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Referencia : Expediente No. **12.019.956**
Asunto : Solicitud de patente para: **ANTICUERPOS HUMANOS DE ALTA AFINIDAD PARA LA AGIOPOYETINA-2 HUMANA**
Solicitante : REGENERON PHARMACEUTICALS, INC.
Fecha de solicitud : 6 de Febrero de 2012

1. Yo, Luz Clemencia de PAEZ, en mi condición de apoderada de la sociedad solicitante de la patente de la referencia, dando alcance al memorial radicado ante su Despacho el 13 de Noviembre de 2013, mediante el cual interpusé respuesta de la acción oficial No. **15168**, deseamos poner de presente lo siguiente.

2. Amablemente, suministramos dos artículos en los cuales se encuentra basada la respuesta a la acción oficial No. **15168**. Así, vale la pena resaltar que dichos artículos son importantes para la argumentación de las objeciones relacionadas con la claridad de la presente solicitud de patente.

3. En consecuencia, solicito a ese Despacho tener en cuenta la información aquí suministrada al momento de expedir una resolución con la decisión sobre el memorial de respuesta a la acción oficial presentado.

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4. **Anexos**

- Bending, M. (1995). Humanization of Rodent Monoclonal Antibodies by CDR Grafting. *Methods: a companion to methods in enzymology*. Vol. 8: 83-93.
- Winter, G. & Harris, W. (1993). Antibody-based therapy. Humanized antibodies. *Immunology today*. Vol. 14(6): 243-246.

Señor Director,


LUZ CLEMENCIA DE PAEZ

C.C. 35.456.344 de Usaquén

T.P.A. No. 23.555

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Humanization of Rodent Monoclonal Antibodies by CDR Grafting

Mary M. Bendig

MRC Collaborative Centre, 1-3 Burtonhole Lane, Mill Hill, London NW7 1AD, United Kingdom

Many rodent antibodies with possible therapeutic applications in humans have been isolated and characterized. Clinical results with rodent antibodies, however, have been disappointing primarily because rodent antibodies are highly immunogenic in humans. To help overcome this problem, rodent antibodies have been partially and fully humanized. Partial humanization consists of joining the rodent variable regions to human constant regions to create a chimeric antibody. Full humanization means taking only the portions of the rodent variable regions that are directly involved in antigen-binding, the complementarity determining regions (CDRs), and grafting these regions into human variable regions. The "reshaped human" variable regions are then joined to human constant regions to create a fully humanized, CDR-grafted antibody. Full humanization by CDR grafting is a more complex type of protein engineering than partial humanization by chimerization. Reliable methods for humanization by CDR grafting, however, have been developed. This article describes the methods developed and used at the MRC Collaborative Centre. The methods rely on selecting human variable regions with a high degree of similarity to the rodent variable regions and constructing a molecular model of the rodent variable regions. The model is used to help identify amino acid residues that participate either directly or indirectly in antigen binding and then to decide what amino acid substitutions might be necessary in the human variable regions in order to achieve good binding to antigen. The methods are described in general and then illustrated for humanizing a mouse anti-human IgE antibody. The article also lists 80 examples of rodent antibodies humanized by CDR grafting. © 1995 Academic Press, Inc.

Antibodies have been proposed and tested as therapeutic agents in humans for many years. As early as the 1920s, polyclonal rabbit antisera were used to treat leukemia in humans. With the advent of hybridoma technology in the 1970s (1), it became possible to produce rodent monoclonal antibodies with specificities to a wide range of therapeutic targets. It has proven more difficult to isolate therapeutically promising human monoclonal antibodies using either hybridoma technology or EBV transformation. Reasons for this include

fundamental technological problems, ethical considerations, and tolerance to self-antigens. Most therapeutic work has concentrated, therefore, on a variety of existing rodent monoclonal antibodies that have useful specificities, good affinities, and the ability to be produced in large quantities.

Rodent antibodies, however, have serious disadvantages as therapeutic agents in humans. First, rodent antibodies induce an immune response in human patients that will result in rapid clearance of the rodent antibody and hence interfere with the intended therapeutic benefit. Second, rodent antibodies generally do not efficiently recruit human immune effector functions that enable complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC). These effector functions are often required for therapeutic efficacy. Third, patients sensitized to rodent antibodies may suffer from allergic side effects. These problems can be largely overcome using protein engineering technologies to "humanize" rodent monoclonal antibodies.

There are essentially two methods for humanization. The first method is to take the entire variable domains from the rodent antibody and join these to the constant domains from human antibodies to create a rodent-human "chimeric" antibody (Figs. 1 and 2). This method is a relatively simple protein engineering procedure in which entire unaltered protein domains from one protein (the variable domains from the light and heavy chains of a rodent immunoglobulin) are joined to entire unaltered protein domains from other related proteins (the constant domains from human immunoglobulin light and heavy chains). Although this method of humanization is simple and reliable in terms of generating an antibody that will have the binding characteristics of the rodent antibody, this method will create only a partially humanized antibody, with approximately 35% of the final antibody sequence derived from the rodent antibody. Although chimeric antibodies are less immunogenic in humans than rodent antibodies, they can still induce anti-variable domain immune response in humans (reviewed in Ref. 2).

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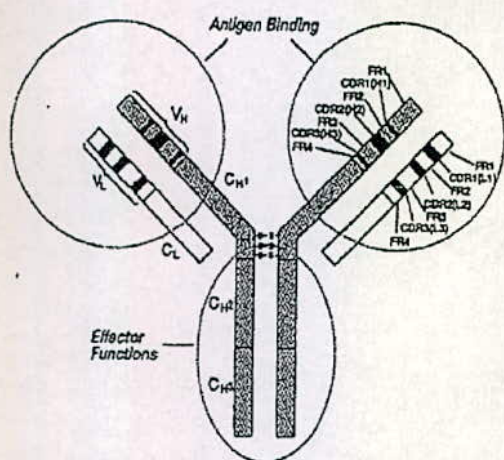


FIG. 1. Diagram of an immunoglobulin molecule. The light and heavy chain variable domains are indicated as V_L and V_H, respectively. The light chain constant domain and the three heavy chain constant domains are indicated by C_L, C_{H1}, C_{H2}, and C_{H3}, respectively. The three complementarity determining regions (CDRs) and four framework regions (FRs) that make up each variable domain are labeled.

The second method for humanization is to take only the antigen-binding loops or complementarity determining regions (CDRs) (Figs. 1 and 3) from the rodent variable domains and to graft these into human

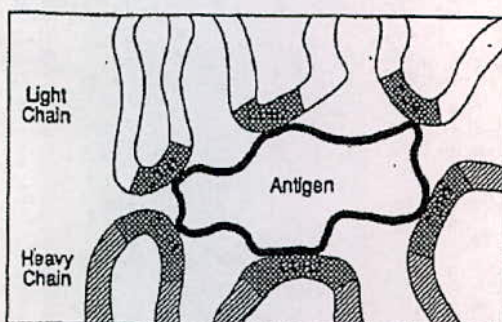


FIG. 3. Diagram illustrating binding to antigen. In a simplified example, the six CDRs of the antibody's light and heavy chain variable regions form loop-like structures that make direct contact with the antigen, holding it in place.

variable domains. These "reshaped" human variable domains are then joined to human constant domains to create a fully humanized antibody (Fig. 2). This method requires considerably more complex protein engineering procedures because major alterations are being made within two interacting protein domains (the light and heavy chain variable regions). Although this method is more complex and is not certain to create a humanized antibody with good binding properties, it will create a fully humanized antibody with only approximately 9% of the final antibody sequence derived from the rodent antibody. At present, there are very

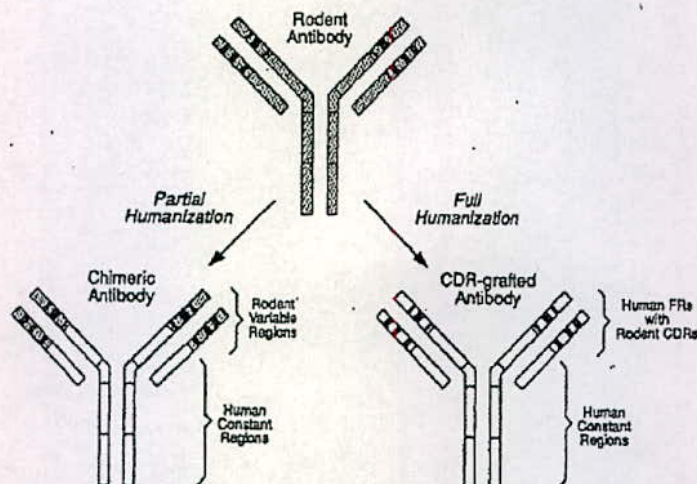


FIG. 2. Diagram illustrating the difference between partial humanization and full humanization. The rodent antibody is shaded with the CDRs in black. In the chimeric and CDR-grafted antibodies, the portions of the antibody that were derived from the rodent antibody are shaded or in black, and the portions that were obtained from a human antibody are in white.

limited data available on the immunogenicity of CDR-grafted antibodies in humans. However, it is likely that full humanization will greatly reduce the immunogenicity of a rodent antibody but that, in some cases, the fully humanized antibody will still elicit an anti-idiotypic response to the antigen-binding site (3, 4).

The concept of humanization of a rodent antibody via CDR grafting was first demonstrated by Dr. Greg Winter at the MRC Laboratory of Molecular Biology in Cambridge, England (5). Since then, the methods for designing and constructing CDR-grafted antibodies have been adopted and further developed by several research groups. A confusing array of terminology has been used to describe variations in methods for humanization, for example, reshaping, hyperchimerization, civilization, veneering, and surface replacement (reviewed in Ref. 2). All of these methods are based on grafting all or part of the rodent CDRs into human variable regions and then making minor alterations in the framework regions (FRs) of the human variable regions in order to achieve good binding to antigen. This paper will describe the methods developed and used at the MRC Collaborative Centre for humanization via CDR grafting. Published results obtained both at the MRC Collaborative Centre and at other research centers will be summarized.

DESCRIPTION OF METHOD

Cloning and Sequencing of the Rodent Variable Regions

In most cases, cDNAs coding for the rodent variable regions are cloned using polymerase chain reaction (PCR) methods to amplify the variable regions. At the MRC Collaborative Centre, we use specially designed PCR primers that hybridize to sequences flanking the variable regions so that the entire variable regions are cloned without the PCR primers influencing potentially important sequence information at the 5'- and 3'-ends of the variable regions (6). In a typical cloning of mouse light and heavy chain variable regions, total RNA is prepared from the hybridoma cells that produce the mouse antibody using a guanidinium thiocyanate/cesium chloride procedure (7). First-strand cDNA is synthesized from the total RNA and used as the template DNA in PCR reactions with the primers designed to amplify mouse light and heavy chain variable regions (6). PCR products of the correct size are cloned into a pUC vector and sequenced. For both the light and heavy chain variable region, at least two independently amplified and cloned DNA fragments are sequenced. If there are any differences between the two sequences, additional clones are sequenced. Care must be taken to recognize errors arising from PCR amplification. Care

must also be taken, particularly with the cloning of the light chain variable region, to recognize aberrant immunoglobulin transcripts arising from the nonsecreting myeloma cell used as the fusion cell partner (8).

Construction of a Chimeric Antibody

The amino acid sequences of the rodent variable regions, as derived from the DNA sequences, can be compared to immunoglobulin variable regions in databases (9) to confirm that the amino acid sequences do code for immunoglobulin variable regions. In order to confirm that these amino acid sequences code for the variable regions of the particular rodent antibody being studied, it is best to construct a chimeric antibody and determine that this chimeric antibody can bind to the antigen recognized by the original rodent antibody.

At the MRC Collaborative Centre, PCR primers were designed to clone the entire mouse variable regions together with their leader sequences (6). Using the cloned leader-variable regions as template DNAs, PCR primers are designed to modify the 5'-ends to contain Kozak sequences for efficient eukaryotic translation (10) and restriction enzyme sites for insertion into the expression vectors and to modify the 3'-ends to contain splice donor sites and restriction enzyme sites for insertion into the expression vector (11). The PCR-amplified mouse light and heavy chain leader-variable regions are then inserted into vectors designed to express partially or fully humanized immunoglobulin light and heavy chains in mammalian cells. These vectors contain the human cytomegalovirus (HCMV) enhancer and promoter for transcription, a human light or heavy chain constant region, a gene such as *neo* for selection of transformed cells, and the SV40 origin of replication for DNA replication in COS cells (12).

In order to produce chimeric antibody for preliminary analyses, COS cells are cotransfected with the two vectors containing the genes coding for the chimeric light and heavy chains. Following a 2- to 3-day period of transient expression, chimeric antibody is present in the culture medium at approximately 0.1 to 1.0 $\mu\text{g}/\text{ml}$. The chimeric antibody and samples of the original mouse antibody are tested, usually by ELISA, for binding to antigen. Since the chimeric antibody contains the entire mouse light and heavy chain variable domains, the chimeric antibody should bind to antigen as well as the parental mouse antibody. This is assuming that both the mouse and the chimeric antibody have two antigen binding sites per antibody molecule, as is usually the case. If, for example, the mouse antibody is an IgM antibody and the chimeric antibody is human IgG1, then the chimeric antibody will be expected to have the same antigen specificity as the mouse antibody but to have a reduced avidity.

Molecular Modeling of the Rodent Variable Regions

In order to assist in the design of the CDR-grafted variable regions, it is very helpful to have a molecular model of the rodent variable regions. Modeling the structures of well-characterized protein families like immunoglobulins is performed using established methods for modeling by homology. A variety of computers, software, and databases are available to assist in such "knowledge-based" modeling. At the MRC Collaborative Centre, molecular modeling is carried out using a Silicon Graphics IRIS 4D workstation, the molecular modeling package QUANTA (Polygen Corp., Waltham, MA), and the Brookhaven database of protein structures with a few additional, as yet unpublished, immunoglobulin structures. As a first step in the modeling exercise, the FRs of the new variable regions are modeled on FRs from similar, structurally solved immunoglobulin variable regions. Most of the CDRs of the new variable regions are modeled based on the canonical structures for CDRs (13). Those CDRs that do not appear to belong to any known group of canonical structures, for example CDR3 of the heavy chain variable region, are modeled based on similar loop structures present in any structurally solved protein. In order to relieve unfavorable atomic contacts and to optimize Van der Waals and electrostatic interactions, the model is subjected to energy minimization using the CHARMM potential (14) as implemented in QUANTA.

Designing the CDR-Grafted Variable Regions

The first step in designing the CDR-grafted variable regions is the selection of the human light and heavy chain variable regions that will serve as the basis of the humanized variable regions. This is one of the most critical steps. At the MRC Collaborative Centre, two approaches for selecting the human variable regions have been tested and compared. In one approach, the human variable regions are selected from the consensus sequences for the different subgroups of human variable regions (9). The rodent light and heavy chain variable regions are compared to the human consensus sequences; the most similar human light and heavy chain consensus sequences are selected from among the six subgroups of human λ light chain variable regions, the four subgroups of human κ light chain variable regions, and the three subgroups of human heavy chain variable regions. In another approach, the human variable regions are selected from all published sequences for human variable regions (9). The amino acid sequences of rodent light and heavy chain variable regions are compared to human sequences, and human variable regions with a high degree of similarity to the rodent variable regions are selected. Some research groups have preferred to use human light and heavy chain variable regions from the same human antibody in order to ensure that the two variable regions will

assemble properly (15). At the MRC Collaborative Centre, the human light and heavy chain variable regions that are selected as the templates are usually derived from two different human antibodies. In this way, it is possible to independently select for human variable regions with the highest degree of similarity to the rodent variable regions. There are many successful examples of CDR-grafted antibodies based on variable regions derived from two different human antibodies. One of the best studied examples is reshaped human CAMPATH-1 antibody (16). This humanized antibody is specific for glycoprotein CDw52, an antigen present on lymphocytes. Significant clinical benefits have been observed after treatment with reshaped human CAMPATH-1 antibody (see Discussion).

The second step in the design process is to insert the rodent CDRs into the selected human light and heavy chain variable regions. At the MRC Collaborative Centre, the entire rodent CDRs, as defined by Kabat *et al.* (9), are joined, on paper, to the human FRs to create a simple CDR graft. In many cases, a rodent antibody that is humanized in a simple CDR graft will show little or no binding to antigen. It is important to study the amino acid sequences of the human FRs to determine whether any of these amino acid residues are likely to adversely influence binding to antigen, either directly through interactions with antigen or indirectly by altering the positioning of the CDR loops.

In the third step, decisions are made as to which amino acid residues in the human FRs should be altered in order to achieve good binding to antigen. This is a difficult and critical step. The model of the rodent variable regions is most useful at this stage in the design process. Also useful are the canonical structures for the CDRs as defined by Chothia *et al.* (13). It is important to conserve in the humanized variable regions any of the rodent amino acid residues that are part of the canonical structures. It is helpful to compare the sequence of the rodent antibody to be humanized to similar sequences from other rodent antibodies to determine whether the amino acids at a certain position are unusual or rare. This might indicate that the rodent amino acid at that position has an important role in antigen binding. By studying the model of the rodent variable regions, it is possible to predict whether amino acids at particular positions could influence antigen binding. If human variable regions from individual human antibodies are being used as the basis of the design; then it is advisable to compare the individual human sequence to the consensus sequence for that subgroup of human variable regions. Any amino acids that are particularly unusual should be noted. In most cases, a few amino acids in the human FRs are identified that should be changed from the amino acid present at that position in the human variable region to the amino acid present in the rodent variable region.

Construction of the Fully Humanized Antibody

Following the design stage, there will be at least two amino acid sequences, one coding for the fully humanized light chain variable region and one coding for the fully humanized heavy chain variable region. There may be a few additional amino acid sequences that are minor modifications of the above two sequences. There are a wide variety of methods that could be used to construct DNA sequences that would code for the amino acid sequences of the newly designed humanized variable regions. Decisions regarding the preferred method of construction are usually made based on whether a DNA sequence coding for a similar amino acid sequence is available for use as a template in the construction of the new DNA sequence. At the MRC Collaborative Centre, most DNA sequences coding for newly designed humanized variable regions are constructed using PCR mutagenesis methods to alter existing DNA sequences (17). For example, PCR primers coding for the new CDRs are hybridized to a DNA template that is a humanized variable region that was designed based on the same, or a very similar, human variable region (18). Alternatively, if a similar DNA sequence is not available to use as a template, the entire DNA sequence coding for the leader-variable region sequence can be constructed from synthetic oligonucleotides (19).

Following the construction and sequencing of the DNA sequences coding for the light and heavy chain leader sequences plus humanized variable regions, the leader-variable regions are inserted into mammalian cell expression vectors as described in the construction of chimeric antibodies. These vectors are designed to join the variable regions to human constant regions via introns (12).

Evaluation of the Humanized Antibodies

As described previously, COS cells are cotransfected with the two vectors designed to express the humanized light and heavy chains. As a positive control for binding to antigen, cells are also transfected with the vectors designed to express the chimeric light and heavy chains. Cells are often also transfected with the vectors that express the chimeric light chain together with the humanized heavy chain and the vectors that express the humanized light chain together with the chimeric heavy chain. If the humanized antibody does not bind to antigen as well as the chimeric antibody, then the experiments with the mixed chimeric/humanized antibodies can be helpful in indicating whether it is the humanized light or heavy chain variable region, or both, that is responsible for poor binding.

Following transient expression in the COS cells, the chimeric and humanized antibodies are analyzed by ELISA to determine the antibody concentrations in the samples. The antibodies are then analyzed for binding

to antigen, usually in an ELISA using purified antigen or a cell line expressing the target antigen on its surface. The original rodent antibody is used as a positive control to confirm that the antigen-binding assay is functioning. Direct comparisons, however, cannot be made between the binding profiles obtained with the rodent antibody and with the humanized antibody because different second antibodies with different sensitivities are employed in the ELISA for the detection of bound rodent and human antibodies. Direct comparisons, however, can be made between the chimeric and the humanized antibodies. The chimeric and humanized antibodies are usually constructed using the same human constant regions, and the same anti-human constant region antibody reagent will detect equally well both chimeric and humanized antibody bound to antigen. As explained previously, the chimeric antibody contains the complete rodent variable regions and is expected to bind to antigen with the same affinity as the original rodent antibody. In the initial analysis of the fully humanized antibodies, binding to antigen is best evaluated by comparing the results obtained using chimeric and humanized antibodies produced using the same human constant regions and the same expression systems.

In most cases several minor variants in the design of the humanized antibody will be constructed and evaluated. Once the best version of the humanized antibody has been identified using a relatively simple antigen-binding assay, more in-depth evaluation can be carried out. For example, competition binding analysis can be used to make direct comparisons between the rodent antibody and the chimeric and humanized antibody. Typically, the rodent antibody is labeled; and unlabeled rodent, chimeric, and humanized antibodies are tested in competition with the labeled rodent antibody for binding to antigen (11). Alternatively, the rodent, chimeric, and humanized antibodies can be tested using a biosensor-based analytical system capable of determining their affinities for antigen by measuring the rates of association and dissociation (19).

RESULTS

An example of a humanized antibody that was designed and constructed at the MRC Collaborative Centre is the mouse TES-C21 (C21) antibody (19). Mouse C21 antibody recognizes an epitope on human IgE and has potential as a therapeutic agent in patients with IgE-mediated allergies such as hay fever, food and drug allergies, and extrinsic asthma. In order to maximize the potential of mouse C21 as a therapeutic agent for use in humans, the mouse antibody was humanized by CDR grafting using the methods outlined. A molecular

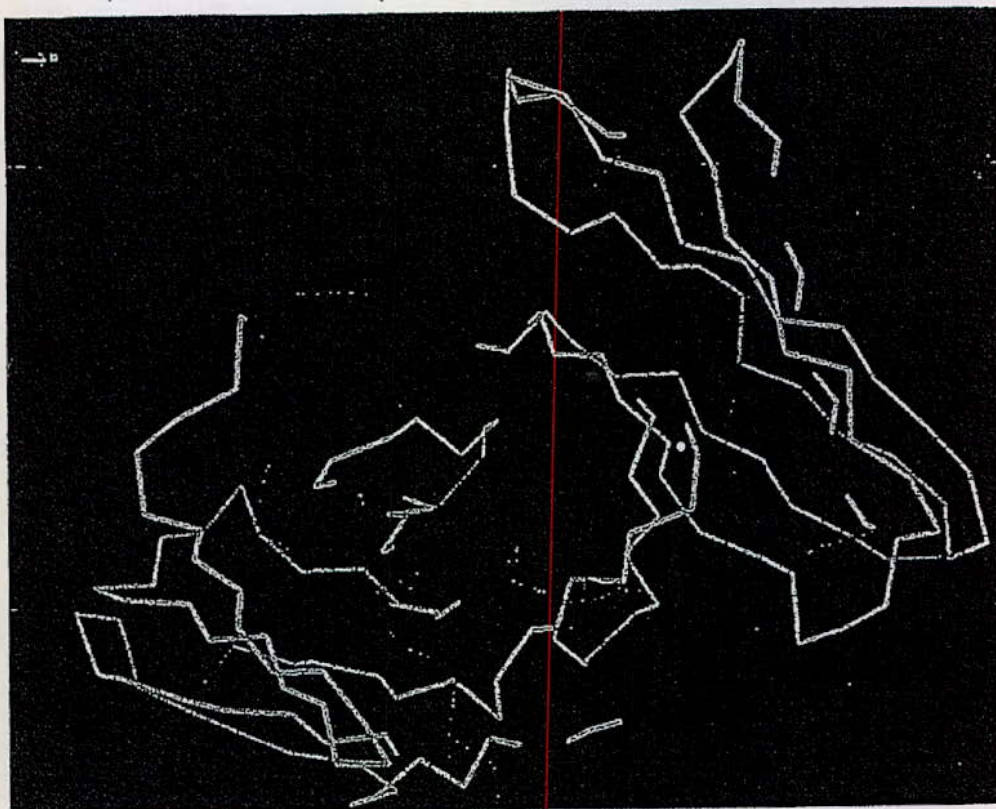


FIG. 4. View of the molecular model of the variable regions of mouse C21 antibody. The model is presented as an α -carbon trace with the CDRs of the light chain in blue and the CDRs of the heavy chain in green. The FRs are mainly in yellow, with seven of the residues in the FRs in red. In one fully humanized C21 antibody, substitutions were made in the FRs of the selected human variable regions at these seven positions (19).

model of the mouse C21 variable regions was built based on portions of the solved structures of three antibodies, the light chain variable region of mouse HyHEL-10 antibody, the heavy chain variable region of mouse HyHEL-5 antibody, and the CDR3 of the Bence-Jones protein RHE (Fig. 4).

Amino acid sequence comparisons were carried out. The mouse C21 light chain variable region was most similar to human κ light chain variable regions from subgroup III and, in particular, to the light chain variable region from human KAF antibody. The mouse C21 heavy chain variable region was most similar to human heavy chain variable regions from subgroup I and, in particular, to the heavy chain variable region from human HAY antibody. Based on these sequence comparisons, three humanized C21 light chain variable regions were designed based on the FRs from human KAF light chain variable region and the CDRs from the mouse

C21 light chain variable region. For the humanized C21 heavy chain variable region, two humanized heavy chain variable regions were designed based on the FRs from the consensus sequence for human subgroup I heavy chain variable regions and CDRs from mouse C21 heavy chain variable region. Another two humanized heavy chain variable regions were designed based on the FRs from human HAY heavy chain variable region and CDRs from mouse C21 heavy chain variable region.

Differences between the amino acid sequences of the human and mouse FRs were noted and examined. As described previously, the model is important at this stage in evaluating whether a given amino acid substitution at a given position in the structure will alter binding to antigen. In the design of humanized C21 light chain variable region, four amino acids in the human FRs were identified as having a possible nega-

tive influence on antigen binding. In the three versions of humanized C21 variable region, the amino acids at these four positions were changed, in various combinations, from the amino acids present in the human FRs to those present in the mouse FRs. Similarly, in the design of humanized C21 heavy chain variable region, 5 or 8 amino acids in the human FRs of the consensus sequence or the human HAY sequence were identified as having a possible negative influence on antigen binding. Again, the amino acids at these positions were changed, in various combinations, from the amino acids present in the human FRs to those present in the mouse FRs. In summary, three versions of humanized C21 light chain variable region that contained from two to four amino acid changes in the human FRs were designed, and four versions of humanized C21 heavy chain variable region that contained from one to eight amino acid changes in the human FRs were designed.

The initial humanized C21 light and heavy chain variable regions were constructed using PCR methods to assemble six synthetic oligonucleotides per leader-variable region. Subsequent versions of the humanized variable regions were constructed using PCR primers to introduce small changes in the initial constructs. Following DNA sequencing to confirm that the sequences were correct, the humanized variable regions were joined via introns to human κ and γ -1 constant regions already present in appropriate mammalian cell expression vectors. The light and heavy chain expression vectors were cotransfected into COS cells and humanized antibody was secreted into the culture medium. In early experiments, humanized C21 antibodies in the culture medium were analyzed by ELISA for binding to human IgE. In later experiments, Protein A-purified humanized C21 antibodies were analyzed for binding to human IgE using a biosensor-based analytical system (Pharmacia BIAcore).

Several combinations of reshaped human C21 light and heavy chain variable regions gave binding to human IgE that was equivalent to the binding observed with the mouse and chimeric C21 antibodies. For the heavy chain variable region, designs based both on the human consensus sequence and on an individual human antibody led to humanized C21 antibodies that bound to antigen as well as the mouse C21 antibody. One of the best humanized C21 antibodies was designed based on the FRs from the light chain variable region of human KAF antibody and the FRs from the heavy chain variable region of human HAY antibody. This humanized C21 antibody had three changes in the human FRs of the light chain variable region and four changes in the human FRs of the heavy chain variable region. In the view of the model of the mouse C21 variable regions shown in Fig. 4, the amino acids at these seven positions in the FRs are colored red.

Humanized C21 antibody is 1 of 12 different mouse

antibodies that have been humanized at the MRC Collaborative Centre. A recent review of published papers and patents identified 80 rodent antibodies that have been humanized by CDR grafting. These fully humanized antibodies are listed by the antigens that they recognize (Table 1). An attempt has been made to evaluate the success of each humanization in terms of how well the humanized antibody bound to antigen in comparison to the original rodent antibody. Given that a wide variety of methods of varying reliability were used to measure binding to antigen, these values must be viewed cautiously and the accuracy examined case by case.

DISCUSSION

Antibodies to a wide range of antigens have been successfully humanized by CDR grafting as judged by the creation of antibodies with human-like sequences that bind to their respective antigens with specificities and affinities that closely mimic those of the original rodent antibodies. The main motivation in humanizing a rodent antibody is to create an antibody that will have improved efficacy when used as a therapeutic agent in humans. It is difficult to cite proven examples where humanization via CDR grafting has created clinically efficacious agents because there is still a very limited amount of published data available on clinical trials with CDR-grafted antibodies. Data are available for reshaped human CAMPATH-1 antibody (16). This antibody recognizes the CDw52 antigen present on all lymphocytes and some monocytes and is proposed as a treatment for lymphoproliferative disorders and as an immunosuppressive agent. The fully humanized CAMPATH-1 antibody has been tested in patients with non-Hodgkin's lymphoma, systemic vasculitis, and refractory rheumatoid arthritis (72, 4, 3). Efficacy was observed in a significant percentage of all three groups of patients. In arthritic patients treated with multiple doses, an anti-idiotypic response was often detected. The anti-idiotypic response, however, did not prevent a beneficial effect (3). Several other fully humanized antibodies, such as anti-IL-2R antibody (15), anti-EGFR antibody (46), and anti-TNF α antibody (57), are in clinical trials but no results have been published to date.

From the limited amount of clinical data available, it is clear that fully humanized antibodies are significantly less immunogenic than rodent antibodies. This means that the humanized antibodies will have longer half-lives in patients and, therefore, will be efficacious at lower doses and with fewer doses. In addition, it will be possible to re-treat patients with the humanized antibodies. The presence of human constant regions

TABLE 1
List of Fully Humanized Antibodies Grouped by the Antigen Recognized

Antigen recognized	Antibody humanized	Place where humanized	Binding to antigen relative to the rodent antibody	Reference
CD2	Rat YTH655	Cambridge University/Wellcome	"Equivalent"	20
CD3	Rat YTH12.5	Cambridge University/Wellcome	87%	21
	Mouse OKT3	Celltech/Ortho	"Similar"	22
	Mouse UCHT1	Genentech	"Close"	23, 24
CD4	Mouse OKT4A	Celltech/Ortho	68%	25
	Rat YNB46.1.8SG2B1.19 (Campath-9)	Cambridge University/Wellcome	33%	26
CD18 ($\beta 2$ integrin)	Mouse 1B4	Merck Sharp Dohme	100%	27
	Mouse H52	Genentech	300%	28
	Mouse 60.3	Bristol-Myers Squibb	"Similar"	29
	Rat YFC51.1.1	Cambridge University/Wellcome	Binds	30
	Mouse antibody	Protein Design Labs	No data	31
CD22	Mouse LL2	Immunomedics	No data	32
CD28	Rat YTH912.13	Wellcome	No data	33
CD33	Mouse M195	Protein Design Labs	300%	34, 35
	CDP771	Celltech/American Cyanamid	No data	31
CD41a (GPIIb/IIIa)	C4G1	Protein Design Labs/Yamanouchi	—	36
CD49a ($\alpha 4$ integrin)	Hpl/2	Scotgen/Biogen	No data	31
$\alpha 4\beta 1$ integrin	Mouse 21.6	MRC Collaborative Centre/Athens Neurosciences	"Equal"	37
CDw62 (Campath-1)	Rat YTH34.5HL	MRC Laboratory of Molecular Biology/Wellcome	33%	16
CD54 (ICAM-1)	Mouse R6-5-D6 (BIRR-0001)	Celltech/Boehringer Ingelheim	50-75%	38
CD56 (NCAM)	N901	University of Bath/ImmunoGen Inc.	"Identical"	39
CD64	O22	Scotgen/Medarex	No data	31
CD68c (NCA90)	MN3	Immunomedics	No data	31
L-selectin (CD62L)	Mouse DREG-200	Protein Design Labs	"Approximately equal"	31
P-selectin	Mouse CY1747	MRC Collaborative Centre/Cytel	"Comparable"	40
IL-2 receptor	Mouse B-B10	Sumitomo	"Nearly the same"	41
IL-2 receptor (CD25)	Anti-Tac	Protein Design Labs/Roche	33%	15
	Rat YTH906.9.21	Wellcome/MRC Laboratory of Molecular Biology	No data	42
IL-2 receptor (CD122)	Mouse M18/1	Protein Design Labs/Roche	"Equivalent"	43
IL-6 receptor	Mouse PM-1	MRC Collaborative Centre/Chugai	100%	44
	Mouse AUK12-20	MRC Collaborative Centre/Chugai	100%	45
EGF receptor	Mouse 425	MRC Collaborative Centre/E. Merck	60%	11
	Mouse mAb4D5	Genentech	300%	46
T-cell receptor (α/β)	Mouse BMA 031	Genzyme Corp./Behringwerke	40%	47
T-cell receptor ($V\beta$ 8.1)	16G8	Scotgen/T Cell Sciences	No data	31
T-cell receptor ($V\beta$ 5.2, 5.3)	TM23	Scotgen/T Cell Sciences	No data	31
CEA (carcinoembryonic antigen)	Mouse BW431/26	MRC Laboratory of Molecular Biology/Behringwerke	40%	48, 49
	Mouse A5B7	Celltech	60%	50
	Mouse CEM231	Hybritech	100%	51
	Mouse MN-14	Scotgen/Immunomedics	—	52
	NP-14	Immunomedics	—	53
Colorectal antigen	CDP833	Celltech/American Cyanamid	No data	31

TABLE 1—Continued

Antigen recognized	Antibody humanized	Place where humanized	Binding to antigen relative to the rodent antibody	Reference
PLAP (human placental alkaline phosphatase)	Mouse H17E2	Unilever	"Less well"	54
PEM/HMPG (polymorphic epithelial mucin of human milk fat globules)	Mouse anti-HMPG	Unilever	"Similar"	55
	Mouse CTM01	Celltech	Close	56
TAG 72 (human mucin)	Mouse B72.3	Celltech	"Similar"	57
MUC-1	Mouse KC4G3	NIH	100%	58
Human mucin	MA5	Immunomedics	—	59
Prostate antigen	PAM4	Merck	—	60
Endosialin	7E11.C6.3	Scotgen/Cytogen	No data	31
	FB5	Scotgen/Ludwig Institute/Sloan Kettering	No data	31
Folate binding protein	LK26	Scotgen/Ludwig Institute/Sloan Kettering	No data	31
HIV	Mouse 0.5 β	MRC Collaborative Centre/Chemo-Sero-Therapeutic Research Institute	50%	12
Herpes	NM-01	Scotgen/Nissin Research Institute	No data	31
	Mouse Fd138-80	Protein Design Labs	"Almost equal"	61
	Mouse Fd79	Protein Design Labs	50%	61
RSV	Mouse RSV19	Scotgen	Reduced binding	62
	Unnamed antibody	MedImmune/Virion Systems	"Close"	63
CMV	Mouse CMV5	Protein Design Labs	"Approximately the same"	64
	CMV16	Scotgen	—	65
	CMV35	Scotgen	No data	31
VZV	206	Scotgen	No data	31
Junin virus	YGD	Scotgen/U.S. Army	No data	31
Vaccinia	VVI	Scotgen/U.S. Army	No data	31
<i>Pseudomonas aeruginosa</i>	PS2	Scotgen	No data	31
<i>Clostridium perfringens</i> α -toxin	3A4D10	Scotgen/Chemical and Biological Defence Establishment	No data	31
<i>Plasmodium falciparum</i>	Unnamed antibody	SmithKline Beecham	No data	66
Human TNF α	Mouse hTNF1	Celltech	"Closely similar"	57
	Mouse hTNF3	Celltech	"Binds well, competes poorly"	57
	Mouse 101.4	Celltech	10% or less	57
	Mouse 61 E71	Celltech	10%	57
Human INF- γ	Mouse AF2	Protein Design Labs	"Approximately the same"	64
IL-5	Mouse SK2	Chugai	100%	67
Lewis B antigen	58-1065	Scotgen/Ludwig Institute/Sloan Kettering	No data	31
Lewis Y antigen	Mouse SDZ ABL 364	Protein Design Labs	50%	68
	3S193	Scotgen/Ludwig Institute/Sloan Kettering	No data	31
Human IgE	Mouse C21	MRC Collaborative Centre/Ciba-Geigy	100%	19
Hapten NP-cap	Mouse MaE11	Genentech	30%	69
	Mouse B1-8	MRC Laboratory of Molecular Biology	Binds	5
Lysozyme	Mouse D1.3	MRC Laboratory of Molecular Biology	25%	70, 71

with human effector functions will also contribute to therapeutic benefit depending on the mode of action proposed for the humanized antibody. Despite the significant reduction in immunogenicity achieved by humanization via CDR grafting, an anti-idiotypic response to the fully humanized antibody will probably still be elicited after multiple administrations. This is not surprising since authentic human antibodies also have the potential to elicit anti-idiotypic responses. It is probable that significant differences in immunogenicity will exist among different fully humanized antibodies. Clinical trials with chimeric antibodies have demonstrated that mouse variable regions differ dramatically in their degree of immunogenicity (73). Ultimately, efficacy will have to be determined for each humanized antibody and each of the therapeutic indications being considered.

In summary, reliable methods exist for humanizing rodent antibodies by grafting the CDRs from rodent antibodies into human antibodies. Although methods for humanization are now well developed and many examples of fully humanized antibodies exist, there are as yet few clinical data demonstrating their efficacy in patients.

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antibody-based therapy



Humanized antibodies

Greg Winter and William J. Harris

Hybridoma technology enabled rodent monoclonal antibodies to be created against human pathogens and cells, but these had limited clinical utility. Protein engineering is now generating antibodies for treatment of infectious disease, autoimmune disease and cancer by 'humanizing' rodent antibodies. Humanized antibodies have improved pharmacokinetics, reduced immunogenicity and have been used to clinical advantage.

The antibody is an adaptor molecule containing binding sites for antigen at one end and for effector molecules at the other, and has evolved to bind to a vast range of antigens. Binding alone may be sufficient to neutralize some toxins and viruses, however, more commonly, the antibody triggers the complement system and cell-mediated killing. Although antibodies are natural therapeutic agents, it has proved difficult to make human monoclonal antibodies (mAbs) by hybridoma technology.

Rodent mAbs unfortunately have serious disadvantages: a short half-life in serum; only some of the different classes can trigger human effector functions; and the mAbs can also elicit an unwanted immune response in patients (human anti-mouse antibodies or HAMA). HAMA can result in enhanced clearance of the antibody from the serum, blocking of its therapeutic effect and hypersensitivity reactions. These problems have prompted the use of protein engineering technologies to 'humanize' rodent mAbs by transplanting antigen-binding sites from rodent to human antibodies. In principle, humanizing allows access to a large pool of well-characterized rodent mAbs for therapy, including those with specificities against human antigens that are difficult to elicit from a human immune response¹.

The engineering of antibodies is facilitated by the modular arrangement of protein domains: the heavy- and light-chain variable (V) domains are responsible for binding to antigen, and the constant domains to effector functions. As both complement and cell-mediated killing require fully glycosylated antibody, the engineered mAbs are expressed in mammalian hosts. Each antibody domain is encoded by a different genetic exon and, to build recombinant antibodies, the exons are pasted together. The exons encoding variable domains (V genes) can be cloned from the genomic DNA of a B-cell hybridoma: more conveniently, the V genes of hybridomas are isolated from the mRNA by use of the polymerase chain reaction (PCR). The V genes are readily linked to those exons encoding constant domains for expression of mAbs² (Fig. 1). Expression vectors have been built with both antibody and viral promoters and enhancers, with V and C genes as different exons ('genomic') or linked together ('cDNA'). Different markers for selection of transformed cells are available and in both myeloma and CHO hosts^{3,4} mAb expression is greatly enhanced by

amplifying the number of integrated copies, resulting in yields of up to 0.7 g/l in fermenters⁵.

Building humanized antibodies

The first generation of humanized antibodies were simple chimaeric mAbs, in which the variable domains of a rodent mAb are transplanted to the constant domains of human antibodies (Fig. 1). This reduces the immunogenicity of the rodent mAb (see below) and allows the effector functions to be selected for the therapeutic application. Thus the human $\gamma 1$ isotype appears to be the most effective for complement and cell-mediated killing, while the human $\gamma 4$ isotype appears more suitable for imaging and blocking⁶.

The second generation of humanized antibodies were the so-called complementarity determining regions (CDR) - grafted antibodies, in which the antigen-binding loops of the rodent mAb were built into a human antibody. The architecture of each antibody V-domain consists of a β -sheet sandwich surmounted by antigen-binding loops in different antibodies, these CDR loops are hypervariable in sequence (Fig. 2). It is this hypervariability that allows the antibody repertoire to bind a potentially vast array of antigens. By transplanting (or grafting) the CDRs from rodent mAb to human antibody, the antigen-binding site can also be transferred⁷; indeed the same human framework can be used for mounting different antigen-binding sites⁷⁻⁹. However, to recreate the antigen-binding site it is also necessary to consider other possible interactions between the β -sheet framework and the loops. With the help of molecular modelling it is possible to design framework substitutions that maintain key contacts with the CDR loops.

For example, with the rat antibody CAMPATH-1 directed against the CDw52 antigen of human lymphocytes, the simple grafting of the CDRs failed to transplant the binding activity to a human antibody. When the three-dimensional folding of the VH-CDR1 loop of the rat antibody and its contacts with the rat framework were modelled by computer graphics, the framework amino acid residue Phe27 was predicted to pack against the loop. However, in the human framework of the CDR-grafted antibody, Phe27 was replaced by Ser27; indeed when Ser27 was mutated to Phe in the CDR-grafted antibody, the binding activity was restored⁸. In other examples, enhancement of antigen affinity was achieved stepwise by combining several

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antibody-based therapy

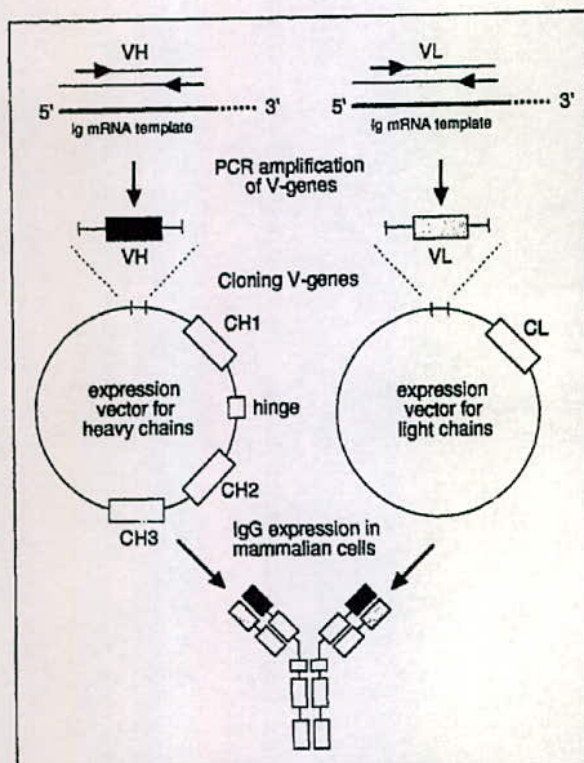


Fig. 1. Cloning of heavy and light chain V genes from the mRNA of a mouse B-cell hybridoma into vectors comprising (human) genes encoding constant domains, for expression of mouse-human chimeric antibodies in mammalian cells.

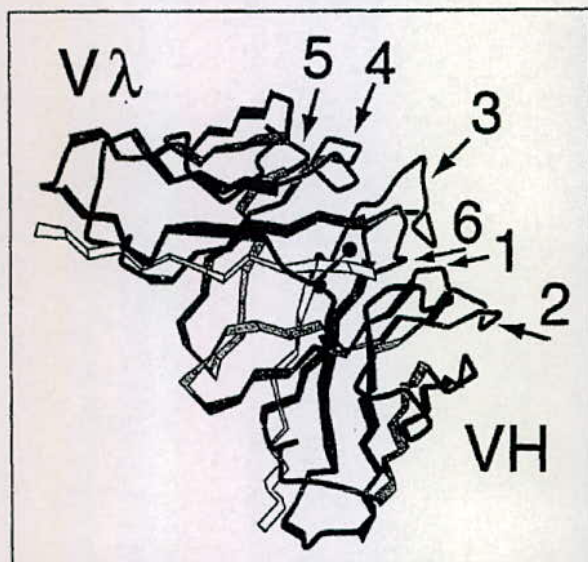


Fig. 2. The β -sheet framework structure of heavy- and light-chain variable domains with hypervariable loops 1-6. (The figure is taken from Winter, G. and Milstein, C. (1991) and is reproduced with permission from Nature 349, 293-299.)

framework substitutions^{10,11}. Indeed analysis of antibody structures is leading towards the identification of sets of framework residues that may exert an influence on CDR structure¹¹ and also on the packing of the strands of the β -sheet¹².

The first CDR-grafted antibodies were based on the known crystallographic structures of the human myeloma proteins⁷⁻⁹. CDR-grafted antibodies have also been built with consensus human frameworks based on several human heavy chains¹³. The use of a single or a limited number of human frameworks offers the prospect of a range of therapeutic antibodies that are almost identical, apart from the CDR sequences. Conversely a range of framework structures should be capable of supporting the CDR loops, and 'hyper-chimaeric' CDR-grafted antibodies have used mouse-human frameworks. For example, to humanize a mouse antibody directed against the human IL-2 receptor (anti-Tac), a human framework sequence was selected by homology. A molecular model was then used to identify those framework residues of the rodent antibody that might interact with the antigen-binding loops, and these were built into the selected human framework¹⁴ (Fig. 3). Chimaeric frameworks have also been proposed in which the internal residues that pack between the domains and with the antigen-binding loops are derived from the rodent sequence and the solvent accessible residues are taken from a human sequence¹⁵.

Most generally, all of the rodent CDRs are transplanted from mouse to human antibody. However, some CDRs are more important than others for binding of antigen, as evident from the crystallographic structures of antibody-antigen complexes. The interaction of antibody loops with antigen involves both main-chain and side-chain contacts: as the CDR loops of mouse and human antibodies fold in a limited number of ways¹⁶, it is possible to maintain some main-chain contacts while varying some of the side chains (and sequence) of the CDRs¹⁷.

There is some loss in binding affinity on CDR-grafting but, in combination with some framework alterations, it is usually possible to obtain a reshaped antibody with an affinity within three fold of the parent monoclonal antibody. High binding affinities may be critical for neutralization of a cytokine or toxin in the serum; they appear to be less important where multiple interactions can occur with high avidities, as with multimeric (cell surface) antibody binding to repeated epitopes on a viral coat⁹. However, small improvements in affinity have been seen for some CDR-grafted antibodies¹⁸, and binding affinities can also be improved *in vitro*, by chain shuffling¹⁹, or random mutation²⁰.

Using humanized antibodies

Both chimaeric and CDR-grafted antibodies appear to have better pharmacokinetics than rodent mAbs, with extended serum half-life (>75 hours) in humans and cynomolgus monkeys. Likewise the immunogenicity is reduced. Much of the HAMA response to mouse antibodies in patients is directed against the constant region; chimaeric antibodies and CDR-grafted antibodies appear to elicit much less response with

antibody-based therapy

the immunogenic epitopes being located in the variable regions. Indeed much of this response is directed against the antigen-binding site (for review see Ref. 4).

No antibody response was detected against the CDR-grafted anti-CDw52 antibody during the treatment of two patients with non-Hodgkin's lymphoma for up to 43 days with escalating doses of antibody ranging from 1 to 20 mg/day²¹. Also, no response was reported when the antibody was used in a single course of therapy for rheumatoid arthritis patients²² or in conjunction with an anti-CD4 antibody in treatment of a patient with intractable systemic vasculitis²³. However, an antibody response was detected on further treatment of the rheumatoid patients²². Antibody responses were not detected against other CDR-grafted antibodies used for radio-imaging in tumour patients²⁴ or treating acute graft-versus-host disease²⁵.

Chimaeric and CDR-grafted antibodies have been constructed against a wide range of viral and bacterial pathogens, and against human cell surface markers including tumour cell antigens. Some of the targets are summarized in Table 1. CDR-grafted antibodies against lymphocyte markers have already been used to clinical advantage. The anti-CDw52 antibody was used to deplete a large tumour mass in two lymphoma patients, and to achieve clinical remission²¹. Likewise, this antibody resulted in significant clinical benefit for a patient with systemic vasculitis²³, and for rheumatoid arthritis patients²². The anti-Tac antibody was used for immunosuppression following allogeneic marrow transplantation, and resulted in improvement in several cases of acute graft-versus-host disease²⁵.

Future prospects

As shown above, humanized antibodies can be engineered from rodent mAbs. Their use has been demonstrated in the clinic, and they have a longer serum half-life and reduced immunogenicity compared to rodent mAbs. They therefore appear to be promising as therapeutics, especially for a single course of treatment. It is not yet clear whether humanized (or even human) antibodies will elicit a blocking immune response over longer or several courses of treatment.

The immunogenicity of humanized antibodies is likely to depend on several factors, including the immune state of the patients, and the dose and regime of antibody administration. The target is also likely to be important; antibodies directed against cell-bound antigens might be expected to be more immunogenic than those binding to soluble antigen. Furthermore, since foreign framework regions can elicit an immune response²⁶, we might also expect that the differing strategies to select and mutate human frameworks, as above, could lead to reshaped antibodies with differing immunogenicity. For example, human antibody frameworks that are mutated (*in vivo* or *in vitro*) with respect to the germ-line segments could prove immunogenic: even buried residues could form the critical element of a T-cell epitope if presented as a denatured peptide by a class II MHC molecule²⁷. Indeed it may be desirable to design CDR-grafted antibodies by using framework regions based on human germ-line V-gene segments.

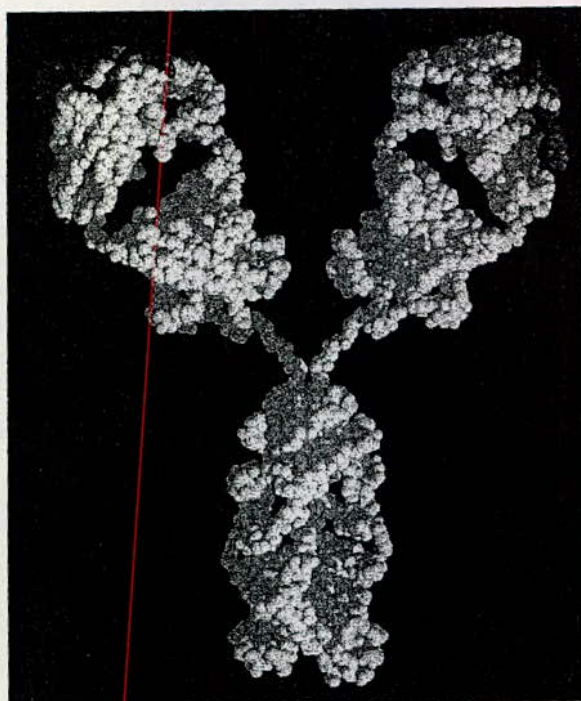


Fig. 3. Computer model of the main-chain backbone of the humanized anti-Tac antibody, with the CDRs in red and altered framework residues in blue (the figure is from prospectus 28 Jan. 1992 of Protein Design Laboratories and reproduced with permission by L. Korn).

The design of antibodies for therapy would certainly be revolutionised if *in vitro* assays were available to test the immunogenicity of different constructs. However the key practical issues are not whether the immune response can be avoided entirely, but how it

Table 1. CDR-grafted antibodies for therapy (see also Ref. 3)

Target	Clinical potential
CDw52	lymphomas, systemic vasculitis, rheumatoid arthritis
CD3	organ transplantation
CD4	organ transplants, rheumatoid arthritis, Crohn's disease
IL-2 receptor	leukaemias and lymphomas, organ transplants, graft-versus-host disease
TNF- α	septic shock
HIV	AIDS
RSV	respiratory syncytial virus infection
HSV	neonatal, ocular and genital herpes infection
Lewis-Y	cancer
p185 ^{HER2}	cancer
PLAP	cancer
CEA	cancer

TNF: tumour necrosis factor; HIV: human immunodeficiency virus; RSV: rous sarcoma virus; HSV: herpes simplex virus; p185-HER-2: human epidermal growth factor receptor 2; PLAP: placental alkaline phosphatase; CEA: carcinoembryonic antigen.

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can be bypassed, for example by changing the idiotype of the engineered antibody, and whether the antibodies can be used for long enough to achieve clinical benefit.

So far we have focussed almost entirely on the construction and use of glycosylated antibodies expressed in mammalian cells. However, the use of antibody fragments may be advantageous in some applications, since they penetrate tissues more readily, and are cleared more rapidly from the serum. This may help in neutralizing and clearing drugs from the serum, or in imaging tumours with radioactive entities coupled to the fragments¹. Although antibody fragments, lacking the glycosylated Fc portion, cannot trigger effector functions, they could in principle be equipped to do so, for example by chemically linking Fab fragments together²⁸ as bispecific antibody fragments (with one arm binding a tumour cell antigen and the other binding and triggering effector cells such as cytotoxic T cells or monocytes).

Furthermore, antibody fragments can be expressed by secretion from bacteria^{29,30}, and can be readily derived from the V genes of hybridomas, or from V gene repertoires. The repertoires are cloned for display on the surface of filamentous bacteriophage by fusion of the encoded antibody fragment to a coat protein of the phage, and phage with the desired activities selected by binding to antigen. Indeed this technology mimics the strategy of immune selection, and human antibody fragments with specificities against many different foreign and human self-antigens have been isolated from the same 'single pot' of phages (see Refs 31, 32 for review).

Over the past century we have seen three generations of antibody therapeutics: polyclonal animal antibodies, rodent monoclonal antibodies and now humanized antibodies. We anticipate that the use of 'repertoire selection' technologies to make human antibodies and fragments will provide the next generation. However in the meantime it seems likely that humanized antibodies will prove clinically useful for treating several diseases, and the experience should prove valuable for designing and formulating the next generation of antibody therapeutics.

Greg Winter is at the MRC Laboratory of Molecular Biology and the MRC Centre for Protein Engineering, Hills Road, Cambridge, UK CB2 2QH; William Harris is at the Dept of Molecular and Cell Biology, Marischal College, University of Aberdeen, Aberdeen, UK AB9 1AS.

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