CHAPTER ONE

Humanization of Recombinant Antibodies

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Since 1890, when von Behring and Kitasato reported that animal antitoxin serum could protect against lethal doses of toxins in humans, antisera have been used to neutralize pathogens in acute disease as well as in prophylaxis. Antisera are also used *in vitro* as diagnostic tools to establish and monitor disease. However, antisera invariably induce an immune response resulting in joint pains, fevers, and sometimes life-threatening anaphylactic shock. Various proteins contribute to the immunogenicity, as the serum is a crude extract containing not only the antibodies against the disease-causing antigen (often at low concentration), but also other antibodies and proteins.

FULLY MOUSE TO FULLY HUMAN

In 1975, Köhler and Milstein (1975) at the Medical Research Council's (MRC) Laboratory of Molecular Biology in Cambridge (UK) reported their discovery of a way to produce custom-built antibodies *in vitro* with relative ease. They fused rodent antibody-producing cells with immortal tumor cells (myelomas) from the bone marrow of mice to produce hybridomas. A hybridoma combines the cancer cell's ability to reproduce almost indefinitely with the immune cell's ability to produce antibodies. Once screened to isolate the hybridomas yielding antibodies of the required antigen specificity and affinity – and given the right nutrients – a hybridoma will grow and divide, mass-producing antibodies of a single type (monoclonals). Nearly a century before, the German scientist Paul Ehrlich envisaged that such entities could be used as magic bullets to target and destroy human diseases, and hybridomas seemed like a production line of batch consistency for these magic bullets.

Although monoclonal antibodies (mAbs) from hybridoma technology have proved to be immensely useful scientific research and diagnostic tools, they have not completely fulfilled the possibilities inherent in Ehrlich's vision. The problems include identifying better antigenic targets of therapeutic value with which to raise mAbs; making useful fragments of mAbs that can be produced using microbial expression systems and are better, for instance, at penetrating solid cancerous tumors; and attaching toxic payloads, such as radioisotopes or immunotoxins, to the mAbs since animal antibodies are not as effective as human in recruiting the other cells of the immune system to complete their therapeutic function. The major

hurdle has proven to be similar to that of antisera therapy – namely, that when animal mAbs are administered in multiple doses, the patient almost invariably raises an immune response to the mAbs causing attenuation of their biological activity and clinical symptoms similar to serum sickness and sometimes serious enough to endanger life. This anti-antibody response (AAR), also known as the human anti-mouse antibody response (HAMA) (Schroff et al., 1985) (since rodents are the most common source of animal mAbs), can develop shortly after initiation of treatment and precludes long-term therapy. The HAMA response can be of two types: anti-isotypic and anti-idiotypic. In actual fact, when the murine antibody OKT3 was administered to human patients, much of the resulting antibody response was directed to the variable domains, making it anti-idiotypic (Jaffers et al., 1986). Despite this difficulty, several murine antibodies or their Fab fragments have been approved for diagnostic and therapeutic use by the Food and Drug Administration (FDA) of the United States (Table 1.1A).

The obvious solution to overcome this hurdle would be to raise human mAbs to the therapeutic targets, but this has been difficult both practically and ethically using the route of immortalization of human antibody-producing cells. Human hybridomas, besides being difficult to prepare, are unstable and secrete low levels of mAbs of the IgM class with low affinity although *ex vivo* immunization and immortalization of human B cells is becoming possible (Li et al., 2006). Two other approaches to producing fully human mAbs from phage libraries (McCafferty et al., 1990) (see section titled ''Phage Libraries'' later in this chapter) or transgenic animals (Brüggemann et al., 1991) have been possible since the early 1990s, and a couple of these have been approved by the FDA (Table 1E): Humira (adalimumab) developed from a phage library for the treatment of rheumatoid arthritis, Crohn's disease, and plaque psoriasis; and Vectibix (panitumumab) obtained from a transgenic mouse and used in the treatment of colorectal cancer.

HUMANIZED ANTIBODIES

Chimeric Antibodies

In an effort to realize Ehrlich's dream of a magic bullet with high binding affinity, reduced immunogenicity (reduced AAR), increased half-life in the human body, and adequate recruitment of human effector functions, scientists have used techniques to design, engineer, and express mAbs from hybridoma technology to produce humanized antibodies. These approaches are possible because of the segmented structure of the antibody molecule, which allows functional domains carrying antigen-binding or effector functions to be exchanged (Figure 1.1: Mouse). One interpolative step between fully mouse and fully human antibodies is to construct a chimeric antibody by coupling the animal antigen-binding variable domains to human constant domains (Figure 1.1: Chimeric) (Boulianne et al., 1984; Morrison et al., 1984; Neuberger et al., 1985) and expressing the engineered, recombinant antibodies in myeloma cells. Transgenic animals have also been bred whose

TABLE 1.1. Antibody drugs approved by the Food and Drug Administration (FDA)

rINN[a]	Trade name	Antigen[b]	Therapeutic area[c]	Approval date (U.S.)	Isotype subtype[d]
A. MURINE					
Muromonab-CD3	Orthoclone OKT3	CD3	AIID	1986	mIgG2a
Tositumomab	Bexxar	CD20 radiolabel I-131	Onco	2003	mIgG2a
Arcitumomab	CEA-Scan	CEA radiolabel Tc-99m	Onco (D)	1996	mIgG1 (Fab)
Imciromab Pentetate	Myoscint	cardiac myosin radiolabel In-111	Card (D)	1996	mIgG2a (Fab)
Capromab Pendetide	Prostascint	PSMA radiolabel In-111	Onco (D)	1996	mIgG1
Technetium nofetumomab merpentan	Verluma	NR-LU-10 (40kd gp) radiolabel Tc-99m	Onco (D)	1996	mIgG2b (Fab)
Ibritumomab Tiuxetan	Zevalin	CD20 radiolabel Y-90/In-111	Onco	2002	mIgG1
B. CHIMERIC					
Cetuximab	Erbitux	EGFR	Onco	2004	hIgG1
Infliximab	Remicade	TNFα	AIID	1998	hIgG1
Abciximab	ReoPro	gpIIb/IIIa Receptor	Card	1994	hIgG1 (Fab)
Rituximab	Rituxan/MabThera	CD20	Onco	1997	hIgG1
Basiliximab	Simulect	CD25	AIID	1998	hIgG1
C. CDR-GRAFTED					
Eculizumab[e]	Soliris	Complement C5	PNH	2007	hIgG2/4
D. RESHAPED					
Bevacizumab	Avastin	VEGF	Onco	2004	hIgG1
Alemtuzumab	Campath	CD52	Onco	2001	hIgG1
Trastuzumab	Herceptin	HER2	Onco	1998	hIgG1
Ranibizumab	Lucentis	VEGF	Ophth	2006	hIgG1 (Fab)
Gemtuzumab Ozogamicin	Mylotarg	CD33 cytotoxic calicheamicin	Onco	2000	hIgG4
Efalizumab	Raptiva	CD11a	AIID	2003	hlgG1
Palivizumab	Synagis	RSV F	Infec	1998	hlgG1
Natalizumab[f]	Tysabri	integrin-α4	AIID	2004	hIgG4
Omalizumab	Xolair	IgE	Resp	2003	hIgG1
Daclizumab	Zenapax	CD25	AIID	1997	hIgG1
Tocilizumab	Actemra	IL-6R	CD	2005 (Japan)	hIgG1
E. HUMAN					
Adalimumab	Humira	TNFα	AIID	2002	hIgG1
Panitumumab	Vectibix	EGFR	Onco	2006	hIgG2

Note: Not all the murine antibodies are commercially available.

[a] rINN, recommended International Nonproprietary Name.

[b] CD, cluster of differentiation; CEA, carcinoembryonic antigen; PSMA, prostate specific membrane antigen; kd, kilodalton; gp, glycoprotein; EGFR, epidermal growth factor receptor; TNFα, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor receptor; HER2, human epidermal growth factor receptor 2; RSV F, respiratory syncytial virus F protein; IgE, immunoglobulin E; IL-6R, interleukin 6 receptor.

[c] (D), diagnostic; AIID, arthritis, inflammation, immune disease; Onco, oncological disease; Card, cardiovascular disease; Ophth, ophthalmic disease; PNH, paroxysmal nocturnal hemoglobinuria; Infec, infectious disease; Resp, respiratory disease; CD, Castleman's disease.

[d] m, mouse; h, human; Fab, fragment antigen binding.

[e] Since Eculizumab (Soliris) used the structural (Chothia) loops for CDR-H1, it arguably contains backmutations and therefore is not strictly a pure CDR graft.

[f] Voluntary suspension in 2005; granted restricted approval 2006.

immune systems produce such chimeric antibodies (Jensen et al., 2007). Variations on this theme have also been attempted, for instance Lv et al. (2007) have recently attached a mouse single chain Fv (scFv) to the CH3 domain of human IgG1 using a redesigned human IgG1 hinge region resulting in what they call a bivalent ''partial chimeric'' antibody.

The main decision in producing a chimeric antibody is the choice of human constant regions (Figure 1.1: Mouse) that provide an isotype relevant to the desired biological function; IgG1 and IgG3 subtypes are most effective for complement and cell-mediated lysis-triggering effector cascades whereas IgG2 and IgG4 are preferred for target neutralization. In fact, most of the humanized antibodies on the market are of the IgG1 subtype (Table 1.1). In some cases, the effector functions of the constant regions are removed by modifying the Fc so that it does not bind its receptor, thereby minimizing T cell activation and cytokine release, and other modifications could also lead to IgG subtypes with better biological properties. Chimeric antibodies with the same antigen-binding domains fused to different subtypes of constant regions can show different binding affinities (Morelock et al., 1994) and also immunogenicity on repeated administration – the so-called human antichimeric antibody (HACA) response (Brüggemann et al., 1989). This HACA response varies depending on the chimeric antibody and therefore some have still been approved by the FDA (Table 1.1B).

CDR-Grafted Antibodies

Going one step further on the path between fully mouse and fully human antibodies, Greg Winter (Jones et al., 1986), also at the MRC's Cambridge Laboratory of Molecular Biology, realized that only the antigen-binding site from the human antibody (the tip of the variable domains) needed to be replaced by the antigen-binding site from the rodent antibody using genetic engineering techniques. Since the antigen-binding site consists mainly of the six complementarity determining region (CDR) peptide loops, only these were grafted into the human variable (V) regions (Figure 1.1: CDR-grafted). Antibodies generated this way are called CDR-grafted, and in some cases pure CDR-grafting can produce a humanized antibody with roughly the same antigen specificity and affinity as the original animal (usually mouse) antibody. The only choices required are which human V regions to graft the CDRs into and the isotype required to provide the desired biological function. The isotype choice is governed as for chimeric antibodies (see above), but the choice of human V regions is more demanding.

Choice of Human Variable Regions

The early literature on humanization showed a preference for the same human V regions, known as the ''fixed frameworks'' approach. Usually REI for the variable light (VL) and NEW or KOL for the variable heavy (VH) were chosen since their three-dimensional structures had been solved. This was the case for the

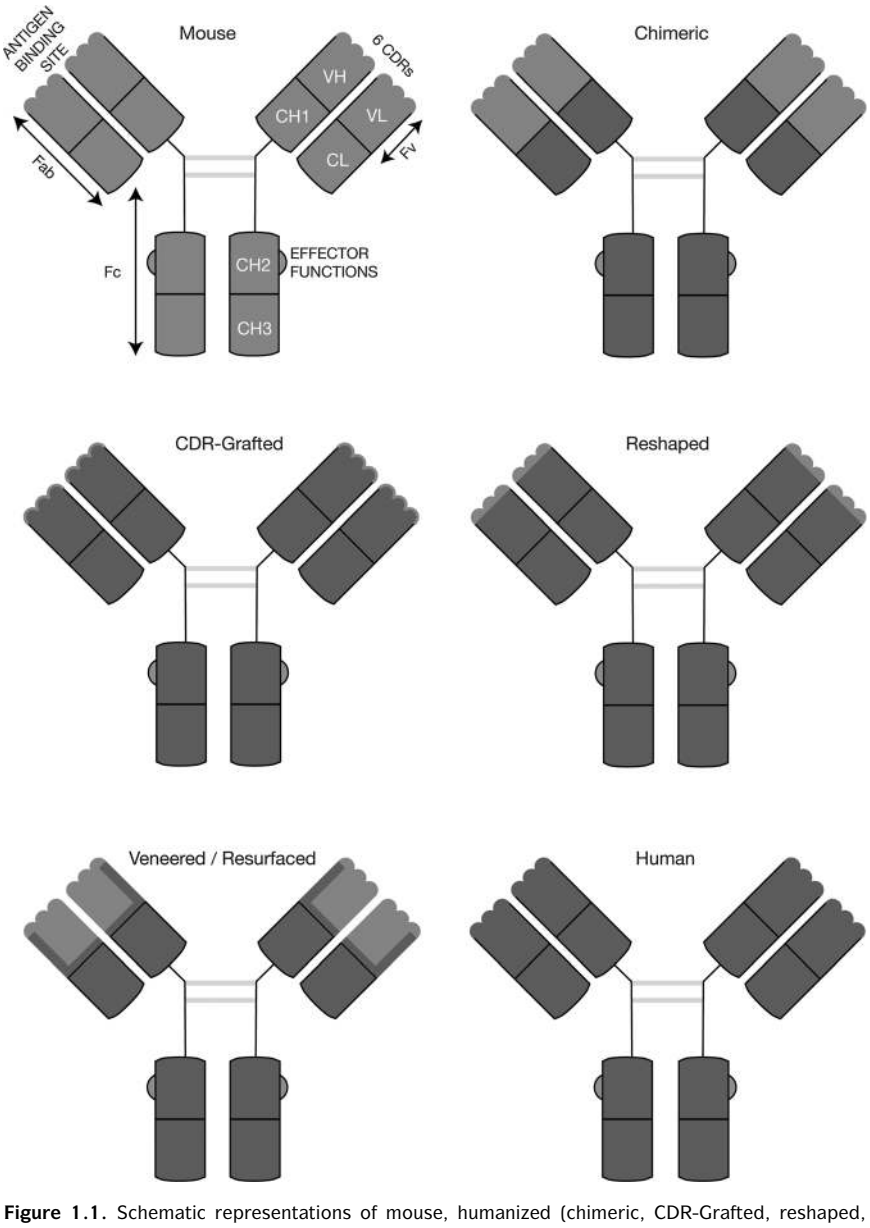

Figure 1.1. Schematic representations of mouse, humanized (chimeric, CDR-Grafted, reshaped, veneered/resurfaced) and human antibodies. Blue, mouse content; red, human content; yellow, disulphide bridges; green, carbohydrate moieties. VH, variable heavy domain; VL, variable light domain; CH1 to 3, constant heavy domains 1 to 3; CL, constant light domain; Fab, fragment antigen binding; Fc, fragment of crystallization; Fv, fragment variable. [See color plate.]

humanized antibody, Campath (Alemtuzumab) (Riechmann et al., 1988) for the treatment of B cell chronic lymphocytic leukemia and later for Actemra (Tocilizumab) (Sato et al., 1993), which is licensed in Japan for the treatment of Castleman's disease and rheumatoid arthritis. Other groups tried to use human acceptor V regions that showed the closest similarity to their mouse donor V regions in an approach known as ''homology matching'' (also called ''best fit'' (Gorman et al.,

1991)). This was the approach taken by Queen et al. (1989) for the VH of the anti-TAC antibody, now marketed as Zenapax (Daclizumab) for the prophylaxis of acute organ rejection in patients receiving renal transplants. In this case, the VL was chosen to match the VH, that is, the same human antibody for both chains, while others have used the most similar VL and VH from different human antibodies (e.g., Daugherty et al., 1991). Variations on the best fit approach take into consideration the extent of sequence similarity (either the whole V region or the individual frameworks between the CDRs – chosen from either single or different human antibodies) and matching lengths of CDRs between the mouse and human V regions. Human V regions can also be chosen based on sequence similarity but with particular amino acid types fixed at positions deemed to be important for affinity, specificity, or stability. A subtle comparison of the fixed frameworks and best fit methods, in terms of the ease of producing a functional humanized antibody, can be found in the humanization of antibody M22 (Graziano et al., 1995). There the preferential choice appeared to be the latter, where the more sequence similar human KOL VH gave better binding than NEW. It has been argued that the advantage of the best fit approach might be outweighed by the wealth of experience that has been assembled using fixed frameworks (Hamilton et al., 1997). However, when the crystal structures of two humanized forms of antibody AF2 were determined, which differed in the sequence identity of the mouse VH to the humanized, the form with greater identity was significantly more structurally similar to the mouse antibody (Bourne et al., 2004). Since structure determines function, the more structurally similar humanized antibody would presumably reproduce the function of the mouse antibody more faithfully.

Both the fixed frameworks and best fit approaches to human V region selection can be limited to the processed V regions found in protein sequence databases, giving the advantage that the humanized molecule is more likely to be stable and expressed. However, this runs the risk of somatic mutations in these V regions creating immunogenic epitopes, even though human sequences are used. An alternative approach is to use the V regions from human consensus sequences where idiosyncratic somatic mutations will have been evened out (Shearman et al., 1991). It is still uncertain whether fixed frameworks, best fit, or consensus selection for humanized antibodies are best in terms of binding. Best fit and consensus selection were compared, in one case showing better binding with best fit (Sato et al., 1994) and in another case showing no difference (Kolbinger et al., 1993). Comparison of fixed frameworks and consensus selection (Maeda et al., 1991) showed loss of binding with the fixed frameworks. In several humanized antibodies, best fit V regions have been chosen from a protein sequence database, only to then exchange the residues in some positions for those of a consensus amino acid (e.g., Hakimi et al., 1993). Consensus sequences can also be selected in a fixed frameworks type of approach, regardless of sequence similarity to the mouse V region, using the knowledge that the most abundant human subgroups are VH subgroup III for the heavy chain and VL subgroup I for the kappa light chain.

Consensus sequences are artificial, having been created by taking the most frequent amino acid at a particular sequence position from a collection of sequences in

a human V region subgroup. Although they have no unusual residues, they may contain unnatural sequence motifs that are immunogenic. An alternative approach is to use human germline sequences, originally suggested by Shearman et al. (1991), that do not contain the somatic hypermutations found in the protein- and cDNA-derived sequences in the databanks. Consensus and germline sequences can be selected in a best fit approach, with high sequence similarity to the mouse V region. The variations considered in the best fit approach are also possible with germline sequences – for instance, multiple individual germline frameworks corresponding to different segments of the V region can be used in an essentially mix-and-match procedure. So called ''superhumanized'' antibodies are humanized antibodies using germlines matched to the canonical templates (see section titled ''Sequence Analysis'' later in the chapter) of the mouse antibody (Tan et al., 2002).

Reshaped Antibodies

The first reported CDR-graft was performed using the donor VH CDRs of a murine anti-hapten antibody B1-8 grafted into acceptor VH frameworks from human antibody NEW (Jones et al., 1986) to determine whether the CDRs were independent of the framework. Although the binding of the hemi-CDR-grafted antibody was two-to-three-fold lower than the donor mouse antibody, the result was encouraging. This initial work was followed by a CDR graft using the VH CDRs of murine anti-lysozyme antibody D1.3 (Verhoeyen et al., 1988). The binding was 10-fold lower than the donor antibody and it was apparent that CDR loops were not stand-alone entities independent of the framework. The first humanized antibody of therapeutic interest using this approach took the six CDRs from rat antibody Campath-1R and grafted them into human VL from antibody REI and VH from NEW (Riechmann et al., 1988). For the first time, framework reversions (known as ''backmutations'') from human to rat were incorporated into the engineered antibody, now known as a ''reshaped'' antibody since the CDR-grafting was accompanied by backmutations to reshape the CDR loops (Figure 1.1: Reshaped). This was followed shortly after by the first reshaped mouse antibody, anti-TAC (Queen et al., 1989). In this case, the human VH framework was chosen based on similarity to the mouse VH, and the partner VL from the same human antibody was also used. Several backmutations were introduced based on a three-dimensional molecular model of the mouse variable regions. The authors proposed to call an antibody prepared in this manner ''hyperchimeric'' (Junghans et al., 1990) and certainly some reshaped antibodies contain so many framework backmutations that they might be considered almost chimeric. Later work (Schneider et al., 1993) showed the immunogenicity of the reshaped antibody was mainly caused by the CDRs rather than the modified human frameworks, supporting the validity of this approach. The backmutations are necessary to reproduce the affinity and specificity of the mouse antibody in the reshaped molecule. Sometimes they are also necessary to improve the expression yields, although these are rarely reported in the literature. In one case, an improvement in both the affinity and expression was accomplished by a single VL backmutation that improved not only the expression but also the affinity of the molecule (Saldanha et al., 1999). From the

early humanizations, it was clear that any strategy for producing reshaped antibodies would require careful analysis of sequence and structure to determine the backmutations required in the final genetically engineered molecule.

Sequence Analysis

The CDRs are six highly variable sequence regions, three each in the VL and VH, of the donor antibody that structurally make up the antigen binding site (Figure 1.1: Mouse). The preponderance of backmutations reported in the literature at position 73 in VH suggests that the structural loop encompassing this residue may also be part of the antigen binding site in some antibodies. The CDRs contain the residues most likely to bind antigen and are defined by sequence according to Kabat (Wu & Kabat, 1970). The extents of the structural loops have also been defined by Chothia (Chothia et al., 1989) although this definition varies in the literature. The CDRs are grafted from the donor antibody into the acceptor human antibody. The Chothia structural loop extents are shorter than the Kabat sequence definition, thus resulting in less mouse sequence in the humanized antibody. However, the structural extent of the CDR-H1 loop encompasses residue positions 28–30 in VH, which are known to exacerbate the immunogenicity in humans (Tempest et al., 1995) whereas the Kabat sequence definition does not. The sequence definition of CDR-H1 often requires backmutations in the region covered by the structural extent of this loop, leading some to combine the definitions for CDR-H1 (e.g., Thomas et al., 1996). Conversely, the structural extent of the CDR-H2 loop usually requires several backmutations in the region covered by the sequence definition (e.g., Rodrigues et al., 1992).

Canonical residues are key residues in the CDR and/or framework that determine the conformation of the structural loop. Chothia and Lesk (1987) originally defined the canonical templates for each CDR conformation, which they later extended (Al-Lazikani et al., 1997). Canonical residues should be retained in the reshaped antibody if they are different from those in the chosen human frameworks. Note that particular CDRs or canonical residues might have no effect on the specificity or affinity of the reshaped antibody if that CDR does not contact the antigen, but there is no way of knowing this by consideration of the sequence alone.

Residues at the interface between VL and VH are also often analyzed in reshaped antibodies since they govern the packing of the variable domains, thus affecting the binding site. Their influence might also be functional since Nakamura et al. (2000) reported that although affinity was not affected, complement-dependent cytotoxicity (CDC), one of the effector functions of mAbs, was improved by a backmutation at the VL/VH interface. The interface residues were defined by Chothia et al. (1985) and have been improved by Vargas-Madrazo and Paz-García (2003).

Rare residues in the donor sequence can be determined by comparison with the Kabat subgroup (Kabat et al., 1987; 1991). Atypical residues near the antigen binding site, as determined from the crystal structure or molecular model (see section titled

''Structure Analysis'' later in the chapter), may possibly contact the antigen and therefore should be backmutated. If they are not close to the binding site, then it is desirable to humanize them because they may represent immunogenic epitopes in the reshaped antibody. Sometimes unusual residues in the donor sequence are actually common residues in the human acceptor (Queen et al., 1989) and cause no such difficulties. Atypical residues in the human acceptor frameworks are not desirable because of the possibility of immunogenicity, unless of course they correspond to unusual residues in the donor and thus may be important functionally. Rarely occurring amino acids in the human frameworks have been mutated to human consensus residues (Co et al., 1991).

Potential N-glycosylation sites are specific to the consensus pattern asparagine-X-serine/threonine, where X can be any amino acid except proline, and most patterns on the surface of the protein are glycosylated. They may occur as part of the germline or arise through somatic hypermutation in the frameworks or CDRs. It was expected that addition or removal of N-glycosylation sites in reshaped antibodies might affect their binding or immunogenicity. Their removal has not destroyed the affinity thus far (Léger et al., 1997), even when they are found at canonical residues (Sato et al., 1996), and in some cases the affinity was even increased (Co et al., 1993). Any additional N-glycosylation sites introduced through the humanization procedure should be checked on a model to ensure that they do not interfere with the CDRs. O-glycosylation sites are usually found in helices and are therefore not common in the beta-sheet structure of antibodies.

Structure Analysis

Humanization using the fixed frameworks approach usually meant that the crystal structures of the human acceptor regions were available. These structures could be inspected to identify potential backmutations. Although some humanizations used only sequence analysis (e.g., Poul et al., 1995), other approaches to framework selection (best fit, consensus, germline) relied on a carefully built model of the mouse variable regions (e.g., Kettleborough et al., 1991) and in some cases the humanized variable regions, particularly when inspecting introduced N-glycosylation sites. Superposition of the mouse and humanized three-dimensional models and analysis of the size, charge, hydrophobicity, or hydrogen-bond potential between topologically equivalent residues allowed potential backmutations to be revealed. In some cases, a model of the antigen was also built (Nishihara et al., 2001). A model of the donor antibody docked to the antigen would be ideal for the design of a humanized antibody, in the absence of a crystal structure for the complex, and has been achieved by computer-guided docking (Zhang et al., 2005). The advantage is that CDRs not contacting antigen can be determined, and unnecessary grafting and backmutations avoided. Computer modeling can only be an interim measure on the way to determining the structure of the antibody or antibody-antigen complex by X-ray crystallography or nuclear magnetic resonance (NMR).

Antibody modeling is relatively simple compared to the modeling of other proteins, since the framework is so well conserved. This reduces the problem to modeling the CDRs, and the conformation of many of these CDRs can be inferred from the canonical templates (see section titled ''Sequence Analysis'' earlier in the chapter). It is rarely necessary to apply sophisticated loop modeling techniques to more than a couple of CDRs per antibody. Although there are no canonical templates available for CDR-H3, Shirai et al. (1996; 1999) showed that in many cases these loops exhibit ''kinked'' or ''extended'' C terminal regions predicted by sequence-based rules. These rules can be applied to determine additional features of CDR-H3 and aid its conformational prediction. It is possible to build a model completely automatically using programs such as Modeller (http://www.salilab.org/modeller/) and academic servers such as Swiss-Model (http://swissmodel.expasy.org/). However, the pitfall of allowing a computer to make all the decisions is highlighted in the humanization of antibody AT13/5 (Ellis et al., 1995), where the interaction between VH residues at positions 29 and 78 was not modeled correctly.

OTHER APPROACHES TO ANTIBODY HUMANIZATION

SDR-Transfer

CDR-grafting and reshaping do not necessarily eliminate the immunogenicity of the resultant molecule due to residual responses directed against the murine CDRs. An analysis of antibody structures determined that antigen binding usually involves only 20% to 33% of the CDR residues (Padlan, 1994) that have been given the label ''specificity determining residues'' (SDRs). Padlan et al. (1995) extended this work to determine the boundaries of the potential SDRs in different antigen-combining sites and called the segments thus found ''abbreviated CDRs.'' The SDRs are commonly located at positions of high variability and are unique to each mAb. However, they can be identified by site-directed mutagenesis or determination of the 3D structure of the variable regions, or, in the absence of this information, the variability of positions within the abbreviated CDRs can be used to suggest which residues are SDRs. Transfer of SDRs only has been used successfully in the humanization of anticarcinoma mAb CC49, which specifically recognizes tumor-associated glycoprotein (TAG)-72 (Yoon et al., 2006). In that case, the lower affinity of the SDR-transferred antibody was improved by random mutagenesis of CDR-H3 (*in vitro* affinity maturation). SDR-transfer into human germline frameworks has been utilized in the humanization of murine mCOL-1, which specifically recognizes carcinoembryonic antigen (CEA). In this case, the SDR-transferred antibody had comparable binding activity to the reshaped equivalent and significantly higher activity compared with the abbreviated CDR-grafted antibody. It also showed decreased reactivity for anti-V region antibodies present in the sera of patients treated with mCOL-1 (Gonzales et al., 2004).