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Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*

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IN antibodies, a heavy and a light chain variable domain, VH and VL, respectively, pack together and the hypervariable loops on each domain contribute to binding antigen[1-4]. We find, however, that isolated VH domains with good antigen-binding affinities can also be prepared. Using the polymerase chain reaction[5], diverse libraries of VH genes were cloned from the spleen genomic DNA of mice immunized with either lysozyme or keyhole-limpet haemocyanin. From these libraries, VH domains were expressed and secreted from *Escherichia coli*. Binding activities were detected against both antigens, and two VH domains were characterized with affinities for lysozyme in the 20 nM range. Isolated variable domains may offer an alternative to monoclonal antibodies and serve as the key to building high-affinity human antibodies. We suggest the name 'single domain antibodies (dAbs)' for these antigen binding demands.

We have analysed the interactions with antigen of individual domains of the anti-lysozyme antibody, D1.3 (ref. 1). The VH domain was expressed in *E. coli* and secreted into the periplasm[6,7], alone or in association with the Vκ domain (fig. 1). Analysis of culture medium, by passage through a lysozyme-Sepharose affinity column[8], followed by SDS-PAGE[9] revealed that both the isolated VH domain, or the associated Fv fragment, could bind to lysozyme, and could be purified to homogeneity in a single step, with yields of ~200 μg l^{-1} and 10 mg l^{-1}, respectively. The VH domain appears to be monomeric by FPLC (Pharmacia, Superose 12 column). The N-terminal sequences of both domains were checked by gas-phase protein sequencing[10,11].

As shown in Table 1, the affinity of Fv fragment for lysozyme and the stoichiometry of binding of the VH domain to lysozyme were determined by titration using fluorescence quench techniques. The affinity of VH domain for lysozyme was determined from the kinetics of binding. The affinity of the Fv fragment (3 nM) is similar to the parent antibody (2 nM). The VH domain binds lysozyme tightly in an equimolar complex with an affinity for lysozyme (19 nM) which is only 10-fold weaker. Separated heavy and light chains have previously been identified with antigen[12] or hapten binding activities[13] although the affinities were poor, with no evidence for binding by single chains[13,14] rather than dimers[15].

In the D1.3 antibody, lysozyme interacts extensively with both domains, and forms three hydrogen bonds to the Vκ domain, and nine hydrogen bonds to the VH domain. Binding of lysozyme buries ~300 $Å^2$ of Vκ domain away from solvent, and 400 $Å^2$ of the VH domain[1]. Our results show, however, that the Vκ domain makes only a small net contribution to the energetics of binding. This is surprising as the removal of a single hydrogen bond[16] or a single van der Waals contact[17] can lead to tenfold loss in affinity. The VH domain presumably binds to lysozyme in a similar way to the antibody and this is consistent with inhibition of binding of the Fv fragment by the VH domain (data not shown, but see Fig. 1 legend). It is possible that the whole surface of interaction might reorientate slightly, perhaps by rocking on side chains, to create a new set of contacts[18].

The result prompted us to obtain VH domains with antigen-binding activities from antibody-producing cells. Previously we have demonstrated the cloning of immunoglobulin variable regions from hybridoma mRNA for expression of chimaeric antibodies, using the polymerase chain reaction (PCR)[5,19]. We now used PCR to amplify the rearranged VH genes from the spleen DNA of a mouse immunized with lysozyme (Fig. 2). The amplified DNA was cloned into the vector M13VHPCR1 (ref. 19) for sequencing. The complete sequences of 48 VH gene clones were determined (data not shown). All but two of the mouse VH gene families[20] were represented, with frequencies of: VA (1), IIIC (1), IIIB (8), IIIA (3), IIB (17), IIA (2), IB (12) and IA (4). In 30 clones the D segments could be assigned to families SP2 (14), FL16 (11) and Q52 (5), and in 38 clones the JH minigenes to families JH1 (3), JH2 (7), JH3 (14) and JH4 (14). The different sequences of CDR3 marked each of the 48 clones as unique. Nine pseudogenes and 16 unproductive rearrangements were identified; of the clones sequenced, 27 have open reading frames. Clearly we can generate a diverse repertoire of VH genes using PCR, but cannot rule out a systematic bias due to our choice of primers and hybridization conditions. VH gene libraries have also been generated using PCR from mRNA of human peripheral blood lymphocytes (J. Marks, D.G. & G.W., unpublished data) and from mRNA of mouse spleen[21].

Amplified DNA was then cloned for expression into a vector which incorporates a C-terminal peptide tag to facilitate detection of expressed VH domains (Fig. 1*f*). Bacterial supernatants were analysed by SDS-PAGE followed by western blotting[22], and bands of the expected size ($M_r \approx 14{,}000$) were detected for 14 of the 17 clones by probing with antibody directed against the tag[23,24]. To screen for lysozyme binding activities, about two thousand colonies were toothpicked in groups of five into wells of enzyme-linked immunosorbent assay (ELISA) plates, and the supernatants tested for binding to lysozyme-coated plates. Twenty-one supernatants were shown to have lysozyme-binding activity, and some of the corresponding individual clones were prepared.

Two of the clones (VH3 and VH8) with lysozyme-binding activities were sequenced (Fig. 3). They belonged to the same VH-gene (Kabat IIB) families and D-segment families (FL16) but had different J segments (JH2 and JH4). There were only six amino-acid differences between the (unrearranged) VH genes, but the sequences of CDR3 were completely different. The VH domains were purified and affinities for lysozyme determined (Table 1). The affinities, in the 20 nM range, are similar to those of the VH domain of the D1.3 antibody. To check the specificity of binding, the three VH domains were also screened

TABLE 1 Affinities of Fv fragment and VH domains for hen egg lysozyme

	Stoichiometry	Affinity (nM)	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})	k_{off}/k_{on} (nM)
Fv-D.1.3	ND	3	1.9×10^6	ND	ND
VH-D1.3	1.2	ND	3.8×10^6	0.075	19
VH3	ND	ND	2.9×10^6	0.036	12
VH8	ND	ND	3.3×10^6	0.088	27

Cultures of 500 ml were grown and induced (see Fig. 1 methods), and the supernatant passed through a 0.45 μm filter (Nalgene), then through a 5 ml lysozyme-Sepharose affinity column. After washing with phosphate buffered saline (PBS), the Fv fragment or VH domains were eluted with 50 mM diethylamine, and analysed for purity by SDS-PAGE[9]. The proteins were titrated with lysozyme at 25 °C using fluorescence quench (Perkin Elmer LS 5B Luminescence Spectrometer)[27] to determine the number of active binding sites, to measure the affinity of the Fv fragment and the stoichiometry of binding of the VH domain (mole lysozyme per mole domain). The concentration of the VH domain of the D1.3 antibody was determined by hydrolysis followed by quantitative amino-acid analysis. The kinetics of lysozyme binding were determined by stopped-flow (HI Tech Stop Flow SHU) at 20 °C under pseudo-first order conditions with binding sites in five to ten fold excess over lysozyme[28]. For the kinetics, the concentration of binding sites, not protein, was measured ND, not determined. k_{on} is the second order rate constant for association, and k_{off} is the first order rate constant for dissociation.

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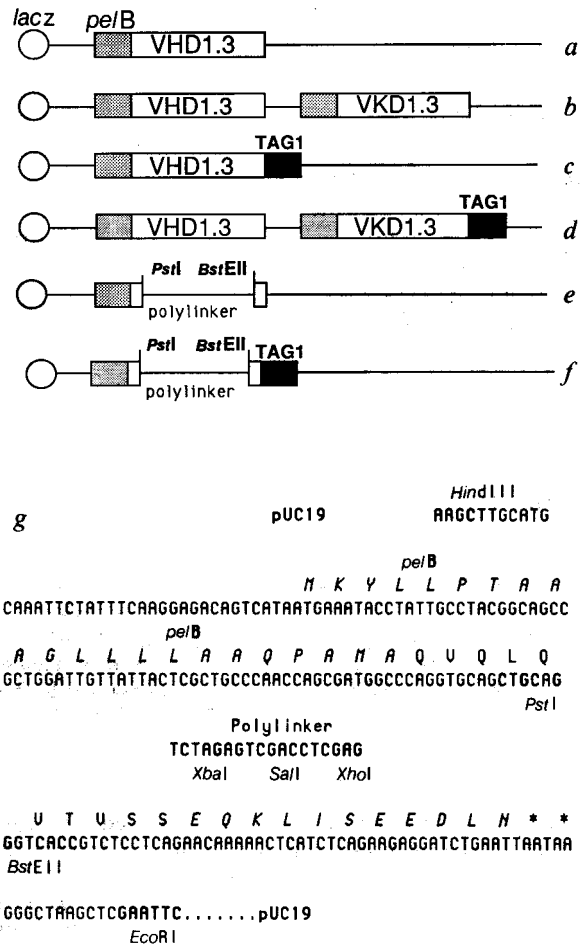

FIG. 1 Vectors for expression of VH and Vκ domains. Scheme showing inserts in expression vectors. *a*, pSW1-VHD1.3; *b*, pSW1-VHD1.3-VKD1.3; *c*, pSW1-VHD1.3-TAG1; *d*, pSW1-VHD1.3-VKD1.3-TAG1; *e*, pSW1-VHPOLY; *f*, pSW1-VHPOLY-TAG1; and *g*, nucleotide sequence of pSW1-VHPOLY-TAG1 insert. The amino acid sequence of the pelB leader and TAG1 are shown in italics.

METHODS. The vectors were assembled from pUC19 (ref. 29), synthetic oligonucleotides encoding the pelB signal sequence[30] peptide tag[24], restriction site polylinker, and (as appropriate) cloned cDNA of the VH and Vκ domains of the D1.3 antibody (M. E. Verhoeyen, C. Berek and G. W., unpublished data). Recombinant plasmids were transformed into *E. coli* BMH71-18 (ref. 31), colonies selected on TYE plates[32] with 100 μg ml^{-1} ampicillin (AMP) and 1% glucose (GLU), and toothpicked into 200 μl 2×TY medium[32], AMP, GLU in wells of ELISA plates. Colonies were grown at 37 °C for 16–24 h. Cells were pelleted, washed twice in 50 mM NaCl (200 μl) and resuspended in 200 μl 2×TY medium, AMP and 1 mM isopropylthiogalactoside (to induce expression) and grown for a further 16–24 h. The cells were cooled, pelleted and supernatants screened for secretion of VH domains (by western blotting) or antigen binding activity (by direct ELISA). Western blot methods were essentially as in ref. 22: supernatant (10 μl) from the cultures was subjected to SDS-PAGE[9] and proteins then transferred electrophoretically to nitrocellulose. The VH domains were detected by means of the peptide tag with 9E10 antibody[24]. However, the tag can be lost by proteolytic cleavage in culture, especially after prolonged growth of the bacteria. Bound antibody was detected using horseradish peroxidase conjugated rabbit anti-mouse antibody at a dilution of 1:1000. 4-chloro-1-naphthol (Sigma) was used as the peroxidase substrate. For direct ELISA, wells of Falcon ELISA plates were coated with antigen in phosphate buffered saline (PBS) overnight (3 mg ml^{-1} lysozyme, or 50 μg ml^{-1} KLH), then blocked with 2% skimmed milk powder in PBS for 2 h at 37 °C. Bacterial supernatant was added and incubated at 37 °C for 2 h. D1.3-VH domains were detected with rabbit polyclonal antiserum raised against the D1.3 Fv fragment, using peroxidase conjugated goat anti-rabbit immunoglobulin. Tagged VH domains were detected as described in western blotting except with 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) as the peroxidase substrate. Three washes of 0.05% Tween 20 in PBS, were followed by three washes of PBS between each step (only PBS washes before addition of blocker or bacterial supernatants). Competition ELISA was as above except that the binding of the Fv fragment tagged on the Vκ domain was competed against by untagged VH domain.

for binding to four other purified proteins (bovine serum albumin, insulin, keyhole-limpet haemocyanin (KLH) and cytochrome c), and to foetal calf serum, milk powder and plastic of microtitre plates. No binding was detected. (However, other cross-reactive VH domains have been found.) The VH-D1.3, VH3 and VH8 domains appear to bind to the same region of lysozyme, as they inhibit the binding of the D1.3 Fv fragment.

To test whether VH domains with other binding activities could be made, and whether immunization was necessary, a new VH-gene library was prepared from a mouse immunized with KLH. Culture supernatants from the two libraries were tested for binding to lysozyme or to KLH. The first library (immunization with lysozyme) had yielded 21 supernatants with lysozyme- and two with KLH-binding activities, whereas the second library (immunization with KLH, screening ~2,000 colonies) yielded two supernatants with lysozyme- and 14 with KLH-binding activities. We conclude that VH domains can be derived, preferably after immunization, with binding activities to lysozyme and KLH and presumably other antigens. VH domains lack the cavity which can be formed with the Vκ partner however, and this might bias the binding activities against hapten binding[2,15]. The affinity of the VH domains (20 nM or 5×10^7 M^{-1}) for lysozyme lies within the range expected for the affinities of monoclonal antibodies for protein antigens, and can be improved by site-directed mutagenesis (unpublished data).

VH domains with binding activities can be generated in a matter of days without recourse to tissue culture, and may also have other advantages over monoclonal antibodies. For example, the smaller molecule should penetrate tissues more

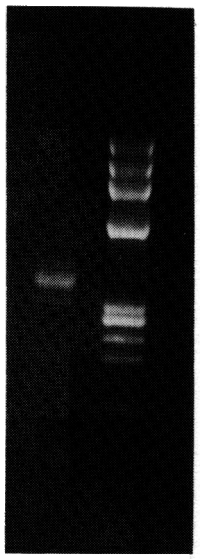

FIG. 2 PCR amplification of VH genes from mouse genomic DNA. Agarose gel electrophoresis of amplified mouse VH DNA (left-hand lane) with markers ΦX174 *Hae*III fragments (right-hand lane). METHODS. Balb/c mice were hyperimmunized with hen egg-white lysozyme (100 μg antigen, intraperitonally on day 1 in complete Freunds adjuvant and on day 14 in incomplete Freunds adjuvant; followed by 50 μg antigen intravenously on day 35; mice were killed on day 39) or similarly with KLH. DNA was prepared from the spleen and the rearranged mouse VH genes were amplified. Conditions were chosen to minimize annealing between the 3′ ends of the two primers. The sample (50–100 μl) included 50–200 ng DNA, VH1FOR-2 (5′ TGA GGA GAC GGT GAC CGT GGT CCC TTG GCC CC 3′) and VH1BACK primers[19] (25 pm of each) 250 μM of each dNTP, 10 mM Tris-HCl pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 100 μg ml^{-1} gelatine. The sample was overlaid with paraffin oil, heated to 95 °C for 2 min, 65 °C for 2 min, and then to 72 °C; *Taq* polymerase (2 units, Cetus) was added after the sample had reached the elongation temperature and the reaction continued for 2 min at 72 °C. The sample was subjected to a further 29 rounds of temperature cycling using the Techne PHC-1 programmable heating block. The amplified DNA was digested with *Pst*I and *Bst*EII and fractionated on an agarose gel. A band of about 350 base pairs was extracted and cloned.

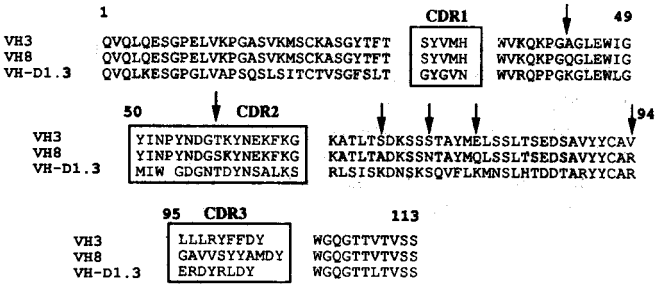

FIG. 3 Sequences of VH domains with lysozyme-binding activities. The sequences of the VH domains are aligned with that of the D1.3 antibody.

readily, could permit the blocking of 'canyon' sites on viruses[25,26] and allow epitope mapping at higher resolution. However, VH domains are relatively 'sticky', presumably due to the exposed hydrophobic surface normally capped by the Vκ or Vλ domains. It should be possible to design VH domains with improved properties. We also envisage that VH domains with binding activities could serve as the building blocks for making Fv fragments or complete antibodies. For example, such VH domains could be co-expressed with a repertoire of Vκ domains, derived by PCR amplification of Vκ genes[19] and screened for association of the domains and antigen binding. This approach could prove valuable for building human antibodies of therapeutic value, and even for catalytic antibodies; for example, making Fv fragments in which the VH domain binds substrate, and side chains or prosthetic groups in the Vκ partner stabilize the transition state or attack the substrate[33]. □

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