The EMBO Journal vol.12 no.2 pp.725-734, 1993

Human anti-self antibodies with high specificity from phage display libraries

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Communicated by G.Winter

Recently we demonstrated that human antibody fragments with binding activities against foreign antigens can be isolated from repertoires of rearranged V-genes derived from the mRNA of peripheral blood lymphocytes (PBLs) from unimmunized humans. The heavy and light chain V-genes were shuffled at random and cloned for display as single-chain Fv (scFv) fragments on the surface of filamentous phage, and the fragments selected by binding of the phage to antigen. Here we show that from the same phage library we can make scFv fragments encoded by both unmutated and mutated V-genes, with high specificities of binding to human self-antigens. Several of the affinity purified scFv fragments were shown to be a mixture of monomers and dimers in solution by FPLC gel filtration and the binding kinetics of the dimers were determined using surface plasmon resonance ($k_{on} = 10^5-10^6\ M^{-1}s^{-1}$, $k_{off} = 10^{-2}\ s^{-1}$ and $K_a = 10^7\ M^{-1}$). The kinetics of association are typical of known Ab-protein interactions, but the kinetics of dissociation are relatively fast. For therapeutic application, the binding affinities of such antibodies could be improved *in vitro* by mutation and selection for slower dissociation kinetics.

Key words: human antibodies/phage display/self

Introduction

Human monoclonal antibodies (mAbs) have huge potential for therapy, but are difficult to make by immortalizing B-lymphocytes (for reviews see James and Bell, 1987; Winter and Milstein, 1991). Furthermore, it is especially difficult to generate human mAbs directed against human antigens (anti-self antibodies), for example antibodies against soluble TNF to block septic shock (Spooner *et al.*, 1992), against membrane bound carcinoembryonic antigen to image colorectal carcinoma (Mach *et al.*, 1980) or against lymphocyte antigens to destroy tumour in lymphoma (Hale *et al.*, 1988). This difficulty results from immunological tolerance mechanisms that prevent the antigen-driven expansion of B-cell clones with self specificities (Burnet, 1959; Nossal, 1989). After antibody gene rearrangement, virgin B-cells may display antibodies with self-reactivity, but tolerance mechanisms can lead to their deletion (Nossal, 1989; Nemazee *et al.*, 1991; Russell *et al.*, 1991) or to their anergy (Nossal, 1989; Basten *et al.*, 1991; Erikson *et al.*, 1991). It has been suggested that cells may be anergized if the antigen is soluble, but deleted if the antigen is membrane bound (Hartley *et al.*, 1991). B-cell tolerance does not seem to occur when concentrations of soluble antigen are low (in contrast to T-cell tolerance) and B-cells with poor affinities for antigen are not tolerized, even at higher antigen concentrations (Adelstein *et al.*, 1991). Such non-tolerized B-cells are not usually expanded because they lack T-cell help (Bretscher and Cohn, 1970; Adelstein *et al.*, 1991), although proliferation can be induced artificially by using polyclonal B-cell activators (reviewed in Nossal, 1987).

It is estimated that 10-30% of B-lymphocytes in normal, healthy individuals are engaged in making autoantibodies (Cohen and Cooke, 1986). However, the 'natural autoantibodies' produced do not lend themselves to therapeutic use as they are often IgM, low affinity and polyreactive (see Nakamura *et al.*, 1988; Tomer and Schoenfeld, 1988; Casali and Notkins, 1989; Rossi *et al.*, 1990; Avrameas, 1991). An immune response against self can arise in autoimmune disease (see Smith and Steinberg, 1983) or after infections (see Bona, 1988) and a few human mAbs directed against self-antigens have been isolated from patients with active autoimmune disease (see James and Bell, 1987). These autoantibodies are frequently specific, but may bind to only a restricted range of epitopes on the antigen (see Bouanani *et al.*, 1991).

Recently monoclonal antibody fragments have been generated and expressed in bacteria using phage antibody technology (McCafferty *et al.*, 1990) by cloning repertoires of V-genes into filamentous bacteriophage and selecting the recombinant phage with antigen (for review, see Hoogenboom *et al.*, 1992). The repertoires comprised random combinatorial libraries (Huse *et al.*, 1989) of the rearranged heavy and light chain V-genes of immunized animals or human donors. Immunization leads to clonal expansion and production of mRNA by plasma cells: as a result, derived V-gene repertoires are enriched for sequences of heavy and light chains encoding part of an antigen binding site (Hawkins and Winter, 1992). The selected antibody fragments can have good affinities for antigen, for example at least $10^8\ M^{-1}$ for the hapten phOx (Clackson *et al.*, 1991). However, because it is difficult to raise an immune response to self-antigens, we have sought to extend the technology to the generation of human antibodies without the use of immunization.

In principle, a range of binding specificities could be isolated from a single huge and diverse phage library by selection with either self or foreign antigens (for review, see

Table I. Frequency of binding clones isolated from the unimmunized scFv library after selection

Antigen	Rounds of selection					Number of unique clones
	1	2	3	4	5	
Thyroglobulin (bovine)	–	–	18/40	–	–	12
Thyroglobulin (human): selected on bovine	–	–	10/40	–	–	4
Fog1 (human IgGlκ antibody)	–	–	–	94/96	–	4
TNFα (human)	–	122/1920	83/192	92/96	–	7
CEA (human)		–	0/96	1/96	2/96	1
MUC1 (human): selected with peptide	–	–	–	0/96	2/96	1
rsCD4 (human)	–	–	–	–	8/96	1

The ratios indicate the frequency of binding clones after each round of selection. Phagemids were rescued with M13ΔgIII helper phage, except for the CEA, MUC1 and rsCD4 selections, where VCS-M13 helper phage was used.

Marks *et al.*, 1992a). To this end, from a large phage antibody library, we first isolated antibody fragments with a high specificity of binding to foreign antigens (turkey lysozyme, bovine serum albumin and the hapten phenyloxazolone) (Marks *et al.*, 1991) and with affinities (K_a) in the range 10^6-10^7 M^{-1}. Repertoires of rearranged heavy and light chain V-genes were provided by PCR amplification from the μ, κ and λ mRNA of peripheral blood lymphocytes from unimmunized, healthy human donors. The V-genes were assembled (Clackson *et al.*, 1991) at random (Huse *et al.*, 1989) to encode repertoires of single-chain Fv (scFv) fragments (Bird *et al.*, 1988; Huston *et al.*, 1988). The fragments were displayed on the surface of the filamentous bacteriophage (McCafferty *et al.*, 1990) by fusion to the minor coat protein pIII (Smith, 1985), and phage encoding scFv fragments with binding activities were selected by binding of the phage to antigen. On infection of bacteria with the selected phage (Hoogenboom *et al.*, 1991), soluble scFv fragments produced from individual clones by secretion into the bacterial periplasm (Glockshuber *et al.*, 1990) were screened for binding activity. We now demonstrate the use of the same phage library to isolate antibody fragments with high specificity against self-antigens.

Results

The selected human antibody fragments show high specificity against self-antigens

The unimmunized library was subjected to affinity enrichment on a range of antigens (see Materials and methods and Table I). After 2–5 rounds of selection, *Escherichia coli* cells were infected with eluted phage and antibody fragments produced by individual clones were screened for binding by ELISA. Phage selected with the 20 amino acid MUC1 peptide (Price *et al.*, 1990), which corresponds to a repeated motif in human MUC1 mucin (tumour-associated polymorphic epithelial mucin or PEM) (Gendler *et al.*, 1988; Gum *et al.*, 1990), were screened for binding to human PEM and hence bind to both peptide and the protein. The V-genes of clones with binding activities were sequenced and between 1–12 different clones identified for each antigen (Table I). The appearance of only low numbers of clones binding to CEA, PEM and human recombinant soluble CD4 (rsCD4), even after several rounds of selection, may reflect the use of VCS-M13 as helper phage (instead of M13ΔgIII helper used for the other antigens). Populations of phage(mid) particles produced by rescue with M13ΔgIII (which cannot produce pIII) have higher average avidities than those produced by rescue with VCS-M13 (where the wild-type pIII encoded by the helper phage can compete with scFv–pIII fusions).

The scFv fragments were then screened for binding to a panel of other protein antigens and were found to be highly specific. This is illustrated in Figure 1 with the three clones with strongest ELISA signals for bovine thyroglobulin, human TNFα and the human mAb Fog-1, and in Figure 2 with the single clones with binding activity to human CEA, MUC1 and human rsCD4. However for a few clones with poor ELISA signals on the target antigen, we found signals with some of the other proteins of the panel (not shown).

The antibody fragments are derived from a range of unmutated and somatically mutated V-genes

The sequences of several clones with self-specificity are given in Table II and contain both kappa and lambda light chains (Table III). Comparison with the sequences of the nearest germline V-gene segments indicates that several different families are used (VH1, 3, 4 and 5; Vκ1 and 4, Vλ1, 2 and 3). In a few cases the V-genes are completely germline, for example both the VH and Vλ genes of αThy-29. However, most of the V-genes have several differences from the nearest germline V-gene segments, both at the nucleotide and amino acid level (Table III), suggesting that they are derived from somatically mutated B-cells (Berek and Milstein, 1987). Some mutations may have arisen during the PCR amplification and assembly process, for example the VH-genes of αFOG1-G8 and αMUC1-1, and the Vκ-gene of αThy-33 probably arose from cross-overs between two V-genes during PCR amplification (Table III). Furthermore, large differences (for example the Vκ of αFOG1-H6, which differs by 36 nucleotides) may be due to the use of unknown V-gene segments. There is a striking homology in the CDR3 of the heavy chain between αTNF-A1 and αTNF-E1: the germline V-genes are different but the same JH segments are used and 11 out of 16 residues of CDR3 are identical. This suggests that both scFv fragments may bind to the same epitope of TNF.

The antibody fragments are directed to different epitopes on the same protein

The scFv fragments directed against bovine thyroglobulin were screened for binding to human thyroglobulin, which differs by only six single amino acid residues in the protomer (Malthiéry and Lissitzky, 1987). Four of the twelve clones (including αThy-29) bound to human thyroglobulin, whereas

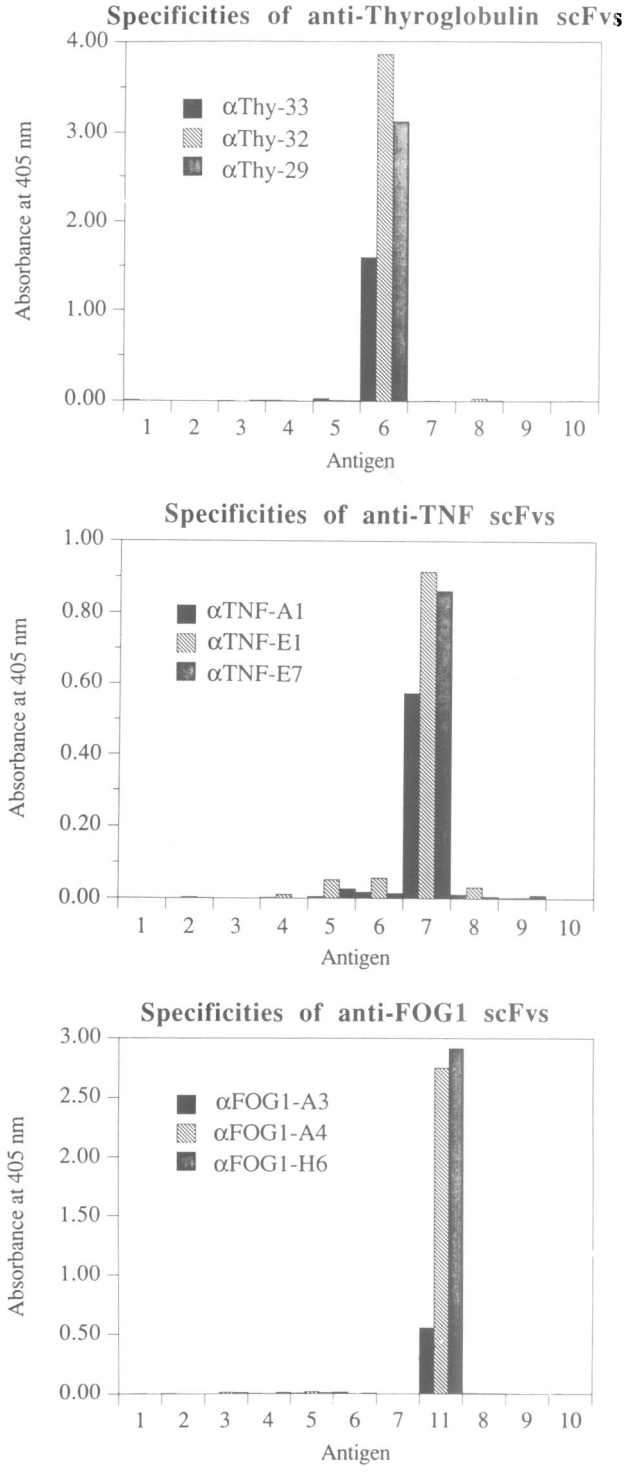

Fig. 1. Specificities of soluble single-chain Fvs (scFvs) isolated from the unimmunized library by selection on bovine thyroglobulin (**upper panel**), human TNFα (**centre panel**) or the human mAb Fog-1 (γ1,κ) (Melamed *et al.*, 1987) (**lower panel**). Binding was determined by ELISA to a panel of proteins: 1, plastic; 2, hen egg trypsin inhibitor; 3, chymotrypsinogen A; 4, hen egg ovalbumin; 5, keyhole limpet haemocyanin; 6, bovine thyroglobulin; 7, human TNFα; 8, turkey egg-white lysozyme; 9, horse heart cytochrome c; 10, bovine serum albumin; 11, mAb Fog-1.

the rest (including αThy-32 and αThy-33) did not (data not shown). Likewise the fragments binding to the human antibody Fog-1 were screened for binding to a range of other antibodies differing in heavy and light chain isotype (Figure 3). The fragment αFOG1-A4 bound to all heavy

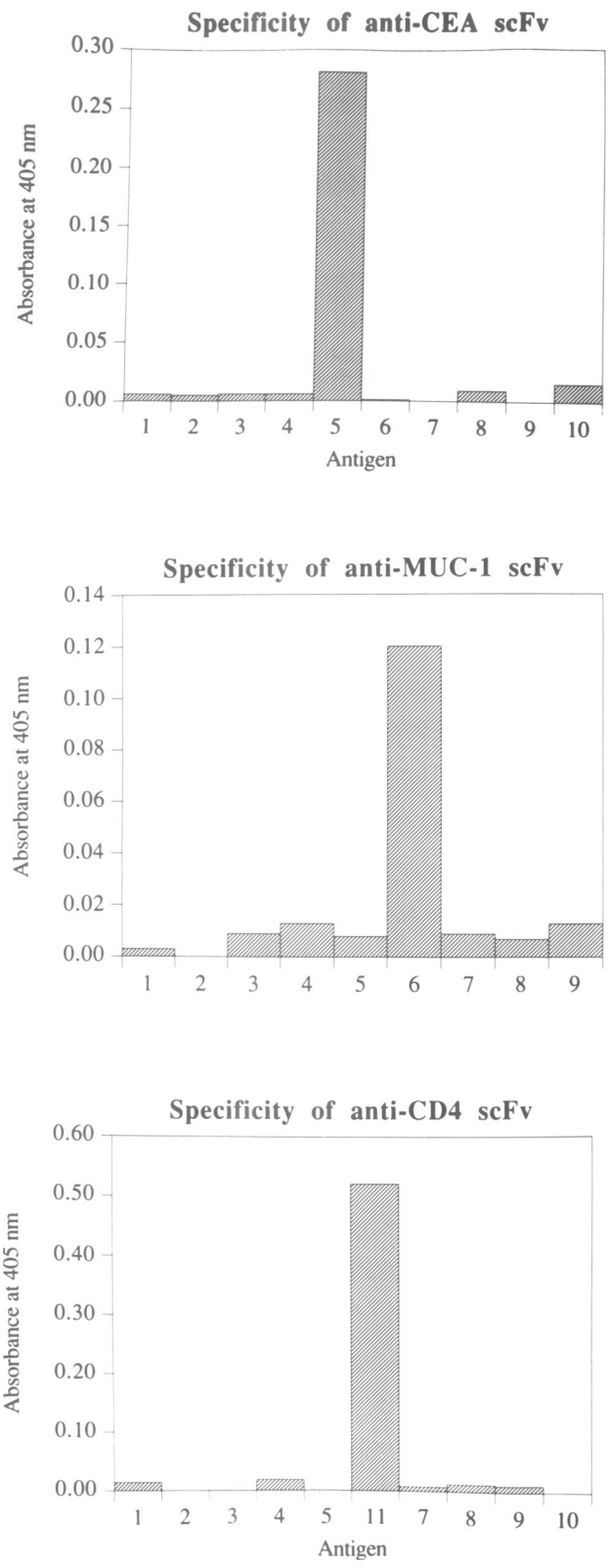

Fig. 2. Specificities of soluble single-chain Fvs (scFvs) isolated from the unimmunized library by selection on human CEA (**upper panel**), the MUC1 peptide (Price *et al.*, 1990) (**central panel**) or human CD4 (**lower panel**). Binding was determined by ELISA to a panel of proteins: 1, hen egg trypsin inhibitor; 2, chymotrypsinogen A; 3, hen egg ovalbumin; 4, keyhole limpet haemocyanin; 5, CEA; 6, urine extract containing human polymorphic epithelial mucin (PEM); 7, bovine thyroglobulin; 8, hen egg-white lysozyme; 9, bovine serum albumin; 10, chicken γ globulin coupled to 4-hydroxy-3-nitrophenyl acetic acid; 11, human recombinant soluble CD4.

Table II. Deduced protein sequences of several antigen-specific scFv fragments isolated from the unimmunized library

A. *Heavy chains*

scFv	FR 1	CDR 1	FR 2	CDR 2	FR 3	CDR 3	FR 4
αThy-23	QVQLQQSGGGVVQPGRSMRLSCAASGFNFR	SYGMH	WVRQAPGKGLEWVS	GISGSGGSTYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK	GSMIVVARYFDY	WGQGTLVTVSS
αThy-29	QVQLVQSGAEVKKPGASVKVSCKASGYTFT	SYGIS	WVRQAPGQGLEWMG	WISAYNGNTNYAQKLQG	RVTMTTDTSTSTAYMELRSLRSDDTAVYYCAA	DTGRIDDFWSGYNFDY	WGQGTLVT
αThy-32	QVQLVQSGGGLVQPGRSLRLSCAASGFTFD	DYAMH	WVRQAPGKGLEWVS	GISSNSGSIGYADSVKG	RFTISRDNAKNSLYLQMNSLRAEDTALYYCAR	GIAVAGAYYFDY	WGQGTLVTVSS
αThy-33	QVQLVQSGGGVVQPGGSLRLSCAASGLSIR	TNGMH	WVRQAPGKGLEWVA	AISYDGRSVYYADSVQG	RVTISRDNSKNTVHLQITSLKSEDTAVYYCAK	DSSSSWFLDS	WGQGTLV
αFOG1-A3	EVQLVESGGGLVQPGGSLRLSCAASGFTFS	SYWMS	WVRQAPGKGLEWVA	NIKQDGSEKYYVDSVKG	RFTISRDNAKNTLYLQMNSLRDEDTAVYYCAK	NPRDSGSYYYFDY	WGQ
αFOG1-A4	QVQLQESGGSVVQPGRSLRLSCAASGFTFS	NYAIH	WVRQAPGKGLEWVA	VISYDGGYEYYADSVKG	RSTISRDNSKNTLYLQMNSLRAEDTAVYYCAR	DASVHTAPYYYMDV	WGK
αFOG1-H6	QVQLQQSGGSLVQPGGSLRLSCAASGFTFS	NSGMN	WVRQAPGKGLEWVS	YISSSSSTIYYADSVKG	RFTISRDNAKNSLYLQMNSLRDEDTAVYYCAR	EEGGLMDV	WGKG
αFOG1-G8	QVQLQESGAGLLKPSETLSLTCAVYGGSFS	GYYWIG	WVRQMPGKGLEWMG	IINPGDSDTRYSPSFQG	QVTISVDKSVSTAYLQWSSLKPSDSAVYFCAR	HDVGYCSSPNCAKRPEYFQH	WGQ
αTNF-A1	EVQLVESGGGLVQPGGSLRLSCAASGFTFS	SYGMH	WVLQAPGKGLEWVA	FIRYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCVR	EDHVITTGRYHYYMDV	WGK
αTNF-E1	QVQLQESGGGLVQPGGSLRLSCAASGLTFS	SYAMH	WVRQAPGKGLEWVA	VISYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMDNLRAEDTAVYYCVR	EDYVITSGFYYYHMDV	WGK
αTNF-E7	QVQLQESGAEVKKPGSSVKVSCKASGGTFS	SYAIS	WVRQAPGQGLEWMG	GIIPIFGTANYAQKFQG	RVTITADESTSTAYMELSSLRSEDTAVYYCAR	GPLRGYDYYYYYMDV	WGK
αTNF-H9G1	QVQLVQSGAEVKKTGSSVKVSCKTSGYTFT	YRYLH	WVRQAPGQALEWMG	WITPFNGNTNYAQKFQD	RVTITRDRSMSTAYMELSSLRSEDTAMYYCAR	SGDLYSGYED	WGQ
αCEA4-8A	QVQLQQSGAEVKKPGASVKVSCKASGYTFT	SYGIS	WVRQAPGQGLEWMG	WISAYNGNTNYAQKLQG	RVTMTTDTSTSTAYMELRSLRSDDTAVYYCAR	DSFGYCSSTSCPYYYYYMDV	WGKGTTVTVSS
αMUC1-1	QVQLVQSGAEVKKPGASVKVSCKASGYTFT	GYYMH	WVRQAPGQGLEWMG	WINPNSGGTNYAQKFQG	RVTITRDTSASTAYMELSSLRSEDTAVYYCAR	DFLSGYLDY	WGQGTLVTVSS
αCD4-74	QVQLQQSGAEMKKPGESLKISCKGFGYDFS	TYWIG	WVRQMPGKGLEYMG	LIYPGDSDTKYSPSFQG	QVTISADKSISTAYLQWSSLKASDTAMYYCAR	VSGYCSSTSSYDYYYYYMDV	WG

B. *Light chains*

scFv	FR 1	CDR 1	FR 2	CDR 2	FR 3	CDR 3	FR 4
αThy-23	DIQMTQSPSSLSASVGDSVTITC	QASQGIRNDLA	WYQQKPGKAPKLLIY	AASTLQS	GVPSRFSGSGSGTEFTLTINGLQPEDFATYYC	QQLGAYPLT	FGGGTKLEIKR
αThy-29	SSELTQDPAVSVALGQTVRITC	QGDSLRSYYAS	WYQQKPGQAPVLVIY	GKNNRPS	GIPDRFSGSSSGNTASLTITGAQAEDEADYYC	NSRDSSGNLYVV	FGGGTKLTVLG
αThy-32	QSVLTQPPSVSGAPGQRVTISC	TGSSNIGAGYDVH	WYQQLPGAAPKLLIY	GNSNRPS	GVPDRFSGSKSGTSASLAITGLQAEDEADYYC	QSYDSSLSGWV	FGGGTKVTVLG
αThy-33	DVVMTQSPSTVSASVGDRVTLTC	RASQSISRWLA	WYQQKPGQPPKLLIY	WASTRKS	GVPDRFSGSGSGTDFTLTISSLQADDFATYYC	QHYDSFSPS	FGQGTKVEIK
αFOG1-A3	QSALTQPASVSGSPGQSITISC	TGTSSDVGGYNYVS	WYQQYPGKAPKLLIY	EVSKRPS	GVSNRFSGSKSGNTASLTISGLQTEDEADYFC	SAYAPTGIMM	FGGGTKLTVLG
αFOG1-A4	DIQMTQSPSSLSASVGDRVTITC	RASQGISNYLA	WYQQKPGKVPKLLIY	AASTLQS	GVPSRFSGSGSGTDFTLTISSLQPEDVAVYYC	QQYYSTPT	FGQGTKVEIKR
αFOG1-H6	DIQMTQSPSTLSASIGDRVTITC	RASQNIDSWLA	WFQHKPGKAPKPLIY	GAFTLQN	GVPSRFSGSGSGTEFSLTISSLQPDDFATYFC	QQAHSFPPT	FGGGTRLEIKR
αFOG1-G8	DIQMTQSPSTLSASIGDRVTITC	RASQSIGLWLA	WYQQKPGKAPKLLIY	AASTLQT	GVPSTFSGSGSGTEFTLTISSLRPEDFATYYC	QQLISYPLT	FGGGTKVEIKR
αTNF-A1	DIQMTQSPSSLSASVGDRVTITC	RESQGIRNDLG	WYQQKPGKAPKLLIY	GTSSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQTTSFPLT	FGGGTKLEIK
αTNF-E1	EIVLTQSPSSVSASVGDRVTITC	RASQGIRSWLA	WYQQKPGKAPNLLIY	AASTLET	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQANSFPLT	FGGGTKLE
αTNF-E7	DIVMTQSPSSLSASIGDRVTITC	RESQGIRNDLG	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYFC	QQANSLPPT	FGGGTKVEIKR
αTNF-H9G1	QSVLTQPPSVSGPPGQRVTISC	TGTSSNIGAGYDVY	WYQQLPDTSPRVLIY	RNSNRPS	GVPDRFSGSKSGTSASLAITGLQAEDEADYYC	QSYDSRLIRV	FGGGTKLTVLG
αCEA4-8A	EIVLTQSPSSLSASVGDRVTITC	RASQSISSYLN	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQYSNYPLT	FGGGTKVDIK
αMUC1-1	QSVLTQPASVSGSPDQSITISC	TGTSSDVGGYKYVS	WYQQHPDKAPKVMIY	DVTNRPS	GGSNRFSGSKSGNTASLTISGLQAEDEADYYC	SSYAGAQSLV	FGGGTKLTVLG
αCD4-74	HVILTQPPSASVAPGQRVTISC	SGSRSNIGSNPVS	WYRQFPGAAPKLLIY	IDDRRSS	GVPDRVSGSRSGTSASLAISGLQSEDEADYYC	VAWDDSLKGWV	FGEGTKLTVL

FR, framework region; CDR, complementarity-determining region. Bovine thyroglobulin binders; αThy-23, αThy-29, αThy-32 and αThy-33. Human thyroglobulin binders; αThy-23 and αThy-29. Fog-1 (a human γ1/κ mAb) binders; αFOG1-A3, αFOG1-A4, αFOG1-H6 and αFOG1-G8. Human TNFα binders; αTNF-A1, αTNF-E1, αTNF-E7 and αTNF-H9G1. Human CEA binder; αCEA4-8A. Human MUC1-1 binder; αMUC1-1. Human rsCD4 binder; αCD4-74.

chain γ1, 2 and 3 isotypes, but not to γ4 or μ. By contrast, the fragments αFOG1-H6 and αFOG1-A3 did not bind to any of the other antibodies, including those of the same isotype as Fog-1, suggesting that they are directed to the variable domain of Fog-1.

Two of the antibody fragments are directed against idiotopes of human mAb Fog-1

The binding of ^{125}I-Fog-1 antibody to human red blood cells bearing the Rh D antigen could be inhibited by both αFOG1-H6 and αFOG1-A3 scFv fragments. Hence, both αFOG1-H6 and αFOG1-A3 are site-associated anti-idiotype antibodies, complexing with the antigen-binding site of Fog-1. The extent of inhibition of ^{125}I-Fog-1 binding to the Rh D antigen (on human R_1R_2 red blood cells) was determined by titration with affinity purified αFOG1-H6 and αFOG1-A3 scFv fragments. [As control, no inhibition of ^{125}I-Fog-1 binding was observed using a scFv fragment (αTEL9) (Marks *et al.*, 1991) directed against turkey egg-white lysozyme.] With the maximum of 16 μg scFv (1000-fold molar excess to ^{125}I-Fog-1), the binding was inhibited by 14.2% (αFOG1-H6) and 20.9% (αFOG1-A3), suggesting that the affinities of these fragments for Fog-1 are much lower than the affinity of Fog-1 for the Rh D antigen ($K_a = 2.2\times10^9$ M^{-1}) which binds monovalently (Gorick *et al.*, 1988). If 100% of the fragments are active, the affinities of the two fragments for binding to Fog-1 could be estimated as $K_a = 3\times10^5$ M^{-1} for αFOG1-H6 and 6×10^5 M^{-1} for αFOG1-A3 and this is consistent with other kinetic measurements (see below and Table IV).

The scFv fragments can form both monomers and dimers in solution

Soluble antibody fragments were purified from bacterial supernatants by affinity chromatography, by binding of the C-terminal peptide tag to the mAb 9E10 (Munro and Pelham, 1986; Clackson *et al.*, 1991; Marks *et al.*, 1991). After ultrafiltration, the fragments were further purified by FPLC gel filtration (Pharmacia) on Superdex 75 (Pharmacia), and detected on-line both by UV absorption (280 nm) and by binding to antigen immobilized on a sensor chip in BIAcore (Pharmacia Biosensor AB) (Jönsson *et al.*, 1991; Jönsson and Malmqvist, 1992). This showed that the scFv fragments emerged in two peaks, corresponding in size to monomers and dimers (Figure 4). The dimers bind more strongly to the immobilized antigen than monomers due to their greater avidity of binding. The scFv dimers run as monomers on non-reducing SDS gels (Laemmli, 1970) (not shown) and are therefore not linked by disulphide bonds. As two peaks are seen in gel filtration, it appears that in this case the monomers and dimers do not interconvert rapidly (for discussion and references for gel filtration equilibria see Jones *et al.*, 1985). Presumably the dimers are scFv fragments interlocked through the flexible linker joining the heavy and light chains, or with the heavy chain of one scFv molecule associated with the light chain of the other. We have preliminary evidence that antibody Fab fragments made in bacteria can also multimerize (unpublished data).

The scFv fragments have micromolar affinities

The presence of both scFv monomers and dimers could lead to an overestimate of affinity of binding using solid phase

Table III. V-gene family, germline derivation and extent of somatic hypermutation of several antigen-specific scFv fragments isolated from the unimmunized library

scFv	Family	Germline genes of closest nucleotide sequence	Differences from germline	
			Nucleotide	Aminoacid
Heavy chains				
αThy-23	VH3	DP-47	13	8
αThy-29	VH1	DP-14	0	0
αThy-32	VH3	DP-31	5	2
αThy-33	VH3	DP-49	32	19
αFOG1-A3	VH3	DP-54	7	3
αFOG1-A4	VH3	DP-46	7	7
αFOG1-H6	VH3	DP-51	10	4
αFOG1-G8[a]	VH4	DP-63 (FR1)	2	0
	VH5	DP-73 (CDR1 to FR3)	15	7
αTNF-A1	VH3	DP-50	9	6
αTNF-E1	VH3	DP-46	14	6
αTNF-E7	VH1	DP-10	0	0
αTNF-H9G1	VH1	DP-4	1	1
αCEA4-8A	VH1	DP14	1	0
αMUC1-1[a]	VH1	VI-2 (FR1 to CDR2)	2	0
	VH1	DP-25 (FR3)	0	0
αCD4-74	VH5	DP-73	13	8
Light chains				
αThy-23	Vκ1	L8	20	9
αThy-29	Vλ3	IGLV3S1	0	0
αThy-32	Vλ1	IGLV1S2	1	1
αThy-33[a]	Vκ1	L12 (FR1 and CDR1)	6	3
	Vκ4	B3 (FR2 to FR3)	5	5
αFOG1-A3	Vλ2	VL2.1	16	9
αFOG1-A4	Vκ1	O4	25	12
αFOG1-H6	Vκ1	L5	36	17
αFOG1-G8	Vκ1	L8	25	10
αTNF-A1	Vκ1	L11	12	8
αTNF-E1	Vκ1	L5	5	5
αTNF-E7	Vκ1	L11	17	8
αTNF-H9G1	Vλ1	IGLV1S2	18	9
αCEA4-8A	Vκ1	O2	4	0
αMUC1-1	Vλ2	VL2.1	18	12
αCD4-74	Vλ1	Humlv1L1	23	17

References for all the heavy chain germline genes can be found in Tomlinson *et al.* (1992). The references for the light chains are VL2.1 (Brockly *et al.*, 1989); IGLV1S2 (Bernard *et al.*, 1990); IGLV3S1 (Frippiat *et al.*, 1990); L8(Vd) and L5(Vb)(Pech *et al.*, 1984); L12(HK102) (Bentley and Rabbits, 1980); B3(VKIV) (Klobeck *et al.*, 1985); 02 and 04 (Pargent *et al.*, 1991); L11 (Scott *et al.*, 1991); Humlv1L1 (Daley *et al.*, 1992). Alternative names are given in parenthesis. a) These genes appear to have been created by cross-overs between two V-genes during PCR amplification and therefore matches have been determined using the two putative germline segments: FR, framework; CDR, complementarity-determining region.

methods. To determine the affinity and kinetics of binding of scFv fragments to the antigen-coated chip using surface plasmon resonance, we therefore purified the fragments by gel filtration (Table IV). For the dimers, the off-rate constants were determined as $\sim 10^{-2}$ s^{-1} and the on-rate constants for the scFv dimers as $\sim 10^5 - 10^6$ M^{-1} s^{-1} (assuming the sample is completely active). In the case of αFOG1-H6, the antigen (the mAb Fog-1) was immobilized on the sensor chip in two ways, either directly (Figure 5) or via a rabbit anti-mouse IgG1 antibody. The results were almost identical by either method (see Table IV). However the active fraction of scFv fragments varies considerably and could lead to an underestimate of the on-rate (and affinity of binding); for example using fluorescence quench titration with several scFv fragments directed against phenyloxazolone we detected only 0.06−0.38 functional binding sites per scFv molecule (unpublished data). Indeed the on-rate constants calculated for the association of the αFOG1-H6 fragment and Fog-1 antibody depend on whether the antibody (k_{on} 2.2×10^5 M^{-1} s^{-1}) or scFv fragment (k_{on} 1.0×10^6 M^{-1} s^{-1}) is immobilized on the sensor chip (Table IV), indicating that the αFOG1-H6 fragment is less active than the Fog-1 antibody. For the scFv monomers, the binding signals were low and it was difficult to follow the kinetics of binding to the surface, except for the dissociation of the αThy-29 monomer (k_{off} = 2×10^{-2} s^{-1}). However, the 4-fold stabilization of the αThy-29 fragment dimer (see below), suggests that the off-rate constants of the other monomers are $>10^{-2}$ s^{-1}, perhaps 10^{-1} s^{-1}.

The greater stability of the scFv dimers on the sensor chip

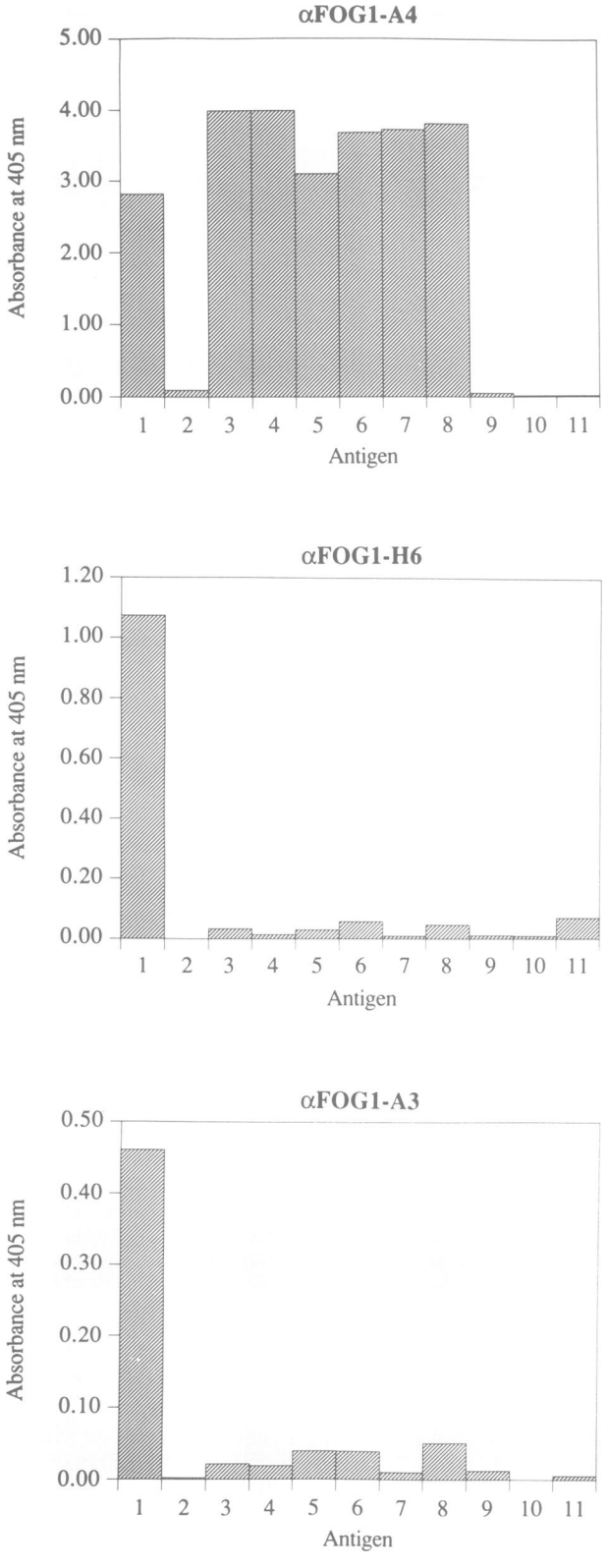

Fig. 3. ELISA to assay the binding of three scFvs, isolated by selection on a human monoclonal antibody Fog-1 (IgG1, κ) (Melamed *et al.*, 1987), to a panel of human antibodies of varying isotype: 1, Fog-1; 2, the Fv fragment of Hulys11 (Foote and Winter, 1992); 3, Hulys11 antibody (IgG1, κ); 4, RegA (IgG1, κ) (Melamed *et al.*, 1987); 5, FogC (IgG3, κ) (N.C.Hughes-Jones, unpublished); 6, Pag1 (IgG1, λ) (Thompson *et al.*, 1986); 7, IgG2, λ antibody purified from myeloma plasma (Sigma); 8, Oak3, (IgG3, λ) (Bye *et al.*, 1992); 9, IgG4, λ purified from myeloma plasma (Sigma); 10, Fom1 (IgM, λ) (Melamed *et al.*, 1987); 11, FomA (IgM, λ) (Melamed *et al.*, 1987).

compared with monomers indicates that the dimers are bivalent. The scFv dimers are therefore analogous to the two heads of the antibody IgG, but with different spacing between the heads, and their binding avidities were estimated as $\sim 10^7$ M^{-1} from k_{on}/k_{off} (Table IV). The affinities of the monomers must be lower by virtue of their faster dissociation from the surface. For the αThy-29 monomer, and assuming that the on-rate constant is the same as for the dimer (Mason and Williams, 1986), we can estimate an affinity of $\sim 3\times10^6$ M^{-1}. These affinities, calculated from the rate constants measured by surface plasmon resonance, appear to be similar to those measured in solution by fluorescence quench techniques. For example the affinity of binding of the monomer scFv fragment αTEL9 (Marks *et al.*, 1991), which binds to turkey lysozyme (and was derived from the same library), was estimated as 3.9×10^7 M^{-1} using surface plasmon resonance (Table IV), and as 1.2×10^7 M^{-1} by fluorescence quench (Marks *et al.*, 1991).

Discussion

We had demonstrated previously that highly specific human antibody fragments (scFv), directed against 'foreign' antigens, both protein and hapten, could be isolated from a large phage display library composed of the rearranged V-genes of peripheral blood lymphocytes from unimmunized donors (Marks *et al.*, 1991). Here we have shown that antibody fragments directed against human antigens (self-antigens), including idiotopes of a human antibody, a cytokine (TNFα), two tumour markers (CEA and MUC1) and the T-lymphocyte marker CD4 can be derived from the same library. Immunological tolerance would make immunization of humans with these antigens difficult; TNFα is also extremely toxic and raising an immune response against CD4 would be an act of suicide by the immune system. The antibody fragments we have isolated show a high specificity of binding to antigen. This contrasts with the poor specificity of binding of fragments isolated from a phage display library in which mouse Fab fragments were fused to the major coat protein (pVIII) of filamentous phage (Gram *et al.*, 1992). The use of different V-genes and heavy and light chain combinations for each antigen suggested that each fragment was likely to bind to different epitopes and this was shown directly for the fragments against thyroglobulin and the human mAb Fog-1.

The affinity of antibodies isolated from a library is thought to be proportional to the library size (Perelson and Oster, 1979) and in this case the size of the phage library is comparable to the number of B-cells in a mouse, and the affinities of antibodies isolated are typical of antibodies from the mouse primary immune response (Foote and Milstein, 1991). The kinetics of association of the antibody fragments to the protein self-antigens (10^5-10^6 M^{-1} s^{-1}) are also typical of previously characterized Ab−protein interactions. However the kinetics of dissociation (10^{-2} s^{-1}) are relatively fast for Ab−protein interactions (but both rates are slow compared with many Ab−hapten interactions) (Smith and Skubitz, 1975; Pecht, 1982; Mason and Williams, 1986; Foote and Milstein, 1991; Foote and Winter, 1992). At first sight, it is surprising that we can isolate scFv fragments with such fast off-rates, as a 'monomeric' phage should not be retained on the solid support during washing. However, scFv fragments are

Table IV. Affinities and kinetics of antigen binding by monomeric and dimeric scFv fragments

scFv	(M/D)[a]	Immobilized species	k_{on}[b] (BIAcore) $M^{-1}s^{-1}/10^4$	k_{off}[b](BIAcore) $s^{-1}/10^{-2}$	$K_a=k_{on}/k_{off}$ (BIAcore) $M^{-1}/10^6$	K_a by FQ[c] or inhibition[d] $M^{-1}/10^6$
αTNF-E7	D	HumanTNFα	9.0 (±1.2)	1.4 (±0.054)	6.4	ND
αFOG1-H6	D	Fog-1 (direct)	22.2 (±0.4)	1.8 (±0.23)	12.3	ND
αFOG1-H6	D	Fog-1 (via RAMIgG1)	22.1 (±1.9)	2.4 (±0.045)	9.3	ND
αFOG1-H6	D	αFOG1-H6 scFv	104 (±2.4)	ND[e]	ND	ND
αFOG1-H6	M + D	(Measured by inhibition)	ND	ND	ND	0.3[d]
αFOG1-A3	M + D	(Measured by inhibition)	ND	ND	ND	0.6[d]
αThy-29	D	Human thyroglobulin	6.6 (±1.2)	0.46 (±0.063)	14.3	ND
αThy-29	M	Human thyroglobulin	ND	2.0 (±0.37)	ND	ND
αTEL9	M	Turkey egg lysozyme	39.2 (±2.6)	1.0 (±0.97)	39.2	11.6[c]

[a] M, monomeric fraction; D, dimeric fraction.
[b] Numbers in brackets are standard deviations.
[c] FQ, fluorescence quench titration.
[d] Calculated from the extent of inhibition of ^{125}I-Fog-1 binding to the Rh D antigen.
[e] Not determined because the dissociation curves were very badly bent.

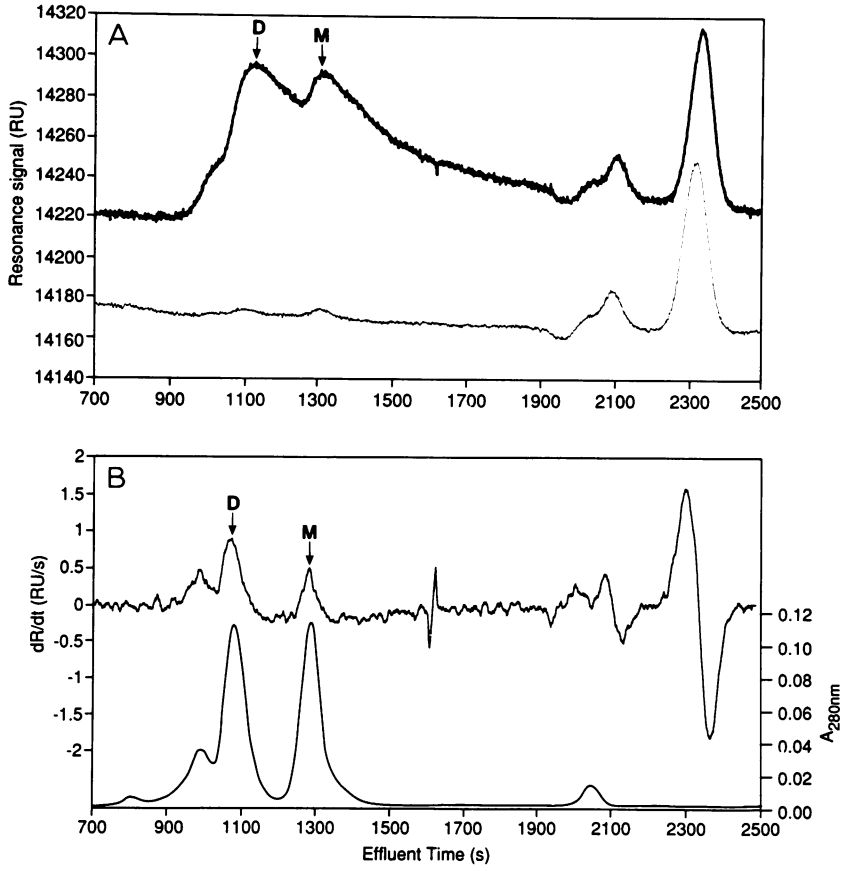

Fig. 4. Gel filtration of affinity purified soluble scFv αThy-29 on Superdex 75 analysed by UV absorption and on-line specific detection of the active component on BIAcore. **A.** BIAcore sensorgram [resonance signal (RU) as a function of time] showing adsorption of scFv in the column effluent passing over a sensor chip with immobilized human thyroglobulin (thick line) and the same sample run over a bare CM5 sensor chip surface without any antigen (thin line). **B.** UV profile of the gel filtration (lower line) and the derivatized sensorgram (upper line) which illustrates the rate of change in mass of protein bound to the sensor chip as a function of time. M, scFv monomer; D, scFv dimer.

displayed multivalently on the phage, especially using the M13ΔgIII helper phage, and some of the scFvs that tend to form dimers in solution may also form dimers on phage. The multivalent interactions with antigen help retain the phage, allowing the encoded scFv phage to be isolated.

Random combinatorial V-gene repertoires derived from the mRNA of immunized animals are enriched for heavy or light chain V-genes encoding part of an antigen binding site (Winter and Milstein, 1991; Hawkins and Winter, 1992), and this facilitates the isolation of antigen-binding fragments (Clackson *et al.*, 1991; Persson *et al.*, 1991) although the combinations of V-genes of each B-lymphocyte appear to be largely destroyed (Winter and Milstein, 1991; Gherardi and Milstein, 1992). Antigen binding sites can also be generated *de novo* by the random combination of chains, as illustrated by the isolation of scFv fragments against

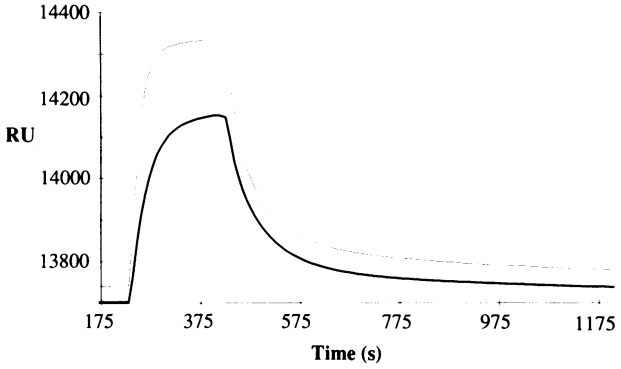

Fig. 5. BIAcore sensorgram [resonance signal (RU) as a function of time] of the interaction of soluble αFOG1-H6 scFv dimer with immobilized mAb Fog-1 (Melamed *et al.*, 1987). A 35 μl pulse of 200 nM (thin line) or 80 nM (thick line) scFv protein was passed, with a flow rate of 10 μl/min over a sensor chip to which mAb Fog-1 was coupled directly.

foreign antigens from unimmunized human donors (Marks *et al.*, 1991). However, the origins of the V-genes of scFv fragments directed against self-antigens are less clear. Self-reactive antibodies, including those with specificities against human thyroglobulin (Ruf *et al.*, 1985), human TNFα (Bendtzen *et al.*, 1990) and human IgG (Welch *et al.*, 1983), are common in healthy individuals and indeed 10–30% of B-lymphocytes appear to be engaged in making autoantibodies (Cohen and Cooke, 1986). Therefore the V-genes could be derived from B-cells that are autoreactive, or those that are not. Since somatic hypermutation of antibody genes is triggered only after antigen-induced B-cell proliferation (Griffiths *et al.*, 1984), the isolation of scFv fragments encoded by somatically mutated V-genes (Table III) indicates that the V-genes have been derived from lymphocytes that have been stimulated by antigen; for example from B-cells with self-specificities that have been stimulated with cross-reactive foreign antigen, or from B-cells encoding antibodies of other (foreign) specificities. Conversely those scFv fragments encoded by V-genes with little or no somatic mutation (see Table III) may well have been derived from virgin B-cells or those involved in early immune responses.

'Natural autoantibodies' (self-reactive antibodies isolated from healthy donors) tend to be of low affinity and polyspecific and may well be produced by a discrete subset of B-cells, the internal activity set (Holmberg and Coutinho, 1985), contributed in part by $CD5^+$ B-cells (Casali and Notkins, 1989). In contrast, these anti-self scFv fragments are highly specific in binding to antigen despite only having micromolar affinities. However, their affinities could presumably be improved *in vitro*, for example, the affinity of an scFv fragment for the hapten phenyloxazolone derived from the phage library (and like the anti-self antibodies described here with a relatively fast off-rate) was improved from $K_a = 3.1\times10^6\ M^{-1}$ to $9.1\times10^8\ M^{-1}$ by chain shuffling (Marks *et al.*, 1992b). This would allow the creation of highly specific, high affinity human antibodies directed against self-antigens for use in human therapy.

Materials and methods

Selection of phage library

The construction and selection of the library of phages displaying scFv fragments has been described previously by Marks *et al.* (1991) and as briefly summarized in the Introduction. The library used in this work contained 2.9×10^7 clones and the VH genes were amplified from cDNA primed with an IgM-specific constant region primer.

To rescue the library 50 ml of 2×TY broth (Miller, 1972) containing 100 μg ampicillin/ml, 1% glucose (2×TY-AMP-GLU) were inoculated with 10^9 *E.coli* TG1; (Gibson, 1984) of the library stock (~10 μl) and grown, shaking at 37°C until the culture reached an $OD_{600\ nm}$ of 0.5. 5 ml of this culture (~2.5×10^9 cells) were added to 50 ml 2×TY-AMP-GLU (pre-warmed to 37°C) containing 5×10^{10} p.f.u. VCS-M13 helper phage (Stratagene) or 5×10^8 pfu M13ΔgIII helper phage (unpublished data). M13ΔgIII helper phage does not encode pIII hence the phage(mid) displaying antibody fragments have a greater avidity of binding to antigen. Infectious M13ΔgIII particles were made by growing the helper phage in cells harbouring a pUC19 derivative supplying the wild-type gIII protein during phage morphogenesis. The culture was incubated for 1 h at 37°C without shaking and then a further 1 h at 37°C with shaking. Cells were spun down (IEC-Centra 8, 4000 r.p.m. for 10 min), resuspended in 300 ml 2×TY broth containing 100 μg/ml ampicillin and 25 μg/ml kanamycin (2×TY-AMP-KAN) and grown overnight shaking at 37°C (or 25°C for phage selected with rsCD4). Phage particles were purified and concentrated from the culture medium by two PEG precipitations (Sambrook *et al.*, 1990), resuspended in 2 ml PBS and passed through a 0.45 μM filter (Minisart NML; Sartorius) to give a final concentration of ~10^{13} transducing units/ml (ampicillin-resistant clones).

The phage were panned for binding using immuno tubes (Nunc; Maxisorp) coated with antigen essentially as Marks *et al.* (1991), or were selected on a column of antigen (McCafferty *et al.*, 1990). Six antigens were used: a human mAb Fog-1 (γ1, κ) (Melamed *et al.*, 1987); recombinant human tumour necrosis factor-α (TNFα) (expressed in yeast); bovine thyroglobulin (Sigma); human recombinant soluble CD4 (rsCD4) (expressed in baculovirus by American Biotechnologies Inc. and supplied by the MRC AIDS Reagent Project [ADP608]); human carcinoembryonic antigen (CEA); and a 20 amino acid peptide (Price *et al.*, 1990), which corresponds to a repeated motif in human MUC1 mucin (tumour-associated polymorphic epithelial mucin or PEM) (Gendler *et al.*, 1988; Gum *et al.*, 1990). All antigens, except the MUC1 peptide, were coated on immuno tubes overnight at room temperature. 10 μg/ml TNFα was coated in 50 mM $NaHCO_3$ (pH 9.6), whilst the other antigens were coated in PBS at a concentration of 10 μg/ml Fog1 and rsCD4, 1 mg/ml bovine thyroglobulin or 20 μg/ml CEA. For the first two rounds of selection tubes were washed 10 times with PBS, 0.1% (v/v) Tween 20 and 10 times with PBS. For subsequent rounds of selection tubes were washed 20 times with PBS, 0.1% (v/v) Tween 20 and 20 times with PBS. Phage were eluted with 100 mM triethylamine as Marks *et al.* (1991). Eluted phage (usually 10^6-10^7 transducing units) were used to infect *E.coli* TG1 cells. Approximately 10^9 infected bacteria were used as an inoculum for the next rescue. The library was subjected to 3–5 rounds of rescue and selection for each antigen.

For selection of phage binding to the MUC1 peptide, the peptide was coupled chemically to Sepharose 4B (provided by M.R.Price). A 1 ml column was prepared and phage was selected as described by McCafferty *et al.* (1990). Briefly, the Sepharose–MUC1 column was washed with PBS containing 2% skimmed milk powder (MPBS) and the phage loaded in 1ml of the same buffer. After washing the column successively with 10 ml volumes of MPBS, PBS (pH 7.2), 50 mM Tris–HCl–500 mM NaCl (pH 8.0) and 50 mM Tris–HCl–500 mM NaCl (pH 9.0), phage was eluted with 5 ml 100 mM triethylamine and neutralized with 0.5 M sodium phosphate buffer (pH 6.8). Five rounds of selection were carried out.

Screening and sequencing of clones

Single ampicillin resistant colonies from infection of *E.coli* TG1 with eluted phage, were screened either for binding of phage (Clackson *et al.*, 1991) or soluble scFv fragments (Marks *et al.*, 1991). Since the gene encoding the antibody fragment is linked to that encoding the phage coat protein by an amber codon, soluble fragments can be secreted from a non-suppressor strain of bacteria infected by the phage (Hoogenboom *et al.*, 1991). The binding to antigen of soluble scFvs in bacterial supernatant was detected with the mouse mAb 9E10 (1 μg/ml), which recognizes the C-terminal peptide tag (Munro and Pelham, 1986) and peroxidase-conjugated anti-mouse Fc antibody (Sigma), as described by Ward *et al.* (1989). Plates were coated with the antigens Fog1, TNFα, bovine thyroglobulin and rsCD4 as described for immuno tubes above and with CEA at 5 mg/ml. A urine extract containing human polymorphic epithelial mucin (PEM) was used at a protein concentration of ~10 mg/ml.

The specificity of the isolated clones was checked by ELISA of the soluble scFv fragments using plates coated with various proteins. Plates were coated with the antigens Fog-1, TNFα, bovine thyroglobulin, rsCD4, CEA and PEM as described above. Other proteins were coated overnight at room temperature at a concentration of 1 mg/ml in PBS (cytochrome *c* [Sigma]) or in 50 mM $NaHCO_3$ (pH 9.6) and bovine serum albumin, turkey egg-

white lysozyme, hen egg-white lysozyme, hen ovalbumin, keyhole limpet haemocyanin (CalBiochem), chymotrypsinogen A, chicken egg-white trypsin inhibitor (Sigma) and chicken γ globulin coupled to 4-hydroxy-3-nitrophenyl acetic acid. The Fog-1 specific clones were screened by binding to a panel of different human antibodies (see legend to Figure 3). The antibodies were coated overnight at room temperature in PBS at a concentration of 10 μg/ml.

Clones found to give a positive ELISA signal were screened by PCR (Gussow and Clackson, 1989) and 'fingerprinted' with the restriction enzyme *Bst*NI (Clackson *et al.*, 1991) as in Marks *et al.* (1991) to identify different clones. Examples of clones with different restriction patterns were selected and the heavy and light chains sequenced (Sanger *et al.*, 1977) using a Sequenase kit (USB) or using a Taq DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems) and an Applied Biosystems 373A DNA sequencer.

Sequenced clones were further analysed using the program MacVector 3.5 (IBI Kodak, New Haven, CT). The VH genes were compared with the 83 germline gene segments present in the VH directory compiled by Tomlinson *et al.* (1992). VL genes were compared with 34 published kappa germline gene segments (Bentley and Rabbits, 1980, 1983; Jaenichen *et al.*, 1984; Pech *et al.*, 1984, 1985; Pech and Zachau, 1984; Klobeck *et al.*, 1985a,b; Stavnezer *et al.*, 1985; Chen *et al.*, 1986, 1987a,b; Lorenz *et al.*, 1988; Straubinger *et al.*, 1988a,b; Scott *et al.*, 1989, 1991; Pargent *et al.*, 1991; Lautner *et al.*, 1992) and 13 published lambda gene segments (Anderson *et al.*, 1984; Alexandre *et al.*, 1989; Brockly *et al.*, 1989; Siminovitch *et al.*, 1989; Bernard *et al.*, 1990; Frippiat *et al.*, 1990; Combriato and Klobeck, 1991; Marks *et al.*, 1991; Daley *et al.*, 1992; Winkler *et al.*, 1992). Regions of the V-genes encoded by PCR primers were not included in the analysis.

Characterization of selected scFv fragments

The following clones were chosen for large scale purification and further characterization: αFOG1-H6, αFOG1-A3, αTNF-E7 and αThy-29. Colonies of the non-suppressor *E.coli* strain HB2151 harbouring the appropriate phagemid were used to inoculate 2 l of 2×TY containing 100 μg/ml ampicillin and 0.1% glucose. The cultures were grown and induced (De Bellis and Schwartz, 1990) and the tagged scFv fragments purified using the mAb 9E10 as in Clackson *et al.* (1991).

The inhibition of ^{125}I-Fog-1 binding to human Rh D antigen by the affinity purified scFv fragments αFOG1-H6 and αFOG1-A3 was essentially as performed earlier (Gorick *et al.*, 1988) with the following modifications. 0.0148 μg of ^{125}I-FOG1 was pre-incubated with varying amounts of purified αFOG1-H6 or αFOG1-A3 scFv fragments (0–16 μg) at 37°C for 1.5 h, before adding 0.5 μl of R_1R_2 cells (or rr cells as control). The mixture was then incubated for a further 1.5 h at 37°C with constant mixing and finally cells separated from the supernatant. As a control, a titration was also performed with a purified scFv fragment directed against turkey egg white lysozyme (αTEL9) (Marks *et al.*, 1991).

Kinetic measurements were made using surface plasmon resonance (BIAcore, Pharmacia Biosensor AB) (Jönsson *et al.*, 1991; Jönsson and Malmqvist, 1992). In order to separate monomeric and multimeric species, the purified scFv fragments were concentrated by ultrafiltration and then fractionated on a calibrated Superdex 75 FPLC column (Pharmacia) in PBS, 0.2 mM EDTA. Gel filtration was monitored both by the absorbance at 280 nm and on-line to BIAcore with immobilized antigen on the sensor chip (Johnsson *et al.*, 1991).

Kinetic experiments were performed in two different configurations. First, to analyse the binding of soluble scFv, the different antigens were covalently immobilized on the sensor chip (in the case of mAb Fog-1, the antibody was also immobilized via a mouse anti-human kappa light chain mAb using a sensor chip coated with rabbit anti-mouse IgG1). Secondly, to analyse the binding of the soluble mAb FOG-1, the αFOG1-H6 scFv was immobilized on the chip surface.

The antigens were coupled to the CM5 sensor chip through their amine groups using the Amine Coupling Kit (Pharmacia Biosensor AB) (Johnsson *et al.*, 1991). The antigens were diluted in 10 mM acetate buffer (pH 5.0) to ~25 μg/ml and 3805 resonance units (RU) of TNF, 6249 RU of human thyroglobulin and 5279 RU of FOG1 were immobilized. For the biospecific presentation of Fog-1, affinity purified rabbit anti-mouse IgG1 (Pharmacia Biosensor AB) was coupled to the surface followed by a mouse mAb anti-human kappa (2300 RU) and then Fog-1 (2050 RU). As binding of the rabbit anti-mouse IgG1 to the mouse mAb was reversible by 10 mM HCl the complex was rebuilt for each analytical cycle. ScFv anti-Fog-1 was coupled to the CM5 surface to 1538 RU. All determinations were performed at 25°C in PBS, 0.2 mM EDTA, 0.05% BIAcore surfactant P20 with a constant flow rate of 10 μl/min and an injected vol sample of 35 μl. It was not necessary to regenerate the antigen as the scFv fragments rapidly dissociate, with the exception of the biospecific presentation of antigen via rabbit anti-mouse IgG1 which was regenerated with 10 mM HCl for 3 min.

Analyses of scFv monomer were performed in the concentration range 100–500 nM and dimers in the range 40–200 nM except for the biospecifically presented Fog-1 where the concentration of dimeric scFv was 0.25–1.26 μM. Fog-1 was analysed on the αFOG1-H6 scFv surface in the concentration range 10–200 nM. All concentrations were calculated from U.V. absorption at 280 nm [assuming that 0.7 mg/ml scFv gives an A_{280} = 1 (Mach *et al.*, 1992) and that M_r of a scFv monomer is 30 kDa and of a dimer is 60 kDa]. No correction was made for the fraction of active protein, and therefore the on-rates are an underestimate. The kinetic evaluation of data was performed according to Karlsson *et al.* (1991) and evaluated on the program Origin 1.1 (Microcal inc., Northampton, MA, USA).

Acknowledgements

We thank M.R.Price for providing CEA, MUC1 peptide coupled to Sepharose 4B and PEM, A.Nissim, C.Chothia and S.Williams for help with sequence analysis, and Peptide Technology Ltd (Sydney, Australia) for a gift of recombinant TNFα. Recombinant soluble CD4 was provided by the MRC AIDS Reagent Project. A.D.G. and M.J.E. were supported by the Cancer Research Campaign, M.M. by Pharmacia Biosensor AB, J.D.M. by the MRC AIDS Directed Programme and the MRC, J.M.B., B.D.G. and N.C.H.-J. by a grant from the International Blood Group Reference Laboratory, J.McC. by Cambridge Antibody Technology Ltd., M.B. by the AIDS Program of the Federal Ministry for Research and Technology Germany, K.P.H. by ETH Zurich, and H.R.H. by the D.Collen Research Foundation, Leuven and the European Molecular Biology Organization.

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Received on September 29, 1992; revised on November 11, 1992

Note added in proof

Nucleotide sequences of all the V-genes whose deduced protein sequences are given in Table II have been submitted to the EMBL Data Library and assigned the following accession numbers: FOG1VHA3, Z18822; FOG1VLA3, Z18823; FOG1VHH6, Z18824; FOG1VLH6, Z18825; FOG1VHA4, Z18826; FOG1VLA4, Z18827; FOG1VHG8, Z18828; FOG1VLG8, Z18829; THYVH23, Z18830; THYVL23, Z18831; THYVH29, Z18832; THYVL29, Z18833; THYVH32, Z18834; THYVL32. Z18835; THYVH33, Z18836; THYVL33, Z18837; TNFVLA1, Z18838; TNFVHE1, Z18839; TNFVLE1, Z18840; TNFVHE7, Z18841; TNFVLE7, Z18842; TNFVHH9, Z18843; TNFVLH9, Z18844; CEAVL8A, Z18845; MUC1VH1, Z18846; MUC1VL1, Z18847; CD4VH74, Z18848; CD4VL74, Z18849; TNFVHA1, Z18850; CEAVH8A, Z18851.