Protein engineering techniques for antibody humanization

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Few proteins have the therapeutic potential of antibodies, which can be developed against a wide variety of targets and genetically or chemically manipulated to further enhance their activities. A number of approaches have been taken in order to render these proteins pharmaceutically useful. Either the amino acid sequence of an antibody can be genetically altered (i.e. reshaped or resurfaced) or the antibody can be conjugated to other proteins or toxins.

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Introduction

The biotechnology revolution has had a major impact on the pharmaceutical industry. Although most drugs in use are small multi-cyclic molecules, some recombinant proteins, such as growth hormone and tissue plasminogen activator, have also become quite useful as therapeutics. Antibodies have the potential to be even more beneficial because of their long half-life and ability to recognize a diverse array of targets. Interestingly, antibodies act in a manner similar to that of small molecules, as they can bind to specific receptors and enzymes. Once a target is identified, an antibody can be generated and developed as rapidly as a small molecule.

Antibodies are presently being developed against three types of targets: infectious agents, secreted molecules and cell-surface receptors. Pre-clinical and clinical studies have already been initiated to assess the utility, safety and efficacy of a number of antibodies against several infectious agents. These include the surface F protein of respiratory syncytial virus (RSV) [1], human immunodeficiency virus (HIV) [2,3], cytomegalovirus [4] and parasitic organisms [5]. A number of anti-cytokine and anti-cytokine receptor antibodies are also being produced, including those directed against tumor necrosis factor [6] and the receptor for interleukin (IL)-2 [7].

In this review, we examine the characteristics of antibodies that can be addressed by protein engineering techniques during the development process, with an emphasis on those properties that influence antibody affinity and immunogenicity.

Antibody humanization

Although other approaches appear promising [8••,9–13], most antibodies targeted against human proteins are raised predominantly from mice or other non-human sources. The conversion of an antibody to a drug useful for man typically requires modification of the molecule such that the human immune response to the murine protein is reduced. The term for this type of modification is humanization, in which as many amino acid residues of the murine antibody as necessary are replaced with those of a typical human antibody. Till now, the challenge has been to do this without affecting the affinity of an antibody for its target antigen.

A number of reviews on humanization and related subjects have appeared recently [14••,15]. Several lines of evidence seem to indicate that the humanization procedure itself is now fairly straightforward, probably due to the increasing accumulation of knowledge concerning the structure and function of antibodies [16,17].

The most common method of humanization involves grafting the regions of the antibody that bind antigen [i.e. the complementarity determining regions (CDRs)] from the variable region of the original murine antibody onto the framework region (FR) of a human antibody (see Fig. 1). Since greater than 90% of the original murine residues are replaced by residues of human origin, one hopes that the immunogenicity is greatly reduced. However, because the affinity for an antigen is controlled by both the CDR sequences and their conformations, changes in the framework might also affect antigen affinity. Depending on the original antibody, the

Abbreviations

CDR—complementarity determining region; **C_H**—heavy-chain constant region; **C_L**—light-chain constant region; **FR**—framework region; **HIV**—human immunodeficiency virus; **IL**—interleukin; **mAb**—monoclonal antibody; **PDB**—Protein Data Bank; **RSV**—respiratory syncytial virus; **V_H**—heavy-chain variable region; **V_L**—light-chain variable region.

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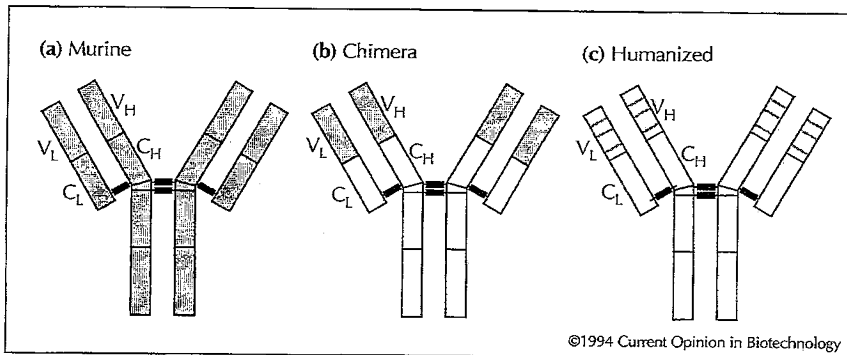

Fig. 1. The structure of murine, chimeric and humanized antibodies. The location of the original murine regions (shaded) is shown. Variable regions of light (V_L) and heavy (V_H) chains, constant regions of the light (C_L) and heavy (C_H) chains, and the disulfide bonds linking these chains are also shown.

mouse and human FR sequences can differ at 5–50 positions per chain. Many of these changes are at exposed sites well removed from the CDRs and thus should not affect CDR conformation.

Differences that occur at FR sites contacting one or more residues of the CDR do, however, have the potential to alter antigen affinity. The importance of these residues was postulated by Chothia and Lesk [17], who used structural information to classify CDR loop conformations into 'canonical structures'. Fortunately, because of the conserved nature of antibody structure, it is feasible to model the novel sequences onto one or more known structures in order to predict the particular residues in the FR that contact the CDRs in a novel antibody [18–20,21•]. If the murine and human sequences differ at these important positions, the human residue in the framework should be replaced by the original murine residue to maintain the original murine CDR conformation. The meaning of the term reshaping has grown from simple CDR grafting to encompass the grafting of both CDRs and CDR contact regions (Fig. 2).

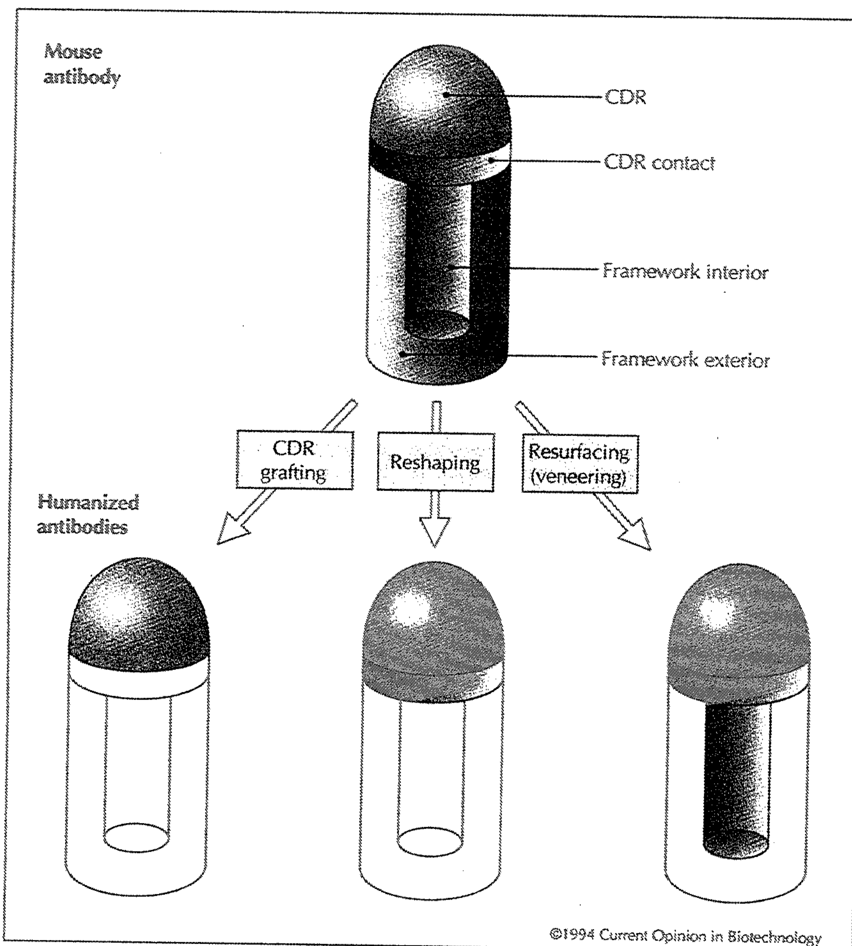

Fig. 2. Diagram illustrating the various techiques of antibody humanization. Regions of the antibody acquired from the original murine source are shaded; those derived from a human framework are unshaded. (The shape of the antibody is altered for ease of comprehension.)

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Because the main goal is to maintain antigen affinity during the humanization process, it may only be necessary to identify the residues that affect CDR conformation, and not the exact manner of their interaction. In general, as antibodies have such similar structures, the identification of residues that contact CDRs in known structures also allows identification of most, if not all, CDR contact residues in a novel sequence. Thus, in many cases, a detailed structural model of the antibody does not need to be constructed.

Fig. 3 demonstrates that the general tendencies of residues that contact the CDR complicate matters. The CDRs, of course, are poorly conserved, but they vary in their exposure. Most of the residues that can potentially contact the CDRs are buried, but vary considerably in their conservation. Finally, many additional framework residues occur that are also buried, yet not well conserved. Because of the sequence variability of these interior residues, simple transplantation of the CDRs and their contact regions onto a single human framework is sometimes difficult.

In some cases, however, this process has been accomplished. For instance, the human NEW-based heavy-chain variable region (V_H) and REI-based light variable region (V_L) have been used as frameworks for humanized antibodies against RSV [1], CD18 [22] and IL-6 [23], among others. Even so, this approach does not always yield a highly potent antibody because the original and human frameworks may be so dissimilar that even mutation of CDR contact regions may not result in a recovery of activity [24]. Presumably, differences in the buried residues of the two antibodies result in changes to the general shape of the antibody.

One approach to minimize this problem is to select as a scaffold a human framework that is as homologous as possible to the original murine framework [18], especially at the CDR-contact regions. These frameworks can be selected from known structures, known V_H/V_L pairs [25], single mature sequences [26], germline sequences or even synthetically constructed subgroup consensus sequences [19,27]. Typically, up to five further changes in the framework region are required for activity to be restored to within threefold that of the original antibody. The greater the similarity between the two framework sequences, the less likely it is that a key framework residue will need to be changed.

Further changes sometimes involve the elimination of residues that rarely occur in human antibodies. The rationale for this approach is to eliminate potential immunogenicity, but recent results demonstrate that the presence of such rare residues can also have effects on the stability, and possibly the amyloidgenicity, of the variable domains [28•].

Veneering, which is also know as resurfacing, approaches this same problem, but from the opposite direction [29,30•,31••]. With this approach, one tries to reduce the immunogenicity of the variable domain directly by altering only the surface-exposed residues of the framework to those seen in human molecules (see Fig. 2). Again, these residues in the novel sequence can be defined by analysis of available antibody structures [29,31••]. It has been shown that two resurfaced antibodies, one containing nine and the other containing 13 amino acid substitutions, do appear to have binding affinities equal to the original murine antibodies [30•]. Although the interior of the domain remains of murine origin, many of these residues are identical to the human antibody; thus, the immunogenicity of these residues is likely to be much less than that of the CDRs themselves. Only clinical evaluation of veneered antibodies will determine if they are indeed tolerated by the immune system.

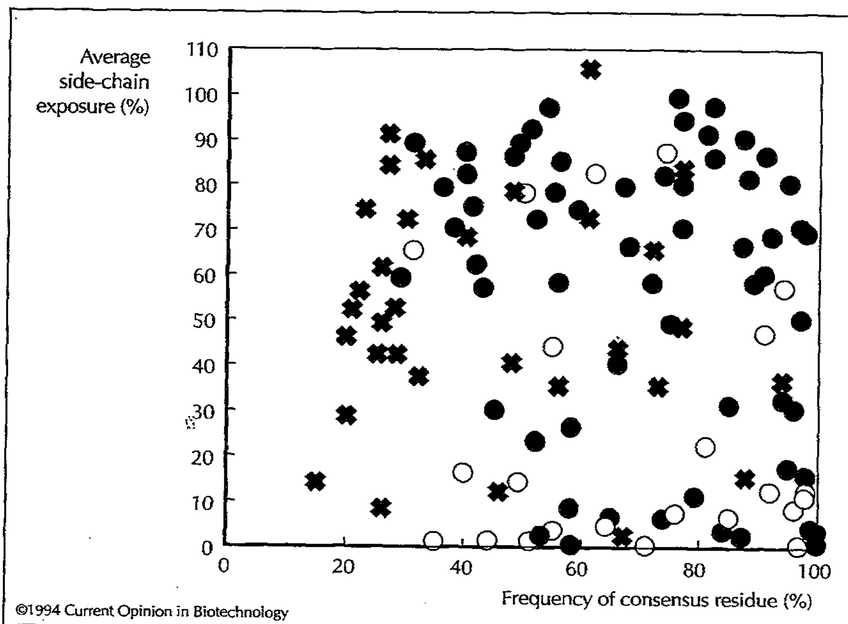

Fig. 3. Variability and exposure of the CDR (✱), CDR contact (○) and all other variable residues (●) of immunoglobulin heavy chains. CDR residues and residue frequency are defined according to Kabat *et al.* [46]. CDR contact residues are defined as those residues that approach within 3.5 Å of a CDR residue in at least one of seven antibody structures (Protein Data Bank (PDB) codes 1F19, 1FDL, 2FB4, 2FBJ, 2HFL, 2MCP and 4FAB). Average side-chain exposure is computed from these seven PDB structures using the algorithm of Connolly [47].

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Antigenicity of antibodies

On the basis of the above observations, the construction of resurfaced or reshaped antibodies with antigen affinities approaching those of the parent molecules is becoming a more and more realistic goal. For both reshaped and resurfaced molecules, one key question remains: how immunogenic are they? Fig. 3 illustrates that, as expected, the CDRs contain a large number of exposed residues that are not conserved. Thus, one might hypothesize that anti-idiotypic responses (i.e. immunoreactivity against the hybrid CDR) occur due to changes in these residues, irrespective of the source or type of antibody. The anti-idiotypic response may also be enhanced if the dose of antibody adminstered during treatment is large (e.g. >50 mg per dose).

The sparse data regarding immunogenicity of engineered antibodies is, however, far from definitive. A limited number of clinical trials using chimeric antibodies, in which murine variable domains were fused to human constant domains (Fig. 1), have also yielded data on immunogenicity. Although, in all of these studies, it was apparent that chimeric monoclonal antibodies (mAbs) were less immunogenic than their murine parents, significant differences were observed among the immune responses that they elicited. In one study, only one out of 16 subjects receiving single and multiple doses of the chimeric mAb 17-1A [32,33] appeared to produce an anti-idiotypic response. In a second study, an anti-idiotypic response was seen in six out of 24 patients treated with the chimeric mAb 72.3 [34,35]. The nature of these varied responses is not well understood. We might speculate that murine variable regions with higher homology to known human variable regions may be more immunotolerated than other variable regions unique to the murine repertoire.

Studies on the immunogenicity of humanized antibodies have largely been focused on CAMPATH-1H, which binds to an antigen that is most frequently present on lymphocytes and, to a lesser-extent, monocytes. A low level of anti-CAMPATH-1H antibodies has been detected in a small percentage of patients undergoing treatment for non-Hodgkin's lymphoma or chronic lymphocytic leukemia [36].

Future clinical trials with other humanized and human mAbs will determine if an inherent immunological difference exists between reshaped and human mAbs. It is important to note that due to diversity, even purely human antibodies may be immunogenic in certain individuals.

Further modifications of antibody structure

Exciting and innovative modifications of antibody structure have recently been developed that increase the variety of potential applications further still. Because the antibody–antigen interaction actually occurs over only a small portion of the entire antibody, other features of the antibody can be altered either to lend additional functionality or to affect susceptibility to clearance mechanisms. Bispecific antibodies (reviewed in [37•]) are antibodies in which two Fv or Fab regions of differing specificities are linked by covalent or non-covalent means. These antibodies are especially useful in recognizing molecules on cellular surfaces, as both targets of the hybrid are expected to be in proximity. Note that other proteins (e.g. β-lactamase [38]) can be attached to antibodies by similar means.

The immunotoxins, in which toxic agents are chemically coupled to antibodies, are another example of a bifunctional antibody. Immunotoxins combine the specificity of an antibody with the toxicity provided by one or more attached molecules derived from bacterial [39], plant [40] or fungal [41] sources. Radioisotopes [42] and drugs [43] have also been successfully coupled to antibodies. In theory, immunotoxins should deliver the toxin to a specific cell, undergo internalization [44], and ultimately destroy the targeted cell. In practice, however, this has not been easily achieved due to a number of factors, including non-specific toxicity and difficulty in tumor penetration. Recently, the administration of Fv and Fab fragments coupled to toxins has been shown to be more effective at penetrating solid tumors than that of whole antibodies [45]. Presumably, elimination of the Fc region reduces the ability of recipients to effectively clear the antibody from their system.

Conclusions

Although many of the protein engineering techniques for creating therapeutically useful antibodies are nearing maturity, antibodies still have not fulfilled their therapeutic potential. If their true utility can be established by clinical data, these molecules will undoubtedly become an integral part of the pharmaceutical arsenal.

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