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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.

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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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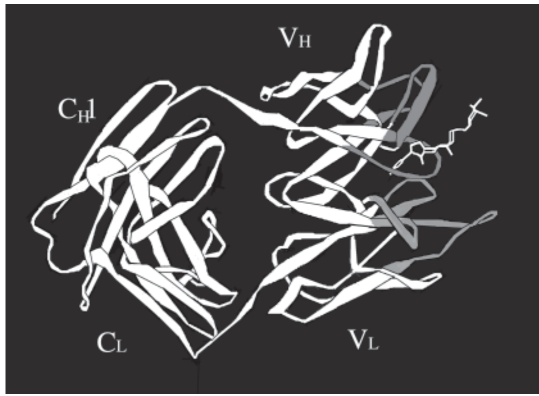

Figure 1 Ribbon diagram from the crystal structure of the antidigoxin Fab, shown in complex with the small molecule steroid, digoxin. Framework variable regions are shown in white, CDR regions in light gray, digoxin in white. Note that only the CDR regions mediate antigen contact. [From PDB file 1IGJ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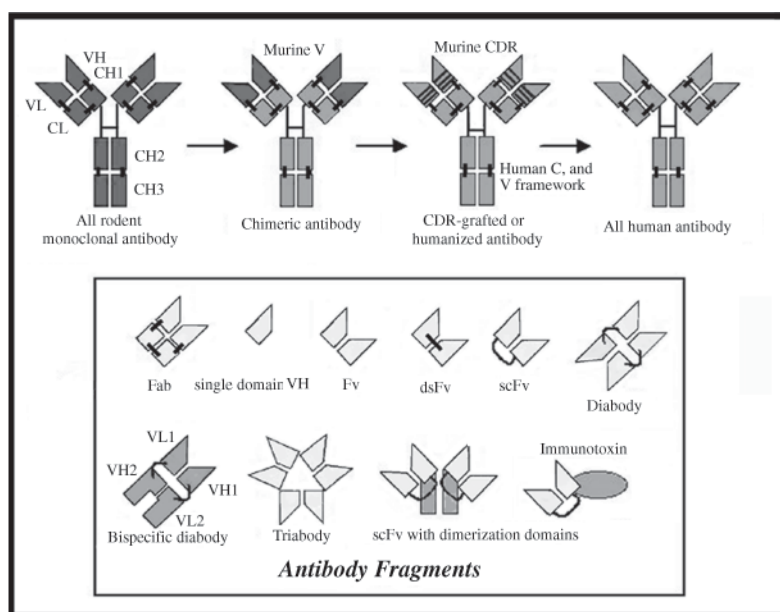

Figure 2 Schematic showing various antibody fragments of biotechnological and clinical interest. Each block represents one antibody domain with a characteristic immunoglobulin fold. Black bars, inter-chain di-sulfide bonds (horizontal) or intra-domain linkages (vertical); longer curved lines, genetically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

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