing potential of some scFv with a high level of antigen binding capacity demonstrated that only some of them had a potent anti-viral activity, whereas others hardly reached 50% neutralization even at the highest concentration tested (Figs. 5 and 6). Thus, good binding,

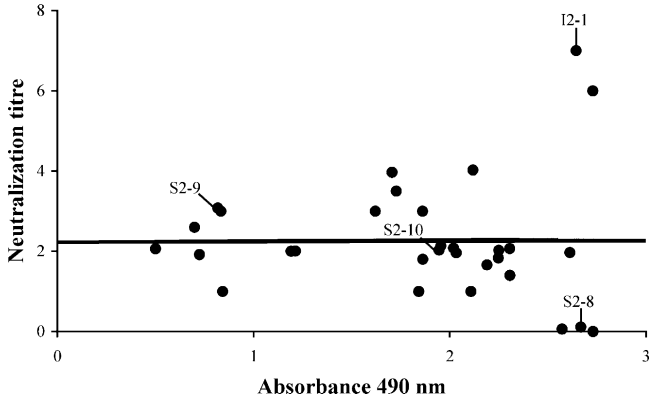

Fig. 5. Neutralization potential of scFv variants as a function of immunoreactivity. The titer of 31 dimerized preparations giving rise to a 50% reduction of input infectivity with CMV strain AD169 is shown as a function of their recognition of AD-1 as determined by ELISA. The slope of the line calculated by linear regression is 0.02 (confidence interval: −0.82 to 0.87 at $p = 0.05$) suggesting no correlation between binding activity and neutralizing potential. The activities of the four scFv that were further analyzed after purification are indicated in the graph.

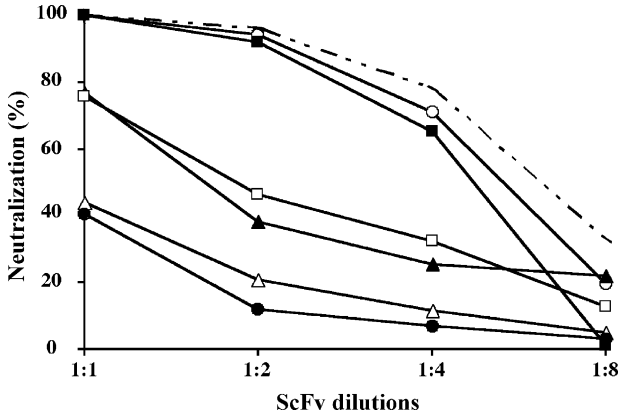

Fig. 6. Neutralizing activity of a group of highly gB-reactive scFv ($OD_{490\,nm} = 2.2–2.8$, as defined in Fig. 5) as well as scFv ITC52, which has a similar reactivity in that binding assay. Antibody fragments include I2-1 (open circles), I2-2 (closed squares), I2-5 (closed triangles), I2-6 (open squares), S2-7 (open triangles) and S2-8 (closed circles), and ITC52 scFv (broken line).

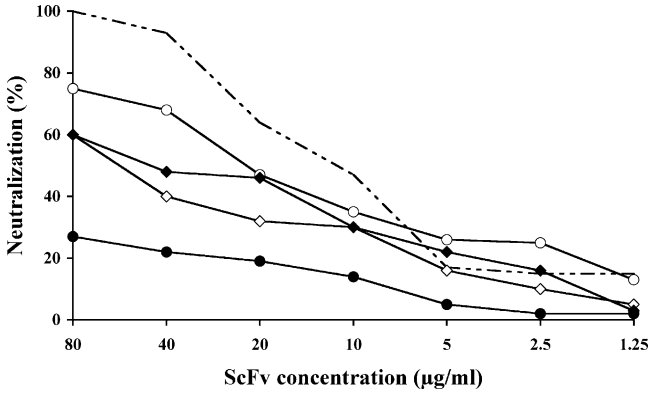

Fig. 7. Neutralization of CMV by purified, monovalent scFv following dimerization with an antibody specific for the peptide tag located at the C-terminus of each scFv. Antibody fragments included the original ITC52 scFv (broken line), the I2-1 scFv (open circles), the S2-8 scFv (closed circles), the S2-9 scFv (open diamonds), and the S2-10 scFv (closed diamonds). In tests employing unpurified antibody fragments (Fig. 5), scFv S2-8 displayed very poor neutralizing activity, mimicking the outcome of the test with these purified preparations.

as demonstrated by a high ELISA reactivity, although apparently required for the most potent anti-viral activity (Fig. 5), does not as a rule imply good biological activity, even when all specificities originate from a single clonotype. The validity of the procedure using crude supernatants, a critical parameter in this study, was tested by analyzing the virus-neutralizing capacity of a set of selected scFv after purification. Indeed, while purified scFv I2-1 displayed a potency close to that of ITC52 scFv (Fig. 7) in agreement with the analysis of the crude supernatant (Fig. 5), purified S2-8 was unable to effectively mediate virus neutralization. Other investigated scFv had an intermediate virus-neutralizing ability (Fig. 7). In all, this assessment demonstrated the validity of the approach taken above. We thus conclude that not only does a single set of clonally related antibody specificities contain members representing diverse recognition of the conformational AD-1 epitope, but it also harbours members with different abilities to mediate CMV neutralization. Thus, diversification of a single clonotype easily results in epitope-specific variants with both beneficial and non-beneficial recognition of CMV gB AD-1.

4. Discussion

Antibodies are potent effector molecules that may limit the consequences or establishment of many infectious diseases either through natural processes or after active or passive immune intervention. For some types of infection the role of antibodies is, however, not all that obvious, or they may even be detrimental. Many infectious agents have also developed strategies to prevent the induction of efficient immune responses including the development of efficiently neutralizing humoral immunity. It has for instance been observed that a majority of antibodies against HIV-1 membrane glycoprotein targets improperly folded protein, while the active form of the protein evokes much less antibody recognition (Parren et al., 1997). Thus, due to massive induction of non-protective antibodies, access of antibodies to neutralization-relevant sites might be efficiently prevented. A similar inefficiency is also seen in antibody responses against CMV. Although antibodies have indeed been shown to confer some protection or to improve prognosis in certain patient groups (Chatterjee et al., 2001; Fowler et al., 1992; Plotkin, 2002; Snydman et al., 1995; Zaia, 1993) many antibodies do not confer protection. Futhermore, some CMV epitopes that are targets of efficient neutralizing antibodies are only poorly immunogenic in humans (Britt and Mach, 1997) while the polyclonal immune response against one of the immunodominant epitopes, AD-1, is unable to completely prevent infection, even at high antibody concentrations (Speckner et al., 1999). Together, these facts naturally limit the beneficial effects of passively administered or actively induced antibodies in prophylaxis or therapy.

To improve on the quality of antibodies targeting potentially protective epitopes in infectious agents such as viruses, several strategies have been proposed. For instance, in the case of HIV-1, efforts have been made to design antigens that would induce highly potent and broadly neutralizing antibodies, while limiting the induction of other specificities (Burton et al., 2004; Pantophlet and Burton, 2003). From the discussion above, it is obvious that the induction of efficient neutralizing antibodies against CMV suffers from similar problems as those encountered in the efforts to raise protective immunity against HIV-1 and much remains to be learned about antibody specificities targeting neutralization epitopes, such as AD-1. It has for example not been previously defined if the diversity with respect to recognition and functional activity relates to a single clonotype and its variants as they develop during somatic hypermutation, or if it occurs as a consequence of the recruitment of different, clonally unrelated B lymphocytes into the pool of cells producing AD-1-specific antibodies. To address this matter, we used combinatorial library technology and phage display selection to identify molecular variants of a previously identified human antibody specific for AD-1. We believe that this mode of evolution is very similar to what could be encountered in an active immune response as it occurs in a secondary lymphoid germinal centre *in vivo*. Firstly, the diversity introduced into this system was based on sequence variability originating from B cells found *in vivo* that produce antibodies with heavy or light chains closely related to those found in the original human antibody against AD-1. Secondly, a selection system identifying AD-1-specific sequence variants was employed, eliminating those variants that evolved to display poor recognition of the epitope. Thus, although this approach does not strictly predict routes that *in vivo* evolution will take, it demonstrates routes that it may take as many of the components characterizing *in vivo* antibody evolution are present.

With access to this library we were able to select several sequence variants using different antigens that display the AD-1 epitope. This was achieved in a manner not necessarily improving the affinity of the antibody. The monoclonal antibody used as a starting point for these studies, ITC52, is by itself a high affinity antibody. In fact, it was the highest affinity binder in a set of human antibodies specific for AD-1 (Ohlin et al., 1993) obtained from a chronically infected individual. It was also already from the beginning of this study highly somatically mutated, carrying only 94% homology to the germline H chain variable gene

IGHV5–51 (Ohlin et al., 1994). In conclusion, it had already prior to this study gained a high affinity for gB through somatic hypermutation and further evolution, as performed here, identified alternative solutions as they may emerge through processes of somatic evolution. Firstly, it was observed that although the selected variants retained the overall specificity for AD-1, the ultra-fine specificity changed in several of the variants as evidenced by the diverse recognition of some AD-1 mutants. This finding agrees with previous studies that have demonstrated that evolution of antibodies may be accompanied by slight modifications of fine specificity (Lantto et al., 2003; Ohlin et al., 1996). Importantly, mutations of the cysteines at position 573 and 610 making up the disulphide bond within AD-1 were not allowed just as they are not allowed for the binding of antibodies developed *in vivo* (Speckner et al., 1999). This suggests that *in vitro* selection did not enrich for variants recognizing grossly misfolded or polymerized antigen. Furthermore, the observed diversity in antigen recognition by antibody fragments belonging to a single diversified clonotype strongly suggests that clonal diversification is sufficient to create much of the diverse recognition of AD-1 observed among antibodies that evolve *in vivo* (Schoppel et al., 1996; Speckner et al., 1999). Thus, it is not sufficient to recruit a restricted set of clonotypes (e.g. those originating from a certain germline variable gene), as even a single clonotype may evolve through somatic hypermutation to give rise to variants with diverse patterns of AD-1 recognition. Importantly, this was demonstrated with a clonotype originating from the antibody heavy chain germline gene (IGHV5–51), which is most frequently used by human AD-1 specific antibodies, thereby showing that this finding is related to a major part of the human humoral response targeting this epitope. Secondly, it was observed that the virus-neutralizing potential of these clonally related specificities differed widely. This agrees well with findings on a different set of antibodies that targets another epitope on gB, AD-2, where closely related antibody variants also displayed very different abilities to neutralize virus infection (Lantto et al., 2003). In similarity with the present case, minor variations in antibody sequence translated into differences in ultra-fine specificity of antigen recognition. In addition to the ability of a single clonotype to diversify its specificity for AD-1, this study, importantly, also demonstrates that one clonotype may evolve in a way that its different members mediate very different anti-viral activities against CMV. Whether or not a single clonotype that mediates anti-viral activity can evolve into a specificity that blocks the activity of other virus-neutralizing antibodies is still an unresolved matter that needs clarification. Nevertheless, recruiting just a limited set of clonotypes into the expanding B cell population cannot prevent the development of diverse levels of anti-viral activity as observed between AD-1-specific antibodies *in vivo*.

5. Conclusions

In conclusion, we propose that evolution of a single clonotype in principal has the potential to create diversity of very different specificity and potency with respect to CMV gB anti-viral activity, a factor that may contribute to the inability of antibody populations that are not strictly clonal in nature to fully neutralize virus infection. This finding has implications for the understanding of dissemination of free virus *in vivo* in the presence of a fully functioning humoral immune response. It will also hamper efforts to develop a vaccine that induces neutralizing antibodies with specificity for CMV gB as it suggests that it will be critical, not only that appropriate specificities are initially recruited into a gB-specific immune response, but also that the evolution of such specificities occurs in a controlled manner maintaining the most appropriate functional specificities at an ultra-fine level. It is thus important that a vaccine strategy, e.g. by selection of proper antigen dosage or administration of optimized antigen variants, ensures that somatic antibody evolution takes appropriate routes that preserves the neutralizing capacity of developing antibodies. Moreover, due to the high degree of amino acid conservation within AD-1 and the similarity of the antibody response against gB from different herpesviruses (Goade et al., 1996; Kropff and Mach, 1997; Xu et al., 1997), humoral immunity against gB molecules from other herpesviruses might suffer from the same problems. In all, it is critical that vaccine formulations in these systems not only prevent the initial selection of inappropriate clonotypes but also the evolution of non-neutralizing variants that are non-protective or at worst counters the activity of those antibodies that mediate neutralization. Such protein variants suitable for vaccination may include those that retain a proper, native fold even after addition of adjuvant and those that through a rigorous test of new, mutated variants of gB or AD-1 itself can be shown to bind neutralizing but not non-neutralizing antibodies.

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