

Design of humanized antibodies: From anti-Tac to Zenapax

Naoya Tsurushita *, Paul R. Hinton, Shankar Kumar

Protein Design Labs, Inc., 34801 Campus Drive, Fremont, CA 94555, USA

Accepted 17 January 2005

Abstract

Since the introduction of hybridoma technology, monoclonal antibodies have become one of the most important tools in the biosciences, finding diverse applications including their use in the therapy of human disease. Initial attempts to use monoclonal antibodies as therapeutics were hampered, however, by the potent immunogenicity of mouse (and other rodent) antibodies in humans. Humanization technology has made it possible to remove the immunogenicity associated with the use of rodent antibodies, or at least to reduce it to an acceptable level for clinical use in humans, thus facilitating the application of monoclonal antibodies to the treatment of human disease. To date, nine humanized monoclonal antibodies have been approved for use as human therapeutics in the United States. In this paper, we describe procedures for antibody humanization with an emphasis on strategies for designing humanized antibodies with the aid of computer-guided modeling of antibody variable domains, using as an example the humanized anti-CD25 monoclonal antibody, Zenapax.

© 2005 Elsevier Inc. All rights reserved.

Keywords: Antibody engineering; Antigen-binding affinity; Daclizumab; Effector functions; Immunogenicity; Molecular biology; Molecular modeling; Protein expression; Sequence homology

1. Introduction

Monoclonal antibodies form an important class of human therapeutics. Since the approval of Orthoclone OKT3 for treatment of allograft rejection in 1986, a total of 18 monoclonal antibodies, including nine humanized antibodies (Table 1), have been approved to date by the Food and Drug Administration (FDA) for therapeutic use in the United States [1]. The utility of monoclonal antibodies as therapeutics was recognized soon after the introduction of hybridoma technology in 1975 [2]. Due to their high affinity and exquisite specificity, monoclonal antibodies can recognize even small quantities of antigen in complex mixtures and neutralize the function of antigens responsible for the onset or maintenance of disease. In addition, effector functions associated with

the Fc region, such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), efficiently trigger immune responses that result in the elimination of antibody-bound cells [3]. A number of rodent monoclonal antibodies with potential clinical applications have now been generated, of which the majority are derived from mice.

The development of monoclonal antibodies as human therapeutics, however, was hampered by the problem that mouse antibodies are strongly immunogenic in humans [4–6]. In the vast majority of clinical studies, potent human anti-mouse antibody (HAMA) responses were observed in human subjects who were administered mouse monoclonal antibodies. As a result, mouse antibodies were neutralized and rapidly cleared from the body, resulting in limited efficacy for such antibodies. Although the invention of mouse–human chimeric antibodies, which are composed of mouse variable regions and human constant regions [7], helped reduce the immunogenicity of mouse monoclonal antibodies in

* Corresponding author. Fax: +1 510 574 1500.

E-mail address: ntsurushita@pdl.com (N. Tsurushita).

Table 1
Humanized antibodies approved for therapeutic use in the US

Generic name	Product name	Antigen	Indication	Approval	Company
Daclizumab	Zenapax	CD25	Renal allograft rejection	1997	Protein Design Labs/Roche
Palivizumab	Synagis	RSV gpF	RSV infection	1998	MedImmune
Trastuzumab	Herceptin	HER2/neu	Breast cancer	1998	Genentech
Gemtuzumab Ozogamicin ^a	Mylotarg	CD33	AML	2000	Celltech/Wyeth
Alemtuzumab	CamPath	CD52	CLL	2001	Millennium/ILEX
Omalizumab	Xolair	IgE	Asthma	2003	Tanox/Genentech/Novartis
Efalizumab	Raptiva	CD11a	Psoriasis	2003	Xoma/Genentech
Bevacizumab	Avastin	VEGF	Colorectal cancer	2004	Genentech
Natalizumab	Tysabri	α 4-integrin	Multiple sclerosis	2004	Biogen Idec/Elan

Abbreviations used: RSV gpF, respiratory syncytial virus glycoprotein F; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; VEGF, vascular endothelial growth factor.

^a Conjugated to calicheamicin.

humans, chimeric antibodies still induced HAMA, or human anti-chimeric antibody (HACA), responses since the mouse-derived variable regions were sufficient to trigger immune responses in humans [5,6,8].

Each of the heavy and light chain variable (V) regions forms a domain structure, composed of three complementarity-determining regions (CDRs 1–3) and four framework regions (FRs 1–4), which belongs to the immunoglobulin superfamily. The CDRs of the heavy and light chain V domains together form the antigen-binding site, while the framework regions constitute a scaffold for the antigen-binding site. The concept of CDR grafting [9] for generating less immunogenic antibodies originated from the hypothesis that the CDRs of a mouse monoclonal antibody (the donor antibody) may replace those of a human antibody (the acceptor antibody) without affecting the structure of the antigen-binding site formed by the mouse CDRs. Although CDR grafting was successful in some cases [10,11], most CDR-grafted antibodies have been found not to retain the antigen-binding affinity of the parental mouse antibody. This is because certain framework residues intimately interact with CDR residues in the V domains, thereby affecting the structure of the antigen-binding site. Thus, as pointed out by Queen et al. [12], the transfer of mouse CDR residues alone into human frameworks may alter the structure of the CDRs, resulting in a loss of antigen-binding affinity. Queen and co-workers went on to propose that key framework residues interacting with the CDRs, and therefore important for the integrity of the antigen-binding site, should be transferred from the donor to the acceptor antibody along with the CDRs. To identify such residues, Queen and co-workers used computer-generated three-dimensional models of V domains. By transferring CDR residues together with key framework amino acids from a mouse antibody into human frameworks, it became possible to routinely generate engineered antibodies, generally referred to as humanized antibodies, which retain the binding affinity and specificity of the parental mouse antibodies. Since the introduction of computer-guided

humanization technology, a large number of humanized antibodies have been successfully generated [13]. Clinical studies have indicated that humanized antibodies are, in general, much less immunogenic than mouse or chimeric antibodies, and are safe and well tolerated in humans [4,14,15]. Thus, the application of mouse antibodies to human therapy has become feasible through the use of humanization technology.

Zenapax (generic name, daclizumab) is the first humanized antibody approved by the FDA for human therapeutic use in the United States. It is a humanized IgG1 form of the mouse monoclonal antibody anti-Tac [16], an anti-human IL-2 receptor α chain (CD25) antibody that blocks the interaction of IL-2 with IL-2 receptor and thus prevents activation of T cells. Zenapax was approved in 1997 for prevention of renal allograft rejection in the United States. To date Zenapax has been administered to over 20,000 patients, and has been found to be safe and effective, thus fulfilling the concept of humanized antibodies.

In this paper, we use the humanization of anti-Tac as an example to describe the process of antibody humanization. Since the theoretical background of the humanization methodology has been discussed elsewhere [17], we focus on technical aspects of antibody humanization in this paper. It should be noted that Zenapax is the trade name used by Roche Pharmaceuticals. We will hereafter use its generic name, daclizumab, to refer to the humanized form of the anti-Tac monoclonal antibody.

2. Antibody humanization procedure

The ultimate goal of antibody humanization is to generate human-like V regions by transferring CDR residues and a minimal number of key framework amino acids from a donor mouse monoclonal antibody onto an acceptor human framework without losing antigen-binding affinity and specificity. The computer-guided antibody humanization technique requires expertise primarily in two areas: three-dimensional modeling of

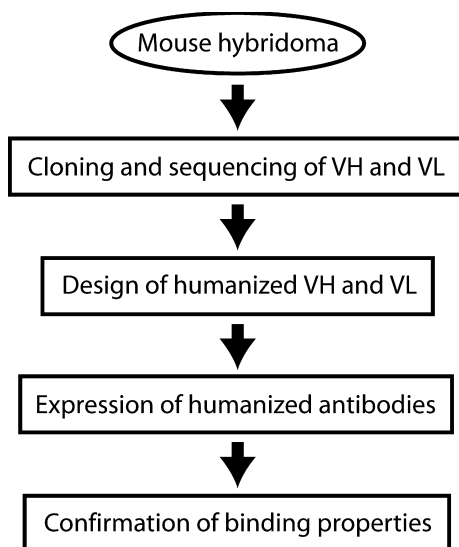


Fig. 1. Overall scheme for antibody humanization.

protein structures and genetic engineering. The overall flow of a typical antibody humanization project is shown in Fig. 1. Since the experimental techniques required for genetic engineering are well documented in various methods books, such as *Molecular Cloning: A Laboratory Manual* [18], and have been successfully applied to antibody engineering, we focus in this section only on the key considerations for successful humanization without describing the detailed experimental procedures. Regarding the computer-guided design of humanized antibodies, we describe the principle and application of our procedure in detail using daclizumab as an example.

2.1. Cloning and sequencing mouse V genes

A humanization project typically begins by cloning and sequencing the V genes from a rodent hybridoma, which is usually of mouse origin, but may also be derived from rat or hamster B cells. Before initiating V gene cloning, however, it is important to ensure that the hybridoma has been subcloned, ideally by single cell subcloning using a cell sorter, rather than by limiting dilution. Without establishing the clonality of the hybridoma, multiple productive heavy and/or light chain mRNA sequences are occasionally observed even though the hybridoma may appear to be producing a single kind of antibody. It is formally possible for a clonal hybridoma to express more than one kind of productive heavy or light chain mRNA, although it is more likely in this case that the hybridoma is not clonal. While it is possible to experimentally determine which of the multiple productive heavy or light chain mRNAs encode the mouse antibody to be humanized, for example, by expressing all heavy and light chain combinations and examining their binding to antigen, it is usually more expeditious to perform one round of single cell sorting if

there is any doubt about the clonality of the starting hybridoma.

A number of methods are available and have been successfully used for cloning cDNAs encoding the VH and VL regions of the target monoclonal antibody. The 5' RACE (rapid amplification of cDNA ends) method using, for example, the SMART RACE cDNA Amplification Kit (BD Biosciences, San Jose, CA) or the GeneRacer Kit (Invitrogen, Carlsbad, CA) is a common choice. To synthesize gene-specific primers for 5' RACE, information about the isotype (and subtype) of the produced monoclonal antibody is helpful. Isotyping of mouse monoclonal antibodies can be readily performed using a commercially available kit, for example, the IsoStrip Mouse Monoclonal Antibody Isotyping Kit (Roche Applied Science, Indianapolis, IN) or the Mouse Immunoglobulin Isotyping Cytometric Bead Array Kit (BD Biosciences). Depending on the isotype of the heavy and light chains of the target monoclonal antibody, a gene-specific primer can be designed that binds immediately downstream of the variable region for each of the heavy and light chains. In the case of γ heavy chains, a 5' RACE primer may be designed to be specific for each subtype ($\gamma 1$, $\gamma 2a$, $\gamma 2b$ or $\gamma 3$ in mice) or alternatively a region identical or highly homologous among all the γ subtypes may be chosen as a basis for designing a common primer. The following set of 5' RACE primers has been successfully used in our laboratory for cDNA cloning of the variable regions of mouse γ heavy and κ light chains: 5'-GCCAGTGGATAGACTGATGG-3' (for mouse $\gamma 1$, $\gamma 2a$, and $\gamma 2b$ heavy chains) and 5'-GATGGA TACAGTTGGTGCAGC-3' (for mouse κ light chains). PCR-amplified V gene fragments can be directly cloned into a plasmid vector, for example, using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen). The cloned fragments are then subjected to DNA sequencing to determine the nucleotide sequences of the VH and VL regions. Since nucleotide substitutions are occasionally introduced by PCR, it is important to sequence several clones to obtain correct V region sequences.

It should be noted that hybridoma cells often produce two kinds of light chain mRNAs, one encoding the productive light chain and another encoding a non-productive form. The latter type of mRNA usually carries a frameshift and/or non-sense mutation(s) in the V region, typically near or in CDR3. Since identical, or very similar, non-productive VL sequences have been observed in many hybridomas generated using the same fusion partner, it is likely that the non-productive light chain mRNA originated from the fusion partner and its gene was retained in the hybridoma. The ratio between productive and non-productive VL cDNAs observed during cloning varies substantially among hybridomas. If the antibody production level of a hybridoma is very low (less than 1 $\mu\text{g/ml}$), more than

90% of VL cDNA clones might encode the non-productive type. Therefore, to obtain several cDNA clones encoding a productive type of VL, sequencing of a few dozen clones might be necessary. However, if a non-productive type of VL is exclusively obtained during cDNA cloning, an alternative approach is to design a pair of PCR primers to identify and *exclude* the non-productive VL gene. This is typically done using CDR1 to design a forward primer and CDR3 to design a reverse primer for the non-productive gene. The primers are then used for PCR amplification of the plasmid clones containing VL genes; clones amplified by the primers are excluded from further analysis, while the remaining clones are sequenced to identify productive VL genes.

Although the authenticity of the cloned VH and VL genes can be confirmed by demonstrating antigen binding using recombinant mouse or chimeric forms of the antibody, N-terminal amino acid sequencing of the target mouse monoclonal antibody is usually more expedient. Sequencing of N-terminal amino acids may be achieved with a protein sequencer such as a Model 241 Protein Sequencer (Hewlett–Packard, Palo Alto, CA) using a standard protocol, or more typically by sending the sample to a contract laboratory for a reasonable fee. Heavy and light chains may be separated before amino acid sequencing by polyacrylamide gel electrophoresis under reducing conditions, or alternatively whole antibody may be subjected to sequencing. In the latter case, two amino acid residues, one each from the heavy and light chains, are usually observed in most of the sequencing cycles, unless the N-terminus of the heavy and/or light chains is blocked by conversion of glutamine to pyroglutamine [19]. The assignment of two amino acids detected at each position to either VH or VL can be performed relatively easily by comparison to the V region sequences in the Kabat antibody database [20,21]. When only one amino acid is detected in a given cycle, it is likely that the heavy and light chains share the same amino acid at that position. A typical amino acid sequence determination by Edman degradation provides at least 15–20 amino acids from the N-terminus, which is usually sufficient to confirm the authenticity of the VH and VL sequences obtained by cDNA cloning.

It is important to note that glutamine is one of the two most common N-terminal amino acids of mouse heavy chain variable regions [20]. Thus, if only the light chain sequence is obtained from standard amino acid sequencing of whole antibody, deblocking the N-terminus of the heavy chain is necessary to obtain its sequence [22]. Also, the N-terminus of a mature mouse light chain is occasionally blocked since mouse germline V segments belonging to subgroup IV often start with glutamine [20]. Rarely, both the heavy and light chains of a mouse antibody start with glutamine.

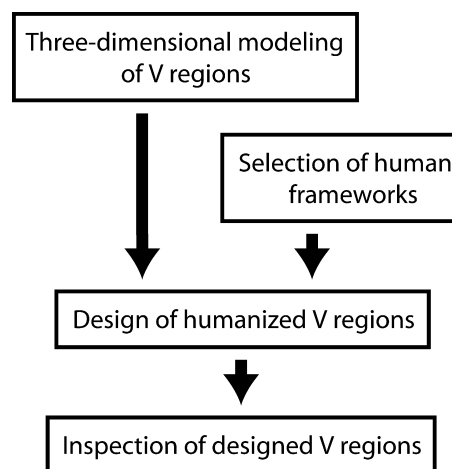


Fig. 2. Procedure for designing humanized V regions.

2.2. Designing humanized antibodies

For successful humanization of a mouse (or other rodent) antibody, the set of mouse framework residues potentially important for maintaining the structure of the CDRs is first identified and then transferred onto a human framework that is selected based on homology to the mouse framework, together with the mouse CDR residues. Building a reliable three-dimensional model of the variable regions is an important first step in the design of a humanized antibody. The process of designing humanized V regions is outlined in Fig. 2.

2.2.1. Three-dimensional modeling of V regions

A detailed all-atom model of the heavy and light chain variable regions of a mouse antibody can be constructed following the method described by Levitt and co-workers [23,24] using the algorithm ABMOD and the ENCAD set of programs and molecular mechanics energy function. The principle of the model building is as follows. Each of the heavy and light variable domains is divided into 14 structurally meaningful segments; these segments are β strands and loop-like structures comprising the domain structure of the immunoglobulin superfamily. The amino acid sequence of each of the 28 segments (14 from each of the heavy and light chain variable regions) of the mouse antibody is aligned with the corresponding segments of antibodies of known structure in the PDB database [25]. For this purpose, we choose a small number of antibodies (typically under 30) that best match the murine antibody to be humanized, and these sequences along with the murine sequences are then subjected to multiple sequence alignment following Levitt's method [23]; in our experience, CLUSTALW [26,27] has also worked well for this purpose. For each of the 28 segments, a corresponding segment having the highest sequence homology is selected from the multiple

Table 2
Variable region segments used for modeling of the mouse anti-Tac V regions

Sequence	Structure	Segment used for modeling ^a
<i>VH residues</i>		
1–10		1JHL
11–15		1NMB
16–26	β strand	1NGQ
27–32		1NGQ
33–43	β strand	1NMB
44–52	β strand	1NGQ
52a–55		1NGQ
56–66		1JHL
67–76		1NMB
77–82c	β strand	1NMB
83–88		1NMB
89–95	β strand	1NMB
96–99		1NMB
100–113	β strand	1NMB
<i>VL residues</i>		
1–2		1BAF
3–9	β strand	1BAF
10–15		1BAF
16–25	β strand	1BAF
26–32		1CLO
33–40	β strand	1FOR
41–49	β strand	1BAF
50–56		1BAF
57–68	β strand	1BAF
69–77	β strand	1BAF
78–82		1BAF
83–90	β strand	1BAF
91–96		1FOR
97–107		1BAF

^a Identification code in the PDB database.

sequence alignment. To illustrate the procedure described above, a three-dimensional model of the mouse anti-Tac variable regions was built. The crystal structures shown in Table 2 were used for the 28

segments. Even though 13 crystal structures were initially chosen for alignment in this example, only six were used to model the individual segments of the heavy and light chains. This is typical for building antibody models of the variable regions from the primary sequences alone. The resulting three-dimensional model of the anti-Tac variable regions is shown in Figs. 3A and B.

The structural parameters of the selected segments are then combined to build a model of the variable regions. At this stage, the resulting structure is not considered to be optimal, because the selected segments are likely to belong to multiple antibodies with different structures. Furthermore, some of the segments, particularly the CDRs, may have deletions or insertions compared to the original mouse sequences. To obtain a reliable structure, the model must be subjected to multiple cycles of conjugate gradient energy minimization (e.g., using ENCAD, or as described by Press et al. [28]) to relax the structure until the energy and the energy gradient reach an acceptable level.

For antibody humanization in our laboratory, ABMOD and ENCAD (with our own modifications) are routinely used for modeling the structures of variable regions; however, other model building software and energy functions, for example, AMBER [29], could also be used with similar results. In addition, there are currently many websites that allow one to build three-dimensional models from primary sequences; for instance, 3D-JIGSAW (<http://www.bmm.icnet.uk/servers/3djigsaw>) and SWISS-MODEL (<http://www.expasy.org/swissmod/>) are available to the interested reader.

2.2.2. Selection of human frameworks

In parallel with modeling the structure of the variable regions, each of the mouse VH and VL region amino acid sequences deduced from cDNA cloning is compared to human V region sequences in the database.

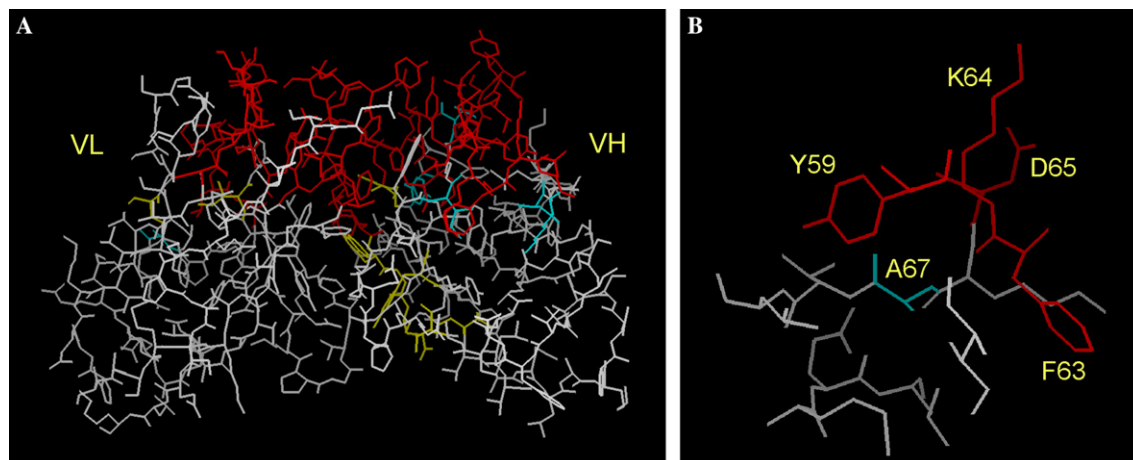


Fig. 3. (A) Three-dimensional model of the mouse anti-Tac V regions. Color scheme: white, framework residues; red, CDR residues; blue, mouse-specific residues retained in the humanized form due to contact with the CDRs; yellow, substituted with human consensus residues in the humanized form. (B) Spatial location of an alanine at position 67 (blue) in VH, which was determined to be a key framework residue for proper formation of the CDR structure. Framework (white) and CDR (red) residues located within about 5 Å of the alanine at position 67 in VH are shown.

Currently, the Kabat database [21] provides a wide selection of variable region sequences that are suitable for this purpose. Although an updated database can be created by including more variable region sequences recently introduced into GenBank and/or identified within each laboratory, inspection of the Kabat database is normally sufficient to find a human framework sequence suitable for antibody humanization. As an acceptor for humanization, a human V region framework sequence with high homology to the mouse sequence is preferred. The Smith–Waterman algorithm [30], or other standard methods such as BLAST [31], can be used to scan the database for sequences having high, e.g., at least 65%, homology to the mouse sequence.

For humanization of the mouse anti-Tac monoclonal antibody, the human Eu antibody [20] was chosen to provide VH and VL frameworks. The amino acid identities in the framework regions between mouse anti-Tac and human Eu are 67% for VH and 65% for VL. The VH and VL amino acid sequences of the mouse anti-Tac and human Eu antibodies are aligned in Fig. 4. Note that

although VH and VL frameworks are often chosen from the same human antibody, this is not essential unless a three-dimensional model of the designed humanized V region indicates conformational changes at the interface of VH and VL domains (discussed below in Section 2.2.4). Indeed, many humanized antibodies have been successfully generated using human VH and VL frameworks that originated from different antibodies.

Although a human acceptor framework can generally be chosen from V region sequences expressed in B cells, i.e., cDNA-based and protein-derived sequences, the recent completion of the sequencing of the human genome allows the use of germline V segments for this purpose. The advantage of using a germline V segment is, at least in theory, to eliminate potential immunogenicity associated with somatic hypermutations in cDNA-based and protein-derived sequences. The use of a consensus framework sequence is another approach to this issue. When using a cDNA-based or protein-derived V region sequence as an acceptor for humanization, the identification and removal of hypermutated residues in

A		1	2	3	4
	123456789	0123456789	0123456789	0123456789	0123456789
Mouse anti-Tac	<u>QVQLQQSGA</u>	<u>ELAKPGASVK</u>	<u>MSCKASGYTF</u>	<u>TSYRMHWVKQ</u>	<u>RPGQGLEWIG</u>
Daclizumab	<u>QVQLVQSGA</u>	<u>EVKKPGSSVK</u>	<u>VSCKASGYTF</u>	<u>TSYRMHWVRQ</u>	<u>APGQGLEWIG</u>
Eu	<u>QVQLVQSGA</u>	<u>EVKKPGSSVK</u>	<u>VSCKASGGTF</u>	<u>S-----WVRQ</u>	<u>APGQGLEWIG</u>
	5	6	7	8	
	01223456789	0123456789	0123456789	0122223456789	
	a			abc	
Mouse anti-Tac	<u>YINPSTGYTEY</u>	<u>NQKFKDKATL</u>	<u>TADKSSSTAY</u>	<u>MQLSSLTFEDSAV</u>	
Daclizumab	<u>YINPSTGYTEY</u>	<u>NQKFKDKATI</u>	<u>TADESTNTAY</u>	<u>MELSSLRSEDATY</u>	
Eu	-----	-----RVTI	<u>TADESTNTAY</u>	<u>MELSSLRSEDATF</u>	
	9	1	1		
	0123456789	0123456789	0123		
Mouse anti-Tac	<u>YYCARGGGVF</u>	<u>-DYWGQGTTL</u>	<u>TVSS</u>		
Daclizumab	<u>YYCARGGGVF</u>	<u>-DYWGQGTLV</u>	<u>TVSS</u>		
Eu	<u>YFCAG-----</u>	<u>---EYNGGLV</u>	<u>TVSS</u>		
B		1	2	3	4
	123456789	0123456789	0123456789	0123456789	0123456789
Mouse anti-Tac	<u>QIVLTQSPA</u>	<u>IMSASPGEKV</u>	<u>TITCSASS-S</u>	<u>ISYMHWFQOK</u>	<u>PGTSPKLIWY</u>
Daclizumab	<u>DIQMTQSPS</u>	<u>TLSASVGDV</u>	<u>TITCSASS-S</u>	<u>ISYMHWFQOK</u>	<u>PGKAPKLLIY</u>
Eu	<u>DIQMTQSPS</u>	<u>TLSASVGDV</u>	<u>TITC-----</u>	<u>-----WYQOK</u>	<u>PGKAPKLLMY</u>
	5	6	7	8	9
	0123456789	0123456789	0123456789	0123456789	0123456789
Mouse anti-Tac	<u>TTSNLASGVP</u>	<u>ARFSGSGSGT</u>	<u>SYSLTISRME</u>	<u>AEDAATYYCH</u>	<u>QRSTYPLTFG</u>
Daclizumab	<u>TTSNLASGVP</u>	<u>ARFSGSGSGT</u>	<u>EFTLTISLQ</u>	<u>PDDFATYYCH</u>	<u>QRSTYPLTFG</u>
Eu	-----GVP	<u>SRFIGSGSGT</u>	<u>EFTLTISLQ</u>	<u>PDDFATYYC-</u>	-----FG
	1				
	0				
	01234567				
Mouse anti-Tac	<u>SGTKLELK</u>				
Daclizumab	<u>QGTKVEVK</u>				
Eu	<u>QGTKVEVK</u>				

Fig. 4. Alignment of mouse, humanized, and human Eu acceptor amino acid sequences of anti-Tac VH (A) and VL (B). Amino acid sequences are shown in single-letter code. Vertical numbers show amino acid location according to Kabat [20]. CDR residues according to Kabat are underlined in the mouse anti-Tac VH and VL sequences. The CDR residues in the human Eu V region are omitted. Single-underlined residues in the daclizumab sequences are mouse-specific amino acids retained in the humanized form due to contact with the CDRs. Double-underlined residues are substitutions to human consensus amino acids.

the framework, as practiced in Queen et al.'s method [12] and described below in Section 2.2.3.2, also produces a satisfactory result in solving the potential immunogenicity problem. Therefore, the use of a cDNA-based, protein-derived, germline, or consensus framework sequence may result in a similar, if not identical, humanized V region sequence.

When choosing a germline VH segment as an acceptor framework, careful consideration must be given to its chromosomal location. Human germline VH segments are encoded on chromosomes 14, 15, and 16; however, only the VH segments on chromosome 14 are actually used for VDJ recombination to form functional VH regions, because the germline JH segments and heavy chain constant regions are also encoded on chromosome 14 [32]. The use of germline VH segments on chromosomes 15 or 16 in designing a humanized antibody should thus be avoided since this might result in unexpected structural defects in the framework.

2.2.3. Design of humanized V regions

Once a three-dimensional model of the mouse variable regions has been built, and human frameworks have been selected, several considerations are relevant in designing humanized V regions. Foremost among these is the identification of mouse framework residues that could potentially contribute to antigen binding. It is also advisable to identify atypical residues in the acceptor frameworks that might represent a potential source of immunogenicity. In addition, it is prudent to examine the variable region sequences for the presence of potential N-linked glycosylation signals since carbohydrate groups may affect antigen binding. Finally, selection of an appropriate heavy chain isotype is an important consideration in designing humanized antibodies.

2.2.3.1. Identification of key framework residues. Using a three-dimensional structure model of the variable regions of the mouse antibody, mouse framework residues that could potentially influence the conformation of the antigen-binding site or directly interact with the antigen, termed key framework residues, need to be identified. According to the humanization method of Queen et al. [12], framework amino acids within about 4–6 Å of the CDRs are candidates for designation as key framework residues. For example, Fig. 3B shows that the side chain of an alanine residue at position 67 (all amino acid positions are numbered according to the Kabat numbering system) in the anti-Tac VH is located within 5 Å of four CDR residues. This process can be achieved using a computer program, such as RASMOL (<http://www.umass.edu/microbio/rasmol/>), that calculates interatomic distances from the atomic coordinates or, though tedious, through manual inspection of a computer model. If amino acids at key framework positions differ between mouse donor and human acceptor sequences,

they are considered for replacement in the humanized form of the antibody. For this purpose, the location and orientation of the side chains of the candidate key framework residues relative to the CDR residues are examined, along with the extent of their solvent interactions. From such analyses, the impact on the CDR structure of replacing a mouse residue with its human counterpart can be determined for each of the candidate key framework residues. If the impact is considered to be minimal, for example, a serine occurs in the human framework and a threonine at the corresponding mouse framework position appears to have a relatively small interaction with the CDR, it is recommended to choose the human amino acid. This process should be carried out judiciously to minimize the number of mouse-specific residues that are retained in the humanized form.

For humanization of mouse anti-Tac, following the criteria described above, a total of nine amino acids were identified in the human Eu frameworks for substitution with the corresponding mouse residues due to their potential contributions to the formation of the antigen-binding site. These amino acids are at positions 27, 30, 48, 66, 67, 94, and 103 in the VH, and at positions 48 and 60 in the VL (Fig. 4) [12].

2.2.3.2. Potential immunogenicity. While a humanized antibody designed according to the procedures described above would be expected to maintain the affinity and specificity of the parental antibody, additional factors ought to be considered for humanization. One of the additional steps we employ is to find atypical, or rare, amino acid residues in the selected human acceptor framework sequences [12]. This process is particularly important when a protein- or cDNA-derived human framework sequence is used as an acceptor for humanization. An amino acid residue that occurs in less than about 10–20% of the variable region sequences belonging to the same subgroup is labeled “atypical” in our standard procedure. If these atypical amino acids in the selected human framework sequence are different from their counterparts in its potential ancestral germline V segment, they are considered to be the result of somatic hypermutation during affinity maturation. A somatically hypermutated amino acid in the human acceptor framework that has little contribution to the CDR structure is a potential source of immunogenicity in humans and therefore we replace it with the donor amino acid at that position if it happens to be a common amino acid in human V regions, or alternatively with a consensus amino acid residue from the same human V region subgroup, or as a third alternative with an amino acid at the corresponding location of the ancestral human germline V segment.

In the case of humanized anti-Tac [12], nine amino acids in the Eu frameworks, at positions 89, 91, 94, 103, 104, 105, and 107 in VH, and at positions 48 and 63 in

VL, were determined to be atypical and were therefore replaced with the corresponding amino acids from the donor antibody, which are common or consensus amino acids at those positions in human V regions. Among these nine positions, three amino acids, at positions 94 and 103 in VH, and at position 48 in VL, were also identified as key framework residues important for maintaining the structure of the CDRs. The amino acid residues at these three positions of the mouse V regions happened to be the same as human consensus amino acids. Therefore, in the humanization of mouse anti-Tac, although a total of 15 amino acids were replaced in the frameworks of the human acceptor sequences, only six of them (at positions 27, 30, 48, 66, and 67 in VH and at position 60 in VL) were changed to mouse-specific residues; the rest had the effect of making the humanized form more generically human-like (Figs. 3A and 4).

2.2.3.3. Glycosylation. Another important consideration for designing humanized V regions is potential N-linked glycosylation signals (Asn-X-Ser/Thr, where X is any amino acid other than Pro [33]) that might exist in the heavy or light chain variable regions of the monoclonal antibody to be humanized. A potential glycosylation signal may be generated by somatic hypermutation in either the frameworks or the CDRs during the process of affinity maturation. Alternatively, a glycosylation signal may have derived from a germline V segment sequence; for example, the majority of mouse VH sequences belonging to the III(B) subgroup naturally carry a glycosylation signal (Asn-Tyr-Thr) in CDR2 [20]. Since most occurrences of the Asn-X-Ser/Thr signal in secreted proteins are actually glycosylated [34], it is safer to assume that an N-linked glycosylation signal in a variable region is used for attachment of carbohydrates. If an N-linked glycosylation signal exists in the framework and the attached carbohydrate is not predicted to contribute to the structure of the CDRs, it is recommended that a human framework without a glycosylation signal at the corresponding site be used as an acceptor for humanization. In the case of humanizing a mouse anti-CD33 monoclonal antibody, removal of a glycosylation site in the heavy chain FR3 actually increased the affinity of the humanized antibody [35]. This is presumably because the carbohydrate in the mouse antibody interfered with antigen binding, whereas removal of the carbohydrate in the humanized form restored binding to the CD33 antigen.

If an N-linked glycosylation signal exists in the CDRs or at a framework position spatially close to the antigen-binding site, removal of the signal could affect the affinity of the antibody for its antigen either positively or negatively, or it might have no effect on affinity. It has been shown that glycosylation in the heavy chain CDR2 of a murine antibody increased the affinity of an antidextran antibody for its antigen [36,37]. In one case, glycosylation in the CDRs reduced the antigen-binding

affinity of an antibody; removal of the carbohydrate attachment site in the heavy chain CDR2 of the humanized form of an antibody significantly increased its antigen-binding affinity (unpublished results). Although the most conservative approach to successful humanization is to retain any potential glycosylation sites in the humanized form, it is desirable to have variable regions free of carbohydrates so that purified humanized antibodies will be less heterogeneous. Moreover, V region glycosylation can result in poor antibody production [38]. A typical method of eliminating the glycosylation signal is to substitute the asparagine at the first position, or the serine or threonine at the third position of the Asn-X-Ser/Thr signal by site-directed mutagenesis, using another amino acid, often Ala, predicted to minimally alter the structure of the CDRs. However, the impact of mutagenesis on the antigen-binding affinity of an antibody must be confirmed empirically, so we recommend testing both glycosylated and non-glycosylated versions of the humanized V region.

2.2.3.4. Isotype selection. For therapeutic applications, antibodies with human constant regions are preferred due to their lower immunogenicity, longer half-life, and improved ability to elicit effector mechanisms compared to rodent antibodies. To date, all non-rodent therapeutic antibodies approved for clinical use are of the human IgG isotype, and the majority of these are of the IgG1 subtype [1]. In general, the IgG1 subtype is the preferred choice when antibody effector functions are desired for efficient cell killing (e.g., anti-cancer antibodies) or when the antigen is a soluble protein (e.g., anti-cytokine antibodies). On the other hand, the IgG2 or IgG4 subtype is typically chosen when effector functions could cause undesired damage to antigen-expressing cells (e.g., anti-adhesion molecule antibodies). Moreover, advances in antibody engineering promise to deliver modified forms of the naturally occurring IgG subtypes with enhanced biological properties [39], such as improved ADCC activity [40–42], more potent CDC activity [43], or longer serum half-lives [44]. In some cases, however, it is desirable to minimize the effector mechanisms of therapeutic antibodies in order to eliminate undesired side effects; for example, several humanized anti-CD3 antibodies have been described with modified Fc regions that are deficient in Fc γ receptor binding, thus minimizing T cell activation and the resulting cytokine release syndrome [45,46].

While it is typical for the binding affinity of the parental mouse antibody to be substantially retained following humanization (discussed below in Section 2.4.1), the binding avidity of IgG2 or IgG4 antibodies is occasionally lower than that of the IgG1 form of chimeric or humanized antibodies [46–48], presumably since the IgG1 subtype has a more flexible hinge than the other IgG subtypes [49]. Despite this, the IgG2 or IgG4

subtypes may be preferred to IgG1 in certain therapeutic applications where antibody effector mechanisms are not needed in order to achieve the desired biological effect.

2.2.4. Inspection of designed V regions

After designing final humanized sequences, a model of the humanized variable regions may be constructed. This can be accomplished by making any necessary amino acid substitutions in the model of the murine antibody, and subjecting the resulting model to a few hundred cycles of energy optimization. If there remain any unusual steric or non-bonded high-energy hot spots that cannot be removed by energy optimization, this indicates the designed humanized V regions may not form the proper structure to retain the antigen-binding affinity. In this case, the design of new humanized sequences may be necessary.

It is often strategically important to decide how many heavy and light chain variable region sequences should be designed to obtain a successfully humanized antibody. If the procedure described above is carefully followed, only one pair of VH and VL sequences is usually sufficient to reproduce the desired antigen-binding characteristics in the humanized form. Our experience shows that, following the procedure described above, a single design generally produces a humanized antibody that retains antigen-binding affinity within threefold (and often better) of the parental antibody.

2.3. Expression of humanized antibodies

The strategy for expressing humanized antibodies depends on several factors including the form of antibody to be expressed (e.g., whole antibody, Fab, or single chain Fv), the preferred host organism for expression (e.g., mammalian cells, yeast, or *Escherichia coli*), and the required amount. No matter which system is chosen, it is important to be able to quickly determine whether the designed humanized antibody retains the binding affinity and specificity of the parental antibody. In this section, we describe the procedure for expression of whole antibody in mammalian cells since most humanized antibodies have been expressed in this host.

2.3.1. Synthesis of designed V genes

Once the amino acid sequences of the humanized V regions have been determined, the genes encoding the humanized V regions need to be designed. The nucleotide sequences of the coding regions of the humanized V regions can be generated artificially by picking a codon for each amino acid location, ideally one of the more preferentially used mammalian codons [50]. Alternatively, as performed routinely in our laboratory, the nucleotide sequences encoding the donor mouse V regions, including the signal peptides, can be used as templates. At positions where amino acids

differ between the mouse and humanized sequences, codons in the mouse V gene template are replaced with those of the corresponding amino acids in the humanized form.

The nucleotide sequences flanking the coding regions of the humanized V genes are determined based on which expression vector is used. It is typical to add appropriate restriction enzyme sites at the 5' and 3' ends for subsequent subcloning. In addition, the Kozak sequence [51] is often placed immediately upstream of the translation initiation codon for efficient ribosomal entry. The design of the 3' end depends on whether the expression vector is based on the cDNA or genomic sequence of the constant region. If the vector uses the cDNA sequence for expression, the 3' end needs to be designed in such a way that the reading frame at the C-terminus of the V region continues in frame to the N-terminus of the constant region in the vector without altering the natural amino acid sequence at the junction. On the other hand, if the vector carries the genomic sequence of the heavy or light chain gene, the coding region of the humanized V gene must be followed by a donor splicing site. Although we routinely use the donor splicing signal adjacent to the germline JH (or J κ) segment used in the humanized V region, any donor splicing signal (or a consensus splicing signal) known to work efficiently for splicing can be used. The designed nucleotide sequence encoding the humanized V region should also preferably be examined for the presence of a stretch of rarely used codons. If such a stretch occurs, one or more codons in the stretch should be converted to more commonly used mammalian codons [50] to avoid low expression of the humanized antibody in mammalian cells.

Fig. 5 shows the nucleotide sequences for the heavy (panel A) and light (panel B) chain variable region genes of the humanized form of anti-Tac (daclizumab). Both the VH and VL genes are flanked by *Xba*I sites for cloning into the appropriate expression vectors. Designed humanized V genes, which are usually 400–450 nucleotides in length, can either be constructed in a research laboratory or ordered through a commercial service. A standard method is to construct a V gene by extension and PCR amplification of several overlapping synthetic oligonucleotides. Fig. 6 shows an example of constructing a V gene with eight overlapping oligonucleotides, each ranging from 60 to 80 nucleotides, and two flanking PCR primers [52]. The number of overlapping oligonucleotides may be more than eight; a total of 20 or more overlapping oligonucleotides, each approximately 40 nucleotides, can be joined and amplified by PCR [53].

2.3.2. Choice of expression vectors

A variety of mammalian expression vectors have been generated and successfully used for the expression of humanized antibodies. In general, any mammalian

A 1 TCTAGACCACCATGGGATGGAGCTGGATCTTTCTCTCCTCCTGTCAGGTACCGCGGGCG
M G W S W I F L F L L S G T A G
 61 TGC ACTCTCAGGTCCAGCTTGTCCAGTCTGGGGCTGAAGTCAAGAAACCTGGCTCGAGCG
V H S Q V Q L V Q S G A E V K K P G S S
 121 TGAAGGTCTCCTGCAAGGCTTCTGGCTACACCTTTACTAGCTACAGGATGCACTGGGTAA
V K V S C K A S G Y T F T S Y R M H W V
 181 GGCAGGCCCTGGACAGGGTCTGGAATGGATGGATATATTAATCCGTCGACTGGGTATA
R Q A P G Q G L E W I G Y I N P S T G Y
 241 CTGAATACAATCAGAAGTTC AAGGACAAGGCAACAATTACTGCAGACGAATCCACCAATA
T E Y N Q K F K D K A T I T A D E S T N
 301 CAGCCTACATGGAAC TGAGCAGCTGAGATCTGAGGACACCGCAGTCTATTACTGTGCAA
T A Y M E L S S L R S E D T A V Y Y C A
 361 GAGGGGGGGGGTCTTTGACTACTGGGGCCAAGGAACCTGGTACAGTCTCCTCAG[^]GTG
R G G G V F D Y W G Q G T L V T V S S
 421 AGTCCTTAAAACCTCTAGA

B 1 TCTAGATGGAGACCGATACCCTCCTGCTATGGGTCTCCTGCTATGGGTCCAGGATCAA
M E T D T L L L W V L L L W V P G S
 61 CCGGAGATATTCAGATGACCCAGTCTCCATCTACCCTCTCTGCTAGCGTCGGGGATAGGG
T G D I Q M T Q S P S T L S A S V G D R
 121 TCACCATAACCTGCTCTGCCAGCTCAAGTATAAGTTACATGCCTGGTACCAGCAAGC
V T I T C S A S S S I S Y M H W Y Q Q K
 181 CAGGCAAAGCTCCCAAGCTTCTAATTTATACCACATCCAACCTGGCTTCTGGAGTCCCTG
P G K A P K L L I Y T T S N L A S G V P
 241 CTCGCTTCAGTGGCAGTGGATCTGGGACCGAGTTCACCCTCACAATCAGCTCTCTGCAQC
A R F S G S G S G T E F T L T I S S L C
 301 CAGATGATTTCCGCACTTATTACTGCCATCAAAGGAGTACTTACCCTCACGTTCCGTC
P D D F A T Y Y C H Q R S T Y P L T F G
 361 AGGGGACCAAGGTGGAGGTCAAAC[^]GTAAGTACACTTTTCTAGA
Q G T K V E V K

Fig. 5. Daclizumab VH and VL genes. Nucleotide sequences of designed daclizumab VH (A) and VL (B) genes are shown. Deduced amino acid sequences are shown below corresponding nucleotide sequences. Signal peptides are in italics. The mature N-termini are in bold and double-underlined. Splicing junctions are indicated by a “[^]”.

expression vector, including a number of commercially available vectors, carrying a strong promoter for expression of a cloned gene and an appropriate selection marker can be used for expression of humanized antibodies in mammalian cells, although the expression level may vary among vector systems. Heavy and light chain genes can be placed on the same vector or separately on two vectors. In the former case, each of the heavy and light chain genes needs to be placed under the control of a strong promoter [54], or the two genes may be translationally linked using the internal ribosome entry site (IRES) sequence [55]. As described below, cotransfection of heavy and light chain expression vectors usually works efficiently for expression of humanized antibodies in both transient and stable transfections.

Fig. 7 shows the structures of the mammalian expression vectors pVg1 for human γ 1 heavy chain expression and pVk for human κ light chain expression [56]. These vectors have been used routinely in our laboratory for production of humanized IgG1 antibodies in the mouse myeloma cell line Sp2/0-Ag14, often referred to as Sp2/0, which can be obtained from the American Type Culture Collection (Manassas, VA). Each vector carries the germline sequence of the respective heavy or light chain gene including its polyadenylation site located downstream of the translation termination codon. Designed humanized V genes, such as the daclizumab VH and VL

genes (Fig. 5), are cloned into the *Xba*I site in the appropriate vector. The human cytomegalovirus early promoter and enhancer drive both the heavy and light chain genes. The gpt gene in the light chain expression vector is used for the isolation of mycophenolic acid-resistant stable transfectants [56]. The dhfr gene in the heavy chain vector can be used for gene amplification to isolate stable transfectants producing higher amounts of humanized antibodies [57]. The pVg1 and pVk vectors as well as their derivatives, such as pVg4 for expression of human γ 4 heavy chain [58], have been successfully used for production of large amounts of humanized antibodies at Protein Design Labs, including the clinical supplies of fontolizumab (HuZAF) [59] and visilizumab (Nuvion) [46].

2.3.3. Expression and purification of humanized antibodies

When a small quantity of humanized antibody (usually up to a few hundred μ g) needs to be expressed for quick evaluation of its binding properties, transient expression is a good choice. Many manufacturers provide a variety of kits for efficient transient transfection of expression vectors into mammalian cells. Our current procedure uses the Lipofectamine 2000 reagent (Invitrogen) and human embryonic kidney cell line 293-H (Invitrogen). Following the protocol recommended by the manufacturer, the production level for humanized anti-

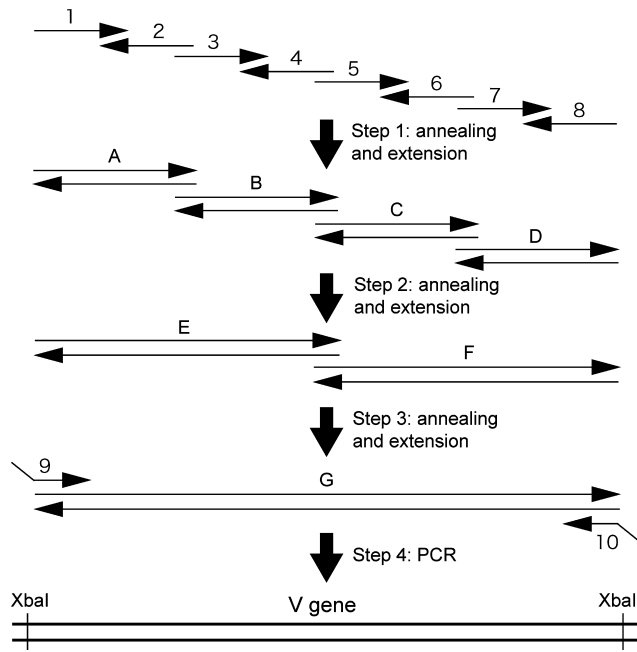


Fig. 6. Scheme for constructing humanized V genes using eight overlapping oligonucleotides. Oligonucleotides 1 and 2, 3 and 4, 5 and 6, and 7 and 8 are separately annealed and extended with the Klenow fragment of DNA polymerase I. The resulting double-stranded DNA fragments, A and B, and C and D, are separately mixed, denatured, annealed, and extended to yield the DNA fragments E and F, respectively, which are then mixed to generate the entire V gene (G) in the third annealing-and-extension step. The V gene is amplified by PCR with primers 9 and 10 to incorporate the flanking *XbaI* sites.

bodies is usually 1–10 $\mu\text{g/ml}$ after 7 days of incubation for transiently transfected 293-H cells in DMEM containing 10% fetal bovine serum (FBS). When transiently expressed humanized antibodies are to be purified, 293-H cells may be incubated in DMEM containing 2% low Ig FBS (HyClone, Logan, UT), in order to minimize copurification of bovine IgG by protein A column chromatography, without significantly reducing antibody production.

The production level can vary substantially among humanized antibodies, ranging from 0.1 to 50 $\mu\text{g/ml}$ in our experience. The differences in expression levels seem to be associated with the amino acid sequences of the heavy and light chain variable regions rather than with the transfection method used. Previous studies showed that a single amino acid substitution in the variable region could significantly change the level of antibody secreted in culture [60–63]. In unusual cases where the antibody expression level is extremely low (less than 0.1 $\mu\text{g/ml}$), culture supernatants should be concentrated using, for example, Vivaspin concentrators (Vivascience, Westford, MA) for binding and competition experiments, or alternatively humanized antibodies may need to be purified following large scale transient transfection.

Several mammalian cell lines have been successfully used for stably producing humanized antibodies, includ-

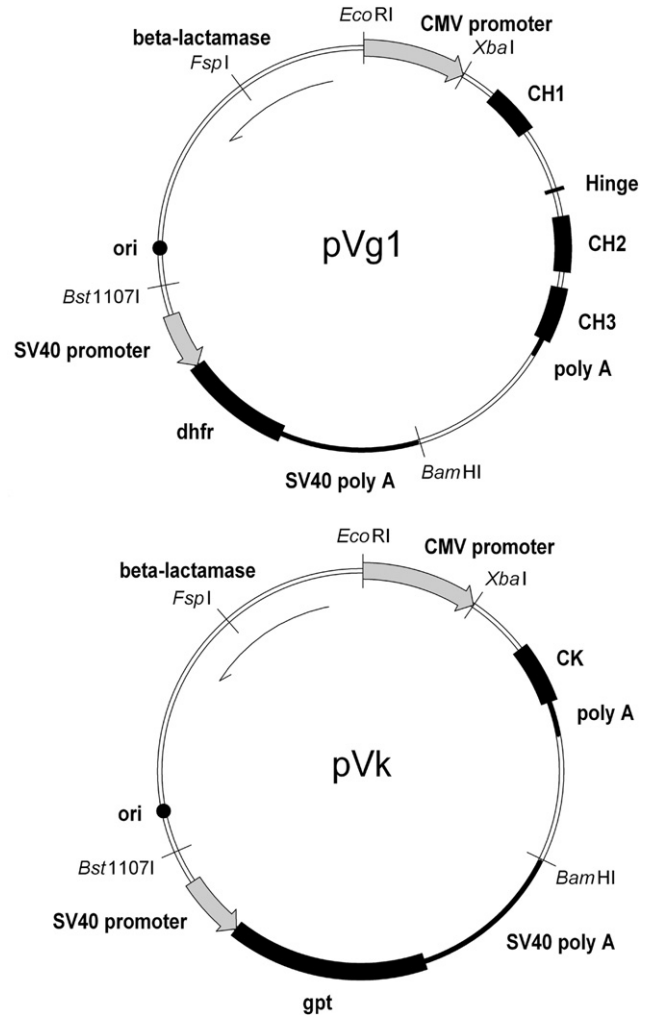


Fig. 7. Restriction maps of expression vectors pVg1 and pVk. The vectors are comprised of the human cytomegalovirus (CMV) major immediate early promoter and enhancer to ensure strong transcription initiation of immunoglobulin genes, an *XbaI* site to allow insertion of humanized VH or VL genes, and the respective human $\gamma 1$ heavy chain or human κ light chain genomic constant region genes as *XbaI*–*BamHI* fragments. The immunoglobulin genes are followed by transcription units encoding the dhfr and gpt selectable markers, respectively, including the simian virus 40 (SV40) promoter and enhancer, and other SV40 regulatory elements. The vectors also contain a bacterial replication origin and the β -lactamase gene to allow propagation of the vectors in *E. coli* and selection of ampicillin-resistant colonies.

ing the Chinese hamster ovary cell line CHO, and the mouse myeloma cell lines Sp2/0 and NS0 [64–67]. Any published method should work reasonably well to generate stable transfectants with these cell lines. When using expression vectors derived from pVg1 and pVk (Fig. 7), such as pHuGTac and pHuLTac for expression of dactlizumab [12], linearized vectors may be cotransfected into Sp2/0 cells by electroporation [56], as performed routinely in our laboratory. Alternatively, the light chain vector may be transfected first to isolate stable transfectants producing high levels of light chain, and then the heavy chain expression vector is transfected to isolate

high antibody producers [12]. The choice of a restriction endonuclease for linearization of expression vectors prior to transfection does not seem to be critical, provided the site is not within the immunoglobulin transcription unit; for example, digestion of pVg1- and pVk-derived vectors with *EcoRI*, *FspI*, *Bst1107I*, or *BamHI* does not seem to significantly effect the efficiency of stable transfection (unpublished results).

When pVg1- and pVk-derived vectors are used, after selection for expression of the gpt gene in mycophenolic acid media, Sp2/0 stable transfectants producing at least a few $\mu\text{g/ml}$ of IgG1/ κ antibody in culture medium can readily be identified by sandwich ELISA using goat anti-human γ chain antibodies for capture and peroxidase-conjugated goat anti-human κ chain antibodies for detection of bound IgG1/ κ antibodies. Purified IgG1/ κ antibody of known concentration should be used as an ELISA standard. Isolated stable transfectants can then be adapted to growth in serum-free medium by splitting the cells gradually in, for example, Hybridoma SFM (Invitrogen), usually by a 25–50% split each time, until the serum level is below 0.1%. Thereafter, the transfectants are maintained in serum-free medium. The antibody production level should be monitored by sandwich ELISA as described above. Though not all Sp2/0 stable transfectants retain their antibody production levels after adaptation to serum-free medium, we routinely observe that at least one quarter of them maintain stable production levels. It should also be noted that some of the high producers gradually lose antibody production over time while others do not, even after adaptation to serum-free medium. Antibody-producing stable transfectants may be subcloned at least once, preferably using a cell sorter, to ensure clonality. In our experience with Sp2/0 and NS0 cells, it is not difficult to isolate primary stable transfectants producing up to 50 $\mu\text{g/ml}$ humanized antibody in exhausted culture when grown in serum-free medium. Although the production level of humanized antibody can ultimately reach more than 1 mg/ml in bioreactors, a series of process and media optimization steps is required [68,69].

Purification of humanized IgG antibodies is typically achieved using protein A affinity column chromatography. To remove any IgG aggregates, an additional size exclusion chromatography step is preferred. Purified humanized antibodies are typically stored in a neutral pH buffer such as phosphate-buffered saline. To avoid copurification of bovine IgG originating from FBS in the culture medium, it is desirable to grow stable transfectants in serum-free medium. If the presence of FBS is required for growth of a stable transfectant, the use of FBS containing low levels of bovine IgG, such as low Ig FBS (Hyclone), is recommended. Stable Sp2/0 and NS0 transfectants usually grow well and maintain antibody production in the presence of 2–5% low IgG FBS.

2.4. Confirmation of binding properties

The initial goal in designing a humanized antibody is to retain the antigen-binding affinity of the parental mouse (or other rodent) antibody. Of equal importance is maintaining the biological activity of the antibody upon humanization. Methods of confirming the binding properties of the humanized antibody are described below.

2.4.1. Affinity measurement

Measurement of the antigen-binding affinity of monoclonal antibodies can be achieved in various ways including equilibrium dialysis, fluorescence quenching, and Scatchard analysis. The details of these methods are described elsewhere [70]. In this section, two popular methods for assessing the affinity of humanized antibodies, namely, competitive binding and biosensor methods utilizing surface plasmon resonance are described.

2.4.1.1. Competitive binding. In the competitive binding method, the interaction of a labeled reference antibody (typically the parental mouse antibody) with the antigen is analyzed in the presence of various amounts of competitor antibody (typically the parental and humanized antibodies). When the antigen is a soluble protein, an ELISA method is used for measuring binding of the reference antibody. When the antigen is expressed on the cell surface, a flow cytometry method based on a fluorescence activated cell sorter (FACS) instrument can be used. The reference antibody may be radiolabeled with ^{125}I , conjugated with peroxidase (for ELISA), or labeled with a fluorochrome such as fluorescein or phycoerythrin (for FACS). Biotinylation of the reference antibody is an alternative for use in either ELISA or FACS, although the use of a secondary reagent, such as streptavidin-conjugated peroxidase (for ELISA) or fluorescein (for FACS), is required for detection of bound reference antibody. Various commercial kits for conjugation of reference antibodies are available from many manufacturers.

The relative affinity difference between the humanized antibody and the parental antibody can be assessed by comparing the IC_{50} values (concentration of competitor antibody required for 50% inhibition of the binding of reference antibody) between the two antibodies. The difference in IC_{50} values is a reliable indicator of the difference in binding affinities between the two antibodies. Thus, when the IC_{50} values of the two antibodies are identical (or very similar) to each other, the affinities of the two antibodies are identical (or very similar) to each other. Alternatively, the absolute binding affinities of the parental and humanized antibodies can be independently determined by Scatchard analysis.

The antigen-binding affinity of daclizumab was determined by competitive binding of ^{125}I -labeled mouse anti-

Tac to the human T lymphoma cell line HuT-102, which expresses IL-2 receptor α chain on its surface, in the presence of various concentrations of either mouse anti-Tac or daclizumab as competitors [12]. In initial experiments by Scatchard plot analysis, the apparent antigen-binding affinity (K_a) of daclizumab was measured to be $3 \times 10^9 \text{ M}^{-1}$, about one-third the published affinity of mouse anti-Tac ($9 \times 10^9 \text{ M}^{-1}$) but still a very high affinity. However, competition experiments conducted subsequently suggested that the affinity difference is smaller than this, and in fact the affinity of the mouse and humanized forms is almost indistinguishable (unpublished results).

One caveat regarding the competition method using ELISA or FACS is that the avidity (bivalent interaction between antibody and antigen) rather than the affinity (monovalent interaction between antigen-binding site and antigen) of the monoclonal antibody is measured, unless antigen density is very low on the cell surface or on the antigen-coated ELISA plate. The apparent affinities of mouse and humanized anti-Tac described above actually reflect their avidities. Although an antibody with a high affinity in its monovalent interaction with antigen would be expected to have a high avidity, the relationship between affinity and avidity is not necessarily linear. In some cases, a low affinity antibody could have a high avidity in antigen binding. To measure the affinity between antibody and antigen, the interaction between antibody and monovalent antigen in solution needs to be analyzed using, for example, equilibrium dialysis or fluorescence quenching. The development of biosensors, as described below, has made it easier to accurately measure the monovalent interaction between antibody and antigen. Of course, the affinity is primarily of academic interest; for therapeutic applications of bivalent antibodies, the avidity may well be the more relevant number.

2.4.1.2. Biosensor methods. A biosensor apparatus, such as a Biacore 3000 (Biacore, Piscataway, NJ) or an ABI 8500 Affinity Chip Analyzer (Applied Biosystems, Foster City, CA), quantitatively measures the interaction between molecules, e.g., antigen–antibody interactions, on a sensor chip in real time by monitoring surface plasmon resonance [71,72]. As a result, both the dissociation and association constants (k_d and k_a , respectively) can be measured, and in turn the affinity equilibrium constants (K_d and K_a) can be calculated. The advantage of using a biosensor is that labeling of antibody and antigen is unnecessary. Therefore, the interaction between antibody and antigen can be analyzed with both molecules in their native forms. An additional advantage is that only a small amount of material is typically required for affinity measurement. Provided that the antigen is monomeric, the affinity of a humanized antibody can be measured by immobilizing the antibody on the sensor chip,

and then using antigen as the analyte in a physiological solution. If the antigen is immobilized on the sensor chip and the antibody is used as the analyte, then the avidity is obtained instead. It is therefore desirable to immobilize the antibody on the sensor chip to reliably measure the antigen-binding affinity.

2.4.2. Biological activity

The final goal in designing a humanized antibody is to retain the biological activity of the parental antibody in the humanized form. Which assay system ought to be used for this purpose, however, must be appropriately chosen for each antibody. Since the biological activity of the parental antibody must have been characterized in certain assays before being chosen for humanization, evaluation of the humanized antibody can be conducted using the same assays. In the case of daclizumab, its biological activity was examined by analyzing IL-2-dependent antigen-induced T cell proliferation [73]. The results indicated that daclizumab is as potent as the parental mouse anti-Tac antibody in blocking the function of IL-2 in this assay. Similar results were obtained in mixed lymphocyte reaction assays [73]. Thus, daclizumab retained both the antigen-binding affinity and biological activity of the parental mouse antibody within acceptable limits.

While a successfully humanized antibody usually retains both the antigen-binding affinity and specificity as well as the same level of the biological activity, e.g., neutralization activity, as the parental antibody, this is not guaranteed. In rare instances, a disassociation between the antigen-binding affinity and biological function of a monoclonal antibody has been observed. For example, when humanizing a mouse anti-IFN γ monoclonal antibody, an amino acid at position 11 in the mouse VH region was found to be critical for retaining high IFN γ neutralization activity in the humanized form while it had no contribution to its antigen-binding affinity [59]. Similarly, a disassociation between affinity and biological activity was reported with the anti-HER2/neu Fab [74] and with the anti-RSV single-chain Fv antibody [75]. It is thus imperative to characterize the biological activity of the humanized antibody at an early stage of the humanization project. Although each antibody displaying such a disassociation between its antigen-binding affinity and biological function will require a unique solution, systematic mutagenesis will allow the identification of mouse residues necessary to restore full biological activity in the humanized form.

3. Concluding remarks

Humanization technology, supported by advances in genetic engineering and three-dimensional modeling of protein structures, has made it possible to apply mouse and other rodent monoclonal antibodies to human ther-

apy. In addition to the nine humanized monoclonal antibodies approved as human therapeutics in the United States, more than 50 humanized antibodies are being evaluated in human clinical studies [1,76]. Because humanized antibodies are, in general, safe and well tolerated in humans, many more humanized antibodies are expected to be generated for a variety of antigens and will be tested in preclinical and clinical studies. The application of humanization technology is not limited to rodent antibodies, however. Since the basic framework structure of the variable regions is conserved among species, humanization of non-rodent, and even non-mammalian, antibodies is theoretically possible. Indeed, humanization of rabbit [77] and chicken [78] antibodies has been successfully accomplished. The procedure for antibody humanization described in this paper has been successfully used in our laboratory for over 50 antibodies, including several non-mammalian antibodies. Although each humanization is unique and challenging, the general guidelines described here should lead to successful generation of humanized antibodies that retain the antigen-binding affinities and specificities as well as the biological activities of parental monoclonal antibodies.

Acknowledgments

We thank Drs. Cary Queen, Man Sung Co, and David B. Powers for their careful review of the manuscript. We also thank all current and former members of the antibody engineering group at Protein Design Labs for improvements to the humanization procedure and for their contributions to the many successful antibody humanizations over the past 15 years.

References

- [1] J. Reichert, A. Pavlou, *Nat. Rev. Drug Discov.* 3 (2004) 383–384.
- [2] G. Kohler, C. Milstein, *Nature* 256 (1975) 495–497.
- [3] D.R. Burton, J.M. Woof, *Adv. Immunol.* 51 (1992) 1–84.
- [4] R. Fagnani, *Immunol. Ser.* 61 (1994) 3–22.
- [5] M.B. Khazaeli, R.M. Conry, A.F. LoBuglio, *J. Immunother.* 15 (1994) 42–52.
- [6] K. Kuus-Reichel, L.S. Grauer, L.M. Karavodin, C. Knott, M. Krusemeier, N.E. Kay, *Clin. Diagn. Lab. Immunol.* 1 (1994) 365–372.
- [7] S.L. Morrison, M.J. Johnson, L.A. Herzenberg, V.T. Oi, *Proc. Natl. Acad. Sci. USA* 81 (1984) 6851–6855.
- [8] A. Mountain, J.R. Adair, *Biotechnol. Genet. Eng. Rev.* 10 (1992) 1–142.
- [9] P.T. Jones, P.H. Dear, J. Foote, M.S. Neuberger, G. Winter, *Nature* 321 (1986) 522–525.
- [10] P.R. Tempest, P. Bremner, M. Lambert, G. Taylor, J.M. Furze, F.J. Carr, W.J. Harris, *Biotechnology* 9 (1991) 266–271.
- [11] L. Riechmann, M. Clark, H. Waldmann, G. Winter, *Nature* 332 (1988) 323–327.
- [12] C. Queen, W.P. Schneider, H.E. Selick, P.W. Payne, N.F. Landolfi, J.F. Duncan, N.M. Avdalovic, M. Levitt, R.P. Junghans, T.A. Waldmann, *Proc. Natl. Acad. Sci. USA* 86 (1989) 10029–10033.
- [13] J.M. Reichert, *Nat. Biotechnol.* 19 (2001) 819–822.
- [14] C. Mateo, E. Moreno, K. Amour, J. Lombardero, W. Harris, R. Perez, *Immunotechnology* 3 (1997) 71–81.
- [15] S. Stephens, S. Emtage, O. Vetterlein, L. Chaplin, C. Bebbington, A. Nesbitt, M. Sopwith, D. Athwal, C. Novak, M. Bodmer, *Immunology* 85 (1995) 668–674.
- [16] T. Uchiyama, S. Broder, T.A. Waldmann, *J. Immunol.* 126 (1981) 1393–1397.
- [17] N. Tsurushita, M. Vasquez, in: T. Honjo, F.W. Alt, M.S. Neuberger (Eds.), *Molecular Biology of B Cells*, Elsevier Academic Press, San Diego, CA, 2004, pp. 533–545.
- [18] J. Sambrook, D.W. Russell, *Molecular Cloning: a Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York, NY, 2001.
- [19] D.I. Stott, A.J. Munro, *Biochem. J.* 128 (1972) 1221–1227.
- [20] E.A. Kabat, T.T. Wu, H.M. Perry, K.S. Gottesman, C. Foeller, *Sequences of Proteins of Immunological Interest*, U.S. Dept. of Health and Human Services, Bethesda, MD, 1991.
- [21] G. Johnson, T.T. Wu, *Nucleic Acids Res.* 28 (2000) 214–218.
- [22] J. Mozdanzowski, J. Bongers, K. Anumula, *Anal. Biochem.* 260 (1998) 183–187.
- [23] R. Levy, O. Assulin, T. Scherf, M. Levitt, J. Anglister, *Biochemistry* 28 (1989) 7168–7175.
- [24] B. Zilber, T. Scherf, M. Levitt, J. Anglister, *Biochemistry* 29 (1990) 10032–10041.
- [25] H.M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T.N. Bhat, H. Weissig, I.N. Shindyalov, P.E. Bourne, *Nucleic Acids Res.* 28 (2000) 235–242.
- [26] D.G. Higgins, J.D. Thompson, T.J. Gibson, *Methods Enzymol.* 266 (1996) 383–402.
- [27] J.D. Thompson, D.G. Higgins, T.J. Gibson, *Nucleic Acids Res.* 22 (1994) 4673–4680.
- [28] W. Press, B. Flannery, S. Teukolsky, W. Vetterling, *Numerical Recipes*, Cambridge University Press, Cambridge, 1990.
- [29] P.K. Weiner, P.A. Kollman, *J. Comp. Chem.* 2 (1981) 287–303.
- [30] D. Gusfield, *Algorithms on Strings, Trees, and Sequences*, Cambridge University Press, Cambridge, 1997.
- [31] S. Karlin, S.F. Altschul, *Proc. Natl. Acad. Sci. USA* 87 (1990) 2264–2268.
- [32] G.P. Cook, I.M. Tomlinson, *Immunol. Today* 16 (1995) 237–242.
- [33] R. Kornfeld, S. Kornfeld, *Annu. Rev. Biochem.* 54 (1985) 631–664.
- [34] Y. Gavel, G. von Heijne, *Protein Eng.* 3 (1990) 433–442.
- [35] M.S. Co, D.A. Scheinberg, N.M. Avdalovic, K. McGraw, M. Vasquez, P.C. Caron, C. Queen, *Mol. Immunol.* 30 (1993) 1361–1367.
- [36] S.C. Wallick, E.A. Kabat, S.L. Morrison, *J. Exp. Med.* 168 (1988) 1099–1109.
- [37] A. Wright, M.H. Tao, E.A. Kabat, S.L. Morrison, *EMBO J.* 10 (1991) 2717–2723.
- [38] F.A. Gala, S.L. Morrison, *J. Immunol.* 172 (2004) 5489–5494.
- [39] L.G. Presta, *Curr. Pharm. Biotechnol.* 3 (2002) 237–256.
- [40] P. Umana, J. Jean-Mairet, R. Moudry, H. Amstutz, J.E. Bailey, *Nat. Biotechnol.* 17 (1999) 176–180.
- [41] R.L. Shields, J. Lai, R. Keck, L.Y. O’Connell, K. Hong, Y.G. Meng, S.H. Weikert, L.G. Presta, *J. Biol. Chem.* 277 (2002) 26733–26740.
- [42] T. Shinkawa, K. Nakamura, N. Yamane, E. Shoji-Hosaka, Y. Kanda, M. Sakurada, K. Uchida, H. Anazawa, M. Satoh, M. Yamasaki, N. Hanai, K. Shitara, *J. Biol. Chem.* 278 (2003) 3466–3473.
- [43] E.E. Idusogie, P.Y. Wong, L.G. Presta, H. Gazzano-Santoro, K. Totpal, M. Uitsch, M.G. Mulkerrin, *J. Immunol.* 166 (2001) 2571–2575.
- [44] P.R. Hinton, M.G. Johlfs, J.M. Xiong, K. Hanestad, K.C. Ong, C. Bullock, S. Keller, M.T. Tang, J.Y. Tso, M. Vasquez, N. Tsurushita, *J. Biol. Chem.* 279 (2004) 6213–6216.
- [45] M.L. Alegre, L.J. Peterson, D. Xu, H.A. Sattar, D.R. Jeyarajah, K. Kowalkowski, J.R. Thistlethwaite, R.A. Zivin, L. Jolliffe, J.A. Bluestone, *Transplantation* 57 (1994) 1537–1543.

- [46] M.S. Cole, K.E. Stellrecht, J.D. Shi, M. Homola, D.H. Hsu, C. Anasetti, M. Vasquez, J.Y. Tso, *Transplantation* 68 (1999) 563–571.
- [47] M.M. Morelock, R. Rothlein, S.M. Bright, M.K. Robinson, E.T. Graham, J.P. Sabo, R. Owens, D.J. King, S.H. Norris, D.S. Scher, J.L. Wright, J.R. Adair, *J. Biol. Chem.* 269 (1994) 13048–13055.
- [48] G.R. McLean, M. Torres, N. Elguezabal, A. Nakouzi, A. Casadevall, *J. Immunol.* 169 (2002) 1379–1386.
- [49] J.L. Dangl, T.G. Wensel, S.L. Morrison, L. Stryer, L.A. Herzenberg, V.T. Oi, *EMBO J.* 7 (1988) 1989–1994.
- [50] Y. Nakamura, T. Gojobori, T. Ikemura, *Nucleic Acids Res.* 28 (2000) 292.
- [51] M. Kozak, *Nucleic Acids Res.* 15 (1987) 8125–8148.
- [52] X.Y. He, Z. Xu, J. Melrose, A. Mullowney, M. Vasquez, C. Queen, V. Vexler, C. Klingbeil, M.S. Co, E.L. Berg, *J. Immunol.* 160 (1998) 1029–1035.
- [53] W.P. Stemmer, A. Cramer, K.D. Ha, T.M. Brennan, H.L. Heyneker, *Gene* 164 (1995) 49–53.
- [54] S.A. Kostelny, B.K. Link, J.Y. Tso, M. Vasquez, B.H. Jorgensen, H. Wang, W.C. Hall, G.J. Weiner, *Int. J. Cancer* 93 (2001) 556–565.
- [55] E. Martinez-Salas, *Curr. Opin. Biotechnol.* 10 (1999) 458–464.
- [56] M.S. Co, N.M. Avdalovic, P.C. Caron, M.V. Avdalovic, D.A. Scheinberg, C. Queen, *J. Immunol.* 148 (1992) 1149–1154.
- [57] M.M. Bendig, *Genet. Eng.* (1988) 91–127.
- [58] M.S. Co, N.F. Landolfi, J.O. Nagy, J.H. Tan, V. Vexler, M. Vasquez, L. Roark, S. Yuan, P.R. Hinton, J. Melrose, C. Klingbeil, C. Queen, E.L. Berg, *Immunotechnology* 4 (1999) 253–266.
- [59] N.F. Landolfi, A.B. Thakur, H. Fu, M. Vasquez, C. Queen, N. Tsurushita, *J. Immunol.* 166 (2001) 1748–1754.
- [60] A.H. Horwitz, R. Nadell, F. Preugschat, M. Better, *Mol. Immunol.* 31 (1994) 683–692.
- [61] J.W. Saldanha, A.C. Martin, O.J. Leger, *Mol. Immunol.* 36 (1999) 709–719.
- [62] J.L. Dul, Y. Argon, *Proc. Natl. Acad. Sci. USA* 87 (1990) 8135–8139.
- [63] G.D. Wiens, A. Lekkerkerker, I. Veltman, M.B. Rittenberg, *J. Immunol.* 167 (2001) 2179–2186.
- [64] C.R. Bebbington, G. Renner, S. Thomson, D. King, D. Abrams, G.T. Yarranton, *Biotechnology (NY)* 10 (1992) 169–175.
- [65] P.P. Sanna, *Methods Mol. Biol.* 178 (2002) 389–395.
- [66] E.M. Yoo, K.R. Chintalacharuvu, M.L. Penichet, S.L. Morrison, *J. Immunol. Methods* 261 (2002) 1–20.
- [67] J.J. Trill, A.R. Shatzman, S. Ganguly, *Curr. Opin. Biotechnol.* 6 (1995) 553–560.
- [68] L. Xie, D.I. Wang, *Trends Biotechnol.* 15 (1997) 109–113.
- [69] P.W. Sauer, J.E. Burky, M.C. Wesson, H.D. Sternard, L. Qu, *Biotechnol. Bioeng.* 67 (2000) 585–597.
- [70] M. Steward, D. Chargelegue, in: L. Herzenberg (Ed.), *Weir's Handbook of Experimental Immunology*, Blackwell Science, Cambridge, MA, 1996 Chapter 38.
- [71] A. Huber, S. Demartis, D. Neri, *J. Mol. Recognit.* 12 (1999) 198–216.
- [72] J.M. McDonnell, *Curr. Opin. Chem. Biol.* 5 (2001) 572–577.
- [73] R.P. Junghans, T.A. Waldmann, N.F. Landolfi, N.M. Avdalovic, W.P. Schneider, C. Queen, *Cancer Res.* 50 (1990) 1495–1502.
- [74] R.F. Kelley, M.P. O'Connell, P. Carter, L. Presta, C. Eigenbrot, M. Covarrubias, B. Snedecor, J.H. Bourell, D. Vetterlein, *Biochemistry* 31 (1992) 5434–5441.
- [75] S. Delagrave, J. Catalan, C. Sweet, G. Drabik, A. Henry, A. Rees, T.P. Monath, F. Guirakhoo, *Protein Eng.* 12 (1999) 357–362.
- [76] L. Stockwin, S. Holmes, *Expert Opin. Biol. Ther.* 3 (2003) 1133–1152.
- [77] C. Rader, G. Ritter, S. Nathan, M. Elia, I. Gout, A.A. Jungbluth, L.S. Cohen, S. Welt, L.J. Old, C.F. Barbas 3rd, *J. Biol. Chem.* 275 (2000) 13668–13676.
- [78] N. Tsurushita, M. Park, K. Pakabunto, K. Ong, A. Avdalovic, H. Fu, A. Jia, M. Vasquez, S. Kumar, *J. Immunol. Methods* 295 (2004) 9–19.