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ANTIBODY ENGINEERING

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■ **Abstract** Antibodies are unique in their high affinity and specificity for a binding partner, a quality that has made them one of the most useful molecules for biotechnology and biomedical applications. The field of antibody engineering has changed rapidly in the past 10 years, fueled by novel technologies for the in vitro isolation of antibodies from combinatorial libraries and their functional expression in bacteria. This review presents an overview of the methods available for the de novo generation of human antibodies, for engineering antibodies with increased antigen affinity, and for the production of antibody fragments. Select applications of recombinant antibodies are also presented.

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INTRODUCTION

The significance of antibodies as diagnostic and analytical reagents has been known and exploited for almost a century. In recent years, antibodies have become increasingly accepted as therapeutic reagents, particularly for cancer but also for numerous other disorders. An indication of the emerging significance of antibody-based therapeutics is that over a third of the proteins currently undergoing clinical testing in the United States are antibodies. Until the late 1980s, antibody technology relied primarily on animal immunization or the expression of engineered antibodies in a eukaryotic host. However, the development of methods for the expression of antibody fragments in bacteria, together with the emergence of powerful techniques for screening combinatorial libraries and an expanding structure-function data base has opened unlimited opportunities for the engineering of antibodies with tailor-made properties for specific applications. Antibodies of low immunogenicity, suitable for human therapeutic or diagnostic purposes, can now be engineered with relative ease. Such reagents can greatly enhance and complement other biomedical engineering technologies. This chapter presents an overview of the current methodologies for antibody isolation and functional optimization. Select applications of possible relevance to biomedical engineering are also discussed. However, we apologize in advance to the reader because, due to space limitations, it was not possible to cover numerous current or emerging areas of antibody technology.

ANTIBODY STRUCTURE AND THE RECOGNITION OF ANTIGENS

There are five classes of immunoglobulins: IgM, IgG, IgA, IgD, and IgE (3). From a biotechnology perspective, by far the most important class of antibodies is IgG and to a lesser extent IgM and IgA. IgMs are pentamers, and their large size results in rather poor pharmacokinetic properties, whereas their low specificity renders them less desirable than IgG antibodies for diagnostic applications. Secretory IgAs can potentially be very important as a means of passive immunization against genital, gastrointestinal, and oral pathogens. However, until recently production of useful amounts of monoclonal secretory IgAs has been problematic (4, 5).

Antibodies belonging to the IgG class are homodimers of two identical polypeptide chains of 450 amino acids (heavy chains) and two identical chains of 250 amino acids (light chains). The structure of each of the four heavy and two light chain domains has the characteristic immunoglobulin fold consisting of two antiparallel β-sheets with an intramolecular disulfide bond. The N-terminal domains of each chain are unique in that the three loops connecting the β-sheets are highly variable in length and sequence. The six hypervariable loops, or complementarity-determining regions (CDRs) form a unique surface that specifically recognizes and binds an antigen (see Figure 1).

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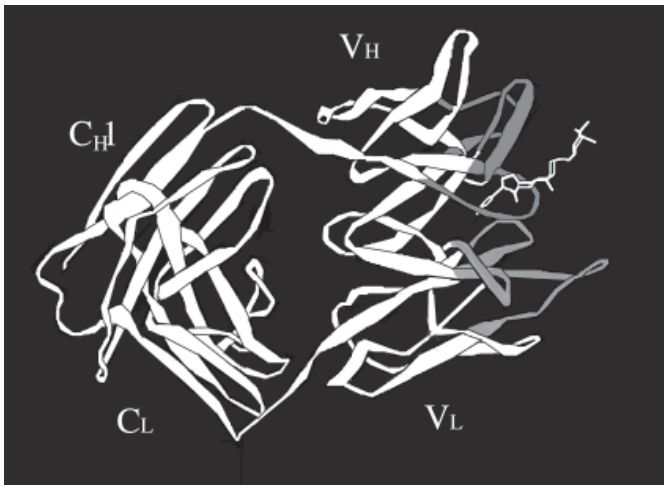

Figure 1 Ribbon diagram from the crystal structure of the antidigoxin Fab, shown in complex with the small molecule steroid, digoxin. Framework variable regions are shown in white, CDR regions in light gray, digoxin in white. Note that only the CDR regions mediate antigen contact. [From PDB file 1IGJ (1).]

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The recognition of antigens by high affinity antibodies is generally described as a "lock and key" fit, in that the conformation of the antibody is generally not greatly perturbed upon antigen binding. However, it should be kept in mind that not all antibody:antigen complexes fit this description, and large conformational changes in antibody binding pockets are not uncommon. MacCallum et al (6) have classified antibody binding sites into four classes: concave and moderately concave (mostly small molecule binders), ridged (mostly peptide binders), and planar (mostly protein binders). Because antigen binding is thermodynamically favorable, it is accompanied by a decrease in free energy, and numerous biochemical and crystallographic studies have explored the energetics of protein-ligand binding (7). In general, antigen recognition is enthalpically driven by van der Waals interactions, salt bridges, and hydrogen bonds, whereas the entropic cost of forming an antibody:antigen complex is partially compensated by desolvation effects. Water molecules play a critical role in stabilizing the interaction, as they fill cavities where the geometric complementarity is imperfect and ensure that all hydrogen bond donors and acceptors are compensated (8). Within the binding pocket, "hot spots," a small number of residues within the binding site that account for a large fraction of the interaction energy, can often be found (9) and are a common theme in biological recognition (10).

ANTIBODY ENGINEERING TECHNOLOGIES

Antibody Cloning

Antibody engineering became possible with the development of hybridoma technology in 1975, relying on the fusion of a myeloma cell line with B-cells from an immunized animal (11). Monoclonal antibody technology remains one of the core technologies of biotechnology, and thousands of medically and diagnostically relevant hybridomas have been developed.

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A revolution in antibody technology began in the late 1980s when efficient systems for the cloning and expression of antibody genes in bacteria were developed (12). Cloning the genes of monoclonal antibodies from murine hybridomas was greatly simplified by the discovery of the polymerase chain reaction (PCR), and bacterial expression systems allowed the rapid and facile production of functional recombinant antibody fragments for analysis (13).

To clone antibodies, mRNA is isolated from hybridoma, spleen, or lymph cells, reverse transcribed into DNA, and antibody genes are amplified by PCR. This strategy requires oligonucleotide primers that can recognize any antibody gene. Numerous primer sets have been published, with 5′ primers based on the N-terminal sequence of purified antibodies (14), rapid amplification of cDNA ends (15), antibody leader sequences (16), and most popularly, primers based on known variable region framework amino acid sequences from the Kabat (17) and V-base databases (18–20).

An artifact of the process used to generate hybridomas is that many myeloma cell lines express irrelevant heavy or light chains, in addition to the desired monoclonal antibodies (21, 22). The general cloning strategy outlined above will amplify all antibody genes present at the mRNA level, and multiple heavy and light chain genes in addition to the desired ones may thus be cloned (20). Although techniques have been developed to selectively remove aberrant mRNA (23), it is critical that cloned antibody genes are expressed in functional form to confirm their ability to bind antigen (20). In addition, mutations are often introduced by the degenerate primers themselves, even when the correct chain is cloned (24), further underscoring the need to screen cloned genes for function.

Once the correct genes encoding V_H and V_L domains have been identified, they can be assembled in a number of forms suitable for expression or further manipulation. The smallest antibody-derived polypeptide that can bind antigen with reasonable affinity is a single V_H chain. V_H chains are very unstable and prone to aggregation in vivo because the largely hydrophobic area that normally forms the interface with the V_L domain is exposed to the solvent. However, one class of V_H chains derived from camelids, such as camels or llamas, are naturally found without a light chain, yet exhibit high antigen affinities (25). Sequence and structural analysis of the camelid V_H chains (26, 27) has been used to guide the rational design of mutations in a human V_H chain that rendered it stable in the absence of a V_L chain (28–30). Alternatively, a unique murine V_H chain found to be naturally stable without a V_L chain has been engineered to recognize different antigens (31).

In general, the presence of both the V_H and V_L chains is needed for high stability and antigen affinity. V_H and V_L chains can be expressed as separate polypeptides in bacteria where they assemble into Fv fragments (see Figure 2). However, in these dimeric proteins the two polypeptide chains are held together by noncovalent interactions and are therefore prone to dissociation and aggregation. The two chains can be covalently assembled by engineering an interchain disulfide bond to give a dsFv antibody. This design is more stable than an Fv fragment, but is difficult

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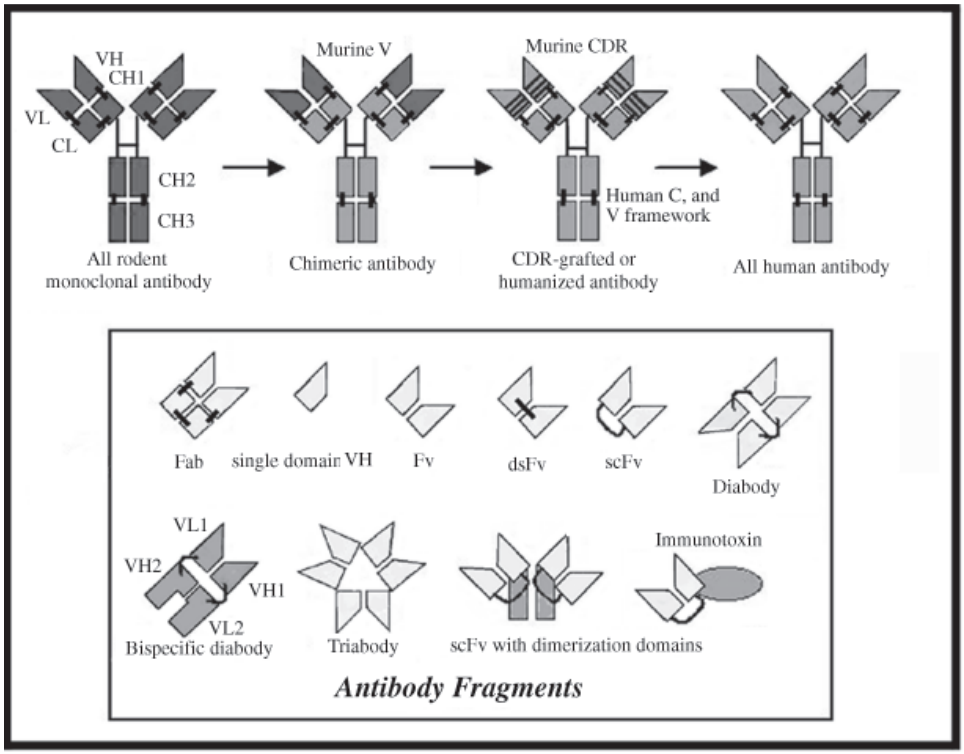

Figure 2 Schematic showing various antibody fragments of biotechnological and clinical interest. Each block represents one antibody domain with a characteristic immunoglobulin fold. Black bars, inter-chain di-sulfide bonds (horizontal) or intra-domain linkages (vertical); longer curved lines, genetically engineered polypeptide linkers. [Adapted from (2).]

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to produce by fermentation, and the disulfide bond can be reduced under mild conditions. Recombinant DNA techniques can introduce a short polypeptide linker to fuse the V_H and V_L chains together into a scFv antibody fragment (see Figure 2). scFvs are relatively small (26–27 kDa), generally quite stable, and are encoded by a single gene, which simplifies genetic manipulations. The most common linker is a flexible $(Gly_4Ser)_3$ decapentapeptide (32). The two variable domains can be connected either as V_H-linker-V_L or V_L-linker-V_H, with the former being more common. The order of the two domains can affect expression efficiency, stability, and the tendency to form dimers in solution (33, 34). If a scFv is found to have poor stability or low affinity compared with the parental antibody, engineering the linker sequence may improve function. A variety of linkers have been designed based on structural considerations, screening of combinatorial linker libraries, or natural linker sequences occurring in multi-domain polypeptides (35–40).

In addition to scFvs, the other commonly used recombinant antibody fragments are Fabs (see Figure 2). Fabs consist of two polypeptide chains, one containing the light chain variable and constant domains, V_L-$C_{\kappa \text{ or } \lambda}$, the other a truncated heavy chain containing the variable domain and one constant domain, V_H-C_{H1}. Just as in intact IgG immunoglobulins, the two chains are linked together by a disulfide bond. The more extensive interface between the two chains and the presence of the

disulfide bond confer increased stability to denaturation. Although the expression of Fab requires the association of two chains, it often occurs quite efficiently in bacteria (41, 42). On the other hand, the presence of two chains somewhat complicates genetic manipulations, and the larger size of these proteins may limit their bioavailability for certain therapeutic applications.

Measuring Antibody Affinity

The most important performance criterion for immunoglobulins or recombinant antibody fragments is the equilibrium binding constant for the formation of an antibody:antigen complex:

$$\mathrm{Ab} + \mathrm{Ag} \underset{\mathrm{k_{off}}}{\overset{\mathrm{k_{on}}}{\rightleftharpoons}} \mathrm{Ab} - \mathrm{Ag}$$

where, $K_D = k_{off}/k_{on}$.

Accurate determination of the equilibrium constant, K_D, is not trivial, as it requires the quantitation of the concentration of both the complex and either free antibody or antigen in solution under conditions that do not perturb the equilibrium (43, 44). One of the most reliable and relatively easy equilibrium techniques is competition ELISA (45). Techniques requiring greater sophistication and specialized equipment include (*a*) fluorescence quenching, whereby the binding of antibodies to fluorescent antigens or to fluorescent conjugates quenches the fluorescence, providing a convenient way to determine equilibrium concentrations; (*b*) isothermal titration microcalorimetry used to determine the enthalpy and equilibrium constant from which the entropy of binding can calculated (46); (*c*) flow cytometry, a convenient way to measure the dissociation rate constant k_{off} for recombinant antibodies displayed on the surface of microbial cells (47–49); and (*d*) surface plasmon resonance (SPR).

In recent years, SPR has emerged as the method of choice for measuring the kinetics of protein-ligand interactions (50, 51) and can be used to analyze antibodies having K_D values from 10^{-5} to 10^{-11} M (52). Although not without pitfalls, SPR is a relatively simple and fast technique. Briefly, the antigen is immobilized on a derivatized dextran matrix on a sensor chip, and the amount of antibody that binds under laminar flow conditions is detected as a change in resonance angle of incident light, which is reported as "response units" (see Figure 3). The kinetics of the bimolecular association reaction are calculated from experiments with different antibody concentrations. The dissociation rate is measured as a decrease in the amount of bound protein when the solution flowing over the chip surface contains free antigen (as a competitor) and no antibody. Usually, the key parameter that determines the K_D is the rate of dissociation of the antibody:antigen complex, which can be determined readily by SPR without needing a purified protein preparation. k_{off} can be determined using antibodies displayed on the surface of bacteriophages, in crude bacterial lysates, and even with antibodies synthesized by in vitro transcription-translation (53, 54; G Georgiou, unpublished results).

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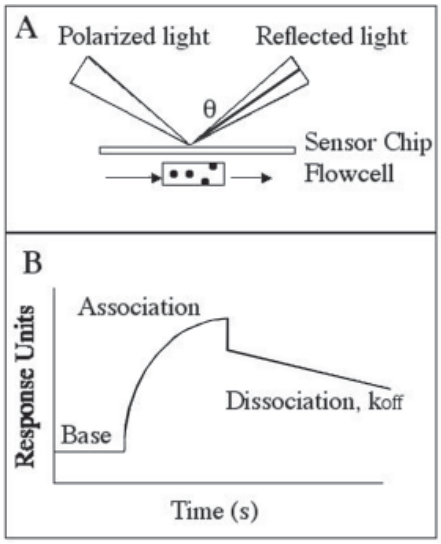

Figure 3 Affinity measurement by surface plasmon resonance. (*A*) Physical configuration: antigen is covalently bound to a sensor chip; the antibody flows over the surface. Binding is detected by a decrease in light intensity for a given incident angle. (*B*) A typical sensorgram. During the association phase, antibody flows over the chip and binds immobilized antigen; response units (RUs) increase. During dissociation, buffer with free antigen flows over the chip, and antibody dissociates from antigen, resulting in exponential decay of the RU signal with a characteristic rate constant k_{off}.

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Antibody Library Screening

The in vitro isolation and engineering of antibodies hinges on the ability to screen large combinatorial libraries of antibody genes to isolate clones that exhibit specific binding to a desired target molecule. Efficient high throughput screening technologies had to be developed because library sizes between 10^5–10^{11} clones have to be screened for most immunotechnology applications. During the last 10 years, a variety of techniques of increasing sophistication and sensitivity have been developed to aid in the isolation of high affinity antibodies from such large libraries. These have been reviewed recently (56–58), and therefore the topic is covered here only briefly.

The simplest library screening strategy is to clone an antibody gene repertoire into a λ bacteriophage expression vector (59). Upon infection of a lawn of bacterial cells growing on a plate, zones of clearance (plaques) containing antibodies released from lysed cells, as well as bacteriophage progeny, are formed. The plate is overlayed with a membrane filter that has a high binding capacity for proteins. The filter is then reacted with a radiolabeled antigen, and spots corresponding to the position of plaques that produce specific antibodies are visualized by autoradiography. The bacteriophage encoding the specific antibodies are then isolated from the plaques and used for further analysis. This technique has been used successfully for the de novo isolation of antibodies and for affinity improvement applications (59, 60). However, it is more cumbersome than other library screening techniques, particularly when large libraries are being used.

The screening of large libraries of recombinant proteins can be greatly simplified by establishing a direct physical link between a gene, the protein it encodes, and the molecule recognized by the protein. Such a link can be established using a variety of display technologies that are exploited for the screening of libraries in an efficient, high throughput manner (56, 58). Both in vivo and in vitro library

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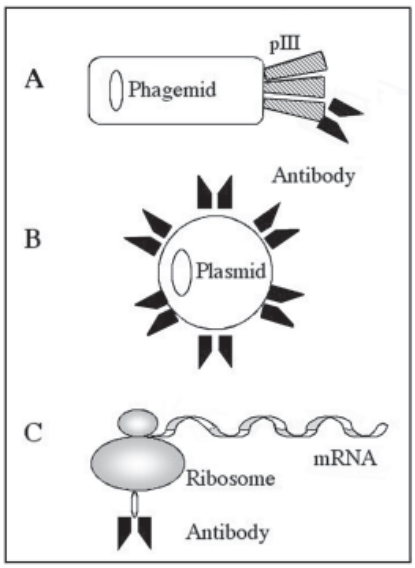

Figure 4 The three major antibody display systems. (*A*) Antibody display on filamentous bacteriophage as a gpIII fusion. Usually one fusion protein is displayed per phage. (*B*) Cell surface display. In this case, the gene is maintained on a plasmid. (*C*) Ribosome display. During an in vitro protein synthesis reaction, a complex is formed between the ribosomes, mRNA, and a properly folded antibody.

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display systems have been developed. For in vivo methods, the protein is encoded by a recombinant gene and displayed on the surface of a biological particle such as a virus or a whole cell (see Figure 4). Currently, the most widely used technique for library screening is based on the display of antibodies on the surface of filamentous bacteriophages (57). Antibody genes are fused in-frame to phage genes encoding surface-exposed proteins, most commonly pIII. The gene fusions are translated into chimeric proteins in which the two domains fold independently. Phage displaying an antibody specific for a ligand can be readily enriched by selective adsorption onto immobilized ligand, a process known as panning (see Figure 5). The bound phage is desorbed from the surface, usually by acid elution, and amplified through infection of *Escherichia coli* cells. Usually, 5–8 rounds of panning, desorption, and amplification are sufficient to select for phage displaying specific antibodies, even from very large libraries (up to 10^{11} clones). Once specific phage have been isolated, they can be used directly to obtain an estimate of antibody ligand affinity by ELISA or SPR. Also, phage infection of certain *E. coli* host strains can be employed to produce the antibody protein alone without being tethered to the pIII protein.

A wide variety of techniques for addressing specific technical issues in the screening of libraries have been developed. These include (*a*) systems for generating phage that predominantly display only one copy of antibody per phage particle, thus eliminating avidity effects during selection (57); (*b*) methods for guaranteeing the release of bound phage from the solid support by disrupting the chemical linkage that is used for ligand immobilization onto the matrix; (*c*) phage enrichment by binding to soluble rather than immobilized ligands, to allow better discrimination of high affinity clones (61); (*d*) selectively infective phage (SIP), a technique for obtaining very efficient enrichment of the desired clones by requiring that only phage bound to ligand are able to infect bacteria for amplification

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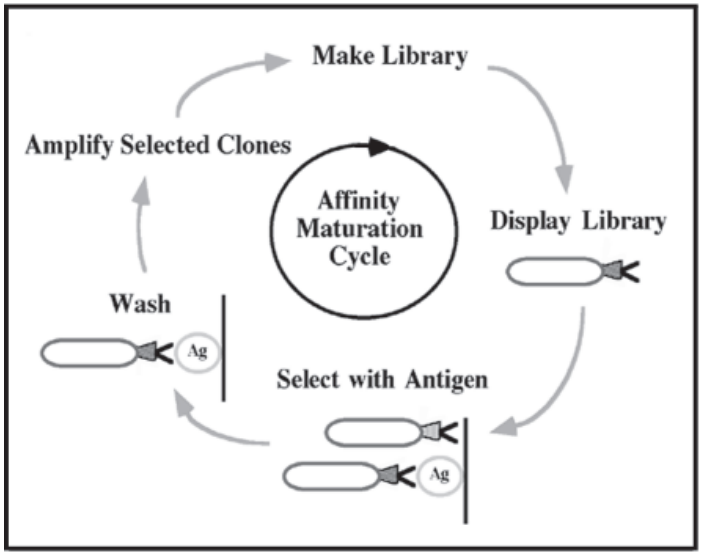

Figure 5 Antibody affinity maturation and library screening cycle. This cycle is similar for all antibody display systems discussed in the text, but monovalent phage display is shown here as an example. The steps of the cycle are (*a*) a library is created and displayed as a phage fusion protein; (*b*) the phage are allowed to bind the immobilized antigen; (*c*) nonspecific and low affinity phage are removed by stringent washing; (*d*) bound phage are eluted and allowed to infect *E. coli* cells to amplify the gene by phage production; and (*e*) the panning cycle is repeated 5–8 times to identify a small number of high affinity antibody genes.

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(62, 63); and (*e*) display on lytic phages that assemble in the bacterial cytoplasm, a strategy especially suited to the production of antibodies that can fold in the cytosol and are thus useful for gene therapy applications (64, 65). A number of excellent reviews describe these and other advances in the applications of the phage display technology for antibody library screening (56, 57, 63, 66).

Examples of some of the most successful applications of the phage display technology in antibody engineering include (*a*) the de novo isolation of high affinity human antibodies from repertoire libraries; (*b*) isolation of genes encoding antigen-specific antibodies from immunized animals and from hybridomas; (*c*) affinity maturation: antibodies with extremely low K_D binding constants, in the low picomolar range, have been generated; (*d*) isolation of antibodies with increased resistance to chemical or thermal denaturation; and (*e*) antibodies specific to cell surface markers or receptors that cannot be produced in purified form have been isolated by panning directly against whole cells and tissue samples.

Antibodies can also be displayed on the surface of microbial cells such as *E. coli* and *Saccharomyces cerevisiae* (47, 49, 67–70). For screening purposes, a library of cells, each displaying multiple copies of a different antibody variant, is incubated with a fluorescently tagged ligand in buffer. Cells displaying antibodies that bind the ligand become fluorescently labeled and are isolated by fluorescence-activated

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cell sorting (FACS). With flow cytometry, the binding of each clone in the library to a particular ligand is determined quantitatively. Parameters such as ligand concentration or time for the dissociation of antibody:ligand complexes can be easily optimized to ensure the isolation of only the highest affinity antibodies. These features are particularly significant for antibody affinity maturation, and in fact, the limited data reported so far indicate that cell surface display may be a superior screening technology for that purpose (47, 48, 71). In addition, the screening of cell-displayed libraries always results in the isolation of antibodies that can be expressed well in the host cell. This is because, in order for a cell to be detected and sorted by the flow cytometer, it must have a minimum threshold fluorescence (roughly equivalent to the binding of at least 1000 ligand molecules conjugated to a small-molecule fluorescent dye). This means that the cell must be able to display at least 1000 correctly folded antibody molecules. If an antibody is expressed poorly, it will not be represented in a sufficiently high number of copies to allow the isolation of the corresponding cell. In contrast, the screening of phage-displayed libraries by panning poses no requirement for good expression, as one copy is displayed per particle. As a result, antibodies isolated from phage-displayed libraries often have very poor expression characteristics.

At present, cell surface display and FACS cannot be used for the de novo isolation of antibodies from natural or synthetic repertoire libraries because the maximum library diversity that can be realistically screened by FACS is about 5×10^8. This is about two orders of magnitude smaller than the library size needed for the isolation of high-affinity antibodies from repertoire libraries (66). However, repertoire libraries can be screened by 1–2 rounds of panning to reduce the library diversity followed by cell display technologies that guarantee the isolation of high-affinity, well expressed antibodies. The isolation of antihapten antibodies from repertoire libraries by such a tandem phage-cell surface display technology has been demonstrated (G Chen, A Hayhurst, J Thomas, B Iverson, Georgiou, in preparation).

In vitro protein synthesis is an excellent means for the facile analysis of large numbers of site-specific protein mutants (73, 74; JA Maynard, B Iverson, G Georgiou, in preparation). In addition, several exciting in vitro library screening techniques have been developed that completely circumvent the need to use a host cell for protein synthesis. In vitro screening technologies employ RNA polymerase for mRNA synthesis from a DNA template. The mRNA is then translated into protein using ribosomal extracts, amino acids, and various cofactors. The advantage of in vitro library screening methods is that the library size that can be screened is vast, potentially 10^{15} different sequences. In theory at least, screening such highly diverse libraries could result in the discovery of entirely new protein functions that cannot be found using cell or bacteriophage libraries. However, this point has not yet been demonstrated experimentally, perhaps because in vitro library screening techniques have been developed only within the last few years (76).

Ribosome display, originally described by Mattheakis et al (77), and adopted to the screening of antibody libraries by Pluckthun (78, 79) and Taussig (80),

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relies on the formation of a ternary complex between ribosomes, mRNA, and the polypeptide (see Figure 4). Complexes containing folded antibodies with a desired specificity are enriched by panning against immobilized ligand. The mRNA of the ribosome-mRNA-protein complexes is reverse transcribed to produce the DNA encoding the antibodies responsible for the binding of the complexes to the immobilized ligand. The DNA is then transcribed by RNA polymerase to begin another cycle of ternary complex formation and selection. Alternatively, a covalent link may be established directly between mRNA and the protein it encodes (81). Covalent mRNA-protein complexes are formed via the reactive amino acid analogue puromycin. The advantage of this technology is that covalent mRNA-protein is more stable and can be subjected to harsh screening conditions to enrich antibodies or other proteins with increased stability.

The screening of combinatorial libraries of antibody genes is one of the most important tools in antibody engineering. The ability to screen large libraries has enabled the development of technologies that mimic the mammalian immune response, allowing the isolation of antibodies without animal immunization, and the engineering of antibodies with very high affinity, increased stability and improved effector functions.

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Bypassing Immunization: Isolation of Antibodies from Large Libraries

In the mammalian immune system, antibodies with high affinities are created in two stages. First, combinatorial recombination and other diversity-generating mechanisms result in the formation of a large collection of assembled, germline antibody genes. Second, B-lymphocytes expressing antibodies of low affinity but high specificity for a target antigen undergo a process of somatic hypermutation in which a high number of mutations accumulate in antibody genes, preferentially within the CDRs (82, 83). B-lymphocytes expressing affinity-matured antibodies exhibiting slower antigen dissociation kinetics are then selected and expanded (3).

Antibody technology can successfully mimic the process by which antibodies are selected by the immune system. This is accomplished by cloning large libraries of antibody genes and selecting for binding to a desired target. If the library is large enough (many contain 10^9 different clones, similar to the number of distinct rearranged human antibodies) an antibody with specificity for almost any antigen should be represented and can be selected.

Immune libraries were first developed as an alternative to hybridoma technology. Immunized rodents or humans exposed to a desired antigen through vaccination or disease have high levels of circulating antibodies to the antigen, and the corresponding mRNAs are highly represented among total antibody mRNAs. Therefore, even small libraries ($\sim 10^5$) from immunized donors give rise to specific antibodies, and because the antibody genes have experienced affinity maturation in vivo, antibodies can potentially be isolated that do not require further affinity improvement. Screening of libraries from immunized animals has been used to

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isolate antibodies specific for viral surface antigens (84), bacterial toxins (85, 86), and cell-surface tumor antigens (87–89) with K_Ds as low as 10^{-10} M. However, the need for immunization poses a number of serious limitations: (*a*) Human antibodies in general cannot be generated by immunization for ethical reasons. (*b*) Immunological tolerance mechanisms make it very difficult and often impossible to isolate antibodies against self antigens, many of which are potentially important therapeutic targets, particularly in cancer. Such antibodies can only be produced by in vitro techniques, as discussed below. (*c*) Antibodies cannot be generated against extremely toxic or chemically unstable antigens that are unsuited for animal immunization.

In principle, antibodies with specificity toward any antigen may be isolated through the screening of a sufficiently diverse pre-existing repertoire of antibody genes. There are two basic strategies for generating such diverse antibody gene repertoires: naïve libraries and semi-synthetic libraries. Naïve libraries representative of the V-gene repertoire are created by cloning the antibody genes found in nonimmunized individuals. mRNA is isolated from peripheral lymphocytes, bone marrow, tonsils, and cadaver spleens. Subsequently, IgM and/or IgG variable regions are amplified using degenerate oligonucleotide primer sets and PCR and are cloned into vectors suitable for screening, typically by phage display. IgM repertoires are preferred to IgG because they have not been subjected to tolerance or antigen selection and are thus more diverse. In contrast, IgG chains can be biased by host immune responses and will not react to self antigens (66). The affinity and specificity of antibodies that can be isolated through the screening of naïve libraries is intrinsically linked to library size. For example, a library consisting of 10^{10} different clones yielded antibodies with affinities in the low nanomolar range (90), whereas a similar library of 3×10^7 clones only resulted in antibodies with micromolar affinities (91, 92). Large libraries contain greater antibody sequence diversity and multiple antibodies against a desired target. Furthermore, antibodies may be present that do not require affinity maturation for in vivo applications (93). Considerable effort is required to generate highly diverse libraries, but once a library has been made, it can be propagated and used repeatedly to isolate antibodies against numerous antigens.

One problem with naïve libraries is that there is very little information on the exact nature of the V-gene repertoire contained in the library. As a result, the usefulness of the library in terms of obtaining antibodies to different antigens can only be determined experimentally on a trial and error basis. Moreover, since the antibody genes are representative of the human immunological repertoire, there is no guarantee that the clones obtained by library screening will encode antibodies amenable to expression in bacteria. In fact, poor expression and toxicity to the host bacteria are often issues with antibodies isolated from naïve libraries. These problems may be circumvented by using synthetic antibody repertoire libraries. In principle, synthetic libraries are not constrained by the forces of selection in the natural immune system and are therefore more likely to contain novel specificities ideal for generating antibodies to self-antigens. Fully synthetic repertoires are

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created by cloning the germline DNA antibody gene segments that are normally arranged by the immune system to generate the natural immunological diversity. V_H, D, and J or $V_{\kappa/\lambda}$ and $J_{\kappa/\lambda}$ gene segments are arranged combinatorially in vitro so as to reconstitute genes encoding complete V_H and V_L chains (94). This synthetic repertoire is subsequently cloned into an expression vector to generate a library of 10^7–10^{10} clones. Antibodies with specificity to protein and hapten antigens, including self- and highly conserved antigens, have been isolated from such synthetic libraries (95–98), but their affinities are generally not high (66).

Semi-synthetic libraries have also been generated by selecting one or more antibody frameworks as a scaffold and randomizing sequences within the CDR loops. Randomization of all the CDR residues simultaneously would require the creation of an immense library ($>10^{30}$ clones) and is not practical. Because CDR3 of the heavy chain contributes most of the antigen-binding contacts, it has been predominantly exploited as a target for randomization. Sequences encoding the randomized residues in CDR3 are generated using appropriately designed randomized oligonucleotides (99). Early semi-synthetic libraries gave rise to antibodies with rather low K_D values for antigen binding, typically in the 100 nM range (100). Randomization of key residues in the light chain CDRs, in addition to the heavy chain CDR3, has been employed to improve diversity, resulting in the recovery of higher affinity antibodies (101). Recently, Knappik and coworkers developed the Human Combinatorial Antibody Library (HuCal), which employs "master" frameworks representative of the human V gene subclasses and randomization of CDR3 heavy and light chain residues (102). This highly diverse library has been used to isolate numerous antibodies with high affinities and good expression characteristics in *E. coli*.

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Affinity Maturation

Foote & Eisen have argued that in the mammalian immune response the process of somatic hypermutation results in an antibody affinity floor limiting K_D values to no lower than 10^{-10} M^{-1} (103). The maximum rate of the formation of the antibody:antigen complex is determined by diffusion and is of the order 10^6 M^{-1} s^{-1}, whereas the first order rate constant for complex dissociation is dictated by in vivo selection pressures and can be as low as 10^{-4} s^{-1}. This analysis suggests that while the maximum association rate for the formation of antibody:antigen complexes is dictated by physical considerations, the dissociation rate is determined by biological factors and may be amenable to improvement by molecular engineering. In fact, antibody affinity has been increased by hundreds to thousands of times, and K_D values in the 10^{-11} M range or even subpicomolar have been reported (104–106; KD Wittrup, personal communication).

As a general rule, antibodies generated either by animal immunization or by repertoire library screening exhibit antigen affinities (K_D) in the 10^{-6} to 10^{-9} M range. Highly hydrophilic antigens such as carbohydrates result in lower affinity antibodies, whereas higher affinities are found with hydrophobic small molecules and

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proteins. A number of strategies have been used to modulate antibody:antigen interactions. These include (*a*) site specific mutagenesis based on the crystal structure or homology models of the antibody:antigen complex, (*b*) chain shuffling, (*c*) screening of libraries in which one or more CDRs are subjected to combinatorial mutagenesis, (*d*) extension of the heavy chain CDR3 loop to introduce additional contacts between the antibody and the antigen, (*e*) random mutagenesis over the entire V-gene, and (*f*) recombination of beneficial mutations isolated from (*a*)–(*e*) above.

Rational structure-based mutagenesis requires a crystal structure of the antibody complexed with the antigen or a computer model (106, 108–110). Chain shuffling is based on the observation that during affinity maturation multiple light chains can be paired with heavy chains to give rise to functional antibodies (111), and light chain promiscuity has been shown to modulate affinity (112). In early studies, the light chain of a known antibody was replaced with a repertoire of light chains, and clones with increased affinity were obtained by phage display (113, 114). For example, pairing the heavy chain of a murine anti-2-phenyloxazol-5-one antibody with a library of nonimmune human light chains improved the K_D 20-fold, from 3.2×10^7 M to 1.5×10^{-8} M (115). In addition to improving affinity, chain shuffling has also been used to generate completely humanized antibodies starting from a murine antibody (116).

Combinatorial mutagenesis of amino acid residues within the CDR loops has proven to be a very effective, albeit time consuming, route to affinity improvement. In short, libraries of mutant antibodies are created by randomizing a select set of CDR residues to all 20 possible amino acids. Randomization can be easily accomplished using degenerate oligonucleotides. However, it is not possible to randomize all CDR residues at the same time; even randomization of six residues (using an NNG/C randomization scheme, where N is any of the four nucleotides) gives rise to a library of over a billion clones. Because there are well over 40 CDR residues in antibodies, subsets of the ones deemed most important must be selected for randomization. The heavy and light chain CDR3 residues usually contribute the majority of binding interactions, so residues within these regions are targeted for randomization (117). Alternatively, subsets of residues within different CDRs are randomized, the libraries screened for improved affinity, and the best mutations combined, often resulting in even greater functional improvement (104, 105). If a crystal structure of the antibody:antigen complex is available the selection of residues for randomization can be greatly facilitated. In one particularly notable example, Lowman and coworkers used structure-guided randomization and selection to improve the affinity of an anti–vascular endothelial growth factor (VEGF) antibody from 2.9 nM to 20 pM at 20°C (106). Interestingly, comparison of the crystal structure of the optimized antibody bound to VEGF relative to the parental antibody revealed that this large affinity improvement resulted from very subtle conformational changes.

A different way to increase affinity is to artificially extend the regions that make contact with the antibody. Lamminmaki et al (118) added a random sequence of two, three, or four amino acids to the heavy CDR2 loop of an antibody specific

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for estradiol because molecular modeling had shown that part of the antibody to be loosely associated with the hapten. Several clones with greatly improved affinity (from $K_D = 2.6 \times 10^{-9}$ to 2.2×10^{-10}) and specificity were obtained in this manner (118). This technique is especially promising for antihapten antibodies, as the immune system appears to have a suboptimal ability to create small molecule binding pockets.

Alternatively, rather than using focused mutagenesis of specific regions, libraries suitable for selection of affinity improved mutants have been constructed by random mutagenesis of the entire gene. Libraries of random mutants can be easily created either by in vitro DNA amplification under conditions that favor misincorporation of nucleotides, or by in vivo propagation of antibody genes within mutator bacterial strains. Error prone DNA amplification by PCR in the presence of Mn^{+2} and/or limiting amounts of dATP and dCTP provides reasonable control over the number of nucleotide substitutions per gene or mutation frequency (119–121). Mutation frequencies as high as 5% can be achieved in this manner, and even higher frequencies can be obtained by doping the DNA amplification reaction with nucleotide analogues (122, 123). Antibody affinity maturation studies have so far employed libraries constructed so as to give low mutation frequencies, typically 1–5 mutations per gene. For example, Hawkins et al reported the isolation of a clone having 4-fold improved affinity from a small library (4×10^4 clones) with an average of 1.7 base changes per V_H gene (61). Similarly, Gram et al isolated a 10-fold improved anti-progesterone antibody from a phage-displayed library screened by three rounds of panning (124).

As mentioned above, flow cytometric screening of cell-displayed libraries is particularly attractive for antibody affinity maturation. The isolation of antibodies with subnanomolar affinities from cell surface–displayed libraries has been reported (48, 71). Recently, it was shown that libraries containing a large number of random mutations per gene (mutation frequencies $\geq$2% nucleotide substitutions per gene) represent a rich source of affinity-improved antibodies (71; R Loo, G Chen, P Daugherty, B Iverson, G Georgiou, unpublished results). Affinity improved antibodies isolated after five rounds of flow cytometric sorting were found to have up to nine amino acid substitutions distributed throughout the protein. Interestingly, most mutations were in framework residues far removed from the antigen-binding site. This finding is consistent with a structural analysis of antibody affinity maturation as it occurs during a secondary immune response (126). In that case, a comparison of the crystal structure of a high-affinity esterolytic antibody (a catalytic antibody with esterase activity) and its germ-line progenitor revealed that beneficial amino acid substitutions were far removed from the binding site and presumably served to rigidify the overall antibody structure.

Libraries of randomly mutated genes can also be generated in vivo by propagating a gene within a mutator bacterial strain that has an impaired mismatch-repair system. Mutation frequencies on the order of 0.2–1 substitutions per 1000 nucleotides are typically obtained for each cycle of propagation (roughly 25 generations) though a mutator strain and are predominantly transitions (127). After

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three cycles of mutagenesis of a low-affinity antibody ($K_D = 320$ nM) in the *E. coli* mutator strain *mut*D5, a clone containing four CDR mutations and four framework mutations and exhibiting a 100-fold improvement was obtained (127). Similarly, Hudson and coworkers reported a striking improvement in the affinity of an antibody specific for glycophorin from libraries generated by in vivo mutagenesis (128). In vivo mutagenesis is a straightforward procedure; however, it is difficult to control the mutation frequency.

Affinity maturation studies can result in the isolation of a number of clones with improved properties. It is often the case that the effect of beneficial mutations is additive, i.e. combining two or more mutations that individually enhance affinity gives rise to an antibody with a proportionally greater improvement (129). Typically, each clone isolated from a screening experiment may contain multiple mutations, and combining such mutations from multiple clones one at a time is impractical. In vitro recombination or DNA shuffling techniques can be employed to generate libraries of nucleic acid substitutions in a combinatorial fashion (130–132) and have been used to select for antibodies with increased binding affinity (133), thermodynamic stability (134), and stability in the absence of disulfide bonds (135).

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Humanization of Murine Antibodies

The vast majority of hybridomas are of murine origin, and many murine antibodies have been developed and tested for human therapeutic applications. In several recent cases these have been approved for use in humans (136, 137). Nonetheless, murine antibodies are foreign proteins to the human immune system and therefore elicit an immune response in the form of human anti-mouse antibodies (HAMA). In the worst case, this response leads to serious allergic hypersensitivity, but at best limits antibody efficacy by resulting in very rapid clearance. Indeed, a gradual decrease in the half-life with repeated dosage of injected murine antibodies has been demonstrated (138).

A variety of humanization techniques for replacing major portions of murine antibody sequences with ones of human origin have been developed. Early studies revealed that replacement of the murine constant regions with those from human antibodies results in chimeras with dramatically reduced immunogenenicity (see Figure 2) (139, 140). In one notable example, the immunogenicity of an anti-platelet glycoprotein antibody was reduced from 17% of patients developing HAMA to 1% after chimerization (141). Nonetheless, clinical trials have revealed that chimeric mouse-human antibodies are still capable of eliciting human anti-mouse antibodies specific for the murine variable regions. It has been estimated that the constant regions contribute 90% of the immunogenicity of chimeric antibodies, with the variable regions including the hyper-variable binding site contributing the remaining 10% (for review, see 142).

A further reduction in immunogenicity can be obtained by transplanting the murine CDR regions, which are primarily responsible for antigen contact and binding, to a homologous human β-sheet framework (see Figures 1 and 2) (143, 144).

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This strategy, termed CDR grafting, decreases the amount of murine sequence in the chimera from about 30% to 3%, and reduces, but still does not eliminate, the risk of HAMA (144). In CDR grafting, it is important to use a human antibody framework region with high sequence homology to the murine region to help preserve the conformation of the CDR loops (145). Nevertheless, CDR grafting often results in a severe reduction in antigen-binding affinity. The substitution of key residues in the human framework by the respective ones from the murine antibody can restore activity by modulating the conformation of the CDRs. In the well-studied anti-lysozyme antibody, D1.3 ($K_D = 3.7$ nM), CDR grafting resulted in a chimera with decreased binding ($K_{D,\ CDR\ graft} = 260$ nM), but the subsequent re-introduction of four murine residues restored the $K_{D,\ engineered}$ to 14 nM (146).

Identifying which murine framework residues are essential for maintaining affinity is the most challenging issue in CDR grafting. Analysis of the antibody-antigen structure, based on crystallographic data or computer modeling, can suggest residues to be substituted by site-specific mutagenesis (146, 147). Unfortunately, this is mostly a trial and error exercise that can be very time consuming. As an alternative, Padlan (148) suggested that the entire murine variable regions be retained, and only framework residues that are exposed to solvent (and represent accessible targets for an immune response) should be replaced by human residues from a homologous framework (148, 149). Thus, residues at the heavy-light chain interface necessary for the stability of the antibody are retained from the original mouse monoclonal, as are affinity- and specificity-determining residues.

Combining rational design and library screening approaches is probably the most robust method for antibody humanization. Wells and coworkers isolated a humanized anti-VEGF antibody from libraries in which the 11 framework residues that most often influence binding were subjected to mutagenesis. A humanized variant with 125-fold greater affinity than the initial CDR-grafted antibody was selected (150). Alternatively, Barbas and coworkers (151) proposed a two-step chain shuffling approach to humanization: First, a rodent light CDR3 grafted into a human light chain library is combined with a rodent heavy chain exhibiting desired specificity, and the human light chain conferring the highest affinity is selected. Second, the heavy CDR3 is grafted into a human heavy chain library, combined with the selected light chain, and thus a human antibody that contains only the murine CDR3 regions is generated. Recently, Huse and coworkers developed an antibody humanization procedure that prevented loss of antigen-binding affinity via the simultaneous mutagenesis of both CDR and framework residues. Specifically, they used combinatorial libraries of eight potentially important framework positions concomitantly with focused CDR3 libraries, and selected an anti-CD40 Fab with 500-fold greater affinity than the initial chimeric antibody (60).

Human Antibodies from Transgenic Mice

A radically different approach to the problem of antibody humanization is to develop murine hybridomas that produce fully human antibodies. For this purpose, transgenic mice in which the native immunoglobulin repertoire has been

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replaced with human V-genes inserted in the murine chromosome have been generated (152–154). These mice appear to carry out VDJ recombination and somatic hypermutation of the human germline antibody genes in a normal fashion, thus producing high-affinity antibodies with completely human sequences and differing only in glycosylation patterns. The transgenic mice can be injected with a desired antigen (including human proteins), and the resulting antibody genes recovered by cloning and screening an immune library, or by conventional hybridoma technology. Antibodies have been isolated with mid-picomolar affinities and appear promising for therapeutic applications (153, 155, 156). Importantly, their half-life in *Cynomologus* monkeys was found to be similar to antibodies recovered from humans [9.5–11 days for transgenic mouse–derived human antibodies, versus 9.6 days for human IgG, 1.9 days for murine IgG, and 7.1 days for chimeric mouse-human IgG (156)]. These mice present a major advance for hybridoma technology, as human hybridomas and immortalized cell lines do not stably produce high levels of antibody, and in vivo immunization of humans is not feasible for many antigens.

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Engineering Antibody Avidity

Although scFv antibodies are designed to be monovalent, single polypeptide proteins, they often form dimers and even higher molecular weight species in solution because of the pairing of the V_H chain of one scFv polypeptide with the V_L chain of another (see Figure 2). This domain swapping phenomenon, i.e. the exchange of one subdomain of one polypeptide with that of another, is common in recombinant antibodies. The formation of multimers appears to be determined primarily by the length of the linker joining the variable heavy and light domains. Linkers greater than 15 amino acids favor the formation of monomeric scFvs, and shorter linkers between 5–12 amino acids result predominantly in the formation of dimers or diabodies, whereas joining the V_H and V_L regions without a linker favors the formation of trimeric species (157, 158). In one recent case, the formation of tetrameric antibodies was reported for a scFv construct in which only one amino acid was added as a spacer between the two domains (159). In addition to linker length, the order in which the two domains are linked in scFvs (V_H-V_L or V_L-V_H) can also affect their tendency to form multimers (160).

Quite remarkably, high-resolution crystal structures of di- and triabodies reveal that even though the interacting V_H and V_L are not covalently linked and are part of separate polypeptide units, the interface between the two domains is not perturbed (161, 162). Interestingly, the crystal structure of a trimeric scFv antibody revealed a triangular arrangement of three fully folded V_H-V_L binding sites (163). As a result, the multimeric forms of recombinant antibodies are multivalent with each V_H-V_L able to bind the antigen. For cell surface antigens or antigens immobilized onto a solid surface at a high density, multivalency results in a significantly higher-binding affinity relative to that exhibited by a monomeric binding site. The affinity enhancement due to multivalency depends on the orientation of the binding sites, the density and orientation of the antigens, and mass transport effects,

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and in general is difficult to predict accurately (164). Nonetheless, numerous experimental studies have unequivocally demonstrated that multivalent antibodies exhibit superior binding to cell surface markers and improved pharmacokinetics (160, 165, 166).

Rather than relying on the intrinsic propensity of scFvs to self-associate, multimeric recombinant antibodies can also be generated by protein engineering techniques (160). For example, scFv molecules with a long linker and little or no ability to multimerize on their own can be fused to a polypeptide domain that will promote association (see Figure 2). A number of short polypeptides that have a strong tendency to associate in a defined fashion can be used, but the most common are sequences that form coiled-coil structures (such as leucine zippers). Fusion of an appropriate association domain to the end of a scFv protein can be employed to form multimeric antibodies with a well-defined stoichiometry (dimers, trimers, or tetramers). Alternatively, high valency scFv complexes can form by fusing each antibody to a short polypeptide that is a ligand for a multivalent protein, which thus forms a complex with several such scFv-peptide tag antibodies (167).

In an analogous manner, two scFvs with different binding specificities can be joined together to form a bispecific diabody (see Figure 2). For example, if the variable heavy V_{H1} region of $scFv_1$ is joined with a short linker to the variable light V_{L2} of the second scFv2 and a similar V_{H2}-V_{L1} construct is made, the two polypeptides will associate to give rise to a scFv1-scFv2 dimer. Bispecific antibodies can be very useful for therapeutic applications because they offer a means to recognize an infected cell via one binding site while providing a second binding factor to recruit cytotoxic lymphocytes (168). Finally, Fab antibody fragments can be engineered to form dimers by introducing a strategically placed cysteine on each fragment, followed by oxidation to link the two proteins with a disulfide bond (41). In summary, protein engineering and heterologous expression in bacteria has simplified the generation of homo- and heteromultimeric antibody fragments for therapeutic and other applications.

PRODUCTION OF RECOMBINANT ANTIBODIES

Mammalian cells including hybridomas and recombinant CHO and COS cell lines can be used to obtain high levels of IgG production (as high as 1 g/L) in large scale (169). However, despite the high titers that can be achieved with mammalian expression systems, the production costs are prohibitive for many applications. In fact, it has been estimated that for many therapeutic applications of monoclonal antibodies to become economical, production levels by tissue culture must be increased between 5- and 10-fold (169).

Therefore, alternative expression systems have been sought. Production of antibodies in plants has been demonstrated and is currently under intense investigation as a possible means for producing very large amounts of full-length immunoglobulins. Heavy and light chains assemble correctly within plant cells, giving rise to

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fully functional, bivalent antibodies. Importantly, the half-life of plant antibodies in mice was found to be nearly identical to that of hybridoma-produced IgGs (170). However, plant antibodies exhibit a greater degree of glycosylation heterogeneity and contain sugars, such as β(1,2) xylose and α(1,3) fucose, not found in animal proteins.

Recombinant antibody fragments (scFv, Fab) can be expressed efficiently in microorganisms, particularly *E. coli*. scFv antibodies contain two disulfide bonds, whereas Fabs contain five disulfides that must all form for stable folding. Normally the oxidation of cysteine thiols into disulfides occurs only after a protein has been exported from the highly reducing environment of the cytoplasm to a more oxidizing secretory compartment. Therefore, in order for antibody fragments to fold into their native, functional state in *E. coli*, they must be expressed in a form compatible with export into the periplasmic space, the compartment between the inner and outer membranes of the bacterial cell. Export into the periplasm is accomplished by attaching in-frame one of several well-characterized bacterial leader peptides (pelB, phoA, ompA) to the N terminus of the antibody fragment (see Figure 6). Proteins expressed into the periplasm can be recovered by osmotic shock or from total cell lysates. Prolonged high-level expression of antibodies at 37°C renders the outer membrane of *E. coli* permeable, and the protein can be recovered from the culture media (171–173).

Very high levels of scFv, exceeding 1–2 g/L in a fermenter, have been achieved by periplasmic expression (41). Unfortunately, the level of expression can vary greatly on a case by case basis. Antibodies, especially single chain antibody fragments, often fail to fold properly and aggregate within the bacterial periplasm, or can be toxic to the host cell. Toxicity most likely occurs because the export of these proteins interferes with the normal function of the secretory machinery of the cell. Protein misfolding and aggregation can be alleviated by growing the cells at 30 or 25°C (174), using tightly regulated promoters such as

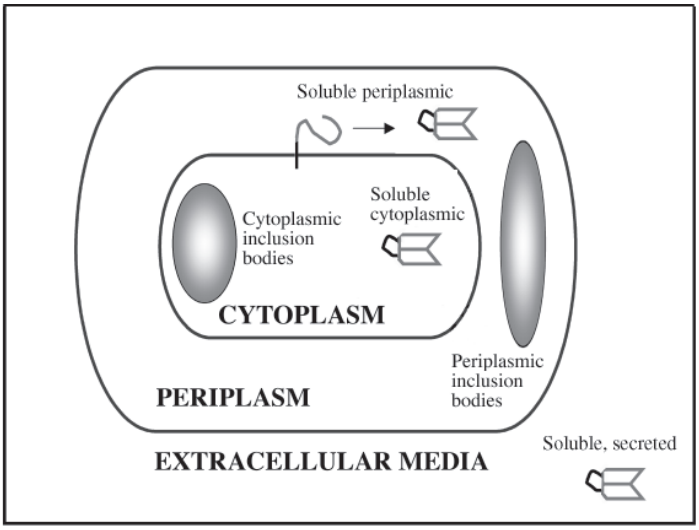

Figure 6 Antibody production in *E. coli* bacteria. Antibodies can be produced in soluble, active form or as inclusion bodies requiring refolding.

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the pBAD arabinose-inducible promoter (175), or by co-expression of proteins that assist folding. Such proteins include enzymes that catalyze disulfide bond formation or isomerization (DsbA, PDI, and DsbC) or chaperones such as Skp (176–179).

Amino acid substitutions can have dramatic effects on protein solubility, and therefore on the level of expression of functional antibody fragments (180, 181). In one particularly noteworthy case, Nieba et al (182) reported a 25-fold increase in expression of the notoriously ill-expressed antifluorescein scFv 4-4-20 upon replacement of a single solvent-exposed hydrophobic residue. Similarly, changing a glutamic acid to glutamine at position V_H-6 has led to a 30-fold increase in soluble scFv (183). Finally, CDR grafting to the framework of well-expressed antibodies has been used to generate scFvs with improved stability that result in better production (184, 185).

High-level expression of antibody fragments in the cytoplasm of *E. coli* results in extensive aggregation. The aggregated proteins form large intracellular particles or inclusion bodies. Owing to their large size and density, inclusion bodies can be readily purified from other cellular components. The aggregated polypeptides can be solubilized by adding strong denaturants (6 M GdmCl or 8 M urea) and a reducing agent, and correctly folded antibody fragments are recovered by refolding upon removal of the denaturant. Determining the best conditions for refolding is an empirical process, but general guidelines have been developed (186). The critical variables are the protein concentration, the buffer composition, and the way and rate with which the denaturant is removed. Typically, the denaturant is removed by dialysis or dilution. The addition of protein stabilizing agents (such as glycerol or arginine) and a proper redox buffer to facilitate the in vitro formation of disulfide bonds greatly affects refolding yields (187, 188). Numerous strategies for improving the recovery of properly folded proteins from inclusion bodies have been devised, and this topic has been the subject of several excellent reviews (189–191).

Although disulfide bonds do not normally form in the cytoplasm of wild-type bacteria, in certain mutant *E. coli* strains the cytoplasm is rendered more oxidizing, allowing limited formation of disulfide bonds (192). Small amounts of correctly folded scFv antibodies have been obtained in such strains (193, 194). Recently, strains with a highly oxidizing cytoplasm, perhaps as oxidizing as the endoplasmic reticulum of eukaryotic cells, have been developed and used to express multi-disulfide proteins in the cytoplasm with high efficiency (195). These strains are being evaluated for cytoplasmic expression of functional Fab antibodies (G Georgiou, unpublished results).

Antibodies that do not require disulfide bonds for folding should in principle be compatible with functional expression in the cytoplasm. The engineering of antibodies that can fold normally in the cytoplasm is important not only as a means of production by bacterial fermentation but also for the development of intracellular antibodies useful in gene therapy applications. The latter application in particular has stimulated great interest in the design of antibodies that can fold properly in

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the reducing environment of the cytoplasm (196). A small number of naturally occurring antibodies lack the conserved cysteines, yet fold correctly because they contain amino acid residues that compensate for the loss of disulfide-mediated stability (197, 198). One such antibody was used as a starting point for the selection of a highly stable disulfide-free framework from a combinatorial library (135). Alternatively, Martineau et al (199) generated libraries by random mutation of a scFv that normally requires disulfide bond formation and, using an ingenious selection system, isolated variants that could fold in the reducing environment of the cytoplasm. Remarkably, this antibody could be produced at yields in excess of 3.0 g/L in a fermentor, more than two-fold greater than the highest yields reported for periplasmic expression of an antibody fragment (41). This scFv variant is both more stable and exhibits faster in vitro folding kinetics under reducing conditions than the parent antibody. Recently, a yeast two-hybrid selection system for in vivo selection of antibodies that can function in the cytoplasm was reported (200). Finally, rational design methods have been employed to create a hyperstable V_L domain that in turn was paired with a mutated V_H to give rise to a Fv antibody fragment that could be expressed in the cytoplasm at moderate levels (201, 202).

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ANTIBODY APPLICATIONS

The key attributes of antibodies, namely high affinity and specificity for a binding partner, have been exploited in a plethora of applications mainly, but by no means exclusively, in the biomedical field and in biotechnology. Antibody-based assays currently represent 30% of the 10 billion dollar per year diagnostics industry. Biomedical applications further include neutralization of toxins in vivo, passive immunization (203), delivery of radionuclides for imaging purposes, immunosuppression, and cancer therapy. Antibodies are also being evaluated for use in the food and environmental industries as biosensors for routine monitoring to detect less than one part per billion of microbial contaminants or organic pesticides. Furthermore, antibodies offer the possibility of efficient removal of microrganisms and organic pollutants from contaminated water and land (204). Due to space limitations, this discussion is limited to four applications of most relevance to bioengineering, namely neutralization of toxins, antibodies as in vivo gene therapy agents, imaging, and cancer therapeutics.

Neutralizing Antibodies

The use of antibodies to bind and inactivate toxins was first reported in 1894 and is still the treatment of choice for snake bites and scorpion stings. Antivenoms are historically heterogeneous immunoglobulins from an immunized animal. They are difficult to prepare reproducibly, and as nonhuman molecules, they have short

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half-lives and a risk of immunogenicity. In contrast, the small size of recombinant scFvs permits rapid diffusion and fast clearance of immunocomplexes. At least in one case, scFvs have proved more effective than the parent monoclonal antibody for neutralization of scorpion toxin (205).

Neutralizing antibodies can also be used to bind viral coat proteins and block viral pathogenesis. High-affinity human antiviral antibodies (e.g. for human immunodeficiency virus type 1, respiratory syncytial virus, and herpes simplex virus) have been selected from immune libraries using a variety of strategies. A small subset of these antibodies shows potent neutralization in vitro and antiviral efficacy in vivo in animal models (84, 206). Gauduin et al (207) found that a high-affinity neutralizing antibody at high dose was able to completely protect immunodeficient mice against HIV, even when given several hours after viral challenge. It is not known how general these findings are, but the results are encouraging for antibody-based prophylaxis.

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Intracellular Antibodies

The intracellular expression of antibodies is a strategy to analyze and manipulate microbial and cellular pathways by inhibiting the function of selected molecules (196, 208). "Intrabodies" must be delivered to the cell by gene therapy methods (209), synthesized within the cell, and targeted to the desired cellular compartment.

The function of many intracellular antigens has been successfully inhibited by intrabodies in the cytoplasm, the nucleus, and the secretory pathway. Notably, scFvs have been targeted to the endoplasmic reticulum to precipitate an HIV envelope protein, decreasing HIV viral production and the infectivity of released particles (210). Similarly, intrabodies have also been used to down-regulate the expression of growth factor receptors (211) and to inactivate oncoproteins (212). Intrabodies are also versatile research tools that can be used for studies including the characterization of in vivo functions of infectious agents and elucidating signal transduction pathways, in addition to their potential as clinical reagents in a gene therapy setting.

Imaging

Radioimmunodetection of tumors and blood clots is widely used to complement other detection approaches. Fab and scFv molecules are preferred over whole antibodies because both targeting and blood clearance are rapid, yielding high tumor to background ratios at early time points (213). Many β and γ-emitting isotopes have been evaluated, but technetium-99m is preferred because it matches the six hour half-life of scFv fragments and is readily and economically available. The most common tumor cell surface target for imaging is carcinoembryonic antigen, and specific antibodies allow the detection of tumors as small as 0.5 cm, which are sometimes missed by other methods (214). Antibody fragments can be labeled with ^{99m}Tc by engineering a C-terminal cysteine (215), by creating an

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antibody fusion with a metal-binding protein such as metallothionein (216), or by chelation via a sequence of six C-terminal histidines (217).

Cancer Therapies

Antibody-based therapies have only recently begun to realize their potential as "magic bullets" in cancer therapeutics, and several promising treatments have recently gained FDA approval (136, 137). Antibodies are useful in cancer therapy because they can recognize tumor-associated antigens that are expressed at higher density on malignant cells than on normal cells. Thus, the antibodies and any associated molecules become concentrated at the site of malignant tissue, 4–50 times relative to other tissues. Antibodies can be used as a single therapy, or in combination with traditional drug therapies, as some combinations are synergistic (218, 219). Antibody binding is exploited to (*a*) specifically deliver toxic molecules to tumor cells, (*b*) recruit cytotoxic immune cells, and (*c*) activate apoptotic signaling pathways.

Radioimmunotherapy has proved extremely successful with non-Hodgkin's lymphoma (NHL) (220). Immunoconjugates are also being evaluated for the delivery of small molecule chemotherapeutic agents such as doxorubicin by covalent attachment or liposomal encapsulation (221, 222). Recombinant immunotoxins (antibody-toxin fusion proteins) are being actively pursued as a means to direct highly cytotoxic bacterial or plant proteins to tumor cells (223, 224). In a recent example, an immunotoxin composed of an antibody recognizing the $p185^{HER2/neu/c\text{-}erb\text{-}B2}$ oncogene product (a transmembrane tyrosine kinase of the epidermal growth factor receptor family that is up-regulated in certain breast cancers) and the ribosome-inhibiting plant toxin gelonin was shown to have an IC_{50} of 97 pM in a human tumor zenograft model. In contrast, antibody or toxin alone showed no cytotoxic effects (225). The selectivity of drug targeting can be further improved with antibody-dependent enzyme/prodrug therapy (ADEPT) (226).

The presence of a complete antibody with an F_c region bound to the surface of a tumor cell has been shown to be sufficient for stimulating host immune responses (218). More recent strategies have focused on bispecific antibody fragments with one binding site recognizing a tumor-associated antigen and the other recognizing cytotoxic T-cells or the $F_c\gamma$ receptor on effector cells (227–229).

Antibody binding to malignant cells can induce a variety of intracellular signals. Antibodies to certain epitopes of the $p185^{HER2/neu/c\text{-}erb\text{-}B2}$ protein have been shown to act as antiproliferative agents, at least in vitro (230). Divalent antibodies may also act by crosslinking receptors, and numerous studies have shown that crosslinking of B-cell receptors initiates cell-cycle arrest and apoptosis in tumor cells (218).

Antibody-mediated therapy has been much more successful for circulating than for solid tumors, primarily because there are few diffusion boundaries, and targeted antigens are present at high density. This is demonstrated by the success of

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Rituximab, an FDA-approved chimeric antibody recognizing the CD20 antigen on B cells, used in the treatment of non-Hodgkins lymphoma (136). For these treatments, the engineering goal is to produce high-affinity molecules with long half-lives in vivo, for example by generating multivalent fragments ≥65 kDa to avoid renal clearance.

The treatment of solid tumors presents a much greater challenge. Antibodies must diffuse into the tumor against hydrostatic pressure and into disordered vasculature (231). Low-molecular weight fragments have better diffusion properties and are more successful at penetrating solid tumors. However, the antibody fragment must stay in circulation long enough to diffuse from the blood stream to the tumor without being cleared by the kidneys. The residence time once within the tumor mass is controlled by affinity and avidity (232). A solution is to use very high affinity scFvs (233), or to use divalent diabodies that have longer half-lives in vivo. The $t_{1/2}$ for cell surface retention is 5 min for a monovalent scFv compared with >5 h for diabody against p185 (166). Thus, engineering antibodies for solid tumor therapy requires finding a balance between a size small enough to allow efficient diffusion but large enough to avoid renal clearance, and high affinity and avidity for retention by the tumor (165, 234, 235).

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CONCLUSIONS

The recent developments of library screening and bacterial expression strategies have permitted the rapid generation of fully human antibody fragments with specificity for almost any antigen. Using new, powerful protein engineering methodologies, antibodies exhibiting desired antigen association and dissociation rates can now be generated on demand. Multimeric recombinant scFvs, containing two or more binding sites or fused to effector proteins, are finding uses in a variety of clinical applications. However, many challenges still need to be met. The in vivo use of many antibodies will require the engineering of protein stability as well as antigen affinity. The use of antibodies in gene therapy will require the development of more general methods for the construction of intrabodies. The production of recombinant antibodies at a large scale is still not a trivial matter and will require further advances in microbial expression technologies. These and other related issues are currently under active investigation, and there is no doubt that in years to come antibodies will become indispensable as clinical and research reagents.

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