Chapter 2

CDR Repair: A Novel Approach to Antibody Humanization

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Abbreviations

CDRs	Complementarity determining regions
Fab	Antigen binding fragment consisting of the light chain and the variable and first constant domains of the heavy chain
HAMA	Human anti-mouse antibodies
VH	Variable heavy domain
VL	Variable light domain

Hybridoma technology has enabled the rapid production of a large number of monoclonal antibodies with interesting biological properties. Their use in a therapeutic setting, however, can lead to the generation of a human anti-mouse antibody (HAMA) response in patients despite the high degree of sequence similarity shared between human and mouse antibodies. This has prompted efforts to make hybridoma antibodies appear more human through the construction of chimeras, (Morrison et al. 1984) and through a process known as antibody humanization (Riechmann et al. 1988; Verhoeyen et al. 1988).

The modular nature of antibodies makes the swapping of domains a relatively simple process. A chimera consisting of the mouse variable heavy (VH) and variable light (VL) domains recombinantly fused to human heavy and light constant domains is a simple way to reduce HAMA response. Yet, despite 60–75% homology to human, murine variable domains may still elicit a HAMA response.

Humanization is a process used to further reduce the content of murine residues in the variable domains. Each VL and VH domain adapts the immunoglobulin fold and presents three loops protruding from one end, called complimentarity determining regions or CDRs, for interaction with antigen. The rest of the variable domain functions as a framework to support and stabilize the conformation of these CDRs. The transfer of the six CDR loops from murine variable domains to human variable frameworks is considered a CDR graft (Jones et al. 1986). Compared to the chimera, this

From: *Current Trends in Monoclonal Antibody Development and Manufacturing,*
Biotechnology: Pharmaceutical Aspects, DOI 10.1007/978-0-387-76643-0_2,
Edited by: S.J. Shire et al.

step further reduces the amount of murine sequence present. Unfortunately, a loss in binding affinity is generally incurred during this process and so additional engineering steps may be required (Riechmann et al. 1988; Foote and Winter 1992).

Fig. 2-1a provides a simple conceptual image of the differences between murine, chimeric and humanized antibodies. Due to the high homology

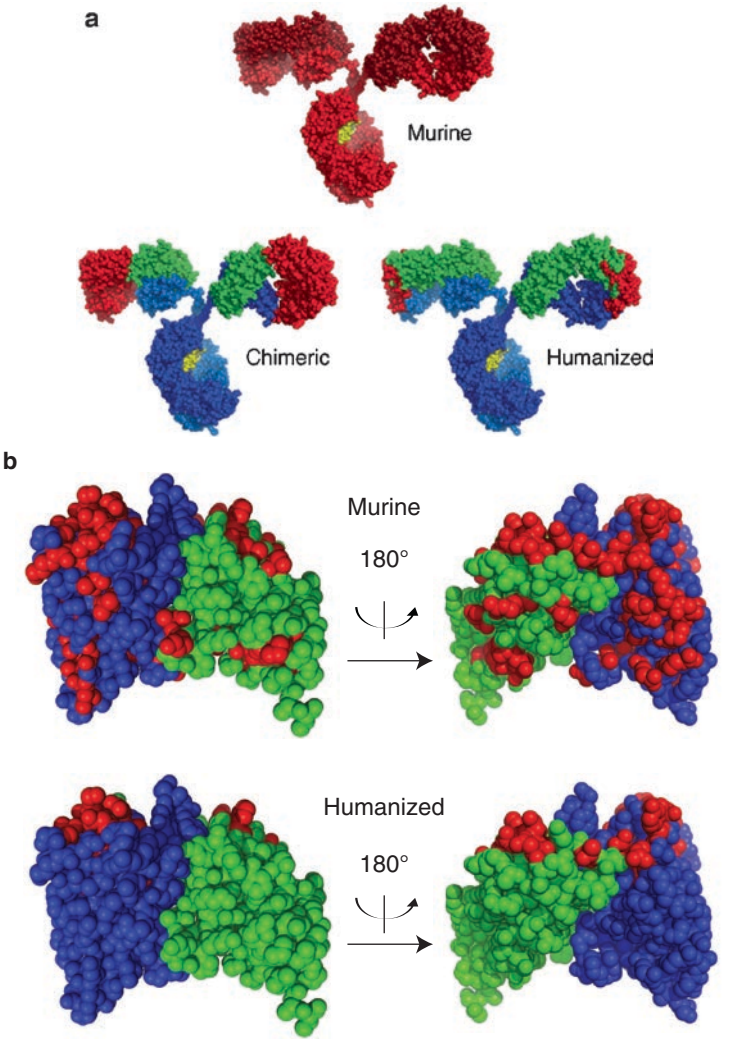

Fig. 2-1. A comparison of murine, chimeric and humanized antibodies. (**a**) A conceptual representation of murine, chimeric and humanized antibodies with amino acid residues derived from the murine antibody are depicted in *red*. The chimera consists of murine VL and VH domains (*red*) fused to human constant light (*green*) and constant heavy (*blue*) domains. The humanized antibody consists entirely of human light (*green*) and heavy (*blue*) chain sequence with exception of the six CDR sequences that have been transferred from the murine antibody (*red*). (**b**) Differences between murine 4D5 and humanized 4D5 (1FVC (Berman et al. 2000)) variable domains are depicted in a way that takes into account sequence identity between murine and human sequences. In this representation, the CDR sequences are oriented at the *top* of the image. The sequence of CDR-H3 is highly variable and is not included in this depiction

between murine and human variable domains, however, this representation is somewhat misleading. Residues that are identical between mouse and human should ideally be excluded from a calculation of an antibody's "human-ness." The variable domains of murine 4D5 and humanized 4D5 (trastuzumab) are depicted in Fig. 2-1b. Both the murine and humanized sequences are compared to their closest human germline, respectively, and residues that differ are colored in red. Here, the degree to which murine residues are reduced by humanization is actually greater than what is suggested in Fig. 2-1a, since many of the CDR residues from the respective germline are identical. In either representation, however, humanization clearly reduces the number of murine residues.

Multiple approaches, discussed below, have been described for improving the success of making a CDR graft, that retains the original antigen binding properties or for restoring binding affinity to the CDR graft. Each of these methods requires an appreciation for the structural components inherent in the antibody variable domains.

1. Important Considerations

Antibody variable domains share a high degree of sequence and structural homology across species and across germlines (Padlan 1994); however, while a few changes in variable domain sequence can have only a very subtle influence on the structure, they can have a profound impact on antigen binding (Eigenbrot et al. 1993). When humanizing an antibody, there are three important factors to consider, each of which can influence antigen binding: delineation of the CDRs, the choice of a human acceptor framework and positions that differ between murine and human frameworks that can influence CDR structure and affect antigen binding. These components are also important when humanizing antibodies from other species (e.g. rat, rabbit or hamster). How each of these factors is utilized can depend upon the humanization method used, nevertheless each should be considered.

Historically and conceptually, there have been three approaches that define the CDRs. The first, a sequence based definition, arose as antibody sequences became available. Kabat and Wu compared multiple variable domain sequences and recognized that the hypervariable regions in antibodies were likely to determine antigen specificity (Wu and Kabat 1970; Kabat and Wu 1971). As antibody X-ray structures were determined, it became apparent that these hypervariable regions mapped to loops with a limited number of conformations extending from the immunoglobulin variable domain β-sandwich. This led Chothia and Lesk to develop a structural definition for CDRs, and propose a set of canonical CDR conformations that were based upon loop length and a few key residues directing main-chain conformation (Chothia and Lesk 1987; Chothia et al. 1989). Later, as multiple antibody–antigen complex structures were determined, yet another definition of the CDRs emerged based upon residues found to be in contact with antigen (MacCallum et al. 1996). While all three of these CDR definitions generally map to similar locations within the VL and VH domains, there are slight differences as to where each CDR starts and stops (Fig. 2-2). The largest discrepancies are at the beginning of CDR-L1, where both the sequence and structural definitions include residues, that are not commonly observed to be in contact with antigen, and the

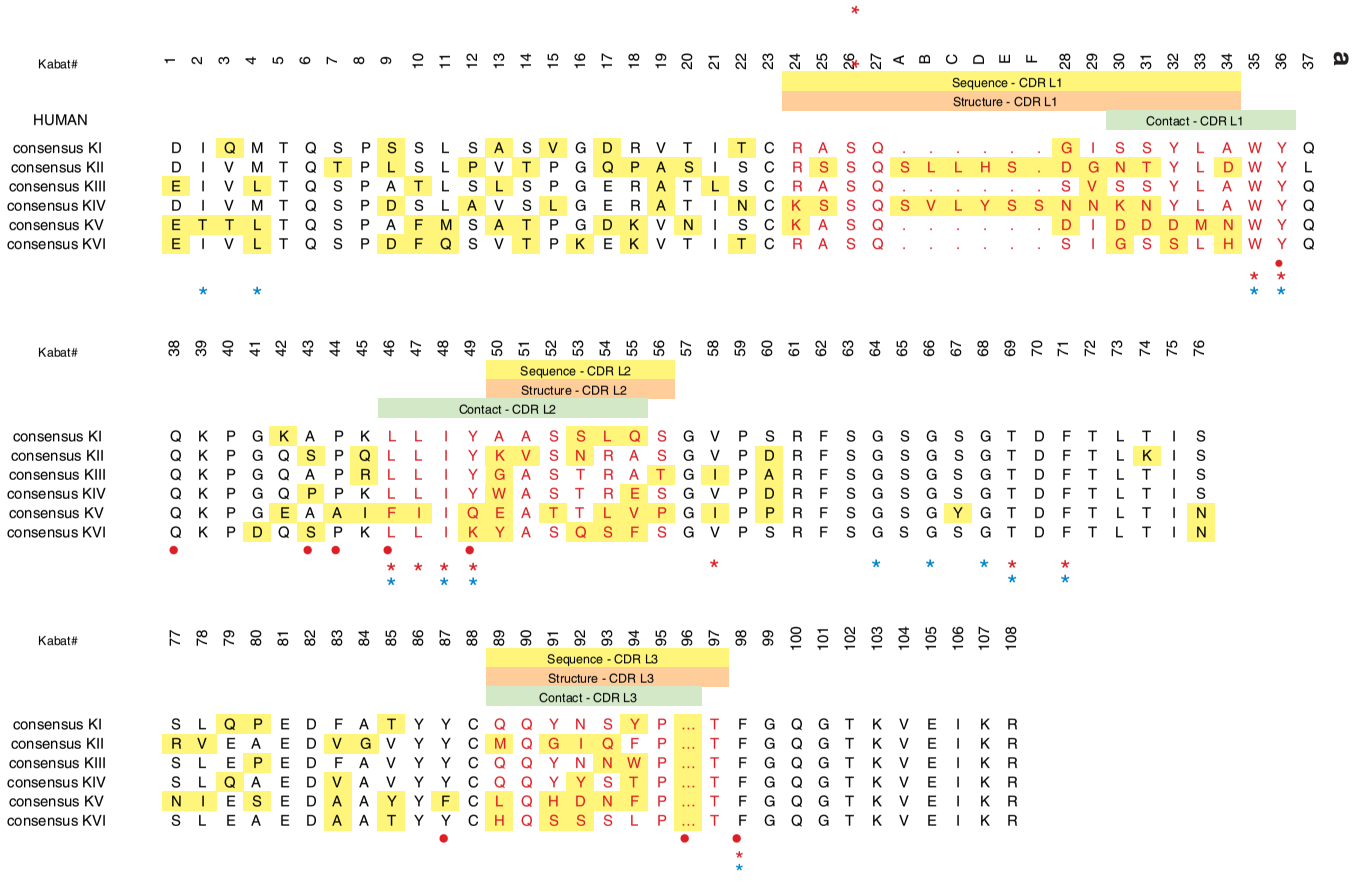

Fig. 2-2. CDR delineation, residues in the VL–VH interface and vernier positions in human consensus variable domains. Human consensus variable domain sequences representing each VL_{kappa} and VH subgroup (derived from Honegger 2007) are shown numbered according to Kabat (Kabat et al. 1991). Non-homologous residues between the consensus sequences are *shaded*. CDR locations in (**a**) VL (CDR-L1, CDR-L2, and CDR-L3) and

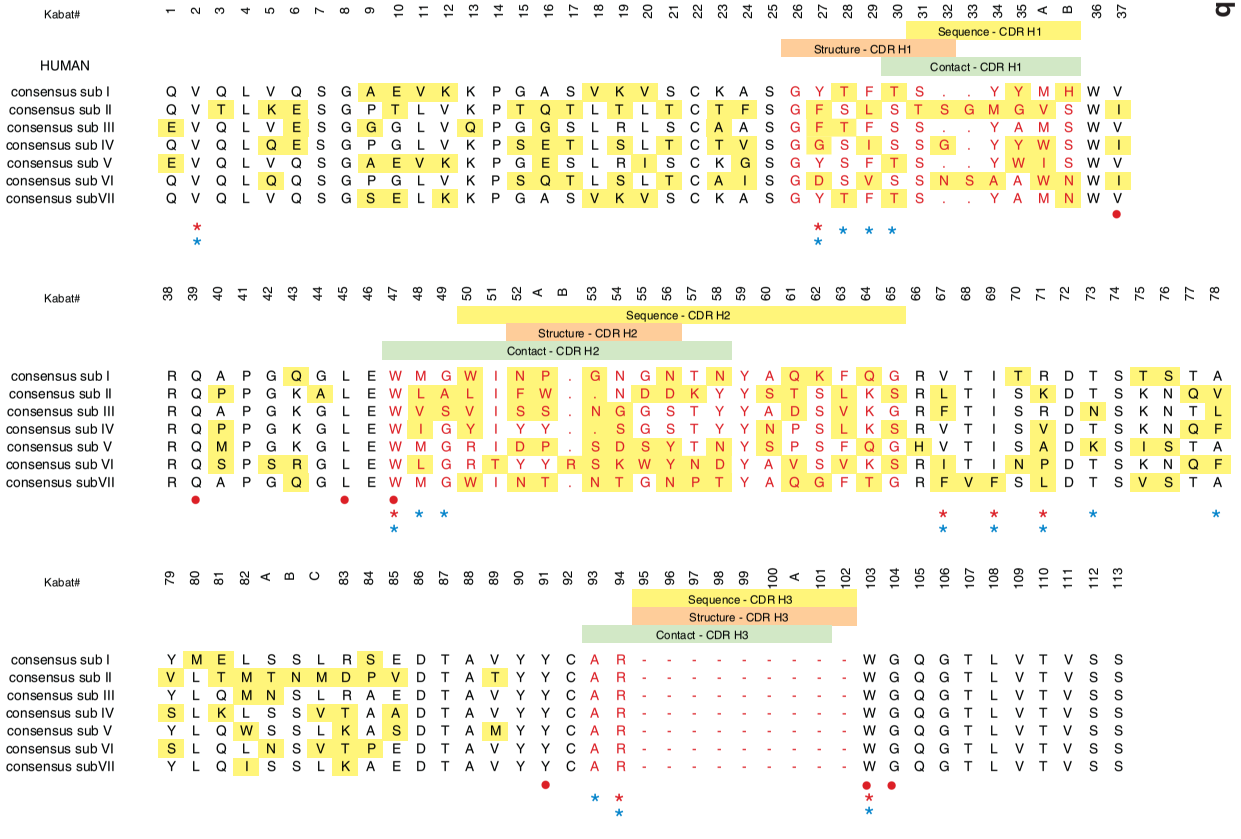

Fig. 2-2. (continued) (**b**) VH (CDR-H1, CDR-H2, and CDR-H3) delineated by sequence hypervariability (*yellow bar* (Kabat and Wu 1971; Kabat et al. 1991)), loop structure (*orange bar* (Chothia and Lesk 1987)) or antigen contact (*green bar* (MacCallum et al. 1996)) are depicted, as well as the location of vernier positions defined by Foote and Winter (1992) (*blue star*) or Padlan (limited to those observed in greater than 70% of studied antibody structures and having at least six van der Waals contacts, *red star* (Padlan 1994)). Residues potentially affecting the VL–VH interface are indicated by a *red dot* (those observed in greater than 80% of studied antibody structures (Padlan 1994))

end of CDR-H2, which contains hypervariable sequence, but is neither part of the protruding loop nor commonly found in antigen contacts.

Another important consideration is the human acceptor framework to be used for a particular humanization for which there are several schools of thought. A common approach is to identify and graft the CDRs into the human germline, that is most homologous to the murine sequence. This has an advantage that the framework environment presenting the CDRs is minimally changed. Related to this, the CDRs may also be grafted into a calculated human consensus framework sequence, based upon the most homologous human germline subgroup (Fig. 2-2). In either case, the choice of framework can be made based upon the overall homology of the variable domain (Queen et al. 1989) or just homology within the framework (Wu et al. 1999) or just within the CDRs (Tan et al. 2002; Hwang et al. 2005). Using homology to select a human framework has a disadvantage in that each humanization can result in a new VL and VH combination. Additional engineering steps may be required to optimize the large VL/VH interface for each combination.

An alternate strategy is to utilize a single stable framework that has been validated in the clinic for generating the CDR graft, regardless of the parent antibody sequence. For example, the $VL_{kappa\ I}$ and VH_{III} consensus frameworks are derived from the most abundant human VL and VH subclasses and has been used to humanize a number of murine antibodies (Carter et al. 1992; Presta et al. 1993, 1997, 2001; Werther et al. 1996; Adams et al. 2006). Utilizing a previously validated framework may reduce the likelihood of protein stability or manufacturing problems and, thereby facilitate clinical development.

CDRs do not function independently on the rest of the antibody. They consist of residues that interact with antigen, but also include residues that interact with the framework and neighboring CDRs. How CDRs are presented and structurally supported by the framework is critical to their ability to interact with antigen. The VL–VH interface is mostly composed of framework residues, yet this interface is also influenced by certain CDR positions. Vernier positions provide a foundation for the CDRs; they can directly influence framework–CDR interactions and as a result can affect antigen binding (Foote and Winter 1992). In addition, other positions that influence VL/VH domain interactions or on occasion are involved in unusual antigen contacts can also play an important role. An analysis of antibody crystal structures has suggested that there are about 30 positions distributed throughout the variable domains that have the potential to influence CDR packing and function (Foote and Winter 1992; Padlan 1994). These are noted in Fig. 2-2 and are illustrated in Fig. 2-3. Depending upon the human acceptor framework selected, these positions will differ from the parent antibody. Further, the importance of any particular vernier position will vary depending on the antibody/antigen system. The interaction between vernier positions and CDR anchor residues (CDR residues that interact with the framework) is often the source of humanization problems, and the identification of the optimal combination of vernier positions can be a major challenge.

2. Humanization Approaches

Typically, the first step during humanization is to generate a CDR graft in which, the CDRs (or some portion of the CDRs (Kashmiri et al. 2005)) are grafted onto a human acceptor framework. As mentioned previously, this

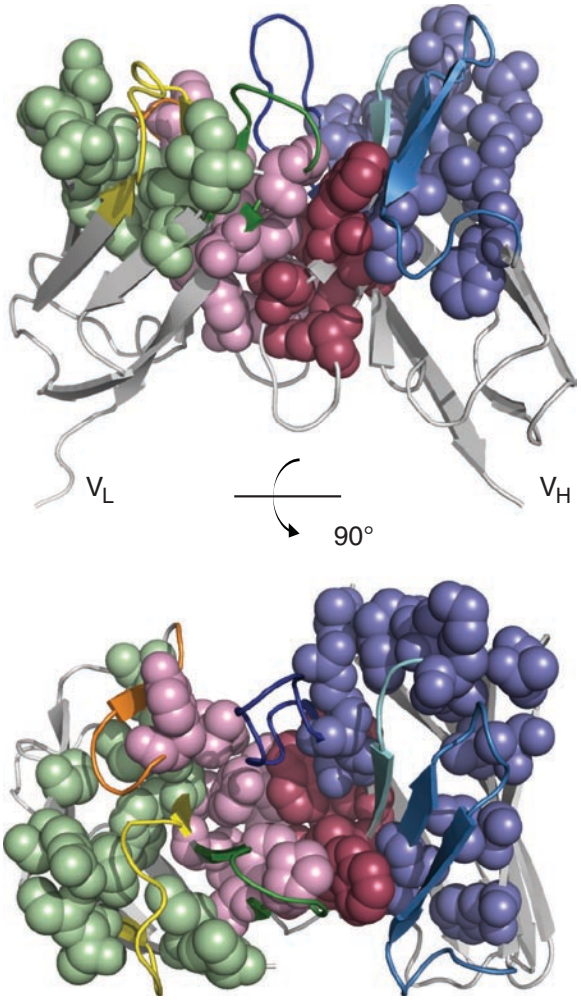

Fig. 2-3. A structural representation of variable domain vernier positions. CDRs are colored (CDR-L1 is *yellow*, CR-L2 is *orange*, CDR-L3 is *dark green*, CDR-H1 is *light blue*, CDR-H2 is *blue* and CDR-H3 is *dark blue*) on the VL and VH framework (*white*). The side chains of vernier positions (*green* for VL and *blue* for VH) and domain interface positions (*pink* for VL and *salmon* for VH) from Fig. 2-2 are depicted using spheres. Humanized 4D5 (1FVC (Berman et al. 2000)) was used as the model (Eigenbrot et al. 1993)

often results in partial or complete loss of antigen binding. The most common approach to restoring high affinity binding is to identify and replace key residues in the human acceptor framework with residues from the parent antibody. Molecular modeling has frequently been used to identify potentially inappropriate packing between CDR anchor residues and vernier positions. Alternatively, the appropriate combination of framework changes can be derived empirically through combinatorial techniques (Baca et al. 1997; Rader et al. 2000; Lee et al. 2004). These approaches attempt to reestablish the original CDR/framework environment by utilizing information gained from the parent murine framework to incorporate changes into the human acceptor framework. This approach to repair the acceptor framework (framework repair)

has been used quite successfully (Kettleborough et al. 1991; Carter et al. 1992; Presta et al. 1993, 1997, 2001; Werther et al. 1996; Tsurushita et al. 2005; Adams et al. 2006) and has been relied on careful modeling of the human and murine variable domains. Several iterations may be required to identify the minimal set of murine positions that need to be substituted into the human framework.

Framework repair can be facilitated by the selection of an appropriate framework from the most homologous human germline, or a consensus framework (Fig. 2-2) that is derived from the most homologous human subgroup (Tsurushita et al. 2005). This method enables the retention of many potentially important vernier residues by default. An alternative approach is to utilize only CDR sequence homology for framework selection, thus identifying a framework that is capable of presenting the proper canonical CDR structure, regardless of sequence variations in the framework (Tan et al. 2002; Hwang et al. 2005).

In contrast to framework repair, recent work in our laboratory suggests that, affinity can also be reestablished in the CDR graft by making changes within the CDRs; thus, this approach is termed CDR repair. CDR repair seeks to identify changes within the CDRs that can alleviate inappropriate CDR anchor residue interaction with framework vernier positions, and thus restore favorable interactions with antigen. Alternatively, rather than resolving inappropriate CDR interactions with the framework, CDR repair may also identify CDR changes that interact directly or modify interactions with antigen to improve binding. The challenge of this approach is that unlike framework repair, where solutions are derived from the parent framework, the solutions required for CDR repair are not immediately obvious and thus a combinatorial approach is required.

CDR repair was used to facilitate the humanization of antibodies in the examples that follow. For each humanization, a single consensus VL ($VL_{kappa\ I}$) and VH (VH_{III}) framework has been used even though the parent antibodies have higher homology to other human germline subgroups. The consensus $VL_{kappa\ I}/VH_{III}$ framework is stable and suitable for manufacturing and has been clinically validated in a number of marketed therapeutics (Carter et al. 1992; Presta et al. 1993, 1997; Werther et al. 1996). A combination of the sequence (Kabat and Wu 1971; Kabat et al. 1991), structural (Chothia and Lesk 1987) and contact (MacCallum et al. 1996) CDR definitions were used for the CDR graft. Thus in VL, CDR-L1 is defined as positions 24–36, CDR-L2 includes positions 46–56, and CDR-L3 includes positions 89–97. In VH, CDR-H1 consists of positions 26–35b, CDR-H2 includes 47–65 and CDR-H3 includes 93–102 (Fig. 2-2)[1] We have found that the inclusion of residues defined by the contact CDR definition is frequently important to restore antigen binding. Following the identification of a suitable humanized candidate, these positions can be changed back to the human sequence to assess their importance.

Following the construction and evaluation of the initial CDR graft, should additional affinity be needed, a number of strategies can be employed and are illustrated in the following examples.

[1]The Kabat numbering system for positions in the variable domain is used throughout (Kabat et al. 1991).

3. Humanization Methods

Antigen binding affinity can be re-established and in many cases it can be improved over the parent antibody, following the generation of a CDR graft by introduction of mutations into the framework or CDRs. Identification of favorable mutations is most easily achieved by combinatorial methods such as phage or ribosome display; however, to maintain the properties inherent in the parent antibody, the introduction of mutations should be minimized. Further, to mitigate immunogenic risk, the choice of acceptable amino acid substitutions should be guided by the diversity of amino acids observed naturally at particular amino acid positions (Kabat et al. 1991; Johnson and Wu 2001).

The vernier positions listed in Fig. 2-2, derived through modeling and experimentation, provide a good starting place but are not meant as an all-inclusive list. The inclusion of murine residues at these positions can improve antibody function, however, due to potential immunogenic risk, the identification of a minimal set is desired. Variants can be generated incorporating one murine vernier position at a time to identify those that influence binding, followed by combinations of those identified as important. Unfortunately, combined vernier position changes are not necessarily additive, and the search for an optimum combination can be difficult. Alternatively, all murine vernier positions can be added and then removed one (or a few) at a time to identify those that are not important. Obviously, this approach can be tedious and modeling is often performed to guide residue selection. Careful framework selection to incorporate many vernier positions a priori, can facilitate this approach and may lead to CDR grafts with higher starting affinities.

Methods reported for introducing diversity into a CDR graft are numerous. For example, libraries can be generated by DNA shuffling using the murine and human DNA to generate hybrid proteins from which, variants with improved binding can be selected; alternatively, error prone PCR can infuse random mutations throughout the CDRs and framework (Maynard et al. 2002; Schlapschy et al. 2004; Wang et al. 2004; Oliphant et al. 2005). CDR diversity can also be introduced in a modular fashion by cassette mutagenesis (Knappik et al. 2000) or site-directed mutatgenesis (Sidhu et al. 2004); however, since the CDR regions comprise approximately 60 residues, the sequence space that can actually be sampled in a diverse library is limited. Further, antigen binding characteristics inherent in the transferred CDRs may be lost upon the introduction of unrestrained CDR diversity.

Soft-randomization is a technique that enables mutation of several positions (such as an entire CDR sequence) while maintaining a bias towards the parent sequence. Soft-randomization is easily accomplished by phage display using Kunkel mutagenesis, where mutation can be introduced using a poisoned oligonucleotide. The flanking regions of the oligonucleotides anneal to the single stranded DNA template, while the region to be soft randomized is synthesized using 70% of the proper base (that coding for the wild-type DNA sequence) and 10% each of the other three bases. Following mutagenesis, the resulting "poisoned" codon will then code for the wild-type amino acid approximately 50% of the time while allowing all other 19 amino acids to be introduced at a lower frequency (Gallop et al. 1994). This mutagenesis approach can be employed on one CDR at a time or on all six CDRs simultaneously.

The following example describes the humanization of a rat anti-β7 antibody (Fib504 (Andrew et al. 1994)). This antibody blocks the adhesion of α4β7 positive lymphocytes to MAdCAM-1, VCAM-1 and fibronectin and may have therapeutic utility in inflammatory bowel disease by blocking lymphocyte migration to the gut (Kelsen et al. 2004).

A CDR graft of Fib504 was generated using the $VL_{kappa\ I}/VH_{III}$ framework (Fig. 2-4a, b); however, this CDR graft had no detectible binding affinity for α4β7 despite the use of a broad definition for the CDRs. Using a Fab form of the CDR graft as a template, a framework toggle phage library was generated by Kunkel mutageneis (Baca et al. 1997). The library was designed to offer either rat or human amino acid residues at vernier positions that differed between the two frameworks. After panning against a detergent solubilized form of α4β7, a change to the rat framework amino acid at position 78 in VH (L78F) was highly selected while the frequency of rat or human amino acids at other positions was unbiased. This single framework change, incorporated into the graft (graft.v2), restored binding to within 23-fold of the chimera.

To further improve the affinity, graft.v2 was used as a template for a soft randomization library that incorporated all six CDRs simultaneously. Random sequences from the initial unselected library are shown in Fig. 2-4c. Note that the library members have mutations localized to the CDR regions, not all CDRs are mutated (many clones have less than six oligonucleotides incorporated) and that introduced mutations reflect an underlying bias towards the initial CDR graft sequence. Unique sequences recovered after four rounds of selection against antigen are shown in Fig. 2-4d. All of the heavily mutated sequences exemplified in Fig. 2-4c were lost, and only clones containing a very similar change in CDR-L1 were selected and remained in the final pool. Surprisingly, the single change Y32L in CDR-L1 nearly restored antigen binding, and an additional change T31D improved binding affinity by threefold compared to the parent antibody. Mutagenesis of the final humanized clone to assess the importance of residues residing within the contact CDR definition suggests that, the inclusion of K49 in VL was critical to the success of this humanization since K49Y resulted in a greater than tenfold loss in binding. The VH mutation M94R resulted in a twofold increase in the dissociation rate for the Fab, however this difference was not detected when reformatted as an IgG.

CDR repair has been successful for many other antibodies. For example, a CDR graft of a mouse anti-IgE antibody (MaE11) also exhibited no affinity for its antigen, but was humanized using CDR repair. The CDR graft of MaE11 was used as a template to generate a soft randomized library of all six CDRs simultaneously from which highly focused changes in CDR-H1 were identified. Many clones incorporating W35L in this CDR were found to completely restore IgE binding (Fig. 2-5). By comparison, the framework repair approach, that was used previously (Presta et al. 1993), required three vernier framework changes (identical regions were transferred in both CDR grafts) to achieve similar results in the marketed Xolair® anti-IgE antibody (omalizumab).

The small number of amino acid changes required to re-establish binding in these two examples was surprising, and suggests that even simpler mutagenesis strategies can be effective. For example, a small combinatorial library that targets each CDR individually is likely to be successful, allowing libraries with higher diversity to be generated. In addition, libraries that target one

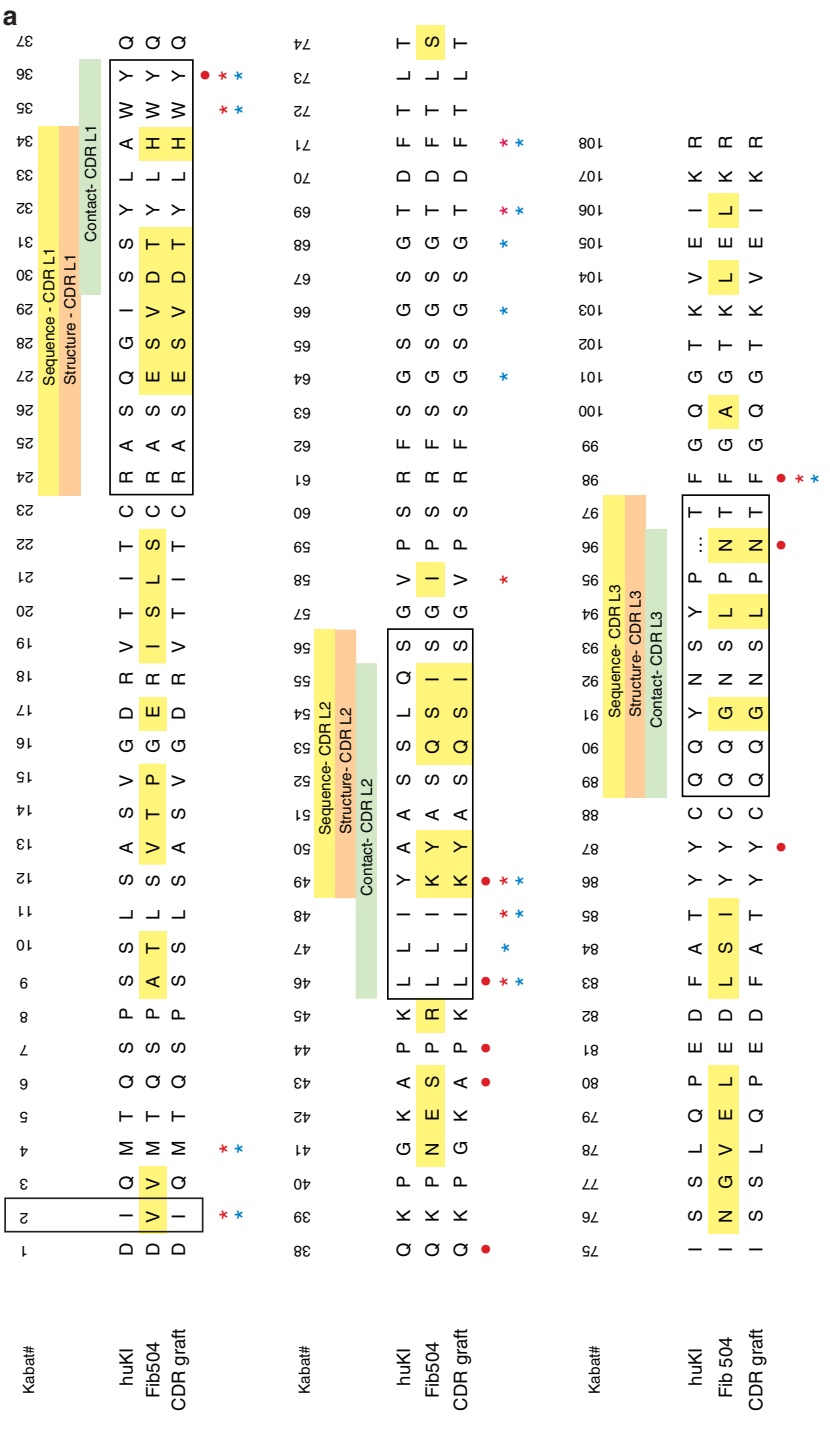

Fig. 2-4. Humanization of the rat monoclonal Fib504. Alignment of the human (**a**) $VL_{kappa\,I}$ consensus or

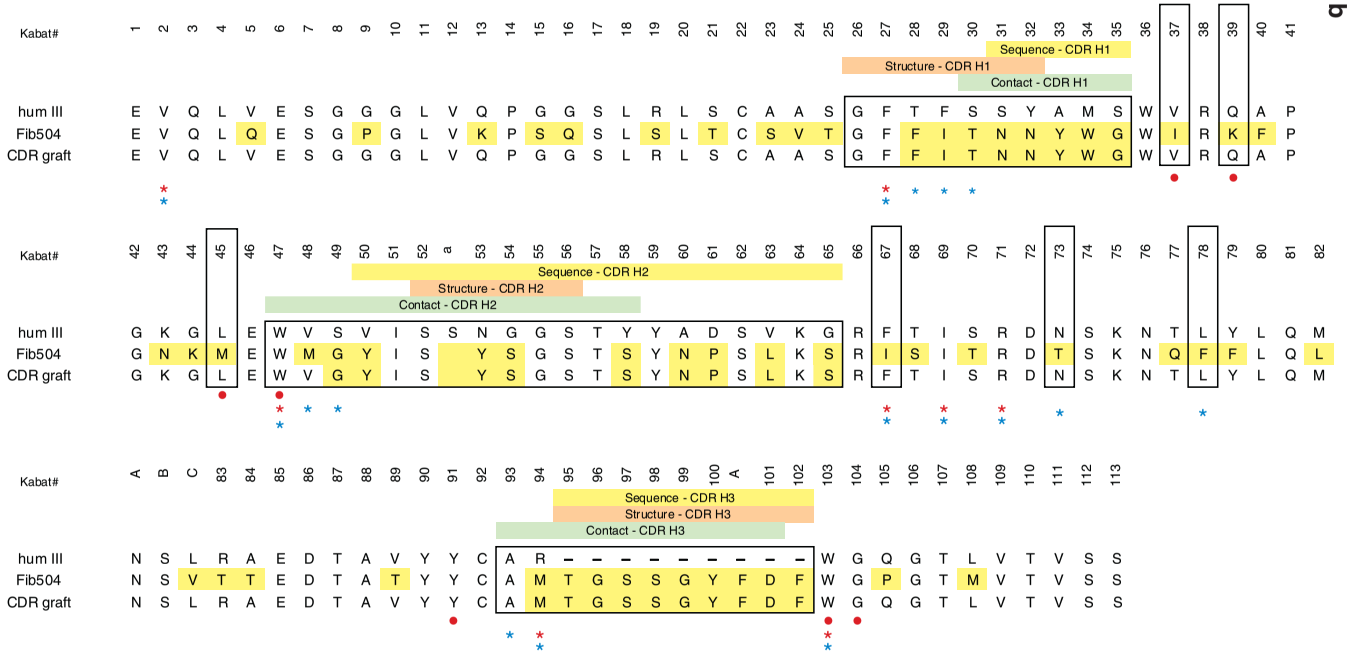

Fig. 2-4. (continued) (**b**) VH_{III} consensus with the variable domain sequences from Fib504, delineation of CDR regions and design of the Fib504 CDR graft. Vernier positions are stared as described in Fig. 2-2. Vernier positions that were toggled between rat and human amino acid residues are *boxed*.

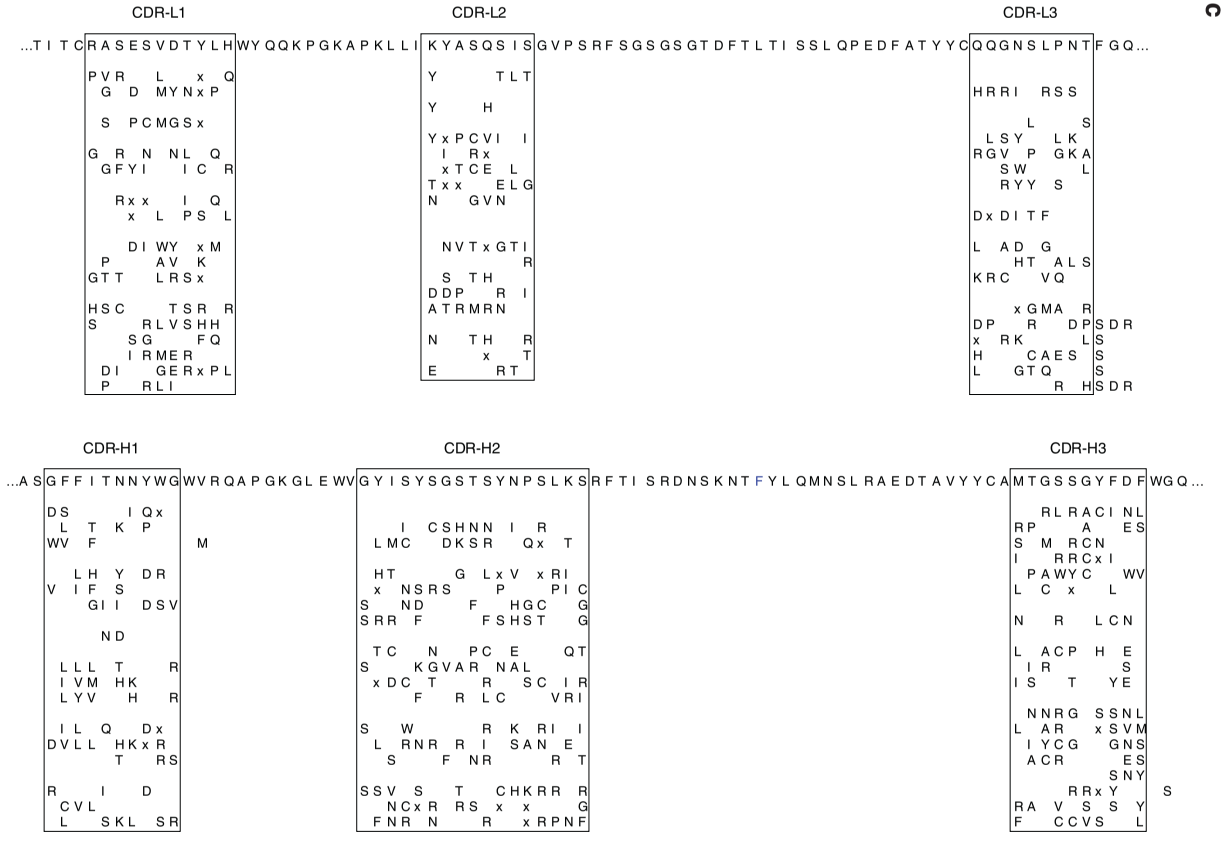

Fig. 2-4. (continued) (**c**) Random sequences selected from an initial unselected soft randomized library that targeted mutations to all six CDRs simultaneously are shown, compared to the amino acid sequence of CDR graft.v2 that served as the mutagenesis template. Targeted CDR sequences are *boxed*. Only positions that were mutated in the library samples during soft randomization are shown.

d

CDR L1

24	25	26	27	28	29	30	31	32	33	34	fold	
R	A	S	E	S	V	D	T	Y	L	H	1	chimera
											ND	graft
											23	graft.v2
								I				
							D	L	V			
							D	L			0.3	
							N	L				
							P	L				
							S	L			0.8	
	S		D				S	L	V			
				D				L				
				N				L				
								L			2.1	

Fig. 2-4. (continued) (**d**) Following four rounds of selection against antigen, ten unique clones were identified. All had changes limited to CDR-L1 and are shown compared with the original CDR-L1 sequence (*top*). Selected clones were expressed as Fab and binding to β7 was assessed using surface plasmon resonance. Affinity is expressed as variant (K_d)/chimera (K_d)

CDR H1

26	27	28	29	30	31	32	33	34	35	35a	fold	
G	Y	S	I	T	S	G	Y	S	W	N	1	chimera
											ND	graft
				S	G			H	L		1.8	
				S	G			R	L		1	
		I						K	L		1.8	
	N							K	L		1.5	
							H	K	L		1.5	
								K	L		0.5	
								N	L		1.5	
									L	H	0.8	

Fig. 2-5. Changes identified during CDR repair of an E25 CDR graft that restore binding to IgE. All six CDRs in the MaE11 CDR graft were soft randomized simultaneously. Following four rounds of selection against IgE, eight unique clones were identified each of which had sequence differences limited to CDR-H1. Phage clones were assessed for IgE binding using a competitive phage ELISA (Li et al. 2000). Affinity is expressed as variant (IC_{50})/chimera (IC_{50})

position at a time have the potential to identify the single most useful change. These can be generated through multiple small-scale mutagenesis reactions, that target each of the approximately 60 CDR residues individually offering all 20 possible amino acids; 60 mutagenesis reactions can then be pooled to form a "single position library" and panned against antigen. A single position library ensures that any CDR repair solution selected from the phage library

will have only a single mutation. Like any humanization approach, introduced changes have the potential to modify antibody–antigen interaction, thus careful subsequent analysis of antibody function is important.

Assigning the effect of CDR mutations to those that repair interactions with the framework, or to those that provide more favorable interactions with antigen can be difficult, and will require a significant structural investigation. Nevertheless, antibodies humanized by CDR repair in these two examples recapitulate the properties, both affinity and function, of the parent antibody and demonstrate that very small changes in the CDR have profound effects (Kettleborough et al. 1991; Eigenbrot et al. 1993). Comparison of the CDR sequences in these examples to homologous variable domains whose tertiary structures have been determined (2D7T was used for Fib504, 1CF8 and 1KB9 were used for MaE11 (Berman et al. 2000)) suggests that these selected mutations affect a remodeling of CDR interactions both with the framework and potentially with antigen. In the Fib504 example, both newly selected positions are anticipated to be solvent exposed. While this suggests the modification of interactions with β7, an influence of these changes on the conformations of CDR-L2 (at position 50) and CDR-L3 (at positions 91 and 92) cannot be ruled out. In contrast, W35L in CDR-H1 of the MaE11 CDR graft should be completely buried and interacting with vernier positions 24 and 78 in VH as well as CDR-H2 (at position 51) and CDR-H3 (at position 94). Thus, this selected mutation likely modifies framework-CDR interactions. Other antibodies humanized by this method in our laboratory have resulted in the selection of multiple solutions, often targeting different locations that restore, or in many cases improve binding affinity. These examples suggest that for the most part, grafted CDRs can be transferred from one framework to another without problem; however a slight mismatch, even as little as a hydroxyl group, can affect binding by ten or more fold. CDR repair enables the rapid identification of solutions to these disruptive interactions, allowing them to be fixed with surprisingly few sequence changes.

In situations where humanization by CDR repair alone is unable to restore binding affinity, or the loss of some other property of the parent antibody, a scan of the vernier residues that differ between the CDR graft and the parent antibody can identify one or two framework positions that cannot be compensated by CDR changes. The framework toggle library approach discussed above is a rapid way to identify these positions. In addition, not all antigens lend themselves to phage selection. Multi-spanning transmembrane proteins, for example, may only be available in a native form on a cell surface. Here, an analysis of differing vernier framework positions in VL and VH (i.e. framework repair) may be the only approach available to restore binding.

By combining both CDR repair and framework repair approaches, we have found that the overall number of changes incorporated into a humanized antibody can be reduced. To date, over a dozen antibodies have been humanized using this approach with a single $VL_{kappa\ I}/VH_{III}$ framework. For 13 antibodies humanized by framework repair alone using the same $VL_{kappa\ I}/VH_{III}$ framework, an average of 7.5 ± 3.4 residues were incorporated into the CDR graft whereas, the combined approach required alteration of only 4.4 ± 2.5 residues. The broader definition of CDRs that incorporates sequence hypervariability, structural considerations and regions that are known to contact antigen resulted in roughly one third of the CDR grafts, having no loss in binding while, the

rest suffered a tenfold or greater loss in binding affinity. CDR repair alone restored antigen binding affinity in the majority of these with less than three amino acid changes in a particular CDR. In fact, binding affinity was improved an average of sixfold. The location of the amino acid changes was antibody dependent with a nearly equal distribution across all six CDRs. Importantly, a wide selection of murine antibodies of distant homology have all been humanized using the same $VL_{kappa\ I}/VH_{III}$ framework containing a nearly constant set of vernier residues.

4. Antibody Properties

Aside from binding affinity, there are many other properties of antibodies that must be monitored during humanization. The equilibrium constant (K_d) for example, is related to the kinetics of association (k_a) and dissociation (k_d). While the success of humanization is often reported as restoring the equilibrium constant, the underlying kinetics may differ, and may be very important in particular in vivo systems. Binding affinity is also influenced by avidity for some antigens; bivalent binding may boost the apparent affinity by over 1,000-fold or cooperative binding may have little affect on apparent affinity. Avidity can be critical depending upon whether the target is a soluble antigen, or on the cell surface. Monitoring the ability of a Fab displayed on phage to bind antigen can be misleading, if the humanized clones behave differently when reformatted into IgG. Thus, performance of the IgG should also be monitored during the humanization process.

Generally, antibodies are selected for humanization due to some function they provide. They may act as agonists that dimerize a receptor or antagonists that block ligand–receptor interactions; they may be used to deliver a therapeutic agent through conjugation or induce ADCC or CDC through Fc effector functions. Occasionally, these properties can be lost during humanization, if the combining site of the parent antibody is not fully reproduced on the newly humanized antibody. These properties must also be carefully monitored during the humanization process.

5. Minimizing Immunogenic Potential

The purpose of antibody humanization is to make a murine antibody appear human, but what does the sequence of a human antibody look like? This is a particularly important consideration for CDR repair, where often multiple solutions, each having different amino acid changes that improve binding, may be obtained. Only changes that are compatible with canonical CDR structure should be considered (Chothia et al. 1989). In addition, comparisons to human germline sequences can be used as a guide for selecting a variant with the lowest immunogenic risk (Kabat et al. 1991). When considering the origin of a mature naturally occurring antibody, it becomes apparent that the CDR regions, while initially appearing hypervariable across a group of aligned variable domain sequences, are in fact well defined. Each CDR sequence is linked to its originating germline and as a result CDR sequences (e.g. CDR-L1, CDR-L2 and part of CDR-L3) are generally linked until the joining region in VL or the diversity segment in VH. Thus when considering which amino

acids are commonly observed at a given position within a particular CDR, the context of the rest of the CDR should also be considered.

Positions that differ in sequence between a mouse and human variable domain are typically distributed evenly with about 30 differences per variable domain. Humanization can reduce these by half with the remaining differences concentrated in the CDR regions (Fig. 2-1b). In comparison, mature antibodies have only about six sporadically distributed somatic mutations per variable domain with half of these targeted towards CDR regions (Clark et al. 2006). While the entire humanized variable domain may not match any specific germline, peptides that may be presented on MHC molecules should look relatively similar to those from naturally occurring antibodies.

Aside from the antibody itself, many additional factors may significantly influence immunogenic potential. These include route of administration, dose and dose frequency, formulation, degree of aggregation, antibody stability and pharmacokinetics, the targeted antigen, therapeutic indication, and the patient's immune status. While these problems cannot be anticipated, it is incumbent upon the antibody engineer to eliminate as much potential risk from the antibody sequence as possible.

Biological research is greatly facilitated by the ability to rapidly generate and screen large numbers of high affinity antibodies from hybridomas. While the advent of synthetic antibody phage libraries and "fully human" antibodies from genetically engineered mice have the potential to further reduce differences from germline, once having generated and validated a murine hybridoma antibody with desired properties, humanization is likely to remain the simplest path forward. Further, humanization and antibody engineering enable the ability to choose well-characterized frameworks that are stable for manufacturing and storage.

6. Conclusions

Monoclonal antibodies are an increasingly important class of therapeutics that can be recruited to target specific antigens and engineered to perform specific functions in vivo. Humanization technologies have made it possible to use murine antibodies, that can be easily generated as a starting point for developing these therapeutics. Despite the high homology between mouse and human antibodies, the transfer of variable domain CDRs to a new human framework often results in a loss of antigen binding affinity due to changes in the environment supporting the CDRs. Alterations, most easily identified through combinatorial means, are frequently required in order to repair the interactions between the human acceptor framework and CDRs. Framework repair and CDR repair can both be used to alter CDR-framework interactions from either side of this interface. CDR repair has an added potential to modify antigen interactions that can improve binding affinity.

In practice, each antibody humanization is unique and presents its own challenges. Both framework repair and CDR repair are effective approaches to restore antigen binding following the transfer of CDRs to a human framework, and should lead to the successful generation of humanized antibodies, that retain antigen binding affinity and biological properties of parental monoclonal antibodies.

Acknowledgment. I gratefully recognize the contributions made by the oligonucleotide synthesis and DNA sequencing groups at Genentech who have made this work possible. I also thank all current and former members of the Protein Engineering and Antibody Engineering Departments at Genentech for discussions and their contributions to the development of phage display methods, and antibody humanization technologies and especially, Henry Lowman for his support.

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http://www.springer.com/978-0-387-76642-3

Current Trends in Monoclonal Antibody Development and Manufacturing

Shire, S.J.; Gombotz, W.; Bechtold-Peters, K.; Andya, J. (Eds.)

2010, XIV, 354 p., Hardcover
ISBN: 978-0-387-76642-3