



LIGHT CHAIN SHUFFLING OF A HIGH AFFINITY ANTIBODY RESULTS IN A DRIFT IN EPITOPE RECOGNITION

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(First received 25 May 1995; accepted in revised form 29 August 1995)

Abstract—Human polyclonal and monoclonal antibodies against pathogens and toxins are potentially useful in the treatment of various diseases. A number of human monoclonal antibodies with protective capacity *in vitro* have been established by conventional hybridoma technology. However, with the development of phage-display technology, the possibility of specifically tailoring antigen-binding properties has improved substantially. We show here that the reactivity of a high affinity, virus-neutralizing human antibody against the AD-2 epitope of cytomegalovirus gB can be modified by introducing other V κ sequences together with the original V H sequence. The fine specificity, as determined by the requirement of particular amino acid residues in the epitope, is shifted in these new antibody fragments. It was also evident that the V H /V κ pairing was not promiscuous, since antibody fragments selected by phage display retained light chain sequences very similar to the original hybridoma-derived light chain, proving that a high affinity interaction was very dependent on a co-operativity between both variable domains. These findings show that phage display technology might modify the binding properties of pre-existing, high affinity antibodies.

Key words: cytomegalovirus, glycoprotein B (gB), human monoclonal antibody, phage-display technology, antibody specificity.

INTRODUCTION

Establishing human monoclonal antibody specificities has proven very labour intensive using conventional hybridoma technology. Recently, however, the introduction of antibody libraries (Huse *et al.*, 1989) and the use of antibody-display on filamentous phage (McCafferty *et al.*, 1990) have opened new possibilities. Thus, it has been possible to obtain specific antibodies against a variety of antigens from both pre-immunized (see e.g. Burton *et al.*, 1991 and Williamson *et al.*, 1993) and also from apparently non-immune donors (Gram *et al.*, 1992; Hoogenboom and Winter, 1992; Marks *et al.*, 1991). Synthetic (Barbas *et al.*, 1992; Griffiths *et al.*, 1994) repertoires have also recently been used for the same purpose. Furthermore, the phage technique has been used to select for antibody fragments exhibiting specific characteristics with respect to kinetic parameters (Haw-

kins *et al.*, 1992; Dueñas *et al.*, submitted) and to evaluate repertoires developed *in vivo* (Rapoport *et al.*, 1995).

In this report, we demonstrate the use of phage-display technology to modify the reactivity of high-affinity human antibodies. Specifically, a monoclonal antibody recognizing the AD-2 epitope expressed by human cytomegalovirus (CMV) glycoprotein B (gB) (Ohlin *et al.*, 1993) was used for this purpose. The AD-2 epitope, which is conserved among different isolates of CMV (Lehner *et al.*, 1991; Meyer *et al.*, 1992), is an important target for complement-independent CMV-neutralizing antibodies (Meyer *et al.*, 1990; Ohlin *et al.*, 1993) and might consequently be a useful target for passive immunotherapy. Clinical trials have already been initiated with one human antibody specific for CMV AD-2 (Azuma *et al.*, 1991). Although the two human monoclonal antibodies which have been well characterized for their binding to the AD-2 epitope show essentially identical reactivity patterns (Meyer *et al.*, 1992; Ohlin *et al.*, 1993), it appears that a polyclonal response can recognize the AD-2 epitope in different ways (Silvestri *et al.*, 1993). Since the gB antigen is also a leading CMV vaccine candidate (Spaete *et al.*, 1994), an understanding of antibody recognition of the most important neutralization epitopes is crucial to evaluate the benefits of an antibody response *in vivo*. Furthermore, development of antibodies with improved affinity (in particular reduced dissociation rate constant)

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Abbreviations: CDR, complementarity-determining region, CMV, cytomegalovirus, ELISA, enzyme-linked immunosorbent assay, FR, framework region, gB, CMV glycoprotein B, k_{diss} , dissociation rate constant, PBMC, peripheral blood mononuclear cells, scFv, single chain variable region fragment (V H -linker-V κ), SPR, surface plasmon resonance.

and fine specificity would permit the development of more effective therapeutic modalities.

MATERIALS AND METHODS

Antibodies, antigens and phagemid vectors

The hybridoma producing the neutralizing, human IgG1 antibody ITC88 (Ohlin *et al.*, 1993; Silvestri *et al.*, 1993) specific for the AD-2 epitope of CMV gB was used in this investigation. The antibody produced by this cell line has been shown to recognize recombinant gB with a $K_a = 2 \times 10^9 \text{ M}^{-1}$ (Ohlin *et al.*, 1993). The cell line was cultured in RPMI 1640 supplemented with 10% fetal calf serum, 4 mM glutamine, 1% non-essential amino acids and 50 µg gentamycin/ml. Antibody was obtained in tissue culture supernatant and was used directly or following purification on a protein A column.

Proteins and peptides related to CMV gB all originated from the AD169 strain (Cranage *et al.*, 1986). The recombinant protein HM90-5 consists of CMV gB residues 28–101 fused to a β -galactosidase fragment (aa 1–375) (Meyer *et al.*, 1990). The synthetic peptide T7-13 (CMV gB residues 67–86) and fragments thereof (Silvestri *et al.*, 1993) were prepared by solid phase synthesis (Sällberg *et al.*, 1991). Synthetic peptides derived from the sequence of HIV-1 gp120 (Broliden *et al.*, 1990, 1991) were kindly provided by B. Wahren (Swedish Institute for Infectious Disease Control, Stockholm, Sweden).

The phagemid vector pEXmide4 (engineered by Dr J. Sundelin, Lund, Sweden) was obtained from pEXmide3 (Söderlind *et al.*, 1993) by removal of the *NotI/EcoRI* fragment, containing an antibody light chain-encoding sequence. This vector allows cloning of scFv-encoding gene fragments. The scFv may be produced either as a soluble product or fused to a C-terminal fragment of bacteriophage M13 coat protein3.

Antibody variable region genes

The mRNA was prepared from the ITC88 hybridoma cell line, using an ultracentrifugation technique as previously described (Larrick *et al.*, 1989). Similarly, mRNA was extracted from bone-marrow derived cells or from pokeweed mitogen-activated (42 hr stimulation period, using 2.5 µg PWM/ml) peripheral blood mononuclear cells (PBMC) obtained from CMV-seropositive healthy donors (Lund University Hospital, Lund, Sweden), using the RNagents Total RNA Isolation Kit (Promega, Madison, WI, U.S.A.). The cDNA was prepared from these lymphocyte-derived sources, as described (Larrick *et al.*, 1989) or using the Reverse Transcription System (Promega) from 10–15 µg total RNA with primers annealing to constant domains of the heavy and kappa light chains. The cDNA synthesis of RNA derived from hybridoma cells was primed with oligo-dT.

Polymerase chain reaction (PCR) and assembly of genes encoding scFv

The PCR amplification of cDNA and subsequent cloning of the relevant variable region genes used by the

ITC88 hybridoma was conducted as previously described, using primers annealing to signal peptide/constant region-encoding sequences (Ohlin *et al.*, 1994). The PCR of variable region genes for incorporation into scFv-encoding constructs was conducted by standard procedures using primers specific for FR1 and FR4 sequences (Table 1) (denaturation: 1 min, 94°C; annealing: 1 min, 55°C; extension: 3 min, 72°C). The use of these primers resulted in a few amino acid substitutions in heavy chain framework regions (leu²→val² and glu⁶→gln⁶ in scFv; gln¹→glu¹, leu²→val² and ser¹⁰⁷→thr¹⁰⁷ in Fab) in comparison to the original hybridoma sequence. For the amplification of light chain libraries, relevant mixtures of primers specific for different variable gene families (Table 1) were used in equimolar amounts. The amplified VH and VL sequences were mixed and extended using each other as templates. The complete VH-linker-VL sequences were finally amplified with primers specific for the terminal parts of the original 5'-VH and 3'-VL primers. The final product was purified following electrophoresis in a low-melting agarose gel using Magic PCR Preps DNA Purification System (Promega). Following cloning in an expression vector these constructs will produce scFv carrying the previously described (gly₄ser)₃-linker (Huston *et al.*, 1988).

Cloning of antibody fragment-encoding genes

The PCR amplified scFv genes were cleaved with *NcoI* and *SalI* (Boehringer Mannheim) and the purified fragments were ligated at an 8:1 ratio to cleaved and purified pEXmide4. Ligated material was transformed into electrocompetent *E. coli* SURE (Stratagene, La Jolla, CA, U.S.A.) essentially as described (Potter, 1993). Titration of the ampicillin-resistant clones indicated that libraries with approximately 2×10^6 members were achieved. Bacteriophage stock solutions were prepared following superinfection with VCSM13 helper phage (Stratagene) by culture in the presence of 0.1 mM IPTG at 30°C overnight.

In order to allow bacterial expression of some specificities as Fab, the variable region genes of some specific scFv were cloned into the pEXmide3 vector (Söderlind *et al.*, 1993), using primers outlined in Table 1. Similarly, some scFv were produced with an N-terminal streptavidin-binding peptide sequence (Strep-tag) (Schmidt and Skerra, 1993) to facilitate their use in ELISA.

Panning of antibody-displaying phages

Antigen (HM90-5) in PBS containing 6 M urea was coated onto polystyrene microtitre plates over night at room temperature. Antigen-specific phages were purified on this matrix, recovered and used to prepare new phage-stock preparations essentially as previously described (Rosenblum and Barbas, 1995). The panning procedure was repeated once or twice prior to analysis of recovered clones. Clones producing HM90-5-specific scFv were identified by surface plasmon resonance (SPR)-analysis (see below) or by ELISA (Ohlin and Borrebaeck, 1993;

Table 1. Primers used for the amplification of antibody variable region genes with the purpose of preparing scFv- or Fab-encoding fragments for cloning in the pEXmide4 or pEXmide3 phagemid vectors, respectively^a

Primer	Sequence
VH-FR1 (<i>NcoI</i>) (scFv)	5'-GTCCTCGAACTGCCCATGCCAGGTGACGCTGGTGCAGTCTGG-3'
VH-FR1 (<i>NcoI</i>) (Fab)	5'-GTCCTCGCAACTGCCCATGCCAGGTGACGCTGGTGCAGTCTGG-3'
VH-FR4 (<i>scFv</i>)	5'-CCGCCGGATCCACCTCCGCTGAACCCCTCCACCTGAGGAGACGGTGAC-3'
VH-FR4 (<i>Apal</i>) (Fab)	5'-GAGTCATTCTCGACTGGGCCCTTGGTACCGGCTGAGGAGACGGTGACCGGGTTCC-3'
Vκ I-FR1 (scFv)	5'-GGAGGCGGTTCAAGCGGAGGTGGATCCGGCGGTGGCGGATCGGACATCCAGATGACCCAGTCTCC-3'
Vκ III-FR1 (scFv)	5'-GGAGGCGGTTCAAGCGGAGGTGGATCCGGCGGTGGCGGATCGGAAATTGTTGACGCGAGTCTCC-3'
Vκ III-FR1 (<i>NotI</i>) (Fab)	5'-GTCCTCGCAACTGCAGCGGCCGACGCGATGGCCGAAATTGTTGACGCGAGTCTCC-3'
Vκ-FR4 (<i>SaII</i>) (scFv)	5'-GAGTCATTCTCGACTGCTAGTCGACCTAACGTTTGAATCTCCACCTTGGTCCC-3'
Vκ-FR4 (<i>SaII</i>) (scFv/Strep-tag)	5'-GAGTCATTCTCGACTGCTAGTCGACCTAACCCACCACTCGGGGTGACGCCAAGCGCTGCCTGCACGTTTGAATCTCCACCTTGGTCCC-3'
Vκ-FR4 (<i>NheI</i>) (Fab)	5'-GAGTCATTCTCGACTGCTAGTCGACACAGTACGTTTGAATCTCCACCTTGGTCCC-3'
Reamplification 5'VH (scFv, Fab)	5'-GTCTCTCGCAACTGCCCCAT-3'
Reamplification 3'VH (Fab)	5'-GAGTCATTCTCGACTGGGCCCTT-3'
Reamplification 5'Vκ (Fab)	5'-GTCTCTCGCAACTGCAGCGG-3'
Reamplification 3'Vκ (scFv, Fab)	5'-GAGTCATTCTCGACTGCTA-3'

^aIncorporated cloning sites and the fragment type amplified with each primer are indicated in the primer name. Underlined sequences correspond to bases encoding FR1- and FR4-related structures. Nucleotides encoding the scFv linker are bold. Note that the coding region of Vκ in scFv is immediately followed by an amber stop codon. Although Vκ-FR1 primers were designed to primarily fit VκI and VκII, they have also been shown independently to amplify sequences belonging to the more rarely used VκII and VκIV protein subgroups (unpublished observations and M. Dueñas, personal communication).

Söderlind *et al.*, 1993) using a sheep-anti-M13 antiserum provided by Dr M. Hobart (MRC, Cambridge, U.K.).

Production of antibody fragments

Exponentially growing cultures of ampicillin-resistant clones (of SURE or of transformed *E. coli* strain Y1089) were induced with 1 mM IPTG and cultured overnight at 30°C. The antibody fragment-containing supernatant was recovered and concentrated and washed using ultrafiltration (cutoff: 10 kDa).

Sequence determination of variable region genes

Variable region genes derived from library-derived, purified (Magic Miniprep DNA Purification System, Promega) plasmids were sequenced in both the forward and reverse direction using the fmol DNA Sequencing System (Promega) and the CircumVent Thermal Cycle DNA Sequencing Kit (New England BioLabs, Beverly, MA, U.S.A.). Three independent clones of each of the original ITC88 hybridoma-derived genes were similarly sequenced. Determination of sequence similarities with known germline sequences (derived from the V BASE sequence directory (release 1) provided by Dr I. M. Tomlinson *et al.*, MRC Centre for Protein Engineering, Cambridge, U.K.) was performed using the GeneWorks computer software (IntelliGenetics Inc., Mountain View, CA, U.S.A.).

Evaluation of antibody fine specificity

Antibody specificity was evaluated by inhibition of the binding of antibody fragments to immobilized antigen with synthetic peptides, using SPR analysis (BIAcore™, Pharmacia Biosensor AB, Uppsala, Sweden), as described previously (Malmborg *et al.*, 1992). Briefly, HM90-5 (approximately 3000 resonance units) was immobilized on the N-hydroxy succinimide ester-activated surface as recommended by the manufacturer. Antibody fragments at a concentration giving less than maximum response were pre-incubated with various concentrations of soluble peptides prior to determination of the residual capacity to bind immobilized antigen. Peptides included the sequence covering the AD-2 epitope (residues 67–86) of CMV gB and fragments thereof, as well as control peptides derived from human immunodeficiency virus type 1 gp120. The cross-reactive properties of scFv carrying a Strep-tag were also evaluated in ELISA (Ohlin and Borrebaeck, 1993). Their reactivity against various proteins and nucleic acid preparations (obtained from Sigma Chemical Co., St Louis, MO, U.S.A.) in comparison to their binding to HM90-5 was thus investigated. Bound scFv was detected with peroxidase-labelled streptavidin (DAKO A/S, Glostrup, Denmark) using orthophenylenediamine as the chromogen.

RESULTS

The variable region genes encoding ITC88 IgG belong to the VHIII and VκIIIa families linked to JH4b

(Schroeder *et al.*, 1987) and Jκ4 (Hieter *et al.*, 1982) joining segments, respectively (Genome Sequence Data Base, accession codes L37310 and L37311). The most closely related V-region germline genes were hv3005f3 and b32g (both with 92% homology) (Olee *et al.*, 1991) and L6 (Vg) (97% homology) (Pech and Zachau, 1984), respectively. In addition, the D-segment of the heavy chain variable region showed homology to the DXP1 germline sequence (Ichihara *et al.*, 1988).

Since the specificity of many antibodies is believed mainly to be determined by the heavy chain variable region, we selected to combine the original VH-region gene with a library of new light chain sequences. Kappa light chain variable region genes were PCR-amplified from cDNA prepared from lymphocytes/plasma cells obtained from CMV-infected healthy individuals. These genes were shuffled with the heavy chain variable region of the AD-2 specific ITC88 antibody to allow production of scFv genes. A substantial enrichment of specific clones could be observed following three rounds of panning of one of the libraries (1/7 specific clones after the second round of panning; 7/8 specific clones after the third round of panning), containing PBMC-derived light chain genes. Additional specific clones (AC11, AC15 and AC18), using PBMC-derived light chain sequences, were identified using ELISA-based screening of phages expressing scFv. A specific clone (AG21) was also obtained from the library of bone-marrow derived κ chain variable region genes. Their specific reaction with the HM90-5 protein containing the AD-2 epitope was also confirmed by SPR-based analysis.

Sequence analysis of the new clones (obtained after three rounds of selection) showed that all the light chains were exclusively members of the VκIIIa germline family, although they differed from the original ITC88 Vκ in various positions. Just as the original ITC88 Vκ, these sequences were linked to a Jκ4 sequence (Fig. 1). This high frequency of VκIIIa/Jκ4 did not, however, reflect an over-representation of this variable region gene family in the original library. Thus, among 10 randomly selected clones in this library, VκI, VκIIIa and VκIIIb sequences were represented by five, one and four members, respectively. Similarly, only 1/10 clones in the unselected repertoire used Jκ4 (7/10 and 2/10 used Jκ1 and Jκ2, respectively).

The expressed antibody fragments were evaluated regarding their fine specificity. The binding to the recombinant protein HM90-5 could be specifically blocked by a synthetic peptide (residues 67–86) representing the AD-2 epitope. This inhibition was specific since none of five investigated HIV-1 gp120-related control peptides had any such inhibitory activity. Subsequent evaluation of the specificity of fragments AE11, AC33 and AG21, carrying a Strep-tag to facilitate detection in ELISA, showed that none of them recognized any of 11 unrelated antigens (Fig. 2). Since short, synthetic peptides were able to mimic the structure of the native, neutralization-inducing epitope, a detailed evaluation of the fine specificity of the various fragments was conducted. The reactivity of all investigated scFv was inhibited by a peptide covering

Clone	Light chain amino acid sequence													
	10	20	30	a	40	50	60							
ITC88/AE11 (scFv)	EIVLTQSPAT	LSLSPGERAT	LSCRASQSVG	G-YLAWYQQKP	GOAPRLIIYD	ASNRAAGIPS								
L6 (germline)			S	S-.....		T...A								
AC11 (scFv)		T....N	S-R.V.....	L..	T...A									
AC15 (scFv)			S-S.....	V..	T....T...A									
AC18 (scFv)		S	-.....		TF...T...A									
AC31 (scFv)	D.QM.....	M.....	N-	L..	T...A									
AC32, AC34, AC38 (scFv)			S	S-.....	T...A									
AC33, AC36 (scFv)			S-S	V..	T....T...A									
AC37 (scFv)			S	S-.....	T.T.A									
AC41 (scFv)	D.QM....G.		S	SS.....	G	..S..T...D								
AG21 (scFv)	D.QM.....		S	S-.....	T...A									
AG21B (scFv)			S	S-.....	T...A									
***** *****														
Clone	Light chain amino acid sequence										*****			
											Homology	Homology	JK-	
											to L6	to ITC88	family	
											(DNA)	(protein)		
	70	80	90	a	100									
ITC88 / AE11 (scFv)	RFSGSGSGTD	FTLTISLAP	EDFAVYFCQQ	RSSWPPLTFGG	GTKVEIK	97.2					JK4			
L6 (germline)	E.	Y...	..N..-----	-----										
AC11 (scFv)	E.	Y...	..NN..-....A		96.1 %	87 %					JK4			
AC15 (scFv)	..N.....	E.	D.Y...	..E.-.....	96.1 %	89 %					JK4			
AC18 (scFv)	E.	Y...	..Y.....		96.1 %	93 %					JK4			
AC31 (scFv)	E.	Y...	..YN.....		95.8 %	89 %					JK4			
AC32, AC34, AC38 (scFv)	E.	Y...	..N.....		99.6 %	94 %					JK4			
AC33, AC36 (scFv)	E.	D.Y...	..E.-.....		96.5 %	90 %					JK4			
AC37 (scFv)	E.	Y...			98.2 %	94 %					JK4			
AC41 (scFv)	R.E.	Y...	..YGSS.-G...Q		91.3 %	81 %					JK1			
AG21 (scFv)	E.	Y...	..N..-.....		97.9 %	90 %					JK4			
AG21B (scFv)	E.	Y...	..N..-.....		100.0 %	93 %					JK4			
** *****														

Fig. 1. The V_{κ} sequences selected following panning on HM90-5 antigen of phages displaying scFv carrying the ITC88-derived VH segment. Clones beginning with AC and AG use PBMC- and bone marrow-derived light chain variable region sequences, respectively. Dots indicate identity with the ITC88 V_{κ} sequence, and dashes indicate absent residues. The CDR regions are indicated by stars. The differences observed in three of the first four positions of AC31, AC41 and AG21 V_{κ} sequences probably originate from the primer (V_{κ} I related) which had been used to amplify these particular V_{κ} genes (AG21B is a modified version of AG21 using the normal V_{κ} IIIa-related FR1 sequence). The light chain of the only clone (AC41) isolated following two rounds of panning belonged to the V_{κ} IIIb subgroup (288 bp) while all other clones used V_{κ} IIIa light chain variable regions (285 bp). The per cent homology to the L6 germline nucleotide sequence and the ITC88 light chain variable domain is shown as is the proposed J κ genes used by the various constructs. In the case of AC31, AC41 and AG21 scFv, the homology values are somewhat low as a consequence of the primer-associated differences affecting FR1 described above. Numbering of residues follows the conventions established by Kabat *et al.* (1991) with the exception that the additional residue found in the V_{κ} IIIb-related sequence AC41 was introduced at position 31^a in order to optimize the alignment. The nucleotide sequences of scFv light chain variable region genes are available in the Genome Sequence Data Base under accession codes L37300–L37309 and L37311.

residues 67–86 of gB, but some shorter peptides also blocked the antigen-specific reactivity [Fig. 3(A)–(E)]. This agrees with the peptide reactivity of the original ITC88 antibody (14, 16). However, as shown by titration experiments, the relative ability of shorter fragments of the AD-2 epitope to inhibit scFv binding to immobilized

antigen differed substantially between the different scFv [Fig. 3(F)–(J)]. In particular, AC15 and AC36 scFv showed a much reduced reactivity to some of the decapeptides despite the fact that the residue 67–86 peptide had similar inhibitory potency against these fragments in comparison to the originally hybridoma-derived

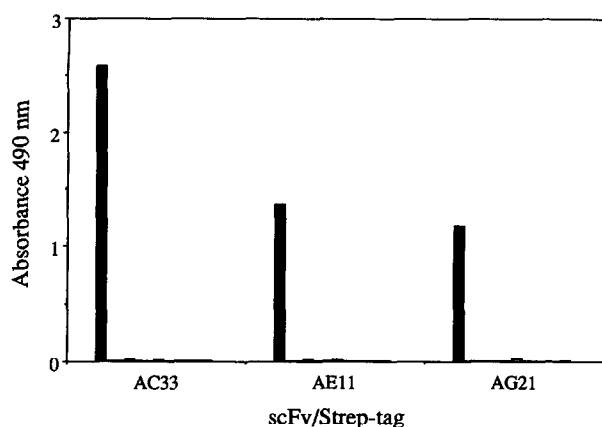


Fig. 2. The ELISA evaluation of cross-reactivity of Strep-tag-carrying scFv AC33, AE11 and AG21 against (columns from left to right) HM90-5 (recombinant protein expressing the CMV AD-2 epitope of gB), human transferrin, keyhole limpet hemocyanin, acid-soluble collagen, bovine insulin, human serum albumin, calf thymus histones, ovalbumin, bovine serum albumin, porcine thyroglobulin, double-stranded DNA and single-stranded DNA.

sequence (AE11). Some other scFv also exhibited modified relative reactivity with the various peptides investigated, indicating a drift in fine epitope recognition induced by the chain-shuffling procedure.

It was observed that only some chain-shuffled scFv recognizing the AD-2 epitope retained residue pro^{95a} which was found in ITC88 V κ (Fig. 1), but which is normally found only in a minority of all κ chains. This residue is obviously not required for maintaining a functional binding site in this antigen-antibody system, although its presence might affect the fine specificity. AC38 and AG21, which differ in fine specificity to the AD-2 epitope (Fig. 3) only differ in three of the first four residues of V κ FR1 and in the presence of pro^{95a} in AC38. In order to investigate the importance of this additional residue on the fine specificity, we replaced the modified, primer-encoded amino acids in FR1 of AG21 with those found in most V κ IIIa-encoded light chains, including AC38 and AE11. It was subsequently shown by SPR analysis that the fine specificity profile of this new scFv (AG21B; Fig. 1), as determined by the relative potency of the inhibitory peptides, was more similar to those displayed by the AC38 and AE11 scFv than to AG21 scFv (data not shown). The relative difference in reactivity to the different AD-2-related peptides observed between AC38 and AG21 was thus not dependent on the residue pro^{95a}, but rather seemed to depend on residues found in FR1.

DISCUSSION

We have studied chain-shuffled scFv derived from the ITC88 antibody specific for the AD-2 epitope of CMV gB and involved in virus neutralization. The new AD-2-binding scFv were obtained by combining the ITC88 heavy chain variable region gene with libraries of κ chain variable region genes. These clones selectively retained light chain variable domains belonging to the V κ IIIa/J κ 4

subgroups, which were highly homologous to the light chain used by the original antibody. Such a high degree of representation of V κ IIIa/J κ 4 in phage library-selected clones is generally not observed when selecting specific fragments by phage-display technology (see e.g. Griffiths *et al.* (1993)). Thus, it seemed that this type of light chain variable region structure was required to recreate the functional binding site of this high affinity antibody. This is also supported by the finding that the only, but weakly reactive scFv (AC41) identified after two rounds of selection used a V κ IIIb-related variable region gene (a subgroup highly represented in the original library). This type of clone was, however, not found following one additional round of panning, despite the fact that V κ IIIb genes are quite similar to the L6 (V κ IIIa) germline gene. Our observations agree with a previous study, which suggested that some heavy chains prefer to pair with similar light chain variable regions to form high affinity binders (Barbas *et al.*, 1993). Similarly, thyroid peroxidase-specific autoantibodies using particular heavy chain variable regions were substantially restricted in light chain variable region gene usage in order to create a functional binding site (Jaume *et al.*, 1994). Together these studies support the fact that light chain promiscuity is restricted in the case of many high affinity antibodies. Furthermore, we conclude that certain specific clones retrieved from combinatorial libraries may be under-represented in sequences exhibiting limited light chain promiscuity, since such heavy chains are less likely to encounter a suitable light chain counterpart in the construction of the library.

When investigating the various scFv obtained from our recombinatorial, light-chain shuffled library, it was evident that the new light chains affected only the fine specificity of the antibody fragments, without modifying the overall specificity as determined by binding to the AD-2 structure itself. Some of the new fragments also appeared to mimic the specificity against the AD-2 epitope (as determined by peptide reactivity) found among some polyclonal human sera which do not exactly match the specificity recognized by ITC88 (Silvestri *et al.*, 1993). Thus, this neutralization epitope can give rise to an array of antibodies exhibiting different fine specificities *in vivo* as well as by the chain-shuffling process used here. However, these findings also suggest that using recombinatorial library approaches one may identify fine specificities that do not match that of the original antibody. Although this technology is frequently used for investigating *in vivo*-developed repertoires [for review see e.g. Rapoport *et al.* (1995)], these results demonstrate the difficulties in interpreting such studies both with respect to, for example, light chain promiscuity and fine specificity. However, the observation that all clones binding the recombinant HM90-5 antigen retained an overall specificity for the AD-2 epitope suggests that a heavy chain variable sequence does not greatly modify its overall specificity as a consequence of the light chain-shuffling procedure. Consequently, in the case of this antibody, a combinatorial library approach would have provided a approximate but not exact mirror of the specificity developed *in vivo*.

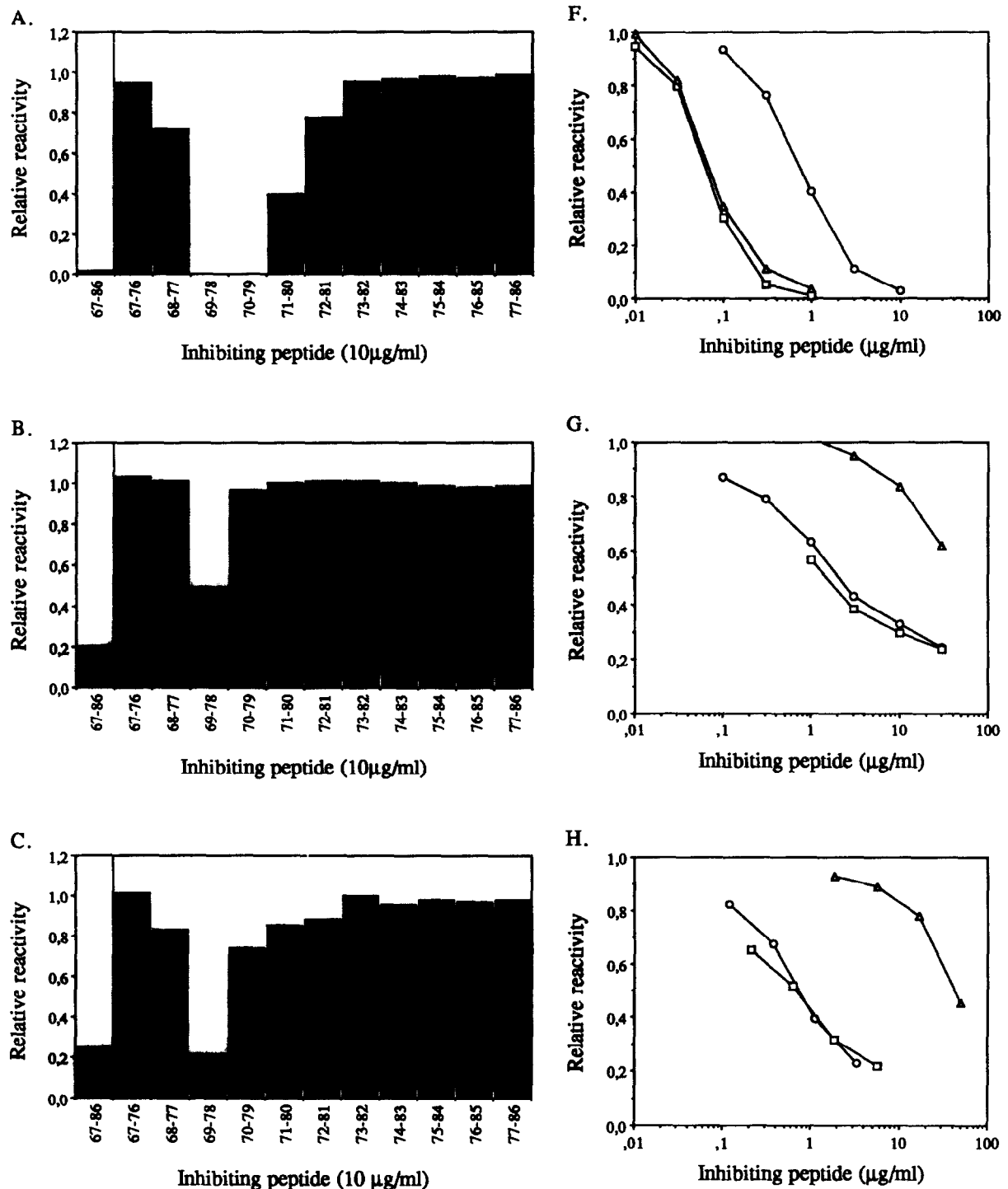


Fig. 3. The SPR-based analysis of the inhibition of the binding of antibody fragments to immobilized antigen (HM90-5) in the presence of competing soluble peptides derived from the AD-2 epitope of CMV gB. The inhibition of (A) AE11 (scFv with the original ITC88 V κ sequence), (B) AC15, (C) AC18, (D) AC37 and (E) AG21 in the presence of peptides (10 µg/ml) displaying different parts of the entire epitope are shown to identify the sequences that were mainly responsible for antigen binding of each scFv. Titration of the blocking activities of the peptides T7-13 (aa 67–86) (○), T7-13:3 (aa 69–78) (□) and T7-13:4 (aa 70–79) (△) (each expressing sequences recognized by the original antibody ITC88) against (F) AE11, (G) AC15, (H) AC18, (I) AC37 and (J) AG21 is also shown scFv. The AC36 and AC38 (figures on p. 54) behaved similarly to AC33 and AC37, respectively (data not shown).

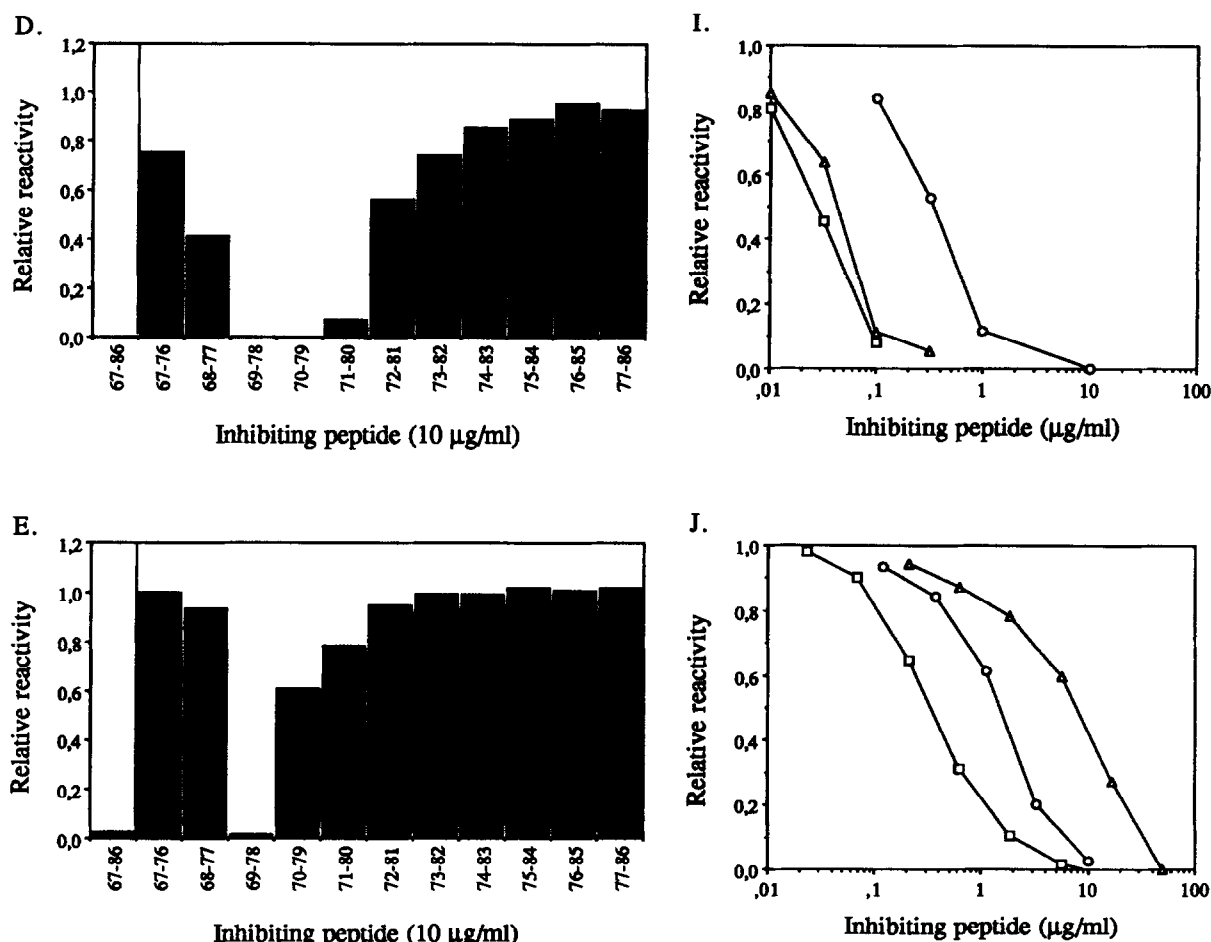


Fig. 3—continued.

As shown above, the selection of functional light chain fragments was not random, but rather suggested that a proper binding site required the presence of particular light chain structures. It seems very likely that the light chain of this antibody played an important role in the recognition of the antigen, in particular since small differences in light chain sequence influenced the absolute fine specificity of the interaction. Many of these differences were located in the parts of the CDR which are believed to influence the specificity (Padlan *et al.*, 1995). As expected, scFv with highly similar light chain protein sequences (such as AC15/AC36 or AC37/AC38 exhibiting 99% and 98% homology at the amino acid level of their variable domains, respectively) also appeared to have very similar fine specificities. In our case, scFv carrying light chain variable domains most homologous (e.g. AC37 and AC38) to the original clone also retained a similar relationship of reactivity between the investigated peptides, while less similar clones (e.g. AC15 and AC36) exhibited a modified reactivity pattern.

The effect of these light chain modifications on the biological activity (virus neutralization) of the scFv is being investigated further and they may be used to determine the importance of different recognition patterns as described previously by Silvestri *et al.* (1993) for virus neutralization. Such studies will also provide information on the importance of dissociation rate on this protective activity against CMV as previously reported for HIV-1-

specific antibodies (VanCott *et al.*, 1994). This comparison is particularly valid, since both the AD-2 epitope of CMV gB and the V3-epitope detected by the antibodies described by VanCott *et al.* (1994) are believed to be involved not in virus attachment as such, but rather later events of the infectivity process (Ohizumi *et al.*, 1992; Skinner *et al.*, 1988). It currently appears that it will be necessary to develop bivalent binding units to create a neutralizing activity for the AD-2 specificity, since no neutralizing activity has been observed even with the AE11 scFv carrying the original ITC88 light chain (Dr V.-A. Sundqvist, unpublished observations). Such dependence on antibody di- or multivalency for efficient viral neutralization would agree with similar observations of neutralizing antibodies of other specificities (Cavacini *et al.*, 1994).

In conclusion, we have shown by light chain-shuffling of a high affinity antibody established *in vivo* that the light chain variable regions capable of recreating a functional binding site in this case was very restricted to sequences closely resembling the sequence used by the original antibody. It was evident, furthermore, that minor modifications of the light chain sequences in this case changed the fine specificity without introducing unwanted multi-reactive properties to the scFv. Consequently, it may be suggested that in some cases it will be possible by this technology, for example, to reduce virus isolate restriction of antibodies occurring as a consequence of binding

to variable residues located in or in close proximity of conserved epitopes. Finally, it was evident that the recognition of the neutralization-inducing AD-2 epitope may vary between different specific antibodies and a further evaluation of the biological importance of such minor differences in specificity is warranted.

Acknowledgements—This study was supported by a grant from NUTEK. Information on the neutralizing activity of scFv was kindly provided by Dr Vivi-Anne Sundqvist. Useful suggestions by Ann Catrin Simonsson Lagerkvist are gratefully acknowledged.

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