Antibody Engineering Using Escherichia coli *as Host*

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I. Introduction

The development of hybridoma technology (Köhler and Milstein, 1975) has generated an enormous expansion in the use of antibodies in medicine and biology. Furthermore, developments in recombinant DNA techniques, in addition to the use of suitable expression hosts, have allowed the production of a new generation of "designer" antibodies (Williams, 1988; Morrison and Oi, 1989). Thus, it is relatively straightforward to produce immunoglobulins or immunoglobulin fragments in genetically tailored form for use in therapy and diagnosis. In addition, ways of expressing repertoires of genes encoding antibody fragments in *Escherichia coli* have been developed (Ward *et al.*, 1989a; Sastry *et al.*, 1989; Huse *et al.*, 1989;

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Mullinax *et al.*, 1990; Caton and Koprowski, 1990; Persson *et al.*, 1991), and "surface" expression systems for the selection of antigen binding activities from such repertoires have been reported (McCafferty *et al.*, 1990; Kang *et al.*, 1991). These technologies open up new avenues for the generation and selection of antigen binding activities *in vitro*. Expression of immunoglobulin fragments in *E. coli* also offers a rapid, convenient route for their large-scale production, as the fragments can be produced in milligram quantities (Skerra and Plückthun, 1988; Better *et al.*, 1988; Ward *et al.*, 1989a), or even gram quantities in fermentation vats in a matter of days.

In the longer term, it may be possible to use genetic manipulation and prokaryotic expression systems to mimic both the affinity maturation (somatic mutation; for reviews see Berek and Milstein, 1988; French *et al.*, 1989) and selection mechanisms of the immune response *in vitro*. Such an "*in vitro* immune system" is attractive as it could avoid the need to immunize animals for the generation of specific antibodies. It will, however, probably be difficult to develop an *in vitro* system that is as efficient in generating high-affinity antibodies as the *in vivo* immune system.

The main focus of this article is the expression and genetic manipulation of antibody fragments in *E. coli*. The use of the polymerase chain reaction (PCR) (Saiki *et al.*, 1985, 1988) for the isolation of antibody variable domain genes is also briefly covered.

II. Immunoglobulin Structure

The antibody molecule comprises two heavy chains linked by one or more sulfhydryl bridges to two light chains (Porter, 1973). Each heavy and light chain can be divided into discrete domains (Fig. 1). The domain organization of an antibody molecule is mirrored at the gene level, as each domain is encoded in a separate exon (Maki *et al.*, 1980; Alt *et al.*, 1984). The domains have distinct functions; for example, the C_H2 domain is involved in C1q binding and also in interacting with the high-affinity FcR receptor and, therefore, this domain plays a key role in the effector functions of an antibody molecule (Burton, 1985, 1987; Woof *et al.*, 1986; Duncan and Winter, 1988; Duncan *et al.*, 1988). The variable domains (designated V_H and V_L for heavy- and light-chain variable domains, respectively) are of particular interest as they carry the determinants of specificity and affinity for antigen binding (Inbar *et al.*, 1972; Isenman *et al.*, 1975; Davies and Metzger, 1983; Amit *et al.*, 1986; Satow *et al.*, 1986; Colman *et al.*, 1987; Sheriff *et al.*, 1987; Padlan *et al.*, 1989). The V_H and V_L domains have a structure similar to that of the domains of all proteins that fall into the

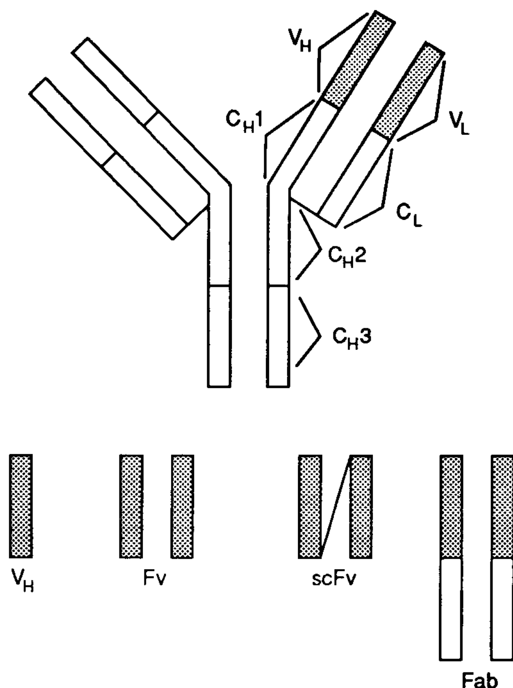

Fig. 1 Schematic representation of antibody structure. The immunoglobulin G (IgG) molecule consists of four polypeptide chains, two heavy and two light (Porter, 1973), and these chains consist of discrete domains. The framework structure of each domain is conserved and is made up of β sheets which pack together and are linked by peptide loops at the ends of each β strand (Marquart *et al.*, 1980; Davies and Metzger, 1983). For the variable domains, three loops that are hypervariable in sequence (Kabat *et al.*, 1987) protrude from the top of each domain, and it is the residues in and flanking these loops that confer the antigen binding specificity of the immunoglobulin molecule. The hinge region links the C_H1 domain to the C_H2 domain, and one or more sulfhydryl bridges covalently link the two heavy chains in this region. In addition, the light chain (V_L–C_L) is linked to the heavy chain by a sulfhydryl bridge. The heavy- and light-chain domains also pack against each other through noncovalent interactions. The immunoglobulin molecule can be proteolytically cleaved in the hinge region to produce an Fab fragment and an Fc fragment. For the other fragments shown in this figure, it is now simpler to produce them by genetic engineering rather than by proteolysis. V_H domains, Fv fragments (heavy- and light-chain variable domains, noncovalently associated by interdomain interactions), single-chain (sc) Fv fragments (V_H and V_L domains linked to each other by synthetic peptide linker sequences), and Fab fragments can be expressed as either secreted or intracellular proteins, using *Escherichia coli* as a host (Better *et al.*, 1988; Skerra and Plückthun, 1988; Huston *et al.*, 1988; Bird *et al.*, 1988, Ward *et al.*, 1989a, b).

immunoglobulin superfamily, namely, a β-sheet framework (Davies and Metzger, 1983). This β-sheet framework supports the hypervariable loops of the immunoglobulin variable domains (or CDR loops, for complementarity determining regions). Residues within these loops play a major role in determining the binding specificity and affinity of an antibody. Canonical structures for the conformation of these loops have recently been modeled (Chothia and Lesk, 1987; Chothia *et al.*, 1989). The CDR loops, of which there are three per V_H or V_L domain, exhibit high sequence variability, particularly in the third CDR loop which is the site of V, (D), and J joining during somatic recombination (Alt *et al.*, 1984). The potential for the generation of almost unlimited numbers of different V_H and V_L domain sequences is a prerequisite for the recognition of the extremely diverse array of antigens with which the immune system is continually being challenged.

Thus, because of the high sequence variability and antigen binding specificity of the variable domains, there is considerable interest in developing methods of rapidly isolating and expressing diverse repertoires of immunoglobulin V_H and V_L domain genes in a suitable host. There is additional interest in the development of selection systems to avoid the tedious screening for clones that produce immunoglobulins (or immunoglobulin fragments) with antigen binding activities. To date, two developments in recombinant DNA techniques have made substantial contributions toward these aims; (1) the use of the PCR (Saiki *et al.*, 1985, 1988) to isolate V_H and V_L domain genes (Orlandi *et al.*, 1989; Larrick *et al.*, 1989a, b) and (2) the development of expression systems for the secretion of antibody fragments in functional form, using *E. coli* as a host (Skerra and Plückthun, 1988; Better *et al.*, 1988).

III. Isolation of Variable Domain Genes Using the Polymerase Chain Reaction

The PCR (Saiki *et al.*, 1985, 1988) allows the specific and rapid isolation of genes or members of gene families. The genes can be isolated with a high degree of sensitivity by primer-directed amplification from highly heterogeneous DNA preparations. The requirement for their isolation, using the PCR, is that there is some preexisting knowledge of the gene sequences at either, or both, of the 5′ and 3′ ends. For the immunoglobulin variable domains, there are databases such as the Kabat classification (Kabat *et al.*, 1987) that document the sequences of V_H and V_L genes for nine different species, including mouse, rabbit, rat, and human. The homologies shared by the V_H and V_L genes within a given species allow the design of primers

that can be used in the PCR to isolate the variable domain genes (Orlandi *et al.*, 1989; Larrick *et al.*, 1989a, b; Chiang *et al.*, 1989; Marks *et al.*, 1991). Thus, primers have been designed to hybridize to the 5′ and 3′ ends of V_H and V_L genes and used to isolate both "clonal" antibody genes from hybridomas and diverse V_H and V_L gene repertoires from antibody producing cells. There are nucleotide bases at the 5′ and 3′ ends of V_H and V_L genes that are not particularly well conserved, and at these positions redundancy is incorporated into the primers during oligonucleotide synthesis (Orlandi *et al.*, 1989), or as an alternative, family-specific primers can be used (Marks *et al.*, 1991). The PCR primers can instead be designed to anneal to the immunoglobulin leader sequences and constant domains (Larrick *et al.*, 1989a, b; Andris *et al.*, 1991). The primers can also be designed to have "internal" restriction sites to facilitate forced cloning into vectors for expression (Orlandi *et al.*, 1989; Ward *et al.*, 1989a; Huse *et al.*, 1989; Mullinax *et al.*, 1990; Persson *et al.*, 1991).

By judicious design of species-specific primers using sequences published and documented in existing databases, it is now possible to isolate repertoires of genes encoding immunoglobulin variable domains from antibody-producing cells [peripheral blood lymphocytes (PBLs), spleen cells] from a number of different species. These genes can be cloned for expression in *E. coli,* and with the procedures described in the following sections and references therein, clones that produce fragments with antigen binding activities can be identified. This technology could be particularly useful for the isolation of human monoclonal antibodies as the isolation of human monoclonals has proved technically to be more difficult than that of rodent monoclonals (Carson and Freimark, 1986; Thompson, 1988; Borrebaeck *et al.*, 1988; Borrebaeck, 1988). It can also be applied to "rescue" clonal V_H and V_L genes from potentially unstable hybridomas at an early stage in cloning. The use of the PCR facilitates not only the isolation of specific antigen binding activities by expression cloning, but also the analysis at the nucleotide sequence level, for example, of affinity maturation (Berek and Milstein, 1988) of the immune response against a particular antigen.

IV. Cloning Immunoglobulin Variable Domain Genes for Expression

A. Expression Using *E. coli* as Host

In the early to mid-1980s, attempts to express antibody fragments in *E. coli* resulted in the accumulation of recombinant proteins as intracellular inclu-

sion bodies (Kurokawa *et al.*, 1983; Boss *et al.*, 1984; Cabilly *et al.*, 1984; Liu *et al.*, 1984; Ishizaka *et al.*, 1986). Recombinant protein was isolated by denaturation and resolubilization of the inclusions. A similar approach has since been used to express and purify single-chain Fv (scFv) fragments from *E. coli* (Bird *et al.*, 1988; Huston *et al.*, 1988; Gibbs *et al.*, 1991; Whitlow and Filpula, 1991), and improved methodology for the isolation of functional Fab fragments from inclusion bodies has been reported (Cabilly, 1989; Buchner and Rudolph, 1991).

Alternative approaches, in which immunoglobulin Fv and Fab fragments are secreted from recombinant *E. coli* cells in fully functional form have recently been described (Fig. 1) (Better *et al.*, 1988; Skerra and Plückthun, 1988). These secretion systems greatly facilitate purification and have led to the use of *E. coli* as a host of choice for the production of antibody fragments. The use of *E. coli* has several advantages over that of mammalian cell transfectomas; for example, *E. coli* grows at a much greater rate than mammalian cells, so that the expressed, recombinant proteins can be analyzed in a much shorter time. In addition, in economic terms the use of *E. coli* is more favorable. There are drawbacks to the expression of eukaryotic proteins in *E. coli,* however, as the recombinant proteins will not be glycosylated. This is generally not a problem for the production of antibody variable domains, which are rarely glycosylated; however, for the expression of the C_H2 domains this may be a concern, as these domains have N-linked oligosaccharides that are thought to be structurally important (Leatherbarrow *et al.*, 1985). In this respect, recombinant Fc regions expressed in *E. coli* have recently been shown to bind to Fc receptors with 500 times lower affinity than Fc regions isolated from mammalian cell transfectomas (Nose *et al.*, 1990). A possible reason for this, as suggested by Nose and colleagues, is that the presence of the oligosaccharides on the C_H2 domains is critical for Fc receptor recognition.

B. Secretion of Fab and Fv Fragments in *E. coli*

The expression plasmids that have been designed for the secretion of immunoglobulin domains in *E. coli* are shown in Fig. 2. The constructions have signal sequences for secretion of the recombinant polypeptides across the inner membrane of *E. coli* into the periplasmic space. After prolonged induction (6–30 hr) of expression and secretion, the recombinant protein leaks into the culture supernatant (Skerra and Plückthun, 1988; Ward *et al.*, 1989a). Thus, the protein can be either purified directly from the culture supernatant or, alternatively, isolated from the periplasm by osmotically shocking the cells. The latter route for protein isolation is

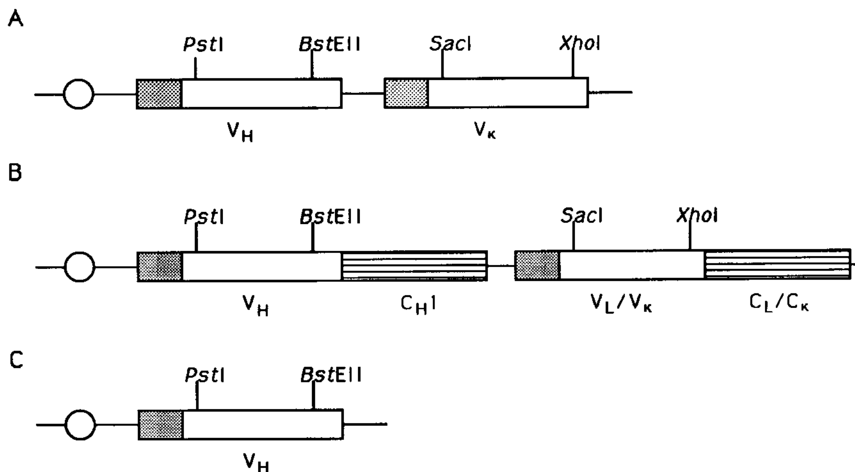

Fig. 2 Vectors used for the expression and secretion of immunoglobulin fragments in *Escherichia coli,* for the production of (A) Fv fragments, (B) Fab fragments, and (C) single V_H domains (Ward *et al.*, 1989a). Restriction sites used for cloning the genes are shown, and these sites are also incorporated into polymerase chain reaction primers. Open circle, lacz promoter; stippled box, pelB leader sequence. The vector backbone for all the constructs is pUC19 (Yanisch-Perron *et al.,* 1985). Adapted, with permission, from Ward (1992).

preferable for Fv fragments for which the association of the V_H and V_L domains is not particularly stable, as dilution into the culture supernatant may result in dissociation of the heterodimer (A. Skerra, personal communication). In this respect, an advantage of a scFv fragment compared with an Fv fragment is that the synthetic linker between the V_H and V_L domains stabilizes the noncovalent V_H–V_L interaction. A number of different single-chain linker peptides have now been described (Whitlow and Filpula, 1991). It may therefore be preferable to express V_H–V_L pairs as scFv fragments, despite the observation that the binding affinity of the scFv may be slightly lower than that of the Fv (Bird *et al.*, 1988; Huston *et al.*, 1988). In addition to being expressed as intracellular inclusions, scFv fragments can be secreted from recombinant *E. coli* cells, albeit in lower yields than V_H–V_L heterodimers (our observations). An alternative way to stabilize the V_H–V_L association in Fv fragments is to genetically engineer cysteines into the V_H and V_L domains, so that an intermolecular –S–S– bridge is formed, as recently reported by Glockshuber *et al.* (1990).

Initially the plasmids shown in Fig. 2 were used to produce Fv and Fab fragments derived from well-characterized monoclonal antibodies. The yields of expressed protein vary from 0.5 to 10 mg/liter of culture for Fv

fragments (Skerra and Plückthun, 1988; Ward *et al.*, 1989a) and from 2 to 5 mg/liter for Fab fragments (Better *et al.*, 1988; E. S. Ward, unpublished). On a molar basis these yields compare favorably with those obtained with mammalian cell transfectomas. The bacterially expressed Fv fragment of the antilysozyme D1.3 antibody has been crystallized with and without antigen (Boulot *et al.*, 1990), and the high-resolution structure solved by X-ray crystallography (Bhat *et al.*, 1990). This study indicates that the bacterially expressed Fv fragment folds in the same way as the variable domains of the complete antibody expressed by the appropriate hybridoma cells (Amit *et al.*, 1986; Bhat *et al.*, 1990). Thus, it is likely that by extension, correct folding of V_H and V_L domains will be a general feature for antibody variable domains secreted from *E. coli*.

C. Secretion of V_H Domains in *E. coli*

The plasmids shown in Fig. 2, or derivatives thereof, have been used to express single V_H domains (Ward et al., 1989a, 1989b). The V_H domain is another single-chain antibody fragment, of about half the size of a scFv. The observation that a V_H domain, or single-domain antibody (dAb), derived from the antilysozyme D1.3 monoclonal antibody retains high affinity for antigen binding in the absence of the V_LD1.3 domain led to the generation of repertoires of V_H domain genes from the spleen DNA of hyperimmunized mice (Ward *et al.*, 1989a). These gene repertoires were isolated by the PCR and cloned into an *E. coli* expression/secretion vector, and the resulting clones screened for the production of dAbs that have antigen binding activities. This resulted in the identification of dAbs with specificities toward two antigens, namely, hen egg lysozyme and keyhole limpet hemocyanin. Characterization of several of the antilysozyme V_H domains by stopped-flow kinetics and fluorescence quench indicated that these single domains have surprisingly high antigen binding affinities, namely, of the order of 10^{-8} *M* (Ward *et al.*, 1989a, b). In addition, a V_H domain derived from an antineuraminidase monoclonal antibody (Colman *et al.*, 1987) has recently been expressed in *E. coli* and retains antigen binding affinity (P. Hudson, personal communication). Further indirect evidence that the V_H domains of antibodies can bind antigens in the absence of paired V_L domains comes from several studies.

1. Chimeric T-cell receptors (TCRs) have been constructed in which the V_H domain of antihapten antibodies has been used to replace the V_α or V_β domain of the TCR (Becker *et al.*, 1989; Goverman *et al.*, 1989; Gross *et al.*, 1989). In all three cases, the chimeric TCR immunoglobulin has been shown to have binding specificity for the appropriate hapten, despite the absence of a paired V_L domain.

2. X-Ray crystallographic studies have now been reported for a number of antibodies complexed with protein antigens. For a number of these structures, the residues of the V_H domain make a greater number of contacts with antigen than those of the V_L domain (Amit *et al.*, 1986; Colman *et al.*, 1987; Sheriff *et al.*, 1987; Padlan *et al.*, 1989). In addition, the observation that V_H domains can bind antigen without a paired light chain is consistent with earlier reports that isolated immunoglobulin heavy chains can have antigen binding affinities (Fleischmann *et al.*, 1963; Utsumi and Karush, 1964; Jaton *et al.*, 1968). In these early reports, however, the affinities are low and there is no evidence for binding by single chains. Thus, the surprising result with the antilysozyme V_H domains is their retention of high affinity. From the X-ray structure of the D1.3 antibody complexed with antigen hen egg lysozyme, it is clear that the V_H domain makes the majority of contacts, but the V_L domain still makes a significant number (for example, 3 of 12 of the hydrogen bonding interactions).

1. Potential Uses of Single-Domain Antibodies

Single-domain antibodies are approximately 1/12th the size of a complete antibody molecule, and this may make them preferable reagents in situations where small size is advantageous. For example, single domains may be able to penetrate the conserved (receptor binding) "canyon" sites of viruses such as rhinoviruses (Kim *et al.*, 1989). The size of this crevice is, by molecular modeling, too small to allow penetration by a complete antibody molecule. Additionally, dAbs may have greater penetration into solid tumors because of their small size. A disadvantage of using dAbs, however, is that they do not carry immunoglobulin constant domains and, therefore, have no effector functions (for reviews, see Burton, 1985, 1987). This could be overcome by linking, at the genetic level, toxins such as ricin A chain or *Pseudomonas* exotoxin to produce potent immunotoxins (Vitetta and Uhr, 1985; Chaudhary *et al.*, 1989, 1990; Ahmad and Law, 1988; O'Hare *et al.*, 1990; Chovnick *et al.*, 1991). Alternatively, as the high-affinity FcRI binding site has been localized on the C_H2 domain (Woof *et al.*, 1986; Duncan *et al.*, 1988), it may be possible to use protein engineering to incorporate this binding site as a module onto the single V_H domain.

2. Properties of Single-Domain Antibodies

a. Specificity The specificity of dAbs is a key issue that should be addressed if they are to be used for *in vivo* therapy. It is conceivable that the lack of the additional surface area of an antibody–antigen interaction, which in an Fv fragment is provided by the presence of the V_L domain,

may reduce the specificity of the single domain. In addition, the V_H domain of an antibody has hydrophobic residues that normally interact with the paired V_L domain (Chothia *et al.*, 1985; Padlan *et al.*, 1986); thus, the exposure of these residues in a monomeric V_H domain may produce a "sticky" patch which results in nonspecific binding. To date, the evidence suggests that the antilysozyme V_H domains are specific, but the assays used were rather limited in that only four different antigens and two mixed protein antigens were tested (Ward *et al.*, 1989a). Clearly, there is a need for rigorous testing of the specificity of dAbs, by using, for example, murine tissue sections, before a definitive answer to this question can be given.

b. Immunogenicity The *in vivo* immunogenicity of dAbs is as yet unknown. It is probable that dAbs will be cleared rapidly from the circulation, and this may be an advantage as it may reduce their ability to evoke an immune response. In this respect, scFv fragments, which are twice the size of a dAb, are cleared extremely rapidly (Colcher *et al.*, 1990). These smaller immunoglobulin fragments may therefore, after tagging with radionuclides, have advantages as imaging reagents. The rapid clearance could, on the other hand, be a disadvantage in that maintenance of high serum levels of the dAb may be desirable in some therapies. Thus, there is a clear need to address these issues, as it is currently only possible to speculate about the relative merits of dAbs over complete antibodies for therapy prior to this being carried out.

V. Combinatorial Repertoires of V_H and V_L Domains

One of the advantages of dAbs, in a technical sense, is that the isolation of single V_H domains with antigen binding activities from repertoires of variable domain genes overcomes the problem of obtaining matched V_H and V_L genes within the same expression host. In some cases, however, it may be preferable to obtain (sc)Fv or Fab fragments with antigen binding activities rather than V_H domains. During the isolation of genomic DNA from the antibody-producing cells the V_H–V_L matching that existed within a single cell is lost. To isolate (sc)Fv or Fab fragments with antigen binding affinities, therefore, systems have to be developed for randomly combining the isolated V_H and V_L genes in vectors that are designed to coexpress the domains as either (sc)Fv or Fab fragments. Several such systems have been reported. One involves the use of a bacteriophage lambda vector system, and is designed to secrete Fab fragments from transfected *E. coli* cells (Huse *et al.*, 1989). With this system, V_H and V_L domain genes were

isolated by the PCR from the spleens of immunized mice and ligated into appropriate light- and heavy-chain vectors, to generate libraries of light-chain and Fd fragment genes. These libraries were then randomly combined by ligation at a unique asymmetric restriction site, and recombinant clones expressing Fab fragments with antigen binding activities toward the immunogen *p*-nitrophenyl phosphonamidate were identified by screening (Huse *et al.*, 1989).

This "lambda zap" system has now been extended to express Fab fragments derived from human PBLs, and antibody fragments that have anti-tetanus toxoid binding activities have been isolated (Mullinax *et al.*, 1990; Persson *et al.*, 1991). The isolation of immunoglobulin fragments from human antibody-producing cells that have high affinities for a particular antigen is an attractive route for the production of human monoclonal antibodies. This goal is, however, impeded by ethical barriers, because even if sufficient antigen is available for immunization, it is clearly not usually possible to immunize humans. The isolation of anti-tetanus toxoid antibodies, for example, was made possible only by the fortuitous and necessary immunization of human volunteers (Mullinax *et al.*, 1990; Persson *et al.*, 1991). Thus, three key issues need to be addressed for the isolation of human-derived antibodies from, for example, human PBLs, which have binding activities against an antigen of choice:

1. How many randomly combined V_H and V_L genes derived from naive repertoires (with respect to the antigen under study) need to be screened to isolate the desired activity?
2. As the number in (1) is probably extremely large, can systems be devised that allow the *selection* of clones that have antigen binding activities, so as to considerably reduce the amount of screening that needs to be carried out?
3. Can such a selection system be coupled to *in vitro* mutagenesis, so that sequential rounds of mutagenesis can be followed by selection? This would mimic the *in vivo* situation, where surface-bound immunoglobulin molecules on antigen-specific B cells are somatically mutated and selected in response to antigenic challenge (Berek and Milstein, 1988).

A step toward the development of a selection system has recently been reported, namely, the expression of scFv or Fab fragments in functional form on the surface of filamentous bacteriophage (McCafferty *et al.*, 1990; Kang *et al.*, 1991). McCafferty and colleagues achieved this by linking the genes encoding an antilysozyme scFv fragment [in which the V_H domain is linked by a synthetic Gly–Ser-rich linker (Huston *et al.*, 1988) to the matched V_L domain] into the gene III coat protein of phage fd. The scFv

fragment is linked in translational frame, so that the gene III protein, as a coat protein, acts as a vehicle to transport the scFv fragment to the phage surface. A similar approach was taken by Kang and colleagues, who linked the genes encoding an antibody Fd fragment to the gene VIII coat protein of bacteriophage M13. The corresponding light chain was also ligated into the same phagemid (Kang *et al.*, 1991). Thus, phage particles are produced that expose "surface-bound" immunoglobulin fragments. The phage that express antigen binding activities can be selected with about 10^6-fold enrichment, using antigen affinity columns, from a pool of phage that do not express antigen-specific scFv fragments (McCafferty *et al.*, 1990).

More recently, repertoires of V_H and V_L genes have been isolated by the PCR from the spleens of immunized mice, and combined genetically as scFv fragments in this selection system (Fig. 3). Phage expressing these repertoires of randomly combined V_H and V_L genes were passed down antigen columns, and phage displaying antigen binding activities were isolated with high efficiency (Clackson *et al.*, 1991). This system, or the M13-Fab system, opens up new avenues for the *in vitro* generation and selection of antigen binding activities from immunoglobulin repertoires. The systems could be coupled to *in vitro* mutagenesis, and in theory could allow the generation and selection of fragments of high affinity and specificity for any antigen. Use of such systems may, in the longer term, avoid the need for immunization of either humans or rodents for the isolation of antigen-specific scFv or Fab fragments. To date it is unclear, however, whether this synthetic approach will produce higher-affinity antibodies with the same efficiency as the *in vivo* immune system undergoing antigenic challenge (for review, see Winter and Milstein, 1991).

VI. Therapeutic Potential of Single-Chain Fv and Fab Fragments

The generation of high-affinity antibody fragments (scFv, Fab) by expression cloning of V_H and V_L genes from immunoglobulin-producing cells should lead to the production of reagents for therapy. There are several questions related to the use of these antibody fragments:

1. What are the pharmacokinetics of these smaller fragments? Recent data indicate that the fragments are cleared extremely rapidly and, thus, are not suitable for therapy in their own right (Covall *et al.*, 1986; Colcher *et al.*, 1990). This feature may be an advantage in imaging, for

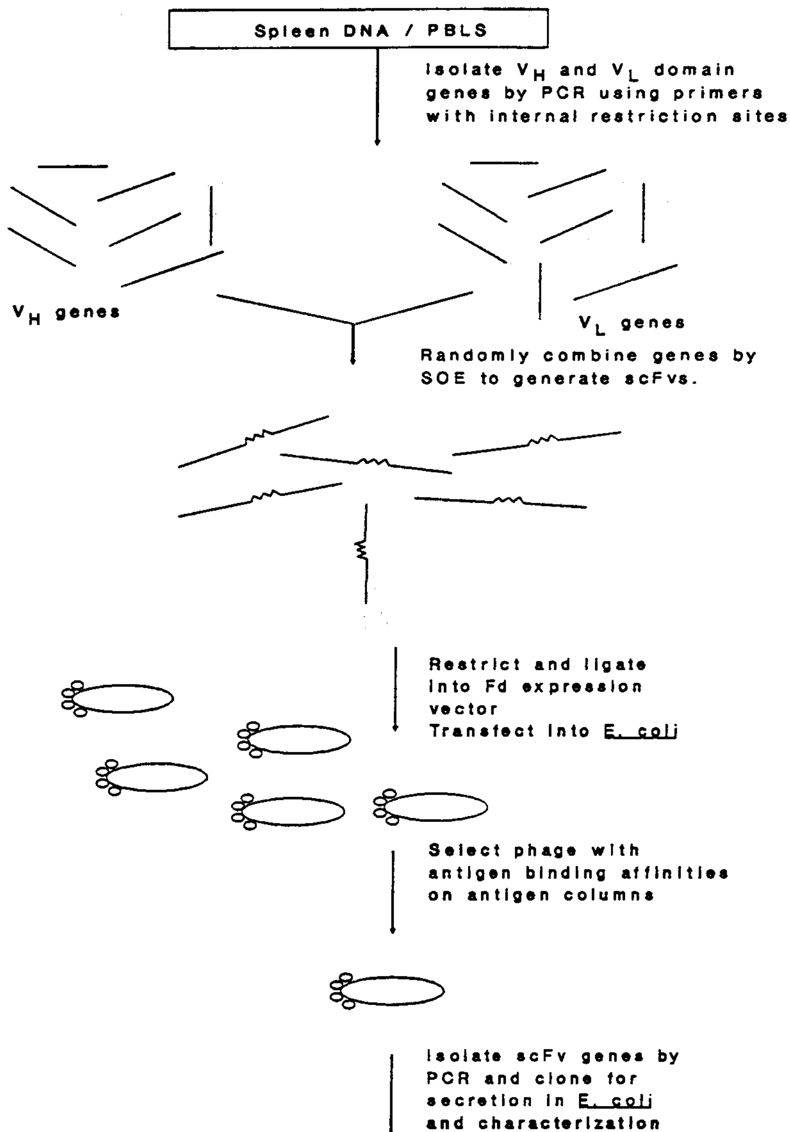

Fig. 3 Scheme for the production of combinatorial libraries followed by selection for antigen binding activities (Horton *et al.*, 1989). SOE, splicing by overlap extension; PBLs, peripheral blood lymphocytes; PCR, polymerase chain reaction.

example, but for prolonged therapy could be a disadvantage. Thus, for therapy, it may be desirable to link the variable domains to immunoglobulin constant domains, for which the genes are now available (Brüggeman *et al.*, 1987).

2. If *in vitro* mutagenesis is used to generate higher-affinity antibodies by using sequential rounds of mutagenesis followed by selection, will the use of these semisynthetic antibodies in therapy result in stronger anti-idiotypic responses than antibodies generated by *in vivo* somatic mutation? It is also conceivable that these semisynthetic antibodies may have autoreactivities.

3. If smaller antibody fragments are desirable for the treatment of solid tumors because of their greater penetrance (Sutherland *et al.*, 1987), which are the most effective ways to arm these with antibody effector functions? It is conceivable that functions such as antibody-dependent cell-mediated cytotoxicity and C1q binding can be linked on as smaller modules at the genetic level, but to date, there is no evidence to suggest that the C_H2 domain can be fragmented and still retain functional activity. An attractive alternative, if small size is desirable, is to construct antibody fragment–toxin fusions (Chaudhary *et al.*, 1989, 1990; Chovnick *et al.*, 1991).

4. What is the optimal affinity of an antibody fragment for use in therapy? It is conceivable that antibodies with very high affinities for binding to tumor cell markers, for example, may not be as effective in penetrating the tumor as lower-affinity variants, because of tight binding to the exterior of the tumor (Eshhar, presented at UCLA Meeting on Monoclonal Antibodies, Denver, Colorado, March 1991). Thus, the optimal fragment for use in therapy may not be the one with the highest affinity.

VII. Summary

The expression of immunoglobulin fragments with antigen binding activities in *E. coli* is now routinely possible. Using such expression systems, Fv, Fab, and scFv fragments and single V_H domains can be produced as secreted proteins in yields of the order of milligrams per liter. Moreover, expression systems are being rapidly developed for the production of antibody scFv or Fab fragments by repertoire cloning followed by selection. Diverse repertoires of genes encoding V_H and V_L domains can be isolated by the PCR and cloned for expression using these systems, which allow the selection of recombinants that produce fragments with the de-

sired antigen binding specificities. This technology is rapidly evolving and, coupled with the development of systems for the random mutagenesis and selection of higher-affinity antibody fragments, could, in the longer term, provide an alternative rapid route to hybridoma technology.

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