Antibody engineering: the use of *Escherichia coli* as an expression host

E. SALLY WARD

Cancer Immunobiology Center and Department of Microbiology, The University of Texas Southwestern Medical Center, Dallas, Texas 75235-9048, USA

ABSTRACT **The hypervariable loops of an antibody molecule are supported on the relatively conserved β-sheeted frameworks of the heavy- and light-chain variable domains (designated VH and VL domains, respectively). Residues within and flanking these loops interact with antigen and confer the specificity and affinity of antigen binding on the immunoglobulin molecule. Thus, the isolation and expression of VH and VL domain genes are of particular interest both for analysis of the determinants of antibody specificity and for generation of fragments with binding affinities for use in therapy and diagnosis. The PCR can now be used to isolate diverse repertoires of antibody VH and VL domain genes from antibody-producing cells from different species, including humans and mice. The genes can be expressed as either secreted or surface-bound Fv or Fab fragments, using *Escherichia coli* expression systems, and the desired antigen-binding specificity screened for or, preferably, selected. The use of *E. coli* as an expression host allows the required antigen-binding specificity to be isolated in clonal form in a matter of days. The VH and VL domain genes can also be hypermutated and higher-affinity variants isolated by screening or selection. Thus, the use of this technology should allow the isolation of novel binding specificities or specificities that are difficult to generate by hybridoma technology. It will also facilitate the isolation of human-derived Fv/Fab fragments that may be less immunogenic in therapy. This approach therefore has almost unlimited potential in the generation of therapeutics with binding specificities to order. The fragments can be used either alone or linked to effector functions in the form of antibody-constant domains or toxins. The new technology could prove to be a method of choice for the rapid and convenient production of designer antibodies.—Ward, E. S. Antibody engineering: the use of *Escherichia coli* as an expression host. *FASEB J.* 6: 2422–2427; 1992.**

Key Words: VH and VL genes • polymerase chain reaction • recombinant antibody fragments • bacteriophage surface expression

THE DEVELOPMENT OF HYBRIDOMA TECHNOLOGY (1) in the middle 1970s has resulted in an enormous expansion of the use of antibodies in medicine and biology. This technology allows the production of antibodies of defined specificity in clonal form. More recently, developments in genetic manipulation facilitate the isolation of diverse repertoires of antibody variable-domain genes from immunoglobulin-producing cells followed by their expression using *Escherichia coli* as a host (2–4). Thus, the polymerase chain reaction (PCR)[1] (5) can be routinely applied to the isolation of diverse repertoires of antibody genes from different species, including mouse and human (2–4, 6, 7). These repertoires can be cloned in *E. coli* for expression, and recombinant clones producing immunoglobulin fragments with desired binding specificities screened for (2, 4, 8–10) or, more recently, selected for (11, 12). This review will focus on these recent developments and discuss possible applications of the new technology in the production of engineered antibodies for therapy and diagnosis.

USE OF THE POLYMERASE CHAIN REACTION TO ISOLATE IMMUNOGLOBULIN GENES

The PCR (5) can be used to isolate individual genes and members of gene families; the only requirement is some preexisting knowledge of the sequences of either the gene (by protein sequencing, for example) or members of the family. The sequence knowledge does not have to be complete, as the PCR can be used with partially degenerate oligonucleotide primers. For the immunoglobulin variable-domain sequences, databases exist that document the known sequences for different species. For example, the Kabat database (13) contains sequence information for immunoglobulin genes for nine species, including human, mouse, rat, and rabbit. Initially, a comparison of the nucleotide sequences of murine immunoglobulin variable-domain genes was used to design universal oligonucleotide primers that anneal to the 5' and 3' ends of antibody heavy-chain variable (VH) and light-chain variable (VL) domain genes (**Fig. 1**). Moreover, these primers were designed with internal restriction sites, which allows the forced cloning of the PCR products directly for expression (6). Alternatively, for human and mouse VH and VL genes, PCR primers have been designed that are specific for different VH and VL families at the 5' ends and/or anneal to the highly conserved constant-domain genes (4, 7, 14). A further approach is to design primers that are complementary to the leader sequences rather than the 5′ ends of the mature VH or VL genes (15–17) (Fig. 1).

Thus, a variety of primers exist that can be used in the PCR to isolate either diverse repertoires of antibody variable-domain genes from heterogeneous populations of antibody-producing cells or, more simply, the variable-domain genes from a particular hybridoma. The repertoires of VH and VL genes can be cloned for expression in *E. coli* as VH domains (2), Fv fragments (2, 18), single-chain Fv (scFv) fragments (19, 20), or Fab fragments (21) (**Fig. 2**). The challenges now lie in the development of systems for the screening, and preferably selection, of clones producing antibody fragments of the desired antigen-binding specificity.

[1]Abbreviations: PCR, polymerase chain reaction; scFv, single-chain Fv; CDR, complementarity-determining region; SOE, splicing by overlap extension; phOx, 2-phenyloxazol-5-one; ADCC, antibody-dependent cell-mediated cytotoxicity; VH, heavy-chain variable; VL, light-chain variable.

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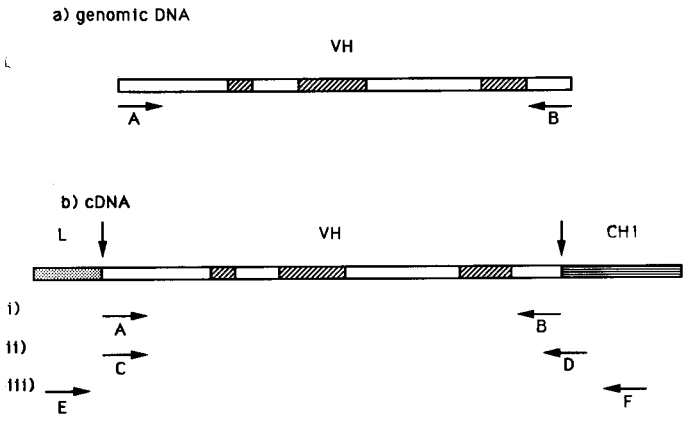

Figure 1. Strategies for PCR amplification of immunoglobulin VH domain genes: *a*) from genomic DNA isolated from antibody-producing cells, primers A (5') and B (3') can be used to isolate the rearranged VH genes (2, 6). These primers anneal to the 5' and 3' ends of the coding sequences of the VH domain genes, and productively and nonproductively rearranged genes will both be isolated; *b*) from cDNA isolated from antibody-producing cells. *i*) primers A (5') and B (3') can be used, as in *a*. In this case, only productively rearranged genes will be isolated; *ii*) primers C (5') and D (3') can be used on cDNA only, as primer D overlaps both the J region and CH1 domain (7); *iii*) primers E (5') and F (3') can be used on cDNA only, because E anneals to the leader sequence (L) and F anneals to the CH1 domain (15–17). All primers can be partially degenerate or family-specific. Hatched boxes represent CDR1, CDR2, and CDR3. The leader exon is represented by a stippled box, and the 5' end of the CH1 domain by horizontal hatching. Vertical arrows indicate the location of intron-exon boundaries in the corresponding genomic DNA that encodes rearranged immunoglobulin genes. Similar strategies can be used for the isolation of VL genes.

EXPRESSION OF IMMUNOGLOBULIN FRAGMENTS IN *E. COLI*

The development of secretion systems for the production of functional Fv and Fab fragments (18, 21) has led to the use of *E. coli* as a host of choice for the expression of immunoglobulin fragments. In these systems, signal sequences such as the pectate lyase (pelB) leader sequence (21), which is derived from the pelB gene of *Erwinia carotovora,* are linked up in translational frame to the genes encoding immunoglobulin fragments. This results in secretion of the expressed protein into the periplasmic space. Fv fragments can be secreted in yields of 2–10 mg/l of culture and Fabs in yields of 2–5 mg/l (2, 18, 21). Although Fv fragments may have advantages over Fabs due to their small size, the stability of the noncovalent VH-VL domain association appears to be variable. This probably results from differences in the sequences of the third hypervariable (CDR3) loops from one antibody to another. These loops form the core of the antigen-binding site that bridges the interface of the VH-VL domain interaction (23–25). These residues may, if not directly involved, affect the stability of the VH-VL domain interaction by modulation of the conformation of the flanking framework residues.

There are several ways of stabilizing the association, as follows.

1) The VH and VL domains can be expressed as a scFv fragment (19, 20, 26), in which the VH domain is linked by a peptide linker to the VL domain. Suitable peptide linkers have now been designed, and the scFvs can be expressed as either intracellular inclusion bodies (19, 20, 26) or as secreted proteins (A. D. Griffiths and E. S. Ward, unpublished

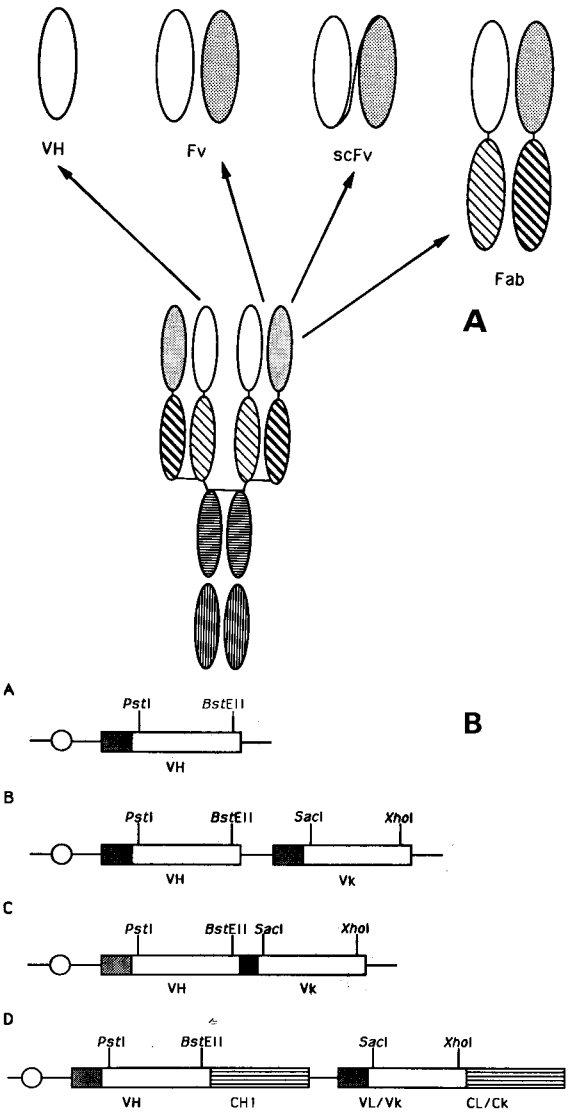

Figure 2A. Schematic representation of the immunoglobulin molecule. The immunoglobulin IgG molecule consists of strings of discrete domains, and comprises two heavy chains and two light chains (22). The heavy and light chains are linked by an intermolecular disulfide bridge (indicated by a horizontal line between the hatched CH1 and CL domains). The heavy chains are also linked to each other by one or more intermolecular disulfide bridges. These -S-S- bridges are in the hinge region, which links the CH1 domain to the CH2 domain (indicated by horizontal hatching). The CH3 domain is indicated by vertical hatching. Immunoglobulin-derived fragments that can be expressed and secreted from recombinant *E. coli* cells are shown at the top of the figure. VH, heavy-chain variable domain; Fv, VH and light-chain variable (VL) domains; scFv, VH and VL domains linked by a synthetic peptide linker (19, 20); Fab, Fd (VH linked to CH1 domain) and paired light chain. *B*) Plasmid vectors for the secretion of immunoglobulin fragments (2, 4). A, VH domains; B, Fv fragments; C, scFv fragments, and D, Fab fragments. The restriction sites in these plasmids are designed for the cloning of murine VH and VL genes, and can be modified to accommodate genes tailored with different restriction sites. VH genes are cloned as *Pst*I-*Bst*EII fragments, and the VL genes as *Sac*I-*Xho*I fragments. These sites are incorporated during the PCR (primers for VH genes are as described in refs 2, 6; for VL genes, VK2BACK, ref 38, and 3' primer: 5'-CCG TTT CAG CTC GAG CTT GGT CCC 3'). The pelB leader sequences (21) are represented by stippled boxes, VH and VL genes by open boxes, the single-chain linker sequence (20) by vertical hatching, and the antibody CH1 and CL domains by horizontal hatching. Open circles represent the *lacz* promoter.

results). The secretion yields may be lower than those using resolubilization from isolated inclusion bodies, but the lower yields may be offset by the relative ease of purification by using a secretion system. If high yields are desirable, for example in industrial scaleup, the isolation from inclusion bodies may be a preferable route. In some cases, a small decrease in binding affinity has been observed for scFvs compared with the corresponding Fv (19, 20), but generally this is not significant. For scFvs isolated from inclusion bodies, the decrease in affinity may be due to incomplete renaturation rather than the presence of the single chain linker.

2) Genetic engineering has been used to insert an intramolecular -S-S- bridge between the two domains (light-chain residue 55 and heavy-chain residue 108; light-chain residue 56 and heavy-chain residue 106) (27). This results in the expression of functionally active Fv fragments that are stably associated.

3) Glutaraldehyde treatment has been used to chemically cross-link the VH and VL domains (27).

ANTIBODY FRAGMENTS WITH ANTIGEN-BINDING ACTIVITIES

Diverse repertoires of antibody VH domain genes, isolated by the PCR, have been cloned for expression in *E. coli* using the pelB leader sequence to direct the expressed protein into the periplasmic space (2). After several hours of induction, the expressed protein starts to leak from the periplasmic space into the culture supernatant (18). Culture supernatants can therefore be conveniently screened for the presence of fragments with antigen-binding specificities by ELISA. Using such a secretion system, VH domains with antigen-binding activities were isolated against two different antigens (lysozyme and keyhole limpet hemocyanin) from repertoires of VH domain genes generated by the PCR from the spleen DNA of immunized mice (2). The anti-lysozyme VH domains were characterized and shown to have high and specific antigen-binding affinities. Subsequently, VH domains with specificities against mucin and influenza virus neuraminidase have been isolated (D. Allen, personal communication; P. Hudson, personal communication).

The rather hydrophobic nature of VH domains, due to the exposed residues that are normally capped off by the paired light chain (28), may offset the advantages of their small size. It may be possible to reduce the hydrophobicity by judicious replacement of the hydrophobic residues by more hydrophilic ones using protein engineering, which would make these small domains attractive reagents for use in therapy and diagnosis.

The smallest units of antigen binding isolated to date are complementarity-determining region (CDR)-derived peptides (29, 30). These peptides were designed on the basis of modeling and/or sequence analysis, and could, if a general feature for a larger number of antibodies, prove to have potential uses in therapy— for example, as blocking reagents.

RANDOM COMBINATIONS OF REPERTOIRES OF VH AND VL DOMAIN GENES

An alternative approach for the isolation of fragments with antigen-binding specificities from repertoires is to randomly combine the isolated VH and VL genes by either restriction at a unique site (4), or by using splicing by overlap extension (SOE, ref 31, **Fig. 3**). Huse and colleagues (4) reported the expression of randomly combined variable-domain genes as Fab fragments using a lambda Zap expression vector. After

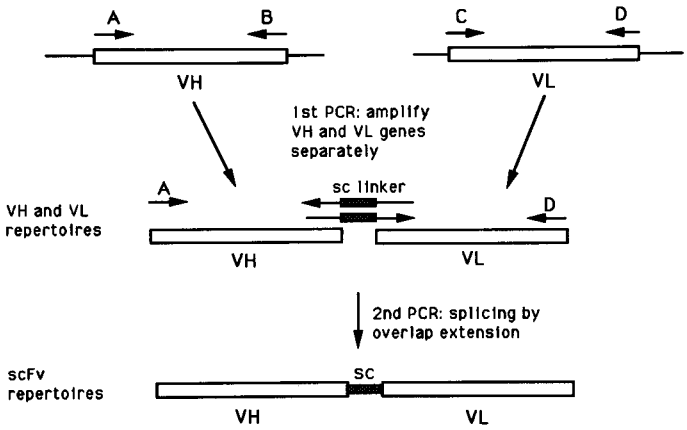

Figure 3. Schematic representation of splicing by overlap extension (SOE, ref 31) for the generation of scFv genes from random combinations of repertoires of VH and VL domain genes (38). The VH genes are isolated from antibody-producing cells using the PCR and primers A (5') and B (3'). The VL genes are isolated from antibody-producing cells in a separate PCR using primers C (5') and D (3'). The VH and VL gene repertoires are randomly combined by splicing together in a PCR with primers A and D, together with single chain (sc) linker primers. These sc linker primers encode a synthetic peptide linker (20) and also have regions of identity or complementarity to primers B (3' of VH genes) and C (5' of VL genes). Open boxes represent the VH and VL domain genes, and stippled boxes represent the sc linker sequence.

transfection, the resulting plaques were screened using radiolabeled antigen. Initially this was carried out for the hapten NPN, and has since been extended to the isolation of antigen-binding activities from repertoires of genes derived from both murine and human antibody-producing cells (8–10). For example, this approach has been used to isolate anti-tetanus toxoid Fabs from repertoires derived from PBLs from humans (who have recently been boosted against tetanus infection), demonstrating the utility of this route for the isolation of 'pure' human antibodies (9, 10). This is significant, as to date the production of human monoclonal antibodies by hybridoma technology has proved to be difficult due to both technical and ethical barriers (32–35). A general problem encountered during the isolation of human antibodies is that for ethical reasons it is not possible to immunize humans. Without immunization the frequency of antigen-specific clones will probably be extremely low, as exposure of the immune system to immunogen results in clonal expansion and affinity maturation of antigen-specific cells with extremely high efficiency (36, 37). Thus, to isolate fragments with antigen-binding specificities from naive repertoires, using *E. coli* as a host, it is necessary to mimic the efficiency of the in vivo immune system in identifying low-frequency, and possibly low-affinity, antigen-specific clones. Thus, the challenges lie in the development of suitable selection systems that will allow this goal to be achieved.

SELECTION VS. SCREENING

The systems described previously involve screening rather than selection. To isolate low-frequency clones from gene repertoires derived from unimmunized animals there is a clear need for the development of selection systems to avoid tedious screening. McCafferty and colleagues (11) have designed such a selection system that involves the expression of

scFvs on the surface of bacteriophage fd. This is achieved by insertion of genes encoding scFv fragments into the gene III coat protein of the bacteriophage. Initially, the anti-lysozyme D1.3 scFv was expressed in this way, and phage expressing antigen-binding activities enriched for, by passage over lysozyme sepharose, by a factor of a million from a pool of irrelevant phage. This system has more recently been modified and extended to the selection of scFvs with anti-2-phenyloxazol-5-one (phOx) binding activities, with a range of affinities, from repertoires of VH and VL domain genes derived from spleen cDNA of immunized mice (38). For combinatorial libraries (of about 10^6 clones in size) derived from unimmunized animals, no phOx binding activities were selected (38). This suggests that to isolate binding activities from unimmunized repertoires, it will be necessary to generate extremely large libraries.

A similar phage surface expression system has been described by Kang and colleagues (12), who expressed Fab fragments on the surface of bacteriophage M13 by linkage to the gene VIII coat protein. This system differs from the fd system insofar as there are 1–24 copies of the gene VIII protein that are expressed over the entire phage surface. In contrast, the gene III coat protein is expressed in three or four copies per phage particle and these are located at the tip of the particle.

The phage systems (11, 12) should prove to be extremely useful tools for the selection of antigen-binding activities from repertoires. Alternatively, these selection systems could be applied to the generation of higher-affinity antibodies. After identification of phage with surface-bound binding activities, it is easy to isolate the VH and VL genes by PCR and clone them for secretion from *E. coli* as either (sc)Fv or Fab fragments, using vectors similar to those shown in Fig. 2*B*. This allows the selection of fragments of the desired specificity to be followed by purification and characterization.

Thus, there are now ways to isolate antibody variable-domain genes by the PCR, to clone these for expression and to select phage which bear fragments with antigen-binding affinities (**Fig. 4**). After isolation of the Fv or Fab of desired binding specificity, there are many potential uses. It may be desirable to increase the affinity of the fragments using random PCR mutagenesis (39) followed by selection (11, 12). The high-affinity fragments may have uses either alone or linked up genetically to effector functions to generate reagents for therapy and diagnosis.

GENERATION OF HIGHER-AFFINITY ANTIBODIES BY IN VITRO MUTAGENESIS

Random mutagenesis can be used, coupled with the PCR, to hypermutate particular regions of an antibody-variable domain. This has been applied to the hypermutation of the anti-lysozyme D1.3 VH domain, and higher-affinity variants have been isolated by tedious screening (39). The phage selection systems now offer an attractive alternative for the selection of higher-affinity variants from many hypermutated clones. This could prove to be an extremely useful route for improvement of the binding affinities of antibody fragments. It may even be possible to hypermutate a germline variable-domain gene and generate novel specificities, and this could be particularly useful when the antigen is not immunogenic, or for the production and isolation of human-derived antibodies for which immunization is not ethical. Questions arise as to how much hypermutation of a human VH or VL domain can be carried out before the immunoglobulin fragments become immunogenic, if they are to be used in human therapy. For example, will hypermutation of

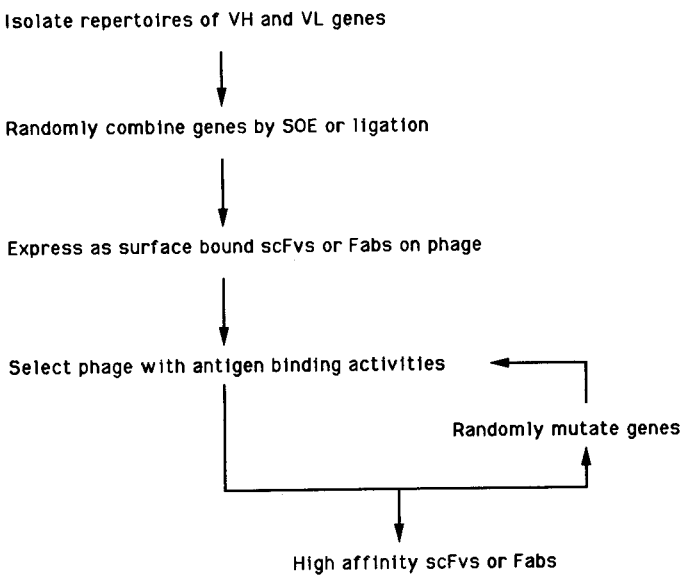

Figure 4. Scheme for the production of high-affinity Fv or Fab fragments using a combination of bacteriophage selection systems (11, 12, 38) and random mutagenesis. For further details, see text.

CDR loops result in sequence motifs that are antigenic in vivo? In addition, will such new specificities have autoreactivities; in other words, will such novel specificities represent self-reactive clones that are normally forbidden in vivo?

USES OF IMMUNOGLOBULIN FRAGMENTS FOR IN VIVO THERAPY AND DIAGNOSIS

Immunoglobulin Fv and Fab fragments have been demonstrated to have rapid clearance rates in vivo, with half-lives on the order of 10–60 min (40; A. J. Cumber, E. S. Ward, G. Winter, G. D. Parnell, and E. J. Wawrzynczak, unpublished results). The absence of the immunoglobulin CH2 domain (41), together with the small size of the fragments, are probably the main factors that result in the short half-lives. In this respect, an $F(ab)_2$ fragment has been shown to have a half-life approximately twice as long as that of the corresponding Fab fragment (40). Fv fragments may also have a tendency to dissociate in vivo, as recently demonstrated for the anti-lysozyme D1.3 Fv (A. J. Cumber et al., unpublished results), and scFv or Fab fragments may therefore be preferable reagents for in vivo applications. Alternatively, a combination of genetic engineering and protein chemistry have been used to construct a bivalent Fv fragment that has improved stability in vivo (A. J. Cumber et al., unpublished results), and the bivalent nature of this protein may be advantageous as it increases the avidity of the interaction with antigen.

Immunoglobulin fragments have uses in clinical situations where rapid clearance is either required or advantageous. Such a situation is the imaging of tumors, as the rapid clearance results in reduced immunogenicity. This is of particular relevance if the imaging is to be followed by therapy with the same antibody as a complete molecule. In addition, Fabs have been shown to give high tumor: normal tissue ratios compared with complete antibodies (42), possibly due to the lack of Fc receptor-mediated binding (43, 44) and rapid clearance. A further application of immunoglobulin fragments is for the treatment of drug overdoses, as they could

be used to 'mop up' the excess drug and clear it rapidly from the circulation.

REBUILDING FV AND FAB FRAGMENTS INTO REAGENTS WITH EFFECTOR FUNCTIONS

For treatment of disease (for example, cancer or infections) there is a need to graft effector functions onto the antibody fragments, such as toxins (45–49) or the Fc portion of the immunoglobulin molecule (43, 44, 50). A truncated form of *Pseudomonas* exotoxin has been genetically linked to an antitumor Fv fragment to generate an immunotoxin that can be expressed in high yields in *E. coli* (48). In addition, an Fab-toxin construction, containing the gene encoding phospholipase C from *Clostridium perfringens,* has been secreted in functional form from recombinant *E. coli* cells (49). A limitation of Fv-toxin conjugates may be that they are highly immunogenic due to the toxin moiety and also have a rapid rate of clearance. It may be possible to avoid an undesirable immune response by using different toxin moieties for cases where repeated doses are necessary. The feasibility of this clearly depends on the availability of different Fv-toxin conjugates with suitable properties for therapy.

For some situations therefore it may be preferable to use the Fv or Fab fragments of desired specificity as building blocks for complete immunoglobulin molecules. The complete immunoglobulin is stable in vivo (half-life of days), and providing the isotype is derived from the same species, it is less immunogenic than toxin moieties. The choice of isotype will depend on the functions required, as some studies have indicated that different isotypes vary considerably in their complement lytic and antibody-dependent cell-mediated cytotoxicity (ADCC) activities (51). It appears that for most clinical situations, the IgG1 isotype is the one of choice (51). This isotype has been used effectively in therapy, linked for example to the humanized variable domains of CAMPATH-I (52). After rebuilding of an Fv or Fab fragment into a complete antibody at the genetic level, the antibody can be expressed using either mammalian cell transfectomas (53) or the baculovirus system (54), which has recently been shown to express high yields of functionally active antibodies. Thus, antibody fragments can be rapidly isolated by selection from VH and VL domain repertoires which are expressed on the surface of bacteriophage, and used to rebuild antibodies for therapy. Such tailor-made antibodies have the advantages of high affinity and specificity (if necessary generated by in vitro mutagenesis), and the effector functions of choice. These recombinant antibodies promise to have extensive uses in therapy and diagnosis. FJ

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