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By-passing Immunization

Human Antibodies from V-gene Libraries Displayed on Phage

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We have mimicked features of immune selection to make human antibodies in bacteria. Diverse libraries of immunoglobulin heavy (V_H) and light (V_κ and V_λ) chain variable (V) genes were prepared from peripheral blood lymphocytes (PBLs) of unimmunized donors by polymerase chain reaction (PCR) amplification. Genes encoding single chain Fv fragments were made by randomly combining heavy and light chain V-genes using PCR, and the combinatorial library ($>10^7$ members) cloned for display on the surface of a phage. Rare phage with "antigen-binding" activities were selected by four rounds of growth and panning with "antigen" (turkey egg-white lysozyme (TEL) or bovine serum albumin) or "hapten" (2-phenyloxazol-5-one (phOx)), and the encoding heavy and light chain genes were sequenced. The V-genes were human with some nearly identical to known germ-line V-genes, while others were more heavily mutated. Soluble antibody fragments were prepared and shown to bind specifically to antigen or hapten and with good affinities, K_a (TEL) $= 10^7$ M^{-1}; K_a (phOx) $= 2 \times 10^6$ M^{-1}. Isolation of higher-affinity fragments may require the use of larger primary libraries or the construction of secondary libraries from the binders. Nevertheless, our results suggest that a single large phage display library can be used to isolate human antibodies against any antigen, by-passing both hybridoma technology and immunization.

Keywords: filamentous phage; human antibodies; combinatorial libraries

1. Introduction

Over the last century animal antiserum, and more recently rodent monoclonal antibodies, have been used clinically to neutralize toxins, and to treat bacterial and viral infections. In the future the specific recognition of human cell-surface markers by antibody fragments should enable functional manipulations of subsets of immuno-competent cells in the fields of, for example, autoimmunity, transplantation, and the inhibition of cell adhesion and of cytokine-stimulated cell proliferation. However, the use of animal antibody can lead to an antiglobulin response and hypersensitivity reactions. Ideally human monoclonal antibodies would be used, but it is difficult to make them. Not only are peripheral blood lymphocytes (PBLs‡) a poor source of the blast cells that are actively involved in the immune response, but it is difficult to immortalize them. The use of mouse myeloma lines as fusion partners for human B-cells leads to a preferential loss of human chromosomes and instability of the hybrids, and Epstein Barr virus infection of B-cells also tends to

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‡ Abbreviations used: PBL, peripheral blood lymphocyte; Ig, immunoglobulin; PCR, polymerase chain reaction; g3p, gene *3* protein; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; TEL, turkey egg-white lysozyme; t.u., transducing unit(s); p.f.u., plaque-forming units(s); IPTG, isopropyl β-D-thiogalactopyranoside.

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produce unstable (IgM) lines with poor antigen affinity (for a review and references, see Winter & Milstein (1991)).

However, there are other ways of tapping the antibody repertoire of immunized humans or animals. Instead of immortalizing B-cells for production of monoclonal antibodies, the antibody heavy and light chain V-genes are immortalized by gene technology, and antibodies or fragments expressed in mammalian cells, yeast or bacteria. For example, recombinant antibodies were rescued from hybridomas by PCR amplification of the V-genes with "universal" primers, and cloning the genes into vectors for expression of complete antibodies (Orlandi *et al.*, 1989). In principle this technique could be extended to the construction of antibodies from the V-genes of single B-cells, thereby bypassing hybridoma technology (Orlandi *et al.*, 1989; Larrick *et al.*, 1989). Alternatively, libraries of V-genes have been used to express soluble antibody fragments, which are then screened for antigen-binding activities (Ward *et al.*, 1989; Huse *et al.*, 1989; Caton & Koprowski, 1990; Mullinax *et al.*, 1990; Persson *et al.*, 1991). For example, from a donor immunized with tetanus toxoid, V-genes from the mRNA of 10^8 human PBLs were combined at random in bacteriophage lambda, so scrambling the original heavy and light chain pairings. When the combinatorial library (10^7 members) was expressed in bacteria and 12,000 plaques were screened on nitrocellulose filters for binding to toxoid, 10 binders were found (Mullinax *et al.*, 1990). Thus, human antibodies can be made by filter screening of combinatorial libraries from immunized donors.

By contrast we have avoided the screening of large numbers of individual clones on filters by mimicking features of immune selection (Milstein, 1990; McCafferty *et al.*, 1990; Winter & Milstein, 1991). In the immune system, diverse combinatorial libraries of antibodies are displayed on the surface of B-cells, and specific recognition with antigen triggers cell proliferation and differentiation into antibody-secreting or memory pathways. We have displayed (Smith, 1985; Parmley & Smith, 1988) antibody fragments on the surface of filamentous bacteriophage by fusion to a minor coat protein at the tip of the phage, the gene *3* protein (g3p) (McCafferty *et al.*, 1990). Phage encoding antibody fragments with binding activities were selected from those encoding non-binders by affinity chromatography. By rounds of growth and selection, rare binders were selected, with an enrichment of one in 10^3 after one round of panning, and one in 10^6 after two rounds (McCafferty *et al.*, 1990). Antibody fragments can be displayed as fusions with g3p as single polypeptide chains in which the heavy and light chain variable domains are linked by a polypeptide spacer (single chain Fv or scFv: McCafferty *et al.*, 1990), or as non-covalently associated heavy and light chains (Fab fragments) (Hoogenboom *et al.*, 1991). Fab fragments have also been displayed as fusions with the major coat protein (gene *8*: Kang *et al.*, 1991). Recently we used phage to display a small random combinatorial library (2×10^5 members) of scFv antibody fragments from the spleen mRNA of immunized mice (Clackson *et al.*, 1991). The mRNA is presumably derived mainly from plasma cells (R. Hawkins & G. Winter, unpublished results), as the level of Ig mRNA in these cells is up to 1000-fold greater than in resting B-cells (Schibler *et al.*, 1978). After only a single round of affinity selection, we isolated numerous different antibodies with affinities in the range of 10^5 M^{-1} to 10^8 M^{-1}.

However, it is rarely possible to immunize humans to order, and the possibility of making human antibodies without prior immunization is particularly appealing. We have therefore applied the phage display technology to making human antibodies from V-gene repertoires from unimmunized donors. We made a large scFv library from the PBLs, and with greater than 10^7 members it was similar in size to the B-cell repertoire of a mouse at any one moment. The library was also made as diverse as possible by using both V_κ and V_λ light chains, as well as V_Hs derived from IgM and IgG mRNA. Diversity was further maximized by using PCR primers based on each of the human heavy and light chain gene families (Marks *et al.*, 1991). Finally, the library was subjected to multiple rounds of affinity selection to ensure that even a single clone in the original library could be isolated.

2. Materials and Methods

(a) *Primer design*

We optimized the design of the PCR primers for the rearranged V-genes to maximize the diversity of the PCR products. The primers were located at the 5′ and 3′ ends (back and forward primers, respectively) of the mature V-regions (Orlandi *et al.*, 1989; Marks *et al.*, 1991; Songsivilai *et al.*, 1990), but did not incorporate internal restriction sites that mismatch the template and bias amplification. The back primers were designed to match each of the families of human V-genes, and forward primers to match each of the human germ-line J-segments (Table 1). Furthermore, sets of PCR primers were designed to optimize the linking of V_H and V_κ or V_λ genes at random, and append restriction sites to the linked genes (Table 1 and Fig. 1).

(b) *Assay of donor serum for presence of IgM antibodies to phOx-BSA and TEL*

Serum from the 2 donors was assayed for the presence of IgM antibodies to phOx-BSA and TEL using an ELISA-based assay kit for detection of human IgM antibodies in serum (Platest, Menarini Diagnostics). Microtiter plates were coated overnight with either 10 μg phOx-BSA/ml or 10 μg TEL/ml. Plates were washed 3 times with PBS (phosphate-buffered saline: 25 mM-NaH_2PO_4, 125 mM-NaCl, pH 7·0) and blocked for 2 h with 2% MPBS (2% (w/v) skimmed milk powder (Marvel) in PBS) at 37°C. Donor serum was diluted 1/40 in PBS and 50 μl was added to the microtiter wells and incubated for 30 min at room temperature. The plates were washed 3 times with PBS and 50 μl horseradish peroxidase-conjugated anti-human IgM antibody was

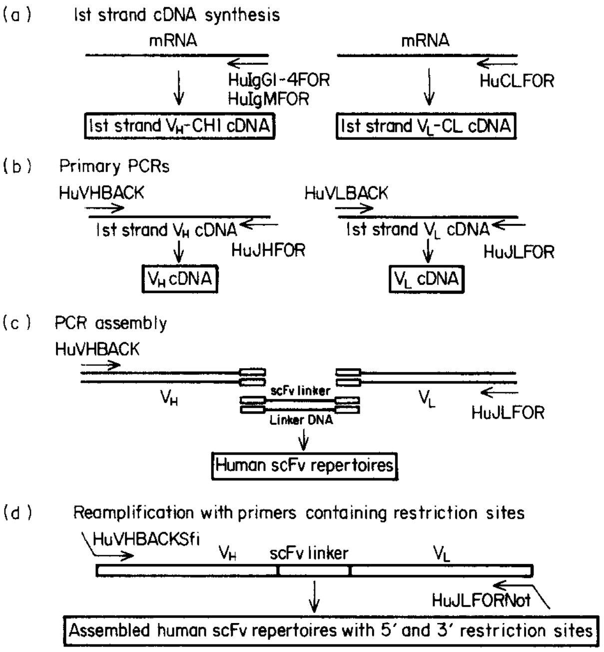

Figure 1. Making scFv gene repertoires. (a) mRNA is primed with constant region-specific oligonucleotides and 1st strand cDNA synthesized. (b) Portions of 1st strand cDNA are PCR amplified with a mixture of V-gene and J-segment primers. (c) The rearranged V_H and V_L PCR products are combined in a 2nd PCR amplification containing linker DNA that overlaps the C terminus of the V_H and the N terminus of the V_L genes. This reaction mixture is subjected to temperature cycling followed by amplification. (d) Finally, the resulting scFv gene repertoires are reamplified with primers containing appended restriction sites.

added to each well and incubated for 30 min. Plates were washed 3 times with PBS, developed as in the kit protocol and the plate read at 450 nm.

(c) *cDNA synthesis, PCR amplication and assembly of scFv genes*

Blood (500 ml) containing approximately 10^8 B-lymphocytes, was obtained from 2 healthy volunteers. The white cells were separated in Ficoll and RNA was prepared using a modified method described by Cathala *et al.* (1983). Heavy chain repertoires were prepared from both IgG and IgM cDNA in order to tap both mature and naive lymphocytes (Roit *et al.*, 1985), and light chain repertoires were prepared from both V_κ and V_λ genes. Thus, 4 first strand cDNA syntheses were made as described (Marks *et al.*, 1991) from RNA corresponding to $2{\cdot}5\times10^7$ B-cells, using either an IgG or an IgM constant region primer for the heavy chains, or a κ or λ constant region primer for light chains (Table 1 and Fig. 1(a)). All of the cDNA was used to generate 4 separate repertoires of scFv genes (V_{H_μ}-V_κ, V_{H_μ}-V_λ, V_{H_γ}-V_κ V_{H_γ}-V_λ) as described below (Figs 1 and 2).

V_H, V_κ and V_λ-genes were amplified separately using an equimolar mixture of the appropriate family-based back and forward primers (Table 1, Figs 1(b) and 2). Reaction mixtures (50 μl) were prepared containing 5 μl of the supernatant from the cDNA synthesis, 20 pmol back primers, 20 pmol forward primers, 250 μM-dNTPs 10 mM-KCl, 10 mM-$(NH_4)_2SO_4$, 20 mM-Tris·HCl (pH 8·8), 2·0 mM-$MgCl_2$, 100 μg BSA/ml and 1 μl (1 unit) Vent DNA polymerase (New England Biolabs). The reaction mixture was overlaid with mineral (paraffin) oil and subjected to 30 cycles of amplification using a Techne thermal cycler. The cycle was 94°C for 1 min (denaturation), 57°C for 1 min (annealing) and 72°C for 1 min (extension). The products were purified on a 2% (w/v) agarose gel, isolated from the gel by Geneclean (Bio-101) and resuspended in 25 μl of water.

To make the scFv linker DNA, 52 separate 50 μl PCR reactions were performed using each of the 4 reverse JH primers in combination with each of the 13 reverse V_κ and V_λ oligonucleotides (Table 1). The template was approximately 1 ng of pSW2scFvD1·3 (McCafferty *et al.*, 1990) containing the short peptide $(Gly_4Ser)_3$ (Huston *et al.*, 1988). The PCR reaction reagents were as described above and the cycle was 94°C for 1 min, 45°C for 1 min and 72°C for 1 min. The linkers were purified on a 2% agarose gel, eluted from the gel on a Spin-X column (Costar) and precipitated with ethanol.

For PCR assembly of the scFv repertoires (Fig. 1(c)), approximately 1 μg of a primary heavy chain amplification (V_{H_μ} or V_{H_γ}) and 1 μg of a primary light chain amplification (V_κ or V_λ) were combined with approximately 250 ng of the appropriate linker DNA (an equimolar mixture of each of the 6 JH-V_κ or 7 JH-V_λ linkers) in a 50 μl PCR reaction mixture and cycled 7 times (94°C for 2 min and 72°C for 2·5 min) to join the fragments. The reaction mixture was then amplified for 25 cycles (94°C for 1 min and 72°C for 3 min) after the addition of 20 pmol of the outer PCR primers (Fig. 1(c)). Finally, the assembled products were gel-purified and reamplified for 25 cycles (94°C for 1 min, 55°C for 1 min, 72°C for 2·5 min) with the flanking oligonucleotides containing the appended restriction sites (Fig. 1(d)). PCR buffers and dNTPs were as described previously. The resulting scFv repertoires (V_{H_μ}-V_κ, V_{H_μ}-V_λ, V_{H_γ}-V_κ, V_{H_γ}-V_λ were purified on a 1·5% agarose gel, electroeluted and precipitated with ethanol (Sambrook *et al.*, 1990). For subsequent cloning, the V_{H_μ}-V_κ and V_{H_μ}-V_λ repertoires were combined (IgM repertoire) as were the V_{H_γ}-V_κ and V_{H_γ}-V_λ repertoires (IgG repertoire).

(d) *Cloning of the scFv gene repertoires*

Purified DNA of the scFv gene repertoires (1 to 4 μg) was digested with *Not*I and either *Sfi*I or *Nco*I restriction enzymes. (The 2 different restriction enzymes were tried in an attempt to increase the cloning efficiency.) After digestion, the fragments were extracted with phenol/chloroform, and ligated into pHEN1 (Hoogenboom *et al.*, 1991) vector that had been digested with either *Sfi*I and *Not*I or *Nco*I and *Not*I and electroeluted from a 0·8% agarose gel (Sambrook *et al.*, 1990). Each scFv gene repertoire was combined in a ligation mixture which included 6 μg of digested vector, in a 100 μl ligation mix with 2000 units of phage T4 DNA ligase (New England Biolabs) overnight at room temperature. The ligation mix was purified by extraction with phenol and precipitation with ethanol. The ligated DNA was resuspended in 10 μl of water, and 2·5 μl samples were electroporated (Dower *et al.*, 1988) into 50 μl *Escherichia coli* TG1 (Gibson, 1984). Cells were grown in 1 ml of SOC (Sambrook *et al.*, 1990) for 1 h and then plated on TYE (Miller, 1972) medium with 100 μg ampicillin/ml and 1% (w/v) glucose (TYE-AMP-GLU), in 243 mm × 243 mm dishes (Nunc). Colonies were scraped off the plates into 10 ml of 2 × TY broth (Miller, 1972) containing 100 μg ampicillin/ml, 1%

Table 1

Oligonucleotide primers used for PCR of human immunoglobulin genes

A. *1st strand cDNA synthesis*	
Human heavy chain constant region primers	
HuIgG1-4CH1FOR	5'-GTC CAC CTT GGT GTT GCT GGG CTT-3'
HuIgMFOR	5'-TGG AAG AGG CAC GTT CTT TTC TTT-3'
Human κ constant region primer	
HuGκFOR	5'-AGA CTC TCC CCT GTT GAA GCT CTT-3'
Human λ constant region primer	
HuCλFOR	5'-TGA AGA TTC TGT AGG GGC CAC TGT CTT-3'
B. *Primary PCRs*	
Human V_H back primers	
HuVH1aBACK	5'-CAG GTG CAG CTG GTG CAG TCT GG-3'
HuVH2aBACK	5'-CAG GTC AAC TTA AGG GAG TCT GG-3'
HuVH3aBACK	5'-GAG GTG CAG CTG GTG GAG TCT GG-3'
HuVH4aBACK	5'-CAG GTG CAG CTG CAG GAG TCG GG-3'
HuVH5aBACK	5'-GAG GTG CAG CTG TTG CAG TCT GC-3'
HuVH6aBACK	5'-CAG GTA CAG CTG CAG CAG TCA GG-3'
Human J_H forward primers	
HuJH1-2FOR	5'-TGA GGA GAC GGT GAC CAG GGT GCC-3'
HuJH3FOR	5'-TGA AGA GAC GGT GAC CAT TGT CCC-3'
HuJH4-5FOR	5'-TGA GGA GAC GGT GAC CAG GGT TCC-3'
HuJH6FOR	5'-TGA GGA GAC GGT GAC CGT GGT CCC-3'
Human V_κ back primers	
HuVκ1aBACK	5'-GAC ATC CAG ATG ACC CAG TCT CC-3'
HuVκ2aBACK	5'-GAT GTT GTG ATG ACT CAG TCT CC-3'
HuVκ3aBACK	5'-GAA ATT GTG TTG ACG CAG TCT CC-3'
HuVκ4aBACK	5'-GAC ATC GTG ATG ACC CAG TCT CC-3'
HuVκ5aBACK	5'-GAA ACG ACA CTC ACG CAG TCT CC-3'
HuVκ6aBACK	5'-GAA ATT GTG CTG ACT CAG TCT CC-3'
Human J_κ forward primers	
HuJκ1FOR	5'-ACG TTT GAT TTC CAC CTT GGT CCC-3'
HuJκ2FOR	5'-ACG TTT GAT CTC CAG CTT GGT CCC-3'
HuJκ3FOR	5'-ACG TTT GAT ATC CAC TTT GGT CCC-3'
HuJκ4FOR	5'-ACG TTT GAT CTC CAC CTT GGT CCC-3'
HuJκ5FOR	5'-ACG TTT AAT CTC CAG TCG TGT CCC-3'
Human λ back primers	
Huλ1BACK	5'-CAG TCT GTG TTG ACG CAG CCG CC-3'
Huλ2BACK	5'-CAG TCT GCC CTG ACT CAG CCT GC-3'
Huλ3aBACK	5'-TCC TAT GTG CTG ACT CAG CCA CC-3'
Huλ3bBACK	5'-TCT TCT GAG CTG ACT CAG GAC CC-3'
Huλ4BACK	5'-CAC GTT ATA CTG ACT CAA CCG CC-3'
Huλ5BACK	5'-CAG GCT GTG CTC ACT CAG CCG TC-3'
Huλ6BACK	5'-AAT TTT ATG CTG ACT CAG CCC CA-3'
Human λ forward primers	
HuJλ1FOR	5'-ACC TAG GAC GGT GAC CTT GGT CCC-3'
HuJλ2-3FOR	5'-ACC TAG GAC GGT CAG CTT GGT CCC-3'
HuJλ4-5FOR	5'-ACC TAA AAC GGT GAG CTG GGT CCC-3'
C. *PCR assembly*	
Reverse J_H for scFv linker	
RHuJH1-2	5'-GCA CCC TGG TCA CCG TCT CCT CAG GTG G-3'
RHuJH3	5'-GGA CAA TGG TCA CCG TCT CTT CAG GTG G-3'
RHuJH4-5	5'-GAA CCC TGG TCA CCG TCT CCT CAG GTG G-3'
RHuJH6	5'-GGA CCA CGG TCA CCG TCT CCT CAG GTG C-3'
Reverse V_κ for scFv linker	
RHuVκ1aBACKFv	5'-GGA GAC TGG GTC ATC TGG ATG TCC GAT CCG CC-3'
RHuVκ2aBACKFv	5'-GGA GAC TGA GTC ATC ACA ACA TCC GAT CCG CC-3'
RHuVκ3aBACKFv	5'-GGA GAC TGC GTC AAC ACA ATT TCC GAT CCG CC-3'
RHuVκ4aBACKFv	5'-GGA GAC TGG GTC ATC ACG ATG TCC GAT CCG CC-3'
RHuVκ5aBACKFv	5'-GGA GAC TGC GTG AGT GTC GTT TCC GAT CCG CC-3'
RHuVκ6aBACKFv	5'-GGA GAC TGA GTC AGC ACA ATT TCC GAT CCG CC-3'

Table 1 *(continued)*

Reverse V_λ for scFv linker	
RHuVλBACK1Fv	5'-GGC GGC TGC GTC AAC ACA GAC TGC GAT CCG CCA CCG CCA GAG-3'
RHuVλBACK2Fv	5'-GCA GGC TGA GTC AGA GCA GAC TGC GAT CCG CCA CCG CCA GAG-3'
RHuVλBACK3aFv	5'-GGT GGC TGA GTC AGC ACA TAG GAC GAT CCG CCA CCG CCA GAG-3'
RHuVλBACK3bFv	5'-GGG TCC TGA GTC AGC TCA GAA GAC GAT CCG CCA CCG CCA GAG-3'
RHuVλBACK4Fv	5'-GGC GGT TGA GTC AGT ATA ACG TGC GAT CCG CCA CCG CCA GAG-3'
RHuVλBACK5Fv	5'-GAC GGC TGA GTC AGC ACA GAC TGC GAT CCG CCA CCG CCA GAG-3'
RHuVλBACK6Fv	5'-TGG GGC TGA GTC AGC ATA AAA TTC GAT CCG CCA CCG CCA GAG-3'
D. *Reamplification with primers containing restriction sites*	
Human V_H back primers	
HuVH1aBACKSfi	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTG GTG CAG TCT GG-3'
HuVH2aBACKSfi	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTC AAC TTA AGG GAG TCT GG-3'
HuVH3aBACKSfi	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAG GTG CAG CTG GTG GAG TCT GG-3'
HuVH4aBACKSfi	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTG CAG GAG TCG GG-3'
HuVH5aBACKSfi	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTG TTG CAG TCT GC-3'
HuVH6aBACKSfi	5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTA CAG CTG CAG CAG TCA GG-3'
Human J_κ forward primers	
HuJκ1BACKNot	5'-GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT TTC CAC CTT GGT CCC-3'
HuJκ2BACKNot	5'-GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT CTC CAG CTT GGT CCC-3'
HuJκ3BACKNot	5'-GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT ATC CAC TTT GGT CCC-3'
HuJκ4BACKNot	5'-GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT CTC CAC CTT GGT CCC-3'
HuJκ5BACKNot	5'-GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT AAT CTC CAG TCG TGT CCC-3'
Human J_λ forward primers	
HuJλ1FORNOT	5'-GAG TCA TTC TCG ACT TGC GGC CGC ACC TAG GAC GGT GAC CTT GGT CCC-3'
HuJλ2-3FORNOT	5'-GAG TCA TTC TCG ACT TGC GGC CGC ACC TAG GAC GGT CAG CTT GGT CCC-3'
HuJλ4-5FORNOT	5'-GAG TCA TTC TCG ACT TGC GGC CGC ACY TAA AAC GGT GAG CTG GGT CCC-3'

glucose (2 × TY-AMP-GLU) and 15% (v/v) glycerol for storage at −70 °C as a library stock.

(e) *Rescue of phagemid libraries*

To rescue phagemid particles from the library, 100 ml of 2 × TY-AMP-GLU was inoculated with 10^9 bacteria taken from the library stock (approx. 10 μl) and grown for 1·5 h, shaking at 37 °C. Cells were spun down (IEC-Centra 8, 4000 revs/min for 15 min) and resuspended in 100 ml of prewarmed (37 °C) 2 × TY broth containing 100 μg ampicillin/ml (2 × TY-AMP), 2×10^{10} plaque-forming units of VCS-M13 (Stratagene) particles were added and the mixture incubated 30 min at 37 °C without shaking. The mixture was then added to 900 ml of 2 × TY broth containing 100 μg ampicillin/ml and 25 μg kanamycin/ml (2 × TY-AMP-KAN), and grown overnight, shaking at 37 °C. Phage particles were purified and concentrated by three PEG-precipitations (Sambrook *et al.*, 1990) and resuspended in PBS to 10^{13} transducing units/ml (ampicillin-resistant clones).

(f) *Selection of phOx:BSA binders using tubes*

For selection, 75 mm × 12 mm immuno tube (Nunc; Maxisorp) was coated with 4 ml of phOx:BSA (1 mg/ml; 14 phOx per BSA: Mäkelä *et al.*, 1978) in PBS overnight at room temperature. After washing 3 times with PBS, the tube was incubated for 2 h and 37 °C with 2% MPBS for blocking. The wash was repeated and phagemid particles (10^{13} t.u.) in 4 ml of 2% MPBS added, incubated 30 min at room temperature, systematically inverting the tube using a rotating turntable, and then left undisturbed for a further 1·5 h at room temperature. Tubes were then washed 20 times with PBS, 0·1% (v/v) Tween 20 and 20 times with PBS (each washing step was performed by pouring buffer in and out immediately). Bound phage particles were eluted from the tube by adding 1 ml of 100 mM-triethylamine, inverting the tube using a rotating turntable for 15 min. The eluted material was immediately neutralized by adding 0·5 ml of 1·0 M-Tris·HCl (pH 7·4). Phage were stored at 4 °C. Eluted phage (in 1·5 ml) were used to infect 8 ml of logarithmic growing *E. coli* TG1 cells in 15 ml of 2 × TY broth, and plated on TYE-AMP-GLU plates as described above, yielding on average 10^7 t.u. For selection of phOx:BSA binders, the rescue–selection–plating cycle was repeated 4 times, after which phagemid clones were analysed for binding to both phOx:BSA and BSA.

(g) *Selection for lysozyme binders by panning and by affinity column*

A circular Petri dish (35 mm × 10 mm Falcon 3001 Tissue culture dish) was used for enrichment by panning. During all steps, the plates were rocked on an A600 rocking plate (Raven Scientific). Plates were coated overnight with 1 ml of TEL (3 mg/ml; Sigma) in 50 mM-sodium hydrogen carbonate (pH 9·6), washed 3 times with 2 ml of PBS, and blocked with 2 ml of 2% MPBS at room temperature for 2 h. Approximately 10^{13} t.u. phage in 1 ml of 2% MPBS were added per plate, and left rocking for 2 h at room temperature. Plates were washed for 5 min with 2 ml of the following solutions: 5 times with PBS; PBS, 0·02% Tween 20; 50 mM-Tris·HCl (pH 7·5), 500 mM-NaCl; 50 mM-Tris·HCl (pH 8·5), 500 mM-NaCl; 50 mM-Tris·HCl (pH 9·5), 500 mM-NaCl and finally 50 mM-sodium hydrogen carbonate (pH 9·6). Bound phage particles were then eluted by adding 1 ml of 100 mM-triethylamine and rocking for 5 min before neutralizing with 0·5 ml of 1 M-Tris·HCl (pH 7·4). Eluted phage was used to infect logarithmic growing *E. coli* TG1 as described above.

Alternatively, TEL-Sepharose columns were used for affinity purification. One ml columns of TEL coupled to Sepharose (as described by Ward *et al.*, 1989) were washed extensively with PBS, blocked with 5 ml of 2% MPBS, and 10^{13} t.u. phage in 1 ml of 2% MPBS loaded. Columns were washed with 50 ml of PBS; 10 ml of PBS, 0·02% Tween 20; 5 ml of 50 mM-Tris·HCl (pH 7·5), 500 mM-NaCl; 5 ml of 50 mM-Tris·HCl (pH 8·5), 500 mM-NaCl; 5 ml of 50 mM-Tris·HCl (pH 9·5) 500 mM-NaCl and finally 5 ml of 50 mM-sodium hydrogen carbonate (pH 9·6), 500 mM-NaCl. Bound phage were eluted using 1·5 ml of 100 mM-triethylamine and neutralized with 0·5 ml 1 M-Tris·HCl (pH 7·4). Eluted phage were used to infect logarithmically growing *E. coli* TG1 as described above.

For selection of lysozyme binders by either method, the rescue–selection–plating cycle was repeated 4 times, after which phagemid clones were analysed for binding by ELISA.

(h) *Rescue of phage or soluble scFv from individual phagemid clones for binding ELISA*

To rescue phage, single ampicillin-resistant colonies, resulting from infection of *E. coli* TG1 with eluted phage, were inoculated into 150 μl of 2 ×TY-AMP-GLU broth in 96-well plates (Cell wells; Corning) and grown with shaking (250 revs/min) overnight at 37 °C. A 96-well plate replicator was used to inoculate approximately 4 μl of the overnight cultures on the master plate into 200 μl fresh 2×TY-AMP-GLU. After 1 h, 50 μl of 2×TY-AMP-GLU broth containing 10^8 p.f.u. of VCS-M13 was added to each well, and the plate incubated at 37 °C for 45 min without agitation. The plate was then shaken at 37 °C for 1 h after which time glucose was removed by spinning down the cells (IEC-Centra 8, 4000 revs/min for 15 min), and aspirating the supernatant with a drawn-out glass Pasteur pipete. Cells were resuspended in 200 μl 2×TY-AMP-KAN broth and grown for 20 h, shaking at 37 °C. Supernatant containing phage was tested for binding by ELISA.

To produce soluble scFvs, single ampicillin-resistant colonies of infected *E. coli* HB2151, a non-suppressor strain (Carter *et al.*, 1985), were inoculated into 150 μl of 2×TY broth containing 100 μg ampicillin/ml and 0·1% glucose in 96-well plates and grown with shaking at 37 °C until an $A_{600\ \mathrm{nm}}$ of 0·9 was reached. Expression of soluble scFv was induced by the addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 1 mM (DeBellis & Schwartz, 1990) and the cultures grown overnight at 30 °C. Supernatant containing soluble scFv was taken for analysis by ELISA.

(i) *ELISA*

Analysis of phage for binding to phOx:BSA, BSA or lysozyme by ELISA was performed on bacterial supernatants containing phage essentially as described by Clackson *et al.* (1991), with 100 μg phOx:BSA or BSA/ml, or 3 mg TEL/ml used for coating. The specificity of isolated clones was checked by ELISA of the soluble scFv fragments using plates coated with various proteins at 1 mg/ml (hen egg ovalbumin, hen egg lysozyme, chymotrypsinogen A, cytochrome *c*, bovine thyroglobin, glyceraldehyde-3-phosphate dehydrogenase, chicken egg white trypsin inhibitor (Sigma), keyhole limpet haemocyanin (CalBiochem)). Binding of soluble scFvs to antigen was detected with the mouse monoclonal antibody 9E10 (1 μg/ml), which recognizes the C-terminal peptide tag (Munro & Pelham, 1986), and peroxidase-conjugated anti-mouse Fc antibody (Sigma), as described (Ward *et al.*, 1989).

(j) *DNA fingerprinting of clones*

The diversity of the original and selected libraries was determined by PCR screening (Güssow & Clackson, 1989). Recombinant clones were screened before and after selection by amplifying the scFv insert using primers LMB3 (5′-CAGGAAACAGCTATGAC, which sits upstream from the pelB leader sequence) and fd-SEQ1 (5′-GAATTTTCTGTATGAGG, which sits in the 5′ end of gene *3*) followed by digestion with the frequent-cutting enzyme *Bst*NI. The heavy and light chain variable regions from at least 2 clones of each restriction pattern were sequenced using a Sequenase kit (USB) by the dideoxy chain termination method (Sanger *et al.*, 1977). The nucleic acid sequences of the V-regions were compared with a database of germline V-genes to determine the family of origin and extent of somatic mutation.

(k) *Frequency of lambda and kappa light chains in the unselected IgM library*

The frequency of lambda and kappa light chains in the unselected IgM library was determined by probing replica-plated colonies with either an equimolar mixture of the V_λ PCR primers (Table 1) or an equimolar mixture of family-specific V_κ framework 1 probes (Marks *et al.*, 1991). One hundred individual colonies from the unselected IgM library were replica-plated on 2×TY-AMP-GLU plates and lifted onto nylon membranes (Hybond-N, 0·45 μm). The membranes were treated as described (Buluwela *et al.*, 1989) and then ultraviolet crosslinked for 5 min (Stratalinker; Stratagene). Membranes were prehybridized for 20 min at 42°C in hybridization solution (0·9 M-NaCl, 0·09 M-Tris (pH 7·5), 6 mM-EDTA (pH 7·4), 1 mM-sodium pyrophosphate, 0·5% (v/v) NP40, 0·6 mg/l rATP, 20 mg/l yeast RNA, 20 mg/l Ficoll 400, 20 mg/l polyvinylpyrrolidone and 20 mg/l BSA) and then hybridized for 2 h at 42°C with 10 pmol of (γ-^{32}P)-labelled oligonucleotide probe. Membranes were washed once at 42°C for 10 min in 6×SSC (900 mM-NaCl, 90 mM-trisodium citrate, pH 7·0), 0·1% (w/v) SDS, 0·1% (w/v) sodium pyrophosphate, once for 15 min at 55°C in 3 M-tetramethylammonium chloride, 50 mM-Tris (pH 8·0), 0·1% SDS, 2 mM-EDTA and exposed for 2 h on Fuji RX film.

(l) *Purification of scFvs and affinity determination*

The phOx binding scFv clone 15 (αphOx15) and the TEL binding scFv clone 9 (αTEL9), which gave the strongest ELISA signals, were chosen for affinity determination. Colonies of *E. coli* HB2151, a non-suppressor strain, harbouring the appropriate phagemid were used to inoculate 10 l of 2×TY containing 100 μg ampicillin/ml and 0·1% glucose. The cultures were grown to an $A_{600\ \mathrm{nm}}$ of 0·9 and expression of soluble scFv induced by the addition of IPTG to a final concentration of 1 mM (DeBellis & Schwartz, 1990). Supernatant was concentrated 8-fold by ultrafiltration (Filtron; Flowgen) and 200 ml loaded onto a 5 ml column of Protein A–Sepharose crosslinked by dimethylpimelidate (Harlow & Lane, 1988) to the monoclonal antibody 9E10 that recognizes the C-terminal peptide tag (Clackson *et al.*, 1991; Munro & Pelham, 1986). The column was washed with 100 ml of PBS; 10 ml of PBS, 0·5 M-NaCl; 10 ml of 0·2 M-glycine (pH 6·0); and

10 ml of 0·2 M-glycine (pH 5·0). The scFv fragment was eluted with 10 ml of 0·2 M-glycine (pH 3·0), neutralized with Tris base and dialysed into PBSE (PBS buffer containing 0·2 mM-EDTA). Supernatant from a separate induction of the αTEL9 scFv was purified on lysozyme–Sepharose (Ward *et al.*, 1989).

Affinities were measured by fluorescence quench techniques, based on the quenching of tryptophan fluorescence by the bound hapten or antigen (Eisen 1964; Foote & Milstein, 1991; J. Foote & G. Winter, unpublished results). All measurements were made with a Perkin–Elmer LS-5B spectrofluorimeter, using an excitation wavelength of 280 nm. Antibody (0·9 ml) in PBSE, was placed in a 4 mm × 10 mm cuvette in the instrument, and held at 20°C.

For determination of the affinity of αphOx15, fluorescence quench titration was performed essentially as described by Foote & Milstein (1991). A regime of hapten excess was used: the antibody concentration (100 nM) was at most equal to the lowest concentration of hapten. Negligible volumes of the hapten 4-γ-amino-butyric acid methylene 2-phenyl-oxazol-5-one (phOx-GABA) were added to αphOx15 protein to cover a concentration range of 0·2 to 4 times the preliminary estimate of the dissociation constant (500 nM), and the fluorescence detemined 1 min after each addition. Emission was monitored at 340 nm. Data were averaged from 3 runs and the value of the equilibrium constant was obtained from a least-squares fit of the data to a hyperbola.

Fluorescence quench titration was also used to determine the affinity of αTEL9 (Eisen, 1964; J. Foote & G. Winter, unpublished results). αTEL9 protein at 200 mM was titrated to 2-fold molar excess with TEL (Sigma) in PBSE, sample fluorescence being determined 1 min after each addition. Emission was monitored at 350 nm and the titration repeated 6 times. Five identical titrations with TEL were also performed on αphOx15 as control. The fluorescence data from each of the 6 titrations of αTEL9 were subtracted from the mean fluorescence values from the 5 control titrations of αphOx15 to account for the fluorescence contributed by the added TEL. To obtain the equilibrium constant, fluorescence data, averaged from the 6 corrected titrations of αTEL9, were fit by least-squares to a hyperbola.

(m) *Western blot*

Western blotting was performed essentially as described by Towbin *et al.* (1979). Samples (10 μg and 1 μg) of TEL were subjected to SDS/PAGE (Laemmli, 1970) and protein transferred by electroblotting to Immobilon-P (Millipore). The blot was blocked with PBS, 3% BSA for 20 min and then incubated with αTEL9 (1 μg/ml) in PBS, 3% BSA for 1·5 h. Binding of αTEL9 to lysozyme was detected with 9E10 (1 μg/ml) and peroxidase-conjugated anti-mouse Fc antibody (Sigma) as described Ward *et al.* (1989).

3. Results

(a) *Generation of scFv gene repertoires and libraries*

Single bands of the correct size for V_H, V_κ and V_λ cDNA were obtained after amplification of first strand cDNA made from RNA primed with the appropriate constant region primer (Table 1). No bands were obtained in the absence of a primer in the first strand cDNA reaction, indicating that the products resulted from the amplification of RNA and not DNA. A major band of the appropriate size for an assembled scFv gene was obtained when the V_H and V_κ, or V_H and V_λ, were combined with linker DNA in a PCR reaction. No product was obtained in the absence of linker DNA (data not shown).

Libraries of $2{\cdot}9\times10^7$ $V_{H\mu}$-V_L scFv clones (IgM library) and $1{\cdot}6\times10^8$ $V_{H\gamma}$-V_L scFv clones (IgG library) were obtained (Fig. 2). Analysis of 100 colonies from the IgM library by probing revealed that 81 carried either kappa or lambda light chains (45 (56%) for lambda and 36 (44%) for kappa). Analysis of 48 clones from each unselected library (IgM and IgG) indicated that greater than 90% of the clones carried an insert, and the libraries appeared to be extremely diverse as judged by the *Bst*NI restriction pattern (Fig. 3(a)).

(b) *Isolation and characterization of binders*

Phagemid particles were rescued from the library by superinfection with helper phage and selected by passing over either immobilized TEL or phOx:BSA. Eluted phage were used to infect *E. coli*, the library was again rescued with helper phage and the phagemid particles were subjected to a second

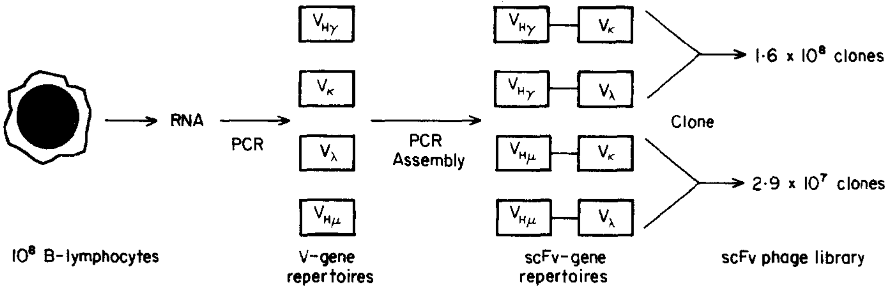

Figure 2. The origin of V-genes in the phage libraries. RNA made from 10^8 B-lymphocytes was primed with constant region-specific primers (for IgM, IgG, Cκ and Cλ) and 1st strand cDNA synthesized. Portions of 1st strand cDNA were used to amplify $V_{H\mu}$ and $V_{H\gamma}$ genes, and V_κ and V_λ genes. The V-genes were assembled together in separate PCR assembly reactions to generate 4 distinct scFv repertoires: $V_{H\mu}$-V_κ, $V_{H\mu}$-V_λ, $V_{H\gamma}$-V_κ and $V_{H\gamma}$-V_λ. The $V_{H\mu}$-V_κ and $V_{H\mu}$-V_λ repertoires were combined and cloned to generate a $V_{H\mu}$ scFv library of $2{\cdot}9\times10^7$ clones. Likewise the $V_{H\gamma}$-V_κ and $V_{H\gamma}$-V_λ repertoires were combined and cloned to generate a $V_{H\gamma}$ scFv library of $1{\cdot}6\times10^8$ clones.

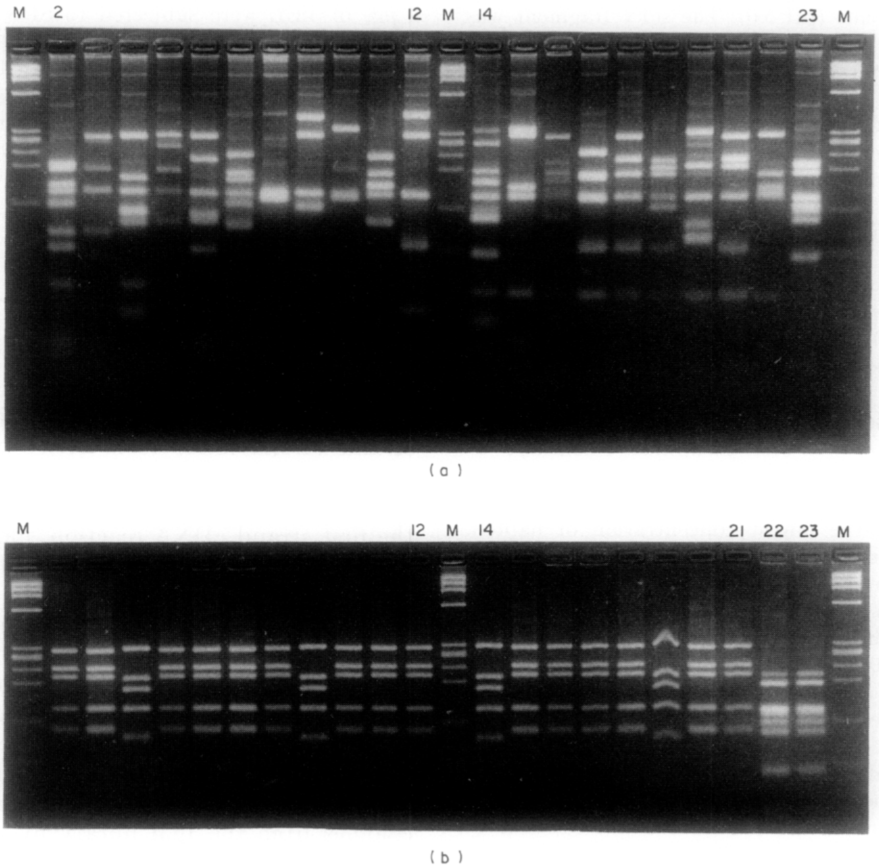

Figure 3. *Bst*N1 fingerprinting of scFv clones. The scFv insert was amplified from individual colonies, the product digested with *Bst*N1 and analysed on an agarose gel. M, ϕX174 DNA *Hae*III-digested molecular weight markers. (a) Lanes 2 to 12 and 14 to 23 are digests from colonies from the library before selection. (b) Lanes 2 to 12 and 14 to 21 are digests from 21 random colonies after 4 rounds of panning of the IgM library on TEL. Lanes 22 and 23 are digests of 2 other TEL binding clones obtained after 4 rounds of selection of the IgM or IgG library on a TEL column, respectively.

round of affinity purification. Four rounds of rescue–selection–infection were performed. Clones binding TEL, BSA and phOx were identified after four rounds of selection of the IgM library (Table 2). In contrast only clones binding TEL were identified after four rounds of selection of the IgG library (Table 2). Unselected clones and clones isolated after one and two rounds of selection showed no binding. Comparison of the frequency of binders to TEL and BSA obtained after three and four rounds of selection indicates up to 50-fold enrichment in the fourth round of selection. Thus, these binders must

Table 2
Frequency of binding clones from scFv libraries before and after selection

	Rounds of selection				
	0	1	2	3	4
A. *IgM library*					
Human anti-TEL: panning	0/864	0/192	0/192	3/192	94/192
Human anti-TEL: columns	–	–		–	19/96
Human anti-BSA: panning	0/192	0/192	0/192	2/192	43/96
Human anti-phOx: panning	0/192	0/192	0/192	0/192	1/96
B. *IgG library*					
Human anti-TEL: panning				–	0/96
Human anti-TEL: columns				–	6/96
Human anti-BSA: panning			–		0/96
Human anti-phOx: panning			–	–	0/96

Panning, antigen coated on Petri dish; columns, antigen covalently linked to Sepharose column; IgM library, single chain Fv library (scFv) with V_H genes derived from IgM mRNA; IgG library, scFv genes with V_H genes derived from IgG mRNA.

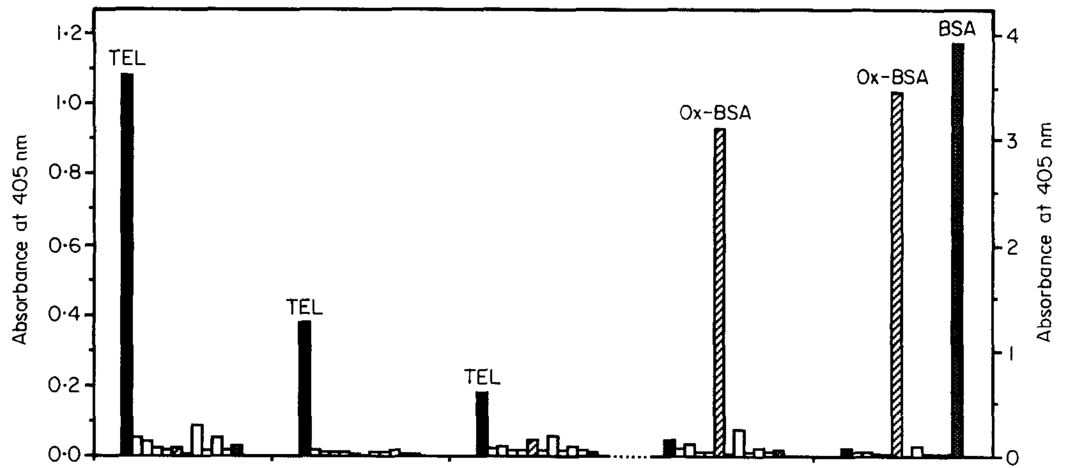

Figure 4. Specificity of soluble single chain Fvs (scFvs). Binding was determined by ELISA to a variety of proteins. αTEL9, αTEL13 and αTEL14 = 3 anti-turkey lysozyme scFvs; αphOx15 = anti-2-phenyloxazole-5-one scFv; αBSA3 = anti-bovine serum albumin scFv. Antigens: TEL (filled box), phOx-BSA (hatched box), BSA (stippled box); other antigens (open box) = keyhole limpet haemocyanin, bovine thyroglobulin, chymotrypsinogen A, hen-egg ovalbumin, cytochrome *c*, hen egg lysozyme, hen egg trypsin inhibitor, glyceraldehyde-3-phosphate dehydrogenase, and plastic.

plastic.

have been present in the original library at a frequency of 1 per $6{\cdot}25 \times 10^6$ clones ($1/50^4$) if enrichment were equal over the four rounds of selection.

*Bst*NI fingerprinting of 23 lysozyme binding clones from the IgM library indicated the presence of three different digestion patterns, whereas the six lysozyme binding clones obtained from the IgG library all had the same restriction pattern (Fig. 3(b), and data not shown). The *Bst*NI fingerprinting of 35 BSA binding clones indicated the presence of only one digestion pattern (data not shown) which was different from the pattern of the phOx binding clone.

The sequences of the variable regions of multiple clones representing the different restriction patterns indicated that there were four unique TEL binders (αTEL9, αTEL13, αTEL14 and αTEL16), one BSA binder (αBSA3) and one phOx binder (αphOx15) (Table 3). The V_Hs were derived from four different V_H families and five different V_H germline genes (Table 5). The light chains were mainly lambda (5/6) and were derived from four different light chain families and germline genes (Table 5). Both V-genes of αBSA3 were unmutated compared to germline (Tables 4 and 5). Similarly, the V-genes of αphOx15 were minimally mutated from germline (4 differences with VH380·6 (Berman *et al.*, 1988) and six with IGLV3S1 (Frippiat *et al.*, 1990)). Two other antibodies (αTEL13 and αTEL16) had heavy chains that are more extensively mutated (11 and 18 changes from VH251 (Sanz *et al.*, 1989)). Only upper estimates of mutation are possible for the other chains (Tables 4 and 5), as the sequences of all the germ-line V-genes from these families are not known. Finally, the TEL binder isolated from the IgG library (αTEL16) was highly related to one of the IgM TEL binders (αTEL13), and with a greater degree of somatic mutation.

(c) *Specificity of binding*

Soluble antibody fragments were readily prepared by growth of *E. coli* HB2151, a non-suppressor strain, carrying the phagemid (Hoogenboom *et al.*, 1991). Soluble scFvs of αphOx15, αBSA3, αTEL9, αTEL13 and αTEL14 were highly specific in an ELISA to test cross-reactivity (Fig. 4). The αTEL16 scFv, isolated from the IgG library, could not be detected in ELISA as a soluble fragment, probably due to its low affinity.

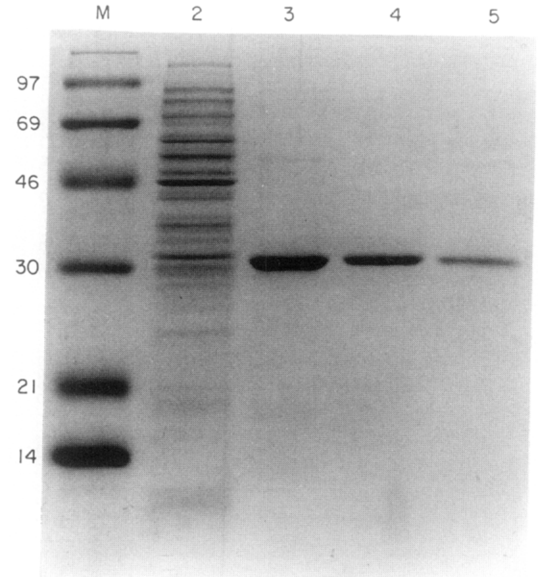

Figure 5. Purification of scFvs protein from a bacterial supernatant. M, molecular weight markers ($\times 10^{-3}$). Lane 2, unpurified bacterial supernatant; lane 3, αTEL9 scFv protein purified on a lysozyme–Sepharose column; lane 4, αTEL9 scFv protein purified on column of antibody 9E10 directed against the c-*myc* tag; lane 5, αphOx15 scFv protein purified as in lane 4.

Table 3

Deduced protein sequences of antigen-specific heavy and light chains selected from unimmunized libraries

A. *Heavy chains*

Clone	FR 1	CDR 1	FR 2	CDR 2	FR 3	CDR 3	FR 4
αphOx15	QVQLVQSGAEVKKPGASVKVSCKASGYTFT	SYGIS	WVRQAPGQGLEWMG	WISAYNGNTKYAQKLQG	RVTMTTDTSTSTAYMELRSLRSDDTAVYYCVR	LLPKRTATLHYYIDV	WGKGTLVTVSS
αBSA3	QVQLVQSGGGVVQPGRSLRLSCAASGFTFS	SYGMH	WVRQAPGKGLEWVA	VISYDGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK	TGYSSGWGYFDY	WGQGTLVTVSS
αTEL9	QVQLQQSGSGLVKPSQTLSLTCSVSGDSIS	SGGYSWS	WIRQPSGKGLEWIG	SVHHSGPTYYNPSLKS	RVTMSVDTSKNQFSLKLKSVTAADTAMYFCAR	EGGSTWRSLYKHYYMDV	WGKGTLVTVSS
αTEL14	QVQLQESGPGLVKPSETLSLVCTVSGGSLS	FSYWG	WIRQPPGKGLEWIG	YISHRGTDYNSSLQS	RVTISADTSKNQFSLKLSSVTAADTAVYYCAR	SFSNSFFFGY	WGQGTLVTVSS
αTEL13	QVQLVQSGAEVKKPGQSLMISCQGSGYSFS	NYWIG	WVRQMPGKGLEWMG	IIYPGDSDTRYSPSFQG	QVTISADKSISTAYLHWSSLKASDTALYYCAR	LVGGTPAY	WGQGTLVTVSS
αTEL16	QVQLVQSGAEVKKPGQSLRISCKGAGYSFS	TYWIG	WVRQMPGKGLEWMG	IIYPDDSDTRYSPSFEG	QVTISVDKSITTAYLHWSSLKASDTAIYYCAR	LVGGAPAY	WGQGTLVTVSS

B. *Light chains*

Clone	FR 1	CDR 1	FR 2	CDR 2	FR 3	CDR 3	FR 4
αphOx15	QSVLTQPPSVSAAPGQKVTISC	SGSSSNIGNNYVS	WYQHLPGTAPNLLIY	DNNKRPS	GIPDRFSGSKSGTSATLGITGLQTGDEADYYC	GTWDGRLTAAV	FGSGTKVTVLG
αBSA3	SSELTQDPAVSVALGQTVRITC	QGDSLRSYYAS	WYQQKPGQAPVLVIY	GKNNRPS	GIPDRFSGSSSGNTASLTITGAQAEDEADYYC	NSRDSSGNHVV	FGGGTKLTVLG
αTEL9	EIVLTQSPSSLSASVGDRVTITC	RASQSISNYLN	WYQQKPGKAPKLLIY	AASTLQS	GVPSRFSGSGSGTDFTLTINSLQPEDFATYYC	QQTNSFPLT	FGGGTKLEIKR
αTEL14	SSELTQDPAVSVAFGQTVRITC	QGDSLRSSYAS	WYQQKPGQAPLLVIY	GENSRPS	GIPDRFSGSSSGNTASLTITGAQAEDEADYYC	NSRDSRGTHLEV	FGGGTKLTVLG
αTEL13	HVILTQPASVSGSPGQSITISC	TGSSRDVGGYNYVS	WYQHHPGKAPKLLIS	EVTNRPS	GVSNRFSGSKSGNTASLTISGLQAEDEADYFC	ASYTSSKTYV	FGRGTKLTVLG
αTEL16	QSALTQPASVSGSPGQSITISC	SGSSSDIGRYDYVS	WYQHYPDKAPKLLIY	EVKHRPS	GISHRFSASKSGNTASLTISELQPGDEADYYC	ASYTESKTYI	FGGGTKVTVLG

FR, framework region; CDR, complementarity-determining region.

Table 4

Nucleotide sequences of antigen specific heavy and light chain V-genes selected from unimmunized libraries compared with the most homologous germline gene

A. *Heavy chains*

	10	20	30	40	50	60	70	80	90	100
VH380.6	CAGGTTCAGC	TGGTGCAGTC	TGGAGCTGAG	GTGAAGAAGC	CTGGGGCCTC	AGTGAAGGTC	TCCTGCAAGG	CTTCTGGTTA	CACCTTTACC	AGCTATGGTA
αphOx15	-----g----	----------	---G------	----------	----------	----------	----------	----------	----------	----------
	110	120	130	140	150	160	170	180	190	200
VH380.6	TCAGCTGGGT	GCGACAGGCC	CCTGGACAAG	GGCTTGAGTG	GATGGATGG	ATCAGCGCTT	ACAATGGTAA	CACAAACTAT	GCACAGAAGC	TCCAGGGCAG
αphOx15	----------	----------	----------	----------	----------	----------	----------	------G---	----------	----------
	210	220	230	240	250	260	270	280	290	
VH380.6	AGTCACCATG	ACCACAGACA	CATCCACGAG	CACAGCCTAC	ATGGAGCTGA	GGAGCCTGAG	ATCTGACGAC	ACGGCCGTGT	ATTACTGTGC	GAGA
αpHOx15	----------	----------	----------	----------	-----A----	----------	----------	----------	---------T	----
	10	20	30	40	50	60	70	80	90	100
VH1.9III	CAGGTGCAGC	TGGTGGAGTC	TGGGGGAGGC	GTGGTCCAGC	CTGGGAGGTC	CCTGAGACTC	TCCTGTGCAG	CCTCTGGATT	CACCTTCAGT	AGCTATGGCA
αBSA3	----------	-----c----	----------	----------	----------	----------	----------	----------	----------	----------
	110	120	130	140	150	160	170	180	190	200
VH1.9III	TGCACTGGGT	CCGCCAGGCT	CCAGGCAAGG	GGCTGGAGTG	GGTGGCAGTT	ATATCATATG	ATGGAAGTAA	TAAATACTAT	GCAGACTCCG	TGAAGGGCCG
αBSA3	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------
	210	220	230	240	250	260	270	280	290	
VH1.9III	ATTCACCATC	TCCAGAGACA	ATTCCAAGAA	CACGCTGTAT	CTGCAAATGA	ACAGCCTGAG	AGCTGAGGAC	ACGGCTGTGT	ATTACTGTGC	GAAA
αBSA3	----------	----------	----------	----------	----------	----------	----------	----------	----------	----
	10	20	30	40	50	60	70	80	90	100
U514A	CAGGTGCAGC	TGCAGGAGTC	GGGCCCAGGA	CTGGTGAAGC	CTTCACAGAC	CCTGTCCCTC	ACCTGCACTG	TCTCTGGTGG	CTCCATCAGC	AGTGGTGGTT
αTEL9†	-----a----	-----c----	a---T-----	----------	----------	----------	------T---	---------A	-------TC-	----------
U514G	----------	----------	----------	----------	----GG----	----------	------G---	--------TA	----------	----------
	110	120	130	140	150	160	170	180	190	200
U514A	ACTACTGGAG	CTGGATCCGG	CAGCCCCCAG	GGAAGGGACT	GGAGTGGATT	GGGTACATCT	ATTACAGTGG	GAGCACCTAC	TACAACCCGT	CCCTCAAGAG
αTEL9	---C------	----------	-----AT---	-------G--	----------	---AGTG--C	--C-------	-CC-------	----------	----------
U514G	--------G-	----------	----------	-------G--	----------	---AGT----	--C-T-----	----------	----------	----------
	210	220	230	240	250	260	270	280	290	
U514A	TCGAGTTACC	ATATCAGTAG	ACACGTCTAA	GAACCAGTTC	TCCCTGAAGC	TGAGCTCTGT	GACTGCCGCG	GACACGGCCG	TGTATTACTG	TGCGAGA
αTEL9	------C---	--G-----T-	-------C--	----------	--------AT	---AG-----	---C-----A	---------A	------TT--	-------
U514G	------C---	----------	-------C--	----------	----------	----------	---C-----A	----------	----------	-------
	10	20	30	40	50	60	70	80	90	100
U4H	CAGGTGCAGC	TGCAGGAGTC	GGGCCCAGGA	CTGGTGAAGC	CTTCGGAGAC	CCTGTCCCTC	ACCTGCACTG	TCTCTGGTGG	CTCCATCAGT	AGTTACTACT
αTEL14	----------	----------	---G------	----------	----------	----------	GT--------	----------	----C-----	TT--C-----
	110	120	130	140	150	160	170	180	190	200
U4.H	GGAGCTGGAT	CCGGCAGCCC	CCAGGGAAGG	GACTGGAGTG	GATTGGGTAT	ATCTATTACA	GTGGGAGCAC	CAACTACAAC	CCCTCCCTCA	AGAGTCGAGT
αTEL14	--G-------	----------	----------	----------	------C--C	----C-C---	-G--- --	-G-------T	T--------C	----------
	210	220	230	240	250	260	270	280	290	
U4.H	CACCATATCA	GTAGACACGT	CCAAGAACCA	GTTCTCCCTG	AAGCTGAGCT	CTGTGACCGC	TGCGGACACG	GCCGTGTATT	ACTGTGCGAG	A
αTEL14	----------	-C--------	----------	----------	---T------	----------	----------	----------	----------	-

Table 4 *(continued)*

	10	20	30	40	50	60	70	80	90	100
VH251	GAGGTGCAGC	TGGTGCAGTC	TGGAGCAGAG	GTGAAAAAGC	CCGGGGAGTC	TCTGAAGATC	TCCTGTAAGG	GTTCTGGATA	CAGCTTTACC	AGCTACTGGA
αTEL13	c---------	----------	----------	----------	-----C----	-----T----	------C---	----------	--------G-	-A--------
αTEL16	c---------	----------	---G------	----------	-----C----	-----G----	----------	--G-------	--------G-	-C--------
	110	120	130	140	150	160	170	180	190	200
VH251	TCGGCTGGGT	GCGCCAGATG	CCCGGGAAAG	GCCTGGAGTG	GATGGGGATC	ATCTATCCTG	GTGACTCTGA	TACCAGATAC	AGCCCGTCCT	TCCAAGGCCA
αTEL13	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------
αTEL16	----------	----------	----------	----------	----------	----------	A---------	----------	--T-------	--G-G-----
	210	220	230	240	250	260	270	280	290	
VH251	GGTCACCATC	TCAGCCGACA	AGTCCATCAG	CACCGCCTAC	CTGCAGTGGA	GCAGCCTGAA	GGCCTCGGAC	ACCGCCATGT	ATTACTGTGC	GAGA
αTEL13	----------	----------	----------	----------	--C--C----	----------	----------	------C-T-	-C--------	---G
αTEL16	----------	--C-T-----	---------C	T-----A---	-----C----	----------	----------	--------T-	----------	----

B. *Light chains*

	10	20	30	40	50	60	70	80	90	100
JMλ1A	CAGTCTGTGT	TGACGCAGCC	GCCCTCAGTG	TCTGCGGCCC	CAGGACAGAA	GGTCACCATC	TCCTGCTCTG	GAAGCAGCTC	CAACATTGGG	AATAATTATG
αphOx15	----------	----------	----------	----------	-------A--	----------	----------	----------	----------	----------
	110	120	130	140	150	160	170	180	190	200
JMλ1A	TATCCTGGTA	CCAGCAGCTC	CCAGGAACAG	CCCCCAAACT	CCTCATTTAT	GACAATAATA	AGCGACCCTC	AGGGATTCCT	GACCGATTCT	CTGGCTCCAA
αphOx15	----------	------C---	----------	-------T--	----------	----------	----------	----------	----------	----------
	210	220	230	240	250	260	270	280	290	
JMλ1A	GTCTGGCACG	TCAGCCACCC	TGGGCATCAC	CGGACTCCAG	ACTGGGGACG	AGGCCGATTA	TTACTGCGGA	ACATGGGATA	GCAGCCTGAG	TGCT
αphOx15	----------	----------	----------	----------	----------	----------	----------	--C------G	--C------C	----

	10	20	30	40	50	60	70	80	90	100
IgLV3S1	TCTTCTGAGC	TGACTCAGGA	CCCTGCTGTG	TCTGTGGCCT	TGGGACAGAC	AGTCAGGATC	ACATGCCAAG	GAGACAGCCT	CAGAAGCTAT	TATGCAAGCT
αBSA3	--g-------	----------	----------	----------	----------	----------	----------	----------	----------	----------
αTEL14	--g-------	----------	----------	----------	-C--------	----------	----------	-------T--	------T-C-	----------
	110	120	130	140	150	160	170	180	190	200
IgLV3S1	GGTACCAGCA	GAAGCCAGGA	CAGGCCCCTG	TACTTGTCAT	CTATGGTAAA	AACAACCGGC	CCTCAGGGAT	CCCAGACCGA	TTCTCTGGCT	CCAGCTCAGG
αBSA3	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------
αTEL14	----------	----------	---------C	----------	-------G--	----G-----	----------	----------	----------	----------
	210	220	230	240	250	260	270	280		
IgLV3S1	AAACACAGCT	TCCTTGACCA	TCACTGGGGC	TCAGGCGGAA	GATGAGGCTG	ACTATTACTG	TAACTCCCGG	GACAGCAGTG	GTAACCAT	
BSA3	----------	----------	----------	----------	----------	----------	----------	----------	--------	
αTEL14	----------	----------	----------	----------	-----A----	----------	----------	--------G-	---C----	

```
                    10         20         30         40         50         60         70         80         90        100
JMVλ2F.1    CAGTCTGCCC TGACTCAGCC TGCCTCCGTG TCTGGGTCTC CTGGACAGTC GATCACCATC TCCTGCACTG GAACCAGCAG TGATGTTGGG AGTTATAACC
αTEL13      --cgt-ata- -------a-- ---------- ---------- ---------- ---------- ---------- ---G------ A--C-----T G--------T
αTEL16      --------t- ---------- ---------- ---------- ---------- ---------- -------G-- ---G------ ---CA----T C-----G--T
                   110        120        130        140        150        160        170        180        190        200
JMVλ2F.1    TTGTCTCCTG GTACCAACAG CACCCAGGCA AAGCCCCCAA ACTCATGATT TATGAGGGCA GTAAGCGGCC CTCAGGGGTT TCTAATCGCT TCTCTGGCTC
αTEL13      A--------- ---T--G--T ---------- ---------- ----C-A--- -C-----T-- C---T----- G--------- --C------- ----------
αTEL16      A--------- ---------T T----T-A-- ---------- ------C--- -------T-- AAC-T----- -------A-A ---C------ ------C---
                   210        220        230        240        250        260        270        280        290
JMVλ2F.1    CAAGTCTGGC AACACGGCCT CCCTGACAAT CTCTGGGCTC CAGGCTGAGG ACGAGGCTGA TTATTACTGC AGCTTATATA CAAGCAGCAG CACT
αTEL13      A--------- ---------- -------C-- T--------- --------A- ---------- -----T---- GC--C----- ----TTC--A G---
αTEL16      ------C--- ---------- -------C-- -----A---- ---C---GA- ---------- ---------- GC--C----- --GAA--T-A G---
```

```
                    10         20         30         40         50         60         70         80         90        100
HK137       GACATCCAGA TGACCCAGTC TCCATCCTCA CTGTCTGCAT CTGTAGGAGA CAGAGTCACC ATCACTTGTC GGGCGAGTCA GGGCATTAGC AATTATTTAG
αTEL9       g-a--tgtgt ----g----- ---------C ---------- ---------- ---------- --------C- ----A----- -A-------- --C------A
                   110        120        130        140        150        160        170        180        190        200
HK137       CCTGGTTTCA GCAGAAACCA GGGAAAGCCC CTAAGCTCCT GATCTATGCT GCATCCAGTT TGCAAAGTGG GGTCCCATCA AGGTTCAGCG GCAGTGGATC
αTEL9       AT----A--- ---------- ---------- ---------- ---------- -------C-- ---------- ---------- ---------- ----------
                   210        220        230        240        250        260        270        280
HK137       TGGGACAGAT TTCACTCTCA CCATCAGCAG CCTGCAGCCT GAAGATTTTG CAACTTATTA CTGCCAACAG TATAATAGTT ACCCT
αTEL9       ---------- ---------- ------A--- ---------- ---------- -------C-- T--T------ AC---C---- TT--G
```

Lower case, differences from germline genes encoded by the PCR primer, complementarity-determining regions (CDRs) are underlined. αBSA3, bovine serum albumin binder; αphOx15, 2-phenyloxazol-5-one binder; αTEL9, αTEL13, αTEL14 and αTEL16, turkey egg lysozyme binders. References for germline genes: VH380·6, U514A, U514G, U4H, JMλ1A and JMλ2F: M. B. Llewelyn, J. D. Marks, I. M. Tomlinson, G. Walter & G. Winter, unpublished results; VH1·9III: Berman *et al.* (1988); VH251: Sanz *et al.* (1989); IgLV3S1: Frippiat *et al.* (1990); HK137: Bentley & Rabbitts (1983). Nucleotide and protein sequences have been deposited with the European Molecular Biology Library (accession numbers X61640 to X61651 inclusive). phOx-binding phage with a mouse heavy chain and human light chains were identified in addition to the entirely human αphOx15, but are not included in this paper. The mouse heavy chain corresponded to the VHB domain of Clackson *et al.* (1991) that had been isolated in the same laboratory, and presumably arose from contamination during the library construction. This demonstrates the importance of completely sequencing all antibody constructs.

† αTEL9 appears to be derived partially from germline genes U514A and U514G, suggesting that it is a result of PCR cross-over between 2 highly related V_Hs.

Table 5

V-gene family, germline derivation and extent of somatic hypermutation of antigen-specific clones isolated from unimmunized libraries

	V_H			V_L		
Clone	Family	Germline gene	Differences from germline	Family	Germline gene	Differences from germline
αBSA3	V_{H3}	VH1·9III	0	$V_{\lambda 3}$	IGLV3S1	0
αphOx15	V_{H1}	VH380·6	4	$V_{\lambda 1}$	JMVλ1A	7
αTEL9	V_{H4}	U514A (U514G)	<22	$V_{\kappa 1}$	HK137	<20
αTEL14	V_{H4}	U4·H	<19	$V_{\lambda 3}$	IGLV3S1	<10
αTEL13	V_{H5}	VH251	11	$V_{\lambda 2}$	JMVλ2F	<31
αTEL16	V_{H5}	VH251	18	$V_{\lambda 2}$	JMVλ2F	<38

αBSA3, bovine serum albumin binder; αphOx15, 2-phenyl-oxazol-5-one binder; αTEL9, αTEL13, αTEL14 and αTEL16, turkey egg lysozyme binders. References for germline genes: see Table 4.

(d) *Protein purification and binding affinity*

Soluble scFv αTEL9 was purified in one step on a TEL-Sepharose column or *via* its c-*myc* peptide tag on a 9E10 antibody column (Fig. 5). Soluble scFv αphOx15 was purified in one step on a 9E10 column (Fig. 5). Typical yields were 2 mg/l after purification on 9E10 and 5 to 10 mg/l after purification on an antigen column. The dissociation constant of the αTEL9 scFv was 86(±61) nM and the dissociation constant of the αphOx15 scFv was 534(±72) nM. The high standard error observed for the dissociation constant of αTEL9 has been observed for hen egg lysozyme binding antibodies using this technique. However, equilibrium constants obtained by fluorescence quench titration are consistent with those deduced by the more precise pseudo-equilibrium relaxation method (J. Foote & G. Winter, unpublished results). Finally, soluble αTEL9 scFv could be used to detect lysozyme (1 μg) in a Western blot (data not shown).

4. Discussion

We used a phage display library utilizing V-gene repertoires to isolate antibody fragments of reasonable affinity against three different (foreign) antigens. The two donors were unimmunized, and their serum IgM antibodies did not appear to bind to the antigens TEL or phOx-BSA as there was no difference in signal intensity in wells coated with antigen compared with control wells not containing antigen. Furthermore most of the VH genes of the binders derive from the IgM (naive and primary response B-cells) rather than the IgG mRNA (secondary response B-cells). Each of the heavy and light chain pairings in Table 5 is unique and contrasts with the promiscuous pairings (in which one chain is associated with more than one partner) noted in libraries from the IgG mRNA from immunized animals (Clackson *et al.*, 1991; Caton & Koprowski, 1990; Persson *et al.*, 1991). Therefore, the library appears to be naive with respect to these antigens.

A recent attempt to isolate human antibodies from an unimmunized donor using a λ phage random combinatorial library failed (Persson *et al.*, 1991). The library (10^6 members) was constructed from IgG mRNA using only PCR primers for V_{H1}, V_{H3}, $V_{\kappa 1}$ and $V_{\kappa 3}$ gene families and was screened for antigen binding using nitrocellulose filters. However, library size, diversity and binding threshold determine the chances of isolating binders. The probability (p) that an epitope is not recognized by at least one antibody in a library depends on the probability ($p[K]$) that an individual antibody recognizes a random epitope with an affinity above a threshold value ($[K]$) and on the number of different antibodies (N) according to the equation $p = e^{-Np[K]}$ (Perelson, 1989).

We attempted to maximize the size of the library by using a pUC-based phagemid (Hoogenboom *et al.*, 1991) that has higher transformation efficiencies than fd vectors. Indeed our library sizes (10^7 to 10^8 members) were at least an order of magnitude greater than with phage fd (Clackson *et al.*, 1991). We also attempted to maximize diversity by using primers optimized for each V-gene family, as well as utilizing IgG and IgM mRNA and both κ and λ light chains. The V_H genes of the binders belong to four different families (V_H families 1, 3, 4 and 5), as do the light chain genes (V_λ families 1, 2 and 3, and V_κ family 1). Furthermore, most (5/6) of the binders were derived from the IgM mRNA, perhaps reflecting the greater diversity of V_H genes. Indeed the only binder from the IgG mRNA (αTEL16) had the poorest binding affinity and/or decreased expression and was barely detectable by ELISA.

The chances of finding a phage with binding activity also depend on its affinity and the efficiency and number of rounds of selection. Both phage (McCafferty *et al.*, 1990; Scott & Smith, 1990) and phagemid (Hoogenboom *et al.*, 1991; Bass *et al.*, 1990) vectors have been used to display peptide or protein fusions with g3p. The phage vectors allow three copies of the g3p fusion protein on each phage particle (Glaser-Wuttke *et al.*, 1989), whereas the g3p fusion protein encoded by phagemid vectors has to compete with the g3p of the helper phage for incorporation into the phagemid particle. Although phage vectors should permit isolation of a greater number of binders, by virtue of the avidity of binding of the multivalent antibody heads, many will have poor affinities. To enrich for the higher-affinity antibodies, we used phagemid vectors. We noted lower selection efficiencies with phagemid (50-fold/round), compared to 675 to 1000-fold per round for phage vectors (Clackson *et al.*, 1991; McCafferty *et al.*, 1990). We found that three or four rounds of selection were required to isolate the binders, and estimate that only one or two copies of each were present in the original library of 3×10^7 members.

The binders utilize both germline and mutated V-genes. Most of the differences are likely to have arisen as a result of somatic mutation of the V-genes in the original B-cells, but some may have arisen during the PCR amplification and assembly process. Indeed the heavy chain of αTEL9 may have arisen from a cross-over during PCR amplification between rearranged V_H-genes from two highly related germline genes U514A and U514G (Table 4). Surprisingly, most of the binders (5/6) utilized V_λ rather than V_κ genes despite their equal representation in the unselected library. However, human hybridomas prepared by EBV immortalization often secrete IgM and λ chains (Thompson *et al.*, 1991), and during maturation of the immune response, the repertoire may shift from IgM, λ antibodies to IgG, κ (Thompson *et al.*, 1991; J. Bye, N. Hughes-Jones, J. D. Marks & G. Winter, unpublished results).

By using phagemid vectors we can mimic the switch of antibody from its display on B-cells to its secretion by plasma cells. By interposing a stop codon between the antibody and g3p, the antibody fragments can be switched between surface display, or secretion as a soluble fragment from bacteria, by growth in suppressor or non-suppressor strains of bacteria (Hoogenboom *et al.*, 1991). The affinities of two of the soluble antibody fragments αphOx15 and αTEL9, prepared in this way, as determined by fluorescence quench ($K_a = 2 \times 10^6\ \mathrm{M}^{-1}$ and $10^7\ \mathrm{M}^{-1}$, respectively), appear similar to those of human IgM antibodies derived from PBLs after immunization. For example the affinities of human IgM antibodies directed against rhesus D antigen, and made by EBV immortalization of PBLs from immunized donors lie in the range of $10^7\ \mathrm{M}^{-1}$ (Hughes-Jones & Gorick, 1991).

The antibody fragments isolated from the library are also highly specific (Fig. 4) to the antigen used in panning. For example, those fragments isolated using TEL did not bind to a range of other protein antigens, including hen egg white lysozyme that differs by only seven amino acids (Imoto *et al.*, 1972). The monovalent αTEL9 fragment could even be used in Western blotting but the sensitivity (1 μg TEL) was poor.

Although we can make human antibodies with reasonable affinity and specificity, a yet more diverse and large library should enable the isolation of even higher-affinity antibodies (Perelson, 1989). For example, the rearranged V_H genes would reflect more the naive B-cell repertoire if they had been prepared from the mRNA of membrane-bound IgM or IgD (for example, by basing primers for cDNA synthesis in the membrane anchor region). Other diverse libraries might be constructed by assembling unrearranged V-genes with synthetic D and J elements, or by assembling diverse antigen binding loops on a common structural framework (Milstein, 1990). Larger libraries could be made by improving transfection and ligation efficiencies and by scale-up, or by encoding repertoires of light chains on one vector and heavy chains on another (Hoogenboom *et al.*, 1991).

Alternatively higher-affinity antibodies might be made by mutating the binders and selecting those with improved affinity (Winter & Milstein, 1991). Point mutants could be made in a variety of ways; for example, using an error-prone polymerase (Liao & Wise, 1990), spiked oligonucleotides (Hermes *et al.*, 1989), or growth of the phage in mutator strains of bacteria (Schaaper, 1988; Yamagishi *et al.*, 1990). For more extensive variation, artificial cross-overs could be induced with related genes using the polymerase chain reaction (Meyerhans *et al.*, 1990), or light or heavy chains replaced by repertoires (Clackson *et al.*, 1991). Selection of antibodies on phage according to affinity has demonstrated that, for example, high-affinity binding phage ($10^8\ \mathrm{M}^{-1}$) can be fractionated 10^4-fold with respect to low-affinity phage ($10^5\ \mathrm{M}^{-1}$) using only two rounds of selection (Clackson *et al.*, 1991). By using several rounds of selection and adjusting the coating density of the antigen used for panning, it is also possible to select between phages bearing antibodies that are much closer in affinity. However, phagemid vectors leading to display of only a single copy of the antibody on the surface of the phage are preferable for selection between phages with closely related affinities when using antigen immobilized on solid phase (T.P.B. & G.W., unpublished results).

For making high-affinity antibodies, phage display libraries built from the spleen mRNA of hyperimmunized animals (Clackson *et al.*, 1991), or PBL mRNA of deliberately immunized humans remain attractive. However, immunization is often difficult, and new libraries have to be constructed for each antigen. In contrast, a single library made without immunization may provide a rich source of antibody specificities, including those directed against "naive" antigens (as described above), common pathogens or self antigens. For example, from the same library as above, we have isolated specificities directed to human blood group B, human tumour necrosis factor-α, and a human monoclonal antibody (our unpublished results). We propose the term "natural" libraries for those derived from unimmunized donors, and envisage that human antibodies of many specificities will be made in the future by panning a single large natural phage display library with antigen.

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