GENERATING A REPERTOIRE OF ANTIBODY V GENES AND THE SELECTION AND EXPRESSION OF ANTIGEN BINDING ACTIVITIES IN *E.COLI*

P.T. Jones, E.S. Ward, D.H. Güssow, A.D. Griffiths, and G. Winter

Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, U.K.

Immunotherapy is hindered by the lack of human antibodies of the desired specificities. Although useful rodent monoclonal antibodies have been made by hybridoma technology (1), the use of such antibodies in therapy is limited by an anti-globulin response. Since it has proved difficult to make human antibodies by hybridoma technology, we and others have tried to make these antibodies indirectly, by "humanising" mouse antibodies.

The Ig molecule consists of two copies of heavy and light chains, each consisting of a string of domains of similar architecture. Each domain is about 100 amino acid residues and consists of a β–sheet sandwich with loops at each end. The variable domains are responsible for binding antigen, while the constant domains carry the effector functions, such as complement mediated lysis, cell mediated lysis and phagocytosis. The antigen binding site is fashioned from both heavy (VH) and light (VL; Vκ or Vλ) chain variable domains, as demonstrated by the solved crystallographic structures of antibody in association with antigen (2-4) or hapten (5). The interactions with antigen are made by the loops at one end of the variable domains: these loops are hypervariable in amino acid sequence (see ref 6).

To humanise mouse antibodies, two approaches have been used. In the first, entire variable domains of mouse antibodies have been pasted onto constant domains of human antibodies to make mouse-human chimaeric antibodies with the same binding specificity as the original mouse antibody (7-9). In the second, only the critical antigen binding loops have been pasted into human antibodies (10-12). One such "reshaped" human antibody, CAMPATH-1, has been used clinically to treat two patients with non-Hodgkins lymphoma (12, 13).

Although both the heavy and light chain variable domains are involved in binding to antigen, we have found recently that single heavy chain variable domains can also bind well to antigen. We had been dissecting the

interactions of the anti-lysozyme antibody, D1.3 with antigen (2), and had expressed the VH and VL domains individually, or in association as an Fv fragment by secretion into *E. coli* periplasm (14). Unexpectedly we found that the VH domain bound to antigen with a high affinity (Table 1). We also showed that this domain binds to lysozyme in an equimolar complex. In previous work, separated heavy and light chains were identified with antigen (15) or hapten binding activities (16), but the affinities were poor with no evidence for binding by single chains (17) rather than dimers (18).

Table 1. *Binding affinities of immunoglobulin fragments for lysozyme*

	Stoichiometry nM	Affinity $M^{-1}s^{-1}$	k_{on} s^{-1}	k_{off} nM	k_{off}/k_{on}
Fv-D1.3	n.d.	3	0.8×10^6	n.d.	n.d.
VHD1.3	1.2	<40	3.8×10^6	0.075	19
VH1	n.d.	<15	n.d.	n.d.	n.d.
VH3	n.d.	n.d.	2.9×10^6	0.036	12
VH8	n.d.	n.d.	3.3×10^6	0.088	27

In the D1.3 antibody, lysozyme makes extensive interactions to both domains, including three H-bonds to the Vκ domain, and nine H-bonds to the VH domain. Binding of lysozyme buries about 300 Å^2 of Vκ domain to solvent, and 400 Å^2 of the VH domain (2, C. Chothia unpublished results). Despite these interactions, the Vκ domain appears to make only a small net contribution to the energetics of binding. The VH domain presumably binds to lysozyme in a similar way as the antibody, although 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 (19), or that the loops of the VH domain could adjust to binding of antigen (20).

Is it likely to be a general observation that VH domains of antibodies will have good affinities for antigen ? In the crystallographic structures of other antibody-antigen complexes, both domains make extensive interactions with the antigen. Like the D1.3 antibody, the contacts between antigen and VH or VL domains appear to vary, and the relative energetics of the contacts are not known. The answer must await a systematic analysis of the binding activities of VH domains from a range of antibodies, preferably those with

solved crystallographic structures. However we have attempted to answer a slightly different question: can we rescue VH domains with good antigen binding activities directly from the genes of antibody-secreting cells ?

Recently we devised a way for cloning immunoglobulin variable domains directly for expression in mammalian or bacterial hosts (Fig. 1). By comparing a data base of nucleotide sequences which encode antibody variable domains (6) we could devise "universal" primers for the amplification of mouse VH and Vκ domains. The primers incorporated restriction sites to enable the forced cloning of these genes into expression vectors (21). We had shown that VH and Vκ genes could be cloned from mRNA of several hybridomas, and we now extended the technique in an attempt to clone a repertoire of VH genes from the chromosomal DNA of mouse spleen. We determined the complete sequences of some 48 clones and have demonstrated a diverse repertoire (Table 2), although we cannot rule out a systematic bias due to our choice of primers or hybridisation conditions (22).

Table 2. *Usage of VH gene, D segment and J-region families in the VH repertoire*

VH genes		D segments		J regions	
Family	Number	Family	Number	Family	Number
IA	4	SP2	14	JH1	3
IB	12	FL16	11	JH2	7
IIA	2	Q52	5	JH3	14
IIB	17			JH4	14
IIIA3					
IIIB8					
IIIC1					
VA1					

From this library, we could derive VH domains with antigen binding activities. For example, from 2000 clones derived from the spleen of a mouse immunised with lysozyme, we find 21 clones with lysozyme-binding activity and two with keyhole limpet haemocyananin (KLH)-binding activity. From 2000 clones from the spleen of a mouse immunised with KLH, we find 14 clones with KLH-binding activity and one with lysozyme-

binding activity. Thus prior immunisation facilitates the isolation of these activities. The affinities of two of the domains, VH3 and VH8, for lysozyme were measured (Table 1), and lie within the range expected for monoclonal antibodies for protein antigens. Thus PCR cloning of VH domains from immunised spleen may offer an alternative to hybridoma technology (22).

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 readily, could permit the blocking of "canyon" sites on viruses (23, 24) and allow epitope mapping at higher resolution. However, we also envisage that VH domains with binding activities could also serve as the building blocks for making Fv fragments or complete antibodies. For example, VH domains could be co-expressed with a repertoire of Vκ domains, derived by PCR amplification of Vκ genes (21) and screened for association of the domains and antigen binding.

Although it is possible to derive "natural" VH domains by cloning a repertoire from the immune system, it should also be possible to make an entirely synthetic repertoire of VH domains. We have therefore hypermutated the third hypervariable region of the VH domain of the D1.3 antibody (Fig. 1). CDR3 was chosen because it is the most diverse portion of sequence in antibodies, derived by the joining of three genetic elements, V, D and J, and would be expected to carry major determinants for binding to antigen. For example in the D1.3 antibody, the three residues Arg 99, Asp 100 and Tyr 101 make six of the nine hydrogen bonds of the heavy chain (2), and presumably have similar roles in the isolated VH domain. When these four residues are changed en bloc by PCR mutagenesis, the vast majority of mutants lose the ability to bind lysozyme. However of 2000 colonies screened, nineteen retain the ability to bind lysozyme, and four acquire the ability to bind to KLH. One of the mutants, (VH1) which binds to lysozyme has a completely different amino acid sequence in CDR3 and has a slightly improved affinity compared with the parent VH domain (Table 1). The interaction of lysozyme with this region (and perhaps also with CDR1 and CDR2) is also likely to differ, as the main chain now incorporates a proline at residue 102.

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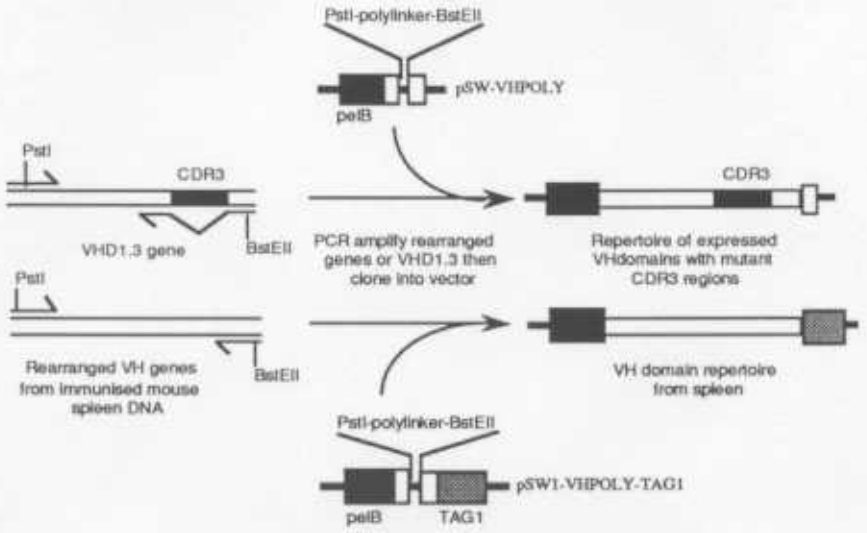

Figure 1. Strategy for the cloning of VH repertoires into *E.coli* expression vectors

In conclusion, recombinant DNA techniques can be used to tailor monoclonal antibodies derived by hybridoma technology, but should also permit antigenic specificities to be cloned directly from the genes of antibody-producing cells as VH domains or complete antibodies.

ACKNOWLEDGEMENTS

A.D.G. was supported by Celltech, D.G. by Behringwerke and E.S.W. by a Stanley Elmore Senior Medical Research Fellowship, Sidney Sussex College, Cambridge.

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