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Antibody Engineering

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Abstract Monoclonal antibodies (Mabs) have been used as diagnostic and analytical reagents since hybridoma technology was invented in 1975. In recent years, antibodies have become increasingly accepted as therapeutics for human diseases, particularly for cancer, viral infection and autoimmune disorders. An indication of the emerging significance of antibody-based therapeutics is that over a third of the proteins currently undergoing clinical trials in the United States are antibodies. Until the late 1980's, antibody technology relied primarily on animal immunization and the expression of engineered antibodies. However, the development of methods for the expression of antibody fragments in bacteria and powerful techniques for screening combinatorial libraries, together with the accumulating structure-function data base of antibodies, have opened unlimited opportunities for the engineering of antibodies with tailor-made properties for specific applications. Antibodies of low immunogenicity, suitable for human therapy and *in vivo* diagnosis, can now be developed with relative ease. Here, antibody structure-function and antibody engineering technologies are described.

Keywords: recombinant antibody, humanization, phage display, library screening, combinatorial libraries

STRUCTURE AND FUNCTION OF ANTIBODIES

There are five classes of immunoglobulins: IgM, IgG, IgE, IgA, and IgD. From a biotechnology perspective, IgG is the most important class of antibodies. IgG antibodies are homodimers of two identical polypeptide chains of 50 kDa heavy chains and two identical chains of 25 kDa light chains. Disulfide bonds link together the heavy and light chain pairs as well as the two heavy chains (Fig. 1). Each chain consists of one domain that is variable (V domain) in sequence and one or more domains that are constant (C). The V domains are responsible for antigen binding, while the C domains mediate effector functions. Light chains consist of one V domain (VL) and a single constant domain (CL), whereas heavy chains comprise one V domain (VH) and three constant domains (CH1, CH2, and CH3). Each domain features the characteristic immunoglobulin fold consisting of two antiparallel β-sheets with an intramolecular disulfide bond. Within each V domain are three regions that are hypervariable in sequence and that form loops at the ends of rigid β-sheets. The hypervariable loops are primarily responsible for antigen recognition and are referred to as complementarity determining regions (CDRs). The remaining V region amino acids act as a scaffold to support the loops and are known as framework residues (FR).

This domain structure of immunoglobulins has facilitated manipulation by protein engineering. Thus, fragments containing effector function (Fc) can be exchanged between antibodies, or CDRs can be transplanted from one antibody to another in order to transfer antigen-binding capability. Furthermore, molecules of only antigen-binding domain have been produced, including Fab, scFv, VH, and diabody (Fig. 1).

HUMANIZATION OF MURINE ANTIBODIES

The vast majority of hybridomas are of murine origin and many murine Mabs have been developed and tested for human therapeutic applications. However, they suffer the limitation of being frequently recognized as foreign by the human immune system and therefore eliciting human anti-mouse antibodies (HAMA). Thus, repeated doses of murine Mabs can lead to serious allergic hypersensitivity or result in a decrease in half life [1,2].

To improve the therapeutic potential of murine Mabs chimeric antibodies have been constructed in which the mouse constant regions are replaced by their human counterparts (Fig. 2) [3,4]. In one notable example, the immunogenicity of an anti-platelet glycoprotein antibody was reduced from 17% to 1% of patients developing HAMA after chimerization [5]. Nonetheless, clinical trials have revealed that chimeric antibodies are still capable of eliciting HAMA. It has been estimated that the constant regions contribute 90% of the immunogeni-

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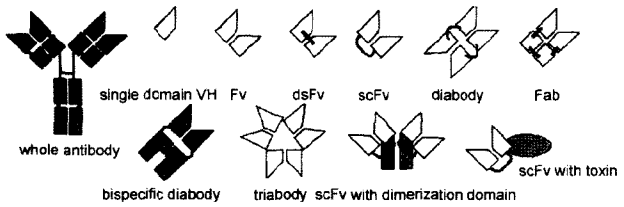

Fig. 1. Various antibody fragments for biotechnological and clinical interest. Each block represents one antibody domain with a characteristic immunoglobulin fold. The black bars represent interchain disulfide bonds (horizontal) or intradomain linkages (vertical); the longer curved lines, genetically engineered polypeptide linkers.

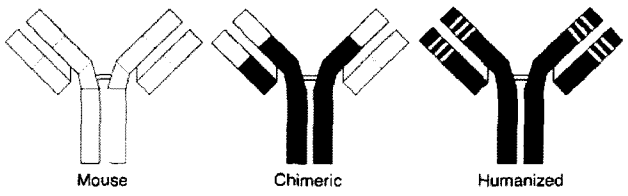

Fig. 2. Humanization of an IgG molecule. The white represents mouse sequences and the gray, human sequences. In a chimeric antibody, the mouse heavy- and light-chain variable region (V-region) sequences are joined onto human heavy-chain and light-chain constant regions (C-regions). In humanized antibodies, the muring CDR sequences (three from the heavy-chain V-region and three from the light-chain V-region) are grafted onto human V-region framework regions and expressed with human C regions.

city of chimeric antibodies, with the variable regions contributing the remaining 10% [6].

A further reduction in immunogenicity can be obtained by transplanting the murine CDRs to a homologous human FR (see Fig. 2) [3,4]. This strategy, termed CDR-grafting, decreases the number/ratio of murine sequences in the chimera from about 30% to 3%, but still does not eliminate the risk of HAMA [4]. CDR-grafting often results in significant loss of antigen-binding affinity. The substitution of key framework residues in the human sequence by original murine residues can restore affinity. Therefore, identifying which murine FR are essential for maintaining affinity is the most challenging issue in the development of CDR-grafting.

Many humanized Mabs have now been subjected to clinical trials for a variety of treatments and indications (Table 1). Among them, the first humanized Mab tested was CAMPATH-1H for the treatment of non-Hodgkin's lymphoma and rheumatoid arthritis. Although evidence of obvious benefit was observed, over 50% of rheumatoid arthritis patients developed HAMA responses to repeated therapy, which lead researchers to drop further clinical testing. However, other humanized Mabs have shown negligible HAMA responses in patients (1 out of 38), such as on multiple dosing with an anti-CD33 Mab in myeloid leukemia patients. Since 1998, six humanized antibodies have been approved by the FDA, and many more will be approved soon. The two approved are Herceptin, a humanized Mab acting against the HER2/neu proto-oncogene, for the treatment of metastatic breast cancer, and Synagis, another humanized Mab acting against the fusion protein of respiratory syncytial virus (RSV) for the prophylaxis and treatment of pediatric RSV infection. Herceptin has been reported to show minimal side effects and no detectable immunogenicity. Soon, humanized anti-CD25 Mab (daclizumab), humanized anti-CEA (carcinoembryonic antigen) Mab, and humanized anti-CD22 Mab will appear on the market.

Recombinant Antibody Fragments

Genetic engineering has enabled the creation of many recombinant antibody fragments with potential uses. For clinical uses, the ideal antibody would probably be small for optimal penetration with high affinity and specificity against its antigens. The fragments should also be perceived as human to avoid immune responses and be stable to reach the target for therapeutic or *in vivo* diagnostic purposes.

The smallest antibody-derived polypeptide that can bind an antigen with specific affinity is a single VH chain [7]. Because the largely hydrophobic area that normally forms the interface with the VL domain is exposed to the solvent, VH chains are very unstable and aggregated *in vivo*. However, one class of VH chains derived from camels or llamas is found naturally without a light chain [8-10]. Sequence and structural analyses of the camel VH chains have guided the rational design for human VH chain engineering to render it stable without a VL chain [11].

Generally, the presence of both the VH and VL chains is needed for high stability and antigen-binding affinity. VH and VL chains can be expressed as separate polypeptides in bacteria where they assemble into Fv fragments. 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 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 VH and VL chains together into an scFv antibody fragment. This scFv fragment is relatively small (26–27 kDa), generally quite stable, and is encoded by a single gene, which simplifies genetic manipulations. The most common linker is a flexible $(Gly_4Ser)_3$ decapentapeptide [12]. The two variable domains can be connected either as VH-linker-VL or VL-linker-VH, 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. If an 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 multidomain polypeptides [13-15].

In addition to scFv, the other commonly used recombinant antibody fragment is Fab. Fab consists of two polypeptide chains, one containing the light chain variable and constant domains, VL-CL or, the other a truncated heavy chain containing the variable domain and one constant domain, VH-CH1. 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 [16]. Different formats of antibody fragments can be created for additional purposes such as diabody, bi-specific antibody, triabody, and scFv with toxin (Fig. 1).

Phage Display Of Antibody Library

The classical antibody selection method, either animal immunization or hybridoma technique, was rather restricted due to its low speed. The concept of molecular display technology was the provision of a physical linkage between genotype and phenotype to allow simultaneous selection of the genes that encode a protein with the desired binding function. The display of functional antibody repertoires on phages required large collections of antibody variable domains and functional expression in bacteria. McCafferty *et al.* showed that antibody fragments could be displayed on the surface of filamentous phage particles by fusion of the antibody variable genes to one of the bacteriophage coat proteins [17]. Antigen-specific phage antibodies were subsequently enriched by multiple rounds of affinity selection, because the phage particle carried the gene encoding the displayed antibody. This was originally reported for single-chain Fv fragments, and later for Fab fragments and other antibody derivatives such as diabodies, as well as extended to various display systems [18-21].

Table 1. Humanized antibodies in clinical use

Antibody type	Specificity	Application	Company	Trade name	FDA approval date
Chimeric Fab	platelet GPIIb/IIIa	Preventing blood clot	Centocor	ReoPro	1994
Humanized antibody	IL-2 receptor	Prevention of acute kidney transplant rejection	Hoffmann-La Roche	Zenapax	1997
Chimeric antibody	CD20 of B cell	Non-Hodgkin's B-cell lymphoma	Genentech	Rituxan	1997
Humanized antibody	Respiratory syncitial virus	Prophylaxis of RSV infection	MedImmune	Synagis	1998
Humanized antibody	TNF-α	Crohn Disease, Rheumatoid artritis	Centocor	Rimicade	1998
Humanized antibody	Her2/neu	Breast cancer	Genentech	Herceptin	1998
Chimeric antibody	CD25	Immunosupression	Novartis	Simulect	1998
Humanized antibody	CD3	Immunosupression	Wyeth-Ayerst	Mylotarg	2000
Humanized antibody	CD52	Chronic Lymphocytic Leukemia	Millennium	Campath	2001
Humanized antibody	CD20	Non-Hodgkin's lymphoma	IDEC	Zevalin	2002

Table 2. Major differences between three types of antibody repertoire

Type of repertoire	Advantages	Disadvantages	Main applications
Biased	High frequency of binders In vivo affinity maturation	Library has to be rebuilt for each antigen Study of humoral response in disease Bias against self-antibodies Antigen has to be immunogenic	Study of humoral response in disease (viral infections, cancer and autoimmunity) High-affinity diagnostic antibodies
Naive	Can be used against any antigen Scaffold and somatic mutations selected by evolution	Has to be very large to obtain good affinities Bias towards certain sequences and against self	Selection of high-affinity human antibody for imaging or therapy
Synthetic	Expand the structural scope of binding-site repertoires Special 'built-in' design possible for specific applications	Has to be very large to obtain good affinities A high percentage of 'non-viable' sequences	More suitable for use in automated selection and in microarray field (genomics or proteomics)

In general, three kinds of antibody libraries have been constructed; a) biased libraries from immunized animals, b) naïve libraries from non-immunized animals, and c) synthetic libraries by random mutagenesis of the CDRs within a single or small set of frameworks [22]. These three types of binding-site repertoire can be exploited according to their own merits (Table 2). Highly diverse naïve libraries consisting of more than 10^{10} clones have been generated and their use in the isolation of antibodies to a variety of targets has been demonstrated. A completely synthetic library, HuCAL, based on a small set of antibody frameworks representing the major families used in the human immune response, has recently been constructed [23,24].

Currently the screening of naïve, semi-synthetic and synthetic repertoire libraries is enabling their development into the most powerful and rapid tools for the isolation of antibodies in a high-throughput manner. However, the effort required to construct and confirm the effectiveness of a diverse antibody library is laborious, time-consuming, and significant. Furthermore, limited amounts of initial library DNA can be produced because every time the library is amplified in bacteria, sequences that affect cell growth adversely are counterselected which thereby reduces the diversity significantly. Consequently, the best libraries are proprietary and not available to the research community. For academic use, the semi-synthetic Griffin library, which is somewhat less diverse than the best proprietary libraries, can be obtained free of charge from the Medical Research Council in the UK.

Fully human Mabs derived from phage display have recently entered clinical trials for rheumatoid arthritis, glaucoma surgery, and fibrosis [25].

ANTIBODY APPLICATIONS AND FUTURE PERSPECTIVES

Antibody-based assays currently represent 30% of the 10 billion dollars per year diagnostics industry. Biomedical applications further include neutralization of toxins or virus *in vivo*, passive immunization, delivery of radioisotopes for *in vivo* imaging purposes, immunosuppression, and cancer therapy [1]. Humanization has proven to be a powerful method for transferring the specificity and affinity of an existing rodent Mab into human format. However, it is thought that humanized antibodies will be gradually replaced by human Mabs, as more and more fully human Mabs are isolated from phage display and transgenic mice [26,27]. Antibodies are also being evaluated for use in the food and environmental industries as biosensors for routine monitoring to detect microbial contaminants or organic pesticides at concentrations of less than one part per billion [28]. In the post-genomic era, recombinant antibodies are becoming increasingly important in the field of proteomics. Coupled with new screening technologies, such as high-density antibody arrays or antibody generation against individual protein spots on 2D gel, this antibody technology promises to become one of the most valuable tools for whole proteome analysis [29-31]. When all these developments are considered together, it is expected that with rapid progress in antibody engineering technologies, Mabs will become indispensable as clinical and research reagents in the near future.

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