

An internal HindIII site was not included in this adapter, to differentiate the two chimeric light chain genes.

The variable region fragment was isolated as a 376 bp EcoRI-AvaI fragment. The oligonucleotide linker was ligated to NarI cut pNW361 and then the adapted 396bp constant region was isolated after recutting the modified pNW361 with EcoRI. The variable region fragment and the modified constant region fragment were ligated directly into EcoRI/C1P treated pEE6hCMVneo to yield pJA137. Initially all clones examined had the insert in the incorrect orientation. Therefore, the insert was re-isolated and recloned to turn the insert round and yield plasmid pJA141. Several clones with the insert in the correct orientation were obtained and the adapter sequence of one was confirmed by DNA sequencing

9.3. HEAVY CHAIN GENE CONSTRUCTION

9.3.1. CHOICE OF HEAVY CHAIN GENE ISOTYPE

The constant region isotype chosen for the heavy chain was human IgG4.

9.3.2. GENE CONSTRUCTION

A The heavy chain cDNA sequence showed a BanI site near the 3' end of the variable region [Fig. 2(a)].^(SER ID NO:6)
The majority of the sequence of the variable region was isolated as a 426bp. EcoRI/C1P/BanI fragment. An oligonucleotide adapter was designated to replace the remainder of the 3' region of the variable region from the BanI site up to and including a unique HindIII site which had been previously engineered into the first two amino acids of the constant region.

The linker was ligated to the V_H fragment and the EcoRI-HindIII adapted fragment was purified from the ligation mixture.

The variable region was ligated to the constant region by cutting pJA91 with EcoR1 and Hind111 removing the intron fragment and replacing it with the V_H to yield pJA142. Clones were isolated after transformation into E.coli JM101 and the linker and junction sequences were confirmed by DNA sequencing. (N.B. The Hind111 site is lost on cloning).

10. CONSTRUCTION OF CHIMERIC EXPRESSION VECTORS

10.1. neo AND gpt VECTORS

The chimeric light chain (version 1) was removed from pJA143 as an EcoR1 fragment and cloned into EcoR1/C1P treated pEE6hCMVneo expression vector to yield pJA145. Clones with the insert in the correct orientation were identified by restriction mapping.

The chimeric light chain (version 2) was constructed as described above.

The chimeric heavy chain gene was isolated from pJA142 as a 2.5Kbp EcoR1/BamH1 fragment and cloned into the EcoR1/Bcl1/C1P treated vector fragment of a derivative of pEE6hCMVgpt to yield plasmid pJA144.

10.2. GS SEPARATE VECTORS

GS versions of pJA141 and pJA144 were constructed by replacing the neo and gpt cassettes by a BamH1/Sall/C1P treatment of the plasmids, isolation of the vector fragment and ligation to a GS-containing fragment from the plasmid pR049 to yield the light chain vector pJA179 and the heavy chain vector pJA180.

10.3. GS SINGLE VECTOR CONSTRUCTION

Single vector constructions containing the cL (chimeric light), cH (chimeric heavy) and GS genes on one plasmid in the order cL-cH-GS, or cH-cL-GS

and with transcription of the genes being head to tail e.g. cL>CH>GS were constructed. These plasmids were made by treating pJA179 or pJA180 with BamH1/C1P and ligating in a Bgl111/Hind111 hCMV promoter cassette along with either the Hind111/BamH1 fragment from pJA141 into pJA180 to give the cH-cL-GS plasmid pJA182 or the Hind111/BamH1 fragment from pJA144 into pJA179 to give the cL-cH-GS plasmid pJA181.

11. EXPRESSION OF CHIMERIC GENES

11.1. EXPRESSION IN COS CELLS

The chimeric antibody plasmid pJA145 (cL) and pJA144 (cH) were co-transfected into COS cells and supernatant from the transient expression experiment was shown to contain assembled antibody which bound to the HUT 78 human T-cell line. Metabolic labelling experiments using ³⁵S methionine showed expression and assembly of heavy and light chains. However the light chain mobility seen on reduced gels suggested that the potential glycosylation site was being glycosylated. Expression in COS cells in the presence of tunicamycin showed a reduction in size of the light chain to that shown for control chimeric antibodies and the OKT3 mouse light chain. Therefore JA141 was constructed and expressed. In this case the light chain did not show an aberrant mobility or a size shift in the presence or absence of tunicamycin. This second version of the chimeric light chain, when expressed in association with chimeric heavy (cH) chain, produced antibody which showed good binding to HUT 78 cells. In both cases antigen binding was equivalent to that of the mouse antibody.

11.2 EXPRESSION IN CHINESE HAMSTER OVARY (CHO) CELLS
Stable cell lines have been prepared from plasmids PJA141/pJA144 and from pJA179/pJA180, pJA181 and pJA182 by transfection into CHO cells.

12. CDR-GRAFTING

The approach taken was to try to introduce sufficient mouse residues into a human variable region framework to generate antigen binding activity comparable to the mouse and chimeric antibodies.

12.1. VARIABLE REGION ANALYSIS

From an examination of a small database of structures of antibodies and antigen-antibody complexes it is clear that only a small number of antibody residues make direct contact with antigen. Other residues may contribute to antigen binding by positioning the contact residues in favourable configurations and also by inducing a stable packing of the individual variable domains and stable interaction of the light and heavy chain variable domains.

The residues chosen for transfer can be identified in a number of ways:

- (a) By examination of antibody X-ray crystal structures the antigen binding surface can be predominantly located on a series of loops, three per domain, which extend from the B-barrel framework.
- (b) By analysis of antibody variable domain sequences regions of hypervariability [termed the Complementarity Determining Regions (CDRs) by Wu and Kabat (ref. 5)] can be identified. In the most but not all cases these CDRs correspond to, but extend a short way beyond, the loop regions noted above.

(c) Residues not identified by (a) and (b) may contribute to antigen binding directly or indirectly by affecting antigen binding site topology, or by inducing a stable packing of the individual variable domains and stabilising the inter-variable domain interaction. These residues may be identified either by superimposing the sequences for a given antibody on a known structure and looking at key residues for their contribution, or by sequence alignment analysis and noting "idiosyncratic" residues followed by examination of their structural location and likely effects.

12.1.1. LIGHT CHAIN ²⁹
 (SEQ ID NO: 8 And 9)

Figure 3 shows an alignment of sequences for the human framework region RE1 and the OKT3 light variable region. The structural loops (LOOP) and CDRs (KABAT) believed to correspond to the antigen binding region are marked. Also marked are a number of other residues which may also contribute to antigen binding as described in 13.1(c). Above the sequence in Figure 3 the residue type indicates the spatial location of each residue side chain, derived by examination of resolved structures from X-ray crystallography analysis. The key to this residue type designation is as follows:

- N - near to CDR (From X-ray Structures)
- P - Packing
- S - Surface
- I - Interface
- Packing/Part Exposed
- ? - Non-CDR Residues which may require to be left as Mouse sequence.
- B - Buried Non-Packing
- E - Exposed
- * - Interface

A S
 A A S
 A S

13964

29
(SEQ ID NO: 8 and 9)

A J
B

Residues underlined in Figure 3₁ are amino acids. (SEQ ID NO: 8 and 9)
REI₁ was chosen as the human framework because the light chain is a kappa chain and the kappa variable regions show higher homology with the mouse sequences than a lambda light variable region, e.g. KOL₁ (see below). (SEQ ID NO: 10) (SEQ ID NO: 8 and 9)
REI₁ was chosen in preference to another kappa light chain because the X-ray structure of the light chain has been determined so that a structural examination of individual residues could be made.

A

12.1.2. HEAVY CHAIN

Similarly Figure 4 shows an alignment of sequences for the human framework region KOL₁ and the OKT3₁ heavy variable region. (SEQ ID NO: 10) (SEQ ID NO: 7)
The structural loops and CDRs believed to correspond to the antigen binding region are marked. Also marked are a number of other residues which may also contribute to antigen binding as described in 12.1(c). The residue type key and other indicators used in Figure 4 are the same as those used in Figure 3.
KOL₁ was chosen as the heavy chain framework because the X-ray structure has been determined to a better resolution than, for example, NEWM and also the sequence alignment of OKT3 heavy variable region₁ showed a slightly better homology to KOL₁ (SEQ ID NO: 10) (SEQ ID NO: 10) than to NEWM.

J A

A

A

12.2. DESIGN OF VARIABLE GENES

The variable region domains were designed with mouse variable region optimal codon usage [Grantham and Perrin (ref. 15)] and used the B72.3 signal sequences [Whittle et al (ref. 13)]. The sequences were designed to be attached to the constant region in the same way as for the chimeric genes described above. Some constructs contained the "Kozak consensus sequence" [Kozak (ref. 16)] directly linked to the 5' of the signal

sequence in the gene. This sequence motif is believed to have a beneficial role in translation initiation in eukaryotes.

12.3. GENE CONSTRUCTION

To build the variable regions, various strategies are available. The sequence may be assembled by using oligonucleotides in a manner similar to Jones et al (ref. 17) or by simultaneously replacing all of the CDRs or loop regions by oligonucleotide directed site specific mutagenesis in a manner similar to Verhoeyen et al (ref. 2). Both strategies were used and a list of constructions is set out in Tables 1 and 2 and Figures 4 and 5. ^{a-c} It was noted in several cases that the mutagenesis approach led to deletions and rearrangements in the gene being remodelled, while the success of the assembly approach was very sensitive to the quality of the oligonucleotides.

13. CONSTRUCTION OF EXPRESSION VECTORS

Genes were isolated from M13 or SP65 based intermediate vectors and cloned into pEE6hCMVneo for the light chains and pEE6hCMVgpt for the heavy chains in a manner similar to that for the chimeric genes as described above.

TABLE 1 CDR-GRAFTED GENE CONSTRUCTS

CODE	MOUSE SEQUENCE CONTENT	METHOD OF CONSTRUCTION	KOZAK SEQUENCE	
			-	+
<hr/>				
LIGHT CHAIN	ALL HUMAN FRAMEWORK REL			
121	26-32, 50-56, 91-96 inclusive	SDM and gene assembly	+	n.d.
121A	26-32, 50-56, 91-96 inclusive +1, 3, 46, 47	Partial gene assembly	n.d.	+
121B	26-32, 50-56, 91-96 inclusive + 46, 47	Partial gene assembly	n.d.	+
221	24-24, 50-56, 91-96 inclusive	Partial gene assembly	+	+
221A	24-34, 50-56, 91-96 inclusive +1, 3, 46, 47	Partial gene assembly	+	+
221B	24-34, 50-56, 91-96 inclusive +1, 3	Partial gene assembly	+	+
221C	24-34, 50-56, 91-96 inclusive	Partial gene assembly	+	+
HEAVY CHAIN	ALL HUMAN FRAMEWORK KOL			
121	26-32, 50-56, 95-100B inclusive	Gene assembly	n.d.	+
131	26-32, 50-58, 95-100B inclusive	Gene assembly	n.d.	+
141	26-32, 50-65, 95-100B inclusive	Partial gene assembly	+	n.d.
321	26-35, 50-56, 95-100B inclusive	Partial gene assembly	+	n.d.
331	26-35, 50-58, 95-100B inclusive	Partial gene assembly	+	
		Gene assembly		+
341	26-35, 50-65, 95-100B inclusive	SDM	+	
		Partial gene assembly		+
341A	26-35, 50-65, 95-100B inclusive +6, 23, 24, 48, 49, 71, 73, 76,	Gene assembly	n.d.	+
	78, 88, 91 (+63 = human) (SER 79 NO: 8-28)			
341B	26-35, 50-65, 95-100B inclusive + 48, 49, 71, 73, 76, 78, 88, 91 (+63 + human)	Gene assembly	n.d.	+

KEY

n.d. not done
SDM Site directed mutagenesis
Gene assembly Variable region assembled entirely from oligonucleotides
Partial gene assembly Variable region assembled by combination of restriction fragments either from other genes originally created by SDM and gene assembly or by oligonucleotide assembly of part of the variable region and reconstruction with restriction fragments from other genes originally created by SDM and gene assembly

14. EXPRESSION OF CDR-GRAFTED GENES

14.1. PRODUCTION OF ANTIBODY CONSISTING OF GRAFTED LIGHT (gL) CHAINS WITH MOUSE HEAVY (mH) OR CHIMERIC HEAVY (cH) CHAINS

All gL chains, in association with mH or cH produced reasonable amounts of antibody.

Insertion of the Kozak consensus sequence at a position 5' to the ATG (kgL constructs) however, led to a 2-5 fold improvement in net expression. Over an extended series of experiments expression levels were raised from approximately 200ng/ml to approximately 500 ng/ml for kgL/cH or kgL/mH combinations.

When direct binding to antigen on HUT 78 cells was measured, a construct designed to include mouse sequence based on loop length (gL121) did not lead to active antibody in association with mH or cH.

A construct designed to include mouse sequence based on Kabat CDRs (gL221) ^(SEQ ID NO: 20) demonstrated some weak binding in association with mH or cH. However, when framework residues 1, 3, 46, 47 were changed from the human to the murine OKT3 equivalents based on the arguments outlined in Section 12.1 antigen binding was demonstrated when both of the new constructs, which were termed 121A and 221A were co-expressed with cH. When the effects of these residues were examined in more detail, it appears that residues 1 and 3 are not major contributing residues as the product of the gL221B ^(SEQ ID NO: 27) gene shows little detectable binding activity in association with cH. The light chain product of gL221C, ^(SEQ ID NO: 28) in which mouse sequences are present at 46 and 47, shows good binding activity in association with cH.

14.2 PRODUCTION OF ANTIBODY CONSISTING OF GRAFTED HEAVY (gH) CHAINS WITH MOUSE LIGHT (mL) OR CHIMERIC LIGHT (cL) CHAINS

Expression of the gH genes proved to be more difficult to achieve than for gL. First, inclusion of the Kozak sequence appeared to have no marked effect on expression of gH genes. Expression appears to be slightly improved but not to the same degree as seen for the grafted light chain.

Also, it proved difficult to demonstrate production of expected quantities of material when the loop choice (amino acid 26-32) for CDR1 is used, e.g. gH121, 131, 141 and no conclusions can be drawn about these constructs.

Moreover, co-expression of the gH341 gene with cL or mL has been variable and has tended to produce lower amounts of antibody than the cH/cL or mH/mL combinations. The alterations to gH341 to produce gH341A and gH341B lead to improved levels of expression.

This may be due either to a general increase in the fraction of mouse sequence in the variable region, or to the alteration at position 63 where the residue is returned to the human amino acid Valine (Val) from Phenylalanine (Phe) to avoid possible internal packing problems with the rest of the human framework. This arrangement also occurs in gH331 and gH321.

When gH321 or gH331 were expressed in association with cL, antibody was produced but antibody binding activity was not detected.

When the more conservative gH341 gene was used antigen binding could be detected in association with cL or mL, but the activity was only marginally above the background level.

When further mouse residues were substituted based on the arguments in 12.1, antigen binding could be clearly demonstrated for the antibody produced when kgH341A and kgH341B were expressed in association with cL.

14.3 PRODUCTION OF FULLY CDR-GRAFTED ANTIBODY

The kgL221A gene was co-expressed with kgH341, kgH341A or kgH341B. For the combination kgH221A/kgH341 very little material was produced in a normal COS cell expression.

For the combinations kgL221A/kgH341A or kgH221A/kgH341B amounts of antibody similar to gL/CH was produced.

In several experiments no antigen binding activity could be detected with kgH221A/gH341 or kgH221A/kgH341 combinations, although expression levels were very low.

Antigen binding was detected when kgL221A/kgH341A or kgH221A/kgH341B combinations were expressed. In the case of the antibody produced from the kgL221A/kgH341A combination the antigen binding was very similar to that of the chimeric antibody.

An analysis of the above results is given below.

15. DISCUSSION OF CDR-GRAFTING RESULTS

In the design of the fully humanised antibody the aim was to transfer the minimum number of mouse amino acids that would confer antigen binding onto a human antibody framework.

15.1. LIGHT CHAIN

15.1.1. EXTENT OF THE CDRs

For the light chain the regions defining the loops known from structural studies of other antibodies to contain the antigen contacting residues, and

those hypervariable sequences defined by Kabat et al (refs. 4 and 5) as Complementarity Determining Regions (CDRs) are equivalent for CDR2. For CDR1 the hypervariable region extends from residues 24-34 inclusive while the structural loop extends from 26-32 inclusive. In the case of OKT3 ^(SEQ ID NO 5) there is only one amino acid difference between the two options, at amino acid 24, where the mouse sequence is a serine and the human framework RE1 has glutamine. For CDR3 the loop extends from residues 91-96 inclusive while the Kabat hypervariability extends from residues 89-97 inclusive. For OKT3 amino acids 89, 90 and 97 ²⁹ are the same between OKT3 and RE1 (Fig. 3) ^(SEQ ID NO 8 And 9). When constructs based on the loop choice for CDR1 (gL121) and the Kabat choice (gL221) were made and co-expressed with mH or cH no evidence for antigen binding activity could be found for gL121, but trace activity could be detected for the gL221, suggesting that a single extra mouse residue in the grafted variable region could have some detectable effect. Both gene constructs were reasonably well expressed in the transient expression system.

15.1.2. FRAMEWORK RESIDUES

The remaining framework residues were then further examined, in particular amino acids known from X-ray analysis of other antibodies to be close to the CDRs and also those amino acids which in OKT3 showed differences from the consensus framework for the mouse subgroup (subgroup VI) to which OKT3 shows most homology. Four positions 1, 3, 46 and 47 were identified and their possible contribution was examined by substituting the mouse amino acid for the human amino acid at each position. Therefore gL221A (gL221 + D1Q, Q3V, L46R, L47W,

see Figure 3 and Table 1) was made, cloned in EE6hCMVneo and co-expressed with cH (pJA144). The resultant antibody was well expressed and showed good binding activity. When the related genes gL221B^(SEQ ID NO:28) (gL221 + D1Q, Q3V) and gL221C (gL221 + L46R, L47W) were made and similarly tested, while both genes produced antibody when co-expressed with cH, only the gL221C/cH combination showed good antigen binding. When the gL121A (gL121 + D1Q, Q3V, L46R, L47W) gene was made and co-expressed with cH, antibody was produced which also bound to antigen.

15.2. HEAVY CHAIN

15.2.1. EXTENT OF THE CDRs

For the heavy chain the loop and hypervariability analyses agree only in CDR3. For CDR1 the loop region extends from residues 26-32 inclusive whereas the Kabat CDR extends from residues 31-35 inclusive. For CDR2 the loop region is from 50-58 inclusive while the hypervariable region covers amino acids 50-65 inclusive. Therefore humanised heavy chains were constructed using the framework from antibody KOL and with various combinations of these CDR choices, including a shorter choice for CDR2 of 50-56 inclusive as there was some uncertainty as to the definition of the end point for the CDR2 loop around residues 56 to 58. The genes were co-expressed with mL or cL initially. In the case of the gH genes with loop choices for CDR1 e.g. gH121, gH131, gH141 very little antibody was produced in the culture supernatants. As no free light chain was detected it was presumed that the antibody was being made and assembled inside the cell but that the heavy chain was aberrant in some way, possibly incorrectly folded, and therefore the antibody was

being degraded internally. In some experiments trace amounts of antibody could be detected in ³⁵S labelling studies.

As no net antibody was produced, analysis of these constructs was not pursued further.

When, however, a combination of the loop choice and the Kabat choice for CDR1 was tested (mouse amino acids 26-35 inclusive) and in which residues 31 (Ser to Arg), 33 (Ala to Thr), and 35 (Tyr to His) were changed from the human residues to the mouse residue and compared to the first series, antibody was produced for gH321, kgH331 and kgH341 when co-expressed with cL. Expression was generally low and could not be markedly improved by the insertion of the Kozak consensus sequence 5' to the ATG of the signal sequence of the gene, as distinct from the case of the gL genes where such insertion led to a 2-5 fold increase in net antibody production. However, only in the case of gH341/mL or kgH341/cL could marginal antigen binding activity be demonstrated. When the kgH341 gene was co-expressed with kgL221A, the net yield of antibody was too low to give a signal above the background level in the antigen binding assay. (SEQ ID NO: 26)

15.2.2. FRAMEWORK RESIDUES

As in the case of the light chain the heavy chain frameworks were re-examined. Possibly because of the lower initial homology between the mouse and human heavy variable domains compared to the light chains, more amino acid positions proved to be of interest. Two genes kgH341A and kgH341B were constructed, with 11 or 8 human residues respectively substituted by mouse residues compared to gH341, and with the CDR2 residue 63 returned to the human amino acid potentially to

improve domain packing. Both showed antigen binding when combined with cL or kgL221A, the kgH341A gene with all 11 changes appearing to be the superior choice.

15.3 INTERIM CONCLUSIONS

It has been demonstrated, therefore, for OKT3 that to transfer antigen binding ability to the humanised antibody, mouse residues outside the CDR regions defined by the Kabat hypervariability or structural loop choices are required for both the light and heavy chains. Fewer extra residues are needed for the light chain, possibly due to the higher initial homology between the mouse and human kappa variable regions.

Of the changes seven (1 and 3 from the light chain and 6, 23, 71, 73 and 76 from the heavy chain) are predicted from a knowledge of other antibody structures to be either partly exposed or on the antibody surface. It has been shown here that residues 1 and 3 in the light chain are not absolutely required to be the mouse sequence; and for the heavy chain the gH341B heavy chain in combination with the 221A light chain generated only weak binding activity. Therefore the presence of the 6, 23 and 24 changes are important to maintain a binding affinity similar to that of the murine antibody. It was important, therefore, to further study the individual contribution of the other 8 mouse residues of the kgH341A gene compared to kgH341.

16. FURTHER CDR-GRAFTING EXPERIMENTS

Additional CDR-grafted heavy chain genes were prepared substantially as described above. With reference to Table 2 the further heavy chain genes were based upon the gh341 (plasmid pJA178) and

(SEQ ID NO: 12)

gH341A (plasmid pJA185) with either mouse OKT3 or human KOL residues at 6, 23, 24, 48, 49, 63, 71, 73, 76, 78, 88 and 91, as indicated. The CDR-

grafted light chain genes used in these further experiments were gL221_λ, gL221A_λ, gL221B_λ and gL221C_λ as described above.

(SEQ ID NO: 15) (SEQ ID NO: 26) (SEQ ID NO: 27) (SEQ ID NO: 28)

TABLE 2

OKT3 HEAVY CHAIN CDR GRAFTS

1. gH341 and derivatives

RES NUM	6	23	24	48	49	63	71	73	76	78	88	91	
OKT3vh	<u>Q</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>F</u>	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>A</u>	<u>Y</u>	
gH341	E	S	S	V	A	F	R	N	N	L	G	F	JA178
gH341A	<u>Q</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>A</u>	<u>Y</u>	JA185
gH341E	<u>Q</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>G</u>	<u>G</u>	JA198
gH341*	<u>Q</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>N</u>	<u>A</u>	<u>G</u>	<u>F</u>	JA207
gH341*	<u>Q</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>R</u>	<u>N</u>	<u>N</u>	<u>A</u>	<u>G</u>	<u>F</u>	JA209
gH341D	<u>Q</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>N</u>	<u>L</u>	<u>G</u>	<u>F</u>	JA197
gH341*	<u>Q</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>R</u>	<u>N</u>	<u>N</u>	<u>L</u>	<u>G</u>	<u>F</u>	JA199
gH341C	<u>Q</u>	<u>K</u>	<u>A</u>	<u>V</u>	<u>A</u>	<u>F</u>	<u>R</u>	<u>N</u>	<u>N</u>	<u>L</u>	<u>G</u>	<u>F</u>	JA184
gH341*	<u>Q</u>	<u>S</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>A</u>	<u>Y</u>	JA203
gH341*	E	S	<u>A</u>	<u>I</u>	<u>G</u>	V	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>A</u>	<u>Y</u>	JA205
gH341B	E	S	S	<u>I</u>	<u>G</u>	V	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>A</u>	<u>Y</u>	JA183
gH341*	<u>Q</u>	<u>S</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>G</u>	<u>F</u>	JA204
gH341*	E	S	<u>A</u>	<u>I</u>	<u>G</u>	V	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>G</u>	<u>F</u>	JA206
gH341*	<u>Q</u>	<u>S</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>N</u>	<u>A</u>	<u>G</u>	<u>F</u>	JA208
KOL	E	S	S	V	A		R	N	N	L	G	F	

AS (SEQ ID NO: ³⁰7, 10 and 11-24)
OKT3 LIGHT CHAIN CDR GRAFTS

2. gL221 and derivatives

RES NUM	1	3	46	47	
OKT3v1	<u>Q</u>	<u>V</u>	<u>R</u>	<u>W</u>	
GL221	D	Q	L	L	DA221
gL221A	<u>Q</u>	<u>V</u>	<u>R</u>	<u>W</u>	DA221A
gL221B	<u>Q</u>	<u>V</u>	L	L	DA221B
GL221C	D	Q	<u>R</u>	<u>W</u>	DA221C
RE1	D	Q ²⁹	L	L	

AS (SEQ ID NO: 5, 8, 9 and 25-28)
 MURINE RESIDUES ARE UNDERLINED

The CDR-grafted heavy and light chain genes were co-expressed in COS cells either with one another in various combinations but also with the corresponding murine and chimeric heavy and light chain genes substantially as described above. The resultant antibody products were then assayed in binding and blocking assays with HPB-ALL cells as described above.

A
S
S
The results of the assays for various grafted heavy chains co-expressed with the gL221C light chain ^(SEQ ID NO: 28) are given in Figures 7 and 8 (for the JA184, JA185, JA197 and JA198 constructs - see Table 2), in Figure 9 (for the JA183, JA184, JA185 and JA197 constructs) in Figure 10 ^{a and b} (for the chimeric, JA185, JA199, JA204, JA205, JA207, JA208 and JA209 constructs) and in Figure 11 ^{a and b} (for the JA183, JA184, JA185, JA198, JA203, JA205 and JA206 constructs).

A
A
A
The basic grafted product without any human to murine changes in the variable frameworks, i.e. gL221 ^(SEQ ID NO: 25) co-expressed with gh341 (JA178) ^(SEQ ID NO: 11) and also the "fully grafted" product, having most human to murine changes in the grafted heavy chain framework, i.e. gL221C ^(SEQ ID NO: 28) co-expressed with gh341A (JA185) ^(SEQ ID NO: 12) were assayed for relative binding affinity in a competition assay against murine OKT3 reference standard, using HPB-ALL cells. The assay used was as described above in section 3.3. The results obtained are given in Figure 12 for the basic grafted product and in Figure 13 for the fully grafted product. These results indicate that the basic grafted product has negligible binding ability as compared with the OKT3 murine reference standard; whereas the "fully grafted" product has a binding ability very similar to that of the OKT3 murine reference standard.

The binding and blocking assay results indicate the following:

The JA198 and JA207 constructs appear to have the best binding characteristics and similar binding abilities, both substantially the same as the chimeric and fully grafted gH341A products. This indicates that positions 88 and 91 and position 76 are not highly critical for maintaining the OKT3 binding ability; whereas at least some of positions 6, 23, 24, 48, 49, 71, 73 and 78 are more important.

This is borne out by the finding that the JA209 and JA199, although of similar binding ability to one another, are of lower binding ability than the JA198 and JA207 constructs. This indicates the importance of having mouse residues at positions 71, 73 and 78, which are either completely or partially human in the JA199 and JA209 constructs respectively.

Moreover, on comparing the results obtained for the JA205 and JA183 constructs it is seen that there is a decrease in binding going from the JA205 to the JA183 constructs. This indicates the importance of retaining a mouse residue at position 23, the only position changed between JA205 and JA183.

These and other results lead us to the conclusion that of the 11 mouse framework residues used in the gH341A (JA185) construct, it is important to retain mouse residues at all of positions 6, 23, 24, 48 and 49, and possibly for maximum binding affinity at 71, 73 and 78.

Similar Experiments were carried out to CDR-graft a number of the rodent antibodies including antibodies having specificity for CD4 (OKT4), ICAM-1 (R6-5), TAG72 (B72.3), and TNF α (61E71, 101.4, hTNF1, hTNF2 and hTNF3).

EXAMPLE 2

CDR-GRAFTING OF A MURINE ANTI-CD4 T CELL
RECEPTOR ANTIBODY, OKT4A

Anti OKT4A CDR-grafted heavy and light chain genes were prepared, expressed and tested substantially as described above in Example 1 for CDR-grafted OKT3. The CDR grafting of OKT4A is described in detail in Ortho patent application PCT/GB 90..... of even date herewith entitled "Humanised Antibodies". The disclosure of this Ortho patent application PCT/GB 90..... is incorporated herein by reference. A number of CDR-grafted OKT4 antibodies have been prepared. Presently the CDR-grafted OKT4A of choice is the combination of the grafted light chain LCDR2 and the grafted heavy chain HCDR10.

THE LIGHT CHAIN

5 The human acceptor framework used for the grafted light chains was REL. ^(see ID NO: 8 and 9) The preferred LCDR2 light chain has human to mouse changes at positions 33, 34, 38, 49 and 89 in addition to the structural loop CDRs. Of these changed positions, positions 33, 34 and 89 fall within the preferred extended CDRs of the present invention (positions 33 and 34 in CDR1 and position 89 in CDR3). The human to murine changes at positions 38 and 49 corresponds to positions at which the amino acid residues are preferably donor murine amino acid residues in accordance with the present invention. A comparison of the amino acid sequences of the donor murine light chain variable domain and the RE1 human acceptor light chain variable further reveals that the murine and human residues are identical at all of positions 46, 48 and 71 and at all of positions 2, 4, 6, 35, 36, 44, 47, 62, 64-69, 85, 87, 98, 99 and 101 and 102. However the amino acid residue at position 58 in LCDR2 is

the human RE1 framework residue not the mouse OKT4 residue as would be preferred in accordance with the present invention.

THE HEAVY CHAIN

A The human acceptor framework used for the grafted heavy chains was KOL_A ^(SEQ ID NO. 10)

The preferred CDR graft HCDR10 heavy chain has human to mouse changes at positions 24, 35, 57, 58, 60, 88 and 91 in addition to the structural loop CDRs.

Of these positions, positions 35 (CDR1) and positions 57, 58 and 60 (CDR2) fall within the preferred extended CDRs of the present invention. Also the human to mouse change at position 24 corresponds to a position at which the amino acid residue is a donor murine residue in accordance with the present invention. Moreover, the human to mouse changes at positions 88 and 91 correspond to positions at which the amino acid residues are optionally donor murine residues.

Moreover, a comparison of the murine OKT4A and human KOL heavy chain variable amino acid sequences reveals that the murine and human residues are identical at all of positions 23, 49, 71, 73 and 78 and at all of positions 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.

Thus the OKT4A CDR-grafted heavy chain HCDR10 corresponds to a particularly preferred embodiment according to the present invention.

EXAMPLE 3

CDR-GRAFTING OF AN ANTI-MUCIN SPECIFIC MURINE ANTIBODY, B72.3

The cloning of the genes coding for the anti-mucin specific murine monoclonal antibody B72.3 and the preparation of B72.3 mouse-human chimeric antibodies has been described previously (ref. 13 and WO 89/01783). CDR-grafted versions of B72.3 were prepared as follows.

(a) B72.3 Light Chain

CDR-grafting of this light chain was accomplished by direct transfer of the murine CDRs into the framework of the human light chain RE1. The regions transferred were:

<u>CDR Number</u>	<u>Residues</u>
1	24-34
2	50-56
3	90-96

The activity of the resulting grafted light chain was assessed by co-expression in COS cells, of genes for the combinations:

- B72.3 cH/B72.3 cL
- and B72.3 cH/B72.3 gL

Supernatants were assayed for antibody concentration and for the ability to bind to microtitre plates coated with mucin. The results obtained indicated that, in combination with the B72.3 cH chain, B72.3 cL and B72.3 gL had similar binding properties.

Comparison of the murine B72.3 and RE1 light chain amino acid sequences reveals that the residues are identical at positions 46, 58 and 71 but are different at position 48.

*and 9
(SEQ ID NO: 8)*

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Thus changing the human residue to the donor mouse residue at position 48 may further improve the binding characteristics of the CDR-grafted light chain, (B72.3 gL) in accordance with the present invention.

(b) B72.3 heavy chain

i. Choice of framework

At the outset it was necessary to make a choice of human framework. Simply put, the question was as follows: Was it necessary to use the framework regions from an antibody whose crystal structure was known or could the choice be made on some other criteria?

For B72.3 heavy chain, it was reasoned that, while knowledge of structure was important, transfer of the CDRs from mouse to human frameworks might be facilitated if the overall homology between the donor and receptor frameworks was maximised.

Comparison of the B72.3 heavy chain sequence with those in Kabat (ref. 4) for human heavy chains showed clearly that B72.3 had poor homology for KOL and NEWM (for which crystal structures are available) but was very homologous to the heavy chain for EU. (SEQ ID NO:10)

On this basis, EU was chosen for the CDR-grafting and the following residues transferred as CDRs.

<u>CDR Number</u>	<u>Residues</u>
1	27-36
2	50-63
3	93-102

Also it was noticed that the FR4 region of EU was unlike that of any other human (or mouse) antibody. Consequently, in the grafted heavy chain genes this was also changed to produce a "consensus" human sequence. (Preliminary experiments showed that grafted heavy chain genes containing the EU FR4 sequence expressed very poorly in transient expression systems.)

- ii. Results with grafted heavy chain genes
Expression of grafted heavy chain genes containing all human framework regions with either gL or cL genes produced a grafted antibody with little ability to bind to mucin. The grafted antibody had about 1% the activity of the chimeric antibody. In these experiments, however, it was noted that the activity of the grafted antibody could be increased to ~10% of B72.3 by exposure to pHs of 2-3.5. This observation provided a clue as to how the activity of the grafted antibody could be improved without acid treatment. It was postulated that acid exposure brought about the protonation of an acidic residue (pKa of aspartic acid = 3.86 and of glutamine acid = 4.25) which in turn caused a change in structure of the CDR loops, or allowed better access of antigen. From comparison of the sequences of B72.3 (ref. 13) and EU (refs. 4 and 5), it was clear that, in going from the mouse to human frameworks, only two positions had been changed in such a way that acidic residues had been introduced. These

positions are at residues 73 and 81, where K to E and Q to E changes had been made, respectively.

Which of these positions might be important was determined by examining the crystal structure of the KOL antibody. In KOL heavy chain, ^(SEQ ID NO: 10) position 81 is far removed from either of the CDR loops.

Position 73, however, is close to both CDRs 1 and 3 of the heavy chain and, in this position it was possible to envisage that a K to E change in this region could have a detrimental effect on antigen binding.

iii. Framework changes in B72.3 gH gene

On the basis of the above analysis, E73 was mutated to a lysine (K). It was found that this change had a dramatic effect on the ability of the grafted Ab to bind to mucin. Further the ability of the grafted B72.3 produced by the mutated gH/gL combination to bind to mucin was similar to that of the B72.3 chimeric antibody.

iv. Other framework changes

In the course of the above experiments, other changes were made in the heavy chain framework regions. Within the accuracy of the assays used, none of the changes, either alone or together, appeared beneficial.

v. Other

All assays used measured the ability of the grafted Ab to bind to mucin and, as a whole, indicated that the single framework change at position 73 is sufficient to generate an antibody with similar binding properties to B72.3.

Comparison of the B72.3 murine and EU heavy chain sequences reveals that the mouse and human residues are identical at positions 23, 24, 71 and 78.

Thus the mutated CDR-grafted B72.3 heavy chain corresponds to a preferred embodiment of the present invention.

EXAMPLE 4

CDR-GRAFTING OF A MURINE ANTI-ICAM-1 MONOCLONAL ANTIBODY

A murine antibody, R6-5-D6 (EP 0314863) having specificity for Intercellular Adhesion Molecule 1 (ICAM-1) was CDR-grafted substantially as described above in previous examples. This work is described in greater detail in co-pending application, British Patent Application No. 9009549.8, the disclosure of which is incorporated herein by reference.

The human EU framework was used as the acceptor framework for both heavy and light chains. The CDR-grafted antibody currently of choice is provided by co-expression of grafted light chain gL221A (SEQ ID NO: 26) and grafted heavy chain gH341D (SEQ ID NO: 16) which has a binding affinity for ICAM 1 of about 75% of that of the corresponding mouse-human chimeric antibody.

LIGHT CHAIN

gL221A has murine CDRs at positions 24-34 (CDR1), 50-56 (CDR2) and 89-97 (CDR3). In addition several framework residues are also the murine amino acid. These residues were chosen after consideration of the possible contribution of these residues to domain packing and stability of the conformation of the antigen binding region. The residues which have been retained as mouse are at positions 2, 3, 48 (?), 60, 84, 85 and 87. Comparison of the murine anti-ICAM 1 and human EU light chain amino acid sequences reveals that the murine and human residues are identical at positions 46, 58 and 71.

HEAVY CHAIN

gH341D has murine CDRs at positions 26-35 (CDR1), 50-56 (CDR2) and 94-100B (CDR3). In addition murine residues were used in gH341D at positions 24, 48, 69, 71, 73, 80, 88 and 91. Comparison of the murine anti-ICAM 1 and human EU heavy chain amino acid sequences are identical at positions 23, 49 and 78.

EXAMPLE 5

CDR-Grafting of murine anti-TNF α antibodies

A number of murine anti-TNF α monoclonal antibodies were CDR-grafted substantially as described above in previous examples. These antibodies include the murine monoclonal antibodies designated 61 E71, hTNF1, hTNF3 and 101.4. A brief summary of the CDR-grafting of each of these antibodies is given below.

61E71

A similar analysis as described above (Example 1, Section 12.1.) was done for 61E71 and for the heavy chain 10 residues were identified at 23, 24, 48, 49, 68, 69, 71, 73, 75 and 88 as residues to potentially retain as murine. The human frameworks chosen for CDR-grafting of this antibody, and the hTNF3 and 101.4 antibodies were RE1 for the light chain and KOL for the heavy chain.

Three genes were built, the first of which contained 23, 24, 48, 49, 71 and 73 [gH341(6)] as murine residues. The second gene also had 75 and 88 as murine residues [gH341(8)] while the third gene additionally had 68, 69, 75 and 88 as murine residues [gH341(10)]. Each was co-expressed with gL221, the minimum grafted light chain (CDRs only). The gL221/gH341(6) and gL221/gH341(8) antibodies both bound as well to TNF as murine 61E71. The gL221/gH341(10) antibody did not express and this combination was not taken further.

Subsequently the gL221/gH341(6) antibody was assessed in an L929 cell competition assay in which the antibody competes against the TNF receptor on L929 cells for binding to TNF in solution. In this assay the gL221/gH341(6) antibody was approximately 10% as active as murine 61E71.

hTNF1

hTNF1 is a monoclonal antibody which recognises an epitope on human TNF- . The EU human framework was used for CDR-grafting of both the heavy and light variable domains.

Heavy Chain

In the CDR-grafted heavy chain (ghTNF1) mouse CDRs were used at positions 26-35 (CDR1), 50-65 (CDR2) and 95-102 (CDR3). Mouse residues were also used in the frameworks at positions 48, 67, 69, 71, 73, 76, 89, 91, 94 and 108. Comparison of the TNF1 mouse and EU human heavy chain residues reveals that these are identical at positions 23, 24, 29 and 78.

Light Chain

In the CDR-grafted light chain (gLhTNF1) mouse CDRs were used at positions 24-34 (CDR1), 50-56 (CDR2) and 89-97 (CDR3). In addition mouse residues were used in the frameworks at positions 3, 42, 48, 49, 83, 106 and 108. Comparison of the hTNF1 mouse and EU human light chain residues reveals that these are identical at positions 46, 58 and 71.

The grafted hTNF1 heavy chain was co-expressed with the chimeric light chain and the binding ability of the product compared with that of the chimeric light chain/chimeric heavy chain product in a TNF binding assay. The grafted heavy chain product appeared to have binding ability for TNF slightly better than the fully chimeric product.

Similarly, a grafted heavy chain/grafted light chain product was co-expressed and compared with the fully chimeric product and found to have closely similar binding properties to the latter product.

hTNF3

hTNF3 recognises an epitope on human TNF- α . The sequence of hTNF3 shows only 21 differences compared to 61E71 in the light and heavy chain variable regions, 10 in the light chain (2 in the CDRs at positions 50, 96 and 8 in the framework at 1, 19, 40, 45, 46, 76, 103 and 106) and 11 in the heavy chain (3 in the CDR regions at positions 52, 60 and 95 and 8 in the framework at 1, 10, 38, 40, 67, 73, 87 and 105). The light and heavy chains of the 61E71 and hTNF3 chimeric antibodies can be exchanged without loss of activity in the direct binding assay. However 61E71 is an order of magnitude less able to compete with the TNF receptor on L929 cells for TNF-a compared to hTNF3. Based on the 61E71 CDR grafting data gL221 and gH341(+23, 24, 48, 49 71 and 73 as mouse) genes have been built for hTNF3 and tested and the resultant grafted antibody binds well to TNF-a, but competes very poorly in the L929 assay. It is possible that in this case also the framework residues identified for OKT3 programme may improve the competitive binding ability of this antibody.

101.4

101.4 is a further murine monoclonal antibody able to recognise human TNF-a. The heavy chain of this antibody shows good homology to KOL and so the CDR-grafting has been based on RE1 for the light chain and KOL for the heavy chain. Several grafted heavy chain genes have been constructed with conservative choices for the CDR's (gH341)^(SEQ ID NO:11) and which have one or a small number of non-CDR residues at positions 73, 78 or 77-79 inclusive, as the mouse amino acids. These have been co-expressed with cL or gL221. In all cases binding to TNF equivalent to the chimeric antibody is seen and when co-expressed with cL the resultant antibodies are able to compete well in the L929 assay. However, with gL221 the resultant antibodies

are at least an order of magnitude less able to compete for TNF against the TNF receptor on L929 cells.

Mouse residues at other positions in the heavy chain, for example, at 23 and 24 together or at 76 have been demonstrated to provide no improvement to the competitive ability of the grafted antibody in the L929 assay.

A number of other antibodies including antibodies having specificity for interleukins e.g. IL1 and cancer markers such as carcinoembryonic antigen (CEA) e.g. the monoclonal antibody A5B7 (ref. 21), have been successfully CDR-grafted according to the present invention.

It will be appreciated that the foregoing examples are given by way of illustration only and are not intended to limit the scope of the claimed invention. Changes and modifications may be made to the methods described whilst still falling within the spirit and scope of the invention.

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CLAIMS

1. A CDR-grafted antibody heavy chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 6, 23 and/or 24, 48 and/or 49, 71 and/or 73, 75 and/or 76 and/or 78 and 88 and/or 91.
2. A CDR-grafted heavy chain according to Claim 1 comprising donor residues at positions 23, 24, 49, 71, 73 and 78, or at positions 23, 24 and 49.
3. A CDR-grafted heavy chain according to Claim 2 comprising donor residues at positions 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.
4. A CDR-grafted heavy chain according to Claim 2 or 3, comprising donor residues at one, some or all of positions:
1 and 3,
69 (if 48 is different between donor and acceptor),
38 and 46 (if 48 is the donor residue),
67,
82 and 18 (if 67 is the donor residue),
91, and
any one or more of 9, 11, 41, 87, 108, 110 and 112.
5. A CDR-grafted heavy chain according to any of the preceding comprising donor CDRs at positions 26-35, 50-65 and 95-100.
6. A CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 1 and/or 3 and 46 and/or 47.

7. A CDR-grafted light chain according to Claim 6 comprising donor residues at positions 46 and 47.
8. A CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 46, 48, 58 and 71.
9. A CDR-grafted light chain according to Claim 8 comprising donor residues at positions 46, 48, 58 and 71.
10. A CDR-grafted light chain according to Claim 8 or 9, comprising donor residues at positions 2, 4, 6, 35, 36, 38, 44, 47, 49, 52, 64-69, 85, 87, 98, 99, 101 and 102.
11. A CDR-grafted light chain according to Claim 9 or 10, comprising donor residues at one, some or all of positions:
1 and 3,
63,
60 (if 60 and 54 are able to form a potential saltbridge),
70 (if 70 and 24 are able to form a potential saltbridge),
73 and 21 (if 47 is different between donor and acceptor),
37 and 45 (if 47 is different between donor and acceptor), and
any one or more of 10, 12, 40, 83, 103 and 105.
12. A CDR-grafted light chain according to any one of Claims 6-11, comprising donor CDRs at positions 24-34, 50-56 and 89-97.

13. A CDR-grafted antibody molecule comprising at least one CDR-grafted heavy chain according to any one of Claims 1-5 and at least one CDR-grafted light chain according to any one of Claims 6-12.
14. A CDR-grafted antibody molecule according to Claim 13, which is a site-specific antibody molecule.
15. A CDR-grafted antibody molecule according to Claim 13 which has specificity for an interleukin, hormone or other biologically active compound or a receptor therefor.
16. A CDR-grafted antibody heavy or light chain or molecule according to any one of the preceding claims comprising human acceptor residues and non-human donor residues.
17. A DNA sequence which codes for a CDR-grafted heavy chain according to Claim 1 or a CDR-grafted light chain according to Claim 6 or Claim 8.
18. A cloning or expression vector containing a DNA sequence according to Claim 17.
19. A host cell transformed with a DNA sequence according to Claim 17.
20. A process for the production of a CDR-grafted antibody sequence according to Claim 17 in a transformed host cell.
21. A process for producing a CDR-grafted antibody product comprising:

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- (a) producing in an expression vector an operon having a DNA sequence which encodes an antibody heavy chain according to Claim 1;
- and/or
- (b) producing in an expression vector an operon having a DNA sequence which encodes a complementary antibody light chain according to Claim 6 or Claim 8;
- (c) transfecting a host cell with the or each vector; and
- (d) culturing the transfected cell line to produce the CDR-grafted antibody product.

22. A therapeutic or diagnostic composition comprising a CDR-grafted antibody heavy chain according to Claim 1, or a CDR-grafted light chain according to Claim 6 or Claim 8, or a CDR-grafted antibody molecule according to Claim 13 in combination with a pharmaceutically acceptable carrier, diluent or excipient.

23. A method of therapy or diagnosis comprising administering an effective amount of a CDR-grafted heavy chain according to Claim 1, or a CDR-grafted light chain according to Claim 6 or Claim 8, or a CDR-grafted antibody molecule according to Claim 13 to a human or animal subject.

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ABSTRACT

CDR-grafted antibody heavy and light chains comprise acceptor framework and donor antigen binding regions, the heavy chains comprising donor residues at at least one of positions (6, 23) and/or (24, 48) and/or (49, 71) and/or (73, 75) and/or (76) and/or (78) and (88) and/or (91). The CDR-grafted light chains comprise donor residues at at least one of positions (1) and/or (3) and (46) and/or (47) or at at least one of positions (46, 48, 58) and (71).

10 The CDR-grafted antibodies are preferably humanised antibodies, having non human, e.g. rodent, donor and human acceptor frameworks, and may be used for *in vivo* therapy and diagnosis. A generally applicable protocol is disclosed for obtaining CDR-grafted antibodies.

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1 GAATTC~~CCAA~~ AGACAAAatg gattttcaag tgcagatttt cagcttctg
51 ctaatcagtg cctcagtcaatatccaga ggc~~caa~~attg ttctcacc~~ca~~
101 gtctccagca atcatgtctg catctccagg ggagaaggte accatgacct
151 gcagtgccag ctcaagtgtg agttacatga actggtagca gcagaagtca
201 ggcacctccc ccaaagatg gatttatgac acatccaaac tggcttctgg
251 agtccctgct cacttcaggg gcagtg~~gg~~tc tgggacctct tactctctca
301 caatcagcgg catggaggct gaagatgctg cca~~ct~~tatta ctgccagcag
351 tggagtagta acccattcac gttcggctcg gggacaaagt tggaaataaa
401 ccgggctgat actgcaccaa ctgtatccat cttcccacca tccagtgagc
451 agttaacatc tggaggtgcc tcagtcgtgt gcttcttgaa caacttctac
501 cccaaagaca tcaatgtcaa gtggaagatt gatggcagtg aacgacaaaa
551 tggcgtcctg aacagttgga ctgatcagga cagcaaagac agcacctaca
601 gcatgagcag caccctcacg ttgaccaagg acgagtatga acgacataac
651 agctatacct gtgaggccac tcacaagaca tcaacttcac ccattgtcaa
701 gagcttcaac aggaatgagt gtTAGAGACA AAGGTCCTGA GACGCCACCA
751 CCAGCTCCCA GCTCCATCCT ATCTTCCCTT CTAAGGTCTT GGAGGCTTCC
801 CCACAAGCGC tTACCACTGT TGCGGTGCTC tAAACCTCCT CCCACCTCCT
851 TCTCCTCCTC CTCCTTTCC TTGGCTTTTA TCATGCTAAT ATTTGCAGAA
901 AATATTCAAT AAAGTGAGTC TTTGCCTTGA AAAAAAAAAA AAA

Fig. 1(a)

1 MDFOVOIFSF LLISASVIIS RGQIVLTQSP AIMSASPGEK VTMTCSASSS
51 VSYMNWYQQK SGTSPKRWIY DTSKLAGVP AHFRGSGSGT SYSLTISGME
101 AEDAATYYCQ QWSSNPFTFG SGTKLEINRA DTAPTVSIFP PSSEQLTSGG
151 ASVVCFLNNF YPKDINVKWK IDGSERQNGV LNSWTDQDSK DSTYSMSSTL
201 TLTKEYERH NSYTCEATHK TSTSPIVKSF NRNEC*

Fig. 1(b)

SUBSTITUTE SHEET

1 GAATTCCCCT CTCCACAGAC ACTGAAAACCT CTGACTCAAC ATGGAAAGGC
 51 ACTGGATCTT TCTACTCCTG TTGTCAGTAA CTGCAGGTGT CCACTCCCAG
 101 GTCCAGCTGC AGCAGTCTGG GGCTGAACTG GCAAGACCTG GGGCCTCAGT
 151 GAAGATGTCC TGCAAGGCTT CTGGCTACAC CTTTACTAGG TACACGATGC
 201 ACTGGGTAAA ACAGAGGCCT GGACAGGGTC TGGAAATGGAT TGGATAACATT
 251 AATCCTAGCC GTGGTTATAC TAATTACAAT CAGAAGTTCA AGGACAAGGC
 301 CACATTGACT ACAGACAAAT CCTCCAGCAC AGCCTACATG CAACTGAGCA
 351 GCCTGACATC TGAGGACTCT GCAGTCTATT ACTGTGCAAG ATATTATGAT
 401 GATCATTACT GCCTTGACTA CTGGGGCCAA GGCACCACTC TCACAGTCTC
 451 CTCAGCCAAA ACAACAGCCC CATCGGTCTA TCCACTGGCC CCTGTGTGTG
 501 GAGATACAAC TGGCTCCTCG GTGACTCTAG GATGCCTGGT CAAGGGTTAT
 551 TTCCCTGAGC CAGTGACCTT GACCTGGAAC TCTGGATCCC TGTCCAGTGG
 601 TGTGCACACC TTCCCAGCTG TCCTGCAGTC TGACCTCTAC ACCCTCAGCA
 651 GCTCAGTGAC TGTAACCTCG AGCACCTGGC CCAGCCAGTC CATCACCTGC
 701 AATGTGGCCC ACCCGGCAAG CAGCACCAAG GTGGACAAGA AAATTGAGCC
 751 CAGAGGGCCC ACAATCAAGC CCTGTCTCTC ATGCAAATGC CCAGCACCTA
 801 ACCTCTTGGG TGGACCATCC GTCTTCATCT TCCCTCCAAA GATCAAGGAT
 851 GTACTCATGA TCTCCCTGAG CCCCATAGTC ACATGTGTGG TGGTGGATGT
 901 GAGCGAGGAT GACCCAGATG TCCAGATCAG CTGGTTTGTG AACAACTGG
 951 AAGTACACAC AGCTCAGACA CAAACCCATA GAGAGGATTA CAACAGTACT
 1001 CTCCGGGTGG TCAGTGCCCT CCCCATCCAG CACCAGGACT GGATGAGTGG
 1051 CAAGGAGTTC AAATGCAAGG TCAACAACAA AGACCTCCCA GCGCCCATCG
 1101 AGAGAACCAT CTCAAACCC AAAGGGTCAG TAAGAGCTCC ACAGGTATAT
 1151 GTCTTGCCTC CACCAGAAGA AGAGATGACT AAGAAACAGG TCACTCTGAC
 1201 CTGCATGGTC ACAGACTTCA TGCCTGAAGA CATTTACGTG GAGTGGACCA
 1251 ACAACGGGAA AACAGAGCTA AACTACAAGA AACTGAACC AGTCCTGGAC
 1301 TCTGATGGTT CTTACTTCAT GTACAGCAAG CTGAGAGTGG AAAAGAAGAA
 1351 CTGGGTGGAA AGAAATAGCT ACTCCTGTTC AGTGGTCCAC GAGGGTCTGC
 1401 ACAATCACCA CACGACTAAG AGCTTCTCCC GGACTIONGG TAAATGAGCT
 1451 CAGCACCCAC AAAACTCTCA GGTCCAAAGA GACACCCACA CTCATCTCCA
 1501 TGCTTCCCTT GTATAAATAA AGCACCCAGC AATGCCTGGG ACCATGTAAA
 1551 AAAAAAAAAA AAAGGAATTC

Fig. 2(a)

SUBSTITUTE SHEET

OKT 3 HEAVY CHAIN PROTEIN SEQUENCE DEDUCED FROM DNA SEQUENCE

1 MERHWIFLLL LSVTAGVHSQ VQLQQSGAEL ARPGASVKMS CKASGYTFTR
 51 YTMHWVKQRP GQGLEWIGYI NPSRGYTNYN QKFKDKATLT TDKSSSTAYM
 101 QLSSLTSEDS AVYCYARYYD DHYCLDYWGQ GTTLTVSSAK TTAPSVYPLA
 151 PVCGDTTGSS VTLGCLVKGY FPEPVTLTWN SGLSSGVHT FPAVLQSDLY
 201 TLSSSVTVTS STWPSQSITC NVAHPASSTK VDKKIEPRGP TIKPCPPCKC
 251 PAPANLLGGPS VFIFPPKIKD VLMISLSPIV TCVVVDVSED DPDVQISWFV
 301 NNVEVHTAQT QTHREDYNST LRVVSALPIQ HQDWMMSGKEF KCKVNNKDLP
 351 APIERTISKP KGSVRAPQVY VLPPPEEEMT KKQVTLTCMV TDFMPEDIYV
 401 EWTNNGKTEL NYKNTEPULD SDGSYFMYSK LRVEKKNWVE RNSYSCSVVH
 451 EGLHNNHHTTK SPSRTPGK*

Fig. 2(b)

	1		23		42
	NN	N	N	N	N
RES TYPE	SBspSPESssBSbSsSssPSPSPsPSsse*s*p*Pi`ISsSe				
Okt3v1	QIVLTQSPAIMASAPGEXVTMTCSASS.SVSYM N WYQKSGT				
REI	DIQMTQSPSSLASVGDVRTITCQASQDIIKYLNWYQQT P GK				
	?	?			
	CDR1	(LOOP)		*****	
	CDR1	(KABAT)		*****	

		56		85
	N	NN		
RES TYPE	*IsiPpIeesesssSBesePsPSBSSEsPspPssseesSPePb			
Okt3v1	SPKRWIYDTSKLAGVPAHFRGSGSGTYSYSLTISGMEAEADAAT			
REI	APKLLIYEASNLAQGVPSRFSGSGSGTDY T P T ISSLQPEDIA T			
	?	??		? ?
	***** CDR2 (LOOP/KABAT)			

		102	108
RES TYPE	PiPIPIes**iPIIsPPSPSPSS		
Okt3v1	YYCQQWSSNPFTFGG G TKLEI N R		
REIv1	YYCQQYQSLPYTFGQGT K LOI T R		
		?	?
	*****	CDR3 (LOOP)	
	*****	CRD3 (KABAT)	

Fig. 3

SUBSTITUTE SHEET

```

NN N                23 26    32 35 N39  43
RES TYPE  SESPs`SBssS`sSSsSpSpSPsPSEbSBssBePiPiesss
Okt3h     QVQLQQGGAELARPGASVKMSCKASGYTFTRYTMHWVKORPGQ
KOL       QVQLVESGGGVQPGRSLRLSCSSSGFIFSSYAMYWVRQAPGK
          ?                ..??

          ***** CDR1 (LOOP)
          ***** CDR1 (KABAT)

```

```

          52a    60 65    N N N    82abc    89
RES TYPE  IIeIppp`ssssssss`ps`pSSsbSpseSsSseSp`pSpSbSSs`epb
Okt3vh    GLEWIGYINPSRGYTNTNQFKDKATLITDKSSSTAYMQLSSLTSESAV
KOL       GLEWVAIIWDDGSDQHYADSVKGRFTISRDNKSKNTLELQMDSLRPEDTGV
          ??                ? ? ? ?                ?

          ***** CDR2 (LOOP)
          ***** CDR2 (KABAT)

```

```

          92 N                107    113
RES TYPE  PiPIEiSSSSiiSSsbibi*EiPIp*spSBSS
Okt3vh    YYCARYYDDHY.....CLDYWGQGTTLTVSS
KOL       YFCARDGGHGFCSSASCFGPDYWGQGTPEVTVSS
          ***** CRD3 (KABAT/LOOP)

```

Fig. 4

SUBSTITUTE SHEET

OKT 3 HEAVY CHAIN CDR GRAFTS

1. gh341 and derivatives

	1	26	35	39	43	
Okt3vh	QVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPGQ					
gh341	QVQLVESGGGVVQPGRSLRLSCSSSGYTFTRYTMHWVRQAPGK					JA178
gh341A	QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK					JA185
gh341E	QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK					JA198
gh341*	QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK					JA207
gh341*	QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK					JA209
gh341D	QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK					JA197
gh341*	QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK					JA199
gh341C	QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK					JA184
gh341*	QVQLVQSGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK					JA203
gh341*	QVQLVESGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK					JA205
gh341B	QVQLVESGGGVVQPGRSLRLSCSSSGYTFTRYTMHWVRQAPGK					JA183
gh341*	QVQLVQSGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK					JA204
gh341*	QVQLVESGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK					JA206
gh341*	QVQLVQSGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK					JA208
KOL	QVQLVESGGGVVQPGRSLRLSCSSSGFIFSSYAMYWVRQAPGK					

Fig. 5(i)

SUBSTITUTE SHEET

	44	50	65	83	
Okt3vh	GLEWIGYINPSRGYTNYNOKFKDKATLTDDKSSSTAYMQLSSLT				
gH341	GLEWVAYINPSRGYTNYNOKFKDRFTISRDNKNTLFLQMDSL				JA178
gH341A	GLEWIGYINPSRGYTNYNOKVKDRFTISTDKSKSTAFLOMDSL				JA185
gH341E	GLEWIGYINPSRGYTNYNOKVKDRFTISTDKSKSTAFLOMDSL				JA198
gH341*	GLEWIGYINPSRGYTNYNOKVKDRFTISTDKSKNTAFLOMDSL				JA207
gH341*	GLEWIGYINPSRGYTNYNOKVKDRFTISRDNKNTAFLOMDSL				JA209
gH341D	GLEWIGYINPSRGYTNYNOKVKDRFTISTDKSKNTLFLQMDSL				JA197
gH341*	GLEWIGYINPSRGYTNYNOKVKDRFTISRDNKNTLFLQMDSL				JA199
gH341C	GLEWVAYINPSRGYTNYNOKFKDRFTISRDNKNTLFLQMDSL				JA184
gH341*	GLEWIGYINPSRGYTNYNOKVKDRFTISTDKSKSTAFLOMDSL				JA207
gH341*	GLEWIGYINPSRGYTNYNOKVKDRFTISTDKSKSTAFLOMDSL				JA205
gH341B	GLEWIGYINPSRGYTNYNOKVKDRFTISTDKSKSTAFLOMDSL				JA183
gH341*	GLEWIGYINPSRGYTNYNOKVKDRFTISTDKSKSTAFLOMDSL				JA204
gH341*	GLEWIGYINPSRGYTNYNOKVKDRFTISTDKSKSTAFLOMDSL				JA206
gH341*	GLEWIGYINPSRGYTNYNOKVKDRFTISTDKSKNTAFLOMDSL				JA208
KOL	GLEWVAI IWDDGSDQHYADSVKGRFTISRDNKNTLFLQMDSL				

Fig. 5(ii)

SUBSTITUTE SHEET

	84	95	102	113	
Okt3vh	SEDSAVYYCARYYDDHY.....		CLDYWGQGTTLTVSS		
gH341	PEDTGVYFCARYYDDHY.....		CLDYWGQGTTLTVSS		JA178
gH341A	PEDTAVYYCARYYDDHY.....		CLDYWGQGTTLTVSS		JA185
gH341E	PEDTGVYFCARYYDDHY.....		CLDYWGQGTTLTVSS		JA198
gH341*	PEDTGVYFCARYYDDHY.....		CLDYWGQGTTLTVSS		JA207
gH341D	PEDTGVYFCARYYDDHY.....		CLDYWGQGTTLTVSS		JA197
gH341*	PEDTGVYFCARYYDDHY.....		CLDYWGQGTTLTVSS		JA209
gH341*	PEDTGVYFCARYYDDHY.....		CLDYWGQGTTLTVSS		JA199
gH341C	PEDTGVYFCARYYDDHY.....		CLDYWGQGTTLTVSS		JA184
gH341*	PEDTAVYYCARYYDDHY.....		CLDYWGQGTTLTVSS		JA203
gH341*	PEDTAVYYCARYYDDHY.....		CLDYWGQGTTLTVSS		JA205
gH341B	PEDTAVYYCARYYDDHY.....		CLDYWGQGTTLTVSS		JA183
gH341*	PEDTGVYFCARYYDDHY.....		CLDYWGQGTTLTVSS		JA204
gH341*	PEDTGVYFCARYYDDHY.....		CLDYWGQGTTLTVSS		JA206
gH341*	PEDTGVYFCARYYDDHY.....		CLDYWGQGTTLTVSS		JA208
KOL	PEDTGVYFCARDGGHGFCSASCFGPDYWGQGTPTVSS				

Fig. 5(iii)

SUBSTITUTE SHEET

OKT3 LIGHT CHAIN CDR GRAFTING

1. gL221 and derivatives

	1	24	34	42
Okt3v1	QIVLTQSPAIMSASPGEKVTMTCSASS.SVSYMNWYQQKSGT			
gL221	DIQMTQSPSSLSASVGDRTITCSASS.SVSYMNWYQQTPGK			
gL221A	<u>QIV</u> MTQSPSSLSASVGDRTITCSASS.SVSYMNWYQQTPGK			
gL221B	<u>QIV</u> MTQSPSSLSASVGDRTITCSASS.SVSYMNWYQQTPGK			
gL221C	DIQMTQSPSSLSASVGDRTITCSASS.SVSYMNWYQQTPGK			
REI	DIQMTQSPSSLSASVGDRTITCQASQDIIKYLWYQQTPGK			
	43	50	56	85
Okt3v1	SPKRWIYDTSKLAGVPAHFRGSGSGTYSYSLTISGMEAEDAAT			
gL221	APKLLIYDTSKLAGVPSRFSGSGSDYFTFTISSLOPEDIAT			
gL221A	APKRWIYDTSKLAGVPSRFSGSGSDYFTFTISSLOPEDIAT			
gL221B	APKRWIYDTSKLAGVPSRFSGSGSDYFTFTISSLOPEDIAT			
gL221C	APKRWIYDTSKLAGVPSRFSGSGSDYFTFTISSLOPEDIAT			
REI	APKLLIYEASNLAQGVPSRFSGSGSDYFTFTISSLOPEDIAT			
	86	91	96	108
Okt3v1	YQCQWSSNPFTFGSGTKLEINR			
gL221	<u>YQCQWSSNPFTFGQ</u> GTKLQITR			
gL221A	<u>YQCQWSSNPFTFGQ</u> GTKLQITR			
gL221B	<u>YQCQWSSNPFTFGQ</u> GTKLQITR			
gL221C	<u>YQCQWSSNPFTFGQ</u> GTKLQITR			
REI	YQCQYQSLPYTFGQGTKLQITR			

CDR'S ARE UNDERLINED

FRAMEWORK RESIDUES INCLUDED IN THE GENE ARE DOUBLE UNDERLINED

Fig. 6

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18 RES'D BCLN10 17 JUL 1997

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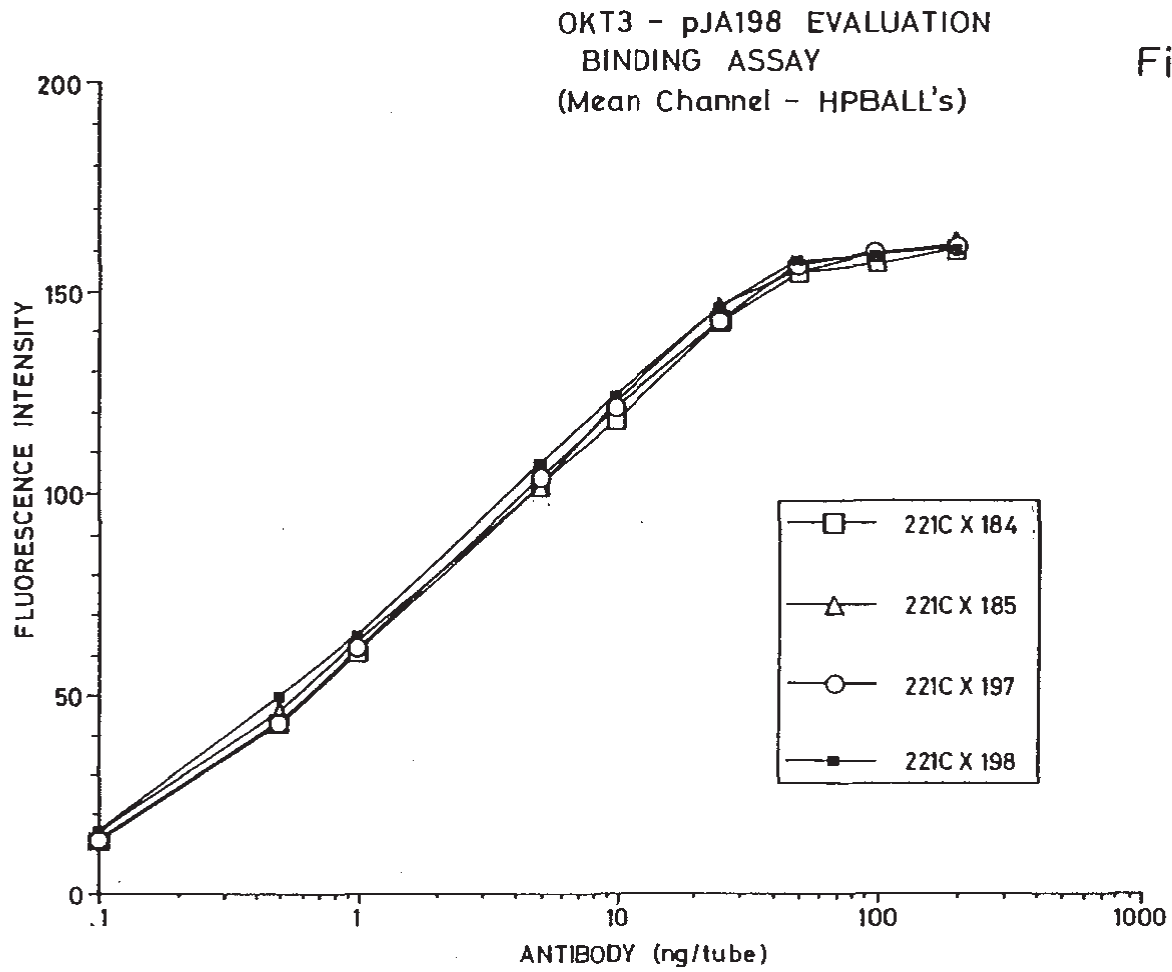
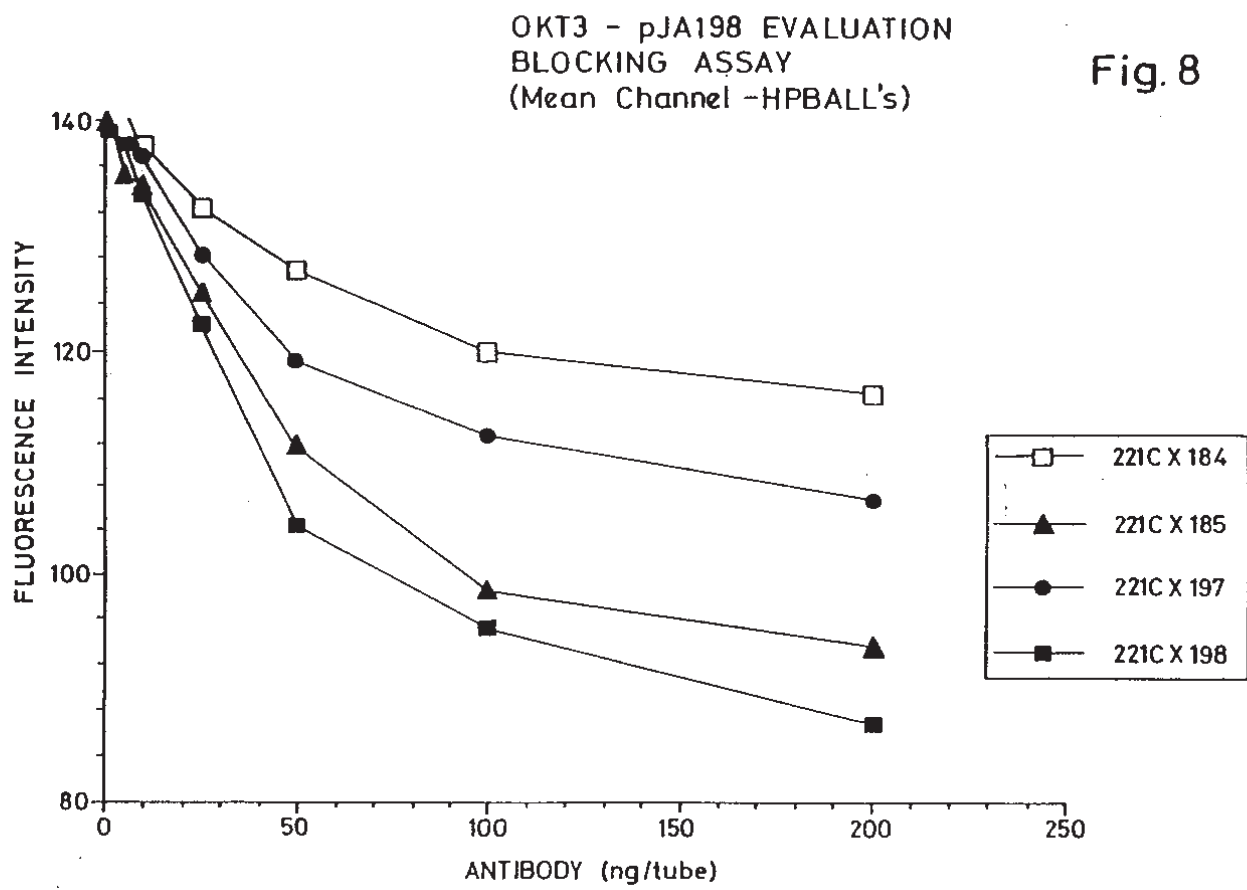


Fig. 7

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08/30/3569

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08/30/3569

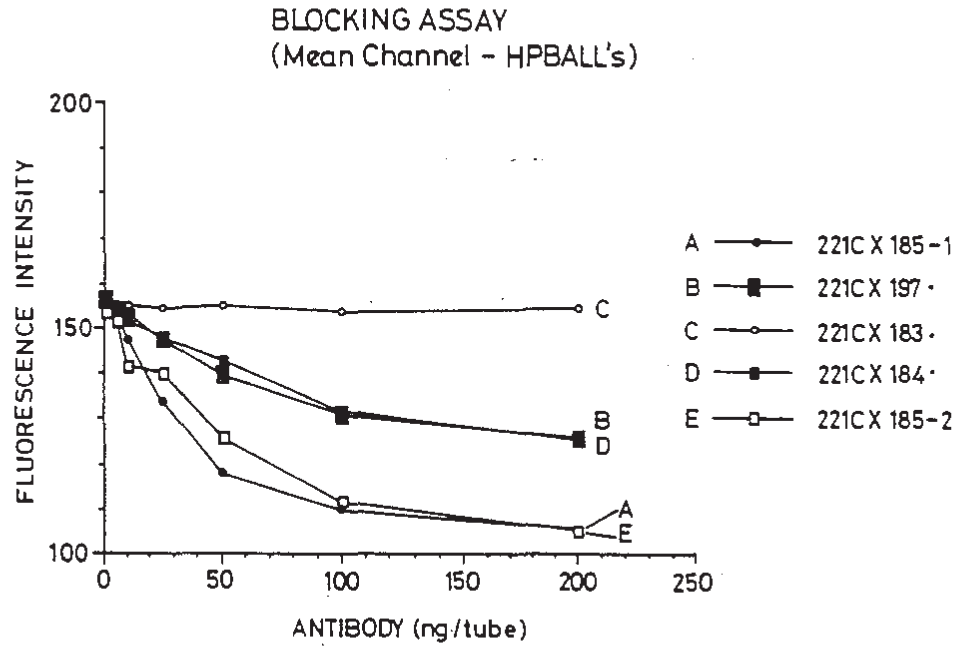
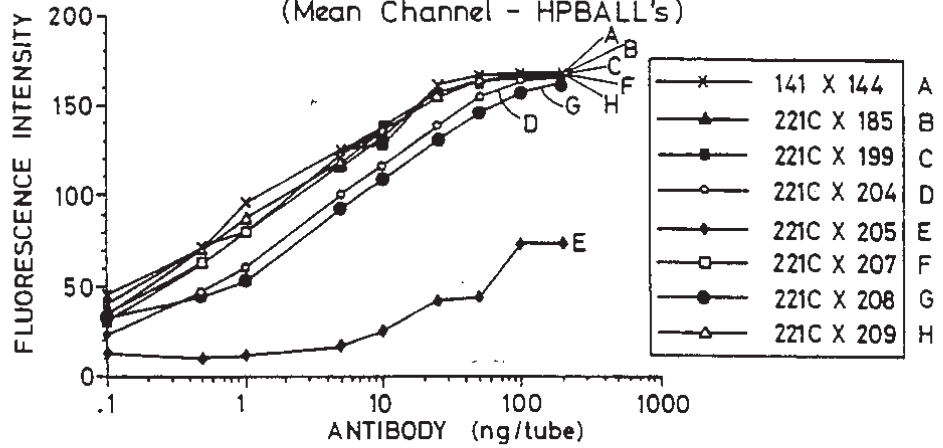


Fig. 9

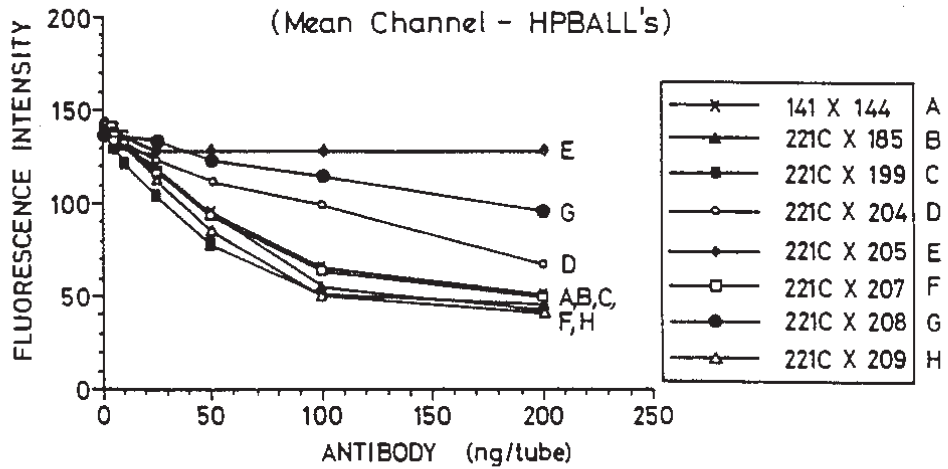
SUBSTITUTE SHEET

Fig.10

OKT3 - GRAFTED HEAVY CHAINS
BINDING ASSAY
(Mean Channel - HPBALL's)



OKT3 - GRAFTED HEAVY CHAINS
BLOCKING ASSAY
(Mean Channel - HPBALL's)



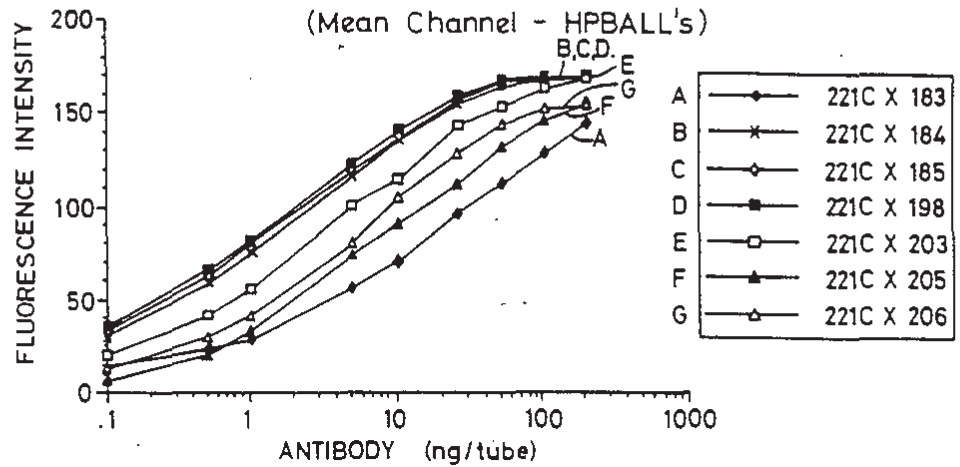
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(199)	6,23,24,48,49,.....,.....,.....,
(207)	6,23,24,48,49,71,73,.....,78,.....,
(185)	6,23,24,48,49,71,73,76,78,88,91,
(209)	6,23,24,48,49,.....,.....,78,.....,
141 X 144	

SUBSTITUTE SHEET

Fig. 11

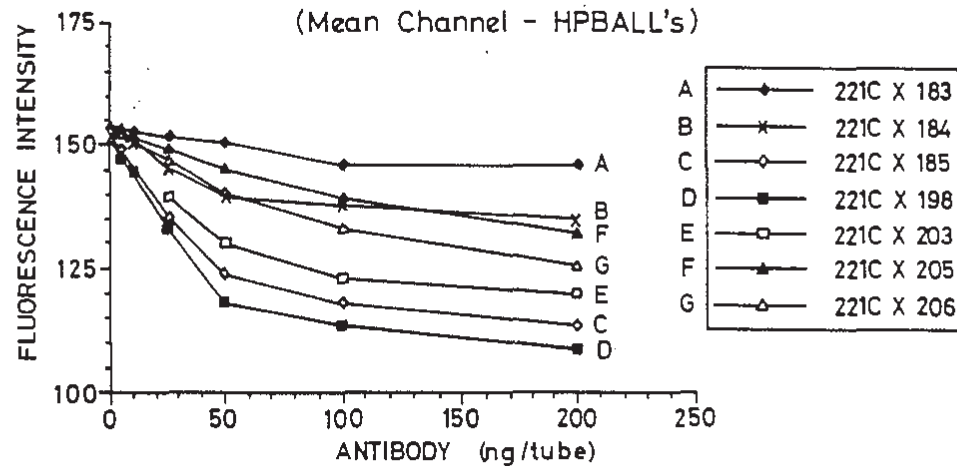
OKT3 - GRAFTED HEAVY CHAINS
BINDING ASSAY

(Mean Channel - HPBALL's)



OKT3 GRAFTED HEAVY CHAINS
BLOCKING ASSAY

(Mean Channel - HPBALL's)

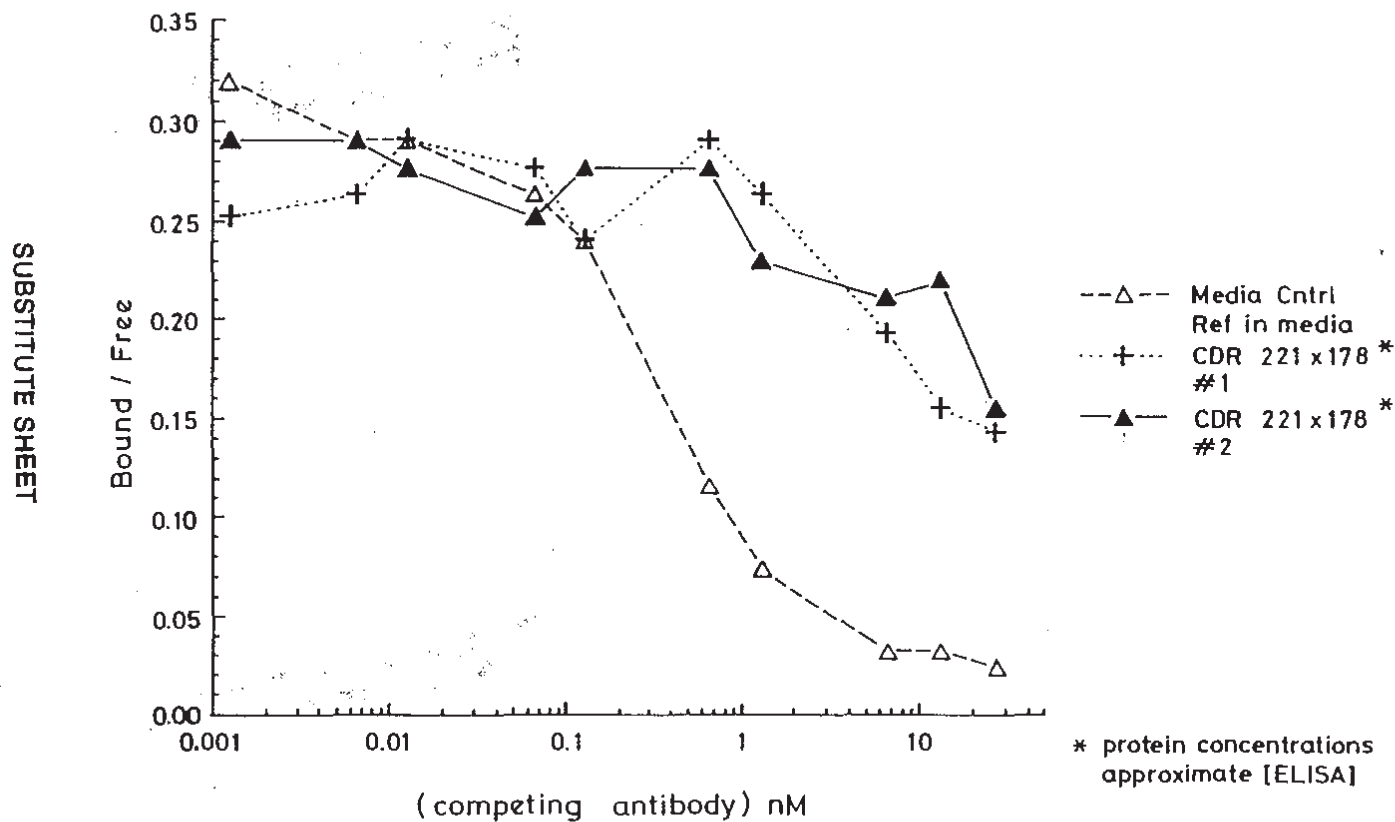


◆	(183)	-----,48,49,71,73,76,78,88,91,
▲	(205)	-----,24,48,49,71,73,76,78,88,91,
×	(184)	6,23,24,-----,-----,
△	(206)	-----,24,48,49,71,73,76,78,-----,
□	(203)	6,-----,24,48,49,71,73,76,78,88,91,
◇	(185)	6,23,24,48,49,71,73,76,78,88,91,
■	(198)	6,23,24,48,49,71,73,76,78,-----,

SUBSTITUTE SHEET

OKT3 Competition
 Murine Ref Std vs. CDR Grafted OKT3

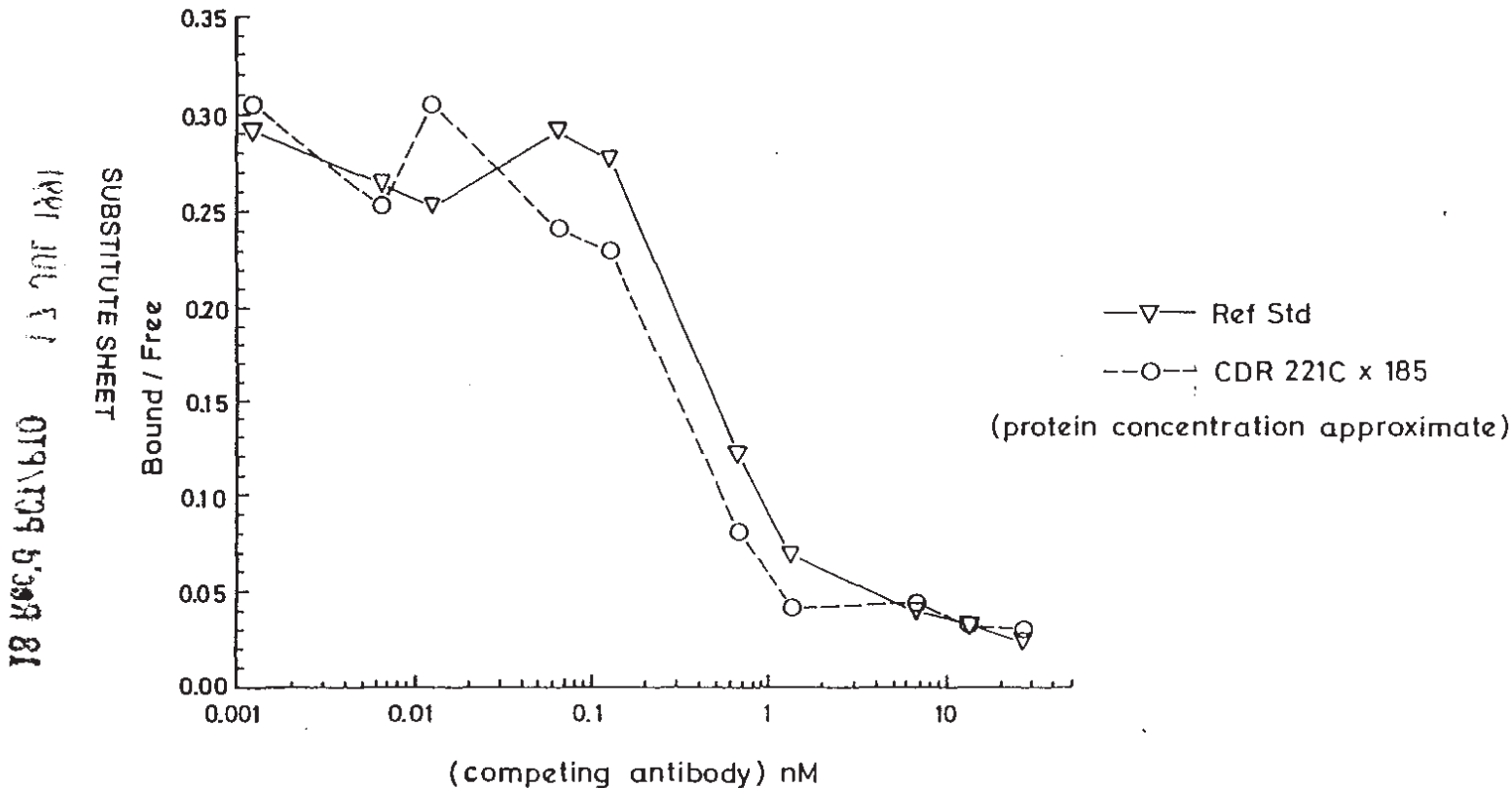
Fig. 12



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OKT3 Competition
Murine Ref Std vs. CDR Grafted OKT3

Fig. 13



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#4

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)

CARP-0009

INTERNATIONAL APPLICATION NO.
PCT/GB90/02017

INTERNATIONAL FILING DATE
21 December 1990

PRIORITY DATE CLAIMED
21 December 1989

TITLE OF INVENTION
HUMANISED ANTIBODIES

APPLICANT(S) FOR DO/EO/US
ADAIR, John, Robert, ATHWAL, Diljeet, Singh, and EMTAGE, John, Spencer

Applicant herewith submits to the United States Designated/ Elected Office (DO/EO/US) the following items under 35 U.S.C. 371:

1. This express request to immediately begin national examination procedures (35 U.S.C. 371(f)).
2. The U.S. National Fee (35 U.S.C. 371(c)(1)) and other fees as follows:

CLAIMS	(1) FOR	(2) NUMBER FILED	(3) NUMBER EXTRA	(4) RATE	(5) CALCULATIONS
TOTAL CLAIMS		-20-		X\$ 20.00	\$
INDEPENDENT CLAIMS		-3-		X\$ 60.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)				+\$ 200.00	
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(4)):					
<input type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482)..... \$ 330					
<input type="checkbox"/> No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445 (a)(2))..... \$ 370					
<input type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO..... \$ 500					
<input type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2) to (4)..... \$ 50					
Surcharge of \$120 for furnishing the National fee or oath or declaration later than <input checked="" type="checkbox"/> 20 <input type="checkbox"/> 30 mos. from the earliest claimed priority date (37 CFR 1.492(e)).					\$120.00
TOTAL OF ABOVE CALCULATIONS					-\$120.00
Reduction by 1/2 for filing by small entity, if applicable. Affidavit must be filed also. (Note 37 CFR 1.9, 1.27, 1.28.)					\$60.00
SUBTOTAL					+\$60.00
Processing fee of \$30 for furnishing the English Translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 mos. from the earliest claimed priority date (37 CFR 1.492(f)).					
TOTAL NATIONAL FEE					\$ 60.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)).					+
TOTAL FEES ENCLOSED					\$ 60.00

- a. A check in the amount of \$ 60.00 to cover the above fees is enclosed.
- b. Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.
- c. The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 23-3050. A duplicate copy of this sheet is enclosed.

10/16/91X060 * 00236-00257*

01143329

25-9

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Date of Deposit September 17, 1991

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, D. C. 20591

Diane M. Kushner

Diane M. Kushner
(SIGNATURE)

- 3. A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. is not required, as the application was filed in the United States Receiving Office (RO/US).
 - c. has been transmitted by the International Bureau.
- 4. A translation of the International Application into English (35 U.S.C. 371 (c)(2)).
- 5. Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. have been transmitted by the International Bureau.
- 6. A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
- 7. An oath or declaration of the inventor (35 U.S.C. 371(c)(4)).
- 8. A translation of the Annexes to the International Preliminary Examination Report under PCT Article 36(35 U.S.C. 371(c)(5)).

Other document(s) or information included:

- 9. An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
- 10. An assignment document for recording.
Please mail the recorded assignment document to:
 - a. the person whose signature, name & address appears at the bottom of this page.
 - b. the following:

- 11. The above checked items are being transmitted
 - a. before the 18th month publication.
 - b. after publication and the Article 20 communication but before 20 months from the priority date.
 - c. after 20 months but before 22 months (surcharge and/or processing fee included).
 - d. after 22 months (surcharge and/or processing fee included).
Note: Petition to revive (37 CFR 1.137(a) or (b)) is necessary if 35 U.S.C. 371 requirements submitted after 22 months and no proper demand for International Preliminary Examination was made by 19 months from the earliest claimed priority date.
 - e. by 30 months and a proper demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
 - f. after 30 months but before 32 months and a proper demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date (surcharge and/or processing fee included).
 - g. after 32 months (surcharge and/or processing fee included).
Note: Petition to revive (37 CFR 1.137(a) or (b)) is necessary if 35 U.S.C. 371 requirements submitted after 32 months and a proper demand for International Preliminary Examination was made by 19 months from the earliest claimed priority date.
- 12. At the time of transmittal, the time limit for amending claims under Article 19
 - a. has expired and no amendments were made.
 - b. has not yet expired.
- 13. Certain requirements under 35 U.S.C. 371 were previously submitted by the applicant on _____, namely:
date

14. Submission of Verified Statement Claiming Small Entity Status and Request for Refund

Francis A. Paintin

NAME

Woodcock Washburn Kurtz Mackiewicz & Norris

ADDRESS

One Liberty Place - 46th Floor

Philadelphia, PA 19103

SIGNATURE

REGISTRATION NUMBER

19,386

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name; and

I verily believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: HUMANISED ANTIBODIES the specification of which:

_____ is attached hereto.

was filed on 21 December 1990 as International Application Serial No. PCT/GB90/02017 and was amended on _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with 37 CFR § 1.56(a).

I hereby claim foreign priority benefits under 35 U.S.C. § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of any application on which priority is claimed:

Country	Number	Date Filed	Priority Claimed
<u>U.K.</u>	<u>8928874.0</u>	<u>21.12.89</u>	<u>yes</u>
_____	_____	_____	_____
_____	_____	_____	_____

18 Rec'd PCT/PTO

17 SEP 1991

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose material information as defined in 37 CFR § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.	Filing Date	Status (patented, pending)
_____	_____	_____
_____	_____	_____
_____	_____	_____

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:
Francis A. Paintin

Registration Nos. 19,386
of the firm of **WOODCOCK WASHBURN KURTZ MACKIEWICZ & NORRIS**, One Liberty Place - 46th Floor, Philadelphia, Pennsylvania 19103, and

Address all telephone calls and correspondence to:
Francis A. Paintin

WOODCOCK WASHBURN KURTZ MACKIEWICZ & NORRIS
One Liberty Place - 46th Floor
Philadelphia, PA 19103
Telephone No. 215-568-3100.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the

18 Rec'd PCT/PTO

17 SEP 1991

United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00 1	Full Name JOHN ROBERT ADAIR	Inventor's Signature <i>John Robert Adair</i>	Date 13/8/91
	Residence 23 George Road, Stokenchurch High Wycombe, Buckinghamshire HP14 3RN, U.K. GBX		Citizenship U.K.
	Post Office Address 23 George Road, Stokenchurch, High Wycombe, Buckinghamshire HP14 3RN, U.K.		
2-00 2	Full Name DILJEET SINGH ATHWAL	Inventor's Signature <i>[Signature]</i>	Date 13/8/91
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Carter et al.

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(54) **METHOD FOR MAKING HUMANIZED ANTIBODIES**

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435/70.21; 435/91; 536/23.53; 424/133.1

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435/70.21, 91, 172.2, 240.1, 240.27, 252.3,
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(57) **ABSTRACT**

Variant immunoglobulins, particularly humanized antibody polypeptides are provided, along with methods for their preparation and use. Consensus immunoglobulin sequences and structural models are also provided.

82 Claims, 9 Drawing Sheets

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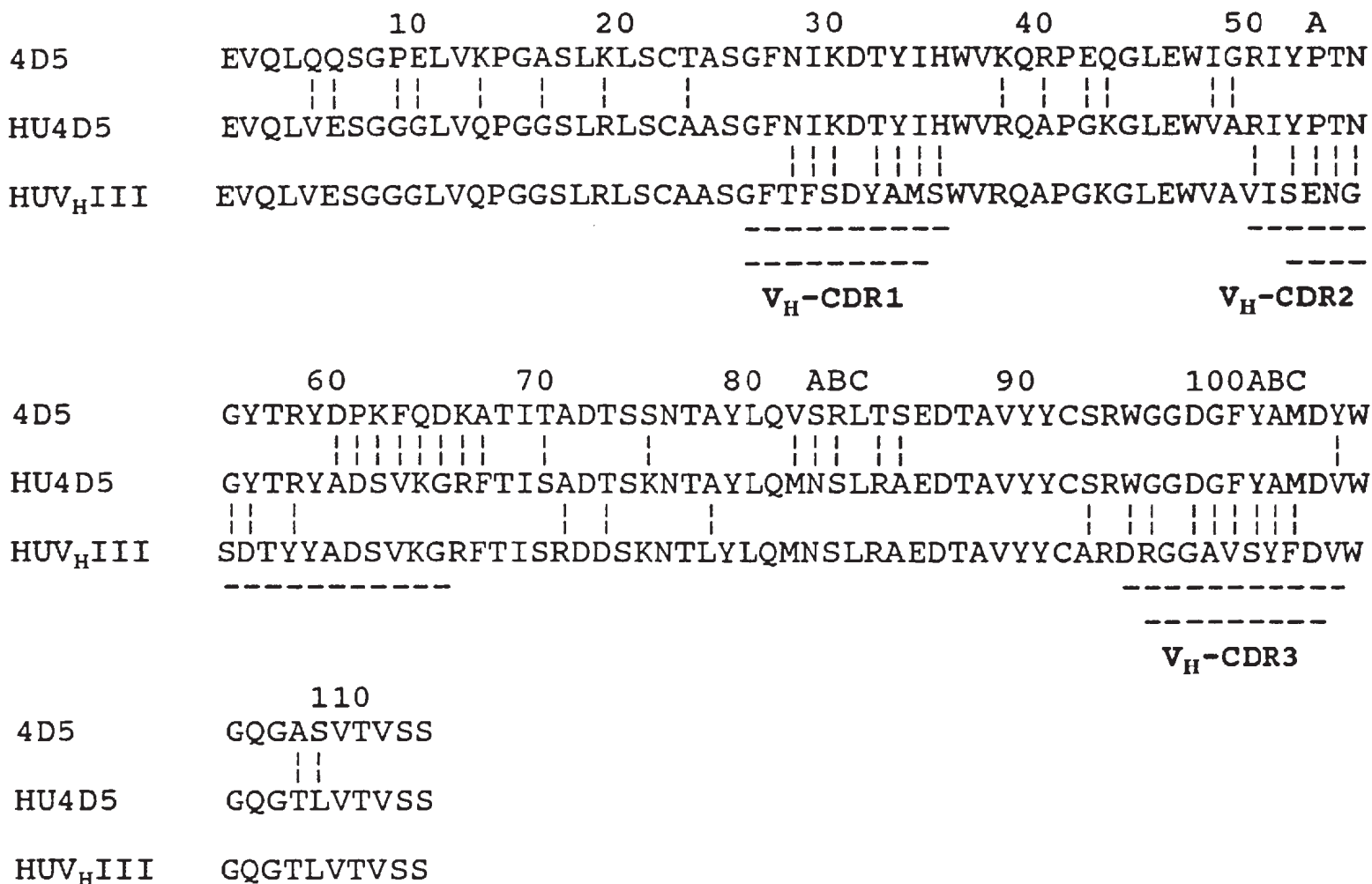
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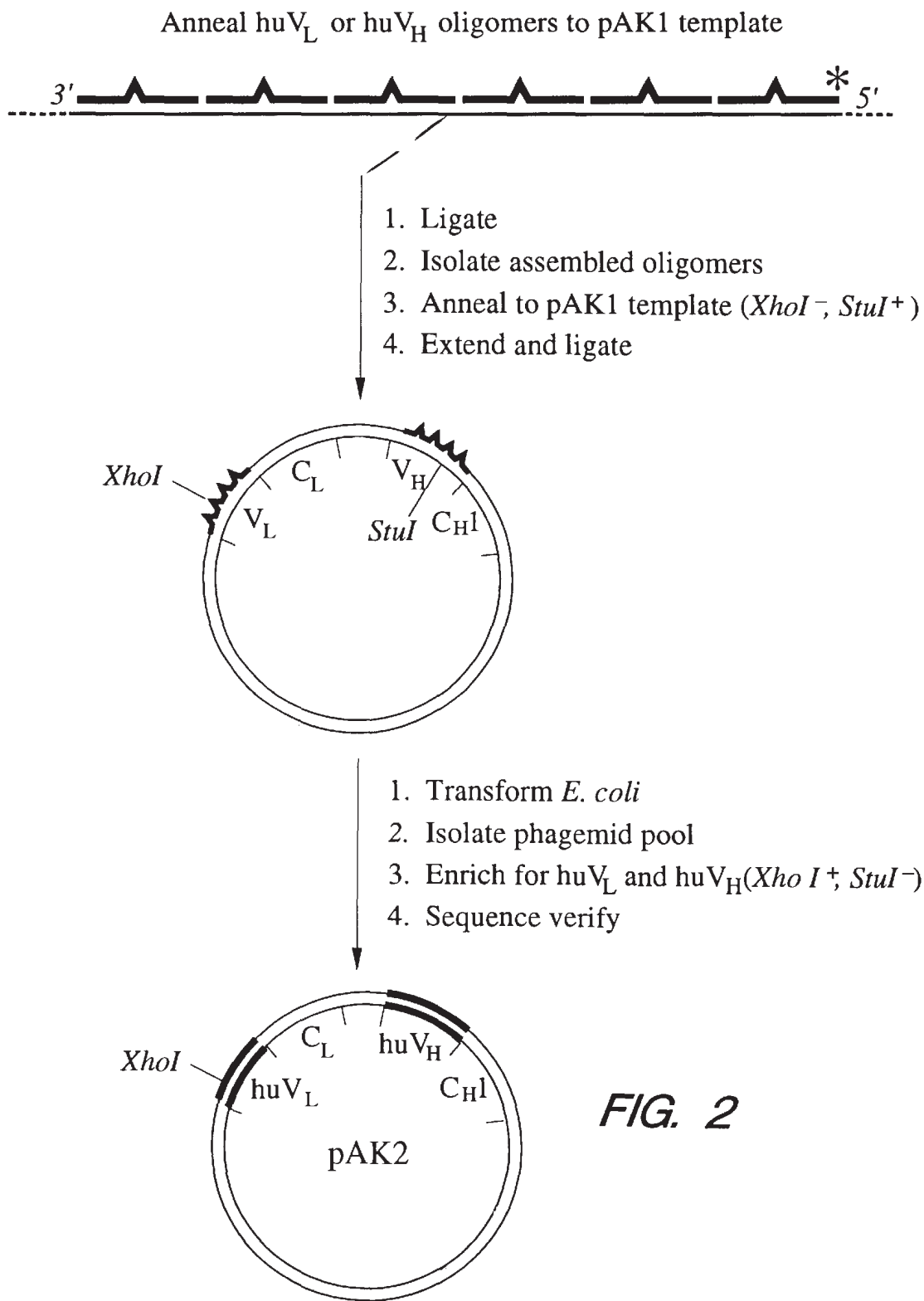
FIG. 1A

	10	20	30	40	50
4D5	DIVMTQSHKFMSTSVGDRVSITCKASQDVNTAVAWYQQKPGHSPKLLIYSASFRYT				
HU4D5	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLES				
HUV _L κI	DIQMTQSPSSLSASVGDRVTITCRASQDVSSYLAWYQQKPGKAPKLLIYAASSLES				
			-----		-----
			-----		---
			V_L-CDR1		V_L-CDR2
	60	70	80	90	100
4D5	GVPDRFTGNRSGTDFFTISSVQAEDLAVYYCQQHYTTPPTFGGGTKLEIKRA				
HU4D5	GVPSRFSGSRSGTDFTLTISLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRT				
HUV _L κI	GVPSRFSGSGSGTDFTLTISLQPEDFATYYCQQYNSLPYTFGQGTKVEIKRT				

			V_L-CDR3		

FIG. 1B





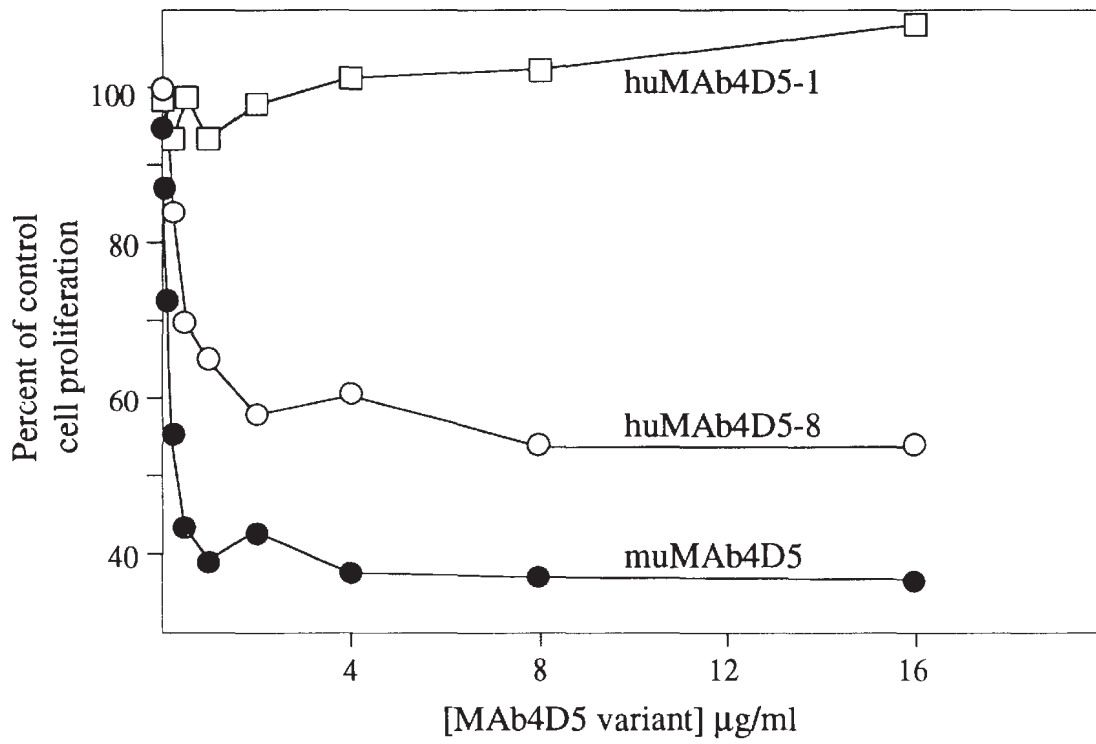


FIG. 3

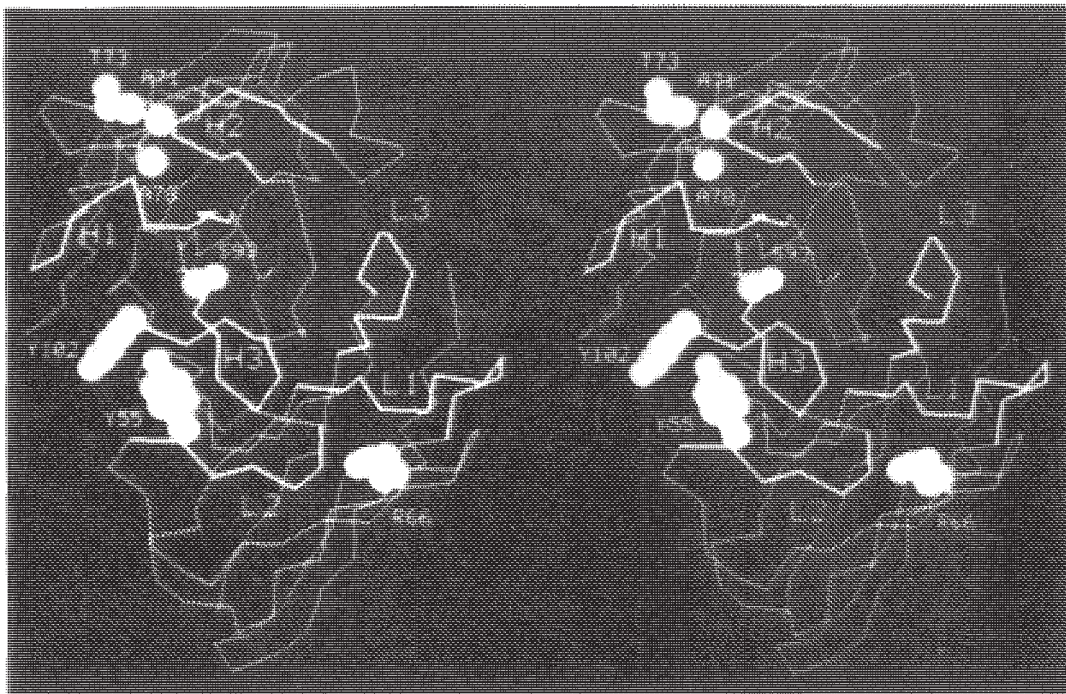


FIG. 4

	10	20	30	40
V_L muxCD3	DIQMTQTSSLSASLGDRVTISCRASQDIRN ^{•••} YL ^{•••} NWYQQKP			
	^{**}	[*]		
huxCD3v1	DIQMTQSPSSLSASVGDRTITCRASQDIRN ^{•••} YL ^{•••} NWYQQKP			
			^{# # #}	
huκI	DIQMTQSPSSLSASVGDRTITCRASQISIN ^{•••} YL ^{•••} AWYQQKP6			
			^{^ ^ ^ ^}	

	50	60	70	80
muxCD3	DGTVKLLI ^{•••} Y ^{•••} T ^{•••} SRL ^{•••} HSGVPSKFSGSGSGTDYSLTISNLEQ			
	^{****}	[*]	[*]	^{**}
huxCD3v1	GKAPKLLIY ^{•••} T ^{•••} SRLESGVPSRFSGSGSGTDYTLT ^{•••} ISSLQP			
	^{# # #}		[#]	
huκI	GKAPKLLIYAASSLESGVPSRFSGSGSGTDFTLT ^{•••} ISSLQP			
	^{^ ^}			

	90	100
muxCD3	EDIATYFCQ ^{•••} Q ^{•••} NTLPWTFAGG ^{•••} TKLEIK	
	[*]	^{**}
huxCD3v1	EDFATYYCQ ^{•••} Q ^{•••} NTLPWTFGQ ^{•••} G ^{•••} TKVEIK	
	^{# #}	
huκI	EDFATYYCQ ^{•••} Y ^{•••} NSLPWTFGQ ^{•••} G ^{•••} TKVEIK	
	^{^ ^ ^ ^}	

	10	20	30	40
V_H muxCD3	EVQLQ ^{•••} QSGPELVKPGASMKISCKASGYSFT ^{•••} G ^{•••} Y ^{•••} TMN ^{•••} WVKQS			
	^{**}	^{**}	[*]	^{**}
huxCD3v1	EVQLVESGGGLVQPGGSLRLSCAASGYSFTG ^{•••} Y ^{•••} TMN ^{•••} WVRQA			
			^{# # # #}	
huIII	EVQLVESGGGLVQPGGSLRLSCAASGFTFSS ^{•••} Y ^{•••} AMSWVRQA			
			^{^ ^ ^ ^}	

	50	60	70
muxCD3	HGKNLEW ^{•••} MGLIN ^{•••} PYK ^{•••} GV ^{•••} STYNQKFKDKATLTVDKSSSTAY		
	[*]	[*]	^{**}
huxCD3v1	PGKGLEWVALINPYKGV ^{•••} TTYADSVKGRFTISVDKSKNTAY		
	^{# # # # #}	[#]	^{# # #}
HuIII	PGKGLEWVSVISGDGGSTYYADSVKGRFTISRDN ^{•••} SKNTLY		
	^{^ ^ ^}		

	80	abc	90	100	abcde	110
muxCD3	MELLSLTSEDSAVYYCAR ^{•••} S ^{•••} G ^{•••} Y ^{•••} Y ^{•••} G ^{•••} S ^{•••} DWYFDVWGAGTTVTVSS					
	^{****}	^{**}	[*]		[*]	[*]
huxCD3v1	LQMNSLRAEDTAVYYCARSGYYGDS ^{•••} DWYFDVWQ ^{•••} GLVTVSS					
			^{#####}	[#]		
huIII	LQMNSLRAEDTAVYYCARGRVGYSLSGLYDYWQ ^{•••} GLVTVSS					
			^{D E T S}			
			^{^ ^ ^ ^ ^}			

FIG. 5

FIG. 6A-1

H52H4-160		10	20	30
	QVQLQQSGPELVKPGASVKISCKTSGYTFTE			
pH52-8.0	MGWSCIILFLVATATGVHSEVQLVESGGGLVQPGGSLRLSCATSGYTFTE			
		10	20	30
		40	50	60
H52H4-160	YTMHWMKQSHGKSLEWIGGFNPKNGGSSHNQRFMDKATLAVDKSTSTAYM			
pH52-8.0	YTMHWMRQAPGKGLEWVAGINPKNGGTSHNQRFMDFRTISVDKSTSTAYM			
		60	70	80
		90	100	110
H52H4-160	ELRSLTSEDSGIYYCARWRGLNYGFDVRYFDVWGAGTTVTVSSASTKGPS			
pH52-8.0	QMNSLRAEDTAVYYCARWRGLNYGFDVRYFDVWGQGLVTVSSASTKGPS			
		110	120	130
		140	150	160
H52H4-160	VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSQVHTFPAVL			
pH52-8.0	VFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSQVHTFPAVL			
		160	170	180
		190	200	210
H52H4-160	QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKEPKSCDKTH			
pH52-8.0	QSSGLYSLSSVVTVTSSNFGTQTYTCNVNVDHKPSNTKVDKKTVERKCC---V			
		210	220	230
		240	250	260
H52H4-160	TCPPCPAPPELLGGPSVFLFPPKPKDTLMIISRTPEVTCVVVDVSHEDPEVK			
pH52-8.0	ECPPCPAPP-VAGPSVFLFPPKPKDTLMIISRTPEVTCVVVDVSHEDPEVQ			
		250	260	270
		280	290	300

FIG. 6A-2

290 300 310 320 330
 H52H4-160 FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV
 *****.*****.***.*****.*****
 pH52-8.0 FNWYVDGMEVHNAKTKPREEQFNSTFRVVSVLTVVHQDWLNGKEYKCKV
 300 310 320 330 340

340 350 360 370 380
 H52H4-160 NKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYP
 .***.*****
 pH52-8.0 NKGLPAPIEKTISKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYP
 350 360 370 380 390

390 400 410 420 430
 H52H4-160 SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFS
 *****.*****
 pH52-8.0 SDIAVEWESNGQPENNYKTTPPMLDSDGSFFLYSKLTVDKSRWQQGNVFS
 400 410 420 430 440

440 450
 H52H4-160 CSVMHEALHNHYTQKSLSLSPGK

 pH52-8.0 CSVMHEALHNHYTQKSLSLSPGK
 450 460

FIG. 6B

		10	20	30
H52L6-158		DVQMTQTTSSLSASLGDRVTINCRASQDINN		
		*.****.*****.*****.*****.*****		
pH52-9.0	MGWSCIIILFLVATATGVHSDIQMTQSPSSLSASVGDRVTITCRASQDINN			
		10	20	30
		40	50	60
H52L6-158	YLNWYQQKPNGTVKLLIYYTSTLHSGVPSRFSGSGSGTDYSLTISNLDQE			
		***** . *****.*****.*****.*. *		
pH52-9.0	YLNWYQQKPGKAPKLLIYYTSTLHSGVPSRFSGSGSGTDYTLTISLQPE			
		60	70	80
		90	100	110
H52L6-158	DIATYFCQQGNTLPPTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTAS			
		*.***.*****.*****.*****.*****.*****.*****		
pH52-9.0	DFATYYCQQGNTLPPTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTAS			
		110	120	130
		140	150	160
H52L6-158	VVCLLNNFYFPREAKVQWKVDNALQSGNSQESVTEQDSKDSYSTLSSTLTL			
		*****.*****.*****.*****.*****.*****.*****.*****		
pH52-9.0	VVCLLNNFYFPREAKVQWKVDNALQSGNSQESVTEQDSKDSYSTLSSTLTL			
		160	170	180
		190	200	210
H52L6-158	SKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC			
		*****.*****.*****.*****.*****.*****.*****.*****		
pH52-9.0	SKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC			
		210	220	230

METHOD FOR MAKING HUMANIZED ANTIBODIES

CROSS REFERENCES

This application is a continuation-in-part of U.S. application Ser. No. 07/715,272 filed Jun. 14, 1991 (abandoned) which application is incorporated herein by reference and to which application priority is claimed under 35 USC §120.

FIELD OF THE INVENTION

This invention relates to methods for the preparation and use of variant antibodies and finds application particularly in the fields of immunology and cancer diagnosis and therapy.

BACKGROUND OF THE INVENTION

Naturally occurring antibodies (immunoglobulins) comprise two heavy chains linked together by disulfide bonds and two light chains, one light chain being linked to each of the heavy chains by disulfide bonds. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain (V_L) at one end and a constant domain at its other end, the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light and heavy chain variable domains, see e.g. Chothia et al., *J. Mol. Biol.* 186:651-663 (1985); Novotny and Haber, *Proc. Natl. Acad. Sci. USA* 82:4592-4596 (1985).

The constant domains are not involved directly in binding the antibody to an antigen, but are involved in various effector functions, such as participation of the antibody in antibody-dependent cellular cytotoxicity. The variable domains of each pair of light and heavy chains are involved directly in binding the antibody to the antigen. The domains of natural light and heavy chains have the same general structure, and each domain comprises four framework (FR) regions, whose sequences are somewhat conserved, connected by three hyper-variable or complementarity determining regions (CDRs) (see Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest*, National Institutes of Health, Bethesda, Md., (1987)). The four framework regions largely adopt a β -sheet conformation and the CDRs form loops connecting, and in some cases forming part of, the β -sheet structure. The CDRs in each chain are held in close proximity by the framework regions and, with the CDRs from the other chain, contribute to the formation of the antigen binding site.

Widespread use has been made of monoclonal antibodies, particularly those derived from rodents including mice, however they are frequently antigenic in human clinical use. For example, a major limitation in the clinical use of rodent monoclonal antibodies is an anti-globulin response during therapy (Miller, R. A. et al., *Blood* 62:988-995 (1983); Schroff, R. W. et al., *Cancer Res.* 45:879-885 (1985)).

The art has attempted to overcome this problem by constructing "chimeric" antibodies in which an animal antigen-binding variable domain is coupled to a human constant domain (Cabilly et al., U.S. Pat. No. 4,816,567; Morrison, S. L. et al., *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984); Boulianne, G. L. et al., *Nature* 312:643-646 (1984); Neuberger, M. S. et al., *Nature* 314:268-270 (1985)). The term "chimeric" antibody is used herein to describe a polypeptide comprising at least the

antigen binding portion of an antibody molecule linked to at least part of another protein (typically an immunoglobulin constant domain).

The isotype of the human constant domain may be selected to tailor the chimeric antibody for participation in antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (see e.g. Brüggemann, M. et al., *J. Exp. Med.* 166:1351-1361 (1987); Riechmann, L. et al., *Nature* 332:323-327 (1988); Love et al., *Methods in Enzymology* 178:515-527 (1989); Bindon et al., *J. Exp. Med.* 168:127-142 (1988).

In the typical embodiment, such chimeric antibodies contain about one third rodent (or other non-human species) sequence and thus are capable of eliciting a significant anti-globulin response in humans. For example, in the case of the murine anti-CD3 antibody, OKT3, much of the resulting anti-globulin response is directed against the variable region rather than the constant region (Jaffers, G. J. et al., *Transplantation* 41:572-578 (1986)).

In a further effort to resolve the antigen binding functions of antibodies and to minimize the use of heterologous sequences in human antibodies, Winter and colleagues (Jones, P. T. et al., *Nature* 321:522-525 (1986); Riechmann, L. et al., *Nature* 332:323-327 (1988); Verhoeven, M. et al., *Science* 239:1534-1536 (1988)) have substituted rodent CDRs or CDR sequences for the corresponding segments of a human antibody. As used herein, the term "humanized" antibody is an embodiment of chimeric antibodies wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The therapeutic promise of this approach is supported by the clinical efficacy of a humanized antibody specific for the CAMPATH-1 antigen with two non-Hodgkin lymphoma patients, one of whom had previously developed an anti-globulin response to the parental rat antibody (Riechmann, L. et al., *Nature* 332:323-327 (1988); Hale, G. et al., *Lancet* i:1394-1399 (1988)). A murine antibody to the interleukin 2 receptor has also recently been humanized (Queen, C. et al., *Proc. Natl. Acad. Sci. USA* 86:10029-10033 (1989)) as a potential immunosuppressive reagent. Additional references related to humanization of antibodies include Co et al., *Proc. Natl. Acad. Sci. USA* 88:2869-2873 (1991); Gorman et al., *Proc. Natl. Acad. Sci. USA* 88:4181-4185 (1991); Daugherty et al., *Nucleic Acids Research* 19(9):2471-2476 (1991); Brown et al., *Proc. Natl. Acad. Sci. USA* 88:2663-2667 (1991); Junghans et al., *Cancer Research* 50:1495-1502 (1990).

In some cases, substituting CDRs from rodent antibodies for the human CDRs in human frameworks is sufficient to transfer high antigen binding affinity (Jones, P. T. et al., *Nature* 321:522-525 (1986); Verhoeven, M. et al., *Science* 239:1534-1536 (1988)), whereas in other cases it has been necessary to additionally replace one (Riechmann, L. et al., *Nature* 332:323-327 (1988)) or several (Queen, C. et al., *Proc. Natl. Acad. Sci. USA* 86:10029-10033 (1989)) framework region (FR) residues. See also Co et al., *supra*.

For a given antibody a small number of FR residues are anticipated to be important for antigen binding. Firstly for example, certain antibodies have been shown to contain a few FR residues which directly contact antigen in crystal structures of antibody-antigen complexes (e.g., reviewed in Davies, D. R. et al., *Ann. Rev. Biochem.* 59:439-473 (1990)).

Secondly, a number of FR residues have been proposed by Chothia, Lesk and colleagues (Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901-917 (1987); Chothia, C. et al., *Nature* 342:877-883 (1989); Tramontano, A. et al., *J. Mol. Biol.* 215:175-182 (1990)) as critically affecting the conformation of particular CDRs and thus their contribution to antigen binding. See also Margolies et al., *Proc. Natl. Acad. Sci. USA* 72:2180-2184 (1975).

It is also known that, in a few instances, an antibody variable domain (either V_H or V_L) may contain glycosylation sites, and that this glycosylation may improve or abolish antigen binding, Pluckthun, *Biotechnology* 9:545-51 (1991); Spiegelberg et al., *Biochemistry* 9:4217-4223 (1970); Wallic et al., *J. Exp. Med.* 168:1099-1109 (1988); Sox et al., *Proc. Natl. Acad. Sci. USA* 66:975-982 (1970); Margni et al., *Ann. Rev. Immunol.* 6:535-554 (1988). Ordinarily, however, glycosylation has no influence on the antigen-binding properties of an antibody, Pluckthun, supra, (1991).

The three-dimensional structure of immunoglobulin chains has been studied, and crystal structures for intact immunoglobulins, for a variety of immunoglobulin fragments, and for antibody-antigen complexes have been published (see e.g., Saul et al., *Journal of Biological Chemistry* 25:585-97 (1978); Sheriff et al., *Proc. Natl. Acad. Sci. USA* 84:8075-79 (1987); Segal et al., *Proc. Natl. Acad. Sci. USA* 71:4298-4302 (1974); Epp et al., *Biochemistry* 14(22):4943-4952 (1975); Marquart et al., *J. Mol. Biol.* 141:369-391 (1980); Furey et al., *J. Mol. Biol.* 167:661-692 (1983); Snow and Amzel, *Protein: Structure, Function, and Genetics* 1:267-279, Alan R. Liss, Inc. pubs. (1986); Chothia and Lesk, *J. Mol. Biol.* 196:901-917 (1987); Chothia et al., *Nature* 342:877-883 (1989); Chothia et al., *Science* 233:755-58 (1986); Huber et al., *Nature* 264:415-420 (1976); Bruccoleri et al., *Nature* 335:564-568 (1988) and *Nature* 336:266 (1988); Sherman et al., *Journal of Biological Chemistry* 263:4064-4074 (1988); Amzel and Poljak, *Ann. Rev. Biochem.* 48:961-67 (1979); Silvertown et al., *Proc. Natl. Acad. Sci. USA* 74:5140-5144 (1977); and Gregory et al., *Molecular Immunology* 24:821-829 (1987). It is known that the function of an antibody is dependent on its three dimensional structure, and that amino acid substitutions can change the three-dimensional structure of an antibody, Snow and Amzel, supra. It has previously been shown that the antigen binding affinity of a humanized antibody can be increased by mutagenesis based upon molecular modelling (Riechmann, L. et al., *Nature* 332:323-327 (1988); Queen, C. et al., *Proc. Natl. Acad. Sci. USA* 86:10029-10033 (1989)).

Humanizing an antibody with retention of high affinity for antigen and other desired biological activities is at present difficult to achieve using currently available procedures. Methods are needed for rationalizing the selection of sites for substitution in preparing such antibodies and thereby increasing the efficiency of antibody humanization.

The proto-oncogene HER2 (human epidermal growth factor receptor 2) encodes a protein tyrosine kinase (p185^{HER2}) that is related to and somewhat homologous to the human epidermal growth factor receptor (see Coussens, L. et al., *Science* 230:1132-1139 (1985); Yamamoto, T. et al., *Nature* 319:230-234 (1986); King, C. R. et al., *Science* 229:974-976 (1985)). HER2 is also known in the field as c-erbB-2, and sometimes by the name of the rat homolog, neu. Amplification and/or overexpression of HER2 is associated with multiple human malignancies and appears to be integrally involved in progression of 25-30% of human breast and ovarian cancers (Slamon, D. J. et al., *Science*

235:177-182 (1987), Slamon, D. J. et al., *Science* 244:707-712 (1989)). Furthermore, the extent of amplification is inversely correlated with the observed median patient survival time (Slamon, supra, *Science* 1989).

The murine monoclonal antibody known as muMAB4D5 (Fendly, B. M. et al., *Cancer Res.* 50:1550-1558 (1990)), directed against the extracellular domain (ECD) of p185^{HER2}, specifically inhibits the growth of tumor cell lines overexpressing p185^{HER2} in monolayer culture or in soft agar (Hudziak, R. M. et al., *Molec. Cell. Biol.* 9:1165-1172 (1989); Lupu, R. et al., *Science* 249:1552-1555 (1990)). MuMAB4D5 also has the potential of enhancing tumor cell sensitivity to tumor necrosis factor, an important effector molecule in macrophage-mediated tumor cell cytotoxicity (Hudziak, supra, 1989; Shepard, H. M. and Lewis, G. D. *J. Clinical Immunology* 8:333-395 (1988)). Thus muMAB4D5 has potential for clinical intervention in and imaging of carcinomas in which p185^{HER2} is overexpressed. The muMAB4D5 and its uses are described in PCT application WO 89/06692 published Jul. 27, 1989. This murine antibody was deposited with the ATCC and designated ATCC CRL 10463. However, this antibody may be immunogenic in humans.

It is therefore an object of this invention to provide methods for the preparation of antibodies which are less antigenic in humans than non-human antibodies but have desired antigen binding and other characteristics and activities.

It is a further object of this invention to provide methods for the efficient humanization of antibodies, i.e. selecting non-human amino acid residues for importation into a human antibody background sequence in such a fashion as to retain or improve the affinity of the non-human donor antibody for a given antigen.

It is another object of this invention to provide humanized antibodies capable of binding p185^{HER2}.

Other objects, features, and characteristics of the present invention will become apparent upon consideration of the following description and the appended claims.

SUMMARY OF THE INVENTION

The objects of this invention are accomplished by a method for making a humanized antibody comprising amino acid sequence of an import, non-human antibody and a human antibody, comprising the steps of:

- a. obtaining the amino acid sequences of at least a portion of an import antibody variable domain and of a consensus variable domain;
- b. identifying Complementarity Determining Region (CDR) amino acid sequences in the import and the human variable domain sequences;
- c. substituting an import CDR amino acid sequence for the corresponding human CDR amino acid sequence;
- d. aligning the amino acid sequences of a Framework Region (FR) of the import antibody and the corresponding FR of the consensus antibody;
- e. identifying import antibody FR residues in the aligned FR sequences that are non-homologous to the corresponding consensus antibody residues;
- f. determining if the non-homologous import amino acid residue is reasonably expected to have at least one of the following effects:
 1. non-covalently binds antigen directly,
 2. interacts with a CDR; or
 3. participates in the V_L - V_H interface; and

g. for any non-homologous import antibody amino acid residue which is reasonably expected to have at least one of these effects, substituting that residue for the corresponding amino acid residue in the consensus antibody FR sequence.

Optionally, the method of this invention comprises the additional steps of determining if any non-homologous residues identified in step (e) are exposed on the surface of the domain or buried within it, and if the residue is exposed but has none of the effects identified in step (f), retaining the consensus residue.

Additionally, in certain embodiments the method of this invention comprises the feature wherein the corresponding consensus antibody residues identified in step (e) above are selected from the group consisting of 4L, 35L, 36L, 38L, 43L, 44L, 46L, 58L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 71 L, 73L, 85L, 87L, 98L, 2H, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 76H, 78H, 91H, 92H, 93H, and 103H (utilizing the numbering system set forth in Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)).

In certain embodiments, the method of this invention comprises the additional steps of searching either or both of the import, non-human and the consensus variable domain sequences for glycosylation sites, determining if the glycosylation is reasonably expected to be important for the desired antigen binding and biological activity of the antibody (i.e., determining if the glycosylation site binds to antigen or changes a side chain of an amino acid residue that binds to antigen, or if the glycosylation enhances or weakens antigen binding, or is important for maintaining antibody affinity). If the import sequence bears the glycosylation site, it is preferred to substitute that site for the corresponding residues in the consensus human if the glycosylation site is reasonably expected to be important. If only the consensus sequence, and not the import, bears the glycosylation site, it is preferred to eliminate that glycosylation site or substitute therefor the corresponding amino acid residues from the import sequence.

Another embodiment of this invention comprises aligning import antibody and the consensus antibody FR sequences, identifying import antibody FR residues which are non-homologous with the aligned consensus FR sequence, and for each such non-homologous import antibody FR residue, determining if the corresponding consensus antibody residue represents a residue which is highly conserved across all species at that site, and if it is so conserved, preparing a humanized antibody which comprises the consensus antibody amino acid residue at that site.

Certain alternate embodiments of the methods of this invention comprise obtaining the amino acid sequence of at least a portion of an import, non-human antibody variable domain having a CDR and a FR, obtaining the amino acid sequence of at least a portion of a consensus antibody variable domain having a CDR and a FR, substituting the non-human CDR for the human CDR in the consensus antibody variable domain, and then substituting an amino acid residue for the consensus amino acid residue at at least one of the following sites:

- a. (in the FR of the variable domain of the light chain) 4L, 35L, 36L, 38L, 43L, 44L, 58L, 46L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 71L, 73L, 85L, 87L, 98L, or
- b. (in the FR of the variable domain of the heavy chain) 2H, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 78H, 91H, 92H, 93H, and 103H.

In preferred embodiments, the non-CDR residue substituted at the consensus FR site is the residue found at the corresponding location of the non-human antibody.

Optionally, this just-recited embodiment comprises the additional steps of following the method steps appearing at the beginning of this summary and determining whether a particular amino acid residue can reasonably be expected to have undesirable effects.

This invention also relates to a humanized antibody comprising the CDR sequence of an import, non-human antibody and the FR sequence of a human antibody, wherein an amino acid residue within the human FR sequence located at any one of the sites 4L, 35L, 36L, 38L, 43L, 44L, 46L, 58L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 2H, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 76H, 78H, 91H, 92H, 93H, and 103H has been substituted by another residue. In preferred embodiments, the residue substituted at the human FR site is the residue found at the corresponding location of the non-human antibody from which the non-human CDR was obtained. In other embodiments, no human FR residue other than those set forth in this group has been substituted.

This invention also encompasses specific humanized antibody variable domains, and isolated polypeptides having homology with the following sequences.

1. SEQ. ID NO. 1, which is the light chain variable domain of a humanized version of muMab4D5: DIQMTOSPSSLSASVGDRTITCRASQDVNTAVAWYQQKPKGKAPKLLIYSASFLESGVPSRFRSGSRSGTDFTLTISLQPEDFA-TYYCQQHYTTPPTFGQGTKVEIKRT

2. SEQ. ID NO. 2, which is the heavy chain variable domain of a humanized version of muMab4D5: EVQLVESGGGLVOPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDVWVGQGLVTVSS

In another aspect, this invention provides a consensus antibody variable domain amino acid sequence for use in the preparation of humanized antibodies, methods for obtaining, using, and storing a computer representation of such a consensus sequence, and computers comprising the sequence data of such a sequence. In one embodiment, the following consensus antibody variable domain amino acid sequences are provided:

SEQ. ID NO. 3 (light chain): DDIOMTOSPSSLSASVGDRTITCRASQDVSSYLAWYQQKPKGKAPKLLIYAASSLESGVPSRFRSGSGTDFTLTISLQPEDFAIYYCQQYNSLPYTFGQGTKVEIKRT, and

SEQ. ID NO. 4 (heavy chain): EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYAMSWVRQAPGKGL EWWAVISENGGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAWYCSRWGGDGFYAMDVWVGQGLVTVSS

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A shows the comparison of the V_L domain amino acid residues of muMab4D5, huMab4D5, and a consensus sequence (FIG. 1A, SEQ.ID NO. 5, SEQ. ID NO. 1 and SEQ. ID NO. 3, respectively). FIG. 1B shows the comparison between the V_H domain amino acid residues of the muMab4D5, huMab4D5, and a consensus sequence (FIG. 1B, SEQ. ID NO. 6, SEQ. ID NO. 2 and SEQ. ID NO. 4, respectively). Both FIGS. 1A and 1B use the generally accepted numbering scheme from Kabat, E. A., et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md. (1987)). In both FIG. 1A

and FIG. 1B, the CDR residues determined according to a standard sequence definition (as in Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) are indicated by the first underlining beneath the sequences, and the CDR residues determined according to a structural definition (as in Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901-917 (1987)) are indicated by the second, lower underlines. The mismatches between genes are shown by the vertical lines.

FIG. 2 shows a scheme for humanization of muMab4D5 V_L and V_H by gene conversion mutagenesis.

FIG. 3 shows the inhibition of SK-BR-3 proliferation by MAb4D5 variants. Relative cell proliferation was determined as described (Hudziak, R. M. et al., *Molec. Cell. Biol.* 9:1165-1172 (1989)) and data (average of triplicate determinations) are presented as a percentage of results with untreated cultures for muMab4D5 (●), huMab4D5-8 (○) and huMab4D5-1 (□).

FIG. 4 shows a stereo view of α -carbon tracing for a model of huMab4D5-8 V_L and V_H . The CDR residues (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) are shown in bold and side chains of V_H residues A71, T73, A78, S93, Y102 and V_L residues Y55 plus R66 (see Table 3) are shown.

FIG. 5 shows an amino acid sequence comparison of V_L (top panel) and V_H (lower panel) domains of the murine anti-CD3 monoclonal Ab UCHT1 (muxCD3, Shalaby et al., *J. Exp. Med.* 175, 217-225 (1992)) with a humanized variant of this antibody (huxCD3v1). Also shown are consensus sequences (most commonly occurring residue or pair of residues) of the most abundant human subgroups, namely V_L κ 1 and V_H III upon which the humanized sequences are based (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest*, 5th edition, National Institutes of Health, Bethesda, Md., USA (1991)). The light chain sequences—muxCD3, huxCD3v1 and huKI—correspond to SEQ.ID. NOs 16, 17, and 18, respectively. The heavy chain sequences—muxCD3, huxCD3v1 and huxI—correspond to SEQ.ID.NOs 19, 26, and 21, respectively. Residues which differ between muxCD3 and huxCD3v1 are identified by an asterisk (*), whereas those which differ between humanized and consensus sequences are identified by a sharp sign (#). A bullet (•) denotes that a residue at this position has been found to contact antigen in one or more crystallographic structures of antibody/antigen complexes (Kabat et al., 1991; Mian, I. S. et al., *J. Mol. Biol.* 217, 133-151 (1991)). The location of CDR residues according to a sequence definition (Kabat et al., 1991) and a structural definition (Chothia and Lesk, supra 1987) are shown by a line and carats (^) beneath the sequences, respectively.

FIG. 6A compares murine and humanized amino acid sequences for the heavy chain of an anti-CD18 antibody. H52H4-160 (SEQ. ID. NO. 22) is the murine sequence, and pH52-8.0 (SEQ. ID. NO. 23) is the humanized heavy chain sequence. pH52-8.0 residue 143S is the final amino acid in the variable heavy chain domain V_H , and residue 144A is the first amino acid in the constant heavy chain domain C_{H1} .

FIG. 6B compares murine and humanized amino acid sequences for the light chain of an anti-CD18 antibody. H52L6-158 (SEQ. ID. NO. 24) is the murine sequence, and pH52-9.0 (SEQ. ID. NO. 25) is the humanized light chain sequence. pH52-9.0 residue 128T is the final amino acid in the light chain variable domain V_L , and residue 129V is the first amino acid in the light chain constant domain C_L .

DETAILED DESCRIPTION OF THE INVENTION

Definitions

In general, the following words or phrases have the indicated definitions when used in the description, examples, and claims:

The murine monoclonal antibody known as muMab4D5 (Fendly, B. M. et al., *Cancer Res.* 50:1550-1558 (1990)) is directed against the extracellular domain (ECD) of p185^{HER2}. The muMab4D5 and its uses are described in PCT application WO 89/06692 published Jul. 27, 1989. This murine antibody was deposited with the ATCC and designated ATCC CRL 10463. In this description and claims, the terms muMab4D5, chMab4D5 and huMab4D5 represent murine, chimerized and humanized versions of the monoclonal antibody 4D5, respectively.

A humanized antibody for the purposes herein is an immunoglobulin amino acid sequence variant or fragment thereof which is capable of binding to a predetermined antigen and which comprises a FR region having substantially the amino acid sequence of a human immunoglobulin and a CDR having substantially the amino acid sequence of a non-human immunoglobulin.

Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are referred to herein as "import" residues, which are typically taken from an "import" antibody domain, particularly a variable domain. An import residue, sequence, or antibody has a desired affinity and/or specificity, or other desirable antibody biological activity as discussed herein.

In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains (Fab, Fab', F(ab')₂, Fabc, Fv) in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. Ordinarily, the antibody will contain both the light chain as well as at least the variable domain of a heavy chain. The antibody also may include the CH1, hinge, CH2, CH3, and CH4 regions of the heavy chain.

The humanized antibody will be selected from any class of immunoglobulins, including IgM, IgG, IgD, IgA and IgE, and any isotype, including IgG1, IgG2, IgG3 and IgG4. Usually the constant domain is a complement fixing constant domain where it is desired that the humanized antibody exhibit cytotoxic activity, and the class is typically IgG₁. Where such cytotoxic activity is not desirable, the constant domain may be of the IgG₂ class. The humanized antibody may comprise sequences from more than one class or isotype, and selecting particular constant domains to optimize desired effector functions is within the ordinary skill in the art.

The FR and CDR regions of the humanized antibody need not correspond precisely to the parental sequences, e.g., the import CDR or the consensus FR may be mutagenized by substitution, insertion or deletion of at least one residue so that the CDR or FR residue at that site does not correspond to either the consensus or the import antibody. Such mutations, however, will not be extensive. Usually, at least 75% of the humanized antibody residues will correspond to those of the parental FR and CDR sequences, more often 90%, and most preferably greater than 95%.

In general, humanized antibodies prepared by the method of this invention are produced by a process of analysis of the parental sequences and various conceptual humanized products using three dimensional models of the parental and humanized sequences. Three dimensional immunoglobulin models are commonly available and are familiar to those

skilled in the art. Computer programs are available which illustrate and display probable three dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen.

Residues that influence antigen binding are defined to be residues that are substantially responsible for the antigen affinity or antigen specificity of a candidate immunoglobulin, in a positive or a negative sense. The invention is directed to the selection and combination of FR residues from the consensus and import sequence so that the desired immunoglobulin characteristic is achieved. Such desired characteristics include increases in affinity and greater specificity for the target antigen, although it is conceivable that in some circumstances the opposite effects might be desired. In general, the CDR residues are directly and most substantially involved in influencing antigen binding (although not all CDR residues are so involved and therefore need not be substituted into the consensus sequence). However, FR residues also have a significant effect and can exert their influence in at least three ways: They may noncovalently directly bind to antigen, they may interact with CDR residues and they may affect the interface between the heavy and light chains.

A residue that noncovalently directly binds to antigen is one that, by three dimensional analysis, is reasonably expected to noncovalently directly bind to antigen. Typically, it is necessary to impute the position of antigen from the spatial location of neighboring CDRs and the dimensions and structure of the target antigen. In general, only those humanized antibody residues that are capable of forming salt bridges, hydrogen bonds, or hydrophobic interactions are likely to be involved in non-covalent antigen binding, however residues which have atoms which are separated from antigen spatially by 3.2 Angstroms or less may also non-covalently interact with antigen. Such residues typically are the relatively larger amino acids having the side chains with the greatest bulk, such as tyrosine, arginine, and lysine. Antigen-binding FR residues also typically will have side chains that are oriented into an envelope surrounding the solvent oriented face of a CDR which extends about 7 Angstroms into the solvent from the CDR domain and about 7 Angstroms on either side of the CDR domain, again as visualized by three dimensional modeling.

A residue that interacts with a CDR generally is a residue that either affects the conformation of the CDR polypeptide backbone or forms a noncovalent bond with a CDR residue side chain. Conformation-affecting residues ordinarily are those that change the spatial position of any CDR backbone atom (N, Ca, C, O, C β) by more than about 0.2 Angstroms. Backbone atoms of CDR sequences are displaced for example by residues that interrupt or modify organized structures such as beta sheets, helices or loops. Residues that can exert a profound affect on the conformation of neighboring sequences include proline and glycine, both of which are capable of introducing bends into the backbone. Other residues that can displace backbone atoms are those that are capable of participating in salt bridges and hydrogen bonds.

A residue that interacts with a CDR side chain is one that is reasonably expected to form a noncovalent bond with a CDR side chain, generally either a salt bridge or hydrogen bond. Such residues are identified by three dimensional positioning of their side chains. A salt or ion bridge could be expected to form between two side chains positioned within

about 2.5–3.2 Angstroms of one another that bear opposite charges, for example a lysinyl and a glutamyl pairing. A hydrogen bond could be expected to form between the side chains of residue pairs such as seryl or threonyl with aspartyl or glutamyl (or other hydrogen accepting residues). Such pairings are well known in the protein chemistry art and will be apparent to the artisan upon three dimensional modeling of the candidate immunoglobulin.

Immunoglobulin residues that affect the interface between heavy and light chain variable regions (“the V_L–V_H interface”) are those that affect the proximity or orientation of the two chains with respect to one another. Certain residues involved in interchain interactions are already known and include V_L residues 34, 36, 38, 44, 46, 87, 89, 91, 96, and 98 and V_H residues 35, 37, 39, 45, 47, 91, 93, 95, 100, and 103 (utilizing the nomenclature set forth in Kabat et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)). Additional residues are newly identified by the inventors herein, and include 43L, 85L, 43H and 60H. While these residues are indicated for IgG only, they are applicable across species. In the practice of this invention, import antibody residues that are reasonably expected to be involved in interchain interactions are selected for substitution into the consensus sequence. It is believed that heretofore no humanized antibody has been prepared with an intrachain-affecting residue selected from an import antibody sequence.

Since it is not entirely possible to predict in advance what the exact impact of a given substitution will be it may be necessary to make the substitution and assay the candidate antibody for the desired characteristic. These steps, however, are per se routine and well within the ordinary skill of the art.

CDR and FR residues are determined according to a standard sequence definition (Kabat et al., *Sequences of Proteins of Immunological Interest*, National Institutes of Health, Bethesda Md. (1987), and a structural definition (as in Chothia and Lesk, *J. Mol. Biol.* 196:901–917 (1987)). Where these two methods result in slightly different identifications of a CDR, the structural definition is preferred, but the residues identified by the sequence definition method are considered important FR residues for determination of which framework residues to import into a consensus sequence.

Throughout this description, reference is made to the numbering scheme from Kabat, E. A., et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md. (1987) and (1991)). In these compendiums, Kabat lists many amino acid sequences for antibodies for each subclass, and lists the most commonly occurring amino acid for each residue position in that subclass. Kabat uses a method for assigning a residue number to each amino acid in a listed sequence, and this method for assigning residue numbers has become standard in the field. The Kabat numbering scheme is followed in this description.

For purposes of this invention, to assign residue numbers to a candidate antibody amino acid sequence which is not included in the Kabat compendium, one follows the following steps. Generally, the candidate sequence is aligned with any immunoglobulin sequence or any consensus sequence in Kabat. Alignment may be done by hand, or by computer using commonly accepted computer programs; an example of such a program is the Align 2 program discussed in this description. Alignment may be facilitated by using some amino acid residues which are common to most Fab

sequences. For example, the light and heavy chains each typically have two cysteines which have the same residue numbers; in V_L domain the two cysteines are typically at residue numbers 23 and 88, and in the V_H domain the two cysteine residues are typically numbered 22 and 92. Framework residues generally, but not always, have approximately the same number of residues, however the CDRs will vary in size. For example, in the case of a CDR from a candidate sequence which is longer than the CDR in the sequence in Kabat to which it is aligned, typically suffixes are added to the residue number to indicate the insertion of additional residues (see, e.g. residues 100abcde in FIG. 5). For candidate sequences which, for example, align with a Kabat sequence for residues 34 and 36 but have no residue between them to align with residue 35, the number 35 is simply not assigned to a residue.

Thus, in humanization of an import variable sequence, where one cuts out an entire human or consensus CDR and replaces it with an import CDR sequence, (a) the exact number of residues may be swapped, leaving the numbering the same, (b) fewer import amino acid residues may be introduced than are cut, in which case there will be a gap in the residue numbers, or (c) a larger number of amino acid residues may be introduced then were cut, in which case the numbering will involve the use of suffixes such as 100abcde.

The terms "consensus sequence" and "consensus antibody" as used herein refers to an amino acid sequence which comprises the most frequently occurring amino acid residues at each location in all immunoglobulins of any particular subclass or subunit structure. The consensus sequence may be based on immunoglobulins of a particular species or of many species. A "consensus" sequence, structure, or antibody is understood to encompass a consensus human sequence as described in certain embodiments of this invention, and to refer to an amino acid sequence which comprises the most frequently occurring amino acid residues at each location in all human immunoglobulins of any particular subclass or subunit structure. This invention provides consensus human structures and consensus structures which consider other species in addition to human.

The subunit structures of the live immunoglobulin classes in humans are as follows:

Class	Heavy Chain Subclasses	Light Chain	Molecular Formula
IgG	γ	$\gamma 1, \gamma 2, \gamma 3, \gamma 4$	κ or λ $(\gamma_2\kappa_2), (\gamma_2\lambda_2)$
IgA	α	$\alpha 1, \alpha 2$	κ or λ $(\alpha_2\kappa_2)_n^8, (\alpha_2\lambda_2)_n^8$
IgM	μ	none	κ or λ $(\mu_2\kappa_2)_5, (\mu_2\lambda_2)_5$
IgD	δ	none	κ or λ $(\delta_2\kappa_2), (\delta_2\lambda_2)$
IgE	ϵ	none	κ or λ $(\epsilon_2\kappa_2), (\epsilon_2\lambda_2)$

⁸_n may equal 1, 2, or 3)

In preferred embodiments of an IgG γ 1 human consensus sequence, the consensus variable domain sequences are derived from the most abundant subclasses in the sequence compilation of Kabat et al., *Sequences of Proteins of Immunological Interest*, National Institutes of Health, Bethesda Md. (1987), namely V_L κ subgroup I and V_H group III. In such preferred embodiments, the V_L consensus domain has the amino acid sequence:

DIQMTQSPSSLSASVGDRTTITCRASQD-VSSYLAWYQQKPGKAPKLLIYAASSLES-GVPSRFSGSGSDFTLTISLQPEDFA-TYYCQQYNSLPYTFGQGTKVEIKRT (SEQ. ID NO. 3);

the V_H consensus domain has the amino acid sequence:
 EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYAMSW
 VRQAPGKGLEWVAIVSENGGYTRYADSVKGRFT
 ISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGD
 GFYAMDVWGQGLVTVSS (SEQ. ID NO. 4).

These sequences include consensus CDRs as well as consensus FR residues (see for example in FIG. 1).

While not wishing to be limited to any particular theories, it may be that these preferred embodiments are less likely to be immunogenic in an individual than less abundant subclasses. However, in other embodiments, the consensus sequence is derived from other subclasses of human immunoglobulin variable domains. In yet other embodiments, the consensus sequence is derived from human constant domains.

Identity or homology with respect to a specified amino acid sequence of this invention is defined herein as the percentage of amino acid residues in a candidate sequence that are identical with the specified residues, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and not considering any conservative substitutions as part of the sequence identity. None of N-terminal, C-terminal or internal extensions, deletions, or insertions into the specified sequence shall be construed as affecting homology. All sequence alignments called for in this invention are such maximal homology alignments. While such alignments may be done by hand using conventional methods, a suitable computer program is the "Align 2" program for which protection is being sought from the U.S. Register of Copyrights (Align 2, by Genentech, Inc., application filed Dec. 9, 1991).

"Non-homologous" import antibody residues are those residues which are not identical to the amino acid residue at the analogous or corresponding location in a consensus sequence, after the import and consensus sequences are aligned.

The term "computer representation" refers to information which is in a form that can be manipulated by a computer. The act of storing a computer representation refers to the act of placing the information in a form suitable for manipulation by a computer.

This invention is also directed to novel polypeptides, and in certain aspects, isolated novel humanized anti-p185^{HER2} antibodies are provided. These novel anti-p185^{HER2} antibodies are sometimes collectively referred to herein as huMAB4D5, and also sometimes as the light or heavy chain variable domains of huMAB4D5, and are defined herein to be any polypeptide sequence which possesses a biological property of a polypeptide comprising the following polypeptide sequence:

DIQMTQSPSSLSASVGDRTTITCRASODVNTAVAWY
 QQKPGKAPKLLIYSASFLESGVPSRFSGSRSGT
 DFTLTISLQPEDFATYYCQQHYTTPPTFGQGTK
 VEIKRT (SEQ. ID NO. 1, which is the light chain variable domain of huMAB4D5); or

EVQLVESGGGLVOPGGSLRLSCAASGFNIKDTYIHW
 VRQAPGKGLEWVARIYPTNGYTRYADSVKGRFT
 ISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGD
 GFYAMDVWGQGLVTVSS (SEQ. ID NO. 2, which is the heavy chain variable domain of huMAB4D5).

"Biological property", as relates for example to anti-p185^{HER2}, for the purposes herein means an in vivo effector or antigen-binding function or activity that is directly or indirectly performed by huMAB4D5 (whether in its native or denatured conformation). Effector functions include p185^{HER2} binding, any hormonal or hormonal antagonist activity, any mitogenic or agonist or antagonist activity, any

cytotoxic activity. An antigenic function means possession of an epitope or antigenic site that is capable of cross-reacting with antibodies raised against the polypeptide sequence of huMAB4D5.

Biologically active huMAB4D5 is defined herein as a polypeptide that shares an effector function of huMAB4D5. A principal known effector function of huMAB4D5 is its ability to bind to p185^{HER2}.

Thus, the biologically active and antigenically active huMAB4D5 polypeptides that are the subject of certain embodiments of this invention include the sequence of the entire translated nucleotide sequence of huMAB4D5; mature huMAB4D5; fragments thereof having a consecutive sequence of at least 5, 10, 15, 20, 25, 30 or 40 amino acid residues comprising sequences from muMAB4D5 plus residues from the human FR of huMAB4D5; amino acid sequence variants of huMAB4D5 wherein an amino acid residue has been inserted N- or C-terminal to, or within, huMAB4D5 or its fragment as defined above; amino acid sequence variants of huMAB4D5 or its fragment as defined above wherein an amino acid residue of huMAB4D5 or its fragment as defined above has been substituted by another residue, including predetermined mutations by, e.g., site-directed or PCR mutagenesis; derivatives of huMAB4D5 or its fragments as defined above wherein huMAB4D5 or its fragments have been covalently modified, by substitution, chemical, enzymatic, or other appropriate means, with a moiety other than a naturally occurring amino acid; and glycosylation variants of huMAB4D5 (insertion of a glycosylation site or deletion of any glycosylation site by deletion, insertion or substitution of suitable residues). Such fragments and variants exclude any polypeptide heretofore identified, including muMAB4D5 or any known polypeptide fragment, which are anticipatory order 35 U.S.C. 102 as well as polypeptides obvious thereover under 35 U.S.C. 103.

An "isolated" polypeptide means polypeptide which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, for example, a polypeptide product comprising huMAB4D5 will be purified from a cell culture or other synthetic environment (1) to greater than 95% by weight of protein as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a gas- or liquid-phase sequenator (such as a commercially available Applied Biosystems sequenator Model 470, 477, or 473), or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated huMAB4D5 includes huMAB4D5 *in situ* within recombinant cells since at least one component of the huMAB4D5 natural environment will not be present. Ordinarily, however, isolated huMAB4D5 will be prepared by at least one purification step.

In accordance with this invention, huMAB4D5 nucleic acid is RNA or DNA containing greater than ten bases that encodes a biologically or antigenically active huMAB4D5, is complementary to nucleic acid sequence encoding such huMAB4D5, or hybridizes to nucleic acid sequence encoding such huMAB4D5 and remains stably bound to it under stringent conditions, and comprises nucleic acid from a muMAB4D5 CDR and a human FR region.

Preferably, the huMAB4D5 nucleic acid encodes a polypeptide sharing at least 75% sequence identity, more

preferably at least 80%, still more preferably at least 85%, even more preferably at 90%, and most preferably 95%, with the huMAB4D5 amino acid sequence. Preferably, a nucleic acid molecule that hybridizes to the huMAB4D5 nucleic acid contains at least 20, more preferably 40, and most preferably 90 bases. Such hybridizing or complementary nucleic acid, however, is further defined as being novel under 35 U.S.C. 102 and unobvious under 35 U.S.C. 103 over any prior art nucleic acid.

Stringent conditions are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0/1% NaDodSO₄ at 50° C.; (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0/1% Ficoll/0/1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42° C.; or (3) employ 50% formamide, 5×SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5×Denhardt's solution, sonicated salmon sperm DNA (50 g/ml), 0.1% SDS, and 10% dextran sulfate at 42 C., with washes at 42 C. in 0.2×SSC and 0.1% SDS.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, and possibly, other as yet poorly understood sequences. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. However enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

An "exogenous" element is defined herein to mean nucleic acid sequence that is foreign to the cell, or homologous to the cell but in a position within the host cell nucleic acid in which the element is ordinarily not found.

As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

"Oligonucleotides" are short-length, single- or double-stranded polydeoxynucleotides that are chemically synthesized by known methods (such as phosphotriester, phosphite, or phosphoramidite chemistry, using solid phase techniques such as described in EP 266,032 published May

4, 1988, or via deoxynucleoside H-phosphonate intermediates as described by Froehler et al., *Nucl. Acids Res.*, 14: 5399-5407 [1986]). They are then purified on polyacrylamide gels.

The technique of "polymerase chain reaction," or "PCR," as used herein generally refers to a procedure wherein minute amounts of a specific piece of nucleic acid, RNA and/or DNA, are amplified as described in U.S. Pat. No. 4,683,195 issued Jul. 28, 1987. Generally, sequence information from the ends of the region of interest or beyond needs to be available, such that oligonucleotide primers can be designed; these primers will be identical or similar in sequence to opposite strands of the template to be amplified. The 5' terminal nucleotides of the two primers may coincide with the ends of the amplified material. PCR can be used to amplify specific RNA sequences, specific DNA sequences from total genomic DNA, and cDNA transcribed from total cellular RNA, bacteriophage or plasmid sequences, etc. See generally Mullis et al., *Cold Spring Harbor Symp. Quant. Biol.*, 51: 263 (1987); Erlich, ed., *PCR Technology*, (Stockton Press, N.Y., 1989). As used herein, PCR is considered to be one, but not the only, example of a nucleic acid polymerase reaction method for amplifying a nucleic acid test sample, comprising the use of a known nucleic acid (DNA or RNA) as a primer and utilizes a nucleic acid polymerase to amplify or generate a specific piece of nucleic acid or to amplify or generate a specific piece of nucleic acid which is complementary to a particular nucleic acid.

Suitable Methods for Practicing the Invention

Some aspects of this invention include obtaining an import, non-human antibody variable domain, producing a desired humanized antibody sequence and for humanizing an antibody gene sequence are described below. A particularly preferred method of changing a gene sequence, such as gene conversion from a non-human or consensus sequence into a humanized nucleic acid sequence, is the cassette mutagenesis procedure described in Example 1. Additionally, methods are given for obtaining and producing antibodies generally, which apply equally to native non-human antibodies as well as to humanized antibodies.

Generally, the antibodies and antibody variable domains of this invention are conventionally prepared in recombinant

cell culture, as described in more detail below. Recombinant synthesis is preferred for reasons of safety and economy, but it is known to prepare peptides by chemical synthesis and to purify them from natural sources; such preparations are included within the definition of antibodies herein.

Molecular Modeling

An integral step in our approach to antibody humanization is construction of computer graphics models of the import and humanized antibodies. These models are used to determine if the six complementarity-determining regions (CDRs) can be successfully transplanted from the import framework to a human one and to determine which framework residues from the import antibody, if any, need to be incorporated into the humanized antibody in order to maintain CDR conformation. In addition, analysis of the sequences of the import and humanized antibodies and reference to the models can help to discern which framework residues are unusual and thereby might be involved in antigen binding or maintenance of proper antibody structure.

All of the humanized antibody models of this invention are based on a single three-dimensional computer graphics structure hereafter referred to as the consensus structure. This consensus structure is a key distinction from the approach of previous workers in the field, who typically begin by selecting a human antibody structure which has an amino acid sequence which is similar to the sequence of their import antibody.

The consensus structure of one embodiment of this invention was built in five steps as described below.

Step 1

Seven Fab X-ray crystal structures from the Brookhaven Protein Data Bank were used (entries 2FB4, 2RHE, 3FAB, and 1REI which are human structures, and 2MCP, 1FBJ, and 2HFL which are murine structures). For each structure, protein mainchain geometry and hydrogen bonding patterns were used to assign each residue to one of three secondary structure types: alpha-helix, beta-strand or other (i.e. non-helix and non-strand). The immunoglobulin residues used in superpositioning and those included in the consensus structure are shown in Table 1.

TABLE I

Immunoglobulin Residues Used in Superpositioning and Those Included in the Consensus Structure								
Ig ^a	2FB4	2RHE	2MCP	3FAB	1FBJ	2HFL	1REI	Consensus ^b
<u>V_Lκ domain</u>								
	18-24	18-24	19-25	18-24	19-25	19-25	19-25	2-11
	32-37	34-39	39-44	32-37	32-37	32-37	33-38	16-27
								41-49
	60-66	62-68	67-72	53-66	60-65	60-65	61-66	59-77
	69-74	71-76	76-81	69-74	69-74	69-74	70-75	
	84-88	86-90	91-95	84-88	84-88	84-88	85-89	82-91
								101-105
RMS ^c		0.40	0.60	0.53	0.54	0.48	0.50	
<u>V_H domain</u>								
	18-25		18-25	18-25	18-25	18-25		3-8
	34-39		34-39	34-39	34-39	34-39		17-23
	46-52		46-52	46-52	46-52	46-52		33-41
	57-61		59-63	56-60	57-61	57-61		45-51
	68-71		70-73	67-70	68-71	68-71		57-61
	78-84		80-86	77-83	78-84	78-84		66-71
	92-99		94-101	91-98	92-99	92-99		75-82
								88-94
								102-108

TABLE I-continued

Ig ^a	Immunoglobulin Residues Used in Superpositioning and Those Included in the Consensus Structure							
	2FB4	2RHE	2MCP	3FAB	1FBJ	2HFL	1REI	Consensus ^b
RMS ^c			0.43	0.85	0.62	0.91		
RMS ^d	0.91		0.73	0.77	0.92			

^aFour-letter code for Protein Data Bank file.

^bResidue numbers for the crystal structures are taken from the Protein Data Bank files. Residue numbers for the consensus structure are according to Kabat et al.

^cRoot-mean-square deviation in Å for (N, Cα, C) atoms superimposed on 2FB4.

^dRoot-mean-square deviation in Å for (N, Cα, C) atoms superimposed on 2HFL.

Step 2

Having identified the alpha-helices and beta-strands in each of the seven structures, the structures were superimposed on one another using the INSIGHT computer program (Biosym Technologies, San Diego, Calif.) as follows: The 2FB4 structure was arbitrarily chosen as the template (or reference) structure. The 2FB4 was held fixed in space and the other six structures rotated and translated in space so that their common secondary structural elements (i.e. alpha-helices and beta-strands) were oriented such that these common elements were as close in position to one another as possible. (This superpositioning was performed using accepted mathematical formulae rather than actually physically moving the structures by hand.)

Step 3

With the seven structures thus superimposed, for each residue in the template (2FB4) Fab one calculates the distance from the template alpha-carbon atom (Cα) to the analogous Cα atom in each of the other six superimposed structures. This results in a table of Cα-Cα distances for each residue position in the sequence. Such a table is necessary in order to determine which residue positions will be included in the consensus model. Generally, is if all Cα-Cα distances for a given residue position were ≤ 1.0 Å, that position was included in the consensus structure. If for a given position only one Fab crystal structure was >1.0 Å, the position was included but the outlying crystal structure was not included in the next step (for this position only). In general, the seven β-strands were included in the consensus structure while some of the loops connecting the β-strands, e.g. complementarity-determining regions (CDRs), were not included in view of Cα divergence.

Step 4

For each residue which was included in the consensus structure after step 3, the average of the coordinates for individual mainchain N, Cα, C, O and Cβ atoms were calculated. Due to the averaging procedure, as well as variation in bond length, bond angle and dihedral angle among the crystal structures, this "average" structure contained some bond lengths and angles which deviated from standard geometry. For purposes of this invention, "standard geometry" is understood to include geometries commonly accepted as typical, such as the compilation of bond lengths and angles from small molecule structures in Weiner, S. J. et. al., *J. Amer. Chem. Soc.*, 106: 765-784 (1984).

Step 5

In order to correct these deviations, the final step was to subject the "average" structure to 50 cycles of energy minimization (DISCOVER program, Biosym Technologies) using the AMBER (Weiner, S. J. et. al., *J. Amer. Chem. Soc.*, 106: 765-784 (1984)) parameter set with only the Cα coordinates fixed (i.e. all other atoms are allowed to move) (energy minimization is described below). This allowed any

deviant bond lengths and angles to assume a standard (chemically acceptable) geometry. See Table II.

TABLE II

	Average Bond Lengths and Angles for "Average" (Before) and Energy-Minimized Consensus (After 50 Cycles) Structures				Standard Geometry (Å)
	V _L K before (Å)	V _L K after (Å)	V _H before (Å)	V _H after (Å)	
N—Cα	1.459(0.012)	1.451(0.004)	1.451(0.023)	1.452(0.004)	1.449
Cα—C	1.515(0.012)	1.523(0.005)	1.507(0.033)	1.542(0.005)	1.522
O=C	1.208(0.062)	1.229(0.003)	1.160(0.177)	1.231(0.003)	1.229
C—N	1.288(0.049)	1.337(0.002)	1.282(0.065)	1.335(0.004)	1.335
Cα—Cβ	1.508(0.026)	1.530(0.002)	1.499(0.039)	1.530(0.002)	1.526
	(*)	(*)	(*)	(*)	(*)
C—N—Cα	123.5(4.2)	123.8(1.1)	125.3(4.6)	124.0(1.1)	121.9
N—Cα—C	110.0(4.0)	109.5(1.9)	110.3(2.8)	109.5(1.6)	110.1
Cα—C—N	116.6(4.0)	116.6(1.2)	117.6(5.2)	116.6(0.8)	116.6
O=C—N	123.1(4.1)	123.4(0.6)	122.2(4.9)	123.3(0.4)	122.9
N—Cα—Cβ	110.3(2.1)	109.8(0.7)	110.6(2.5)	109.8(0.6)	109.5
Cβ—Cα—C	111.4(2.4)	111.1(0.7)	111.2(2.2)	111.1(0.6)	111.1

Values in parentheses are standard deviations. Note that while some bond length and angle averages did not change appreciably after energy-minimization, the corresponding standard deviations are reduced due to deviant geometries assuming standard values after energy-minimization. Standard geometry values are from the AMBER forcefield as implemented in DISCOVER (Biosym Technologies).

The consensus structure might conceivably be dependent upon which crystal structure was chosen as the template on which the others were superimposed. As a test, the entire procedure was repeated using the crystal structure with the worst superposition versus 2FB4, i.e. the 2HFL Fab structure, as the new template (reference). The two consensus structures compare favorably (root-mean-squared deviation of 0.11 Å for all N, Cα and C atoms).

Note that the consensus structure only includes mainchain (N, Cα, C, O, Cβ atoms) coordinates for only those residues which are part of a conformation common to all seven X-ray crystal structures. For the Fab structures, these include the common β-strands (which comprise two β-sheets) and a few non-CDR loops which connect these β-strands. The consensus structure does not include CDRs or sidechains, both of which vary in their conformation among the seven structures. Also, note that the consensus structure includes only the V_L and V_H domains.

This consensus structure is used as the archetype. It is not particular to any species, and has only the basic shape without side chains. Starting with this consensus structure

the model of any import, human, or humanized Fab can be constructed as follows. Using the amino acid sequence of the particular antibody V_L and V_H domains of interest, a computer graphics program (such as INSIGHT, Biosym Technologies) is used to add sidechains and CDRs to the consensus structure. When a sidechain is added, its conformation is chosen on the basis of known Fab crystal structures (see the Background section for publications of such crystal structures) and rotamer libraries (Ponder, J. W. & Richards, F. M., *J. Mol. Biol.* 193: 775-791 (1987)). The model also is constructed so that the atoms of the sidechain are positioned so as to not collide with other atoms in the Fab.

CDRs are added to the model (now having the backbone plus side chains) as follows. The size (i.e. number of amino acids) of each import CDR is compared to canonical CDR structures tabulated by Chothia et al., *Nature*, 342:877-883 (1989) and which were derived from Fab crystals. Each CDR sequence is also reviewed for the presence or absence of certain specific amino acid residues which are identified by Chothia as structurally important: e.g. light chain residues 29 (CDR1) and 95 (CDR3), and heavy chain residues 26, 27, 29 (CDR1) and 55 (CDR2). For light chain CDR2, and heavy chain CDR3, only the size of the CDR is compared to the Chothia canonical structure. If the size and sequence (i.e. inclusion of the specific, structurally important residues as denoted by Chothia et al.) of the import CDR agrees in size and has the same structurally important residues as those of a canonical CDR, then the mainchain conformation of the import CDR in the model is taken to be the same as that of the canonical CDR. This means that the import sequence is assigned the structural configuration of the canonical CDR, which is then incorporated in the evolving model.

However, if no matching canonical CDR can be assigned for the import CDR, then one of two options can be exercised. First, using a program such as INSIGHT (Biosym Technologies), the Brookhaven Protein Data Bank can be searched for loops with a similar size to that of the import CDR and these loops can be evaluated as possible conformations for the import CDR in the model. Minimally, such loops must exhibit a conformation in which no loop atom overlaps with other protein atoms. Second, one can use available programs which calculate possible loop conformations, assuming a given loop size, using methods such as described by Bruccoleri et al., *Nature* 335: 564-568 (1988).

When all CDRs and sidechains have been added to the consensus structure to give the final model (import, human or humanized), the model is preferably subjected to energy minimization using programs which are available commercially (e.g. DISCOVER, Biosym Technologies). This technique uses complex mathematical formulae to refine the model by performing such tasks as checking that all atoms are within appropriate distances from one another and checking that bond lengths and angles are within chemically acceptable limits.

Models of a humanized, import or human antibody sequence are used in the practice of this invention to understand the impact of selected amino acid residues of the activity of the sequence being modeled. For example, such a model can show residues which may be important in antigen binding, or for maintaining the conformation of the antibody, as discussed in more detail below. Modeling can also be used to explore the potential impact of changing any amino acid residue in the antibody sequence.

Methods for Obtaining a Humanized Antibody Sequence

In the practice of this invention, the first step in humanizing an import antibody is deriving a consensus amino acid sequence into which to incorporate the import sequences. Next a model is generated for these sequences using the methods described above. In certain embodiments of this invention, the consensus human sequences are derived from the most abundant subclasses in the sequence compilation of Kabat et al. (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)), namely V_L κ subgroup I and V_H group III, and have the sequences indicated in the definitions above.

While these steps may be taken in different order, typically a structure for the candidate humanized antibody is created by transferring the at least one CDR from the non-human, import sequence into the consensus human structure, after the entire corresponding human CDR has been removed. The humanized antibody may contain human replacements of the non-human import residues at positions within CDRs as defined by sequence variability (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) or as defined by structural variability (Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901-917 (1987)). For example, huMab4D5 contains human replacements of the muMab4D5 residues at three positions within CDRs as defined by sequence variability (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) but not as defined by structural variability (Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901-917 (1987)): V_L -CDR1 K24R, V_L -CDR2 R54L and V_L -CDR2 T56S.

Differences between the non-human import and the human consensus framework residues are individually investigated to determine their possible influence on CDR conformation and/or binding to antigen. Investigation of such possible influences is desirably performed through modeling, by examination of the characteristics of the amino acids at particular locations, or determined experimentally through evaluating the effects of substitution or mutagenesis of particular amino acids.

In certain preferred embodiments of this invention, a humanized antibody is made comprising amino acid sequence of an import, non-human antibody and a human antibody, utilizing the steps of:

- a. obtaining the amino acid sequences of at least a portion of an import antibody variable domain and of a consensus human variable domain;
- b. identifying Complementarity Determining Region (CDR) amino acid sequences in the import and the human variable domain sequences;
- c. substituting an import CDR amino acid sequence for the corresponding human CDR amino acid sequence;
- d. aligning the amino acid sequences of a Framework Region (FR) of the import antibody and the corresponding FR of the consensus antibody;
- e. identifying import antibody FR residues in the aligned FR sequences that are non-homologous to the corresponding consensus antibody residues;
- f. determining if the non-homologous import amino acid residue is reasonably expected to have at least one of the following effects:
 1. non-covalently binds antigen directly,
 2. interacts with a CDR; or
 3. participates in the V_L - V_H interface; and
- g. for any non-homologous import antibody amino acid residue which is reasonably expected to have at least

one of these effects, substituting that residue for the corresponding amino acid residue in the consensus antibody FR sequence.

Optionally, one determines if any non-homologous residues identified in step (e) are exposed on the surface of the domain or buried within it, and if the residue is exposed but has none of the effects identified in step (f), one may retain the consensus residue.

Additionally, in certain embodiments the corresponding consensus antibody residues identified in step (e) above are selected from the group consisting of 4L, 35L, 36L, 38L, 43L, 44L, 46L, 58L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 71 L, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 76H, 78H, 91H, 92H, 93H, and 103H (utilizing the numbering system set forth in Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)).

In preferred embodiments, the method of this invention comprises the additional steps of searching either or both of the import, non-human and the consensus variable domain sequences for glycosylation sites, determining if the glycosylation is reasonably expected to be important for the desired antigen binding and biological activity of the antibody (i.e., determining if the glycosylation site binds to antigen or changes a side chain of an amino acid residue that binds to antigen, or if the glycosylation enhances or weakens antigen binding, or is important for maintaining antibody affinity). if the import sequence bears the glycosylation site, it is preferred to substitute that site for the corresponding residues in the consensus human sequence if the glycosylation site is reasonably expected to be important. If only the consensus sequence, and not the import, bears the glycosylation site, it is preferred to eliminate that glycosylation site or substitute therefor the corresponding amino acid residues from the import sequence.

Another preferred embodiment of the methods of this invention comprises aligning import antibody and the consensus antibody FR sequences, identifying import antibody FR residues which are non-homologous with the aligned consensus FR sequence, and for each such non-homologous import antibody FR residue, determining if the corresponding consensus antibody residue represents a residue which is highly conserved across all species at that site, and if it is so conserved, preparing a humanized antibody which comprises the consensus antibody amino acid residue at that site.

In certain alternate embodiments, one need not utilize the modeling and evaluation steps described above, and may instead proceed with the steps of obtaining the amino acid sequence of at least a portion of an import, non-human antibody variable domain having a CDR and a FR, obtaining the amino acid sequence of at least a portion of a consensus human antibody variable domain having a CDR and a FR, substituting the non-human CDR for the human CDR in the consensus human antibody variable domain, and then substituting an amino acid residue for the consensus amino acid residue at at least one of the following sites:

a. (in the FR of the variable domain of the light chain) 4L, 35L, 36L, 38L, 43L, 44L, 58L, 46L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 71L, 73L, 85L, 87L, 98L, or

b. in the FR of the variable domain of the heavy chain) 2H, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 76H, 78H, 91H, 92H, 93H, and 103H.

Preferably, the non-CDR residue substituted at the consensus FR site is the residue found at the corresponding location

of the non-human antibody. If desired, one may utilize the other method steps described above for determining whether a particular amino acid residue can reasonably be expected to have undesirable effects, and remedying those effects.

If after making a humanized antibody according to the steps above and testing its activity one is not satisfied with the humanized antibody, one preferably reexamines the potential effects of the amino acids at the specific locations recited above. Additionally, it is desirable to reinvestigate any buried residues which are reasonably expected to affect the V_L - V_H interface but may not directly affect CDR conformation. It is also desirable to reevaluate the humanized antibody utilizing the steps of the methods claimed herein.

In certain embodiments of this invention, amino acid residues in the consensus human sequence are substituted for by other amino acid residues. In preferred embodiments, residues from a particular non-human import sequence are substituted, however there are circumstances where it is desired to evaluate the effects of other amino acids. For example, if after making a humanized antibody according to the steps above and testing its activity one is not satisfied with the humanized antibody, one may compare the sequences of other classes or subgroups of human antibodies, or classes or subgroups of antibodies from the particular non-human species, and determine which other amino acid side chains and amino acid residues are found at particular locations and substituting such other residues.

Antibodies

Certain aspects of this invention are directed to natural antibodies and to monoclonal antibodies, as illustrated in the Examples below and by antibody hybridomas deposited with the ATCC (as described below). Thus, the references throughout this description to the use of monoclonal antibodies are intended to include the use of natural or native antibodies as well as humanized and chimeric antibodies. As used herein, the term "antibody" includes the antibody variable domain and other separable antibody domains unless specifically excluded.

In accordance with certain aspects of this invention, antibodies to be humanized (import antibodies) are isolated from continuous hybrid cell lines formed by the fusion of antigen-primed immune lymphocytes with myeloma cells. In certain embodiments, the antibodies of this invention are obtained by routine screening. Polyclonal antibodies to an antigen generally are raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the antigen and an adjuvant. It may be useful to conjugate the antigen or a fragment containing the target amino acid sequence to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, SOCl_2 , or $\text{R}^1\text{N}=\text{C}=\text{NR}$, where R and R^1 are different alkyl groups.

The route and schedule of the host animal or cultured antibody-producing cells therefrom are generally in keeping with established and conventional techniques for antibody stimulation and production. While mice are frequently employed as the test model, it is contemplated that any mammalian subject including human subjects or antibody-producing cells obtained therefrom can be manipulated according to the processes of this invention to serve as the basis for production of mammalian, including human, hybrid cell lines.

Animals are typically immunized against the immunogenic conjugates or derivatives by combining 1 mg or 1 μ g of conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with $\frac{1}{5}$ to $\frac{1}{10}$ the original amount of conjugate in Freund's complete adjuvant (or other suitable adjuvant) by subcutaneous injection at multiple sites. 7 to 14 days later animals are bled and the serum is assayed for antigen titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking agent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are used to enhance the immune response.

After immunization, monoclonal antibodies are prepared by recovering immune lymphoid cells—typically spleen cells or lymphocytes from lymph node tissue—from immunized animals and immortalizing the cells in conventional fashion, e.g. by fusion with myeloma cells or by Epstein-Barr (EB)-virus transformation and screening for clones expressing the desired antibody. The hybridoma technique described originally by Kohler and Milstein, *Eur. J. Immunol.* 6:511 (1976) has been widely applied to produce hybrid cell lines that secrete high levels of monoclonal antibodies against many specific antigens.

It is possible to fuse cells of one species with another. However, it is preferable that the source of the immunized antibody producing cells and the myeloma be from the same species.

The hybrid cell lines can be maintained in culture in vitro in cell culture media. The cell lines of this invention can be selected and/or maintained in a composition comprising the continuous cell line in hypoxanthine-aminopterin thymidine (HAT) medium. In fact, once the hybridoma cell line is established, it can be maintained on a variety of nutritionally adequate media. Moreover, the hybrid cell lines can be stored and preserved in any number of conventional ways, including freezing and storage under liquid nitrogen. Frozen cell lines can be revived and cultured indefinitely with resumed synthesis and secretion of monoclonal antibody. The secreted antibody is recovered from tissue culture supernatant by conventional methods such as precipitation, Ion exchange chromatography, affinity chromatography, or the like. The antibodies described herein are also recovered from hybridoma cell cultures by conventional methods for purification of IgG or IgM as the case may be that heretofore have been used to purify these immunoglobulins from pooled plasma, e.g. ethanol or polyethylene glycol precipitation procedures. The purified antibodies are sterile filtered, and optionally are conjugated to a detectable marker such as an enzyme or spin label for use in diagnostic assays of the antigen in test samples.

While routinely rodent monoclonal antibodies are used as the source of the import antibody, the invention is not limited to any species. Additionally, techniques developed for the production of chimeric antibodies (Morrison et al., *Proc. Natl. Acad. Sci.*, 81:6851 (1984); Neuberger et al., *Nature* 312:604 (1984); Takeda et al., *Nature* 314:452 (1985)) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity (such as ability to activate human complement and mediate ADCC) can be used; such antibodies are within the scope of this invention.

Techniques for creating recombinant DNA versions of the antigen-binding regions of antibody molecules (known as

Fab fragments) which bypass the generation of monoclonal antibodies are encompassed within the practice of this invention. One extracts antibody-specific messenger ANA molecules from immune system cells taken from an immunized animal, transcribes these into complementary DNA (cDNA), and clones the cDNA into a bacterial expressions system. One example of such a technique suitable for the practice of this invention was developed by researchers at Scripps/Stratagene, and incorporates a proprietary bacteriophage lambda vector system which contains a leader sequence that causes the expressed Fab protein to migrate to the periplasmic space (between the bacterial cell membrane and the cell wall) or to be secreted. One can rapidly generate and screen great numbers of functional Fab fragments for those which bind the antigen. Such Fab fragments with specificity for the antigen are specifically encompassed within the term "antibody" as it is defined, discussed, and claimed herein.

Amino Acid Sequence Variants

Amino acid sequence variants of the antibodies and polypeptides of this invention (referred to in herein as the target polypeptide) are prepared by introducing appropriate nucleotide changes into the DNA encoding the target polypeptide, or by in vitro synthesis of the desired target polypeptide. Such variants include, for example, humanized variants of non-human antibodies, as well as deletions from, or insertions or substitutions of, residues within particular amino acid sequences. Any combination of deletion, insertion, and substitution can be made to arrive at the final construct, provided that the final construct possesses the desired characteristics. The amino acid changes also may alter post-translational processes of the target polypeptide, such as changing the number or position of glycosylation sites, altering any membrane anchoring characteristics, and/or altering the intra-cellular location of the target polypeptide by inserting, deleting, or otherwise affecting any leader sequence of the native target polypeptide.

In designing amino acid sequence variants of target polypeptides, the location of the mutation site and the nature of the mutation will depend on the target polypeptide characteristics) to be modified. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first with conservative amino acid choices and then with more radical selections depending upon the results achieved, (2) deleting the target residue, or (3) inserting residues of the same or a different class adjacent to the located site, or combinations of options 1–3. In certain embodiments, these choices are guided by the methods for creating humanized sequences set forth above.

A useful method for identification of certain residues or regions of the target polypeptide that are preferred locations for mutagenesis is called "alanine scanning mutagenesis" as described by Cunningham and Wells (*Science*, 244: 1081–1085 [1989]). Here, a residue or group of target residues are identified (e.g., charged residues such as arg, asp, his, lys, and glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the substitutions then are refined by introducing further or other variants at or for the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For example, to optimize the performance of a mutation at a given site, ala scanning or random mutagenesis may be conducted at the target codon or region and the

expressed target polypeptide variants are screened for the optimal combination of desired activity.

There are two principal variables in the construction of amino acid sequence variants: the location of the mutation site and the nature of the mutation. In general, the location and nature of the mutation chosen will depend upon the target polypeptide characteristic to be modified.

Amino acid sequence deletions of antibodies are generally not preferred, as maintaining the generally configuration of an antibody is believed to be necessary for its activity. Any deletions will be selected so as to preserve the structure of the target antibody.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Intrasequence insertions (i.e., insertions within the target polypeptide sequence) may range generally from about 1 to 10 residues, more preferably 1 to 5, most preferably 1 to 3. Examples of terminal insertions include the target polypeptide with an N-terminal methionyl residue, an artifact of the direct expression of target polypeptide in bacterial recombinant cell culture, and fusion of a heterologous N-terminal signal sequence to the N-terminus of the target polypeptide molecule to facilitate the secretion of the mature target polypeptide from recombinant host cells. Such signal sequences generally will be obtained from, and thus homologous to, the intended host cell species. Suitable sequences include STII or Ipp for *E. coli*, alpha factor for yeast, and viral signals such as herpes gD for mammalian cells.

Other insertional variants of the target polypeptide include the fusion to the N- or C-terminus of the target polypeptide of immunogenic polypeptides, e.g., bacterial polypeptides such as beta-lactamase or an enzyme encoded by the *E. coli* trp locus, or yeast protein, and C-terminal fusions with proteins having a long half-life such as immunoglobulin constant regions (or other immunoglobulin regions), albumin, or ferritin, as described in WO 89/02922 published Apr. 6, 1989.

Another group of variants are amino acid substitution variants. These variants have at least one amino acid residue in the target polypeptide molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include sites identified as the active site(s) of the target polypeptide, and sites where the amino acids found in the target polypeptide from various species are substantially different in terms of side-chain bulk, charge, and/or hydrophobicity. Other sites for substitution are described infra, considering the effect of the substitution of the antigen binding, affinity and other characteristics of a particular target antibody.

Other sites of interest are those in which particular residues of the target polypeptides obtained from various species are identical. These positions may be important for the biological activity of the target polypeptide. These sites, especially those falling within a sequence of at least three other identically conserved sites, are substituted in a relatively conservative manner. If such substitutions result in a change in biological activity, then other changes are introduced and the products screened until the desired effect is obtained.

Substantial modifications in function or immunological identity of the target polypeptide are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or

helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side chain properties:

- (1) hydrophobic: norleucine, met, ala, val, leu, ile;
- (2) neutral hydrophilic: cys, ser, thr;
- (3) acidic: asp, glu;
- (4) basic: asn, gin, his, lys, arg;
- (5) residues that influence chain orientation: gly, pro; and
- (6) aromatic: trp, tyr, phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for another. Such substituted residues may be introduced into regions of the target polypeptide that are homologous with other antibodies of the same class or subclass, or, more preferably, into the non-homologous regions of the molecule.

Any cysteine residues not involved in maintaining the proper conformation of target polypeptide also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking.

DNA encoding amino acid sequence variants of the target polypeptide is prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally occurring amino acid sequence variants) or preparation by oligonucleotide-mediated (or site-directed) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared variant or a non-variant version of the target polypeptide. A particularly preferred method of gene conversion mutagenesis is described below in Example 1. These techniques may utilize target polypeptide nucleic acid (DNA or RNA), or nucleic acid complementary to the target polypeptide nucleic acid.

Oligonucleotide-mediated mutagenesis is a preferred method for preparing substitution, deletion, and insertion variants of target polypeptide DNA. This technique is well known in the art as described by Adelman et al., *DNA*, 2: 183 (1983). Briefly, the target polypeptide DNA is altered by hybridizing an oligonucleotide encoding the desired mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered or native DNA sequence of the target polypeptide. After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer, and will code for the selected alteration in the target polypeptide DNA.

Generally, oligonucleotides of at least 25 nucleotides in length are used. An optimal oligonucleotide will have 12 to 15 nucleotides that are completely complementary to the template on either side of the nucleotide(s) coding for the mutation. This ensures that the oligonucleotide will hybridize properly to the single-stranded DNA template molecule. The oligonucleotides are readily synthesized using techniques known in the art such as that described by Crea et al. (*Proc. Natl. Acad. Sci. USA*, 75: 5765 [1978]).

Single-stranded DNA template may also be generated by denaturing double-stranded plasmid (or other) DNA using standard techniques.

For alteration of the native DNA sequence (to generate amino acid sequence variants, for example), the oligonucleotide is hybridized to the single-stranded template under suitable hybridization conditions. A DNA polymerizing enzyme, usually the Klenow fragment of DNA polymerase 1, is then added to synthesize the complementary strand of the template using the oligonucleotide as a primer for

synthesis. A heteroduplex molecule is thus formed such that one strand of DNA encodes the mutated form of the target polypeptide, and the other strand (the original template) encodes the native, unaltered sequence of the target polypeptide. This heteroduplex molecule is then transformed into a suitable host cell, usually a prokaryote such as *E. coli* JM101. After the cells are grown, they are plated onto agarose plates and screened using the oligonucleotide primer radiolabeled with 32-phosphate to identify the bacterial colonies that contain the mutated DNA. The mutated region is then removed and placed in an appropriate vector for protein production, generally an expression vector of the type typically employed for transformation of an appropriate host.

The method described immediately above may be modified such that a homoduplex molecule is created wherein both strands of the plasmid contain the mutation(s). The modifications are as follows: The single-stranded oligonucleotide is annealed to the single-stranded template as described above. A mixture of three deoxyribonucleotides, deoxyriboadenosine (dATP), deoxyriboguanosine (dGTP), and deoxyribothymidine (dTTP), is combined with a modified thio-deoxyribocytosine called dCTP-(aS) (which can be obtained from Amersham Corporation). This mixture is added to the template-oligonucleotide complex. Upon addition of DNA polymerase to this mixture, a strand of DNA identical to the template except for the mutated bases is generated. In addition, this new strand of DNA will contain dCTP-(aS) instead of dCTP, which serves to protect it from restriction endonuclease digestion.

After the template strand of the double-stranded heteroduplex is nicked with an appropriate restriction enzyme, the template strand can be digested with ExoIII nuclease or another appropriate nuclease past the region that contains the site(s) to be mutagenized. The reaction is then stopped to leave a molecule that is only partially single-stranded. A complete double-stranded DNA homoduplex is then formed using DNA polymerase in the presence of all four deoxyribonucleotide triphosphates, ATP, and DNA ligase. This homoduplex molecule can then be transformed into a suitable host cell such as *E. coli* JM101, as described above.

DNA encoding target polypeptide variants with more than one amino acid to be substituted may be generated in one of several ways. If the amino acids are located close together in the polypeptide chain, they may be mutated simultaneously using one oligonucleotide that codes for all of the desired amino acid substitutions. If, however, the amino acids are located some distance from each other (separated by more than about ten amino acids), it is more difficult to generate a single oligonucleotide that encodes all of the desired changes. Instead, one of two alternative methods may be employed.

In the first method, a separate oligonucleotide is generated for each amino acid to be substituted. The oligonucleotides are then annealed to the single-stranded template DNA simultaneously, and the second strand of DNA that is synthesized from the template will encode all of the desired amino acid substitutions.

The alternative method involves two or more rounds of mutagenesis to produce the desired mutant. The first round is as described for the single mutants: wild-type DNA is used for the template, an oligonucleotide encoding the first desired amino acid substitution(s) is annealed to this template, and the heteroduplex DNA molecule is then generated. The second round of mutagenesis utilizes the mutated DNA produced in the first round of mutagenesis as the template. Thus, this template already contains one or

more mutations. The oligonucleotide encoding the additional desired amino acid substitution(s) is then annealed to this template, and the resulting strand of DNA now encodes mutations from both the first and second rounds of mutagenesis. This resultant DNA can be used as a template in a third round of mutagenesis, and so on.

PCR mutagenesis is also suitable for making amino acid variants of target polypeptide. While the following discussion refers to DNA, it is understood that the technique also finds application with RNA. The PCR technique generally refers to the following procedure (see Erlich, supra, the chapter by R. Higuchi, p. 61-70): When small amounts of template DNA are used as starting material in a PCR, primers that differ slightly in sequence from the corresponding region in a template DNA can be used to generate relatively large quantities of a specific DNA fragment that differs from the template sequence only at the positions where the primers differ from the template. For introduction of a mutation into a plasmid DNA, one of the primers is designed to overlap the position of the mutation and to contain the mutation; the sequence of the other primer must be identical to a stretch of sequence of the opposite strand of the plasmid, but this sequence can be located anywhere along the plasmid DNA. It is preferred, however, that the sequence of the second primer is located within 200 nucleotides from that of the first, such that in the end the entire amplified region of DNA bounded by the primers can be easily sequenced. PCR amplification using a primer pair like the one just described results in a population of DNA fragments that differ at the position of the mutation specified by the primer, and possibly at other positions, as template copying is somewhat error-prone.

If the ratio of template to product material is extremely low, the vast majority of product DNA fragments incorporate the desired mutation(s). This product material is used to replace the corresponding region in the plasmid that served as PCR template using standard DNA technology. Mutations at separate positions can be introduced simultaneously by either using a mutant second primer, or performing a second PCR with different mutant primers and ligating the two resulting PCR fragments simultaneously to the vector fragment in a three (or more)-part ligation.

In a specific example of PCR mutagenesis, template plasmid DNA (1 μ g) is linearized by digestion with a restriction endonuclease that has a unique recognition site in the plasmid DNA outside of the region to be amplified. Of this material, 100 ng is added to a PCR mixture containing PCR buffer, which contains the four deoxynucleotide triphosphates and is included in the GeneAmp® kits (obtained from Perkin-Elmer Cetus, Norwalk, Conn. and Emeryville, Calif.), and 25 pmole of each oligonucleotide primer, to a final volume of 50 μ l. The reaction mixture is overlaid with 35 μ l mineral oil. The reaction is denatured for 5 minutes at 100° C., placed briefly on ice, and then 1 μ l *Thermus aquaticus* (Taq) DNA polymerase (5 units/ μ l, purchased from Perkin-Elmer Cetus, Norwalk, Conn. and Emeryville, Calif.) is added below the mineral oil layer. The reaction mixture is then inserted into a DNA Thermal Cycler (purchased from Perkin-Elmer Cetus) programmed as follows: 2 min. at 55° C., then 30 sec. at 72° C., then 19 cycles of the following: 30 sec. at 94° C., 30 sec. at 55° C., and 30 sec. at 72° C.

At the end of the program, the reaction vial is removed from the thermal cycler and the aqueous phase transferred to a new vial, extracted with phenol/chloroform (50:50:vol), and ethanol precipitated, and the DNA is recovered by standard procedures. This material is subsequently subjected to the appropriate treatments for insertion into a vector.

Another method for preparing variants, cassette mutagenesis, is based on the technique described by Wells et al. (*Gene*, 34: 315 [1985]). The starting material is the plasmid (or other vector) comprising the target polypeptide DNA to be mutated. The codon(s) in the target polypeptide DNA to be mutated are identified. There must be a unique restriction endonuclease site on each side of the identified mutation site(s). If no such restriction sites exist, they may be generated using the above-described oligonucleotide-mediated mutagenesis method to introduce them at appropriate locations in the target polypeptide DNA. After the restriction sites have been introduced into the plasmid, the plasmid is cut at these sites to linearize it. A double-stranded oligonucleotide encoding the sequence of the DNA between the restriction sites but containing the desired mutation(s) is synthesized using standard procedures. The two strands are synthesized separately and then hybridized together using standard techniques. This double-stranded oligonucleotide is referred to as the cassette. This cassette is designed to have 3' and 5' ends that are compatible with the ends of the linearized plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the mutated target polypeptide DNA sequence.

Insertion of DNA into a Cloning Vehicle

The cDNA or genomic DNA encoding the target polypeptide is inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Many vectors are available, and selection of the appropriate vector will depend on 1) whether it is to be used for DNA amplification or for DNA expression, 2) the size of the DNA to be inserted into the vector, and 3) the host cell to be transformed with the vector. Each vector contains various components depending on its function (amplification of DNA or expression of DNA) and the host cell for which it is compatible. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

(a) Signal Sequence Component

In general, the signal sequence may be a component of the vector, or it may be a part of the target polypeptide DNA that is inserted into the vector.

The target polypeptides of this invention may be expressed not only directly, but also as a fusion with a heterologous polypeptide, preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the target polypeptide DNA that is inserted into the vector. Included within the scope of this invention are target polypeptides with any native signal sequence deleted and replaced with a heterologous signal sequence. The heterologous signal sequence selected should be one that is recognized and processed (i.e. cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process the native target polypeptide signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin 11 leaders. For yeast secretion the native target polypeptide signal sequence may be substituted by the yeast invertase, alpha factor, or acid phosphatase leaders. In mammalian cell expression the native signal sequence is satisfactory, although other mammalian signal sequences may be suitable.

(b) Origin of Replication Component

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2 μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

Most expression vectors are "shuttle" vectors, i.e. they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous recombination with the genome and insertion of the target polypeptide DNA. However, the recovery of genomic DNA encoding the target polypeptide is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the target polypeptide DNA.

(c) Selection Gene Component

Expression and cloning vectors should contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g. ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g. the gene encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene express a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin (Southern et al., *J. Molec. Appl. Genet.*, 1: 327 [1982]), mycophenolic acid (Mulligan et al., *Science*: 1422 [1980]) or hygromycin (Sugden et al., *Mol. Cell. Biol.*, 5: 410-413 [1985]). The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the target polypeptide nucleic acid, such as dihydrofolate reductase (DHFR) or thymidine kinase. The mammalian cell transformants are placed under selection pressure which only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants

under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes the target polypeptide. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of the target polypeptide are synthesized from the amplified DNA.

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA*, 77: 4216 [1980]. The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding the target polypeptide. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060). Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding the target polypeptide, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3' phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Pat. No. 4,965,199.

A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7 (Stinchcomb et al., *Nature*, 22: 39 [1979]; Kingsman et al., *Gene*, 7: 141 [1979]; or Tschemper et al., *Gene*, 10: 157 [1980]). The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 (Jones, *Genetics*, 5: 12 [1977]). The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, Leu2-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the Leu2 gene.

(d) Promoter Component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the target polypeptide nucleic acid. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of a particular nucleic acid sequence, such as that encoding the target polypeptide, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g. the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to DNA encoding the target polypeptide by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the

native target polypeptide promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the target polypeptide DNA. However, heterologous promoters are preferred, as they generally permit greater transcription and higher yields of expressed target polypeptide as compared to the native target polypeptide promoter.

Promoters suitable for use with prokaryotic hosts include the β -lactamase and lactose promoter systems (Chang et al., *Nature*, 275: 615 [1978]; and Goeddel et al., *Nature*, 281: 544 [1979]), alkaline phosphatase, a tryptophan (*trp*) promoter system (Goeddel, *Nucleic Acids Res.*, 8: 4057 [1980] and EP 36,776) and hybrid promoters such as the *tao* promoter (deBoer et al., *Proc. Natl. Acad. Sci. USA*, 80: 21-25 [1983]). However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding the target polypeptide (Siebenlist et al., *Cell*, 20: 269 [1980]) using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also generally will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the target polypeptide.

Suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem.*, 255: 2073 [1980]) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg.*, 2: 149 [1968]; and Holland, *Biochemistry*, 17: 4900 [1978]), such as *asenolase*, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in Hitzeman et al., EP 73,657A. Yeast enhancers also are advantageously used with yeast promoters.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into mammalian expression vectors.

Target polypeptide transcription from vectors in mammalian host cells is controlled by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published Jul. 5, 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g. the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with the target polypeptide sequence, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that

also contains the SV40 viral origin of replication. Fiers et al., *Nature*, 273:113 (1978); Mulligan and Berg, *Science*, 209: 1422-1427 (1980); Pavlakis et al., *Proc. Natl. Acad. Sci. USA*, 78: 7398-7402 (1981). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. Greenaway et al., *Gene*, 18: 355-360 (1982). A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Pat. No. 4,419,446. A modification of this system is described in U.S. Pat. No. 4,601,978. See also Gray et al., *Nature*, 29: 503-508 (1982) on expressing cDNA encoding immune interferon in monkey cells; Reyes et al., *Nature*, 297: 598-601 (1982) on expression of human β -interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus, Canaani and Berg, *Proc. Natl. Acad. Sci. USA*, 79: 5166-5170 (1982) on expression of the human interferon β 1 gene in cultured mouse and rabbit cells, and Gorman et al., *Proc. Natl. Acad. Sci. USA*, 79: 6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter.

(e) Enhancer Element Component

Transcription of DNA encoding the target polypeptide of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10-300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent having been found 5' (Laimins et al., *Proc. Natl. Acad. Sci. USA*, 78: 993 [1981]) and 3' (Lusky et al., *Mol. Cell Bio.* 3: 1108 [1983]) to the transcription unit, within an intron (Banerji et al., *Cell*, 33: 729 [1983]) as well as within the coding sequence itself (Osborne et al., *Mol. Cell Bio.*, 4: 1293 [1984]). Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α -fetoprotein and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, *Nature*, 297: 17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the target polypeptide DNA, but is preferably located at a site 5' from the promoter.

(f) Transcription Termination Component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3' untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding the target polypeptide. The 3' untranslated regions also include transcription termination sites.

Construction of suitable vectors containing one or more of the above listed components the desired coding and control sequences employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transfor-

ants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced is by the method of Messing et al., *Nucleic Acids Res.*, 9: 309 (1981) or by the method of Maxam et al., *Methods in Enzymology* 65: 499 (1980).

Particularly useful in the practice of this invention are expression vectors that provide for the transient expression in mammalian cells of DNA encoding the target polypeptide. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying analogs and variants of the target polypeptide that have target polypeptide-like activity.

Other methods, vectors, and host cells suitable for adaptation to the synthesis of the target polypeptide in recombinant vertebrate cell culture are described in Gething et al., *Nature*, 293: 620-625 [1981]; Mantei et al., *Nature*, 281: 40-46 [1979]; Levinson et al., EP 117,060; and EP 117,058. A particularly useful plasmid for mammalian cell culture expression of the target polypeptide is pRK5 (EP pub. no. 307,247) or pSV16B.

Selection and Transformation of Host Cells

Suitable host cells for cloning or expressing the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes include eubacteria, such as Gram-negative or Gram-positive organisms, for example, *E. coli*, Bacilli such as *B. subtilis*, *Pseudomonas* species such as *P. aeruginosa*, *Salmonella typhimurium*, or *Serratia marcescans*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting. Preferably the host cell should secrete minimal amounts of proteolytic enzymes. Alternatively, in vitro methods of cloning, e.g. PCR or other nucleic acid polymerase reactions, are suitable.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable hosts for target polypeptide-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe* [Beach and Nurse, *Nature*, 290: 140 (1981); EP 139,383 published May 2, 1985], *Kluyveromyces* hosts (U.S. Pat. No. 4,943,529) such as, e.g., *K. lactis* [Louvencourt et al., *J. Bacteriol.*, 737 (1983)], *K. fragilis*, *K. bulgaricus*, *K. thermotolerans*, and *K. marxianus*, yarrowia [EP 402,226], *Pichia pastoris* [EP 183,070; Sreekrishna et al., *J. Basic Microbiol.*, 28: 265-278 (1988)], *Candida*, *Trichoderma reesia* [EP 244,2341], *Neurospora crassa* [Case et al., *Proc. Natl. Acad. Sci. USA*, 76: 5259-5263 (1979)], and filamentous fungi such as, e.g. *Neurospora*, *Penicillium*, *Tolypocladium* [WO 91/00357 published Jan. 10, 1991], and *Aspergillus* hosts such as *A. nidulans* [Ballance et al., *Biochem. Biophys. Res. Commun.* 112: 284-289 (1983); Tilburn et al., *Gene*, 26: 205-221 (1983); Yelton et al., *Proc. Natl. Acad. Sci. USA*, 81: 1470-1474 (1984)] and *A. niger* [Kelly and Hynes, *EMBO J.*, 4: 475-479 (1985)].

Suitable host cells for the expression of glycosylated target polypeptide are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* host cells have been identified. See, e.g., Luckow et al., *Bio/Technology* 6: 47-55 (1988); Miller et al., in *Genetic Engineering* Setlow, J. K. et al., eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda et al., *Nature*, 315: 592-594 (1985). A variety of such viral strains are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells. Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens*, which has been previously manipulated to contain the target polypeptide DNA. During incubation of the plant cell culture with *A. tumefaciens*, the DNA encoding target polypeptide is transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the target polypeptide DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences. Depicker et al., *J. Mol. Appl. Gen.*, 1: 561 (1982). In addition, DNA segments isolated from the upstream region of the T-DNA 780 gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue. See EP 321,196 published Jun. 21, 1989.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years [*Tissue Culture*, Academic Press, Kruse and Patterson, editors (1973)]. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., *J. Gen Virol.*, 36: 59 [1977]); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA*, 77: 4216 [1980]); mouse sertoli cells (TM4, Mather, *Biol. Reprod.*, 23: 243-251 [1980]); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HS 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., *Annals N.Y. Acad. Sci.*, 383: 44-68 [1982]); MRC 5 cells; FS4 cells; and a human hepatoma cell line (Hep G2). Preferred host cells are human embryonic kidney 293 and Chinese hamster ovary cells.

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, CaPO₄ and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in section 1.82 of Sambrook et al., supra, is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw et al., *Gene*, 23: 315 (1983) and WO 89/05859 published Jun. 29, 1989. For mammalian cells without such cell walls, the calcium phosphate precipitation method described in sections 16.30-16.37 of Sambrook et al., supra, is preferred. General aspects of mammalian cell host system transformations have been described by Axel in U.S. Pat. No. 4,399,216 issued Aug. 16, 1983. Transformations into yeast are typically carried out according to the method of Van Solingen et al., *J. Bact.*, 130: 946 (1977) and Hsiao et al., *Proc. Natl. Acad. Sci. (USA)*, 76: 3829 (1979). However, other methods for introducing DNA into cells such as by nuclear injection, electroporation, or protoplast fusion may also be used.

Culturina the Host Cells

Prokaryotic cells used to produce the target polypeptide of this invention are cultured in suitable media as described generally in Sambrook et al., supra.

The mammalian host cells used to produce the target polypeptide of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ([MEM], Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ([DMEM], Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace, *Meth. Enz.*, 58: 44 (1979), Barnes and Sato, *Anal. Biochem.* 102: 255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195; U.S. Pat. No. Re. 30,985, may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

The host cells referred to in this disclosure encompass cells in in vitro culture as well as cells that are within a host animal.

It is further envisioned that the target polypeptides of this invention may be produced by homologous recombination, or with recombinant production methods utilizing control elements introduced into cells already containing DNA encoding the target polypeptide currently in use in the field.

For example, a powerful promoter/enhancer element, a suppressor, or an exogenous transcription modulatory element is inserted in the genome of the intended host cell in proximity and orientation sufficient to influence the transcription of DNA encoding the desired target polypeptide. The control element does not encode the target polypeptide of this invention, but the DNA is present in the host cell genome. One next screens for cells making the target polypeptide of this invention, or increased or decreased levels of expression, as desired.

Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, northern blotting to quantitate the transcription of mRNA (Thomas, *Proc. Natl. Acad. Sci. USA*, 77: 5201-5205 [1980]), dot blotting (DNA analysis), or in situ hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, particularly ³²P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionuclides, fluorescers, enzymes, or the like. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like. A particularly sensitive staining technique suitable for use in the present invention is described by Hsu et al., *Am. J. Clin. Path.*, 75: 734-738 (1980).

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native target polypeptide or against a synthetic peptide based on the DNA sequences provided herein as described further in Section 4 below.

Purification of the Target Polypeptide

The target polypeptide preferably is recovered from the culture medium as a secreted polypeptide, although it also may be recovered from host cell lysates when directly expressed without a secretory signal.

When the target polypeptide is expressed in a recombinant cell other than one of human origin, the target polypeptide is completely free of proteins or polypeptides of human origin. However, it is necessary to purify the target polypeptide from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to the target polypeptide. As a first step, the culture medium or lysate is centrifuged to remove particulate cell debris. The membrane and soluble protein fractions are then separated. The target polypeptide may then be purified from the soluble

protein fraction and from the membrane fraction of the culture lysate, depending on whether the target polypeptide is membrane bound. The following procedures are exemplary of suitable purification procedures: fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; and protein A Sepharose columns to remove contaminants such as IgG.

Target polypeptide variants in which residues have been deleted, inserted or substituted are recovered in the same fashion, taking account of any substantial changes in properties occasioned by the variation. For example, preparation of a target polypeptide fusion with another protein or polypeptide, e.g. a bacterial or viral antigen, facilitates purification; an immunoaffinity column containing antibody to the antigen (or containing antigen, where the target polypeptide is an antibody) can be used to adsorb the fusion. Immunoaffinity columns such as a rabbit polyclonal anti-target polypeptide column can be employed to adsorb the target polypeptide variant by binding it to at least one remaining immune epitope. A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native target polypeptide may require modification to account for changes in the character of the target polypeptide or its variants upon expression in recombinant cell culture.

Covalent Modifications of Target Polypeptides

Covalent modifications of target polypeptides are included within the scope of this invention. One type of covalent modification included within the scope of this invention is a target polypeptide fragment. Target polypeptide fragments having up to about 40 amino acid residues may be conveniently prepared by chemical synthesis, or by enzymatic or chemical cleavage of the full-length target polypeptide or variant target polypeptide. Other types of covalent modifications of the target polypeptide or fragments thereof are introduced into the molecule by reacting specific amino acid residues of the target polypeptide or fragments thereof with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues.

Cysteiny residues most commonly are reacted with α -haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteiny residues also are derivatized by reaction with bromotrifluoroacetone, α -bromo- β -(5-imidazolyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl-2-pyridyldisulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Parabromophenacyl bromide also is useful; the reaction is preferably performed in 0.1M sodium cacodylate at pH 6.0.

Lysiny and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysiny residues. Other suitable reagents for derivatizing α -amino-containing residues include imidoesters such as methyl picolinimide; pyridoxal phosphate; pyridoxal;

chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4-pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

The specific modification of tyrosyl residues may be made, with particular interest in so introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidazole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using ^{125}I or ^{131}I to prepare labeled proteins for use in radioimmunoassay, the chloramine T method described above being suitable.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides ($R'-N=C=N-R'$), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Derivatization with bifunctional agents is useful for crosslinking target polypeptide to a water-insoluble support matrix or surface for use in the method for purifying anti-target polypeptide antibodies, and vice versa. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propionimide yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Pat. Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues, respectively. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Other modification include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α -amino groups of lysine, arginine, and histidine side chains, (T. E. Creighton, *Protein: Structure and Molecular Properties*, W. H. Freeman & Co., San Francisco, pp. 79-86 [1983]), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

Another type of covalent modification of the target polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. By altering is meant deleting one or more carbohydrate moieties found in the native target polypeptide, and/or adding one or more glycosylation sites that are not present in the native target polypeptide.

Glycosylation of polypeptides is typically either N-linked or O-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tri-peptide sequences asparagine-X-residue and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tri-peptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-acetylgalactosamine, galactose, or xylose, to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the target polypeptide is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tri-peptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the native target polypeptide sequence (for O-linked glycosylation sites). For ease, the target polypeptide amino acid sequence is preferably altered through changes at the DNA level, particularly by mutating the DNA encoding the target polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s) may be made using methods described above under the heading of "Amino Acid Sequence Variants of Target Polypeptide".

Another means of increasing the number of carbohydrate moieties on the target polypeptide is by chemical or enzymatic coupling glycosides to the polypeptides. These procedures are advantageous in that they do not require production of the polypeptide in a host cell that has glycosylation capabilities for N- or O-linked glycosylation. Depending on the couple mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published Sep. 11, 1987, and in Aplin and Wriston (*CRC Crit. Rev. Biochem.*, pp. 259-306 [1981]).

Removal of carbohydrate moieties present on the native target polypeptide may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin et al. (*Arch. Biochem. Biophys.*, 259:52 [1987]) and by Edge et al. (*Anal. Biochem.*, 118:131 [1981]). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al. (*Meth. Enzymol.* 138:350 [1987]).

Glycosylation at potential glycosylation sites may be prevented by the use of the compound tunicamycin as described by Duskin et al. (*J. Biol. Chem.*, 257:3105 [1982]). Tunicamycin blocks the formation of protein-N-glycoside linkages.

Another type of covalent modification of the target polypeptide comprises linking the target polypeptide to various nonproteinaceous polymers, e.g. polyethylene glycol, polypropylene glycol or polyoxyalkylenes, in the

manner set forth in U.S. Pat. Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

The target polypeptide also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly[methylmethacrylate]microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in *Reminaton's Pharmaceutical Sciences*, 16th edition, Osol, A., Ed., (1980).

Target polypeptide preparations are also useful in generating antibodies, for screening for binding partners, as standards in assays for the target polypeptide (e.g. by labeling the target polypeptide for use as a standard in a radioimmunoassay, enzyme-linked immunoassay, or radioreceptor assay), in affinity purification techniques, and in competitive-type receptor binding assays when labeled with radioiodine, enzymes, fluorophores, spin labels, and the like.

Since it is often difficult to predict in advance the characteristics of a variant target polypeptide, it will be appreciated that some screening of the recovered variant will be needed to select the optimal variant. For example, a change in the immunological character of the target polypeptide molecule, such as affinity for a given antigen or antibody, is measured by a competitive-type immunoassay. The variant is assayed for changes in the suppression or enhancement of its activity by comparison to the activity observed for the target polypeptide in the same assay. Other potential modifications of protein or polypeptide properties such as redox or thermal stability, hydrophobicity, susceptibility to proteolytic degradation, stability in recombinant cell culture or in plasma, or the tendency to aggregate with carriers or into multimers are assayed by methods well known in the art. Diagnostic and Related Uses of the Antibodies

The antibodies of this invention are useful in diagnostic assays for antigen expression in specific cells or tissues. The antibodies are detectably labeled and/or are immobilized on an insoluble matrix.

The antibodies of this invention find further use for the affinity purification of the antigen from recombinant cell culture or natural sources. Suitable diagnostic assays for the antigen and its antibodies depend on the particular antigen or antibody. Generally, such assays include competitive and sandwich assays, and steric inhibition assays. Competitive and sandwich methods employ a phase-separation step as an integral part of the method while steric inhibition assays are conducted in a single reaction mixture. Fundamentally, the same procedures are used for the assay of the antigen and for substances that bind the antigen, although certain methods will be favored depending upon the molecular weight of the substance being assayed. Therefore, the substance to be tested is referred to herein as an analyte, irrespective of its status otherwise as an antigen or antibody, and proteins that bind to the analyte are denominated binding partners, whether they be antibodies, cell surface receptors, or antigens.

Analytical methods for the antigen or its antibodies all use one or more of the following reagents: labeled analyte analogue, immobilized analyte analogue, labeled binding partner, immobilized binding partner and steric conjugates. The labeled reagents also are known as "tracers."

The label used (and this is also useful to label antigen nucleic acid for use as a probe) is any detectable functionality that does not interfere with the binding of analyte and

its binding partner. Numerous labels are known for use in immunoassay, examples including moieties that may be detected directly, such as fluorochrome, chemiluminescent, and radioactive labels, as well as moieties, such as enzymes, that must be reacted or derivatized to be detected. Examples of such labels include the radioisotopes ^{32}P , ^{14}C , ^{125}I , ^3H , and ^{131}I , fluorophores such as rare earth chelates or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, luciferases, e.g., firefly luciferase and bacterial luciferase (U.S. Pat. No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, horseradish peroxidase (HRP), alkaline phosphatase, β -galactosidase, glucoamylase, lysozyme, saccharide oxidases, e.g., glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase, heterocyclic oxidases such as uricase and xanthine oxidase, coupled with an enzyme that employs hydrogen peroxide to oxidize a dye precursor such as HRP, lactoperoxidase, or microperoxidase, biotinavidin, spin labels, bacteriophage labels, stable free radicals, and the like.

Conventional methods are available to bind these labels covalently to proteins or polypeptides. For instance, coupling agents such as dialdehydes, carbodiimides, dimaleimides, bis-imidates, bis-diazotized benzidine, and the like may be used to tag the antibodies with the above-described fluorescent, chemiluminescent, and enzyme labels. See, for example, U.S. Pat. No. 3,940,475 (fluorimetry) and U.S. Pat. No. 3,645,090 (enzymes); Hunter et al., *Nature*, 144: 945 (1962); David et al., *Biochemistry*, 13: 1014-1021 (1974); Pain et al., *J. Immunol. Methods*, 40: 219-230 (1981); and Nygren, *J. Histochem. and Cytochem.*, 30: 407-412 (1982). Preferred labels herein are enzymes such as horseradish peroxidase and alkaline phosphatase.

The conjugation of such label, including the enzymes, to the antibody is a standard manipulative procedure for one of ordinary skill in immunoassay techniques. See, for example, O'Sullivan et al., "Methods for the Preparation of Enzyme-antibody Conjugates for Use in Enzyme Immunoassay," in *Methods in Enzymology*, ed. J. J. Langone and H. Van Vunakis, Vol. 73 (Academic Press, New York, N.Y., 1981), pp. 147-166. Such bonding methods are suitable for use with the antibodies and polypeptides of this invention.

Immobilization of reagents is required for certain assay methods. Immobilization entails separating the binding partner from any analyte that remains free in solution. This conventionally is accomplished by either insolubilizing the binding partner or analyte analogue before the assay procedure, as by adsorption to a water-insoluble matrix or surface (Bennich et al., U.S. Pat. No. 3,720,760), by covalent coupling (for example, using glutaraldehyde cross-linking), or by insolubilizing the partner or analogue afterward, e.g., by immunoprecipitation.

Other assay methods, known as competitive or sandwich assays, are well established and widely used in the commercial diagnostics industry.

Competitive assays rely on the ability of a tracer analogue to compete with the test sample analyte for a limited number of binding sites on a common binding partner. The binding partner generally is insolubilized before or after the competition and then the tracer and analyte bound to the binding partner are separated from the unbound tracer and analyte. This separation is accomplished by decanting (where the binding partner was preinsolubilized) or by centrifuging (where the binding partner was precipitated after the competitive reaction). The amount of test sample analyte is inversely proportional to the amount of bound tracer as measured by the amount of marker substance. Dose-

response curves with known amounts of analyte are prepared and compared with the test results to quantitatively determine the amount of analyte present in the test sample. These assays are called ELISA systems when enzymes are used as the detectable markers.

Another species of competitive assay, called a "homogeneous" assay, does not require a phase separation. Here, a conjugate of an enzyme with the analyte is prepared and used such that when anti-analyte binds to the analyte the presence of the anti-analyte modifies the enzyme activity. In this case, the antigen or its immunologically active fragments are conjugated with a bifunctional organic bridge to an enzyme such as peroxidase. Conjugates are selected for use with antibody so that binding of the antibody inhibits or potentiates the enzyme activity of the label. This method per se is widely practiced under the name of EMIT.

Steric conjugates are used in steric hindrance methods for homogeneous assay. These conjugates are synthesized by covalently linking a low-molecular-weight hapten to a small analyte so that antibody to hapten substantially is unable to bind the conjugate at the same time as anti-analyte. Under this assay procedure the analyte present in the test sample will bind anti-analyte, thereby allowing anti-hapten to bind the conjugate, resulting in a change in the character of the conjugate hapten, e.g., a change in fluorescence when the hapten is a fluorophore.

Sandwich assays particularly are useful for the determination of antigen or antibodies. In sequential sandwich assays an immobilized binding partner is used to adsorb test sample analyte, the test sample is removed as by washing, the bound analyte is used to adsorb labeled binding partner, and bound material is then separated from residual tracer. The amount of bound tracer is directly proportional to test sample analyte. In "simultaneous" sandwich assays the test sample is not separated before adding the labeled binding partner. A sequential sandwich assay using an anti-antigen monoclonal antibody as one antibody and a polyclonal anti-antigen antibody as the other is useful in testing samples for particular antigen activity.

The foregoing are merely exemplary diagnostic assays for the import and humanized antibodies of this invention. Other methods now or hereafter developed for the determination of these analytes are included within the scope hereof, including the bioassays described above.

Immunotoxins

This invention is also directed to immunochemical derivatives of the antibodies of this invention such as immunotoxins (conjugates of the antibody and a cytotoxic moiety). Antibodies which carry the appropriate effector functions, such as with their constant domains, are also used to induce lysis through the natural complement process, and to interact with antibody dependent cytotoxic cells normally present.

For example, purified, sterile filtered antibodies are optionally conjugated to a cytotoxin such as ricin for use in AIDS therapy. U.S. patent application Ser. No. 07/350,895 illustrates methods for making and using immunotoxins for the treatment of HIV infection. The methods of this invention, for example, are suitable for obtaining humanized antibodies for use as immunotoxins for use in AIDS therapy.

The cytotoxic moiety of the immunotoxin may be a cytotoxic drug or an enzymatically active toxin of bacterial, fungal, plant or animal origin, or an enzymatically active fragment of such a toxin. Enzymatically active toxins and fragments thereof used are diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins,

dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcumin, crocin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes.

In another embodiment, the antibodies are conjugated to small molecule anticancer drugs such as cis-platin or 5FU. Conjugates of the monoclonal antibody and such cytotoxic moieties are made using a variety of bifunctional protein coupling agents. Examples of such reagents are SPDP, IT, bifunctional derivatives of imidoesters such as dimethyl adipimidate HCl, active esters such as disuccinimidyl suberate, aldehydes such as glutaraldehyde, bis-azido compounds such as bis (p-azidobenzoyl) hexanediamine, bis-diazonium derivatives such as bis-(p-diazoniumbenzoyl)-ethylenediamine, diisocyanates such as tolylene 2,6-diisocyanate and bis-active fluorine compounds such as 1,5-difluoro-2,4-dinitrobenzene. The lysing portion of a toxin may be joined to the Fab fragment of the antibodies.

Immunotoxins can be made in a variety of ways, as discussed herein. Commonly known crosslinking reagents can be used to yield stable conjugates.

Advantageously, monoclonal antibodies specifically binding the domain of the antigen which is exposed on the infected cell surface, are conjugated to ricin A chain. Most advantageously the ricin A chain is deglycosylated and produced through recombinant means. An advantageous method of making the ricin immunotoxin is described in Vitetta et al., *Science* 238:1098 (1987).

When used to kill infected human cells in vitro for diagnostic purposes, the conjugates will typically be added to the cell culture medium at a concentration of at least about 10 nM. The formulation and mode of administration for in vitro use are not critical. Aqueous formulations that are compatible with the culture or perfusion medium will normally be used. Cytotoxicity may be read by conventional techniques.

Cytotoxic radiopharmaceuticals for treating infected cells may be made by conjugating radioactive isotopes (e.g. I, Y, Pr) to the antibodies. Advantageously alpha particle-emitting isotopes are used. The term "cytotoxic moiety" as used herein is intended to include such isotopes.

In a preferred embodiment, ricin A chain is deglycosylated or produced without oligosaccharides, to decrease its clearance by irrelevant clearance mechanisms (e.g., the liver). In another embodiment, whole ricin (A chain plus B chain) is conjugated to antibody if the galactose binding property of B-chain can be blocked ("blocked ricin").

In a further embodiment toxin-conjugates are made with Fab or F(ab')₂ fragments. Because of their relatively small size these fragments can better penetrate tissue to reach infected cells.

In another embodiment, fusogenic liposomes are filled with a cytotoxic drug and the liposomes are coated with antibodies specifically binding the particular antigen.

Antibody Dependent Cellular Cytotoxicity

Certain aspects of this invention involve antibodies which are (a) directed against a particular antigen and (b) belong to a subclass or isotype that is capable of mediating the lysis of cells to which the antibody molecule binds. More specifically, these antibodies should belong to a subclass or isotype that, upon complexing with cell surface proteins, activates serum complement and/or mediates antibody dependent cellular cytotoxicity (ADCC) by activating effector cells such as natural killer cells or macrophages.

Biological activity of antibodies is known to be determined, to a large extent, by the constant domains or Fc region of the antibody molecule (Uanane and Benacerraf,

Textbook of Immunology, 2nd Edition, Williams & Wilkins, p. 218 (1984)). This includes their ability to activate complement and to mediate antibody-dependent cellular cytotoxicity (ADCC) as effected by leukocytes. Antibodies of different classes and subclasses differ in this respect, as do antibodies from the same subclass but different species; according to the present invention, antibodies of those classes having the desired biological activity are prepared. Preparation of these antibodies involves the selection of antibody constant domains are their incorporation in the humanized antibody by known technique. For example, mouse immunoglobulins of the IgG3 and IgG2a class are capable of activating serum complement upon binding to the target cells which express the cognate antigen, and therefore humanized antibodies which incorporate IgG3 and IgG2a effector functions are desirable for certain therapeutic applications.

In general, mouse antibodies of the IgG2a and IgG3 subclass and occasionally IgG1 can mediate ADCC, and antibodies of the IgG3, IgG2a, and IgM subclasses bind and activate serum complement. Complement activation generally requires the binding of at least two IgG molecules in close proximity on the target cell. However, the binding of only one IgM molecule activates serum complement.

The ability of any particular antibody to mediate lysis of the target cell by complement activation and/or AOCC can be assayed. The cells of interest are grown and labeled in vitro; the antibody is added to the cell culture in combination with either serum complement or immune cells which may be activated by the antigen antibody complexes. Cytolysis of the target cells is detected by the release of label from the lysed cells. In fact, antibodies can be screened using the patient's own serum as a source of complement and/or immune cells. The antibody that is capable of activating complement or mediating ADCC in the in vitro test can then be used therapeutically in that particular patient.

This invention specifically encompasses consensus Fc antibody domains prepared and used according to the teachings of this invention.

Therapeutic and Other Uses of the Antibodies

When used in vivo for therapy, the antibodies of the subject invention are administered to the patient in therapeutically effective amounts (i.e. amounts that have desired therapeutic effect). They will normally be administered parenterally. The dose and dosage regimen will depend upon the degree of the infection, the characteristics of the particular antibody or immunotoxin used, e.g., its therapeutic index, the patient, and the patient's history. Advantageously the antibody or immunotoxin is administered continuously over a period of 1-2 weeks, intravenously to treat cells in the vasculature and subcutaneously and intraperitoneally to treat regional lymph nodes. Optionally, the administration is made during the course of adjunct therapy such as combined cycles of radiation, chemotherapeutic treatment, or administration of tumor necrosis factor, interferon or other cytoprotective or immunomodulatory agent.

For parenteral administration the antibodies will be formulated in a unit dosage injectable form (solution, suspension, emulsion) in association with a pharmaceutically acceptable parenteral vehicle. Such vehicles are inherently nontoxic, and non-therapeutic. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Nonaqueous vehicles such as fixed oils and ethyl oleate can also be used. Liposomes may be used as carriers. The vehicle may contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, e.g., buffers and preser-

vatives. The antibodies will typically be formulated in such vehicles at concentrations of about 1 mg/ml to 10 mg/ml.

Use of IgM antibodies may be preferred for certain applications, however IgG molecules by being smaller may be more able than IgM molecules to localize to certain types of infected cells.

There is evidence that complement activation in vivo leads to a variety of biological effects, including the induction of an inflammatory response and the activation of macrophages (Uananue and Benecerraf, *Textbook of Immunology*, 2nd Edition, Williams & Wilkins, p. 218 (1984)). The increased vasodilation accompanying inflammation may increase the ability of various agents to localize in infected cells. Therefore, antigen-antibody combinations of the type specified by this invention can be used therapeutically in many ways. Additionally, purified antigens (Hakomori, *Ann. Rev. Immunol.* 2:103 (1984)) or anti-idiotypic antibodies (Nepom et al., *Proc. Natl. Acad. Sci.* 81:2864 (1985); Koprowski et al., *Proc. Natl. Acad. Sci.* 81:216 (1984)) relating to such antigens could be used to induce an active immune response in human patients. Such a response includes the formation of antibodies capable of activating human complement and mediating ADCC and by such mechanisms cause infected cell destruction.

Optionally, the antibodies of this invention are useful in passively immunizing patients, as exemplified by the administration of humanized anti-HIV antibodies.

The antibody compositions used in therapy are formulated and dosages established in a fashion consistent with good medical practice taking into account the disorder to be treated, the condition of the individual patient, the site of delivery of the composition, the method of administration and other factors known to practitioners. The antibody compositions are prepared for administration according to the description of preparation of polypeptides for administration, infra.

Deposit of Materials

As described above, cultures of the muMAb4D5 have been deposited with the American Type Culture Collection, 10801 University Blvd., Manassas, Va., USA (ATCC).

This deposit was made under the provisions of the Budapest Treaty on the international Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of viable cultures for 30 years from the date of the deposit. The organisms will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the cultures to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures' availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.12 with particular reference to 886 OG 638).

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

The assignee of the present application has agreed that if the cultures on deposit should die or be lost or destroyed

when cultivated under suitable conditions, they will be promptly replaced on notification with a viable specimen of the same culture. Availability of the deposited strain is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the constructs deposited, since the deposited embodiments are intended to illustrate only certain aspects of the invention and any constructs that are functionally equivalent are within the scope of this invention. The deposit of material herein does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustrations that they represent. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

It is understood that the application of the teachings of the present invention to a specific problem or situation will be within the capabilities of one having ordinary skill in the art in light of the teachings contained herein. Examples of the products of the present invention and representative processes for their isolation, use, and manufacture appear below, but should not be construed to limit the invention.

EXAMPLES

Example 1

Humanization of muMAB4D5

Here we report the chimerization of muMAB4D5 (chMAB4D5) and the rapid and simultaneous humanization of heavy (V_H) and light (V_L) chain variable region genes using a novel "gene conversion mutagenesis" strategy. Eight humanized variants (huMAB4D5) were constructed to probe the importance of several FR residues identified by our molecular modeling or previously proposed to be critical to the conformation of particular CDRs (see Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901-917 (1987); Chothia, C. et al., *Nature* 342:877-883 (1989); Tramontano, A. et al., *J. Mol. Biol.* 215:175-182 (1990)). Efficient transient expression of humanized variants in non-myeloma cells allowed us to rapidly investigate the relationship between binding affinity for p185^{HER2} ECD and anti-proliferative activity against p185^{HER2} overexpressing carcinoma cells.

Materials and Methods

Cloning of Variable Region Genes. The muMAB4D5 V_H and V_L genes were isolated by polymerase chain reaction (PCR) amplification of mRNA from the corresponding hybridoma (Fendly, B. M. et al., *Cancer Res.* 50:1550-1558 (1990)) as described by Orlandi et al. (Orlandi, R. et al., *Proc. Natl. Acad. Sci. USA* 86:3833-3837 (1989)). Amino terminal sequencing of muMAB4D5 V_L and V_H was used to design the sense strand PCR primers, whereas the anti-sense PCR primers were based upon consensus sequences of murine framework residues (Orlandi, R. et al., *Proc. Natl. Acad. Sci. USA* 86:3833-3837 (1989); Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) incorporating restriction sites for directional cloning shown by underlining and listed after the sequences: V_L sense, 5'-TCC

GATATCCAGCTGACCCAGTCTCCA-3' (SEQ. ID NO. 7), EcoRV; V_L anti-sense, 5'-GTTTGATCTCCAGCTT GGTACCHSCDCCGAA-3' (SEQ. ID NO. 8), Asp718; V_H sense, 5'-AGGTSMARCTGCAGSAGTCWGG-3' (SEQ. ID NO. 9), PstI and V_H anti-sense, 5'-TGAGGAGAC GGTGACCGTGGTCCCTTGGCCCCAG-3' (SEQ. ID NO. 10), BstEII; where H=A or C or T, S=C or G, D=A or G or T, M=A or C, R=A or G and W=A or T. The PCR products were cloned into pUC119 (Vieira, J. & Messing, J., *Methods Enzymol.* 153:3-11 (1987)) and five clones for each variable domain sequenced by the dideoxy method (Sanger, F. et al., *Proc. Natl. Acad. Sci. USA* 74:5463-5467 (1977)).

Molecular Modelling. Models for muMAB4D5 V_H and V_L domains were constructed separately from consensus coordinates based upon seven Fab structures from the Brookhaven protein data bank (entries 1FB4, 2RHE, 2MCP, 3FAB, 1FBJ, 2HFL and 1REI). The Fab fragment KOL (Marquart, M. et al., *J. Mol. Biol.* 141:369-391 (1980)) was first chosen as a template for V_L and V_H domains and additional structures were then superimposed upon this structure using their main chain atom coordinates (INSIGHT program, Siosym Technologies). The distance from the template $C\alpha$ to the analogous $C\alpha$ in each of the superimposed structures was calculated for each residue position. If all (or nearly all) $C\alpha$ - $C\alpha$ distances for a given residue were ≤ 1 Å, then that position was included in the consensus structure. In most cases the β -sheet framework residues satisfied these criteria whereas the CDR loops did not. For each of these selected residues the average coordinates for individual N, $C\alpha$, C, O and $C\beta$ atoms were calculated and then corrected for resultant deviations from non-standard bond geometry by 50 cycles of energy minimization using the DISCOVER program (Biosym Technologies) with the AMBER forcefield (Weiner, S. J. et al., *J. Amer. Chem. Soc.* 106:765-784 (1984)) and $C\alpha$ coordinates fixed. The side chains of highly conserved residues, such as the disulfide-bridged cysteine residues, were then incorporated into the resultant consensus structure. Next the sequences of muMAB4D5 V_L and V_H were incorporated starting with the CDR residues and using the tabulations of CDR conformations from Chothia et al. (Chothia, C. et al., *Nature* 342:877-883 (1989)) as a guide. Side-chain conformations were chosen on the basis of Fab crystal structures, rotamer libraries (Ponder, J. W. & Richards, F. M., *J. Mol. Biol.* 193:775-791 (1987)) and packing considerations. Since V_H -COR3 could not be assigned a definite backbone conformation from these criteria, two models were created from a search of similar sized loops using the INSIGHT program. A third model was derived using packing and solvent exposure considerations. Each model was then subjected to 5000 cycles of energy minimization.

In humanizing muMAB4D5, consensus human sequences were first derived from the most abundant subclasses in the sequence compilation of Kabat et al. (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)), namely V_L κ subgroup I and V_H group III, and a molecular model generated for these sequences using the methods described above. A structure for huMAB4D5 was created by transferring the CDRs from the muMAB4D5 model into the consensus human structure. All huMAB4D5 variants contain human replacements of muMAB4D5 residues at three positions within CDRs as defined by sequence variability (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) but not as defined by structural variability (Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901-917 (1987)).

V_L -CDR1 K24R, V_L -CDR2 R54L and V_L -CDR2 T56S. Differences between muMAB4D5 and the human consensus framework residues (FIG. 1) were individually modeled to investigate their possible influence on CDR conformation and/or binding to the p185^{HER2} ECD.

Construction of Chimeric Genes. Genes encoding chMAB4D5 light and heavy chains were separately assembled in previously described phagemid vectors containing the human cytomegalovirus enhancer and promoter, a 5' intron and SV40 polyadenylation signal (Gorman, C. M. et al., *DNA & Prot. Engin. Tech.* 2:3-10 (1990)). Briefly, gene segments encoding muMAB4D5 V_L (FIG. 1A) and REI human κ_1 light chain C_L (Palm, W. & Hilschmann, N., *Z. Physiol. Chem.* 356:167-191 (1975)) were precisely joined as were genes for muMAB4D5 V_H (FIG. 1B) and human γ_1 constant region (Capon, D. J. et al., *Nature* 337:525-531 (1989)) by simple subcloning (Boyle, A., in *Current Protocols in Molecular Biology*, Chapter 3 (F. A. Ausubel et al., eds., Greene Publishing & Wiley-Interscience, New York, 1990)) and site-directed mutagenesis (Carter, P., in *Mutagenesis: A Practical Approach*, Chapter 1 (IRL Press, Oxford, UK 1991)). The γ_1 isotype was chosen as it has been found to be the preferred human isotype for supporting ADCC and complement dependent cytotoxicity using matched sets of chimeric (Bruggemann, M. et al., *J. Exp. Med.* 166:1351-1361 (1987)) or humanized antibodies (Riechmann, L. et al., *Nature* 332:323-327 (1988)). The PCR-generated V_L and V_H fragments (FIG. 1) were subsequently mutagenized so that they faithfully represent the sequence of muMAB4D5 determined at the protein level: V_H Q1E, V_L V₁₀₄L and T109A (variants are denoted by the amino acid residue and number followed by the replacement amino acid). The human γ_1 constant regions are identical to those reported by Ellison et al. (Ellison, J. W. et al., *Nucleic Acids Res.* 13:4071-4079 (1982)) except for the mutations E359D and M361L (Eu numbering, as in Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) which we installed to convert the antibody from the naturally rare A allotype to the much more common non-A allotype (Tramontano, A. et al., *J. Mol. Biol.* 215:175-182 (1990)). This was an attempt to reduce the risk of anti-allotype antibodies interfering with therapy.

Construction of Humanized Genes. Genes encoding chMAB4D5 light chain and heavy chain Fd fragment (V_H and C_{H1} domains) were subcloned together into pUC119 (Vieira, J. & Messing, J., *Methods Enzymol.* 153:3-11 (1987)) to create pAK1 and simultaneously humanized in a single step (FIG. 2). Briefly, sets of 6 contiguous oligonucleotides were designed to humanize V_H and V_L (FIG. 1). These oligonucleotides are 28 to 83 nucleotides in length, contain zero to 19 mismatches to the murine antibody template and are constrained to have 8 or 9 perfectly matched residues at each end to promote efficient annealing and ligation of adjacent oligonucleotides. The sets of V_H and V_L humanization oligonucleotides (5 pmol each) were phosphorylated with either ATP or γ -³²P-ATP (Carter, P. *Methods Enzymol.* 154: 382-403 (1987)) and separately annealed with 3.7 pmol of pAK1 template in 40 μ l 10 mM Tris-HCl (pH 8.0) and 10 mM MgCl₂ by cooling from 100° C. to room temperature over ~30 min. The annealed oligonucleotides were joined by incubation with T4 DNA ligase (12 units; New England Biolabs) in the presence of 2 μ l 5 mM ATP and 2 μ l 0.1 M DTT for 10 min at 14° C. After electrophoresis on a 6% acrylamide sequencing gel the assembled oligonucleotides were located by autoradiography and recovered by electroelution. The assembled oligo-

nucleotides (~0.3 pmol each) were simultaneously annealed to 0.15 pmol single-stranded deoxyuridine-containing pAK1 prepared according to Kunkel et al. (Kunkel, T. A. et al., *Methods Enzymol.* 154:367-382 (1987)) in 10 μ l 40 mM Tris-HCl (pH 7.5) and 16 mM MgCl₂ as above. Heteroduplex DNA was constructed by extending the primers with T7 DNA polymerase and transformed into *E. coli* BMH 71-18 mutL as previously described (Carter, P., in *Mutagenesis: A Practical Approach*, Chapter 1 (IRL Press, Oxford, UK 1991)). The resultant phagemid DNA pool was enriched first for hu V_L by restriction purification using XhoI and then for hu V_H by restriction selection using StuI as described in Carter, P., in *Mutagenesis: A Practical Approach*, Chapter 1 (IRL Press, Oxford, UK 1991); and in Wells, J. A. et al., *Phil. Trans. R. Soc. Lond., A* 317:415-423 (1986). Resultant clones containing both hu V_L and hu V_H genes were identified by nucleotide sequencing (Sanger, F. et al., *Proc. Natl. Acad. Sci. USA* 74:5463-5467 (1977)) and designated pAK2. Additional humanized variants were generated by site-directed mutagenesis (Carter, P., in *Mutagenesis: A Practical Approach*, Chapter 1 (IRL Press, Oxford, UK 1991)). The muMAB4D5 V_L and V_H gene segments in the transient expression vectors described above were then precisely replaced with their humanized versions.

Expression and Purification of MAB4D5 Variants. Appropriate MAB4D5 light and heavy chain cDNA expression vectors were co-transfected into an adenovirus transformed human embryonic kidney cell line, 293 (Graham, F. L. et al., *J. Gen. Virol.* 36:59-72 (1977)) using a high efficiency procedure (Gorman, C. M. et al., *DNA & Prot. Engin. Tech.* 2:3-10 (1990); Gorman, C., in *DNA Cloning*, vol II, pp 143-190 (D. M. Glover, ed., IRL Press, Oxford, UK 1985)). Media were harvested daily for up to 5 days and the cells re-fed with serum free media. Antibodies were recovered from the media and affinity purified on protein A sepharose CL-4B (Pharmacia) as described by the manufacturer. The eluted antibody was buffer-exchanged into phosphate-buffered saline by G25 gel filtration, concentrated by ultrafiltration (Centriprep-30 or Centricon-100, Amicon), sterile-filtered (Millex-GV, Millipore) and stored at 4° C. The concentration of antibody was determined by using both total immunoglobulin and antigen binding ELISAs. The standard used was huMAB4D5-5, whose concentration had been determined by amino acid composition analysis.

Cell Proliferation Assay. The effect of MAB4D5 variants upon proliferation of the human mammary adenocarcinoma cell line, SK-BR-3, was investigated as previously described (Fendly, B. M. et al., *Cancer Res.* 50:1550-1558 (1990)) using saturating MAB4D5 concentrations.

Affinity Measurements. The antigen binding affinity of MAB4D5 variants was determined using a secreted form of the p185^{HER2} ECD prepared as described in Fendly, B. M. et al., *J. Biol. Resp. Mod.* 9:449-455 (1990). Briefly, antibody and p185^{HER2} ECD were incubated in solution until equilibrium was found to be reached. The concentration of free antibody was then determined by ELISA using immobilized p185^{HER2} ECD and used to calculate affinity (K_d) according to Friguet et al. (Friguet, B. et al., *J. Immunol. Methods* 77:305-319 (1985)).

Results

Humanization of muMAB4D5. The muMAB4D5 V_L and V_H gene segments were first cloned by PCR and sequenced (FIG. 1). The variable genes were then simultaneously humanized by gene conversion mutagenesis using preassembled oligonucleotides (FIG. 2). A 311-mer oligonucleo-

otide containing 39 mismatches to the template directed 24 simultaneous amino acid changes required to humanize muMAB4D5 V_L. Humanization of muMAB4D5 V_H required 32 amino acid changes which were installed with a 361-mer containing 59 mismatches to the muMAB4D5 template. Two out of 8 clones sequenced precisely encode huMAB4D5-5, although one of these clones contained a single nucleotide imperfection. The 6 other clones were essentially humanized but contained a small number of errors: <3 nucleotide changes and <1 single nucleotide deletion per kilobase. Additional humanized variants (Table 3) were constructed by site-directed mutagenesis of huMAB4D5-5.

Expression levels of huMAB4D5 variants were in the range of 7 to 15 μg/ml as judged by ELISA using immobilized p185^{HER2} ECD. Successive harvests of five 10 cm plates allowed 200 μg to 500 mg of each variant to be produced in a week. Antibodies affinity purified on protein A gave a single band on a Coomassie blue stained SDS polyacrylamide gel of mobility consistent with the expected M_r of ~150 kDa. Electrophoresis under reducing conditions gave 2 bands consistent with the expected M_r of free heavy (48 kDa) and light (23 kDa) chains (not shown). Amino terminal sequence analysis (10-cycles) gave the mixed sequence expected (see FIG. 1) from an equimolar combination of light and heavy chains (not shown).

huMAB4D5 Variants. In general, the FR residues were chosen from consensus human sequences (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) and CDR residues from muMAB4D5. Additional variants were constructed by replacing selected human residues in huMAB4D5-1 with their muMAB4D5 counterparts. These are V_H residues 71, 73, 78, 93 plus 102 and V_L residues 55 plus 66 identified by our molecular modeling. V_H residue 71 has previously been proposed by others (Tramontano, A. et al., *J. Mol. Biol.* 215:175-182 (1990)) to be critical to the conformation of V_H-CDR2. Amino acid sequence differences between huMAB4D5 variant molecules are shown in Table 3, together with their p185^{HER2} ECD binding affinity and maximal anti-proliferative activities against SK-BR-3 cells. Very similar K_d values were obtained for binding of MAB4D5 variants to either SK-BR-3 cells or to p185^{HER2} ECD (Table 3). However, K_d estimates derived from binding of MAB4D5 variants to p185^{HER2} ECD were more reproducible with smaller standard errors and consumed much smaller quantities of antibody than binding measurements with whole cells.

The most potent humanized variant designed by molecular modeling, huMAB4D5-8, contains 5 FR residues from muMAB4D5. This antibody binds the p185^{HER2} ECD 3-fold more tightly than does muMAB4D5 itself (Table 3) and has comparable anti-proliferative activity with SK-BR-3 cells (FIG. 3). In contrast, huMAB4D5-1 is the most humanized but least potent muMAB4D5 variant, created by simply installing the muMAB4D5 CDRs into the consensus human sequences. huMAB4D5-1 binds the p185^{HER2} ECD 80-fold less tightly than does the murine antibody and has no detectable anti-proliferative activity at the highest antibody concentration investigated (16 μg/ml).

The anti-proliferative activity of huMAB4D5 variants against p185^{HER2} overexpressing SK-BR-3 cells is not simply correlated with their binding affinity for the p185^{HER2} ECD. For example, installation of three murine residues into the V_H domain of huMAB4D5-2 (D73T, L78A and A93S) to create huMAB4D5-3 does not change the antigen binding affinity but does confer significant anti-proliferative activity (Table 3).

The importance of V_H residue 71 (Tramontano, A. et al., *J. Mol. Biol.* 215:175-182 (1990)) is supported by the observed 5-fold increase in affinity for p185^{HER2} ECD on replacement of R71 in huMAB4D5-1 with the corresponding murine residue, alanine (huMAB4D5-2). In contrast, replacing V_H L78 in huMAB4D5-4 with the murine residue, alanine (huMAB4D5-5), does not significantly change the affinity for the p185^{HER2} ECD or change anti-proliferative activity, suggesting that residue 78 is not of critical functional significance to huMAB4D5 and its ability to interact properly with the extracellular domain of p185^{HER2}.

V_L residue 66 is usually a glycine in human and murine κ chain sequences (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) but an arginine occupies this position in the muMAB4D5 κ light chain. The side chain of residue 66 is likely to affect the conformation of V_L-CDR1 and V_L-CDR2 and the hairpin turn at 68-69 (FIG. 4). Consistent with the importance of this residue, the mutation V_L G66R (huMAB4D5-3→huMAB4D5-5) increases the affinity for the p185^{HER2} ECD by 4-fold with a concomitant increase in anti-proliferative activity.

From molecular modeling it appears that the tyrosyl side chain of muMAB4D5 V_L residue 55 may either stabilize the conformation of V_H-CDR3 or provide an interaction at the V_L-V_H interface. The latter function may be dependent upon the presence of V_H Y102. In the context of huMAB4D5-5 the mutations V_L E55Y (huMAB4D5-6) and V_H V102Y (huMAB4D5-7) individually increase the affinity for p185^{HER2} ECD by 5-fold and 2-fold respectively, whereas together (huMAB4D5-8) they increase the affinity by 11-fold. This is consistent with either proposed role of V_L Y55 and V_H Y102.

Secondary Immune Function of huMAB4D5-8. MuMAB4D5 inhibits the growth of human breast tumor cells which overexpress p185^{HER2} (Hudziak, R. M. et al., *Molec. Cell. Biol.* 9:1165-1172 (1989)). The antibody, however, does not offer the possibility of direct tumor cytotoxic effects. This possibility does arise in huMAB4D5-8 as a result of its high affinity (K_d=0.1 μM) and its human IgG₁ subtype. Table 4 compares the ADCC mediated by huMAB4D5-8 with muMAB4D5 on a normal lung epithelial cell line, WI-38, which expresses a low level of p185^{HER2} and on SK-BR-3, which expresses a high level of p185^{HER2}. The results demonstrate that: (1) huMAB4D5 has a greatly enhanced ability to carry out ADCC as compared with its murine parent; and (2) that this activity may be selective for cell types which overexpress p185^{HER2}.

Discussion

MuMAB4D5 is potentially useful for human therapy since it is cytostatic towards human breast and ovarian tumor lines overexpressing the HER2-encoded p185^{HER2} receptor-like tyrosine kinase. Since both breast and ovarian carcinomas are chronic diseases it is anticipated that the optimal MAB4D5 variant molecule for therapy will have low immunogenicity and will be cytotoxic rather than solely cytostatic in effect. Humanization of muMAB4D5 should accomplish these goals. We have identified 5 different huMAB4D5 variants which bind tightly to p185^{HER2} ECD (K_d≤1 nM) and which have significant anti-proliferative activity (Table 3). Furthermore huMAB4D5-8 but not muMAB4D5 mediates ADCC against human tumor cell lines overexpressing p185^{HER2} in the presence of human effector cells (Table 4) as anticipated for a human γ1 isotype (Brcuggemann, M. et al., *J. Exp. Med.* 166:1351-1361 (1987); Riechmann, L. et al., *Nature* 332:323-327 (1988)).

Rapid humanization of huMab4D5 was facilitated by the gene conversion mutagenesis strategy developed here using long preassembled oligonucleotides. This method requires less than half the amount of synthetic DNA as does total gene synthesis and does not require convenient restriction sites in the target DNA. Our method appears to be simpler

direct cytotoxic activity of the humanized molecule in the presence of human effector cells. The apparent selectivity of the cytotoxic activity for cell types which overexpress p185^{HER2} allows for the evolution of a straightforward clinic approach to those human cancers characterized by overexpression of the HER2 protooncogene.

TABLE 3

p185^{HER2} ECD binding affinity and anti-proliferative activities of Mab4D5 variants

Mab4D5 cell Variant proliferation [‡]	V _H Residue*					V _L Residue*			K _d [†] nM	Relative
	71 FR3	73 FR3	78 FR3	93 FR3	102 CDR3	55 CDR2	56 FR3	K _d [†]		
huMab4D5-1	R	D	L	A	V	E	G	25	102	
huMab4D5-2	Ala	D	L	A	V	E	G	4.7	101	
huMab4D5-3	Ala	Thr	Ala	Ser	V	E	G	4.4	66	
huMab4D5-4	Ala	Thr	L	Ser	V	E	Arg	0.82	56	
huMab4D5-5	Ala	Thre	Ala	Ser	V	E	Arg	1.1	48	
huMab4D5-6	Ala	Thr	Ala	Ser	V	Tyr	Arg	0.22	51	
huMab4D5-7	Ala	Thr	Ala	Ser	Tyr	E	Arg	0.62	53	
huMab4D5-8	Ala	Thr	Ala	Ser	Tyr	Tyr	Arg	0.10	54	
muMab4D5	Ala	Thr	Ala	Ser	Tyr	Tyr	Arg	0.30	37	

*Human and murine residues are shown in one letter and three letter amino acid code respectively.
[†]K_d values for the p185^{HER2} ECD were determined using the method of Friguet et al. (43) and the standard error of each estimate is $\leq \pm 10\%$.
[‡]Proliferation of SK-BR-3 cells incubated for 96 hr with Mab4D5 variants shown as a percentage of the untreated control as described (Hudziak, R. M. et al., *Molec. Cell. Biol.* 9: 1165–1172 (1989)). Data represent the maximal anti-proliferative effect for each variant (see FIG. 3A) calculated as the mean of triplicate determinations at a Mab4D5 concentration of 8 $\mu\text{g/ml}$. Data are all taken from the same experiment with an estimated standard error of $\leq \pm 15\%$.

and more reliable than a variant protocol recently reported (Rostapshov, V. M. et al., *FEBS Lett.* 249: 379–382 (1989)). Transient expression of huMab4D5 in human embryonic kidney 293 cells permitted the isolation of a few hundred micrograms of huMab4D5 variants for rapid characterization by growth inhibition and antigen binding affinity assays. Furthermore, different combinations of light and heavy chain were readily tested by co-transfection of corresponding cDNA expression vectors.

The crucial role of molecular modeling in the humanization of muMab4D5 is illustrated by the designed variant huMab4D5-8 which binds the p185^{HER2} ECD 250-fold more tightly than the simple CDR loop swap variant, huMab4D5-1. It has previously been shown that the antigen binding affinity of a humanized antibody can be increased by mutagenesis based upon molecular modelling (Riechmann, L. et al., *Nature* 332:323–327 (1988); Queen, C. et al., *Proc. Natl. Acad. Sci. USA* 86:10029–10033 (1989)). Here we have extended this earlier work by others with a designed humanized antibody which binds its antigen 3-fold more tightly than the parent rodent antibody. While this result is gratifying, assessment of the success of the molecular modeling must await the outcome of X-ray structure determination. From analysis of huMab4D5 variants (Table 3) it is apparent that their anti-proliferative activity is not a simple function of their binding affinity for p185^{HER2} ECD. For example the huMab4D5-8 variant binds p185^{HER2} 3-fold more tightly than muMab4D5 but the humanized variant is slightly less potent in blocking the proliferation of SK-SR-3 cells. Additional huMab4D5 variants are currently being constructed in an attempt to identify residues triggering the anti-proliferative activity and in an attempt to enhance this activity.

In addition to retaining tight receptor binding and the ability to inhibit cell growth, the huMab4D5-8 also confers a secondary immune function (ADCC). This allows for

TABLE 4

Selectivity of antibody dependent tumor cell cytotoxicity mediated by huMab4D5-8

Effector:Target ratio [†]	WI-38*		SK-BR-3	
	muMab4D5	huMab4D5-8	muMab4D5	huMab4D5-8
A. [‡]				
25:1	<1.0	9.3	7.5	40.6
12.5:1	<1.0	11.1	4.7	36.8
6.25:1	<1.0	8.9	0.9	35.2
3.13:1	<1.0	8.5	4.6	19.6
B.				
25:1	<1.0	3.1	6.1	33.4
12.5:1	<1.0	1.7	5.5	26.2
6.25:1	1.3	2.2	2.0	21.0
3.13:1	<1.0	0.8	2.4	13.4

*Sensitivity to ADCC of two human cell lines (WI-38, normal lung epithelium; and SK-BR-3, human breast tumor cell line) are compared. WI-38 expresses a low level of p185^{HER2} (0.6 pg per μg cell protein) and SK-BR-3 expresses a high level of p185^{HER2} (64 pg p185^{HER2} per μg cell protein), as determined by ELISA (Fendly et al., *J. Biol. Resp. Mod.* 9:449–455 (1990)).
[†]ADCC assays were carried out as described in Brüggemann et al., *J. Exp. Med.* 166:1351–1361 (1987). Effector to target ratios were of IL-2 activated human peripheral blood lymphocytes to either WI-38 fibroblasts or SK-BR-3 tumor cells in 96-well microtiter plates for 4 hours at 37° C. Values given represent percent specific cell lysis as determined by ⁵¹Cr release. Estimated standard error in these quadruplicate determinations was $\leq \pm 10\%$.
[‡]Monoclonal antibody concentrations used were 0.1 $\mu\text{g/ml}$ (A) and 0.1 $\mu\text{g/ml}$ (B).

Example 2
Schematic Method for Humanizing an Antibody Sequence

This example illustrates one stepwise elaboration of the methods for creating a humanized sequence described

above. It will be understood that not all of these steps are essential to the claimed invention, and that steps may be taken in different order.

1. ascertain a consensus human variable domain amino acid sequence and prepare from it a consensus structural model.
2. prepare model of import (the non-human domain to be humanized) variable domain sequences and note structural differences with respect to consensus human model.
3. identify CDR sequences in human and in import, both by using Kabat (supra, 1987) and crystal structure criteria. If there is any difference in CDR identity from the different criteria, use of crystal structure definition of the CDR, but retain the Kabat residues as important framework residues to import.
4. substitute import CDR sequences for human CDR sequences to obtain initial "humanized" sequence.
5. compare import non-CDR variable domain sequence to the humanized sequence and note divergences.
6. Proceed through the following analysis for each amino acid residue where the import diverges from the humanized.
 - a. If the humanized residue represents a residue which is generally highly conserved across all species, use the residue in the humanized sequence. If the residue is not conserved across all species, proceed with the analysis described in 6b.
 - b. If the residue is not generally conserved across all species, ask if the residue is generally conserved in humans.
 - i. If the residue is generally conserved in humans but the import residue differs, examine the structural models of the import and human sequences and determine if the import residue would be likely to affect the binding or biological activity of the CDRs by considering 1) could it bind antigen directly and 2) could it affect the conformation of the CDR. If the conclusion is that an affect on the CDRs is likely, substitute the import residue. If the conclusion is that a CDR affect is unlikely, leave the humanized residue unchanged.
 - ii. If the residue is also not generally conserved in humans, examine the structural models of the import and human sequences and determine if the import residue would be likely to affect the binding or biological activity of the CDRs by considering 1) could it bind antigen directly and 2) could it affect the conformation of the CDR. If the conclusion is that an affect on the CDRs is likely, substitute the import residue. If the conclusion is that a CDR affect is unlikely, proceed to the next step.
 - a) Examine the structural models of the import and human sequences and determine if the residue is exposed on the surface of the domain or is buried within. If the residue is exposed, use the residue in the humanized sequence. If the residue is buried, proceed to the next step.
 - (i) Examine the structural models of the import and human sequences and determine if the residue is likely to affect the V_L-V_H interface. Residues involved with the interface include: 34L, 36L, 38L, 43L, 33L, 36L, 85L, 87L, 89L, 91L, 96L, 98L, 35H, 37H, 39H, 43H, 45H, 47H, 60H, 91H, 93H, 95H, 100H, and 103H. If no effect is likely, use the residue in the humanized sequence. If some affect is likely, substitute the import residue.

7. Search the import sequence, the consensus sequence and the humanized sequence for glycosylation sites outside the CDRs, and determine if this glycosylation site is likely to have any affect on antigen binding and/or biological activity. If no effect is likely, use the human sequence at that site; if some affect is likely, eliminate the glycosylation site or use the import sequence at that site.
8. After completing the above analysis, determine the planned humanized sequence and prepare and test a sample. If the sample does not bind well to the target antigen, examine the particular residues listed below, regardless of the question of residue identity between the import and humanized residues.
 - a. Examine particular peripheral (non-CDR) variable domain residues that may, due to their position, possibly interact directly with a macromolecular antigen, including the following residues (where the * indicates residues which have been found to interact with antigen based on crystal structures):
 - i. Variable light domain: 36, 46, 49*, 63-70
 - ii. Variable heavy domain: 2, 47*, 68, 70, 73-76.
 - b. Examine particular variable domain residues which could interact with, or otherwise affect, the conformation of variable domain CDRs, including the following (not including CDR residues themselves, since it is assumed that, because the CDRs interact with one another, any residue in one CDR could potentially affect the conformation of another CDR residue) (L=LIGHT, H=HEAVY, residues appearing in bold are indicated to be structurally important according the Chothia et al., Nature 342:877 (1989), and residues appearing in italic were altered during humanization by Queen et al. (PDL), Proc. Natl. Acad. Sci. USA 86:10029 (1989) and Proc. Natl. Acad. Sci. USA 88:2869 (1991).):
 - i. Variable light domain:
 - a) CDR-1 (residues 24L-34L): 2L, 4L, 66L-69L, 71L
 - b) CDR-2 (residues 50L-56L): 35L, 46L, 47L, 48L, 49L, 58L, 62L, 64L-66L, 71L, 73L
 - c) CDR-3 (residues 89L-97L): 2L, 4L, 36L, 98L, 37H, 45H, 47H, 58H, 60H
 - ii. Variable heavy domain:
 - a) CDR-1 (residues 26H-35H): 2H, 4H, 24H, 36H, 71H, 73H, 76H, 78H, 92H, 94H
 - b) CDR-2 (residues 50H-55H): 49H, 69H, 69H, 71H, 73H, 78H
 - c) CDR-3 (residues 95H-102H): examine all residues as possible interaction partners with this loop, because this loop varies in size and conformation much more than the other CDRs.
9. If after step 8 the humanized variable domain still is lacking in desired binding, repeat step 8. In addition, re-investigate any buried residues which might affect the V_L-V_H interface (but which would not directly affect CDR conformation). Additionally, evaluate the accessibility of non-CDR residues to solvent.

Example 3

Engineering a Humanized Bispecific F(ab')₂ Fragment

This example demonstrates the construction of a humanized bispecific antibody (BsF(ab')₂v1 by separate *E. coli* expression of each Fab' arm followed by directed chemical coupling in vitro. BsF(ab')₂v1 (anti-CD3/anti-p185^{HER2}) was demonstrated to retarget the cytotoxic activity of human

CD3⁺CTL in vitro against the human breast tumor cell line, SK-BR-3, which overexpresses the p185^{HER2} product of the protooncogene HER2. This example demonstrates the minimalistic humanization strategy of installing as few murine residues as possible into a human antibody in order to recruit antigen-binding affinity and biological properties comparable to that of the murine parent antibody. This strategy proved very successful for the anti-p185^{HER2} arm of BsF(ab')₂v1. In contrast BsF(ab')₂v1 binds to T cells via its anti-CD3 arm much less efficiently than does the chimeric BsF(ab')₂ which contains the variable domains of the murine parent anti-CD3 antibody. Here we have constructed additional BsF(ab')₂ fragments containing variant anti-CD3 arms with selected murine residues restored in an attempt to improve antibody binding to T cells. One such variant, SsF(ab')₂v9, was created by replacing six residues in the second hypervariable loop of the anti-CD3 heavy chain variable domain of BsF(ab')₂v1 with their counterparts from the murine parent anti-CD3 antibody. BsF(ab')₂v9 binds to T cells (Jurkat) much more efficiently than does BsF(ab')₂v1 and almost as efficiently as the chimeric BsF(ab')₂. This improvement in the efficiency of T cell binding of the humanized BsF(ab')₂ is an important step in its development as a potential therapeutic agent for the treatment of p185^{HER2}-overexpressing cancers.

Bispecific antibodies (BsAbs) with specificities for tumor-associated antigens and surface markers on immune effector cells have proved effective for retargeting effector cells to kill tumor targets both in vitro and in vivo (reviewed by Fanger, M. W. et al., *Immunol. Today* 10: 92-99 (1989); Fanger, M. W. et al., *Immunol. Today* 12: 51-54 (1991); and Nelson, H., *Cancer Cells* 3: 163-172 (1991)). BsF(ab')₂ fragments have often been used in preference to intact BsAbs in retargeted cellular cytotoxicity to avoid the risk of killing innocent bystander cells binding to the Fc region of the antibody. An additional advantage of BsF(ab')₂ over intact BsAbs is that they are generally much simpler to prepare free of contaminating monospecific molecules (reviewed by Songvilai, S. and Lachmann, P. J., *Clin. Exp. Immunol.* 79: 315-321 (1990) and Nolan, O. and O'Kennedy, R., *Biochim. Biophys. Acta* 1040: 1-11 (1990)).

BsF(ab')₂ fragments are traditionally constructed by directed chemical coupling of Fab' fragments obtained by limited proteolysis plus mild reduction of the parent rodent monoclonal Ab (Brennan, M. et al., *Science* 229, 81-83 (1985) and Glennie, M. J. et al., *J. Immunol.* 139: 2367-2375 (1987)). One such BsF(ab')₂ fragment (anti-glioma associated antigen/anti-CD3) was found to have clinical efficacy in glioma patients (Nitta, T. et al., *Lancet* 335: 368-371 (1990) and another BsF(ab')₂ (anti-indium chelate/anti-carcinoembryonic antigen) allowed clinical imaging of colorectal carcinoma (Stickney, D. R. et al., *Antibody, Immunoconj. Radiopharm.* 2: 1-13 (1989)). Future SsF(ab')₂ destined for clinical applications are likely to be constructed from antibodies which are either human or at least "humanized" (Riechmann, L. et al., *Nature* 332: 323-327 (1988) to reduce their immunogenicity (Hale, G. et al., *Lancet* i: 1394-1399 (1988)).

Recently a facile route to a fully humanized BsF(ab')₂ fragment designed for tumor immunotherapy has been demonstrated (Shalaby, M. R. et al., *J. Exp. Med.* 175: 217-225 (1992)). This approach involves separate *E. coli* expression of each Fab' arm followed by traditional directed chemical coupling in vitro to form the BsF(ab')₂. One arm of the BsF(ab')₂ was a humanized version (Carter, P. et al., *Proc. Natl. Acad. Sci. USA* (1992a) and Carter, P., et al., *Bio/Technology* 10: 163-167 (1992b)) of the murine monoclonal

Ab 4D5 which is directed against the p185^{HER2} product of the protooncogene HER2 (c-erbB-2) (Fendly, B. M. et al. *Cancer Res.* 50: 1550-1558 (1989)). The humanization of the antibody 4D5 is shown in Example 1 of this application. The second arm was a minimalistically humanized anti-CD3 antibody (Shalaby et al. supra) which was created by installing the CDR loops from the variable domains of the murine parent monoclonal Ab UCHT1 (Beverley, P. C. L. and Callard, R. E., *Eur. J. Immunol.* 11: 329-334 (1981)) into the humanized anti-p185^{HER2} antibody. The BsF(ab')₂ fragment containing the most potent humanized anti-CD3 variant (v1) was demonstrated by flow cytometry to bind specifically to a tumor target overexpressing p185^{HER2} and to human peripheral blood mononuclear cells carrying CD3. In addition, BsF(ab')₂v1 enhanced the cytotoxic effects of activated human CTL 4-fold against SK-SR-3 tumor cells overexpressing p185^{HER2}. The example describes efforts to improve the antigen binding affinity of the humanized anti-CD3 arm by the judicious recruitment of a small number of additional murine residues into the minimalistically humanized anti-CD3 variable domains.

Materials and Methods

Construction of Mutations in the Anti-CD3 Variable Region Genes

The construction of genes encoding humanized anti-CD3 variant 1 (v1) variable light (V_L) and heavy (V_H) chain domains in phagemid pUC119 has been described (Shalaby et al. supra). Additional anti-CD3 variants were generated using an efficient site-directed mutagenesis method (Carter, P., *Mutagenesis: a practical approach*, (M. J. McPherson, Ed.), Chapter 1, IRL Press, Oxford, UK (1991)) using mismatched oligonucleotides which either install or remove unique restriction sites. Oligonucleotides used are listed below using lowercase to indicate the targeted mutations. Corresponding coding changes are denoted by the starting amino acid in one letter code followed by the residue numbered according to Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest*, 5th edition, National Institutes of Health, Bethesda, Md., USA (1991), then the replacement amino acid and finally the identity of the anti-CD3 variant:

HX11, 5' GTAGATAAATCCtctAACACAGC-CTAAtCTGCAAATG 3' (SEQ.ID. NO. 11) V_HK75S, v6;
HX12, 5' GTAGATAAATCCAAAtctACAGC-CTAAtCTGCAAATG 3' (SEQ.ID. NO. 12) V_HN76S, v7;
HX13, 5' GTAGATAAATCCtcttctACAGC-CTAAtCTGCAAATG 3' (SEQ.ID. NO. 13) V_HK75S:N76S, v8;
X14, 5' CTTATAAAGGTGTTtCcACCTATaaCcAgAaatTCAAGGatCGTTTCACgATAtcCGTAGATAAATCC 3' (SEQ.ID.NO. 14) V_HT57S:A60N:D61Q:S62K:V63F:G65D, v9;
LX6, 5' CTATACCTCCCGTCTgcatTCTGGAGTCCC 3' (SEQ.ID. NO. 15) V_LE55H, v11.

Oligonucleotides HX11, HX12 and HX13 each remove a site for BspMI, whereas LX6 removes a site for XhoI and HX14 installs a site for EcoRV (bold). Anti-CD3 variant v10 was constructed from v9 by site-directed mutagenesis using oligonucleotide HX13. Mutants were verified by dideoxynucleotide sequencing (Sanger, F. et al., *Proc. Natl. Acad. Sci. USA* 74: 5463-5467 (1977)).

E. coli Expression of Fab' Fragments

The expression plasmid, pAK19, for the co-secretion of light chain and heavy chain Fd' fragment of the most preferred humanized anti-p185^{HER2} variant, HuMab4D5-8, is described in Carter et al., 1992b, *supra*. Briefly, the Fab' expression unit is bicistronic with both chains under the

transcriptional control of the ohoA promoter. Genes encoding humanized V_L and V_H domains are precisely fused on their 5' side to a gene segment encoding the heat-stable enterotoxin II signal sequence and on their 3' side to human $k_1 C_L$ and IgG1C_H1 constant domain genes, respectively. The C_{H1} gene is immediately followed by a sequence encoding the hinge sequence CysAlaAla and followed by a bacteriophage λ t_o transcriptional terminator. Fab' expression plasmids for chimeric and humanized anti-CD3 variants (v1 to v4, Shalaby et al., supra; v6 to v12, this study) were created from pAK19 by precisely replacing anti-p185^{HER2} V_L and V_H gene segments with those encoding murine and corresponding humanized variants of the anti-CD3 antibody, respectively, by sub-cloning and site-directed mutagenesis. The Fab' expression plasmid for the most potent humanized anti-CD3 variant identified in this study (v9) is designated pAK22. The anti-p185^{HER2} Fab' fragment was secreted from *E. coli* K12 strain 25F2 containing plasmid pAK19 grown for 32 to 40 hr at 37° C. in an aerated 10 liter fermentor. The final cell density was 120–150 OD₅₅₀ and the titer of soluble and functional anti-p185^{HER2} Fab' was 1–2 g/liter as judged by antigen binding ELISA (Carter et al., 1992b, supra). Anti-CD3 Fab' variants were secreted from *E. coli* containing corresponding expression plasmids using very similar fermentation protocols. The highest expression titers of chimeric and humanized anti-CD3 variants were 200 mgaliter and 700 mgaliter, respectively, as judged by total immunoglobulin ELISA.

Construction of BsF(ab')₂ Fragments

Fab' fragments were directly recovered from *E. coli* fermentation pastes in the free thiol form (Fab'-SH) by affinity purification on Streptococcal protein G at pH 5 in the presence of EDTA (Carter et al., 1992b supra). Thioether linked BsF(ab')₂ fragments (anti-p185^{HER2}/anti-CD3) were constructed by the procedure of Glennie et al. supra with the following modifications. Anti-p185^{HER2} Fab'-SH in 100 mM Tris acetate, 5 mM EDTA (pH 5.0) was reacted with 0.1 vol of 40 mM N,N'-1,2-phenylenedimaleimide (o-PDM) in dimethyl formamide for ~1.5 hr at 20° C. Excess o-PDM was removed by protein G purification of the Fab' maleimide derivative (Fab'-mal) followed by buffer exchange into 20 mM sodium acetate, 5 mM EDTA (pH 5.3) (coupling buffer) using centrprep-30 concentrators (Amicon). The total concentration of Fab' variants was estimated from the measured absorbance at 280 nm (HuMAb4D5-8 Fab' e^{0.1%}=1.56, Carter et al., 1992b, supra). The free thiol content of Fab' preparations was estimated by reaction with 5,5' -dithiobis (2-nitrobenzoic acid) as described by Creighton, T. E., *Protein structure: a practical approach*, (T. E. Creighton, Ed.), Chapter 7, IRL Press, Oxford, UK (1990). Equimolar amounts of anti-p185^{HER2} Fab'-mal (assuming quantitative reaction of Fab'-SH with o-PDM) and each anti-CD3 Fab'-SH variant were coupled together at a combined concentration of 1 to 2.5 mg/ml in the coupling buffer for 14 to 48 hr at 4° C. The coupling reaction was adjusted to 4 mM cysteine at pH 7.0 and incubated for 15 min at 20° C. to reduce any unwanted disulfide-linked F(ab')₂ formed. These reduction conditions are sufficient to reduce inter-heavy chain disulfide bonds with virtually no reduction of the disulfide between light and heavy chains. Any free thiols generated were then blocked with 50 mM iodoacetamide. BsF(ab')₂ was isolated from the coupling reaction by S100-HR (Pharmacia) size exclusion chromatography (2.5 cm×100 cm) in the presence of PBS. The BsF(ab')₂ samples were passed through a 0.2 mm filter flash frozen in liquid nitrogen and stored at -70° C.

Flow Cytometric Analysis of F(ab')₂ Binding to Jurkat Cells

The Jurkat human acute T cell leukemia cell line was purchased from the American Type Culture Collection (Manassas Va.) (ATCC TIB 152) and grown as recommended by the ATCC. Aliquots of 10⁶ Jurkat cells were incubated with appropriate concentrations of BsF(ab')₂ (anti-p185^{HER2}/anti-CD3 variant) or control mono-specific anti-p185^{HER2} F(ab')₂ in PBS plus 0.1% (w/v) bovine serum albumin and 10 mM sodium azide for 45 min at 4° C. The cells were washed and then incubated with fluorescein-conjugated goat anti-human F(ab')₂ (Organon Teknika, West Chester, Pa.) for 45 min at 4° C. Cells were washed and analyzed on a FACScan® (Becton Dickinson and Co., Mountain View, Calif.). Cells (8×10³) were acquired by list mode and gated by forward light scatter versus side light scatter excluding dead cells and debris.

Results

Design of Humanized anti-CD3 Variants

The most potent humanized anti-CD3 variant previously identified, v1, differs from the murine parent antibody, UCHT1 at 19 out of 107 amino acid residues within V_L and at 37 out of 122 positions within V_H (Shalaby et al., supra 1992). Here we recruited back additional murine residues into anti-CD3 v1 in an attempt to improve the binding affinity for CD3. The strategy chosen was a compromise between minimizing both the number of additional murine residues recruited and the number of anti-CD3 variants to be analyzed. We focused our attentions on a few CDR residues which were originally kept as human sequences in our minimalistic humanization regime. Thus human residues in V_H CDR2 of anti-CD3 v1 were replaced en bloc with their murine counterparts to give anti-CD3 v9: T57S:A60N:D61Q:S62K:V63F:G65D (SEQ ID NO:20). Similarly, the human residue E55 in V_L CDR2 of anti-CD3 v1 was replaced with histidine from the murine anti-CD3 antibody to generate anti-CD3 v11. In addition, V_H framework region (FR) residues 75 and 76 in anti-CD3 v1 were also replaced with their murine counterparts to create anti-CD3 v8: K75S:N76S. V_H residues 75 and 76 are located in a loop close to V_H CDR1 and CDR2 and therefore might influence antigen binding. Additional variants created by combining mutations at these three sites are described below.

Preparation of BsF(ab')₂ Fragments

Soluble and functional anti-p185^{HER2} and anti-CD3 Fab' fragments were recovered directly from corresponding *E. coli* fermentation pastes with the single hinge cysteine predominantly in the free thiol form (75–100% Fab'-SH) by affinity purification on Streptococcal protein G at pH 5 in the presence of EDTA (Carter et al., 1992b, supra). Thioether-linked BsF(ab')₂ fragments were then constructed by directed coupling using o-PDM as described by Glennie et al., supra. One arm was always the most potent humanized anti-p185^{HER2} variant, HuMAb4D5-8 (Carter et al., 1992a, supra) and the other either a chimeric or humanized variant of the anti-CD3 antibody. Anti-p185^{HER2} Fab'-SH was reacted with o-PDM to form the maleimide derivative (Fab'-mal) and then coupled to the Fab'-SH for each anti-CD3 variant. F(ab')₂ was then purified away from unreacted Fab' by size exclusion chromatography as shown for a representative preparation (BsF(ab')₂ v8) in data not shown. The F(ab')₂ fragment represents ~54% of the total amount of antibody fragments (by mass) as judged by integration of the chromatograph peaks.

SDS-PAGE analysis of this BsF(ab')₂ v8 preparation under non-reducing conditions gave one major band with the expected mobility (M, ~96 kD) as well as several very minor

bands (data not shown). Amino-terminal sequence analysis of the major band after electroblotting on to polyvinylidene difluoride 76 are located in a loop close to V_H CDR1 and CDR2 and therefore might membrane Matsudaira, P., *J. Biol. Chem.* 262: 10035–10038 (1987) gave the expected mixed sequence from a stoichiometric 1:1 mixture of light and heavy chains (V_L/V_H : D/E, I/V, Q/D, M/L, T/V, D/E, S/S) expected for BsF(ab')₂. The amino terminal region of both light chains are identical as are both heavy chains and correspond to consensus human FR sequences. We have previously demonstrated that F(ab')₂ constructed by directed chemical coupling carry both anti-p185^{HER2} and anti-CD3 antigen specificities (Shalaby et al., supra). The level of contamination of the BsF(ab')₂ with monospecific F(ab')₂ is likely to be very low since mock coupling reactions with either anti-p185^{HER2} w Fab'-mal or anti-CD3 Fab'-SH alone did not yield detectable quantities of F(ab')₂. Furthermore the coupling reaction was subjected to a mild reduction step followed by alkylation to remove trace amounts of disulfide-linked F(ab')₂ that might be present. SDS-PAGE of the purified F(ab')₂ under reducing conditions gave two major bands with electrophoretic mobility and amino terminal sequence anticipated for free light chain and thioether-linked heavy chain dimers.

Scanning LASER densitometry of a o-PDM coupled F(ab')₂ preparation suggest that the minor species together represent ~10% of the protein. These minor contaminants were characterized by amino terminal sequence analysis and were tentatively identified on the basis of stoichiometry of light and heavy chain sequences and their electrophoretic mobility (data not shown). These data are consistent with the minor contaminants including imperfect F(ab')₂ in which the disulfide bond between light and heavy chains is missing in one or both arms, trace amounts of Fab' and heavy chain thioether-linked to light chain.

Binding of BsF(ab')₂ to Jurkat Cells

Binding of BsF(ab')₂ containing different anti-CD3 variants to Jurkat cells (human acute T cell leukemia) was investigated by flow cytometry (data not shown). BsF(ab')₂v9 binds much more efficiently to Jurkat cells than does our starting molecule, BsF(ab')₂v1, and almost as efficiently as the chimeric BsF(ab')₂. Installation of additional murine residues into anti-CD3 v9 to create v10 (V_H K75S:N76S) and v12 (V_H K75S:N76S plus V_L E55H) did not further improve binding of corresponding BsF(ab')₂ to Jurkat cells. Nor did recruitment of these murine residues into anti-CD3 v1 improve Jurkat binding: V_H K75S (v6), V_H N76S (v7), V_H K75S:N76S (V8), V_L E55H (v11) (not shown). BsF(ab')₂v9 was chosen for future study since it is amongst the most efficient variants in binding to Jurkat cells and contains fewest murine residues in the humanized anti-CD3 arm. A monospecific anti-p185^{HER2} F(ab')₂ did not show significant binding to Jurkat cells consistent with the interaction being mediated through the anti-CD3 arm.

Discussion

A minimalistic strategy was chosen to humanize the anti-p185^{HER2} (Carter et al., 1992a, supra) and anti-CD3 arms (Shalaby et al., supra) of the BsF(ab')₂ in this study in an attempt to minimize the potential immunogenicity of the resulting humanized antibody in the clinic. Thus we tried to install the minimum number of murine CDR and FR residues into the context of consensus human variable domain sequences as required to recruit antigen-binding affinity and biological properties comparable to the murine parent antibody. Molecular modeling was used firstly to predict the murine FR residues which might be important to antigen

binding and secondly to predict the murine CDR residues that might not be required. A small number of humanized variants were then constructed to test these predictions.

Our humanization strategy was very successful for the anti-p185^{HER2} antibody where one out of eight humanized variants (HuMAb4D5-8, IgG1) was identified that bound the p185^{HER2} antigen ~3-fold more tightly than the parent murine antibody (Carter et al., 1992a, supra). HuMAb4D5-8 contains a total of five murine FR residues and nine murine CDR residues, including V_H CDR2 residues 60–65, were discarded in favor of human counterparts. In contrast, BsF(ab')₂v1 containing the most potent humanized anti-CD3 variant out of four originally constructed (Shalaby et al., supra) binds J6 cells with an affinity (K_d) of 140 nM which is ~70-fold weaker than that of the corresponding chimeric BsF(ab')₂.

Here we have restored T cell binding of the humanized anti-CD3 close to that of the chimeric variant by replacing six human residues in V_H CDR2 with their murine counterparts: T57S:A60N:D61Q:S62K:V63F:G65D (anti-CD3 v9, FIG. 5). It appears more likely that these murine residues enhance antigen binding indirectly by influencing the conformation of residues in the N-terminal part of V_H CDR2 rather than by directly contacting antigen. Firstly, only N-terminal residues in V_H CDR2 (50–58) have been found to contact antigen in one or more of eight crystallographic structures of antibody/antigen complexes (Kabat et al., supra; and Mian, I. S. et al., *J. Mol. Biol* 217: 133–151 (1991), FIG. 5). Secondly, molecular modeling suggests that residues in the C-terminal part of V_H CDR2 are at least partially buried (FIG. 5). BsF(ab')₂v9 binds to SK-BR-3 breast tumor cells with equal efficiency to BsF(ab')₂v1 and chimeric BsF(ab')₂ as anticipated since the anti-p185^{HER2} arm is identical in all of these molecules (Shalaby et al., supra, not shown).

Our novel approach to the construction of BsF(ab')₂ fragments exploits an *E. coli* expression system which secretes humanized Fab' fragments at gram per liter titers and permits their direct recovery as Fab'-SH (Carter et al., 1992b, supra). Traditional directed chemical coupling of Fab'-SH fragments is then used to form BsF(ab')₂ in vitro (Brennan et al., supra; and Glennie et al., supra). This route to Fab'-SH obviates problems which are inherent in their generation from intact antibodies: differences in susceptibility to proteolysis and nonspecific cleavage resulting in heterogeneity, low yield as well as partial reduction that is not completely selective for the hinge disulfide bonds. The strategy of using *E. coli*-derived Fab'-SH containing a single hinge cysteine abolishes some sources of heterogeneity in BsF(ab')₂ preparation such as intra-hinge disulfide formation and contamination with intact parent antibody whilst greatly diminishes others, eg. formation of F(ab')₃ fragments.

BsF(ab')₂ fragments constructed here were thioether-linked as originally described by Glennie et al., supra with future in vivo testing of these molecules in mind. Thioether bonds, unlike disulfide bonds, are not susceptible to cleavage by trace amounts of thiol, which led to the proposal that thioether-linked F(ab')₂ may be more stable than disulfide-linked F(ab')₂ in vivo (Glennie et al., supra). This hypothesis is supported by our preliminary pharmacokinetic experiments in normal mice which suggest that thioether-linked BsF(ab')₂ v1 has a 3-fold longer plasma residence time than BsF(ab')₂ v1 linked by a single disulfide bond. Disulfide and thioether-linked chimeric BsF(ab')₂ were found to be indistinguishable in their efficiency of cell binding and in their retargeting of CTL cytotoxicity, which suggests that o-PDM directed coupling does not compromise binding of the

BsF(ab')₂ to either antigen (not shown). Nevertheless the nature of the linkage appears not to be critical since a disulfide-linked BsF(ab')₂ (murine anti-p185^{HER2}/murine anti-CD3) was recently shown by others (Nishimura et al., *Int. J. Cancer* 50: 800-804 (1992) to have potent anti-tumor activity in nude mice. Our previous study (Shalaby et al., supra) together with this one and that of Nishimura, T. et al., supra improve the potential for using BsF(ab')₂ in targeted immunotherapy of p185^{HER2}-overexpressing cancers in humans.

Humanization of an anti-CD18 Antibody

A murine antibody directed against the leukocyte adhesion receptor β-chain (known as the H52 antibody) was humanized following the methods described above. FIGS. 6A and 6B provide amino acid sequence comparisons for the murine and humanized antibody light chains and heavy chains.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 26

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 109 amino acids
 - (B) TYPE: Amino Acid
 - (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
 1           5           10           15
Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val Asn
                20           25           30
Thr Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                35           40           45
Leu Leu Ile Tyr Ser Ala Ser Phe Leu Glu Ser Gly Val Pro Ser
                50           55           60
Arg Phe Ser Gly Ser Arg Ser Gly Thr Asp Phe Thr Leu Thr Ile
                65           70           75
Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
                80           85           90
His Tyr Thr Thr Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu
                95           1 00           1 05
Ile Lys Arg Thr
                109
    
```

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 120 amino acids
 - (B) TYPE: Amino Acid
 - (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
 1           5           10           15
Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Asn Ile Lys
                20           25           30
Asp Thr Tyr Ile His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                35           40           45
Glu Trp Val Ala Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr
                50           55           60
Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Ala Asp Thr Ser
                65           70           75
    
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Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
 80 85 90
 Thr Ala Val Tyr Tyr Cys Ser Arg Trp Gly Gly Asp Gly Phe Tyr
 95 1 00 1 05
 Ala Met Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
 110 115 120

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 109 amino acids
 - (B) TYPE: Amino Acid
 - (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
 1 5 10 15
 Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val Ser
 20 25 30
 Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
 35 40 45
 Leu Leu Ile Tyr Ala Ala Ser Ser Leu Glu Ser Gly Val Pro Ser
 50 55 60
 Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile
 65 70 75
 Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln
 80 85 90
 Tyr Asn Ser Leu Pro Tyr Thr Phe Gly Gln Gly Thr Lys Val Glu
 95 1 00 1 05
 Ile Lys Arg Thr
 109

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 120 amino acids
 - (B) TYPE: Amino Acid
 - (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
 1 5 10 15
 Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
 20 25 30
 Asp Tyr Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
 35 40 45
 Glu Trp Val Ala Val Ile Ser Glu Asn Gly Ser Asp Thr Tyr Tyr
 50 55 60
 Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser
 65 70 75
 Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
 80 85 90
 Thr Ala Val Tyr Tyr Cys Ala Arg Asp Arg Gly Gly Ala Val Ser
 95 1 00 1 05
 Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
 110 115 120

-continued

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 109 amino acids
 - (B) TYPE: Amino Acid
 - (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```

Asp Ile Val Met Thr Gln Ser His Lys Phe Met Ser Thr Ser Val
 1             5             10             15
Gly Asp Arg Val Ser Ile Thr Cys Lys Ala Ser Gln Asp Val Asn
 20             25             30
Thr Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly His Ser Pro Lys
 35             40             45
Leu Leu Ile Tyr Ser Ala Ser Phe Arg Tyr Thr Gly Val Pro Asp
 50             55             60
Arg Phe Thr Gly Asn Arg Ser Gly Thr Asp Phe Thr Phe Thr Ile
 65             70             75
Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr Tyr Cys Gln Gln
 80             85             90
His Tyr Thr Thr Pro Pro Thr Phe Gly Gly Gly Thr Lys Leu Glu
 95             1 00             1 05
Ile Lys Arg Ala
 109
    
```

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 120 amino acids
 - (B) TYPE: Amino Acid
 - (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly
 1             5             10             15
Ala Ser Leu Lys Leu Ser Cys Thr Ala Ser Gly Phe Asn Ile Lys
 20             25             30
Asp Thr Tyr Ile His Trp Val Lys Gln Arg Pro Glu Gln Gly Leu
 35             40             45
Glu Trp Ile Gly Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr
 50             55             60
Asp Pro Lys Phe Gln Asp Lys Ala Thr Ile Thr Ala Asp Thr Ser
 65             70             75
Ser Asn Thr Ala Tyr Leu Gln Val Ser Arg Leu Thr Ser Glu Asp
 80             85             90
Thr Ala Val Tyr Tyr Cys Ser Arg Trp Gly Gly Asp Gly Phe Tyr
 95             1 00             1 05
Ala Met Asp Tyr Trp Gly Gln Gly Ala Ser Val Thr Val Ser Ser
 110            115            120
    
```

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 27 base pairs
 - (B) TYPE: Nucleic Acid
 - (C) STRANDEDNESS: Single
 - (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TCCGATATCC AGCTGACCCA GTCTCCA

-continued

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 31 base pairs
 (B) TYPE: Nucleic Acid
 (C) STRANDEDNESS: Single
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTTTGATCTC CAGCTTGGTA CCHSCDCCGA A 31

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 22 base pairs
 (B) TYPE: Nucleic Acid
 (C) STRANDEDNESS: Single
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AGGTSMARCT GCAGSAGTCW GG 22

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 34 base pairs
 (B) TYPE: Nucleic Acid
 (C) STRANDEDNESS: Single
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TGAGGAGACG GTGACCGTGG TCCCTTGGCC CCAAG 34

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 36 base pairs
 (B) TYPE: Nucleic Acid
 (C) STRANDEDNESS: Single
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GTAGATAAAT CCTCTAACAC AGCCTATCTG CAAATG 36

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 36 base pairs
 (B) TYPE: Nucleic Acid
 (C) STRANDEDNESS: Single
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GTAGATAAAT CCAAATCTAC AGCCTATCTG CAAATG 36

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 36 base pairs
 (B) TYPE: Nucleic Acid
 (C) STRANDEDNESS: Single
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTAGATAAAT CCTCTTCTAC AGCCTATCTG CAAATG 36

-continued

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 68 base pairs
 - (B) TYPE: Nucleic Acid
 - (C) STRANDEDNESS: Single
 - (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CTTATAAAGG TGTTCACC TATAACCAGA AATCAAGGA TCGTTTCACG 50
 ATATCCGTAG ATAAATCC 68

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: Nucleic Acid
 - (C) STRANDEDNESS: Single
 - (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CTATACCTCC CGTCTGCATT CTGGAGTCCC 30

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 107 amino acids
 - (B) TYPE: Amino Acid
 - (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Asp	Ile	Gln	Met	Thr	Gln	Thr	Thr	Ser	Ser	Leu	Ser	Ala	Ser	Leu
1				5						10				15
Gly	Asp	Arg	Val	Thr	Ile	Ser	Cys	Arg	Ala	Ser	Gln	Asp	Ile	Arg
				20					25					30
Asn	Tyr	Leu	Asn	Trp	Tyr	Gln	Gln	Lys	Pro	Asp	Gly	Thr	Val	Lys
				35					40					45
Leu	Leu	Ile	Tyr	Tyr	Thr	Ser	Arg	Leu	His	Ser	Gly	Val	Pro	Ser
				50					55					60
Lys	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Asp	Tyr	Ser	Leu	Thr	Ile
				65					70					75
Ser	Asn	Leu	Glu	Gln	Glu	Asp	Ile	Ala	Thr	Tyr	Phe	Cys	Gln	Gln
				80					85					90
Gly	Asn	Thr	Leu	Pro	Trp	Thr	Phe	Ala	Gly	Gly	Thr	Lys	Leu	Glu
				95					1 00					1 05
Ile	Lys													
	107													

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 107 amino acids
 - (B) TYPE: Amino Acid
 - (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Asp	Ile	Gln	Met	Thr	Gln	Ser	Pro	Ser	Ser	Leu	Ser	Ala	Ser	Val
1				5						10				15
Gly	Asp	Arg	Val	Thr	Ile	Thr	Cys	Arg	Ala	Ser	Gln	Asp	Ile	Arg
				20					25					30
Asn	Tyr	Leu	Asn	Trp	Tyr	Gln	Gln	Lys	Pro	Gly	Lys	Ala	Pro	Lys

-continued

	35		40		45									
Leu	Leu	Ile	Tyr	Tyr	Thr	Ser	Arg	Leu	Glu	Ser	Gly	Val	Pro	Ser
			50						55					60
Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Asp	Tyr	Thr	Leu	Thr	Ile
			65						70					75
Ser	Ser	Leu	Gln	Pro	Glu	Asp	Phe	Ala	Thr	Tyr	Tyr	Cys	Gln	Gln
			80						85					90
Gly	Asn	Thr	Leu	Pro	Trp	Thr	Phe	Gly	Gln	Gly	Thr	Lys	Val	Glu
			95						1	00				1 05
Ile	Lys													
	107													

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 107 amino acids
 - (B) TYPE: Amino Acid
 - (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Asp	Ile	Gln	Met	Thr	Gln	Ser	Pro	Ser	Ser	Leu	Ser	Ala	Ser	Val
1				5					10					15
Gly	Asp	Arg	Val	Thr	Ile	Thr	Cys	Arg	Ala	Ser	Gln	Ser	Ile	Ser
			20						25					30
Asn	Tyr	Leu	Ala	Trp	Tyr	Gln	Gln	Lys	Pro	Gly	Lys	Ala	Pro	Lys
			35						40					45
Leu	Leu	Ile	Tyr	Ala	Ala	Ser	Ser	Leu	Glu	Ser	Gly	Val	Pro	Ser
			50						55					60
Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Asp	Phe	Thr	Leu	Thr	Ile
			65						70					75
Ser	Ser	Leu	Gln	Pro	Glu	Asp	Phe	Ala	Thr	Tyr	Tyr	Cys	Gln	Gln
			80						85					90
Tyr	Asn	Ser	Leu	Pro	Trp	Thr	Phe	Gly	Gln	Gly	Thr	Lys	Val	Glu
			95						1	00				1 05
Ile	Lys													
	107													

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 122 amino acids
 - (B) TYPE: Amino Acid
 - (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Glu	Val	Gln	Leu	Gln	Gln	Ser	Gly	Pro	Glu	Leu	Val	Lys	Pro	Gly
1				5					10					15
Ala	Ser	Met	Lys	Ile	Ser	Cys	Lys	Ala	Ser	Gly	Tyr	Ser	Phe	Thr
			20						25					30
Gly	Tyr	Thr	Met	Asn	Trp	Val	Lys	Gln	Ser	His	Gly	Lys	Asn	Leu
			35						40					45
Glu	Trp	Met	Gly	Leu	Ile	Asn	Pro	Tyr	Lys	Gly	Val	Ser	Thr	Tyr
			50						55					60
Asn	Gln	Lys	Phe	Lys	Asp	Lys	Ala	Thr	Leu	Thr	Val	Asp	Lys	Ser
			65						70					75
Ser	Ser	Thr	Ala	Tyr	Met	Glu	Leu	Leu	Ser	Leu	Thr	Ser	Glu	Asp
			80						85					90

-continued

Ser Ala Val Tyr Tyr Cys Ala Arg Ser Gly Tyr Tyr Gly Asp Ser
 95 1 00 1 05
 Asp Trp Tyr Phe Asp Val Trp Gly Ala Gly Thr Thr Val Thr Val
 110 115 120
 Ser Ser
 122

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 122 amino acids
 - (B) TYPE: Amino Acid
 - (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
 1 5 10 15
 Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Ser Phe Thr
 20 25 30
 Gly Tyr Thr Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
 35 40 45
 Glu Trp Val Ala Leu Ile Asn Pro Tyr Lys Gly Val Ser Thr Tyr
 50 55 60
 Asn Gln Lys Phe Lys Asp Arg Phe Thr Ile Ser Val Asp Lys Ser
 65 70 75
 Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
 80 85 90
 Thr Ala Val Tyr Tyr Cys Ala Arg Ser Gly Tyr Tyr Gly Asp Ser
 95 1 00 1 05
 Asp Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val
 110 115 120
 Ser Ser
 122

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 122 amino acids
 - (B) TYPE: Amino Acid
 - (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
 1 5 10 15
 Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
 20 25 30
 Ser Tyr Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
 35 40 45
 Glu Trp Val Ser Val Ile Ser Gly Asp Gly Gly Ser Thr Tyr Tyr
 50 55 60
 Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser
 65 70 75
 Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
 80 85 90
 Thr Ala Val Tyr Tyr Cys Ala Arg Gly Arg Val Gly Tyr Ser Leu
 95 1 00 1 05
 Ser Gly Leu Tyr Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val
 110 115 120

-continued

Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys
 335 340 345

Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro
 350 355 360

Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu
 365 370 375

Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser
 380 385 390

Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu
 395 400 405

Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp
 410 415 420

Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met
 425 430 435

His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
 440 445 450

Ser Pro Gly Lys
 454

(2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 469 amino acids
 - (B) TYPE: Amino Acid
 - (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr
 1 5 10 15

Gly Val His Ser Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu
 20 25 30

Val Gln Pro Gly Gly Ser Leu Arg Leu Ser Cys Ala Thr Ser Gly
 35 40 45

Tyr Thr Phe Thr Glu Tyr Thr Met His Trp Met Arg Gln Ala Pro
 50 55 60

Gly Lys Gly Leu Glu Trp Val Ala Gly Ile Asn Pro Lys Asn Gly
 65 70 75

Gly Thr Ser His Asn Gln Arg Phe Met Asp Arg Phe Thr Ile Ser
 80 85 90

Val Asp Lys Ser Thr Ser Thr Ala Tyr Met Gln Met Asn Ser Leu
 95 1 00 1 05

Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg Trp Arg Gly
 110 115 120

Leu Asn Tyr Gly Phe Asp Val Arg Tyr Phe Asp Val Trp Gly Gln
 125 130 135

Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser
 140 145 150

Val Phe Pro Leu Ala Pro Cys Ser Arg Ser Thr Ser Glu Ser Thr
 155 160 165

Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val
 170 175 180

Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr
 185 190 195

Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser
 200 205 210

-continued

Val Val Thr Val Thr Ser Ser Asn Phe Gly Thr Gln Thr Tyr Thr
 215 220 225

Cys Asn Val Asp His Lys Pro Ser Asn Thr Lys Val Asp Lys Thr
 230 235 240

Val Glu Arg Lys Cys Cys Val Glu Cys Pro Pro Cys Pro Ala Pro
 245 250 255

Pro Val Ala Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys
 260 265 270

Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val
 275 280 285

Val Asp Val Ser His Glu Asp Pro Glu Val Gln Phe Asn Trp Tyr
 290 295 300

Val Asp Gly Met Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
 305 310 315

Glu Gln Phe Asn Ser Thr Phe Arg Val Val Ser Val Leu Thr Val
 320 325 330

Val His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val
 335 340 345

Ser Asn Lys Gly Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys
 350 355 360

Thr Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro
 365 370 375

Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu
 380 385 390

Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser
 395 400 405

Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Met Leu
 410 415 420

Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp
 425 430 435

Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met
 440 445 450

His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
 455 460 465

Ser Pro Gly Lys
 469

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 214 amino acids
 - (B) TYPE: Amino Acid
 - (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Asp Val Gln Met Thr Gln Thr Thr Ser Ser Leu Ser Ala Ser Leu
 1 5 10 15

Gly Asp Arg Val Thr Ile Asn Cys Arg Ala Ser Gln Asp Ile Asn
 20 25 30

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Asn Gly Thr Val Lys
 35 40 45

Leu Leu Ile Tyr Tyr Thr Ser Thr Leu His Ser Gly Val Pro Ser
 50 55 60

Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr Ser Leu Thr Ile
 65 70 75

-continued

Ser Asn Leu Asp Gln Glu Asp Ile Ala Thr Tyr Phe Cys Gln Gln
 80 85 90

Gly Asn Thr Leu Pro Pro Thr Phe Gly Gly Gly Thr Lys Val Glu
 95 1 00 1 05

Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro Pro
 110 115 120

Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu
 125 130 135

Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val
 140 145 150

Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu
 155 160 165

Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr
 170 175 180

Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu
 185 190 195

Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn
 200 205 210

Arg Gly Glu Cys
 214

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 233 amino acids
 - (B) TYPE: Amino Acid
 - (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Met Gly Trp Ser Cys Ile Ile Leu Phe Leu Val Ala Thr Ala Thr
 1 5 10 15

Gly Val His Ser Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu
 20 25 30

Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser
 35 40 45

Gln Asp Ile Asn Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly
 50 55 60

Lys Ala Pro Lys Leu Leu Ile Tyr Tyr Thr Ser Thr Leu His Ser
 65 70 75

Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr
 80 85 90

Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr
 95 1 00 1 05

Tyr Cys Gln Gln Gly Asn Thr Leu Pro Pro Thr Phe Gly Gln Gly
 110 115 120

Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe
 125 130 135

Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser
 140 145 150

Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val
 155 160 165

Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu
 170 175 180

Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser
 185 190 195

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Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val
                200                205                210
Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr
                215                220                225
Lys Ser Phe Asn Arg Gly Glu Cys
                230                233

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(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 122 amino acids
 (B) TYPE: Amino Acid
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

```

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
 1                5                10                15
Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Ser Phe Thr
                20                25                30
Gly Tyr Thr Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                35                40                45
Glu Trp Val Ala Leu Ile Asn Pro Tyr Lys Gly Val Thr Thr Tyr
                50                55                60
Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser
                65                70                75
Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                80                85                90
Thr Ala Val Tyr Tyr Cys Ala Arg Ser Gly Tyr Tyr Gly Asp Ser
                95                1 00                1 05
Asp Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val
                110                115                120
Ser Ser
                122

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We claim:

1. A humanized antibody variable domain comprising non-human Complementarity Determining Region (CDR) amino acid residues which bind an antigen incorporated into a human antibody variable domain, and further comprising a Framework Region (FR) amino acid substitution at a site selected from the group consisting of: 4L, 38L, 43L, 44L, 58L, 62L, 65L, 66L, 67L, 68L, 69L, 73L, 85L, 98L, 2H, 4H, 36H, 39H, 43H, 45H, 69H, 70H, 74H, and 92H, utilizing the numbering system set forth in Kabat.

2. The humanized variable domain of claim 1 wherein the substituted residue is the residue found at the corresponding location of the non-human antibody from which the non-human CDR amino acid residues are obtained.

3. The humanized variable domain of claim 1 wherein no human Framework Region (FR) residue other than those set forth in the group has been substituted.

4. The humanized variable domain of claim 1 wherein the human antibody variable domain is a consensus human variable domain.

5. The humanized variable domain of claim 1 wherein the residue at site 4L has been substituted.

6. The humanized variable domain of claim 1 wherein the residue at site 38L has been substituted.

7. The humanized variable domain of claim 1 wherein the residue at site 43L has been substituted.

8. The humanized variable domain of claim 1 wherein the residue at site 44L has been substituted.

9. The humanized variable domain of claim 1 wherein the residue at site 58L has been substituted.

10. The humanized variable domain of claim 1 wherein the residue at site 62L has been substituted.

11. The humanized variable domain of claim 1 wherein the residue at site 65L has been substituted.

12. The humanized variable domain of claim 1 wherein the residue at site 66L has been substituted.

13. The humanized variable domain of claim 1 wherein the residue at site 67L has been substituted.

14. The humanized variable domain of claim 1 wherein the residue at site 68L has been substituted.

15. The humanized variable domain of claim 1 wherein the residue at site 69L has been substituted.

16. The humanized variable domain of claim 1 wherein the residue at site 73L has been substituted.

17. The humanized variable domain of claim 1 wherein the residue at site 85L has been substituted.

18. The humanized variable domain of claim 1 wherein the residue at site 98L has been substituted.

19. The humanized variable domain of claim 1 wherein the residue at site 2H has been substituted.

20. The humanized variable domain of claim 1 wherein the residue at site 4H has been substituted.

21. The humanized variable domain of claim 1 wherein the residue at site 36H has been substituted.

22. The humanized variable domain of claim 1 wherein the residue at site 39H has been substituted.

23. The humanized variable domain of claim 1 wherein the residue at site 43H has been substituted.

24. The humanized variable domain of claim 1 wherein the residue at site 45H has been substituted.

25. The humanized variable domain of claim 1 wherein the residue at site 69H has been substituted.

26. The humanized variable domain of claim 1 wherein the residue at site 70H has been substituted.

27. The humanized variable domain of claim 1 wherein the residue at site 74H has been substituted.

28. The humanized variable domain of claim 1 wherein the residue at site 92H has been substituted.

29. An antibody comprising the humanized variable domain of claim 1.

30. An antibody which binds p185^{HER2} and comprises a humanized antibody variable domain, wherein the humanized antibody variable domain comprises non-human Complementarity Determining Region (CDR) amino acid residues which bind p185^{HER2} incorporated into a human antibody variable domain, and further comprises a Framework Region (FR) amino acid substitution at a site selected from the group consisting of: 4L, 38L, 43L, 44L, 46L, 58L, 62L, 65L, 66L, 67L, 68L, 69L, 73L, 85L, 98L, 2H, 4H, 36H, 39H, 43H, 45H, 69H, 70H, 74H, 75H, 76H, 78H and 92H, utilizing the numbering system set forth in Kabat.

31. The antibody of claim 30 wherein the substituted residue is the residue found at the corresponding location of the non-human antibody from which the non-human CDR amino acid residues are obtained.

32. The antibody of claim 30 wherein no human Framework Region (FR) residue other than those set forth in the group has been substituted.

33. The antibody of claim 30 wherein the human antibody variable domain is a consensus human variable domain.

34. The antibody of claim 30 wherein the residue at site 4L has been substituted.

35. The antibody of claim 30 wherein the residue at site 38L has been substituted.

36. The antibody of claim 30 wherein the residue at site 43L has been substituted.

37. The antibody of claim 30 wherein the residue at site 44L has been substituted.

38. The antibody of claim 30 wherein the residue at site 46L has been substituted.

39. The antibody of claim 30 wherein the residue at site 58L has been substituted.

40. The antibody of claim 30 wherein the residue at site 62L has been substituted.

41. The antibody of claim 30 wherein the residue at site 65L has been substituted.

42. The antibody of claim 30 wherein the residue at site 66L has been substituted.

43. The antibody of claim 30 wherein the residue at site 67L has been substituted.

44. The antibody of claim 30 wherein the residue at site 68L has been substituted.

45. The antibody of claim 30 wherein the residue at site 69L has been substituted.

46. The antibody of claim 30 wherein the residue at site 73L has been substituted.

47. The antibody of claim 30 wherein the residue at site 85L has been substituted.

48. The antibody of claim 30 wherein the residue at site 98L has been substituted.

49. The antibody of claim 30 wherein the residue at site 2H has been substituted.

50. The antibody of claim 30 wherein the residue at site 4H has been substituted.

51. The antibody of claim 30 wherein the residue at site 36H has been substituted.

52. The antibody of claim 30 wherein the residue at site 39H has been substituted.

53. The antibody of claim 30 wherein the residue at site 43H has been substituted.

54. The antibody of claim 30 wherein the residue at site 45H has been substituted.

55. The antibody of claim 30 wherein the residue at site 69H has been substituted.

56. The antibody of claim 30 wherein the residue at site 70H has been substituted.

57. The antibody of claim 30 wherein the residue at site 74H has been substituted.

58. The antibody of claim 30 wherein the residue at site 75H has been substituted.

59. The antibody of claim 30 wherein the residue at site 76H has been substituted.

60. The antibody of claim 30 wherein the residue at site 78H has been substituted.

61. The antibody of claim 30 wherein the residue at site 92H has been substituted.

62. A humanized antibody variable domain comprising non-human Complementarity Determining Region (CDR) amino acid residues which bind an antigen incorporated into a consensus human variable domain, and further comprising an amino acid substitution at a site selected from the group consisting of: 4L, 38L, 43L, 44L, 46L, 58L, 62L, 65L, 66L, 67L, 68L, 69L, 73L, 85L, 98L, 2H, 4H, 36H, 39H, 43H, 45H, 69H, 70H, 74H, 75H, 76H, 78H and 92H, utilizing the numbering system set forth in Kabat.

63. A humanized antibody which lacks immunogenicity compared to a non-human parent antibody upon repeated administration to a human patient in order to treat a chronic disease in that patient, wherein the humanized antibody comprises non-human Complementarity Determining Region (CDR) amino acid residues which bind an antigen incorporated into a human antibody variable domain, and further comprises an amino acid substitution at a site selected from the group consisting of: 4L, 38L, 43L, 44L, 46L, 58L, 62L, 65L, 66L, 67L, 68L, 69L, 73L, 85L, 98L, 2H, 4H, 36H, 39H, 43H, 45H, 69H, 70H, 74H, 75H, 76H, 78H and 92H, utilizing the numbering system set forth in Kabat.

64. A humanized variant of a non-human parent antibody which binds an antigen and comprises a human variable domain comprising the most frequently occurring amino acid residues at each location in all human immunoglobulins of a human heavy chain immunoglobulin subgroup wherein amino acid residues forming Complementarity Determining Regions (CDRs) thereof comprise non-human antibody amino acid residues, and further comprises a Framework Region (FR) substitution where the substituted FR residue: (a) noncovalently binds antigen directly; (b) interacts with a CDR; (c) introduces a glycosylation site which affects the antigen binding or affinity of the antibody; or (d) participates in the V_L-V_H interface by affecting the proximity or orientation of the V_L and V_H regions with respect to one another.

65. The humanized variant of claim 63 which binds the antigen up to 3-fold more in the binding affinity than the parent antibody binds antigen.

66. A humanized antibody heavy chain variable domain comprising non-human Complementarity Determining

Region (CDR) amino acid residues which bind antigen incorporated into a human antibody variable domain, and further comprising a Framework Region (FR) amino acid substitution at a site selected from the group consisting of: 24H, 73H, 76H, 78H, and 93H, utilizing the numbering system set forth in Kabat.

67. The humanized variable domain of claim 66 wherein the substituted residue is the residue found at the corresponding location of the non-human antibody from which the non-human CDR amino acid residues are obtained.

68. The humanized variable domain of claim 66 wherein no human Framework Region (FR) residue other than those set forth in the group has been substituted.

69. The humanized variable domain of claim 66 wherein the human antibody variable domain is a consensus human variable domain.

70. The humanized variable domain of claim 66 wherein the residue at site 24H has been substituted.

71. The humanized variable domain of claim 66 wherein the residue at site 73H has been substituted.

72. The humanized variable domain of claim 66 wherein the residue at site 76H has been substituted.

73. The humanized variable domain of claim 66 wherein the residue at site 78H has been substituted.

74. The humanized variable domain of claim 66 wherein the residue at site 93H has been substituted.

75. The humanized variable domain of claim 66 which further comprises an amino acid substitution at site 71H.

76. The humanized variable domain of claim 66 which further comprises amino acid substitutions at sites 71H and 73H.

77. The humanized variable domain of claim 66 which further comprises amino acid substitutions at sites 71H, 73H and 78H.

78. An antibody comprising the humanized variable domain of claim 66.

79. A humanized variant of a non-human parent antibody which binds an antigen, wherein the humanized variant comprises Complementarity Determining Region (CDR) amino acid residues of the non-human parent antibody incorporated into a human antibody variable domain, and further comprises Framework Region (FR) substitutions at heavy chain positions 71H, 73H, 78H and 93H, utilizing the numbering system set forth in Kabat.

80. A humanized antibody variable domain comprising non-human Complementarity Determining Region (CDR) amino acid residues which bind an antigen incorporated into a human antibody variable domain, and further comprising a Framework Region (FR) amino acid substitution where the substituted FR residue:

- (a) noncovalently binds antigen directly;
- (b) interacts with a CDR; or
- (c) participates in the V_L-V_H interface by affecting the proximity or orientation of the V_L and V_H regions with respect to one another, and wherein the substituted FR residue is at a site selected from the group consisting of: 4L, 38L, 43L, 44L, 58L, 62L, 65L, 66L, 67L, 68L, 69L, 73L, 85L, 98L, 2H, 4H, 24H, 36H, 39H, 43H, 45H, 69H, 70H, 73H, 74H, 76H, 78H, 92H and 93H, utilizing the numbering system set forth in Kabat.

81. The humanized variable domain of claim 80 wherein the substituted residue is the residue found at the corresponding location of the non-human antibody from which the non-human CDR amino acid residues are obtained.

82. The humanized variable domain of claim 80 wherein no human Framework Region (FR) residue other than those set forth in the group has been substituted.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 6,407,213 B1
DATED : June 18, 2002
INVENTOR(S) : Carter et al.

Page 1 of 1

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 88,

Line 63, please delete "63" and insert therefor -- 79 --.

Signed and Sealed this

Third Day of December, 2002

A handwritten signature in black ink, appearing to read "James E. Rogan", written over a horizontal line.

JAMES E. ROGAN
Director of the United States Patent and Trademark Office

14042 U.S. PTO
112105

PTO/SB/05 (05-05)
Approved for use through 07/31/2006. OMB 0651-0032
U.S. Patent and Trademark Office, U.S. DEPARTMENT OF COMMERCE

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UTILITY PATENT APPLICATION TRANSMITTAL <small>(Only for new nonprovisional applications under 37 C.F.R. 1.53(b))</small>	Attorney Docket No.	CARP0001-112
	First Inventor	John R. Adair et al.
	Title	HUMANISED ANTIBODIES
	Express Mail Label No.	EV146 601 565US

11/28/2009 U.S. PTO
 11/28/2009
 12105

<p style="text-align: center;">APPLICATION ELEMENTS</p> <p><small>See MPEP chapter 600 concerning utility patent application contents.</small></p> <p>1. <input checked="" type="checkbox"/> Fee Transmittal Form (e.g., PTO/SB/17) <i>(Submit an original and a duplicate for fee processing)</i></p> <p>2. <input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27.</p> <p>3. <input checked="" type="checkbox"/> Specification [Total Pages 70] Both the claims and abstract must start on a new page <i>(For information on the preferred arrangement, see MPEP 608.01(a))</i></p> <p>4. <input checked="" type="checkbox"/> Drawing(s) (35 U.S.C.113) [Total Sheets 18]</p> <p>5. Oath or Declaration [Total Sheets 03]</p> <p>a. <input type="checkbox"/> Newly executed (original or copy)</p> <p>b. <input checked="" type="checkbox"/> Copy from a prior application (37 CFR 1.63 (d)) <i>(for a continuation/divisional with Box 18 completed)</i></p> <p>i. <input type="checkbox"/> DELETION OF INVENTOR(S) Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).</p> <p>6. <input checked="" type="checkbox"/> Application Data Sheet. See 37 CFR 1.76</p> <p>7. <input type="checkbox"/> CD-ROM or CD-R in duplicate, large table or Computer Program <i>(Appendix)</i> <input type="checkbox"/> Landscape Table on CD</p> <p>8. Nucleotide and/or Amino Acid Sequence Submission <i>(if applicable, items a.-c. are required)</i></p> <p>a. Computer Readable Form (CRF)</p> <p>i. <input type="checkbox"/> Computer Readable Form (CRF)</p> <p>ii. <input checked="" type="checkbox"/> Transfer Request (37 CFR 1.821(e))</p> <p>b. Specification Sequence Listing on:</p> <p>i. <input type="checkbox"/> CD-ROM or CD-R (2 copies); or</p> <p>ii. <input checked="" type="checkbox"/> Paper Copy</p> <p>c. <input checked="" type="checkbox"/> Statements verifying identity of above copies</p>	<p>ADDRESS TO: Commissioner for Patents P.O. Box 1450 Alexandria VA 22313-1450</p> <p style="text-align: center;">ACCOMPANYING APPLICATIONS PARTS</p> <p>9. <input type="checkbox"/> Assignment Papers (cover sheet & document(s)) Name of Assignee _____</p> <p>10. <input type="checkbox"/> 37 C.F.R. 3.73(b) Statement <input checked="" type="checkbox"/> Copy of Power of Attorney <i>(when there is an assignee)</i></p> <p>11. <input type="checkbox"/> English Translation Document (if applicable)</p> <p>12. <input type="checkbox"/> Information Disclosure Statement (PTO/SB/08 or PTO-1449) <input type="checkbox"/> Copies of foreign patent documents, publications & other information</p> <p>13. <input checked="" type="checkbox"/> Preliminary Amendment and Request for Interference under 37 C.F.R. § 42.202</p> <p>14. <input checked="" type="checkbox"/> Return Receipt Postcard (MPEP 503) <i>(Should be specifically itemized)</i></p> <p>15. <input checked="" type="checkbox"/> Certified Copy of Priority Document Was Received in Parent Application Serial No. 077743,329, Filed September 17, 1991 (if foreign priority is claimed)</p> <p>16. <input type="checkbox"/> Nonpublication Request under 35 U.S.C. 122(b)(2)(B)(i). Applicant must attach form PTO/SB/35 or equivalent.</p> <p>17. <input checked="" type="checkbox"/> Other: <u>Copy of Change of Correspondence Address - Application dated December 23, 2002, from Application Serial No. 08/846,658, Filed May 1, 1997.</u></p>
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18. If a CONTINUING APPLICATION, check appropriate box, and supply the requisite information below and in the first sentence of the specification following the title, or in an Application Data Sheet under 37 CFR 1.76:

Continuation Divisional Continuation-in-part (CIP)

of prior Application No. Serial No. 08/846,658, Filed May 1, 1997, which is a Continuation of Application Serial No. 08/303,568, Filed September 7, 1994, now U.S. Patent No. 5,859,205, Issued January 12, 1999, which is a File Wrapper Continuation of Application Serial No. 07/743,329, Filed September 17, 1991, Abandoned, which is a 35 U.S.C. 371 National Stage Application of International Application No. PCT/GB90/02017, International Filing Date of December 21, 1989, which claims priority to Great Britain Application No. 8928874.0, Filed December 21, 1989, all applications incorporated by reference herein in their entireties.

Prior application information: Examiner Minh Tam B. Davis Art Unit: 1642

19. CORRESPONDENCE ADDRESS

The address associated with Customer Number **34132** OR Correspondence address below

Name		Address	
City	State	Zip Code	
Country	Telephone	Email address	

Signature	<i>Doreen Yatro Trujillo</i>	Date	November 21, 2005
Name (Print/Type)	Doreen Yatro Trujillo	Registration No. (Attorney/Agent)	35,719

This collection of information is required by 37 CFR 1.53(b). The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Mail Stop Patent Application, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.
If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

EV146601565US



14042 U.S. PTO

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Effective on 12/08/2004.

Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).

**FEE TRANSMITTAL
for FY 2005** Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT (\$) 1,000.00

Complete if Known

Application Number	Not Yet Assigned
Filing Date	November 21, 2005
First Named Inventor	John R. Adair et al.
Examiner Name	Not Yet Assigned
Art Unit	Not Yet Assigned
Attorney Docket No.	CARP0001-112

METHOD OF PAYMENT (check all that apply)

 Check Credit Card Money Order None Other (please identify) : _____ Deposit Account Deposit Account Number: 50-1275 Deposit Account Name: Cozen O'Connor, P.C.

For the above-identified deposit account, the Director is hereby authorized to: (check all that apply)

 Charge fee(s) indicated below Charge fee(s) indicated below, except for the filing fee Charge any additional fee(s) or underpayments of fee(s) Credit any overpayments

Under 37 CFR 1.16 and 1.17

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES

Application Type	FILING FEES		SEARCH FEES		EXAMINATION FEES		Fees Paid (\$)
	Fee (\$)	Small Entity Fee(\$)	Fee(\$)	Small Entity Fee(\$)	Fee(\$)	Small Entity Fee(\$)	
Utility	300	150	500	250	200	100	<u>\$1,000.00</u>
Design	200	100	100	50	130	65	_____
Plant	200	100	300	150	160	80	_____
Reissue	300	150	500	250	600	300	_____
Provisional	200	100	0	0	0	0	_____

2. EXCESS CLAIM FEES

Fee Description

	Fee (\$)	Small Entity Fee (\$)
Each claim over 20 (including Reissues)	50	25
Each independent claim over 3 (including Reissues)	200	100
Multiple dependent claims	360	180

Total Claims	Extra Claims	Fee(\$)	Fee Paid (\$)	Multiple Dependent Claims	Fee (\$)	Fee Paid (\$)
<u>02</u>	<u>- 20</u> or HP=	<u>00</u>	x _____ = _____			

HP = highest number of total claims paid for, if greater than 20.

Indep. Claims	Extra Claims	Fee(\$)	Fee Paid (\$)
<u>01</u>	<u>- 03</u> or HP=	<u>00</u>	x _____ = _____

HP = highest number of independent claims paid for, if greater than 3.

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is \$250 (\$125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets	Extra Sheets	Number of each additional 50 or fraction thereof	Fee (\$)	Fee Paid (\$)
_____	- _____ = _____	/50 = _____ (round up to a whole number) x _____ = _____		

4. OTHER FEE(S)

Non-English Specification, \$130 fee (no small entity discount)

Other (e.g., late filing surcharge): _____

Fees Paid (\$)

SUBMITTED BY

Signature		Registration No. (Attorney/Agent)	35,719	Telephone	(215) 665-5593
Name (Print/Type)	Doreen Yatko Trujillo	Date	November 21, 2005		

This collection of information is required by 37 CFR 1.136. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 30 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing this form, call 1-800-PTO-9199 (1-800-786-9199) and select option 2.



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Effective on 12/08/2004.
Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).

FEE TRANSMITTAL for FY 2005

Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT (\$) 1,000.00

Complete if Known

Application Number	Not Yet Assigned
Filing Date	November 21, 2005
First Named Inventor	John R. Adair et al.
Examiner Name	Not Yet Assigned
Art Unit	Not Yet Assigned
Attorney Docket No.	CARP0001-112

METHOD OF PAYMENT (check all that apply)

Check Credit Card Money Order None Other (please identify) : _____

Deposit Account Deposit Account Number: 50-1275 Deposit Account Name: Cozen O'Connor, P.C.

For the above-identified deposit account, the Director is hereby authorized to: (check all that apply)

Charge fee(s) indicated below Charge fee(s) indicated below, except for the filing fee

Charge any additional fee(s) or underpayments of fee(s) Credit any overpayments

Under 37 CFR 1.16 and 1.17

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FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES

Application Type	FILING FEES		SEARCH FEES		EXAMINATION FEES		Fees Paid (\$)
	Fee (\$)	Small Entity Fee(\$)	Fee(\$)	Small Entity Fee(\$)	Fee(\$)	Small Entity Fee(\$)	
Utility	300	150	500	250	200	100	\$1,000.00
Design	200	100	100	50	130	65	_____
Plant	200	100	300	150	160	80	_____
Reissue	300	150	500	250	600	300	_____
Provisional	200	100	0	0	0	0	_____

2. EXCESS CLAIM FEES

Fee Description	Small Entity Fee (\$)	Fee (\$)
Each claim over 20 (including Reissues)	50	25
Each independent claim over 3 (including Reissues)	200	100
Multiple dependent claims	360	180
Total Claims	Extra Claims	Fee(\$)
<u>02</u> - <u>20</u> or HP= <u>00</u>	x _____	= _____
HP = highest number of total claims paid for, if greater than 20.		
Indep. Claims	Extra Claims	Fee(\$)
<u>01</u> - <u>03</u> or HP= <u>00</u>	x _____	= _____
HP = highest number of independent claims paid for, if greater than 3.		

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is \$250 (\$125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets	Extra Sheets	Number of each additional 50 or fraction thereof	Fee (\$)	Fee Paid (\$)
_____	- _____ = _____	/50 = _____ (round up to a whole number) x _____ = _____	_____	_____

4. OTHER FEE(S)

Non-English Specification, \$130 fee (no small entity discount) _____
Other (e.g., late filing surcharge): _____

SUBMITTED BY

Signature		Registration No. (Attorney/Agent)	35,719	Telephone	(215) 665-5593
Name (Print/Type)	Doreen Yatko Trujillo	Date	November 21, 2005		

This collection of information is required by 37 CFR 1.136. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 30 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing this form, call 1-800-PTO-9199 (1-800-786-9199) and select option 2.

HUMANISED ANTIBODIES

Field of the Invention

The present invention relates to humanised antibody molecules, to processes for their production using recombinant DNA technology, and to their therapeutic uses.

The term "humanised antibody molecule" is used to describe a molecule having an antigen binding site derived from an immunoglobulin from a non-human species, and remaining immunoglobulin-derived parts of the molecule being derived from a human immunoglobulin. The antigen binding site typically comprises complementarity determining regions (CDRs) which determine the binding specificity of the antibody molecule and which are carried on appropriate framework regions in the variable domains. There are 3 CDRs (CDR1, CDR2 and CDR3) in each of the heavy and light chain variable domains.

In the description, reference is made to a number of publications by number. The publications are listed in numerical order at the end of the description.

Background of the Invention

Natural immunoglobulins have been known for many years, as have the various fragments thereof, such as the Fab, (Fab')₂ and Fc fragments, which can be derived by enzymatic cleavage. Natural immunoglobulins comprise a generally Y-shaped molecule having an antigen-binding site towards the end of each upper arm. The remainder of the structure, and particularly the stem of the Y, mediates the effector functions associated with immunoglobulins.

Natural immunoglobulins have been used in assay, diagnosis and, to a more limited extent, therapy. However, such uses, especially in therapy, were hindered until recently by the polyclonal nature of natural immunoglobulins. A significant step towards the realisation of the potential

of immunoglobulins as therapeutic agents was the discovery of procedures for the production of monoclonal antibodies (MAbs) of defined specificity (1).

However, most MAbs are produced by hybridomas which are fusions of rodent spleen cells with rodent myeloma cells. They are therefore essentially rodent proteins. There are very few reports of the production of human MAbs.

Since most available MAbs are of rodent origin, they are naturally antigenic in humans and thus can give rise to an undesirable immune response termed the HAMA (Human Anti-Mouse Antibody) response. Therefore, the use of rodent MAbs as therapeutic agents in humans is inherently limited by the fact that the human subject will mount an immunological response to the MAb and will either remove it entirely or at least reduce its effectiveness. In practice, MAbs of rodent origin may not be used in patients for more than one or a few treatments as a HAMA response soon develops rendering the MAb ineffective as well as giving rise to undesirable reactions. For instance, OKT3 a mouse IgG2a/k MAb which recognises an antigen in the T-cell receptor-CD3 complex has been approved for use in many countries throughout the world as an immunosuppressant in the treatment of acute allograft rejection [Chatenoud et al (2) and Jeffers et al (3)]. However, in view of the rodent nature of this and other such MAbs, a significant HAMA response which may include a major anti-idiotypic component, may build up on use. Clearly, it would be highly desirable to diminish or abolish this undesirable HAMA response and thus enlarge the areas of use of these very useful antibodies.

Proposals have therefore been made to render non-human MAbs less antigenic in humans. Such techniques can be generically termed "humanisation" techniques. These

techniques typically involve the use of recombinant DNA technology to manipulate DNA sequences encoding the polypeptide chains of the antibody molecule.

Early methods for humanising MABs involved production of chimeric antibodies in which an antigen binding site comprising the complete variable domains of one antibody is linked to constant domains derived from another antibody. Methods for carrying out such chimerisation procedures are described in EP0120694 (Celltech Limited), EP0125023 (Genentech Inc. and City of Hope), EP-A-0 171496 (Res. Dev. Corp. Japan), EP-A-0 173 494 (Stanford University), and WO 86/01533 (Celltech Limited). This latter Celltech application (WO 86/01533) discloses a process for preparing an antibody molecule having the variable domains from a mouse MAb and the constant domains from a human immunoglobulin. Such humanised chimeric antibodies, however, still contain a significant proportion of non-human amino acid sequence, i.e. the complete non-human variable domains, and thus may still elicit some HAMA response, particularly if administered over a prolonged period [Begent et al (ref. 4)].

In an alternative approach, described in EP-A-0239400 (Winter), the complementarity determining regions (CDRs) of a mouse MAb have been grafted onto the framework regions of the variable domains of a human immunoglobulin by site directed mutagenesis using long oligonucleotides. The present invention relates to humanised antibody molecules prepared according to this alternative approach, i.e. CDR-grafted humanised antibody molecules. Such CDR-grafted humanised antibodies are much less likely to give rise to a HAMA response than humanised chimeric antibodies in view of the much lower proportion of non-human amino acid sequence which they contain.

The earliest work on humanising MAb's by CDR-grafting was carried out on MAb's recognising synthetic antigens, such as the NP or NIP antigens. However, examples in which a mouse MAb recognising lysozyme and a rat MAb recognising an antigen on human T-cells were humanised by CDR-grafting have been described by Verhoeyen et al (5) and Riechmann et al (6) respectively. The preparation of CDR-grafted antibody to the antigen on human T cells is also described in WO 89/07452 (Medical Research Council).

In Riechmann et al/Medical Research Council it was found that transfer of the CDR regions alone [as defined by Kabat refs. (7) and (8)] was not sufficient to provide satisfactory antigen binding activity in the CDR-grafted product. Riechmann et al found that it was necessary to convert a serine residue at position 27 of the human sequence to the corresponding rat phenylalanine residue to obtain a CDR-grafted product having improved antigen binding activity. This residue at position 27 of the heavy chain is within the structural loop adjacent to CDR1. A further construct which additionally contained a human serine to rat tyrosine change at position 30 of the heavy chain did not have a significantly altered binding activity over the humanised antibody with the serine to phenylalanine change at position 27 alone. These results indicate that changes to residues of the human sequence outside the CDR regions, in particular in the structural loop adjacent to CDR1, may be necessary to obtain effective antigen binding activity for CDR-grafted antibodies which recognise more complex antigens. Even so the binding affinity of the best CDR-grafted antibodies obtained was still significantly less than the original MAb.

Very recently Queen et al (9) have described the preparation of a humanised antibody that binds to the

interleukin 2 receptor, by combining the CDRs of a murine MAb (anti-Tac) with human immunoglobulin framework and constant regions. The human framework regions were chosen to maximise homology with the anti-Tac MAb sequence. In addition computer modelling was used to identify framework amino acid residues which were likely to interact with the CDRs or antigen, and mouse amino acids were used at these positions in the humanised antibody.

In WO 90/07861 Queen et al propose four criteria for designing humanised immunoglobulins. The first criterion is to use as the human acceptor the framework from a particular human immunoglobulin that is unusually homologous to the non-human donor immunoglobulin to be humanised, or to use a consensus framework from many human antibodies. The second criterion is to use the donor amino acid rather than the acceptor if the human acceptor residue is unusual and the donor residue is typical for human sequences at a specific residue of the framework. The third criterion is to use the donor framework amino acid residue rather than the acceptor at positions immediately adjacent to the CDRs. The fourth criterion is to use the donor amino acid residue at framework positions at which the amino acid is predicted to have a side chain atom within about 3 Å of the CDRs in a three-dimensional immunoglobulin model and to be capable of interacting with the antigen or with the CDRs of the humanised immunoglobulin. It is proposed that criteria two, three or four may be applied in addition or alternatively to criterion one, and may be applied singly or in any combination.

WO 90/07861 describes in detail the preparation of a single CDR-grafted humanised antibody, a humanised antibody having specificity for the p55 Tac protein of the

IL-2 receptor. The combination of all four criteria, as above, were employed in designing this humanised antibody, the variable region frameworks of the human antibody Eu (7) being used as acceptor. In the resultant humanised antibody the donor CDRs were as defined by Kabat et al (7 and 8) and in addition the mouse donor residues were used in place of the human acceptor residues, at positions 27, 30, 48, 66, 67, 89, 91, 94, 103, 104, 105 and 107 in the heavy chain and at positions 48, 60 and 63 in the light chain, of the variable region frameworks. The humanised anti-Tac antibody obtained is reported to have an affinity for p55 of $3 \times 10^9 \text{ M}^{-1}$, about one-third of that of the murine MAb.

We have further investigated the preparation of CDR-grafted humanised antibody molecules and have identified a hierarchy of positions within the framework of the variable regions (i.e. outside both the Kabat CDRs and structural loops of the variable regions) at which the amino acid identities of the residues are important for obtaining CDR-grafted products with satisfactory binding affinity. This has enabled us to establish a protocol for obtaining satisfactory CDR-grafted products which may be applied very widely irrespective of the level of homology between the donor immunoglobulin and acceptor framework. The set of residues which we have identified as being of critical importance does not coincide with the residues identified by Queen et al (9).

Summary of the Invention

Accordingly, in a first aspect the invention provides a CDR-grafted antibody heavy chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 6, 23 and/or 24, 48 and/or 49, 71 and/or 73, 75 and/or 76 and/or 78 and 88 and/or 91.

In preferred embodiments, the heavy chain framework comprises donor residues at positions 23, 24, 49, 71, 73 and 78 or at positions 23, 24 and 49. The residues at positions 71, 73 and 78 of the heavy chain framework are preferably either all acceptor or all donor residues.

In particularly preferred embodiments the heavy chain framework additionally comprises donor residues at one, some or all of positions 6, 37, 48 and 94. Also it is particularly preferred that residues at positions of the heavy chain framework which are commonly conserved across species, i.e. positions 2, 4, 25, 36, 39, 47, 93, 103, 104, 106 and 107, if not conserved between donor and acceptor, additionally comprise donor residues. Most preferably the heavy chain framework additionally comprises donor residues at positions 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.

In addition the heavy chain framework optionally comprises donor residues at one, some or all of positions:

1 and 3,
72 and 76,
69 (if 48 is different between donor and acceptor),
38 and 46 (if 48 is the donor residue),
80 and 20 (if 69 is the donor residue),
67,
82 and 18 (if 67 is the donor residue),
91,
88, and
any one or more of 9, 11, 41, 87, 108, 110 and 112.

In the first and other aspects of the present invention reference is made to CDR-grafted antibody products comprising acceptor framework and donor antigen binding regions. It will be appreciated that the invention is widely applicable to the CDR-grafting of antibodies in

general. Thus, the donor and acceptor antibodies may be derived from animals of the same species and even same antibody class or sub-class. More usually, however, the donor and acceptor antibodies are derived from animals of different species. Typically the donor antibody is a non-human antibody, such as a rodent MAb, and the acceptor antibody is a human antibody.

In the first and other aspects of the present invention, the donor antigen binding region typically comprises at least one CDR from the donor antibody. Usually the donor antigen binding region comprises at least two and preferably all three CDRs of each of the heavy chain and/or light chain variable regions. The CDRs may comprise the Kabat CDRs, the structural loop CDRs or a composite of the Kabat and structural loop CDRs and any combination of any of these. Preferably, the antigen binding regions of the CDR-grafted heavy chain variable domain comprise CDRs corresponding to the Kabat CDRs at CDR2 (residues 50-65) and CDR3 (residues 95-100) and a composite of the Kabat and structural loop CDRs at CDR1 (residues 26-35).

The residue designations given above and elsewhere in the present application are numbered according to the Kabat numbering [refs. (7) and (8)]. Thus the residue designations do not always correspond directly with the linear numbering of the amino acid residues. The actual linear amino acid sequence may contain fewer or additional amino acids than in the strict Kabat numbering corresponding to a shortening of, or insertion into, a structural component, whether framework or CDR, of the basic variable domain structure. For example, the heavy chain variable region of the anti-Tac antibody described by Queen et al (9) contains a single amino acid insert (residue 52a) after residue 52 of CDR2 and a three amino

acid insert (residues 82a, 82b and 82c) after framework residue 82, in the Kabat numbering. The correct Kabat numbering of residues may be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence.

The invention also provides in a second aspect a CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 1 and/or 3 and 46 and/or 47. Preferably the CDR grafted light chain of the second aspect comprises donor residues at positions 46 and/or 47.

The invention also provides in a third aspect a CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 46, 48, 58 and 71.

In a preferred embodiment of the third aspect, the framework comprises donor residues at all of positions 46, 48, 58 and 71.

In particularly preferred embodiments of the second and third aspects, the framework additionally comprises donor residues at positions 36, 44, 47, 85 and 87. Similarly positions of the light chain framework which are commonly conserved across species, i.e. positions 2, 4, 6, 35, 49, 62, 64-69, 98, 99, 101 and 102, if not conserved between donor and acceptor, additionally comprise donor residues. Most preferably the light chain framework additionally comprises donor residues at positions 2, 4, 6, 35, 36, 38, 44, 47, 49, 62, 64-69, 85, 87, 98, 99, 101 and 102.

In addition the framework of the second or third aspects optionally comprises donor residues at one, some or all of positions:

1 and 3,

63,

60 (if 60 and 54 are able to form a potential saltbridge),

70 (if 70 and 24 are able to form a potential saltbridge),

73 and 21 (if 47 is different between donor and acceptor),

37 and 45 (if 47 is different between donor and acceptor),

and

any one or more of 10, 12, 40, 80, 103 and 105.

Preferably, the antigen binding regions of the CDR-grafted light chain variable domain comprise CDRs corresponding to the Kabat CDRs at CDR1 (residue 24-34), CDR2 (residues 50-56) and CDR3 (residues 89-97).

The invention further provides in a fourth aspect a CDR-grafted antibody molecule comprising at least one CDR-grafted heavy chain and at least one CDR-grafted light chain according to the first and second or first and third aspects of the invention.

The humanised antibody molecules and chains of the present invention may comprise: a complete antibody molecule, having full length heavy and light chains; a fragment thereof, such as a Fab, (Fab')₂ or FV fragment; a light chain or heavy chain monomer or dimer; or a single chain antibody, e.g. a single chain FV in which heavy and light chain variable regions are joined by a peptide linker; or any other CDR-grafted molecule with the same specificity as the original donor antibody. Similarly the CDR-grafted heavy and light chain variable region may be combined with other antibody domains as appropriate.

Also the heavy or light chains or humanised antibody molecules of the present invention may have attached to them an effector or reporter molecule. For instance, it may have a macrocycle, for chelating a heavy metal atom, or a toxin, such as ricin, attached to it by a covalent bridging structure. Alternatively, the procedures of recombinant DNA technology may be used to produce an immunoglobulin molecule in which the Fc fragment or CH3 domain of a complete immunoglobulin molecule has been replaced by, or has attached thereto by peptide linkage, a functional non-immunoglobulin protein, such as an enzyme or toxin molecule.

Any appropriate acceptor variable region framework sequences may be used having regard to class/type of the donor antibody from which the antigen binding regions are derived. Preferably, the type of acceptor framework used is of the same/similar class/type as the donor antibody. Conveniently, the framework may be chosen to maximise/optimize homology with the donor antibody sequence particularly at positions close or adjacent to the CDRs. However, a high level of homology between donor and acceptor sequences is not important for application of the present invention. The present invention identifies a hierarchy of framework residue positions at which donor residues may be important or desirable for obtaining a CDR-grafted antibody product having satisfactory binding properties. The CDR-grafted products usually have binding affinities of at least 10^5 M^{-1} , preferably at least about 10^8 M^{-1} , or especially in the range 10^8 - 10^{12} M^{-1} . In principle, the present invention is applicable to any combination of donor and acceptor antibodies irrespective of the level of homology between their sequences. A protocol for applying the invention to any particular donor-acceptor antibody pair is given hereinafter. Examples of human frameworks which may be

used are KOL, NEWM, REI, EU, LAY and POM (refs. 4 and 5) and the like; for instance KOL and NEWM for the heavy chain and REI for the light chain and EU, LAY and POM for both the heavy chain and the light chain.

Also the constant region domains of the products of the invention may be selected having regard to the proposed function of the antibody in particular the effector functions which may be required. For example, the constant region domains may be human IgA, IgE, IgG or IgM domains. In particular, IgG human constant region domains may be used, especially of the IgG1 and IgG3 isotypes, when the humanised antibody molecule is intended for therapeutic uses, and antibody effector functions are required. Alternatively, IgG2 and IgG4 isotypes may be used when the humanised antibody molecule is intended for therapeutic purposes and antibody effector functions are not required, e.g. for simple blocking of lymphokine activity.

However, the remainder of the antibody molecules need not comprise only protein sequences from immunoglobulins. For instance, a gene may be constructed in which a DNA sequence encoding part of a human immunoglobulin chain is fused to a DNA sequence encoding the amino acid sequence of a functional polypeptide such as an effector or reporter molecule.

Preferably the CDR-grafted antibody heavy and light chain and antibody molecule products are produced by recombinant DNA technology.

Thus in further aspects the invention also includes DNA sequences coding for the CDR-grafted heavy and light chains, cloning and expression vectors containing the DNA sequences, host cells transformed with the DNA sequences

and processes for producing the CDR-grafted chains and antibody molecules comprising expressing the DNA sequences in the transformed host cells.

The general methods by which the vectors may be constructed, transfection methods and culture methods are well known per se and form no part of the invention. Such methods are shown, for instance, in references 10 and 11.

The DNA sequences which encode the donor amino acid sequence may be obtained by methods well known in the art. For example the donor coding sequences may be obtained by genomic cloning, or cDNA cloning from suitable hybridoma cell lines. Positive clones may be screened using appropriate probes for the heavy and light chain genes in question. Also PCR cloning may be used.

DNA coding for acceptor, e.g. human acceptor, sequences may be obtained in any appropriate way. For example DNA sequences coding for preferred human acceptor frameworks such as KOL, REI, EU and NEWM, are widely available to workers in the art.

The standard techniques of molecular biology may be used to prepare DNA sequences coding for the CDR-grafted products. Desired DNA sequences may be synthesised completely or in part using oligonucleotide synthesis techniques. Site-directed mutagenesis and polymerase chain reaction (PCR) techniques may be used as appropriate. For example oligonucleotide directed synthesis as described by Jones et al (ref. 20) may be used. Also oligonucleotide directed mutagenesis of a pre-existing variable region as, for example, described by Verhoeyen et al (ref. 5) or Riechmann et al (ref. 6) may be used. Also enzymatic filling in of gapped

oligonucleotides using T₄ DNA polymerase as, for example, described by Queen et al (ref. 9) may be used.

Any suitable host cell/vector system may be used for expression of the DNA sequences coding for the CDR-grafted heavy and light chains. Bacterial e.g. E. coli, and other microbial systems may be used, in particular for expression of antibody fragments such as FAb and (Fab')₂ fragments, and especially FV fragments and single chain antibody fragments e.g. single chain FVs. Eucaryotic e.g. mammalian host cell expression systems may be used for production of larger CDR-grafted antibody products, including complete antibody molecules. Suitable mammalian host cells include CHO cells and myeloma or hybridoma cell lines.

Thus, in a further aspect the present invention provides a process for producing a CDR-grafted antibody product comprising:

- (a) producing in an expression vector an operon having a DNA sequence which encodes an antibody heavy chain according to the first aspect of the invention;

and/or

- (b) producing in an expression vector an operon having a DNA sequence which encodes a complementary antibody light chain according to the second or third aspect of the invention;
- (c) transfecting a host cell with the or each vector; and
- (d) culturing the transfected cell line to produce the CDR-grafted antibody product.

The CDR-grafted product may comprise only heavy or light chain derived polypeptide, in which case only a heavy chain or light chain polypeptide coding sequence is used to transfect the host cells.

For production of products comprising both heavy and light chains, the cell line may be transfected with two vectors, the first vector may contain an operon encoding a light chain-derived polypeptide and the second vector containing an operon encoding a heavy chain-derived polypeptide. Preferably, the vectors are identical, except in so far as the coding sequences and selectable markers are concerned, so as to ensure as far as possible that each polypeptide chain is equally expressed. Alternatively, a single vector may be used, the vector including the sequences encoding both light chain- and heavy chain-derived polypeptides.

The DNA in the coding sequences for the light and heavy chains may comprise cDNA or genomic DNA or both. However, it is preferred that the DNA sequence encoding the heavy or light chain comprises at least partially, genomic DNA, preferably a fusion of cDNA and genomic DNA.

The present invention is applicable to antibodies of any appropriate specificity. Advantageously, however, the invention may be applied to the humanisation of non-human antibodies which are used for in vivo therapy or diagnosis. Thus the antibodies may be site-specific antibodies such as tumour-specific or cell surface-specific antibodies, suitable for use in in vivo therapy or diagnosis, e.g. tumour imaging. Examples of cell surface-specific antibodies are anti-T cell antibodies, such as anti-CD3, and CD4 and adhesion molecules, such as CR3, ICAM and ELAM. The antibodies may have specificity for interleukins (including lymphokines, growth factors and stimulating factors), hormones and other biologically active compounds, and receptors for any of these. For

example, the antibodies may have specificity for any of the following: Interferons α , β , γ or δ , IL1, IL2, IL3, or IL4, etc., TNF, GCSF, GMCSF, EPO, hGH, or insulin, etc.

The the present invention also includes therapeutic and diagnostic compositions comprising the CDR-grafted products of the invention and uses of such compositions in therapy and diagnosis.

Accordingly in a further aspect the invention provides a therapeutic or diagnostic composition comprising a CDR-grafted antibody heavy or light chain or molecule according to previous aspects of the invention in combination with a pharmaceutically acceptable carrier, diluent or excipient.

Accordingly also the invention provides a method of therapy or diagnosis comprising administering an effective amount of a CDR-grafted antibody heavy or light chain or molecule according to previous aspects of the invention to a human or animal subject.

A preferred protocol for obtaining CDR-grafted antibody heavy and light chains in accordance with the present invention is set out below together with the rationale by which we have derived this protocol. This protocol and rationale are given without prejudice to the generality of the invention as hereinbefore described and defined.

Protocol

It is first of all necessary to sequence the DNA coding for the heavy and light chain variable regions of the donor antibody, to determine their amino acid sequences. It is also necessary to choose appropriate acceptor heavy and light chain variable regions, of known amino acid sequence. The CDR-grafted chain is then designed

starting from the basis of the acceptor sequence. It will be appreciated that in some cases the donor and acceptor amino acid residues may be identical at a particular position and thus no change of acceptor framework residue is required.

1. As a first step donor residues are substituted for acceptor residues in the CDRs. For this purpose the CDRs are preferably defined as follows:

Heavy chain - CDR1: residues 26-35
 - CDR2: residues 50-65
 - CDR3: residues 95-102
Light chain - CDR1: residues 24-34
 - CDR2: residues 50-56
 - CDR3: residues 89-97

The positions at which donor residues are to be substituted for acceptor in the framework are then chosen as follows, first of all with respect to the heavy chain and subsequently with respect to the light chain.

2. Heavy Chain

- 2.1 Choose donor residues at all of positions 23, 24, 49, 71, 73 and 78 of the heavy chain or all of positions 23, 24 and 49 (71, 73 and 78 are always either all donor or all acceptor).
- 2.2 Check that the following have the same amino acid in donor and acceptor sequences, and if not preferably choose the donor: 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.

2.3 To further optimise affinity consider choosing donor residues at one, some or any of:

- i. 1, 3
- ii. 72, 76
- iii. If 48 is different between donor and acceptor sequences, consider 69
- iv. If at 48 the donor residue is chosen, consider 38 and 46
- v. If at 69 the donor residue is chosen, consider 80 and then 20
- vi. 67
- vii. If at 67 the donor residue is chosen, consider 82 and then 18
- viii. 91
- ix. 88
- x. 9, 11, 41, 87, 108, 110, 112

3. Light Chain

3.1 Choose donor at 46, 48, 58 and 71

3.2 Check that the following have the same amino acid in donor and acceptor sequences, if not preferably choose donor:

2, 4, 6, 35, 38, 44, 47, 49, 62, 64-69 inclusive, 85, 87, 98, 99, 101 and 102

3.3 To further optimise affinity consider choosing donor residues at one, some or any of:

- i. 1, 3
- ii. 63

- iii. 60, if 60 and 54 are able to form potential saltbridge
- iv. 70, if 70 and 24 are able to form potential saltbridge
- v. 73, and 21 if 47 is different between donor and acceptor
- vi. 37, and 45 if 47 is different between donor and acceptor
- vii. 10, 12, 40, 80, 103, 105

Rationale

In order to transfer the binding site of an antibody into a different acceptor framework, a number of factors need to be considered.

1. The extent of the CDRs
The CDRs (Complementary Determining Regions) were defined by Wu and Kabat (refs. 4 and 5) on the basis of an analysis of the variability of different regions of antibody variable regions. Three regions per domain were recognised. In the light chain the sequences are 24-34, 50-56, 89-97 (numbering according to Kabat (ref. 4), Eu Index) inclusive and in the heavy chain the sequences are 31-35, 50-65 and 95-102 inclusive.

When antibody structures became available it became apparent that these CDR regions corresponded in the main to loop regions which extended from the β barrel framework of the light and heavy variable domains. For H1 there was a discrepancy in that the loop was from 26 to 32 inclusive and for H2 the loop was 52 to 56 and for L2 from 50 to 53. However, with the exception of H1 the CDR regions encompassed the loop regions and extended into the β strand

frameworks. In H1 residue 26 tends to be a serine and 27 a phenylalanine or tyrosine, residue 29 is a phenylalanine in most cases. Residues 28 and 30 which are surface residues exposed to solvent might be involved in antigen-binding. A prudent definition of the H1 CDR therefore would include residues 26-35 to include both the loop region and the hypervariable residues 33-35.

It is of interest to note the example of Riechmann et al (ref. 3), who used the residue 31-35 choice for CDR-H1. In order to produce efficient antigen binding, residue 27 also needed to be recruited from the donor (rat) antibody.

2. Non-CDR residues which contribute to antigen binding

By examination of available X-ray structures we have identified a number of residues which may have an effect on net antigen binding and which can be demonstrated by experiment. These residues can be sub-divided into a number of groups.

2.1 Surface residues near CDR [all numbering as in Kabat et al (ref. 7)].

2.1.1. Heavy Chain - Key residues are 23, 71 and 73. Other residues which may contribute to a lesser extent are 1, 3 and 76. Finally 25 is usually conserved but the murine residue should be used if there is a difference.

2.1.2 Light Chain - Many residues close to the CDRs, e.g. 63, 65, 67 and 69 are conserved. If conserved none of the surface residues in the light chain are likely to have a major effect. However, if the murine residue at these positions

is unusual, then it would be of benefit to analyse the likely contribution more closely. Other residues which may also contribute to binding are 1 and 3, and also 60 and 70 if the residues at these positions and at 54 and 24 respectively are potentially able to form a salt bridge i.e. 60 + 54; 70 + 24.

2.2 Packing residues near the CDRs.

2.2.1. Heavy Chain - Key residues are 24, 49 and 78.

Other key residues would be 36 if not a tryptophan, 94 if not an arginine, 104 and 106 if not glycines and 107 if not a threonine.

Residues which may make a further contribution to stable packing of the heavy chain and hence improved affinity are 2, 4, 6, 38, 46, 67 and 69. 67 packs against the CDR residue 63 and this pair could be either both mouse or both human. Finally, residues which contribute to packing in this region but from a longer range are 18, 20, 80, 82 and 86. 82 packs against 67 and in turn 18 packs against 82. 80 packs against 69 and in turn 20 packs against 80. 86 forms an H bond network with 38 and 46. Many of the mouse-human differences appear minor e.g. Leu-Ile, but could have an minor impact on correct packing which could translate into altered positioning of the CDRs.

2.2.2. Light Chain - Key residues are 48, 58 and 71.

Other key residues would be 6 if not glutamine, 35 if not tryptophan, 62 if not phenylalanine or tryosine, 64, 66, 68, 99 and 101 if not glycines and 102 if not a threonine. Residues which make a further contribution are 2, 4, 37, 45 and 47. Finally residues 73 and 21 and 19 may make long distance packing contributions of a minor nature.

- 2.3. Residues at the variable domain interface between heavy and light chains - In both the light and heavy chains most of the non-CDR interface residues are conserved. If a conserved residue is replaced by a residue of different character, e.g. size or charge, it should be considered for retention as the murine residue.
- 2.3.1. Heavy Chain - Residues which need to be considered are 37 if the residue is not a valine but is of larger side chain volume or has a charge or polarity. Other residues are 39 if not a glutamine, 45 if not a leucine, 47 if not a tryptophan, 91 if not a phenylalanine or tyrosine, 93 if not an alanine and 103 if not a tryptophan. Residue 89 is also at the interface but is not in a position where the side chain could be of great impact.
- 2.3.2. Light Chain - Residues which need to be considered are 36, if not a tyrosine, 38 if not a glutamine, 44 if not a proline, 46, 49 if not a tyrosine, residue 85, residue 87 if not a tyrosine and 98 if not a phenylalanine.
- 2.4. Variable-Constant region interface - The elbow angle between variable and constant regions may be affected by alterations in packing of key residues in the variable region against the constant region which may affect the position of V_L and V_H with respect to one another. Therefore it is worth noting the residues likely to be in contact with the constant region. In the heavy chain the surface residues potentially in contact with the variable region are conserved between mouse and human antibodies therefore the variable region contact residues may influence the V-C interaction. In the light chain the amino acids found at a number of the constant

region contact points vary, and the V & C regions are not in such close proximity as the heavy chain. Therefore the influences of the light chain V-C interface may be minor.

- 2.4.1. Heavy Chain - Contact residues are 7, 11, 41, 87, 108, 110, 112.
- 2.4.2. Light Chain - In the light chain potentially contacting residues are 10, 12, 40, 80, 83, 103 and 105.

The above analysis coupled with our considerable practical experimental experience in the CDR-grafting of a number of different antibodies have lead us to the protocol given above.

The present invention is now described, by way of example only, with reference to the accompanying Figures 1 - 13.

Brief Description of the Figures

- Figure 1 shows DNA and amino acid sequences of the OKT3 light chain;
- Figure 2 shows DNA and amino acid sequences of the OKT3 heavy chain;
- Figure 3 shows the alignment of the OKT3 light variable region amino acid sequence with that of the light variable region of the human antibody REI;
- Figure 4 shows the alignment of the OKT3 heavy variable region amino acid sequence with that of the heavy variable region of the human antibody KOL;
- Figure 5 shows the heavy variable region amino acid sequences of OKT3, KOL and various corresponding CDR grafts;
- Figure 6 shows the light variable region amino acid sequences of OKT3, REI and various corresponding CDR grafts;

- Figure 7 shows a graph of binding assay results for various grafted OKT3 antibodies'
- Figure 8 shows a graph of blocking assay results for various grafted OKT3 antibodies;
- Figure 9 shows a similar graph of blocking assay results;
- Figure 10 shows similar graphs for both binding assay and blocking assay results;
- Figure 11 shows further similar graphs for both binding assay and blocking assay results;
- Figure 12 shows a graph of competition assay results for a minimally grafted OKT3 antibody compared with the OKT3 murine reference standard, and
- Figure 13 shows a similar graph of competition assay results comparing a fully grafted OKT3 antibody with the murine reference standard.

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

EXAMPLE 1

CDR-GRAFTING OF OKT3

MATERIAL AND METHODS

1. INCOMING CELLS

Hybridoma cells producing antibody OKT3 were provided by Ortho (seedlot 4882.1) and were grown up in antibiotic free Dulbecco's Modified Eagles Medium (DMEM) supplemented with glutamine and 5% foetal calf serum, and divided to provide both an overgrown supernatant for evaluation and cells for extraction of RNA. The overgrown supernatant was shown to contain 250 ug/mL murine IgG2a/kappa antibody. The supernatant was negative for murine lambda light chain and IgG1, IgG2b, IgG3, IgA and IgM heavy chain. 20mL of supernatant was assayed to confirm that the antibody present was OKT3.

2. MOLECULAR BIOLOGY PROCEDURES

Basic molecular biology procedures were as described in Maniatis et al (ref. 9) with, in some cases, minor modifications. DNA sequencing was performed as described in Sanger et al (ref. 11) and the Amersham International Plc sequencing handbook. Site directed mutagenesis was as described in Kramer et al (ref. 12) and the Anglian Biotechnology Ltd. handbook. COS cell expression and metabolic labelling studies were as described in Whittle et al (ref. 13)

3. RESEARCH ASSAYS

3.1. ASSEMBLY ASSAYS

Assembly assays were performed on supernatants from transfected COS cells to determine the amount of intact IgG present.

3.1.1. COS CELLS TRANSFECTED WITH MOUSE OKT3 GENES

The assembly assay for intact mouse IgG in COS cell supernatants was an ELISA with the following format:

96 well microtitre plates were coated with F(ab')₂ goat anti-mouse IgG Fc. The plates were washed in water and samples added for 1 hour at room temperature. The plates were washed and F(ab')₂ goat anti-mouse IgG F(ab')₂ (HRPO conjugated) was then added. Substrate was added to reveal the reaction. UPC10, a mouse IgG2a myeloma, was used as a standard.

3.1.2. COS AND CHO CELLS TRANSFECTED WITH CHIMERIC OR CDR-GRAFTED OKT3 GENES

The assembly assay for chimeric or CDR-grafted antibody in COS cell supernatants was an ELISA with the following format:

96 well microtitre plates were coated with F(ab')₂ goat anti-human IgG Fc. The plates were washed and samples added and incubated for 1 hour at room temperature. The plates were washed and monoclonal mouse anti-human kappa chain was added for 1 hour at room temperature.

The plates were washed and F(ab')₂ goat anti-mouse IgG Fc (HRPO conjugated) was added. Enzyme substrate was added to reveal the reaction.

Chimeric B72.3 (IgG4) (ref. 13) was used as a standard. The use of a monoclonal anti-kappa chain in this assay allows grafted antibodies to be read from the chimeric standard.

3.2. ASSAY FOR ANTIGEN BINDING ACTIVITY

Material from COS cell supernatants was assayed for OKT3 antigen binding activity onto CD3 positive cells in a direct assay. The procedure was as follows:

HUT 78 cells (human T cell line, CD3 positive) were maintained in culture. Monolayers of HUT 78 cells were prepared onto 96 well ELISA plates using poly-L-lysine and glutaraldehyde. Samples were added to the monolayers for 1 hour at room temperature.

The plates were washed gently using PBS. F(ab')₂ goat anti-human IgG Fc (HRPO conjugated) or F(ab')₂ goat anti-mouse IgG Fc (HRPO conjugated) was added as appropriate for humanised or mouse samples.

Substrate was added to reveal the reaction.

The negative control for the cell-based assay was chimeric B72.3. The positive control was mouse Orthomune OKT3 or chimeric OKT3, when available. This cell-based assay was difficult to perform, and an alternative assay was developed for CDR-grafted OKT3 which was more sensitive and easier to carry out.

In this system CDR-grafted OKT3 produced by COS cells was tested for its ability to bind to the CD3-positive HPB-ALL (human peripheral blood acute lymphocytic leukemia) cell line. It was also tested for its ability to block the binding of murine OKT3 to these cells. Binding was measured by the following procedure: HPB-ALL cells were harvested from tissue culture. Cells were incubated at 4°C for 1 hour with various dilutions of test antibody, positive control antibody, or negative control antibody. The cells were washed once and incubated at 4°C for 1 hour with an FITC-labelled goat anti-human IgG (Fc-

specific, mouse absorbed). The cells were washed twice and analysed by cytofluorography. Chimeric OKT3 was used as a positive control for direct binding. Cells incubated with mock-transfected COS cell supernatant, followed by the FITC-labelled goat anti-human IgG, provided the negative control. To test the ability of CDR-grafted OKT3 to block murine OKT3 binding, the HPB-ALL cells were incubated at 4°C for 1 hour with various dilutions of test antibody or control antibody. A fixed saturating amount of FITC OKT3 was added. The samples were incubated for 1 hour at 4°C, washed twice and analysed by cytofluorography. FITC-labelled OKT3 was used as a positive control to determine maximum binding. Unlabelled murine OKT3 served as a reference standard for blocking. Negative controls were unstained cells with or without mock-transfected cell supernatant. The ability of the CDR-grafted OKT3 light chain to bind CD3-positive cells and block the binding of murine OKT3 was initially tested in combination with the chimeric OKT3 heavy chain. The chimeric OKT3 heavy chain is composed of the murine OKT3 variable region and the human IgG4 constant region. The chimeric heavy chain gene is expressed in the same expression vector used for the CDR-grafted genes. The CDR-grafted light chain expression vector and the chimeric heavy chain expression vector were co-transfected into COS cells. The fully chimeric OKT3 antibody (chimeric light chain and chimeric heavy chain) was found to be fully capable of binding to CD3 positive cells and blocking the binding of murine OKT3 to these cells.

3.3 DETERMINATION OF RELATIVE BINDING AFFINITY

The relative binding affinities of CDR-grafted

anti-CD3 monoclonal antibodies were determined by competition binding (ref. 6) using the HPB-ALL human T cell line as a source of CD3 antigen, and fluorescein-conjugated murine OKT3 (Fl-OKT3) of known binding affinity as a tracer antibody. The binding affinity of Fl-OKT3 tracer antibody was determined by a direct binding assay in which increasing amounts of Fl-OKT3 were incubated with HPB-ALL (5×10^5) in PBS with 5% foetal calf serum for 60 min. at 4°C. Cells were washed, and the fluorescence intensity was determined on a FACScan flow cytometer calibrated with quantitative microbead standards (Flow Cytometry Standards, Research Triangle Park, NC). Fluorescence intensity per antibody molecule (F/P ratio) was determined by using microbeads which have a predetermined number of mouse IgG antibody binding sites (Simply Cellular beads, Flow Cytometry Standards). F/P equals the fluorescence intensity of beads saturated with Fl-OKT3 divided by the number of binding sites per bead. The amount of bound and free Fl-OKT3 was calculated from the mean fluorescence intensity per cell, and the ratio of bound/free was plotted against the number of moles of antibody bound. A linear fit was used to determine the affinity of binding (absolute value of the slope).

For competitive binding, increasing amounts of competitor antibody were added to a sub-saturating dose of Fl-OKT3 and incubated with 5×10^5 HPB-ALL in 200 ml of PBS with 5% foetal calf serum, for 60 min at 4°C. The fluorescence intensities of the cells were measured on a FACScan flow cytometer calibrated with quantitative microbead standards. The concentrations of bound and free Fl-OKT3 were calculated. The affinities of competing anti-

- 30 -

bodies were calculated from the equation $[X]-[OKT3] = (1/Kx) - (1/Ka)$, where Ka is the affinity of murine OKT3, Kx is the affinity of competitor X , $[]$ is the concentration of competitor antibody at which bound/free binding is $R/2$, and R is the maximal bound/free binding.

4. CDNA LIBRARY CONSTRUCTION

4.1. mRNA PREPARATION AND CDNA SYNTHESIS

OKT3 producing cells were grown as described above and 1.2×10^9 cells harvested and mRNA extracted using the guanidinium/LiCl extraction procedure. cDNA was prepared by priming from Oligo-dT to generate full length cDNA. The cDNA was methylated and EcoRI linkers added for cloning.

4.2. LIBRARY CONSTRUCTION

The cDNA library was ligated to pSP65 vector DNA which had been EcoRI cut and the 5' phosphate groups removed by calf intestinal phosphatase (EcoRI/CIP). The ligation was used to transform high transformation efficiency Escherichia coli (E.coli) HB101. A cDNA library was prepared. 3600 colonies were screened for the light chain and 10000 colonies were screened for the heavy chain.

5. SCREENING

E.coli colonies positive for either heavy or light chain probes were identified by oligonucleotide screening using the oligonucleotides:

5' TCCAGATGTTAACTGCTCAC for the light chain, which is complementary to a sequence in the mouse kappa constant region, and 5' CAGGGGCCAGTGGATGGATAGAC for the heavy chain which is complementary to a sequence in the mouse IgG2a constant CH1 domain region. 12 light chain and 9 heavy chain clones

were identified and taken for second round screening. Positive clones from the second round of screening were grown up and DNA prepared. The sizes of the gene inserts were estimated by gel electrophoresis and inserts of a size capable of containing a full length cDNA were subcloned into M13 for DNA sequencing.

6. DNA SEQUENCING

Clones representing four size classes for both heavy and light chains were obtained in M13. DNA sequence for the 5' untranslated regions, signal sequences, variable regions and 3' untranslated regions of full length cDNAs [Figures 1(a) and 2(a)] were obtained and the corresponding amino acid sequences predicted [(Figures 1(b) and 2(b))]. In Figure 1(a) the untranslated DNA regions are shown in uppercase, and in both Figures 1 and 2 the signal sequences are underlined.

7. CONSTRUCTION OF cDNA EXPRESSION VECTORS

Celltech expression vectors are based on the plasmid pEE6hCMV (ref. 14). A polylinker for the insertion of genes to be expressed has been introduced after the major immediate early promoter/enhancer of the human Cytomegalovirus (hCMV). Marker genes for selection of the plasmid in transfected eukaryotic cells can be inserted as BamH1 cassettes in the unique BamH1 site of pEE6 hCMV; for instance, the neo marker to provide pEE6 hCMV neo. It is usual practice to insert the neo and gpt markers prior to insertion of the gene of interest, whereas the GS marker is inserted last because of the presence of internal EcoR1 sites in the cassette.

The selectable markers are expressed from the SV40 late promoter which also provides an origin of replication so that the vectors can be used for expression in the COS cell transient expression system.

The mouse sequences were excised from the M13 based vectors described above as EcoRI fragments and cloned into either pEE6-hCMV-neo for the heavy chain and into EE6-hCMV-gpt for the light chain to yield vectors pJA136 and pJA135 respectively.

8. EXPRESSION OF cDNAS IN COS CELLS

Plasmids pJA135 and pJA136 were co-transfected into COS cells and supernatant from the transient expression experiment was shown to contain assembled antibody which bound to T-cell enriched lymphocytes. Metabolic labelling experiments using ³⁵S methionine showed expression and assembly of heavy and light chains.

9. CONSTRUCTION OF CHIMERIC GENES

Construction of chimeric genes followed a previously described strategy [Whittle et al (ref. 13)]. A restriction site near the 3' end of the variable domain sequence is identified and used to attach an oligonucleotide adapter coding for the remainder of the mouse variable region and a suitable restriction site for attachment to the constant region of choice.

9.1. LIGHT CHAIN GENE CONSTRUCTION

The mouse light chain cDNA sequence contains an Aval site near the 3' end of the variable region [Fig. 1(a)]. The majority of the sequence of the variable region was isolated as a 396 bp. EcoRI-Aval fragment. An oligonucleotide adapter was designed to replace the remainder of the 3'

region of the variable region from the Aval site and to include the 5' residues of the human constant region up to and including a unique NarI site which had been previously engineered into the constant region.

A HindIII site was introduced to act as a marker for insertion of the linker.

The linker was ligated to the VL fragment and the 413 bp EcoRI-NarI adapted fragment was purified from the ligation mixture.

The constant region was isolated as an NarI-BamHI fragment from an M13 clone NW361 and was ligated with the variable region DNA into an EcoRI/BamHI/CIP pSP65 treated vector in a three way reaction to yield plasmid JA143. Clones were isolated after transformation into E.coli and the linker and junction sequences were confirmed by the presence of the HindIII site and by DNA sequencing.

9.2 LIGHT CHAIN GENE CONSTRUCTION - VERSION 2

The construction of the first chimeric light chain gene produces a fusion of mouse and human amino acid sequences at the variable-constant region junction. In the case of the OKT3 light chain the amino acids at the chimera junction are:

.....Leu-Glu-Ile-Asn-Arg/	<u> </u>	-/Thr-Val-Ala	-Ala
	VARIABLE	CONSTANT	

This arrangement of sequence introduces a potential site for Asparagine (Asn) linked (N-linked) glycosylation at the V-C junction. Therefore, a second version of the chimeric light chain oligonucleotide adapter was designed in which the threonine (Thr), the first amino acid of the human constant region, was replaced with the equivalent amino acid from the mouse constant region, Alanine (Ala).

An internal HindIII site was not included in this adapter, to differentiate the two chimeric light chain genes.

The variable region fragment was isolated as a 376 bp EcoRI-AvaI fragment. The oligonucleotide linker was ligated to NarI cut pNW361 and then the adapted 396bp constant region was isolated after recutting the modified pNW361 with EcoRI. The variable region fragment and the modified constant region fragment were ligated directly into EcoRI/CIP treated pEB6hCMVneo to yield pJA137. Initially all clones examined had the insert in the incorrect orientation. Therefore, the insert was re-isolated and recloned to turn the insert round and yield plasmid pJA141. Several clones with the insert in the correct orientation were obtained and the adapter sequence of one was confirmed by DNA sequencing

9.3. HEAVY CHAIN GENE CONSTRUCTION

9.3.1. CHOICE OF HEAVY CHAIN GENE ISOTYPE

The constant region isotype chosen for the heavy chain was human IgG4.

9.3.2. GENE CONSTRUCTION

The heavy chain cDNA sequence showed a BstI site near the 3' end of the variable region [Fig. 2(a)]. The majority of the sequence of the variable region was isolated as a 426bp. EcoRI/CIP/BstI fragment. An oligonucleotide adapter was designated to replace the remainder of the 3' region of the variable region from the BstI site up to and including a unique HindIII site which had been previously engineered into the first two amino acids of the constant region. The linker was ligated to the V_H fragment and the EcoRI-HindIII adapted fragment was purified from the ligation mixture.

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The variable region was ligated to the constant region by cutting pJA91 with EcoRI and HindIII removing the intron fragment and replacing it with the V_H to yield pJA142. Clones were isolated after transformation into E.coli JM101 and the linker and junction sequences were confirmed by DNA sequencing. (N.B. The HindIII site is lost on cloning).

10. CONSTRUCTION OF CHIMERIC EXPRESSION VECTORS

10.1. neo AND gpt VECTORS

The chimeric light chain (version 1) was removed from pJA143 as an EcoRI fragment and cloned into EcoRI/CIP treated pEE6hCMVneo expression vector to yield pJA145. Clones with the insert in the correct orientation were identified by restriction mapping.

The chimeric light chain (version 2) was constructed as described above.

The chimeric heavy chain gene was isolated from pJA142 as a 2.5Kbp EcoRI/BamHI fragment and cloned into the EcoRI/BclI/CIP treated vector fragment of a derivative of pEE6hCMVgpt to yield plasmid pJA144.

10.2. GS SEPARATE VECTORS

GS versions of pJA141 and pJA144 were constructed by replacing the neo and gpt cassettes by a BamHI/SalI/CIP treatment of the plasmids, isolation of the vector fragment and ligation to a GS-containing fragment from the plasmid pRO49 to yield the light chain vector pJA179 and the heavy chain vector pJA180.

10.3. GS SINGLE VECTOR CONSTRUCTION

Single vector constructions containing the cL (chimeric light), cH (chimeric heavy) and GS genes on one plasmid in the order cL-cH-GS, or cH-cL-GS

and with transcription of the genes being head to tail e.g. cL>cH>GS were constructed. These plasmids were made by treating pJA179 or pJA180 with BamHI/CIP and ligating in a BglII/HindIII hCMV promoter cassette along with either the HindIII/BamHI fragment from pJA141 into pJA180 to give the cH-cL-GS plasmid pJA182 or the HindIII/BamHI fragment from pJA144 into pJA179 to give the cL-cH-GS plasmid pJA181.

11. EXPRESSION OF CHIMERIC GENES

11.1. EXPRESSION IN COS CELLS

The chimeric antibody plasmid pJA145 (cL) and pJA144 (cH) were co-transfected into COS cells and supernatant from the transient expression experiment was shown to contain assembled antibody which bound to the HUT 78 human T-cell line. Metabolic labelling experiments using ³⁵S methionine showed expression and assembly of heavy and light chains. However the light chain mobility seen on reduced gels suggested that the potential glycosylation site was being glycosylated. Expression in COS cells in the presence of tunicamycin showed a reduction in size of the light chain to that shown for control chimeric antibodies and the OKT3 mouse light chain. Therefore JA141 was constructed and expressed. In this case the light chain did not show an aberrant mobility or a size shift in the presence or absence of tunicamycin. This second version of the chimeric light chain, when expressed in association with chimeric heavy (cH) chain, produced antibody which showed good binding to HUT 78 cells. In both cases antigen binding was equivalent to that of the mouse antibody.

11.2 **EXPRESSION IN CHINESE HAMSTER OVARY (CHO) CELLS**
Stable cell lines have been prepared from plasmids PJA141/pJA144 and from pJA179/pJA180, pJA181 and pJA182 by transfection into CHO cells.

12. **CDR-GRAFTING**

The approach taken was to try to introduce sufficient mouse residues into a human variable region framework to generate antigen binding activity comparable to the mouse and chimeric antibodies.

12.1. **VARIABLE REGION ANALYSIS**

From an examination of a small database of structures of antibodies and antigen-antibody complexes it is clear that only a small number of antibody residues make direct contact with antigen. Other residues may contribute to antigen binding by positioning the contact residues in favourable configurations and also by inducing a stable packing of the individual variable domains and stable interaction of the light and heavy chain variable domains. The residues chosen for transfer can be identified in a number of ways:

- (a) By examination of antibody X-ray crystal structures the antigen binding surface can be predominantly located on a series of loops, three per domain, which extend from the B-barrel framework.
- (b) By analysis of antibody variable domain sequences regions of hypervariability [termed the Complementarity Determining Regions (CDRs) by Wu and Kabat (ref. 5)] can be identified. In the most but not all cases these CDRs correspond to, but extend a short way beyond, the loop regions noted above.

(c) Residues not identified by (a) and (b) may contribute to antigen binding directly or indirectly by affecting antigen binding site topology, or by inducing a stable packing of the individual variable domains and stabilising the inter-variable domain interaction. These residues may be identified either by superimposing the sequences for a given antibody on a known structure and looking at key residues for their contribution, or by sequence alignment analysis and noting "idiosyncratic" residues followed by examination of their structural location and likely effects.

12.1.1. LIGHT CHAIN

Figure 3 shows an alignment of sequences for the human framework region RE1 and the OKT3 light variable region. The structural loops (LOOP) and CDRs (KABAT) believed to correspond to the antigen binding region are marked. Also marked are a number of other residues which may also contribute to antigen binding as described in 13.1(c). Above the sequence in Figure 3 the residue type indicates the spatial location of each residue side chain, derived by examination of resolved structures from X-ray crystallography analysis. The key to this residue type designation is as follows:

N - near to CDR (From X-ray Structures)	
P - Packing	B - Buried Non-Packing
S - Surface	E - Exposed
I - Interface	* - Interface
- Packing/Part Exposed	
? - Non-CDR Residues which may require to be left as Mouse sequence.	

Residues underlined in Figure 3 are amino acids. RE1 was chosen as the human framework because the light chain is a kappa chain and the kappa variable regions show higher homology with the mouse sequences than a lambda light variable region, e.g. KOL (see below). RE1 was chosen in preference to another kappa light chain because the X-ray structure of the light chain has been determined so that a structural examination of individual residues could be made.

12.1.2. HEAVY CHAIN

Similarly Figure 4 shows an alignment of sequences for the human framework region KOL and the OKT3 heavy variable region. The structural loops and CDRs believed to correspond to the antigen binding region are marked. Also marked are a number of other residues which may also contribute to antigen binding as described in 12.1(c). The residue type key and other indicators used in Figure 4 are the same as those used in Figure 3. KOL was chosen as the heavy chain framework because the X-ray structure has been determined to a better resolution than, for example, NEWM and also the sequence alignment of OKT3 heavy variable region showed a slightly better homology to KOL than to NEWM.

12.2. DESIGN OF VARIABLE GENES

The variable region domains were designed with mouse variable region optimal codon usage [Grantham and Perrin (ref. 15)] and used the B72.3 signal sequences [Whittle et al (ref. 13)]. The sequences were designed to be attached to the constant region in the same way as for the chimeric genes described above. Some constructs contained the "Kozak consensus sequence" [Kozak (ref. 16)] directly linked to the 5' of the signal

sequence in the gene. This sequence motif is believed to have a beneficial role in translation initiation in eukaryotes.

12.3. GENE CONSTRUCTION

To build the variable regions, various strategies are available. The sequence may be assembled by using oligonucleotides in a manner similar to Jones et al (ref. 17) or by simultaneously replacing all of the CDRs or loop regions by oligonucleotide directed site specific mutagenesis in a manner similar to Verhoeven et al (ref. 2). Both strategies were used and a list of constructions is set out in Tables 1 and 2 and Figures 4 and 5. It was noted in several cases that the mutagenesis approach led to deletions and rearrangements in the gene being remodelled, while the success of the assembly approach was very sensitive to the quality of the oligonucleotides.

13. CONSTRUCTION OF EXPRESSION VECTORS

Genes were isolated from M13 or SP65 based intermediate vectors and cloned into pEE6hCMVneo for the light chains and pEE6hCMVgpt for the heavy chains in a manner similar to that for the chimeric genes as described above.

TABLE 1 CDR-GRAFTED GENE CONSTRUCTS

CODE	MOUSE SEQUENCE CONTENT	METHOD OF CONSTRUCTION	KOZAK SEQUENCE	
			-	+
LIGHT CHAIN ALL HUMAN FRAMEWORK RE1				
121	26-32, 50-56, 91-96 inclusive	SDM and gene assembly	+	n.d.
121A	26-32, 50-56, 91-96 inclusive +1, 3, 46, 47	Partial gene assembly	n.d.	+
121B	26-32, 50-56, 91-96 inclusive + 46, 47	Partial gene assembly	n.d.	+
221	24-24, 50-56, 91-96 inclusive	Partial gene assembly	+	+
221A	24-34, 50-56, 91-96 inclusive +1, 3, 46, 47	Partial gene assembly	+	+
221B	24-34, 50-56, 91-96 inclusive +1, 3	Partial gene assembly	+	+
221C	24-34, 50-56, 91-96 inclusive	Partial gene assembly	+	+
HEAVY CHAIN ALL HUMAN FRAMEWORK KOL				
121	26-32, 50-56, 95-100B inclusive	Gene assembly	n.d.	+
131	26-32, 50-58, 95-100B inclusive	Gene assembly	n.d.	+
141	26-32, 50-65, 95-100B inclusive	Partial gene assembly	+	n.d.
321	26-35, 50-56, 95-100B inclusive	Partial gene assembly	+	n.d.
331	26-35, 50-58, 95-100B inclusive	Partial gene assembly	+	
		Gene assembly		+
341	26-35, 50-65, 95-100B inclusive	SDM	+	
		Partial gene assembly		+
341A	26-35, 50-65, 95-100B inclusive +6, 23, 24, 48, 49, 71, 73, 76, 78, 88, 91 (+63 = human)	Gene assembly	n.d.	+
341B	26-35, 50-65, 95-100B inclusive + 48, 49, 71, 73, 76, 78, 88, 91 (+63 + human)	Gene assembly	n.d.	+

KEY

n.d. not done
 SDM Site directed mutagenesis
 Gene assembly Variable region assembled entirely from oligonucleotides
 Partial gene assembly Variable region assembled by combination of restriction fragments either from other genes originally created by SDM and gene assembly or by oligonucleotide assembly of part of the variable region and reconstruction with restriction fragments from other genes originally created by SDM and gene assembly

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14. EXPRESSION OF CDR-GRAFTED GENES

14.1. PRODUCTION OF ANTIBODY CONSISTING OF GRAFTED LIGHT (gL) CHAINS WITH MOUSE HEAVY (mH) OR CHIMERIC HEAVY (cH) CHAINS

All gL chains, in association with mH or cH produced reasonable amounts of antibody.

Insertion of the Kozak consensus sequence at a position 5' to the ATG (kgL constructs) however, led to a 2-5 fold improvement in net expression. Over an extended series of experiments expression levels were raised from approximately 200ng/ml to approximately 500 ng/ml for kgL/cH or kgL/mH combinations.

When direct binding to antigen on HUT 78 cells was measured, a construct designed to include mouse sequence based on loop length (gL121) did not lead to active antibody in association with mH or cH. A construct designed to include mouse sequence based on Kabat CDRs (gL221) demonstrated some weak binding in association with mH or cH. However, when framework residues 1, 3, 46, 47 were changed from the human to the murine OKT3 equivalents based on the arguments outlined in Section 12.1 antigen binding was demonstrated when both of the new constructs, which were termed 121A and 221A were co-expressed with cH. When the effects of these residues were examined in more detail, it appears that residues 1 and 3 are not major contributing residues as the product of the gL221B gene shows little detectable binding activity in association with cH. The light chain product of gL221C, in which mouse sequences are present at 46 and 47, shows good binding activity in association with cH.

14.2 PRODUCTION OF ANTIBODY CONSISTING OF GRAFTED HEAVY (gH) CHAINS WITH MOUSE LIGHT (mL) OR CHIMERIC LIGHT (cL) CHAINS

Expression of the gH genes proved to be more difficult to achieve than for gL. First, inclusion of the Kozak sequence appeared to have no marked effect on expression of gH genes. Expression appears to be slightly improved but not to the same degree as seen for the grafted light chain.

Also, it proved difficult to demonstrate production of expected quantities of material when the loop choice (amino acid 26-32) for CDR1 is used, e.g. gH121, 131, 141 and no conclusions can be drawn about these constructs.

Moreover, co-expression of the gH341 gene with cL or mL has been variable and has tended to produce lower amounts of antibody than the cH/cL or mH/mL combinations. The alterations to gH341 to produce gH341A and gH341B lead to improved levels of expression.

This may be due either to a general increase in the fraction of mouse sequence in the variable region, or to the alteration at position 63 where the residue is returned to the human amino acid Valine (Val) from Phenylalanine (Phe) to avoid possible internal packing problems with the rest of the human framework. This arrangement also occurs in gH331 and gH321.

When gH321 or gH331 were expressed in association with cL, antibody was produced but antibody binding activity was not detected.

When the more conservative gH341 gene was used antigen binding could be detected in association with cL or mL, but the activity was only marginally above the background level.

When further mouse residues were substituted based on the arguments in 12.1, antigen binding could be clearly demonstrated for the antibody produced when kgH341A and kgH341B were expressed in association with cL.

14.3 PRODUCTION OF FULLY CDR-GRAFTED ANTIBODY

The kgL221A gene was co-expressed with kgH341, kgH341A or kgH341B. For the combination kgH221A/kgH341 very little material was produced in a normal COS cell expression.

For the combinations kgL221A/kgH341A or kgH221A/kgH341B amounts of antibody similar to gL/cH was produced.

In several experiments no antigen binding activity could be detected with kgH221A/gH341 or kgH221A/kgH341 combinations, although expression levels were very low.

Antigen binding was detected when kgL221A/kgH341A or kgH221A/kgH341B combinations were expressed.

In the case of the antibody produced from the kgL221A/kgH341A combination the antigen binding was very similar to that of the chimeric antibody.

An analysis of the above results is given below.

15. DISCUSSION OF CDR-GRAFTING RESULTS

In the design of the fully humanised antibody the aim was to transfer the minimum number of mouse amino acids that would confer antigen binding onto a human antibody framework.

15.1. LIGHT CHAIN

15.1.1. EXTENT OF THE CDRs

For the light chain the regions defining the loops known from structural studies of other antibodies to contain the antigen contacting residues, and

those hypervariable sequences defined by Kabat et al (refs. 4 and 5) as Complementarity Determining Regions (CDRs) are equivalent for CDR2. For CDR1 the hypervariable region extends from residues 24-34 inclusive while the structural loop extends from 26-32 inclusive. In the case of OKT3 there is only one amino acid difference between the two options, at amino acid 24, where the mouse sequence is a serine and the human framework RE1 has glutamine. For CDR3 the loop extends from residues 91-96 inclusive while the Kabat hypervariability extends from residues 89-97 inclusive. For OKT3 amino acids 89, 90 and 97 are the same between OKT3 and RE1 (Fig. 3). When constructs based on the loop choice for CDR1 (gL121) and the Kabat choice (gL221) were made and co-expressed with mH or cH no evidence for antigen binding activity could be found for gL121, but trace activity could be detected for the gL221, suggesting that a single extra mouse residue in the grafted variable region could have some detectable effect. Both gene constructs were reasonably well expressed in the transient expression system.

15.1.2. FRAMEWORK RESIDUES

The remaining framework residues were then further examined, in particular amino acids known from X-ray analysis of other antibodies to be close to the CDRs and also those amino acids which in OKT3 showed differences from the consensus framework for the mouse subgroup (subgroup VI) to which OKT3 shows most homology. Four positions 1, 3, 46 and 47 were identified and their possible contribution was examined by substituting the mouse amino acid for the human amino acid at each position. Therefore gL221A (gL221 + D1Q, Q3V, L46R, L47W,

see Figure 3 and Table 1) was made, cloned in EE6hCMVneo and co-expressed with cH (pJA144). The resultant antibody was well expressed and showed good binding activity. When the related genes gL221B (gL221 + D1Q, Q3V) and gL221C (gL221 + L46R, L47W) were made and similarly tested, while both genes produced antibody when co-expressed with cH, only the gL221C/cH combination showed good antigen binding. When the gL121A (gL121 + D1Q, Q3V, L46R, L47W) gene was made and co-expressed with cH, antibody was produced which also bound to antigen.

15.2. HEAVY CHAIN

15.2.1. EXTENT OF THE CDRs

For the heavy chain the loop and hypervariability analyses agree only in CDR3. For CDR1 the loop region extends from residues 26-32 inclusive whereas the Kabat CDR extends from residues 31-35 inclusive. For CDR2 the loop region is from 50-58 inclusive while the hypervariable region covers amino acids 50-65 inclusive. Therefore humanised heavy chains were constructed using the framework from antibody KOL and with various combinations of these CDR choices, including a shorter choice for CDR2 of 50-56 inclusive as there was some uncertainty as to the definition of the end point for the CDR2 loop around residues 56 to 58. The genes were co-expressed with mL or cL initially. In the case of the gH genes with loop choices for CDR1 e.g. gH121, gH131, gH141 very little antibody was produced in the culture supernatants. As no free light chain was detected it was presumed that the antibody was being made and assembled inside the cell but that the heavy chain was aberrant in some way, possibly incorrectly folded, and therefore the antibody was

being degraded internally. In some experiments trace amounts of antibody could be detected in ³⁵S labelling studies.

As no net antibody was produced, analysis of these constructs was not pursued further.

When, however, a combination of the loop choice and the Kabat choice for CDR1 was tested (mouse amino acids 26-35 inclusive) and in which residues 31 (Ser to Arg), 33 (Ala to Thr), and 35 (Tyr to His) were changed from the human residues to the mouse residue and compared to the first series, antibody was produced for gH321, kgH331 and kgH341 when co-expressed with cL. Expression was generally low and could not be markedly improved by the insertion of the Kozak consensus sequence 5' to the ATG of the signal sequence of the gene, as distinct from the case of the gL genes where such insertion led to a 2-5 fold increase in net antibody production. However, only in the case of gH341/mL or kgH341/cL could marginal antigen binding activity be demonstrated. When the kgH341 gene was co-expressed with kgL221A, the net yield of antibody was too low to give a signal above the background level in the antigen binding assay.

15.2.2. FRAMEWORK RESIDUES

As in the case of the light chain the heavy chain frameworks were re-examined. Possibly because of the lower initial homology between the mouse and human heavy variable domains compared to the light chains, more amino acid positions proved to be of interest. Two genes kgH341A and kgH341B were constructed, with 11 or 8 human residues respectively substituted by mouse residues compared to gH341, and with the CDR2 residue 63 returned to the human amino acid potentially to

improve domain packing. Both showed antigen binding when combined with cL or kgL221A, the kgH341A gene with all 11 changes appearing to be the superior choice.

15.3 INTERIM CONCLUSIONS

It has been demonstrated, therefore, for OKT3 that to transfer antigen binding ability to the humanised antibody, mouse residues outside the CDR regions defined by the Kabat hypervariability or structural loop choices are required for both the light and heavy chains. Fewer extra residues are needed for the light chain, possibly due to the higher initial homology between the mouse and human kappa variable regions.

Of the changes seven (1 and 3 from the light chain and 6, 23, 71, 73 and 76 from the heavy chain) are predicted from a knowledge of other antibody structures to be either partly exposed or on the antibody surface. It has been shown here that residues 1 and 3 in the light chain are not absolutely required to be the mouse sequence; and for the heavy chain the gH341B heavy chain in combination with the 221A light chain generated only weak binding activity. Therefore the presence of the 6, 23 and 24 changes are important to maintain a binding affinity similar to that of the murine antibody. It was important, therefore, to further study the individual contribution of the other 8 mouse residues of the kgH341A gene compared to kgH341.

16. FURTHER CDR-GRAFTING EXPERIMENTS

Additional CDR-grafted heavy chain genes were prepared substantially as described above. With reference to Table 2 the further heavy chain genes were based upon the gh341 (plasmid pJA178) and

gH341A (plasmid pJA185) with either mouse OKT3 or human KOL residues at 6, 23, 24, 48, 49, 63, 71, 73, 76, 78, 88 and 91, as indicated. The CDR-grafted light chain genes used in these further experiments were gL221, gL221A, gL221B and gL221C as described above.

TABLE 2

OKT3 HEAVY CHAIN CDR GRAFTS

1. gH341 and derivatives

RES NUM	6	23	24	48	49	63	71	73	76	78	88	91	
OKT3vh	<u>Q</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>F</u>	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>A</u>	<u>Y</u>	
gH341	E	S	S	V	A	F	R	N	N	L	G	F	JA178
gH341A	<u>Q</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>A</u>	<u>Y</u>	JA185
gH341E	<u>Q</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>G</u>	<u>G</u>	JA198
gH341*	<u>Q</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>N</u>	<u>A</u>	<u>G</u>	<u>F</u>	JA207
gH341*	<u>Q</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>R</u>	<u>N</u>	<u>N</u>	<u>A</u>	<u>G</u>	<u>F</u>	JA209
gH341D	<u>Q</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>N</u>	<u>L</u>	<u>G</u>	<u>F</u>	JA197
gH341*	<u>Q</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>R</u>	<u>N</u>	<u>N</u>	<u>L</u>	<u>G</u>	<u>F</u>	JA199
gH341C	<u>Q</u>	<u>K</u>	<u>A</u>	<u>V</u>	<u>A</u>	<u>F</u>	<u>R</u>	<u>N</u>	<u>N</u>	<u>L</u>	<u>G</u>	<u>F</u>	JA184
gH341*	<u>Q</u>	<u>S</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>A</u>	<u>Y</u>	JA203
gH341*	E	S	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>A</u>	<u>Y</u>	JA205
gH341B	E	S	S	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>A</u>	<u>Y</u>	JA183
gH341*	<u>Q</u>	<u>S</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>G</u>	<u>F</u>	JA204
gH341*	E	S	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>G</u>	<u>F</u>	JA206
gH341*	<u>Q</u>	<u>S</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>N</u>	<u>A</u>	<u>G</u>	<u>F</u>	JA208
KOL	E	S	S	V	A		R	N	N	L	G	F	

OKT3 LIGHT CHAIN CDR GRAFTS

2. gL221 and derivatives

RES NUM	1	3	46	47	
OKT3vl	<u>Q</u>	<u>V</u>	<u>R</u>	<u>W</u>	
GL221	D	Q	L	L	DA221
gL221A	<u>Q</u>	<u>V</u>	<u>R</u>	<u>W</u>	DA221A
gL221B	<u>Q</u>	<u>V</u>	<u>L</u>	<u>L</u>	DA221B
GL221C	D	Q	<u>R</u>	<u>W</u>	DA221C
REL	D	Q	L	L	

MURINE RESIDUES ARE UNDERLINED

The CDR-grafted heavy and light chain genes were co-expressed in COS cells either with one another in various combinations but also with the corresponding murine and chimeric heavy and light chain genes substantially as described above. The resultant antibody products were then assayed in binding and blocking assays with HPB-ALL cells as described above.

The results of the assays for various grafted heavy chains co-expressed with the gL221C light chain are given in Figures 7 and 8 (for the JA184, JA185, JA197 and JA198 constructs - see Table 2), in Figure 9 (for the JA183, JA184, JA185 and JA197 constructs) in Figure 10 (for the chimeric, JA185, JA199, JA204, JA205, JA207, JA208 and JA209 constructs) and in Figure 11 (for the JA183, JA184, JA185, JA198, JA203, JA205 and JA206 constructs).

The basic grafted product without any human to murine changes in the variable frameworks, i.e. gL221 co-expressed with gh341 (JA178), and also the "fully grafted" product, having most human to murine changes in the grafted heavy chain framework, i.e. gL221C co-expressed with gh341A (JA185), were assayed for relative binding affinity in a competition assay against murine OKT3 reference standard, using HPB-ALL cells. The assay used was as described above in section 3.3. The results obtained are given in Figure 12 for the basic grafted product and in Figure 13 for the fully grafted product. These results indicate that the basic grafted product has negligible binding ability as compared with the OKT3 murine reference standard; whereas the "fully grafted" product has a binding ability very similar to that of the OKT3 murine reference standard.

The binding and blocking assay results indicate the following:

The JA198 and JA207 constructs appear to have the best binding characteristics and similar binding abilities, both substantially the same as the chimeric and fully grafted gH341A products. This indicates that positions 88 and 91 and position 76 are not highly critical for maintaining the OKT3 binding ability; whereas at least some of positions 6, 23, 24, 48, 49, 71, 73 and 78 are more important.

This is borne out by the finding that the JA209 and JA199, although of similar binding ability to one another, are of lower binding ability than the JA198 and JA207 constructs. This indicates the importance of having mouse residues at positions 71, 73 and 78, which are either completely or partially human in the JA199 and JA209 constructs respectively.

Moreover, on comparing the results obtained for the JA205 and JA183 constructs it is seen that there is a decrease in binding going from the JA205 to the JA183 constructs. This indicates the importance of retaining a mouse residue at position 23, the only position changed between JA205 and JA183.

These and other results lead us to the conclusion that of the 11 mouse framework residues used in the gH341A (JA185) construct, it is important to retain mouse residues at all of positions 6, 23, 24, 48 and 49, and possibly for maximum binding affinity at 71, 73 and 78.

Similar Experiments were carried out to CDR-graft a number of the rodent antibodies including antibodies having specificity for CD4 (OKT4), ICAM-1 (R6-5), TAG72 (B72.3), and TNF α (61E71, 101.4, hTNF1, hTNF2 and hTNF3).

EXAMPLE 2CDR-GRAFTING OF A MURINE ANTI-CD4 T CELL
RECEPTOR ANTIBODY, OKT4A

Anti OKT4A CDR-grafted heavy and light chain genes were prepared, expressed and tested substantially as described above in Example 1 for CDR-grafted OKT3. The CDR grafting of OKT4A is described in detail in Ortho patent application PCT/GB 90..... of even date herewith entitled "Humanised Antibodies". The disclosure of this Ortho patent application PCT/GB 90 is incorporated herein by reference. A number of CDR-grafted OKT4 antibodies have been prepared. Presently the CDR-grafted OKT4A of choice is the combination of the grafted light chain LCDR2 and the grafted heavy chain HCDR10.

THE LIGHT CHAIN

The human acceptor framework used for the grafted light chains was RE1. The preferred LCDR2 light chain has human to mouse changes at positions 33, 34, 38, 49 and 89 in addition to the structural loop CDRs. Of these changed positions, positions 33, 34 and 89 fall within the preferred extended CDRs of the present invention (positions 33 and 34 in CDR1 and position 89 in CDR3). The human to murine changes at positions 38 and 49 corresponds to positions at which the amino acid residues are preferably donor murine amino acid residues in accordance with the present invention. A comparison of the amino acid sequences of the donor murine light chain variable domain and the RE1 human acceptor light chain variable further reveals that the murine and human residues are identical at all of positions 46, 48 and 71 and at all of positions 2, 4, 6, 35, 36, 44, 47, 62, 64-69, 85, 87, 98, 99 and 101 and 102. However the amino acid residue at position 58 in LCDR2 is

the human RE1 framework residue not the mouse OKT4 residue as would be preferred in accordance with the present invention.

THE HEAVY CHAIN

The human acceptor framework used for the grafted heavy chains was KOL.

The preferred CDR graft HCDR10 heavy chain has human to mouse changes at positions 24, 35, 57, 58, 60, 88 and 91 in addition to the structural loop CDRs.

Of these positions, positions 35 (CDR1) and positions 57, 58 and 60 (CDR2) fall within the preferred extended CDRs of the present invention. Also the human to mouse change at position 24 corresponds to a position at which the amino acid residue is a donor murine residue in accordance with the present invention. Moreover, the human to mouse changes at positions 88 and 91 correspond to positions at which the amino acid residues are optionally donor murine residues.

Moreover, a comparison of the murine OKT4A and human KOL heavy chain variable amino acid sequences reveals that the murine and human residues are identical at all of positions 23, 49, 71, 73 and 78 and at all of positions 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.

Thus the OKT4A CDR-grafted heavy chain HCDR10 corresponds to a particularly preferred embodiment according to the present invention.

EXAMPLE 3

CDR-GRAFTING OF AN ANTI-MUCIN SPECIFIC MURINE
ANTIBODY, B72.3

The cloning of the genes coding for the anti-mucin specific murine monoclonal antibody B72.3 and the preparation of B72.3 mouse-human chimeric antibodies has been described previously (ref. 13 and WO 89/01783). CDR-grafted versions of B72.3 were prepared as follows.

(a) B72.3 Light Chain

CDR-grafting of this light chain was accomplished by direct transfer of the murine CDRs into the framework of the human light chain RE1.

The regions transferred were:

<u>CDR Number</u>	<u>Residues</u>
1	24-34
2	50-56
3	90-96

The activity of the resulting grafted light chain was assessed by co-expression in COS cells, of genes for the combinations:

B72.3 cH/B72.3 cL
and B72.3 cH/B72.3 gL

Supernatants were assayed for antibody concentration and for the ability to bind to microtitre plates coated with mucin. The results obtained indicated that, in combination with the B72.3 cH chain, B72.3 cL and B72.3 gL had similar binding properties.

Comparison of the murine B72.3 and RE1 light chain amino acid sequences reveals that the residues are identical at positions 46, 58 and 71 but are different at position 48.

Thus changing the human residue to the donor mouse residue at position 48 may further improve the binding characteristics of the CDR-grafted light chain, (B72.3 gL) in accordance with the present invention.

(b) B72.3 heavy chain

i. Choice of framework

At the outset it was necessary to make a choice of human framework. Simply put, the question was as follows: Was it necessary to use the framework regions from an antibody whose crystal structure was known or could the choice be made on some other criteria?

For B72.3 heavy chain, it was reasoned that, while knowledge of structure was important, transfer of the CDRs from mouse to human frameworks might be facilitated if the overall homology between the donor and receptor frameworks was maximised.

Comparison of the B72.3 heavy chain sequence with those in Kabat (ref. 4) for human heavy chains showed clearly that B72.3 had poor homology for KOL and NEWM (for which crystal structures are available) but was very homologous to the heavy chain for EU.

On this basis, EU was chosen for the CDR-grafting and the following residues transferred as CDRs.

<u>CDR Number</u>	<u>Residues</u>
1	27-36
2	50-63
3	93-102

Also it was noticed that the FR4 region of EU was unlike that of any other human (or mouse) antibody. Consequently, in the grafted heavy chain genes this was also changed to produce a "consensus" human sequence. (Preliminary experiments showed that grafted heavy chain genes containing the EU FR4 sequence expressed very poorly in transient expression systems.)

ii. Results with grafted heavy chain genes

Expression of grafted heavy chain genes containing all human framework regions with either gL or cL genes produced a grafted antibody with little ability to bind to mucin. The grafted antibody had about 1% the activity of the chimeric antibody.

In these experiments, however, it was noted that the activity of the grafted antibody could be increased to ~ 10% of B72.3 by exposure to pHs of 2-3.5.

This observation provided a clue as to how the activity of the grafted antibody could be improved without acid treatment. It was postulated that acid exposure brought about the protonation of an acidic residue (pKa of aspartic acid = 3.86 and of glutamine acid = 4.25) which in turn caused a change in structure of the CDR loops, or allowed better access of antigen.

From comparison of the sequences of B72.3 (ref. 13) and EU (refs. 4 and 5), it was clear that, in going from the mouse to human frameworks, only two positions had been changed in such a way that acidic residues had been introduced. These

positions are at residues 73 and 81, where K to E and Q to E changes had been made, respectively.

Which of these positions might be important was determined by examining the crystal structure of the KOL antibody. In KOL heavy chain, position 81 is far removed from either of the CDR loops.

Position 73, however, is close to both CDRs 1 and 3 of the heavy chain and, in this position it was possible to envisage that a K to E change in this region could have a detrimental effect on antigen binding.

iii. Framework changes in B72.3 gH gene

On the basis of the above analysis, E73 was mutated to a lysine (K). It was found that this change had a dramatic effect on the ability of the grafted Ab to bind to mucin. Further the ability of the grafted B72.3 produced by the mutated gH/gL combination to bind to mucin was similar to that of the B72.3 chimeric antibody.

iv. Other framework changes

In the course of the above experiments, other changes were made in the heavy chain framework regions. Within the accuracy of the assays used, none of the changes, either alone or together, appeared beneficial.

v. Other

All assays used measured the ability of the grafted Ab to bind to mucin and, as a whole, indicated that the single framework change at position 73 is sufficient to generate an antibody with similar binding properties to B72.3.

Comparison of the B72.3 murine and EU heavy chain sequences reveals that the mouse and human residues are identical at positions 23, 24, 71 and 78.

Thus the mutated CDR-grafted B72.3 heavy chain corresponds to a preferred embodiment of the present invention.

EXAMPLE 4

CDR-GRAFTING OF A MURINE ANTI-ICAM-1 MONOCLONAL ANTIBODY

A murine antibody, R6-5-D6 (EP 0314863) having specificity for Intercellular Adhesion Molecule 1 (ICAM-1) was CDR-grafted substantially as described above in previous examples. This work is described in greater detail in co-pending application, British Patent Application No. 9009549.8, the disclosure of which is incorporated herein by reference.

The human EU framework was used as the acceptor framework for both heavy and light chains. The CDR-grafted antibody currently of choice is provided by co-expression of grafted light chain gL221A and grafted heavy chain gH341D which has a binding affinity for ICAM 1 of about 75% of that of the corresponding mouse-human chimeric antibody.

LIGHT CHAIN

gL221A has murine CDRs at positions 24-34 (CDR1), 50-56 (CDR2) and 89-97 (CDR3). In addition several framework residues are also the murine amino acid. These residues were chosen after consideration of the possible contribution of these residues to domain packing and stability of the conformation of the antigen binding region. The residues which have been retained as mouse are at positions 2, 3, 48 (?), 60, 84, 85 and 87. Comparison of the murine anti-ICAM 1 and human EU light chain amino acid sequences reveals that the murine and human residues are identical at positions 46, 58 and 71.

HEAVY CHAIN

gH341D has murine CDRs at positions 26-35 (CDR1), 50-56 (CDR2) and 94-100B (CDR3). In addition murine residues were used in gH341D at positions 24, 48, 69, 71, 73, 80, 88 and 91. Comparison of the murine anti-ICAM 1 and human EU heavy chain amino acid sequences are identical at positions 23, 49 and 78.

EXAMPLE 5

CDR-Grafting of murine anti-TNF α antibodies

A number of murine anti-TNF α monoclonal antibodies were CDR-grafted substantially as described above in previous examples. These antibodies include the murine monoclonal antibodies designated 61 E71, hTNF1, hTNF3 and 101.4. A brief summary of the CDR-grafting of each of these antibodies is given below.

61E71

A similar analysis as described above (Example 1, Section 12.1.) was done for 61E71 and for the heavy chain 10 residues were identified at 23, 24, 48, 49, 68, 69, 71, 73, 75 and 88 as residues to potentially retain as murine. The human frameworks chosen for CDR-grafting of this antibody, and the hTNF3 and 101.4 antibodies were RE1 for the light chain and KOL for the heavy chain. Three genes were built, the first of which contained 23, 24, 48, 49, 71 and 73 [gH341(6)] as murine residues. The second gene also had 75 and 88 as murine residues [gH341(8)] while the third gene additionally had 68, 69, 75 and 88 as murine residues [gH341(10)]. Each was co-expressed with gL221, the minimum grafted light chain (CDRs only). The gL221/gH341(6) and gL221/gH341(8) antibodies both bound as well to TNF as murine 61E71. The gL221/gH341(10) antibody did not express and this combination was not taken further. Subsequently the gL221/gH341(6) antibody was assessed in an L929 cell competition assay in which the antibody competes against the TNF receptor on L929 cells for binding to TNF in solution. In this assay the gL221/gH341(6) antibody was approximately 10% as active as murine 61E71.

hTNF1

hTNF1 is a monoclonal antibody which recognises an epitope on human TNF- . The EU human framework was used for CDR-grafting of both the heavy and light variable domains.

Heavy Chain

In the CDR-grafted heavy chain (ghTNF1) mouse CDRs were used at positions 26-35 (CDR1), 50-65 (CDR2) and 95-102 (CDR3). Mouse residues were also used in the frameworks at positions 48, 67, 69, 71, 73, 76, 89, 91, 94 and 108. Comparison of the TNF1 mouse and EU human heavy chain residues reveals that these are identical at positions 23, 24, 29 and 78.

Light Chain

In the CDR-grafted light chain (gLhTNF1) mouse CDRs were used at positions 24-34 (CDR1), 50-56 (CDR2) and 89-97 (CDR3). In addition mouse residues were used in the frameworks at positions 3, 42, 48, 49, 83, 106 and 108. Comparison of the hTNF1 mouse and EU human light chain residues reveals that these are identical at positions 46, 58 and 71.

The grafted hTNF1 heavy chain was co-expressed with the chimeric light chain and the binding ability of the product compared with that of the chimeric light chain/chimeric heavy chain product in a TNF binding assay. The grafted heavy chain product appeared to have binding ability for TNF slightly better than the fully chimeric product.

Similarly, a grafted heavy chain/grafted light chain product was co-expressed and compared with the fully chimeric product and found to have closely similar binding properties to the latter product.

hTNF3

hTNF3 recognises an epitope on human TNF- α . The sequence of hTNF3 shows only 21 differences compared to 61E71 in the light and heavy chain variable regions, 10 in the light chain (2 in the CDRs at positions 50, 96 and 8 in the framework at 1, 19, 40, 45, 46, 76, 103 and 106) and 11 in the heavy chain (3 in the CDR regions at positions 52, 60 and 95 and 8 in the framework at 1, 10, 38, 40, 67, 73, 87 and 105). The light and heavy chains of the 61E71 and hTNF3 chimeric antibodies can be exchanged without loss of activity in the direct binding assay. However 61E71 is an order of magnitude less able to compete with the TNF receptor on L929 cells for TNF-a compared to hTNF3. Based on the 61E71 CDR grafting data gL221 and gH341(+23, 24, 48, 49 71 and 73 as mouse) genes have been built for hTNF3 and tested and the resultant grafted antibody binds well to TNF-a, but competes very poorly in the L929 assay. It is possible that in this case also the framework residues identified for OKT3 programme may improve the competitive binding ability of this antibody.

101.4

101.4 is a further murine monoclonal antibody able to recognise human TNF-a. The heavy chain of this antibody shows good homology to KOL and so the CDR-grafting has been based on RE1 for the light chain and KOL for the heavy chain. Several grafted heavy chain genes have been constructed with conservative choices for the CDR's (gH341) and which have one or a small number of non-CDR residues at positions 73, 78 or 77-79 inclusive, as the mouse amino acids. These have been co-expressed with cL or gL221. In all cases binding to TNF equivalent to the chimeric antibody is seen and when co-expressed with cL the resultant antibodies are able to compete well in the L929 assay. However, with gL221 the resultant antibodies

are at least an order of magnitude less able to compete for TNF against the TNF receptor on L929 cells.

Mouse residues at other positions in the heavy chain, for example, at 23 and 24 together or at 76 have been demonstrated to provide no improvement to the competitive ability of the grafted antibody in the L929 assay.

A number of other antibodies including antibodies having specificity for interleukins e.g. IL1 and cancer markers such as carcinoembryonic antigen (CEA) e.g. the monoclonal antibody A5B7 (ref. 21), have been successfully CDR-grafted according to the present invention.

It will be appreciated that the foregoing examples are given by way of illustration only and are not intended to limit the scope of the claimed invention. Changes and modifications may be made to the methods described whilst still falling within the spirit and scope of the invention.

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CLAIMS

1. A CDR-grafted antibody heavy chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 6, 23 and/or 24, 48 and/or 49, 71 and/or 73, 75 and/or 76 and/or 78 and 88 and/or 91.
2. A CDR-grafted heavy chain according to Claim 1 comprising donor residues at positions 23, 24, 49, 71, 73 and 78, or at positions 23, 24 and 49.
3. A CDR-grafted heavy chain according to Claim 2 comprising donor residues at positions 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.
4. A CDR-grafted heavy chain according to Claim 2 or 3, comprising donor residues at one, some or all of positions:
1 and 3,
69 (if 48 is different between donor and acceptor),
38 and 46 (if 48 is the donor residue),
67,
82 and 18 (if 67 is the donor residue),
91, and
any one or more of 9, 11, 41, 87, 108, 110 and 112.
5. A CDR-grafted heavy chain according to any of the preceding comprising donor CDRs at positions 26-35, 50-65 and 95-100.
6. A CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 1 and/or 3 and 46 and/or 47.

7. A CDR-grafted light chain according to Claim 6 comprising donor residues at positions 46 and 47.
8. A CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 46, 48, 58 and 71.
9. A CDR-grafted light chain according to Claim 8 comprising donor residues at positions 46, 48, 58 and 71.
10. A CDR-grafted light chain according to Claim 8 or 9, comprising donor residues at positions 2, 4, 6, 35, 36, 38, 44, 47, 49, 62, 64-69, 85, 87, 98, 99, 101 and 102.
11. A CDR-grafted light chain according to Claim 9 or 10, comprising donor residues at one, some or all of positions:
1 and 3,
63,
60 (if 60 and 54 are able to form a potential saltbridge),
70 (if 70 and 24 are able to form a potential saltbridge),
73 and 21 (if 47 is different between donor and acceptor),
37 and 45 (if 47 if different between donor and acceptor), and
any one or more of 10, 12, 40, 83, 103 and 105.
12. A CDR-grafted light chain according to any one of Claims 6-11, comprising donor CDRs at positions 24-34, 50-56 and 89-97.

13. A CDR-grafted antibody molecule comprising at least one CDR-grafted heavy chain according to any one of Claims 1-5 and at least one CDR-grafted light chain according to any one of Claims 6-12.
14. A CDR-grafted antibody molecule according to Claim 13, which is a site-specific antibody molecule.
15. A CDR-grafted antibody molecule according to Claim 13 which has specificity for an interleukin, hormone or other biologically active compound or a receptor therefor.
16. A CDR-grafted antibody heavy or light chain or molecule according to any one of the preceding claims comprising human acceptor residues and non-human donor residues.
17. A DNA sequence which codes for a CDR-grafted heavy chain according to Claim 1 or a CDR-grafted light chain according to Claim 6 or Claim 8.
18. A cloning or expression vector containing a DNA sequence according to Claim 17.
19. A host cell transformed with a DNA sequence according to Claim 17.
20. A process for the production of a CDR-grafted antibody sequence according to Claim 17 in a transformed host cell.
21. A process for producing a CDR-grafted antibody product comprising:

(a) producing in an expression vector an operon having a DNA sequence which encodes an antibody heavy chain according to Claim 1;

and/or

(b) producing in an expression vector an operon having a DNA sequence which encodes a complementary antibody light chain according to Claim 6 or Claim 8;

(c) transfecting a host cell with the or each vector; and

(d) culturing the transfected cell line to produce the CDR-grafted antibody product.

22. A therapeutic or diagnostic composition comprising a CDR-grafted antibody heavy chain according to Claim 1, or a CDR-grafted light chain according to Claim 6 or Claim 8, or a CDR-grafted antibody molecule according to Claim 13 in combination with a pharmaceutically acceptable carrier, diluent or excipient.

23. A method of therapy or diagnosis comprising administering an effective amount of a CDR-grafted heavy chain according to Claim 1, or a CDR-grafted light chain according to Claim 6 or Claim 8, or a CDR-grafted antibody molecule according to Claim 13 to a human or animal subject.

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<p>(21) International Application Number: PCT/GB90/02017 (22) International Filing Date: 21 December 1990 (21.12.90) (30) Priority data: 8928874.0 21 December 1989 (21.12.89) GB (71) Applicant (for all designated States except US): CELLTECH LIMITED [GB/GB]; 216 Bath Road, Slough, Berkshire SL1 4EN (GB). (72) Inventors; and (75) Inventors/Applicants (for US only) : ADAIR, John, Robert [GB/GB]; 23 George Road, Stokanchurch, High Wycombe, Buckinghamshire HP14 3RN (GB). ATHWAL, Diljeet, Singh [GB/GB]; Flat 35, Knollys House, Tavistock Square, London WC1 (GB). EMTAGE, John, Spencer [GB/GB]; 49 Temple Mill Island, Temple, Marlow, Buckinghamshire SL7 1SQ (GB).</p>	<p>(74) Agent: MERCER, Christopher, Paul; Carpmaels & Ransford, 43 Bloomsbury Square, London WC1A 2RA (GB). (81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CM (OAPI patent), DE, DE (European patent), DK, FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GR, HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL, NL (European patent), NO, RO, SD, SE, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent), US. Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> <i>With a request for rectification under Rule 91.1(f).</i></p>	
<p>(54) Title: HUMANISED ANTIBODIES</p> <p>(57) Abstract</p> <p>CDR-grafted antibody heavy and light chains comprise acceptor framework and donor antigen binding regions, the heavy chains comprising donor residues at at least one of positions (6, 23) and/or (24, 48) and/or (49, 71) and/or (73, 75) and/or (76) and/or (78) and (88) and/or (91). The CDR-grafted light chains comprise donor residues at at least one of positions (1) and/or (3) and (46) and/or (47) or at at least one of positions (46, 48, 58) and (71). The CDR-grafted antibodies are preferably humanised antibodies, having non human, e.g. rodent, donor and human acceptor frameworks, and may be used for <i>in vivo</i> therapy and diagnosis. A generally applicable protocol is disclosed for obtaining CDR-grafted antibodies.</p>		

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1  GAATTCCCAA AGACAAAatg gattttcaag tgcagatttt cagcttcctg
51  ctaatcagtg cctcagtcac aatatccaga ggacaaattg ttctcaccca
101  gtctccagca atcatgtctg catctccagg ggagaaggtc accatgacct
151  gcagtgccag ctcaagtgtg agttacatga actggtacca gcagaagtca
201  ggcacctccc ccaaaagatg gatttatgac acatccaaac tggcttctgg
251  agtcccctgct cacttcaggg gcagtgggtc tgggacctct tactctctca
301  caatcagcgg catggaggct gaagatgctg ccacttatta ctgccagcag
351  tggagtagta  accattcac gttcggctcg gggacaaagt tggaaataaa
401  ccgggctgat actgcaccaa ctgtatccat cttcccacca tccagtgagc
451  agttaacatc tggagggtgcc tcagtcgtgt gcttcttgaa caacttctac
501  cccaaagaca tcaatgtcaa gtggaagatt gatggcagtg aacgacaaaa
551  tggcgtcctg aacagttgga ctgatacagga cagcaaagac agcacctaca
601  gcatgagcag caccctcacg ttgaccaagg acgagtatga acgacataac
651  agctatacct gtgaggccac tcacaagaca tcaacttcac ccattgtcaa
701  gagcttcaac aggaatgagt gtTAGAGACA AAGGTCCTGA GACGCCACCA
751  CCAGCTCCCA GCTCCATCCT ATCTTCCCTT CTAAGGTCTT GGAGGCTTCC
801  CCACAAGCGC tTACCACTGT TGCGGTGCTC TAAACCTCCT CCCACCTCCT
851  TCTCCTCCTC CTCCCTTTCC TTGGCTTTTA TCATGCTAAT ATTTGCAGAA
901  AATATTCAAT AAAGTGAGTC TTTGCCTTGA AAAAAAAAAA AAA
(SEQ ID NO: 4)

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FIG. 1a

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1  MDFQVQIFSF LLISASVIIS RGOQIVLTQSP AIMSASPGEK VTMTCSASSS
51  VSYMNWYQQK SGTSPKRWIY DTSKLAGSVP AHFRGSGSGT SYSLTISGME
101  AEDAATYYCQ QWSSNPFTFG SGTKLEINRA DTAPTVSIFP PSSEQLTSGG
151  ASVVCFLNNF YPKDINVKWK IDGSERQNGV LNSWTDQDSK DSTYSMSSTL
201  TLTKDEYERH NSYTCEATHK TSTSPIVKSF NRNEC* (SEQ ID NO:5)

```

FIG. 1b

```

1 GAATTCCCCT CTCCACAGAC ACTGAAAAC TCGACTCAAC ATGGAAAGGC
51 ACTGGATCTT TCTACTCCTG TTGTCAGTAA CTGCAGGTGT CCACTCCCAG
101 GTCCAGCTGC AGCAGTCTGG GGCTGAACTG GCAAGACCTG GGGCCTCAGT
151 GAAGATGTCC TGCAAGGCTT CTGGCTACAC CTTTACTAGG TACACGATGC
201 ACTGGGTA AAA ACAGAGGCCT GGACAGGGTC TGG AATGGAT TGGATACATT
251 ATTCCTAGCC GTGGTTATAC TAATTACAAT CAGAAGTTCA AGGACAAGGC
301 CACATTGACT ACAGACAAAT CCTCCAGCAC AGCCTACATG CAACTGAGCA
351 GCCTGACATC TGAGGACTCT GCAGTCTATT ACTGTGCAAG ATATTATGAT
401 GATCATTACT GCCTTGACTA CTGGGGCCAA GGCACCACTC TCACAGTCTC
451 CTCAGCCAAA ACAACAGCCC CATCGGTCTA TCCACTGGCC CCTGTGTGTG
501 GAGATAACAAC TGGCTCCTCG GTGACTCTAG GATGCCTGGT CAAGGGTTAT
551 TTCCCTGAGC CAGTGACCTT GACCTGGAAC TCTGGATCCC TGTCCAGTGG
601 TGTGCACACC TTCCCAGCTG TCCTGCAGTC TGACCTCTAC ACCCTCAGCA
651 GCTCAGTGAC TGTAACCTCG AGCACCTGGC CCAGCCAGTC CATCACCTGC
701 AATGTGGCCC ACCCGGCAAG CAGCACCAAG GTGGACAAGA AAATTGAGCC
801 ACCTCTTGGG TGGACCATCC GTCTTCATCT TCCCTCCAAA GATCAAGGAT
851 GTACTCATGA TCTCCCTGAG CCCCATAGTC ACATGTGTGG TGGTGGATGT
901 GAGCGAGGAT GACCCAGATG TCCAGATCAG CTGGTTTGTG AACAACTGGG
951 AAGTACACAC AGCTCAGACA CAAACCCATA GAGAGGATTA CAACAGTACT
1001 CTCCGGGTGG TCAGTGCCCT CCCCATCCAG CACCAGGACT GGATGAGTGG
1051 CAAGGAGTTC AAATGCAAGG TCAACAACAA AGACCTCCCA GCGCCCATCG
1101 AGAGAACCAT CTCAAACCC AAAGGGTCAG TAAGAGCTCC ACAGGTATAT
1151 GTCTTGCCCTC CACCAGAAGA AGAGATGACT AAGAAACAGG TCACTCTGAC
1201 CTGCATGGTC ACAGACTTCA TGCTGAAGA CATTTACGTG GAGTGGACCA
1251 ACAACGGGAA AACAGAGCTA AACTACAAGA AACTGAACC AGTCCTGGAC
1301 TCTGATGGTT CTTACTTCAT GTACAGCAAG CTGAGAGTGG AAAAGAAGAA
1351 CTGGGTGGAA AGAAATAGCT ACTCCTGTTC AGTGGTCCAC GAGGGTCTGC
1401 ACAATCACCA CACGACTAAG AGCTTCTCCC GGACTCCGGG TAAATGAGCT
1451 CAGCACCCAC AAAACTCTCA GGTCCAAAGA GAGACCCACA CTCATCTCCA
1501 TGCTTCCCTT GTATAAATAA AGCACCCAGC AATGCCTGGG ACCATGTAAA
1551 AAAAAAAAAA AAAGGAATTC (SEQ ID NO:6)

```

FIG. 2a

DKT 3 HEAVY CHAIN PROTEIN SEQUENCE DEDUCED FROM DNA SEQUENCE

1 MERHWIFLLL LSVTAGVHSQ VQLQQSGAEL ARPGASVKMS CKASGYTFTR
 51 YTMHWVKQRP GQGLEWIGYI NPSRGYTNYN QKFKDKATLT TDKSSSTAYM
 101 QLSSLTSEDS AVYYCARYYD DHYCLDYWGQ GTTLTVSSAK TTAPSVYPLA
 151 PVCGDTTGSS VTLGCLVKGY FPEPVTLTWN SGLSSGVHT FPAVLQSDLY
 201 TLSSSVTVTS STWPSQSITC NVAHPASSTK VDKKIEPRGP TIKPCPPCKC
 251 PAPANLLGGPS VFIFPPKIKD VLMISLSPIV TCVVVDVSED DPDVQISWFV
 301 NNVEVHTAQT QTHREDYNST LRVVSALPIQ HQDWMSGKEF KCKVNNKDLP
 351 APIERTISKP KGSVRAPQVY VLPPPEEEMT KKQVTLTCMV TDFMPEDIYV
 401 EWTNNGKTEL NYKNTEPVLD SDGSYFMYSK LRVEKKNWVE RNSYSCSVVH
 451 EGLHNHHTTK SFSRTPGK* (SEQ ID NO:7)

FIG. 2b

```

1           23           42
NN         N           N     N
RES TYPE   SBspSPESsSBSbSsSsPSPSPsPSsse*s*p*Pi^ISsSe
0kt3vl     QIVLTQSPA IMSASPGEKVTMTCSASS.SVSYMNWYQQKSGT
REI        DIQMTQSPSSLSASVGDVRTITCQASQDI IKYLNWYQQIPGK
           ? ?
           CDR1 (LOOP) *****
           CDR1 (KABAT) *****

           56           85
N   NN
RES TYPE   *IsiPpIeesesssSBEsePsPSBSSEsPspPsseesSPePb
0kt3vl     SPKRWIYDTSKLAGVPAHFRGSGSGTSYSLTISGMEAEDAAT
REI        APKLLIYEASNLQAGVPSRFSGSGSGTDYTETISSLQPEDIAT (SEQ
ID NO:8)   ? ??           ? ?
           ***** CDR2 (LOOP/KABAT)

           102   108
RES TYPE   PiPIPies**iPIIsPPSPSPSS
0kt3vl     YYCQQWSSNPFTFGSGTKLEINR (SEQ ID NO:29)
REIvl      YYCQQYQSLPYTFGQGTKLQITR (SEQ ID NO:9)
           ? ?
           ***** CDR3 (LOOP)
           ***** CRD3(KABAT)

```

FIG. 3

```

NN N                23 26    32 35  N39  43
RES TYPE  SESPs^SBssS^sSSsSpSpSPsPSEbSBssBePi^PIpiesss
Dkt3h     QVQLQQSGAELARPGASVKMSCKASGYTFTRYTMNHVVKQRPGQ
KOL       QVQLVESGGGVVQPGRSLRLSCSSSGFIFSSYAMYWVRQAPGK
           ?                ??

                               ***** CDR1 (LOOP)
                               ***** CDR1 (KABAT)

52a   60   65   N N N   82abc   89
RES TYPE  I IeIppp^ssssss^ps^pSSsbSpseSsSseSp^pSpsSBssS^ePb
Dkt3vh    GLEWIGYINPSRGYTNTNQKFKRKATLTTDKSSSTAYMQLSSLTSEDSAV
KOL       GLEWVAIIWDDGSDQHAYADSVKGRFTISRDNSKNTLFLQMDSLPPEDTGV
           ??                ? ? ? ? ?

                               ***** CDR2 (LOOP)
                               ***** CDR2 (KABAT)

92 N                107   113
RES TYPE  PiPIEissssiissssbibi*EIPiP*spSBSS
Dkt3vh    YYCARYYDDHY.....CLDYWGQGTTLTVSS (SEQ ID NO:30)
KOL       YFCARDGGHGFCSSASCFGPDYWGQGTPVTVSS (SEQ ID NO:10)
           ***** CRD4 (KABAT/LOOP)

```

FIG. 4

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DKT 3 HEAVY CHAIN CDR GRAFTS

1. gh341 and derivatives

	1	26	35	39	43	
Dkt3vh	QVQL	QQSGAELARPGASVKMSCKASGYTF	TRYTMHWVKQRPGQ			
gH341	QVQL	VESSGGGVVQDGRSLRLSCSSSGYTF	TRYTMHWVRQAPGK			JA178
gH341A	QVQL	<u>VQ</u> SGGGVVQPGRSLRLSCK <u>ASGYTF</u>	TRYTMHWVRQAPGK			JA185
gH341E	QVQL	<u>VQ</u> SGGGVVQPGRSLRLSCK <u>ASGYTF</u>	TRYTMHWVRQAPGK			JA198
gH341*	QVQL	<u>VQ</u> SGGGVVQPGRSLRLSCK <u>ASGYTF</u>	TRYTMHWVRQAPGK			JA207
gH341*	QVQL	<u>VQ</u> SGGGVVQPGRSLRLSCK <u>ASGYTF</u>	TRYTMHWVRQAPGK			JA209
gH341D	QVQL	<u>VQ</u> SGGGVVQPGRSLRLSCK <u>ASGYTF</u>	TRYTMHWVRQAPGK			JA197
gH341*	QVQL	<u>VQ</u> SGGGVVQPGRSLRLSCK <u>ASGYTF</u>	TRYTMHWVRQAPGK			JA199
gH341C	QVQL	<u>VQ</u> SGGGVVQPGRSLRLSCK <u>ASGYTF</u>	TRYTMHWVRQAPGK			JA184
gH341*	QVQL	<u>VQ</u> SGGGVVQPGRSLRLSCS <u>ASGYTF</u>	TRYTMHWVRQAPGK			JA203
gH341*	QVQL	<u>VE</u> SSGGGVVQPGRSLRLSCS <u>ASGYTF</u>	TRYTMHWVRQAPGK			JA205
gH341B	QVQL	VESSGGGVVQPGRSLRLSCSSSGYTF	TRYTMHWVRQAPGK			JA183
gH341*	QVQL	<u>VQ</u> SGGGVVQPGRSLRLSCS <u>ASGYTF</u>	TRYTMHWVRQAPGK			JA204
gH341*	QVQL	<u>VE</u> SSGGGVVQPGRSLRLSCS <u>ASGYTF</u>	TRYTMHWVRQAPGK			JA206
gH341*	QVQL	<u>VQ</u> SGGGVVQPGRSLRLSCS <u>ASGYTF</u>	TRYTMHWVRQAPGK			JA208
KDL	QVQL	<u>VE</u> SSGGGVVQPGRSLRLSCSSSGF	IFSSYAMYWVRQAPGK			

FIG. 5a

	44	50	65	83	
Dkt3vh	GLEWIGYINPSRGYTNYNQFKDKATLTTDKSSSTAYMQLSSLT				
gH341	GLEWVAYINPSRGYTNYNQFKDRFTISRDNKNTLFLQMDSLR				JA178
gH341A	GLEWIGYINPSRGYTNYNQVKDRFTISTDKSKSTAFQMDSLR				JA185
gH341E	GLEWIGYINPSRGYTNYNQVKDRFTISTDKSKSTAFQMDSLR				JA198
gH341*	GLEWIGYINPSRGYTNYNQVKDRFTISTDKSKNTAFQMDSLR				JA207
gH341*	GLEWIGYINPSRGYTNYNQVKDRFTISRDNKNTAFQMDSLR				JA209
gH341D	GLEWIGYINPSRGYTNYNQVKDRFTISTDKSKNTLFLQMDSLR				JA197
gH341*	GLEWIGYINPSRGYTNYNQVKDRFTISRDNKNTLFLQMDSLR				JA199
gH341C	GLEWVAYINPSRGYTNYNQFKDRFTISRDNKNTLFLQMDSLR				JA184
gH341*	GLEWIGYINPSRGYTNYNQVKDRFTISTDKSKSTAFQMDSLR				JA207
gH341*	GLEWIGYINPSRGYTNYNQVKDRFTISTDKSKSTAFQMDSLR				JA205
gH341B	GLEWIGYINPSRGYTNYNQVKDRFTISTDKSKSTAFQMDSLR				JA183
gH341*	GLEWIGYINPSRGYTNYNQVKDRFTISTDKSKSTAFQMDSLR				JA204
gH341*	GLEWIGYINPSRGYTNYNQVKDRFTISTDKSKSTAFQMDSLR				JA206
gH341*	GLEWIGYINPSRGYTNYNQVKDRFTISTDKSKNTAFQMDSLR				JA208
KDL	GLEWVAI I WDDGSDQHYADSVKGRFTISRDNKNTLFLQMDSLR				

FIG. 5b

	84	95	102	113		SEQ ID NO:
Okt3vh	SEDSAVYYCARYYDDHY.....			CLDYWGQGTTLTVSS		30
gH341	PEDTGVYFCARYYDDHY.....			CLDYWGQGTTLTVSS	JA178	11
gH341A	PEDTAVYYCARYYDDHY.....			CLDYWGQGTTLTVSS	JA185	12
gH341E	PEDTGVYFCARYYDDHY.....			CLDYWGQGTTLTVSS	JA198	13
gH341*	PEDTGVYFCARYYDDHY.....			CLDYWGQGTTLTVSS	JA207	14
gH341D	PEDTGVYFCARYYDDHY.....			CLDYWGQGTTLTVSS	JA197	15
gH341*	PEDTGVYFCARYYDDHY.....			CLDYWGQGTTLTVSS	JA209	16
gH341*	PEDTGVYFCARYYDDHY.....			CLDYWGQGTTLTVSS	JA199	17
gH341C	PEDTGVYFCARYYDDHY.....			CLDYWGQGTTLTVSS	JA184	18
gH341*	PEDTAVYYCARYYDDHY.....			CLDYWGQGTTLTVSS	JA203	19
gH341*	PEDTAVYYCARYYDDHY.....			CLDYWGQGTTLTVSS	JA205	20
gH341B	PEDTAVYYCARYYDDHY.....			CLDYWGQGTTLTVSS	JA183	21
gH341*	PEDTGVYFCARYYDDHY.....			CLDYWGQGTTLTVSS	JA204	22
gH341*	PEDTGVYFCARYYDDHY.....			CLDYWGQGTTLTVSS	JA206	23
gH341*	PEDTGVYFCARYYDDHY.....			CLDYWGQGTTLTVSS	JA208	24
KOL	PEDTGVYFCARDGGHGFCSASCFGPDYWGQGTPVTVSS					10

FIG. 5c

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OKT3 LIGHT CHAIN CDR GRAFTING

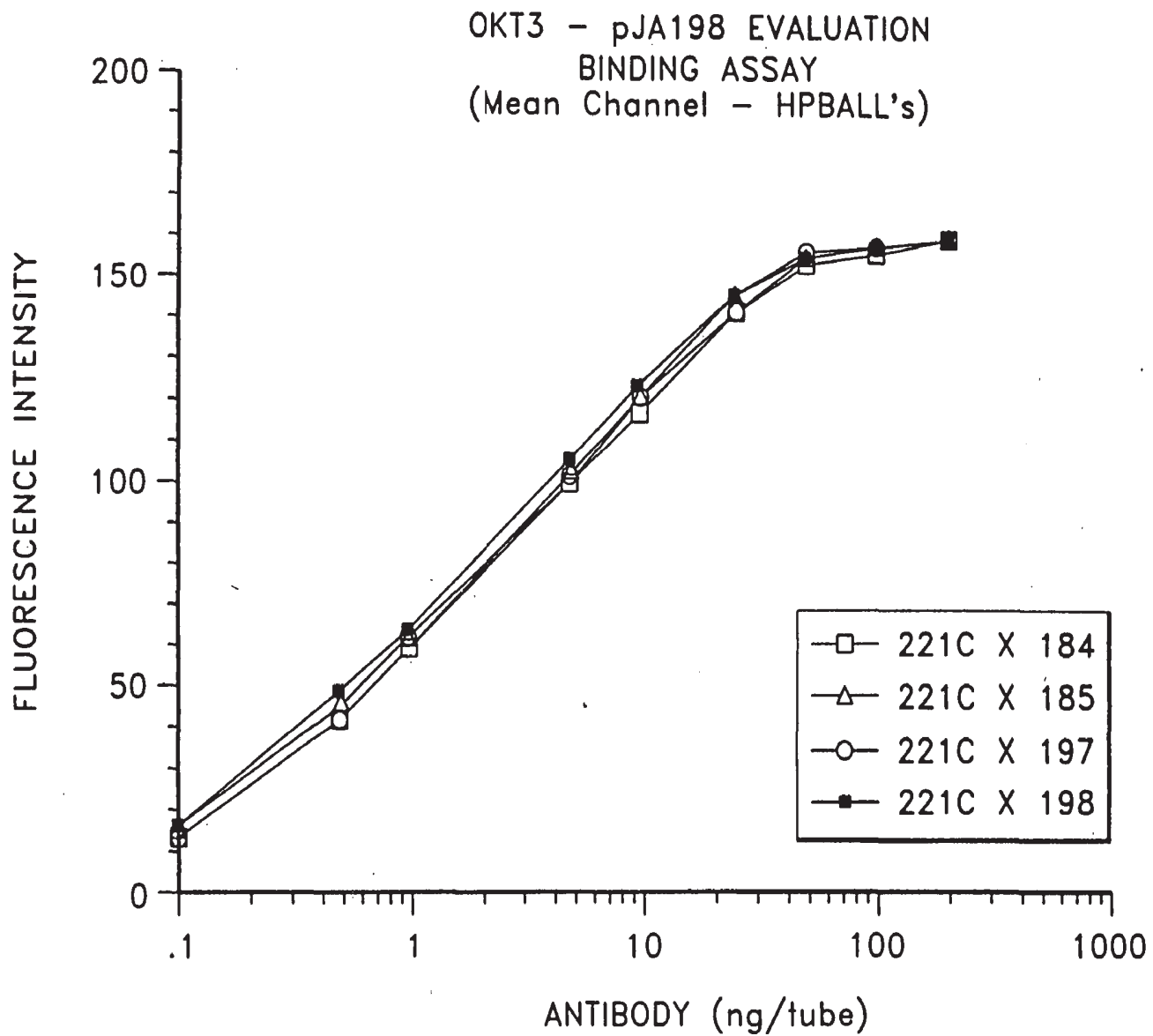
1. gL221 and derivatives

	1	24	34	42	
Okt3v1	<u>QIVLTQSPAOMSAS</u> <u>PG</u> <u>GEKVTMTCSASS</u> .SVSYMNWYQOKSGT				
gL221	DIQMTQSPSSLSASV <u>GDRVTITCSASS</u> .SVSYMNWYQOTPGK				
gL221A	<u>QIV</u> MTQSPSSLSASV <u>GDRVTITCSASS</u> .SVSYMNWYQOTPGK				
gL221B	<u>QIV</u> MTQSPSSLSASV <u>GDRVTITCSASS</u> .SVSYMNWYQOTPGK				
gL221C	DIQMTQSPSSLSASV <u>GDRVTITCSASS</u> .SVSYMNWYQOTPGK				
REI	DIQMTQSPSSLSASV <u>GDRVTITCQASQDI</u> IKYLNWYQOTPGK				
	43	50	56	85	
Okt3v1	SPKRWIYDTSK <u>LASGVPAHFRGSGSGT</u> SYSLTISGMEADAAT				
gL221	APKLLIYDTSK <u>LASGVPSR</u> FGSGSGTDYFTISSLQPEDIAT				
gL221A	APKR <u>WIYDTSK</u> LASGVPSRFGSGSGTDYFTISSLQPEDIAT				
gL221B	APKLLIYDTSK <u>LASGVPSR</u> FGSGSGTDYFTISSLQPEDIAT				
gL221C	APKR <u>WIYDTSK</u> LASGVPSRFGSGSGTDYFTISSLQPEDIAT				
REI	APKLLIYEASN <u>LQAGVPSR</u> FGSGSGTDYFTISSLQPEDIAT (SEQ ID NO:8)				
	86	91	96	108	
Okt3v1	YYC <u>QWSSNP</u> ETFGSGTKLEINR (SEQ ID NO:29)				
gL221	YYC <u>QWSSNP</u> ETFGQGTKLQITR (SEQ ID NO:25)				
gL221A	YYC <u>QWSSNP</u> ETFGQGTKLQITR (SEQ ID NO:26)				
gL221B	YYC <u>QWSSNP</u> ETFGQGTKLQITR (SEQ ID NO:27)				
gL221C	YYC <u>QWSSNP</u> ETFGQGTKLQITR (SEQ ID NO:28)				
REI	YYC <u>QYQSLPYTEFGQ</u> GTKLQITR (SEQ ID NO:9)				

CDR'S ARE UNDERLINED

FRAMEWORK RESIDUES INCLUDED IN THE GENE ARE DOUBLE UNDERLINED

FIG. 6



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FIG. 7

OKT3 - pJA198 EVALUATION
BLOCKING ASSAY
(Mean Channel - HPBALL's)

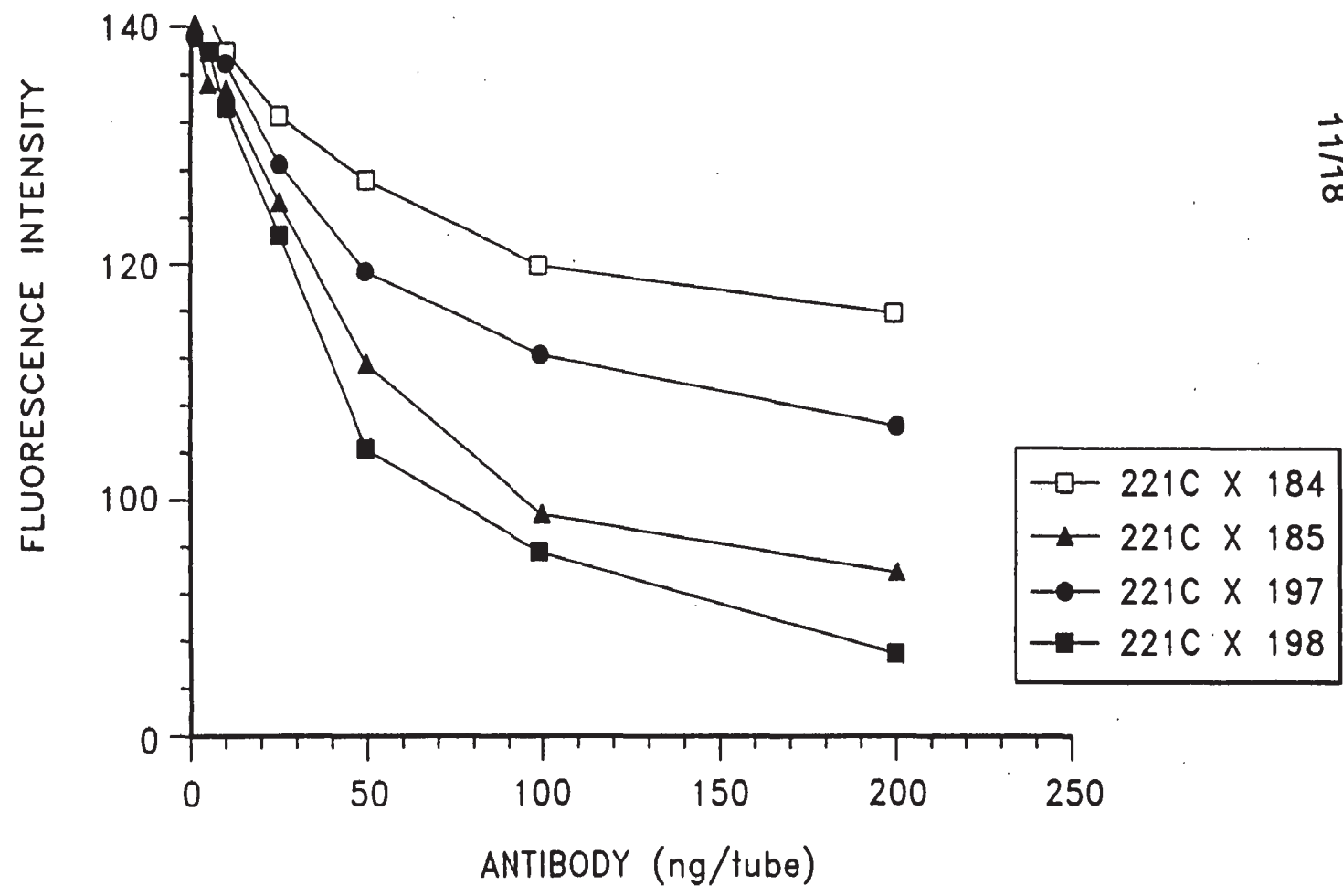


FIG. 8

BLOCKING ASSAY
(Mean Channel - HPBALL's)

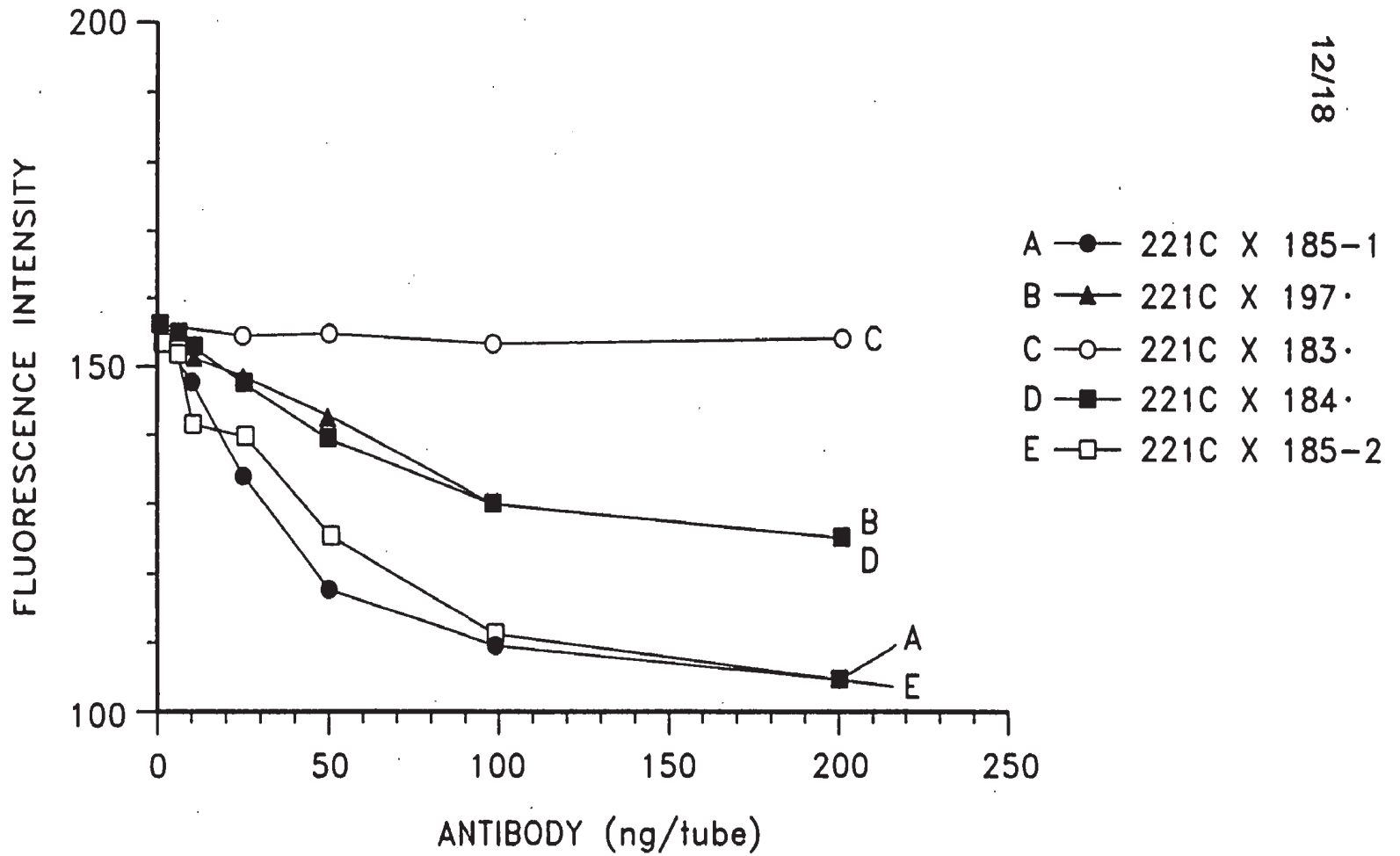
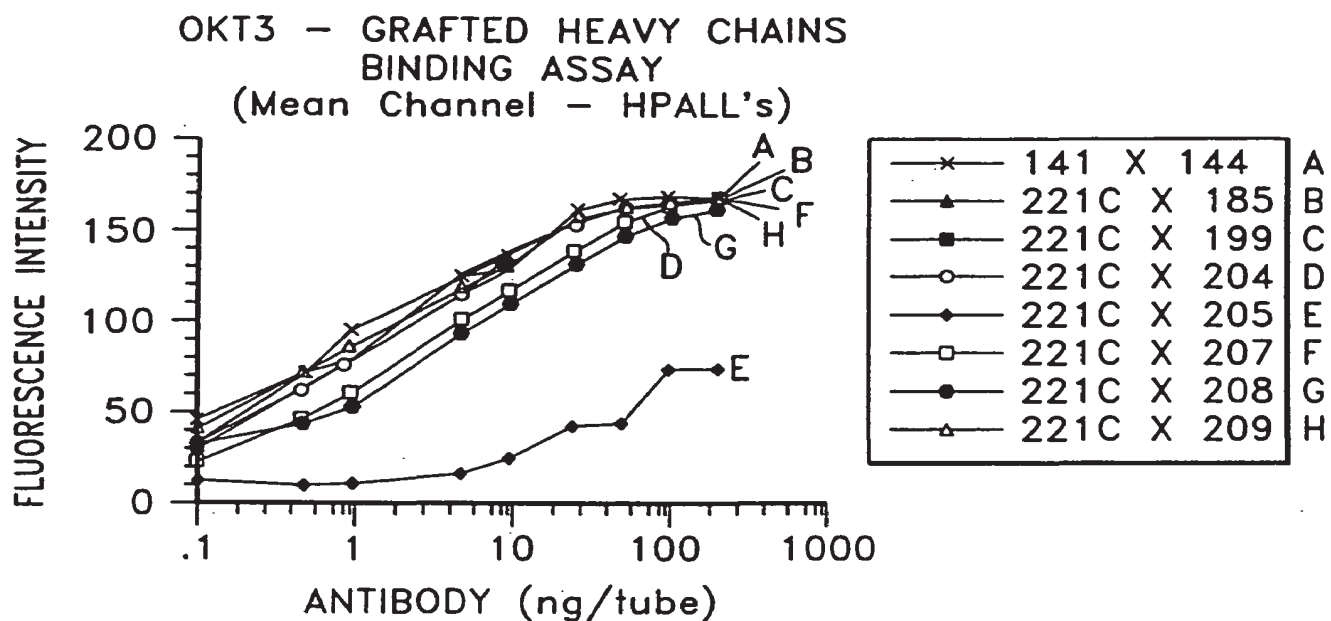


FIG. 9

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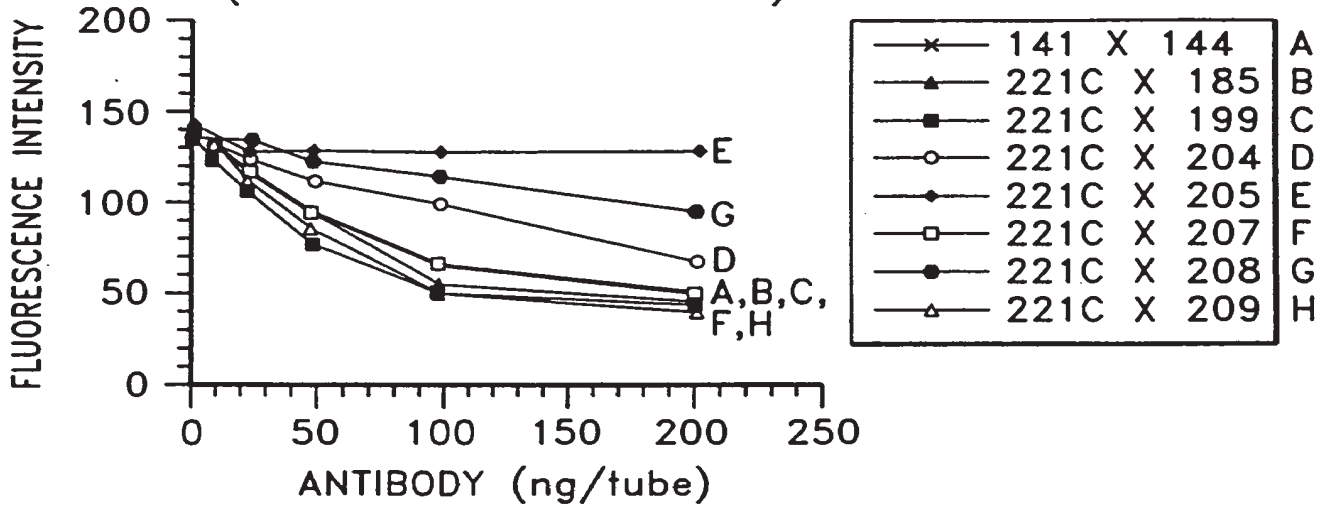


—◆—	(205)	—, —, —, 24, 48, 49, 71, 73, 76, 78, 88, 91,
—●—	(208)	6, —, —, 24, 48, 49, 71, 73, —, 78, —, —,
—○—	(204)	6, —, —, 24, 48, 49, 71, 73, 76, 78, —, —,
—■—	(199)	6, 23, 24, 48, 49, —, —, —, —, —, —,
—□—	(207)	6, 23, 24, 48, 49, 71, 73, —, 78, —, —,
—▲—	(185)	6, 23, 24, 48, 49, 71, 73, 76, 78, 88, 91,
—△—	(209)	6, 23, 24, 48, 49, —, —, —, —, 78, —, —,
—x—	141 X 144	

FIG. 10a

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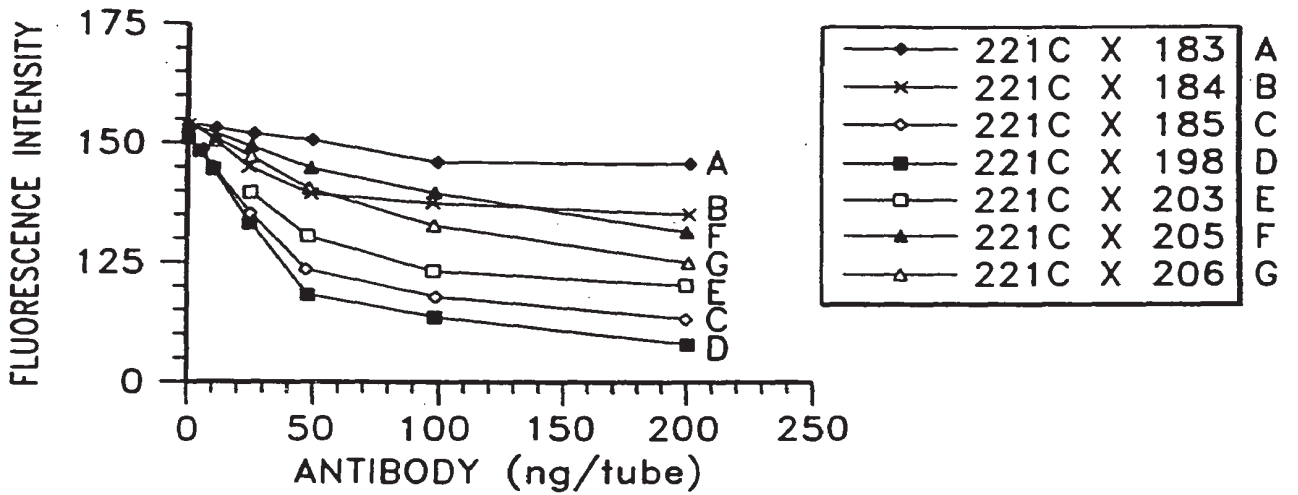
OKT3 - GRAFTED HEAVY CHAINS
 BINDING ASSAY
 (Mean Channel - HPALL's)



—◆—	(205)	—, —, —, 24, 48, 49, 71, 73, 76, 78, 88, 91,
—●—	(208)	6, —, —, 24, 48, 49, 71, 73, —, 78, —, —,
—○—	(204)	6, —, —, 24, 48, 49, 71, 73, 76, 78, —, —,
—■—	(199)	6, 23, 24, 48, 49, —, —, —, —, —, —,
—□—	(207)	6, 23, 24, 48, 49, 71, 73, —, 78, —, —,
—▲—	(185)	6, 23, 24, 48, 49, 71, 73, 76, 78, 88, 91,
—△—	(209)	6, 23, 24, 48, 49, —, —, —, —, 78, —, —,
—×—	141 X 144	

FIG. 10b

OKT3 - GRAFTED HEAVY CHAINS
 BINDING ASSAY
 (Mean Channel - HPALL's)



—◆—	(183)	6, 23, 24, 48, 49, 71, 73, 76, 78, 88, 91,
—▲—	(205)	6, 23, 24, 48, 49, 71, 73, 76, 78, 88, 91,
—×—	(184)	6, 23, 24, 48, 49, 71, 73, 76, 78, 88, 91,
—△—	(206)	6, 23, 24, 48, 49, 71, 73, 76, 78, 88, 91,
—□—	(203)	6, 23, 24, 48, 49, 71, 73, 76, 78, 88, 91,
—◇—	(185)	6, 23, 24, 48, 49, 71, 73, 76, 78, 88, 91,
—■—	(198)	6, 23, 24, 48, 49, 71, 73, 76, 78, 88, 91,

FIG. 11b

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OKT3 COMPETITION
MURIE REF STD vs. CDR GRAFTED OKT3

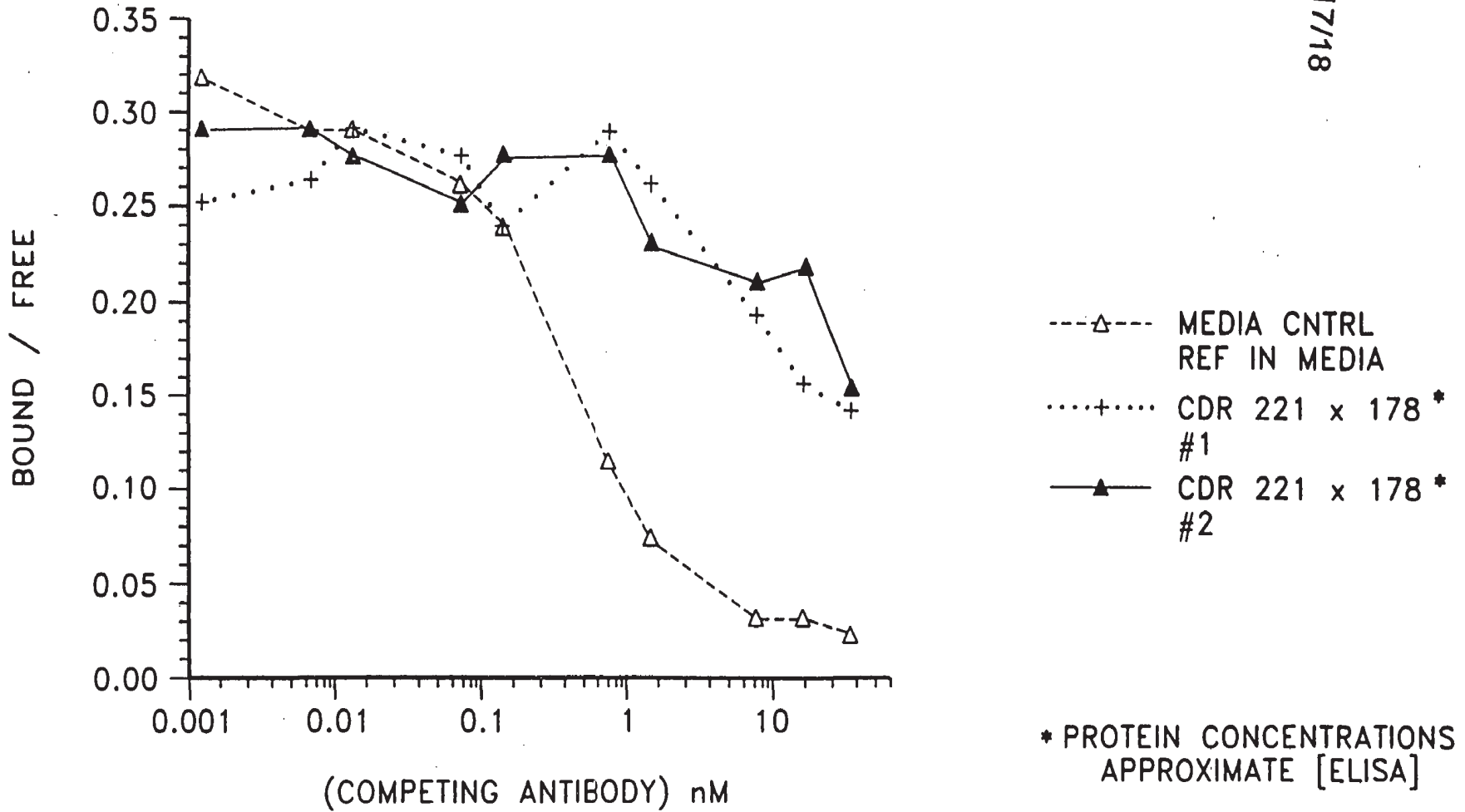


FIG. 12

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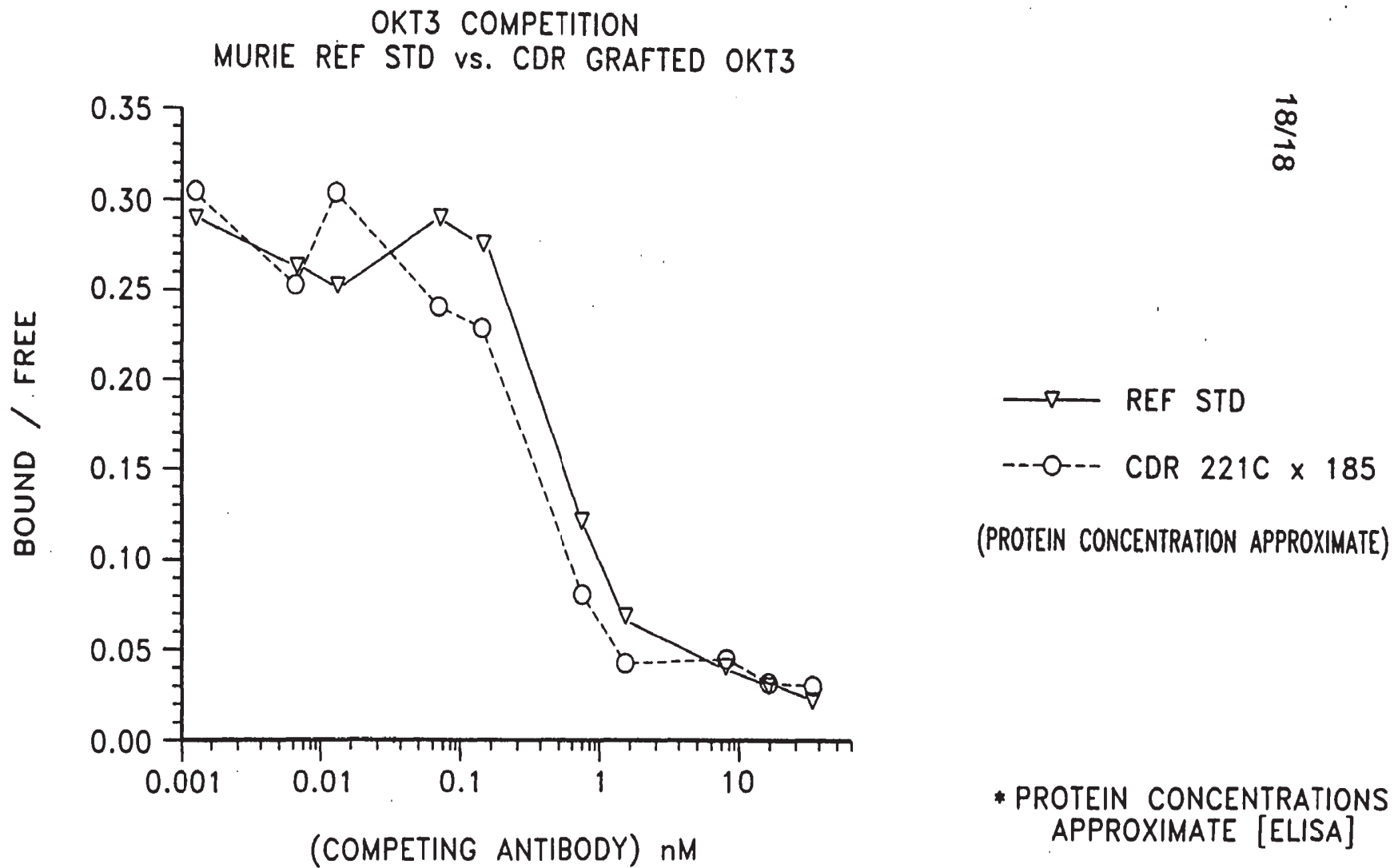


FIG. 13

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name; and

I verily believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: HUMANISED ANTIBODIES

the specification of which:

is attached hereto.

was filed on 21 December 1990 as International Application Serial No. PCT/GB90/02017 and was amended on _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with 37 CFR § 1.56(a).

I hereby claim foreign priority benefits under 35 U.S.C. § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of any application on which priority is claimed:

Country	Number	Date Filed	Priority Claimed
<u>U.K.</u>	<u>8928874.0</u>	<u>21.12.89</u>	<u>yes</u>
_____	_____	_____	_____
_____	_____	_____	_____

BEST AVAILABLE COPY

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose material information as defined in 37 CFR § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.	Filing Date	Status (patented, pending)
_____	_____	_____
_____	_____	_____
_____	_____	_____

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:
Francis A. Paintin

Registration Nos. 19,386
of the firm of **WOODCOCK WASHBURN KURTZ MACKIEWICZ & NORRIS**, One Liberty Place - 46th Floor, Philadelphia, Pennsylvania 19103, and

Address all telephone calls and correspondence to:
Francis A. Paintin

WOODCOCK WASHBURN KURTZ MACKIEWICZ & NORRIS
One Liberty Place - 46th Floor
Philadelphia, PA 19103
Telephone No. 215-568-3100.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the

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United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1	Full Name JOHN ROBERT ADAIR	Inventor's Signature <i>John Robert Adair</i>	Date 13/8/91
	Residence 23 George Road, Stokenchurch High Wycombe, Buckinghamshire HP14 3RN, U.K.		Citizenship U.K.
	Post Office Address 23 George Road, Stokenchurch, High Wycombe, Buckinghamshire HP14 3RN, U.K.		
2	Full Name DILJEET SINGH ATHWAL	Inventor's Signature <i>[Signature]</i>	Date 13/8/91
	Residence Flat 35, Knollys House, Tavistock Square, London WC1, U.K.		Citizenship U.K.
	Post Office Address Flat 35, Knollys House, Tavistock Square, London WC1, U.K.		
3	Full Name JOHN SPENCER EMTAGE	Inventor's Signature <i>John Spencer Emtage</i>	Date 13/8/91
	Residence 49 Temple Mill Island, Temple Marlow, Buckinghamshire, SL7 1SQ, U.K.		Citizenship U.K.
	Post Office Address 49 Temple Mill Island, Temple, Marlow, Buckinghamshire SL7 1SQ, U.K.		
4	Full Name	Inventor's Signature	Date
	Residence	Citizenship	
	Post Office Address		
5	Full Name	Inventor's Signature	Date
	Residence	Citizenship	
	Post Office Address		

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DOCKET NO.: CARP0001-112 PATENT
PRELIMINARY AMENDMENT AND REQUEST FOR INTERFERENCE UNDER 37
C.F.R. § 42.202 DATED NOVEMBER 21, 2005

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: John R. Adair, Diljeet S. Athwal and John S. Emtage

Serial No.: Not Yet Assigned

Art Unit: Not Yet Assigned

Filing Date: November 21, 2005

Examiner: Not Yet Assigned

For: HUMANISED ANTIBODIES

Customer No.: 34132

EXPRESS MAIL LABEL NO.: EV146 601 565US
DATE OF DEPOSIT: November 21, 2005

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

**PRELIMINARY AMENDMENT
AND
REQUEST FOR INTERFERENCE UNDER 37 CFR § 42.202**

Applicant respectfully requests entry of the following amendments prior to the calculation of filing fees:

Amendments To The Specification begin on page 2 of this paper.

Listing of the Claims, reflecting current amendments, begins on page 3 of this paper.

Remarks begin on page 4 of this paper.

The Conclusion is found on page 12.

Appendix A is found on page 14.

Appendix B is found on page 17.

Appendix C is found on page 19.

Appendix D is found on page 20.

Appendix E is found on page 21.

**Carter Exhibit 2003
Carter v. Adair
Interference No. 105,744**

AMENDMENTS TO THE SPECIFICATION

Please replace the paragraph containing the cross-reference to related applications on page 1 of the specification with the following:

This application is a Continuation of U.S. Application Serial No. 08/846,658, Filed May 1, 1997, which is a Continuation of U.S. Application Serial No. 08/303,569, Filed September 7, 1994, now U.S. Patent No. 5,859,205, Issued January 12, 1999, which is a File Wrapper Continuation of U.S. Application Serial No. 07/743,329, Filed September 17, 1991, Abandoned, which is a 35 U.S.C. 371 National Stage Application of International Application No. PCT/GB90/02017, International Filing Date of December 21, 1990, which claims priority to Great Britain Application No. 8928874.0, Filed December 21, 1989, all applications are incorporated by reference herein in their entireties.

LISTING OF CLAIMS

This listing of claims represents the current status of the claims.

Claims 1-23 (cancelled)

Claim 24 (new) A humanised antibody heavy chain variable domain comprising non-human complementarity determining region amino acid residues which bind an antigen and a human framework region wherein said framework region comprises an amino acid substitution at a residue selected from the group consisting of 23, 24, 49, 71, 73, and 78, and combinations thereof, as numbered according to Kabat.

Claim 25 (new) A humanised antibody comprising the variable domain of claim 24.

REMARKS

Claims 24 to 25 are pending. Applicants hereby request an interference in accordance with 37 CFR § 42.202. It is noted that U.S. Patent No. 6,407,213, whose claims present the basis for an interference, is classified in Class 530, and was examined by Examiners Minh-Tam Davis and Anthony C. Caputa.

Compliance with 37 CFR § 42.202

Applicants respectfully submit that all requirements of 37 CFR § 42.202 have been met and respectfully request examination of the present application and declaration of an interference.

(a) Identifying The Patent

Applicants request that an interference be declared between Applicants' above-identified application and U.S. Patent No. 6,407,213 B1 (hereinafter the 213 patent), issued to Carter et al. on June 18, 2002, a copy of which is enclosed herewith.

(b) Compliance with 35 USC § 135(b)

Although the present rules do not require a showing of compliance under 35 USC § 135(b), Applicants submit the following to advance the examination of the present application to allowability. The present application is a Continuation of U.S. Application Serial No. 08/846,658, Filed May 1, 1997, which is a Continuation of U.S. Application Serial No. 08/303,569, Filed September 7, 1994, now U.S. Patent No. 5,859,205, Issued January 12, 1999, which is a File Wrapper Continuation of U.S. Application Serial No. 07/743,329, Filed September 17, 1991, Abandoned, which is a 35 U.S.C. 371 National Stage Application of International Application No. PCT/GB90/02017, International Filing Date of December 21, 1990 (hereinafter "the PCT application," a copy of which is enclosed herewith), which claims priority to Great Britain Application No. 8928874.0, Filed December 21, 1989. Claims 1-23 as filed in the PCT application are attached as Appendix A.

Under 35 USC § 135(b)(1), Applicants must show that they had a claim to the same, or substantially the same, subject matter as a claim of the 213 patent within one year of the issuance of the 213 patent, or June 18, 2003. The 213 patent issued on June 18, 2002. The PCT application was filed on December 21, 1990, over 10 years earlier than the 213 patent issued. The time limit of Section 135(b)(1) has been complied with fully. See *Corbett v. Chisholm*, 196 USPQ 337 (CCPA 1977).

To meet the “same or substantially the same invention” requirement of Section 135(b)(1), Applicants must show that their claim contained all material limitations, i.e. limitations necessary to patentability, of the claim of the 213 patent alleged to be to the same, or substantially the same, invention. *Corbett v. Chisholm*, 196 USPQ 337 (C.C.P.A. 1977), citing *Wetmore v. Miller*, 477 F.2d 960, 177 USPQ 699 (C.C.P.A. 1973).

As is evident from Appendix A, Applicants made a claim for the same, or substantially the same, subject matter as a claim of the 213 patent well before the issuance of the 213 patent. Claim 16 of the PCT application, as depending from claim 8, is to substantially the same subject matter as at least claim 1 of the 213 patent. For the Office’s convenience, all three claims are duplicated below.

Claim 8 of the PCT application: A CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 46, 48, 58 and 71.

Claim 16 of the PCT application: A CDR-grafted antibody heavy or light chain or molecule according to any one of the preceding claims comprising human acceptor residues and non-human donor residues.

Claim 1 of the 213 patent: A humanized antibody variable domain comprising non-human Complementarity Determining Region (CDR) amino acid residues

which bind an antigen incorporated into a human antibody variable domain, and further comprising a Framework Region (FR) amino acid substitution at a site selected from the group consisting of: 4L, 38L, 43L, 44L, **58L**, 62L, 65L, 66L, 67L, 68L, 69L, 73L, 85L, 98L, 2H, 4H, 36H, 39H, 43H, 45H, 69H, 70H, 74H, and 92H, utilizing the numbering system set forth in Kabat.

Both claim 16 of the PCT application, as it depends from claim 8, and claim 1 of the 213 patent are directed to variable domains comprising non-human Complementarity Determining Regions (“CDRs”) that bind antigen, i.e., antigen binding regions, human framework regions, and a non-human framework amino acid at residue 58 of the light chain, using Kabat numbering. Both claims also comprise a human framework region; claim 1 of the 213 patent simply recites it differently -- if one incorporates non-human CDR amino acid residues into a human antibody variable domain, one ends up with a human framework region. Further, the 213 patent defines “humanized antibody” as an antibody having a framework region “having substantially the amino acid sequence of a human immunoglobulin,” i.e., a human framework region (see column 8, lines 11-17, of the 213 patent). “Antigen binding regions,” as recited in claim 8 of the PCT application, refers to CDRs (see page 8, lines 10-13, of the PCT application). As indicated on page 8, lines 22-24 of the PCT application, all numbering is according to Kabat. Claim 16 implicitly contains the recitation that the amino acid be substituted. As indicated on page 17, lines 1-19 of the PCT application, substitution of the non-human framework residues for the human framework residues is required when the donor and framework residue at any of the recited positions differ. While Applicants’ claim recites a “light **chain**” and claim 1 of the 213 patent recites a “variable domain,” Applicants submit that this is merely a difference in scope, and not a material difference. *See Corbett.*

(c) Presentation Of A Proposed Count

Applicants present in Appendix B, attached hereto, a proposed count that is presented in the “alternative” format as claim 30 or claim 80 of the 213 patent or Applicants’ claim 24. All alternatives are to the same patentable invention.

All alternatives recite that the variable domain comprises non-human complementarity determining region amino acid residues which bind an antigen. All alternatives also comprise a human framework region. As discussed above for compliance with Section 135(b), the 213 patent defines “humanized antibody” as an antibody having a framework region “having substantially the amino acid sequence of a human immunoglobulin,” i.e., a human framework region (see column 8, lines 11-17, of the 213 patent). And, if one incorporates non-human CDR amino acid residues into a human antibody variable domain, as recited in claims 30 and 80, one ends up with a human framework region. Further, all alternatives require only that one of the listed framework residues be substituted. Claims 30 and 80 of the 213 patent and Applicants’ claim 24 recite substituting, *inter alia*, residue 78 of the heavy chain.

Although claim 30 of the 213 patent and Applicants’ claim 24 do not recite the alternative recitations (a) through (c) of claim 80 of the 213 patent, e.g., “noncovalently binds antigen directly,” such limitations are clearly implicit for all the framework residue substitutions recited in claim 80.

Although claims 30 and 80 of the 213 patent do not recite that the variable domain is from the light chain, it clearly must be the case when a light chain framework residue, i.e., one designated with an “L,” is to be substituted.

Claim 30 of the 213 patent recites an “antibody” while the remaining alternatives recite a “variable domain.” The antibody recitation is anticipated by and/or obvious over the recitation of variable domain and *vice versa*. “Humanized antibody” is defined in the 213 patent to comprise, *inter alia*, at least one variable domain (see column 8, lines 26-28, of the 213 patent).

Claim 30 of the 213 patent further recites a specific antibody target. The alternatives of the count that do not recite a particular target are clearly anticipated thereby. Considering the target, i.e., the tyrosine kinase receptor for HER2, p185^{HER2}, Applicants submit that claim 30 is clearly obvious over the other alternatives of the count for the reasons that follow.

The import of p185^{HER2} to cancer, particularly breast cancer, had been well documented well before the priority date of the 213 patent. See, for example, Slamon, D.J. et al., *Science*, 235:177-182,1987. The development of antibodies against p185^{HER2} for use in therapy had also

been well documented, as was the use of antibodies other than hybridoma-produced monoclonal antibodies. Applicants respectfully submit that, in view of the knowledge of the art at the time, a humanized antibody against p185^{HER2} would have been obvious over the remaining alternatives of the count.

(d) Identification of claims corresponding to the count

Applicants identify all of the 213 patent claims, claims 1-82, and all of Applicants' pending claims, claims 24-25, as corresponding to the proposed count. All of said claims are either anticipated by, or obvious over, the proposed count as required by 37 CFR § 41.207(b)(2).

More specifically, the alternatives of the proposed count recite framework residue substitutions recited in claims 1, 5-28, 34-63, 66, 70-77, and 79 of the 213 patent.

Claims 2, 31, 67, and 81 of the 213 patent recite that the substituted residue is the residue found at the corresponding location of the non-human antibody from which the non-human CDR amino acids are obtained. Such a claim is anticipated by the proposed count – the goal of substituting framework residues is to improve affinity to approach that of the antibody from which the CDRs are obtained. See, for example, Riechmann, et al., *Nature*, 332:323-327, 1988.

Claims 3, 32, 68, and 82 of the 213 patent recite that no human framework residue other than those set forth in the claim from which they depend has been substituted. Such claims are anticipated by, or clearly obvious over, the proposed count in view of the use of the Markush language “selected from the group consisting of.” The transitional phrase “consisting of” is closed, meaning that it excludes any element not listed. See MPEP 2111.03.

Claims 29 and 78 recite an antibody comprising the humanized variable domains of the claims from which they depend. Such claims are clearly anticipated by claim 30 of the proposed count.

While claims 4, 33, 62 and 69 of the 213 patent further recite that the variable domain is a “consensus” human variable domain, use of such framework regions in making humanized antibodies was known by the priority date of the 213 patent. See, for example, column 13, lines

6-9, of U.S. Patent No. 5,530,101, issued to Queen et al., filed on December 19, 1990. Claims 62 and 69 are, thus, obvious in view of the proposed count and the art.

The recitation in claim 63 that the humanized antibody lacks immunogenicity when compared to the non-human parent upon repeated administration is the very goal of humanization and is, thus, implicit. The motivation for preparing humanized antibodies was to reduce immunogenicity. See column 1, lines 51-58, of the 213 patent, discussing the “Background of the Invention.” References from the early 1980’s are cited therein disclosing the anti-globulin response to rodent monoclonal antibodies. Claim 63 is, at a minimum, obvious over the proposed count and the art.

The alternatives recited in claim 64 include the alternatives recited in claim 80 of the count. Claim 64 is, thus, anticipated by the proposed count.

The recitation in claim 65 that the variant of claim 63 binds the antigen “**up to 3-fold more in the binding affinity than the parent antibody binds antigen**” broadly includes variants that have binding affinities equal to **and less than the parent**. As recognized in the “Background of the Invention” section of the 213 patent, at column 3, lines 50-55, humanizing antibody while **retaining** high affinity for antigen was difficult to achieve; achieving lower affinity was not. This recitation, thus, is also either anticipated by, or obvious over, the proposed count in view of the art.

All of Applicants claims, claims 24-25, are anticipated by or obvious over the proposed count. Claim 25 is anticipated by claim 30 of the count.

(e) Interference-In-Fact

“An interference-in-fact exists if the subject matter of a claim of one party would, if prior art, have anticipated or rendered obvious the subject matter of a claim of the opposing party and vice versa.” 37 CFR § 41.203(a) (2004). Applicants set forth in attached Appendix C a comparison of claim 66 of the 213 patent with Applicants’ claim 24, both of which correspond to the present count.

As is clear from Appendix C, each claim anticipates and/or renders obvious the other. Both claims recite a humanized variable domain. Both claims are directed to a heavy chain

variable domain. Both claims also recite that the variable domain comprises non-human complementarity determining region amino acid residues which bind an antigen. Both claims also comprise a human framework region; claim 66 of the 213 patent simply recites it differently -- if one incorporates non-human CDR amino acid residues into a human antibody variable domain, one ends up with a human framework region. Indeed, as discussed above, the 213 patent defines "humanized antibody" as an antibody having a framework region "having substantially the amino acid sequence of a human immunoglobulin," i.e., a human framework region (see column 8, lines 11-17, of the 213 patent). Both claims recite a single amino acid substitution in the framework region to be selected from a Markush group listed thereafter; both recite that residue 24 of the heavy chain is to be substituted. Finally, both claims recite that numbering is according to Kabat.

(f) Support for Applicants Claims

In attached Appendix D, Applicants illustrate the representative support in their disclosure for the limitations of their claims 24 to 25. There is, of course, additional support in Applicants' application omitted herein for the sake of brevity. In Appendix E, Applicants show support for their claim 24, filed December 21, 1989. Methods for preparing exemplary antibodies having framework substitutions are described, *inter alia*, on pages 18-23, Sections 13.1.1 through 15.3, of the GB priority application.

(g) Applicants Will Prevail on Priority

US Serial No. 08/146,206, which issued as the 213 patent, was filed on June 15, 1992 as a PCT continuation-in-part of U.S. Serial No. 07/715,272, filed June 14, 1991.

The present application is a continuation of Application Serial No. 08/846,658, filed May 1, 1997, which is a continuation of Application Serial No. 08/303,569, filed September 7, 1994, now U.S. Patent No. 5,859,205, which is a continuation of Application Serial No. 07/743,329, filed as PCT/GB90/02017, filed December 21, 1990 and which claims priority benefit of GB 8928874.0, filed December 21, 1989. Applicants' earliest constructive reduction to practice date to which they are entitled is at least as early as December 21, 1989, but not later than December

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C.F.R. § 42.202 DATED NOVEMBER 21, 2005

21, 1990. Regardless, both dates are earlier than the earliest filing date of the 213 patent , or June 14, 1991.

CONCLUSION

The Requested Interference Should Be Declared

Early consideration and indication of allowability of all pending claims is respectfully requested. For an interference to be declared, however, only one claim needs to be allowable. MPEP § 2307.02. Should the present examination involve rejections of applicant's claims that would have been equally applicable against the 213 patent claims, applicants respectfully note MPEP § 2307.02, which requires the approval of the Group Director for such a rejection. Applicants are presumptively the prior inventors of the claimed subject matter and only desire an interference to prove that they are the actual prior inventors. Their opportunity to do so should not be unduly delayed. In view of the foregoing, Applicants respectfully request that an interference be declared between the present application and the 213 patent.

To assist the Examiner, Applicants note the following.

- (1) the proposed count for the interference should be the Proposed Count set forth in Appendix B.
- (2) the claims of the 213 patent which should be designated as corresponding to the count are claims 1-82, all of the issued claims of the patent.
- (3) the claims of Applicants that should be designated as corresponding to the count are claims 24-25, all pending claims of the Applicants' above-identified application.
- (4) no claims of either party should be designated as not corresponding to the count since none are directed to a separate patentable invention when considering the proposed count.

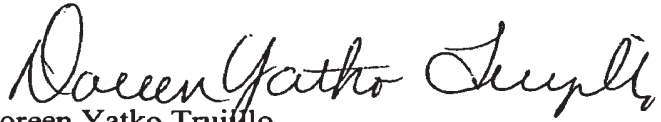
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**PRELIMINARY AMENDMENT AND REQUEST FOR INTERFERENCE UNDER 37
C.F.R. § 42.202 DATED NOVEMBER 21, 2005**

The examiner is requested to contact the undersigned attorney if an interview, telephonic or personal, would facilitate allowance of the claims or declaration of an interference.

Respectfully submitted,



Doreen Yatko Trujillo
Registration No. 35,719

Date: *November 21, 2005*

COZEN O'CONNOR, P.C.
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(215) 665-5593 - Telephone
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Attachments: **Appendices A-E**
Copy of U.S. Patent No. 6,407,213 B1
Copy of PCT Publication WO 91/09967

APPENDIX A

1. A CDR-grafted antibody heavy chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 6, 23 and/or 24, 48 and/or 49, 71 and/or 73, 75 and/or 76 and/or 78 and 88 and/or 91.
2. A CDR-grafted heavy chain according to claim 1 comprising donor residues at positions 23, 24, 49, 71, 73 and 78, or at positions 23, 24 and 49.
3. A CDR-grafted heavy chain according to claim 2 comprising donor residues at positions 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.
4. A CDR-grafted heavy chain according to claim 2 or 3, comprising donor residues at one, some or all of positions: 1 and 3, 69 (if 48 is different between donor and acceptor), 38 and 46 (if 48 is the donor residue), 67, 82 and IS (if 67 is the donor residue), 91, and any one or more of 9, 11, 41, 87, 108, 110 and 112.
5. A CDR-grafted heavy chain according to any of the preceding comprising donor CDRs at positions 26-35, 50-65 and 95-100.
6. A CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 1 and/or 3 and 46 and/or 47.
7. A CDR-grafted light chain according to claim 6 comprising donor residues at positions 46 and 47.
8. A CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor

residues at at least one of positions 46, 48, 58 and 71.

9. A CDR-grafted light chain according to claim 8 comprising donor residues at positions 46, 48, 58 and 71.

10. A CDR-grafted light chain according to claim 8 or 9, comprising donor residues at positions 2, 4, 6, 35, 36, 38, 44, 47, 49, 62, 64-69, 85, 87, 98, 99, 101 and 102.

11. A CDR-grafted light chain according to claim 9 or 10, comprising donor residues at one, some or all of positions: 1 and 3, 63, 60 (if 60 and 54 are able to form a potential saltbridge), 70 (if 70 and 24 are able to form a potential saltbridge), 73 and 21 (if 47 is different between donor and acceptor), 37 and 45 (if 47 is different between donor and acceptor), and any one or more of 10, 12, 40, 83, 103 and 105.

12. A CDR-grafted light chain according to any one of claims 6-11, comprising donor CDRs at positions 24-34, 50-56 and 89-97.

13. A CDR-grafted antibody molecule comprising at least one CDR-grafted heavy chain according to any one of claims 1-5 and at least one CDR-grafted light chain according to any one of claims 6-12.

14. A CDR-grafted antibody molecule according to claim 13, which is a site-specific antibody molecule.

15. A CDR-grafted antibody molecule according to claim 13 which has specificity for an interleukin, hormone or other biologically active compound or a receptor therefor.

16. A CDR-grafted antibody heavy or light chain or molecule according to any one of the preceding claims comprising human acceptor residues and non-human donor residues.

17. A DNA sequence which codes for a CDR-grafted heavy chain according to claim 1 or a

CDR-grafted light chain according to claim 6 or claim 8.

18. A cloning or expression vector containing a DNA sequence according to claim 17.

19. A host cell transformed with a DNA sequence according to claim 17.

20. A process for the production of a CDR-grafted antibody sequence according to claim 17 in a transformed host cell.

21. A process for producing a CDR-grafted antibody product comprising: (a) producing in an expression vector an operon having a DNA sequence which encodes an antibody heavy chain according to claim 1; and/or (b) producing in an expression vector an operon having a DNA sequence which encodes a complementary antibody light chain according to claim 6 or claim 8; (c) transfecting a host cell with the or each vector; and (d) culturing the transfected cell line to produce the CDR-grafted antibody product.

22. A therapeutic or diagnostic composition comprising a CDR-grafted antibody heavy chain according to claim 1, or a CDR-grafted light chain according to claim 6 or claim 8, or a CDR-grafted antibody molecule according to claim 13 in combination with a pharmaceutically acceptable carrier, diluent or excipient.

23. A method of therapy or diagnosis comprising administering an effective amount of a CDR-grafted heavy chain according to claim 1, or a CDR-grafted light chain according to claim 6 or claim 8, or a CDR-grafted antibody molecule according to claim 13 to a human or animal subject.

APPENDIX B**Proposed Count for interference****Claim 30 of the 213 patent**

An antibody which binds p185^{HER2} and comprises a humanized antibody variable domain, wherein the humanized antibody variable domain comprises non-human Complementarity Determining Region (CDR) amino acid residues which bind p185^{HER2} incorporated into a human antibody variable domain, and further comprises a Framework Region (FR) amino acid substitution at a site selected from the group consisting of: 4L, 38L, 43L, 44L, 46L, 58L, 62L, 65L, 66L, 67L, 68L, 69L, 73L, 85L, 98L, 2H, 4H, 36H, 39H, 43H, 45H, 69H, 70H, 74H, 75H, 76H, 78H and 92H, utilizing the numbering system set forth in Kabat.

OR

Claim 80 of the 213 patent

A humanized antibody variable domain comprising non-human Complementarity Determining Region (CDR) amino acid residues which bind an antigen incorporated into a human antibody variable domain, and further comprising a Framework Region (FR) amino acid substitution where the substituted FR residue:

(a) noncovalently binds antigen directly;

(b) interacts with a CDR; or

(c) participates in the $V_L - V_H$ interface by affecting the proximity or orientation of the V_L and V_H regions with respect to one another, and wherein the substituted FR residue is at a site selected from the group consisting of: 4L, 38L, 43L, 44L, 58L, 62L, 65L, 66L, 67L, 68L, 69L, 73L, 85L, 98L, 2H, 4H, 24H, 36H, 39H, 43H, 45H, 69H, 70H, 73H, 74H, 76H, 78H, 92H and 93H, utilizing the numbering system set forth in Kabat.

OR

Applicants' claim 24

A humanised antibody **heavy chain** variable domain comprising non-human complementarity determining region amino acid residues which bind an antigen and a human framework region wherein said framework region comprises an amino acid substitution at a residue selected from the group consisting of 23, 24, 49, 71, 73, and 78, and combinations thereof, as numbered according to Kabat.

APPENDIX C**Comparison of Applicants' claim 24 and claim 66 of the 213 patent**

Applicants' Claim 24	213 patent Claim 66
Claim 24 – A humanised antibody heavy chain variable domain comprising	A humanized antibody heavy chain variable domain comprising
non-human complementarity determining region amino acid residues which bind an antigen and	non-human Complementarity Determining Region (CDR) amino acid residues which bind an antigen
a human framework region wherein said framework region comprises an amino acid substitution at a residue selected from the group consisting of	incorporated into a human antibody variable domain, and further comprising a Framework Region (FR) amino acid substitution at a site selected from the group consisting of:
23, 24, 49, 71, 73, and 78, and combinations thereof,	24H, 73H, 76H, 78H, and 93H
as numbered according to Kabat.	utilizing the numbering system as set forth in Kabat.

APPENDIX D**Support for Applicants' claims in Applicants' Present Specification**

Claim	Present Specification
Claim 24 -- A humanised antibody heavy chain variable domain comprising	page 6, lines 29-31
non-human complementarity determining region amino acid residues which bind an antigen and	page 7, line 29, through page 8, line 7 and page 17, lines 6-7
a human framework region wherein said framework region comprises an amino acid substitution at a residue selected from the group consisting of	page 7, line 29, through page 8, line 7 and page 17, lines 1-19
23, 24, 49, 71, 73, and 78, and combinations thereof,	page 7, lines 1-3
as numbered according to Kabat.	page 8, lines 22-24
Claim 25 -- A humanised antibody comprising the variable domain of claim 24.	see above support for claim 24

APPENDIX E

Support For Applicants' Claims in the GB Application, filed December 21, 1989

Claim	1989 GB Application
Claim 24 – A humanised antibody heavy chain variable domain comprising	page 5, lines 8-10
non-human complementarity determining region amino acid residues which bind an antigen and	page 5, lines 8-21
a human framework region wherein said framework region comprises an amino acid substitution at a residue selected from the group consisting of	page 5, line 10 and page 26, lines 31-33
23, 24, 49, 71, 73, and 78, and combinations thereof,	page 6, lines 8-10 and Table 1
as numbered according to Kabat.	page 6, lines 5-7

DOCKET NO.: CARP0001-112
APPLICATION SERIAL NO. 11/284,261

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re application of: **John R. Adair et al.**

Confirmation No. **5305**

Serial No.: **11/284,261**

Art Unit: **1643**

Filed: **November 21, 2005**

Examiner: **Anne Gussow**

Title: **HUMANISED ANTIBODIES**

Customer No.: **34132**

Via EFS Web:
DATE FILED: **September 9, 2009**

MAIL STOP AMENDMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

REQUEST FOR RECONSIDERATION

Dear Sir:

This paper is being filed in response to the Non-Final Office Action dated as mailed March 9, 2009. Applicants hereby petition for a three-month extension of time to respond to the Non-Final Office Action and request the Commissioner to charge Deposit Account 50-3111 the appropriate extension of time fee.

Listing of the Claims, begin on page 2.

Remarks begin on page 3.

The **Conclusion** is found on page 5.

**Carter Exhibit 2004
Carter v. Adair
Interference No. 105,744**

LISTING OF THE CLAIMS

Claims 1-23 (**cancelled**)

Claim 24 (**currently amended**): A humanised antibody comprising a heavy chain variable domain comprising non-human complementarity determining region amino acid residues which bind an antigen and a human framework region wherein said framework region comprises a non-human amino acid substitution at a residue selected from the group consisting of 23, 24, 49, 71, 73, and 78, and combinations thereof, as numbered according to Kabat.

Claim 25 (**cancelled**)

REMARKS

Claims 24 and 25 were pending. All pending claims were rejected in the Non-Final Rejection. In view of the foregoing amendments and arguments that follow, Applicants respectfully request withdrawal of all rejections upon reconsideration.

Applicants acknowledge with appreciation the Office's withdrawal of the objections to claim 24 under 35 U.S.C. 112, second paragraph, as being indefinite.

Rejection Under 35 U.S.C. § 112, First Paragraph

Claim 24 was amended with the RCE filing and was again rejected as allegedly being indefinite. The Office alleges that the specification is enabling for a humanized antibody comprising a heavy chain variable domain and a light chain variable domain, with all 6 CDRs, and does not provide enablement for a humanized antibody heavy chain variable domain alone. The Office is clearly disregarding the fact that the CDR-grafted chains can be combined with other chains, as disclosed in the specification, including chimeric and mouse chains. Thus, it is not necessary for the claims to recite both chains. Applicants traverse this rejection but have amended claim 24 to recite a humanized antibody comprising a heavy chain variable domain.

Applicants respectfully submit that this rejection has been overcome.

Rejection Under 35 U.S.C. 102(e)

Claims 24 and 25 were rejected under 35 U.S.C. 102(e) as allegedly being anticipated by Queen, et al US Patent 5,585,089 the "'089 patent". Claim 25 has been cancelled. Applicants traverse this rejection as it applies to claim 24.

With all due respect, the Office has apparently misread the claims. Initially, the office states that the claims recite that the framework region comprises a **non-human** amino acid

substitution at a residue selected from 23, 24, 49, 71, 73, and 78, and combinations thereof. The Office then asserts that the “‘089 patent” teaches an antibody comprising a humanized heavy chain variable domain comprising human residues at positions except the CDRs, and the following framework positions—27, 93, 95, 98, 107-109, 11, 30, 67, 48, and 68. The Office concludes, thus, that all the remaining framework positions are the human antibody allegedly taught in the “‘089 patent”. The Office then states that, since the claims recite substitution to **human** residues in the heavy chain in residues 23, 24, 49, 71, 73, and 78, and the “‘089 patent” teaches human residues in all of those positions, the claim limitations are met. As the Office previously acknowledged, however, these residues are **non-human** residues in claim 24. The “‘089 patent”, thus, does not anticipate the Applicants invention.

Applicants respectfully request that this rejection be withdrawn.

CONCLUSION

Applicants respectfully submit that claim 24 is allowable and early allowance of the same. If a telephonic conversation with Applicants' attorney would help expedite the prosecution of the above-identified application, the Examiner is urged to call the undersigned at 215-665-5593.

Respectfully submitted,

/Doreen Yatko Trujillo/

Doreen Yatko Trujillo
Registration No. 35,719

Date: September 9, 2009

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PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

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(54) Title: HUMANISED ANTIBODIES

(57) Abstract

CDR-grafted antibody heavy and light chains comprise acceptor framework and donor antigen binding regions, the heavy chains comprising donor residues at at least one of positions (6, 23) and/or (24, 48) and/or (49, 71) and/or (73, 75) and/or (76) and/or (78) and (88) and/or (91). The CDR-grafted light chains comprise donor residues at at least one of positions (1) and/or (3) and (46) and/or (47) or at at least one of positions (46, 48, 58) and (71). The CDR-grafted antibodies are preferably humanised antibodies, having non human, e.g. rodent, donor and human acceptor frameworks, and may be used for *in vivo* therapy and diagnosis. A generally applicable protocol is disclosed for obtaining CDR-grafted antibodies.

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HUMANISED ANTIBODIESField of the Invention

The present invention relates to humanised antibody molecules, to processes for their production using recombinant DNA technology, and to their therapeutic uses.

The term "humanised antibody molecule" is used to describe a molecule having an antigen binding site derived from an immunoglobulin from a non-human species, and remaining immunoglobulin-derived parts of the molecule being derived from a human immunoglobulin. The antigen binding site typically comprises complementarity determining regions (CDRs) which determine the binding specificity of the antibody molecule and which are carried on appropriate framework regions in the variable domains. There are 3 CDRs (CDR1, CDR2 and CDR3) in each of the heavy and light chain variable domains.

In the description, reference is made to a number of publications by number. The publications are listed in numerical order at the end of the description.

Background of the Invention

Natural immunoglobulins have been known for many years, as have the various fragments thereof, such as the Fab, (Fab')₂ and Fc fragments, which can be derived by enzymatic cleavage. Natural immunoglobulins comprise a generally Y-shaped molecule having an antigen-binding site towards the end of each upper arm. The remainder of the structure, and particularly the stem of the Y, mediates the effector functions associated with immunoglobulins.

Natural immunoglobulins have been used in assay, diagnosis and, to a more limited extent, therapy. However, such uses, especially in therapy, were hindered until recently by the polyclonal nature of natural immunoglobulins. A significant step towards the realisation of the potential

of immunoglobulins as therapeutic agents was the discovery of procedures for the production of monoclonal antibodies (MAbs) of defined specificity (1).

However, most MAbs are produced by hybridomas which are fusions of rodent spleen cells with rodent myeloma cells. They are therefore essentially rodent proteins. There are very few reports of the production of human MAbs.

Since most available MAbs are of rodent origin, they are naturally antigenic in humans and thus can give rise to an undesirable immune response termed the HAMA (Human Anti-Mouse Antibody) response. Therefore, the use of rodent MAbs as therapeutic agents in humans is inherently limited by the fact that the human subject will mount an immunological response to the MAb and will either remove it entirely or at least reduce its effectiveness. In practice, MAbs of rodent origin may not be used in patients for more than one or a few treatments as a HAMA response soon develops rendering the MAb ineffective as well as giving rise to undesirable reactions. For instance, OKT3 a mouse IgG2a/k MAb which recognises an antigen in the T-cell receptor-CD3 complex has been approved for use in many countries throughout the world as an immunosuppressant in the treatment of acute allograft rejection [Chatenoud et al (2) and Jeffers et al (3)]. However, in view of the rodent nature of this and other such MAbs, a significant HAMA response which may include a major anti-idiotypic component, may build up on use. Clearly, it would be highly desirable to diminish or abolish this undesirable HAMA response and thus enlarge the areas of use of these very useful antibodies.

Proposals have therefore been made to render non-human MAbs less antigenic in humans. Such techniques can be generically termed "humanisation" techniques. These

techniques typically involve the use of recombinant DNA technology to manipulate DNA sequences encoding the polypeptide chains of the antibody molecule.

Early methods for humanising MAbS involved production of chimeric antibodies in which an antigen binding site comprising the complete variable domains of one antibody is linked to constant domains derived from another antibody. Methods for carrying out such chimerisation procedures are described in EP0120694 (Celltech Limited), EP0125023 (Genentech Inc. and City of Hope), EP-A-0 171496 (Res. Dev. Corp. Japan), EP-A-0 173 494 (Stanford University), and WO 86/01533 (Celltech Limited). This latter Celltech application (WO 86/01533) discloses a process for preparing an antibody molecule having the variable domains from a mouse MAb and the constant domains from a human immunoglobulin. Such humanised chimeric antibodies, however, still contain a significant proportion of non-human amino acid sequence, i.e. the complete non-human variable domains, and thus may still elicit some HAMA response, particularly if administered over a prolonged period [Begent et al (ref. 4)].

In an alternative approach, described in EP-A-0239400 (Winter), the complementarity determining regions (CDRs) of a mouse MAb have been grafted onto the framework regions of the variable domains of a human immunoglobulin by site directed mutagenesis using long oligonucleotides. The present invention relates to humanised antibody molecules prepared according to this alternative approach, i.e. CDR-grafted humanised antibody molecules. Such CDR-grafted humanised antibodies are much less likely to give rise to a HAMA response than humanised chimeric antibodies in view of the much lower proportion of non-human amino acid sequence which they contain.

The earliest work on humanising MAb's by CDR-grafting was carried out on MAb's recognising synthetic antigens, such as the NP or NIP antigens. However, examples in which a mouse MAb recognising lysozyme and a rat MAb recognising an antigen on human T-cells were humanised by CDR-grafting have been described by Verhoeyen et al (5) and Riechmann et al (6) respectively. The preparation of CDR-grafted antibody to the antigen on human T cells is also described in WO 89/07452 (Medical Research Council).

In Riechmann et al/Medical Research Council it was found that transfer of the CDR regions alone [as defined by Kabat refs. (7) and (8)] was not sufficient to provide satisfactory antigen binding activity in the CDR-grafted product. Riechmann et al found that it was necessary to convert a serine residue at position 27 of the human sequence to the corresponding rat phenylalanine residue to obtain a CDR-grafted product having improved antigen binding activity. This residue at position 27 of the heavy chain is within the structural loop adjacent to CDR1. A further construct which additionally contained a human serine to rat tyrosine change at position 30 of the heavy chain did not have a significantly altered binding activity over the humanised antibody with the serine to phenylalanine change at position 27 alone. These results indicate that changes to residues of the human sequence outside the CDR regions, in particular in the structural loop adjacent to CDR1, may be necessary to obtain effective antigen binding activity for CDR-grafted antibodies which recognise more complex antigens. Even so the binding affinity of the best CDR-grafted antibodies obtained was still significantly less than the original MAb.

Very recently Queen et al (9) have described the preparation of a humanised antibody that binds to the

interleukin 2 receptor, by combining the CDRs of a murine MAb (anti-Tac) with human immunoglobulin framework and constant regions. The human framework regions were chosen to maximise homology with the anti-Tac MAb sequence. In addition computer modelling was used to identify framework amino acid residues which were likely to interact with the CDRs or antigen, and mouse amino acids were used at these positions in the humanised antibody.

In WO 90/07861 Queen et al propose four criteria for designing humanised immunoglobulins. The first criterion is to use as the human acceptor the framework from a particular human immunoglobulin that is unusually homologous to the non-human donor immunoglobulin to be humanised, or to use a consensus framework from many human antibodies. The second criterion is to use the donor amino acid rather than the acceptor if the human acceptor residue is unusual and the donor residue is typical for human sequences at a specific residue of the framework. The third criterion is to use the donor framework amino acid residue rather than the acceptor at positions immediately adjacent to the CDRs. The fourth criterion is to use the donor amino acid residue at framework positions at which the amino acid is predicted to have a side chain atom within about 3 Å of the CDRs in a three-dimensional immunoglobulin model and to be capable of interacting with the antigen or with the CDRs of the humanised immunoglobulin. It is proposed that criteria two, three or four may be applied in addition or alternatively to criterion one, and may be applied singly or in any combination.

WO 90/07861 describes in detail the preparation of a single CDR-grafted humanised antibody, a humanised antibody having specificity for the p55 Tac protein of the

IL-2 receptor. The combination of all four criteria, as above, were employed in designing this humanised antibody, the variable region frameworks of the human antibody Eu (7) being used as acceptor. In the resultant humanised antibody the donor CDRs were as defined by Kabat et al (7 and 8) and in addition the mouse donor residues were used in place of the human acceptor residues, at positions 27, 30, 48, 66, 67, 89, 91, 94, 103, 104, 105 and 107 in the heavy chain and at positions 48, 60 and 63 in the light chain, of the variable region frameworks. The humanised anti-Tac antibody obtained is reported to have an affinity for p55 of $3 \times 10^9 \text{ M}^{-1}$, about one-third of that of the murine MAb.

We have further investigated the preparation of CDR-grafted humanised antibody molecules and have identified a hierarchy of positions within the framework of the variable regions (i.e. outside both the Kabat CDRs and structural loops of the variable regions) at which the amino acid identities of the residues are important for obtaining CDR-grafted products with satisfactory binding affinity. This has enabled us to establish a protocol for obtaining satisfactory CDR-grafted products which may be applied very widely irrespective of the level of homology between the donor immunoglobulin and acceptor framework. The set of residues which we have identified as being of critical importance does not coincide with the residues identified by Queen et al (9).

Summary of the Invention

Accordingly, in a first aspect the invention provides a CDR-grafted antibody heavy chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 6, 23 and/or 24, 48 and/or 49, 71 and/or 73, 75 and/or 76 and/or 78 and 88 and/or 91.

In preferred embodiments, the heavy chain framework comprises donor residues at positions 23, 24, 49, 71, 73 and 78 or at positions 23, 24 and 49. The residues at positions 71, 73 and 78 of the heavy chain framework are preferably either all acceptor or all donor residues.

In particularly preferred embodiments the heavy chain framework additionally comprises donor residues at one, some or all of positions 6, 37, 48 and 94. Also it is particularly preferred that residues at positions of the heavy chain framework which are commonly conserved across species, i.e. positions 2, 4, 25, 36, 39, 47, 93, 103, 104, 106 and 107, if not conserved between donor and acceptor, additionally comprise donor residues. Most preferably the heavy chain framework additionally comprises donor residues at positions 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.

In addition the heavy chain framework optionally comprises donor residues at one, some or all of positions:

1 and 3,
72 and 76,
69 (if 48 is different between donor and acceptor),
38 and 46 (if 48 is the donor residue),
80 and 20 (if 69 is the donor residue),
67,
82 and 18 (if 67 is the donor residue),
91,
88, and
any one or more of 9, 11, 41, 87, 108, 110 and 112.

In the first and other aspects of the present invention reference is made to CDR-grafted antibody products comprising acceptor framework and donor antigen binding regions. It will be appreciated that the invention is widely applicable to the CDR-grafting of antibodies in

general. Thus, the donor and acceptor antibodies may be derived from animals of the same species and even same antibody class or sub-class. More usually, however, the donor and acceptor antibodies are derived from animals of different species. Typically the donor antibody is a non-human antibody, such as a rodent MAb, and the acceptor antibody is a human antibody.

In the first and other aspects of the present invention, the donor antigen binding region typically comprises at least one CDR from the donor antibody. Usually the donor antigen binding region comprises at least two and preferably all three CDRs of each of the heavy chain and/or light chain variable regions. The CDRs may comprise the Kabat CDRs, the structural loop CDRs or a composite of the Kabat and structural loop CDRs and any combination of any of these. Preferably, the antigen binding regions of the CDR-grafted heavy chain variable domain comprise CDRs corresponding to the Kabat CDRs at CDR2 (residues 50-65) and CDR3 (residues 95-100) and a composite of the Kabat and structural loop CDRs at CDR1 (residues 26-35).

The residue designations given above and elsewhere in the present application are numbered according to the Kabat numbering [refs. (7) and (8)]. Thus the residue designations do not always correspond directly with the linear numbering of the amino acid residues. The actual linear amino acid sequence may contain fewer or additional amino acids than in the strict Kabat numbering corresponding to a shortening of, or insertion into, a structural component, whether framework or CDR, of the basic variable domain structure. For example, the heavy chain variable region of the anti-Tac antibody described by Queen et al (9) contains a single amino acid insert (residue 52a) after residue 52 of CDR2 and a three amino

acid insert (residues 82a, 82b and 82c) after framework residue 82, in the Kabat numbering. The correct Kabat numbering of residues may be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence.

The invention also provides in a second aspect a CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 1 and/or 3 and 46 and/or 47. Preferably the CDR grafted light chain of the second aspect comprises donor residues at positions 46 and/or 47.

The invention also provides in a third aspect a CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 46, 48, 58 and 71.

In a preferred embodiment of the third aspect, the framework comprises donor residues at all of positions 46, 48, 58 and 71.

In particularly preferred embodiments of the second and third aspects, the framework additionally comprises donor residues at positions 36, 44, 47, 85 and 87. Similarly positions of the light chain framework which are commonly conserved across species, i.e. positions 2, 4, 6, 35, 49, 62, 64-69, 98, 99, 101 and 102, if not conserved between donor and acceptor, additionally comprise donor residues. Most preferably the light chain framework additionally comprises donor residues at positions 2, 4, 6, 35, 36, 38, 44, 47, 49, 62, 64-69, 85, 87, 98, 99, 101 and 102.

In addition the framework of the second or third aspects optionally comprises donor residues at one, some or all of positions:

1 and 3,

63,

60 (if 60 and 54 are able to form a potential saltbridge),

70 (if 70 and 24 are able to form a potential saltbridge),

73 and 21 (if 47 is different between donor and acceptor),

37 and 45 (if 47 is different between donor and acceptor),

and

any one or more of 10, 12, 40, 80, 103 and 105.

Preferably, the antigen binding regions of the CDR-grafted light chain variable domain comprise CDRs corresponding to the Kabat CDRs at CDR1 (residue 24-34), CDR2 (residues 50-56) and CDR3 (residues 89-97).

The invention further provides in a fourth aspect a CDR-grafted antibody molecule comprising at least one CDR-grafted heavy chain and at least one CDR-grafted light chain according to the first and second or first and third aspects of the invention.

The humanised antibody molecules and chains of the present invention may comprise: a complete antibody molecule, having full length heavy and light chains; a fragment thereof, such as a Fab, (Fab')₂ or FV fragment; a light chain or heavy chain monomer or dimer; or a single chain antibody, e.g. a single chain FV in which heavy and light chain variable regions are joined by a peptide linker; or any other CDR-grafted molecule with the same specificity as the original donor antibody. Similarly the CDR-grafted heavy and light chain variable region may be combined with other antibody domains as appropriate.

Also the heavy or light chains or humanised antibody molecules of the present invention may have attached to them an effector or reporter molecule. For instance, it may have a macrocycle, for chelating a heavy metal atom, or a toxin, such as ricin, attached to it by a covalent bridging structure. Alternatively, the procedures of recombinant DNA technology may be used to produce an immunoglobulin molecule in which the Fc fragment or CH3 domain of a complete immunoglobulin molecule has been replaced by, or has attached thereto by peptide linkage, a functional non-immunoglobulin protein, such as an enzyme or toxin molecule.

Any appropriate acceptor variable region framework sequences may be used having regard to class/type of the donor antibody from which the antigen binding regions are derived. Preferably, the type of acceptor framework used is of the same/similar class/type as the donor antibody. Conveniently, the framework may be chosen to maximise/optimise homology with the donor antibody sequence particularly at positions close or adjacent to the CDRs. However, a high level of homology between donor and acceptor sequences is not important for application of the present invention. The present invention identifies a hierarchy of framework residue positions at which donor residues may be important or desirable for obtaining a CDR-grafted antibody product having satisfactory binding properties. The CDR-grafted products usually have binding affinities of at least 10^5 M^{-1} , preferably at least about 10^8 M^{-1} , or especially in the range 10^8 - 10^{12} M^{-1} . In principle, the present invention is applicable to any combination of donor and acceptor antibodies irrespective of the level of homology between their sequences. A protocol for applying the invention to any particular donor-acceptor antibody pair is given hereinafter. Examples of human frameworks which may be

used are KOL, NEWM, REI, EU, LAY and POM (refs. 4 and 5) and the like; for instance KOL and NEWM for the heavy chain and REI for the light chain and EU, LAY and POM for both the heavy chain and the light chain.

Also the constant region domains of the products of the invention may be selected having regard to the proposed function of the antibody in particular the effector functions which may be required. For example, the constant region domains may be human IgA, IgE, IgG or IgM domains. In particular, IgG human constant region domains may be used, especially of the IgG1 and IgG3 isotypes, when the humanised antibody molecule is intended for therapeutic uses, and antibody effector functions are required. Alternatively, IgG2 and IgG4 isotypes may be used when the humanised antibody molecule is intended for therapeutic purposes and antibody effector functions are not required, e.g. for simple blocking of lymphokine activity.

However, the remainder of the antibody molecules need not comprise only protein sequences from immunoglobulins. For instance, a gene may be constructed in which a DNA sequence encoding part of a human immunoglobulin chain is fused to a DNA sequence encoding the amino acid sequence of a functional polypeptide such as an effector or reporter molecule.

Preferably the CDR-grafted antibody heavy and light chain and antibody molecule products are produced by recombinant DNA technology.

Thus in further aspects the invention also includes DNA sequences coding for the CDR-grafted heavy and light chains, cloning and expression vectors containing the DNA sequences, host cells transformed with the DNA sequences

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and processes for producing the CDR-grafted chains and antibody molecules comprising expressing the DNA sequences in the transformed host cells.

The general methods by which the vectors may be constructed, transfection methods and culture methods are well known per se and form no part of the invention. Such methods are shown, for instance, in references 10 and 11.

The DNA sequences which encode the donor amino acid sequence may be obtained by methods well known in the art. For example the donor coding sequences may be obtained by genomic cloning, or cDNA cloning from suitable hybridoma cell lines. Positive clones may be screened using appropriate probes for the heavy and light chain genes in question. Also PCR cloning may be used.

DNA coding for acceptor, e.g. human acceptor, sequences may be obtained in any appropriate way. For example DNA sequences coding for preferred human acceptor frameworks such as KOL, REI, EU and NEWM, are widely available to workers in the art.

The standard techniques of molecular biology may be used to prepare DNA sequences coding for the CDR-grafted products. Desired DNA sequences may be synthesised completely or in part using oligonucleotide synthesis techniques. Site-directed mutagenesis and polymerase chain reaction (PCR) techniques may be used as appropriate. For example oligonucleotide directed synthesis as described by Jones et al (ref. 20) may be used. Also oligonucleotide directed mutagenesis of a pre-existing variable region as, for example, described by Verhoeyen et al (ref. 5) or Riechmann et al (ref. 6) may be used. Also enzymatic filling in of gapped

oligonucleotides using T₄ DNA polymerase as, for example, described by Queen et al (ref. 9) may be used.

Any suitable host cell/vector system may be used for expression of the DNA sequences coding for the CDR-grafted heavy and light chains. Bacterial e.g. E. coli, and other microbial systems may be used, in particular for expression of antibody fragments such as FAb and (Fab')₂ fragments, and especially FV fragments and single chain antibody fragments e.g. single chain FVs. Eucaryotic e.g. mammalian host cell expression systems may be used for production of larger CDR-grafted antibody products, including complete antibody molecules. Suitable mammalian host cells include CHO cells and myeloma or hybridoma cell lines.

Thus, in a further aspect the present invention provides a process for producing a CDR-grafted antibody product comprising:

- (a) producing in an expression vector an operon having a DNA sequence which encodes an antibody heavy chain according to the first aspect of the invention;

and/or

- (b) producing in an expression vector an operon having a DNA sequence which encodes a complementary antibody light chain according to the second or third aspect of the invention;
- (c) transfecting a host cell with the or each vector; and
- (d) culturing the transfected cell line to produce the CDR-grafted antibody product.

The CDR-grafted product may comprise only heavy or light chain derived polypeptide, in which case only a heavy chain or light chain polypeptide coding sequence is used to transfect the host cells.

For production of products comprising both heavy and light chains, the cell line may be transfected with two vectors, the first vector may contain an operon encoding a light chain-derived polypeptide and the second vector containing an operon encoding a heavy chain-derived polypeptide. Preferably, the vectors are identical, except in so far as the coding sequences and selectable markers are concerned, so as to ensure as far as possible that each polypeptide chain is equally expressed. Alternatively, a single vector may be used, the vector including the sequences encoding both light chain- and heavy chain-derived polypeptides.

The DNA in the coding sequences for the light and heavy chains may comprise cDNA or genomic DNA or both. However, it is preferred that the DNA sequence encoding the heavy or light chain comprises at least partially, genomic DNA, preferably a fusion of cDNA and genomic DNA.

The present invention is applicable to antibodies of any appropriate specificity. Advantageously, however, the invention may be applied to the humanisation of non-human antibodies which are used for in vivo therapy or diagnosis. Thus the antibodies may be site-specific antibodies such as tumour-specific or cell surface-specific antibodies, suitable for use in in vivo therapy or diagnosis, e.g. tumour imaging. Examples of cell surface-specific antibodies are anti-T cell antibodies, such as anti-CD3, and CD4 and adhesion molecules, such as CR3, ICAM and ELAM. The antibodies may have specificity for interleukins (including lymphokines, growth factors and stimulating factors), hormones and other biologically active compounds, and receptors for any of these. For

example, the antibodies may have specificity for any of the following: Interferons α , β , γ or δ , IL1, IL2, IL3, or IL4, etc., TNF, GCSF, GMCSF, EPO, hGH, or insulin, etc.

The the present invention also includes therapeutic and diagnostic compositions comprising the CDR-grafted products of the invention and uses of such compositions in therapy and diagnosis.

Accordingly in a further aspect the invention provides a therapeutic or diagnostic composition comprising a CDR-grafted antibody heavy or light chain or molecule according to previous aspects of the invention in combination with a pharmaceutically acceptable carrier, diluent or excipient.

Accordingly also the invention provides a method of therapy or diagnosis comprising administering an effective amount of a CDR-grafted antibody heavy or light chain or molecule according to previous aspects of the invention to a human or animal subject.

A preferred protocol for obtaining CDR-grafted antibody heavy and light chains in accordance with the present invention is set out below together with the rationale by which we have derived this protocol. This protocol and rationale are given without prejudice to the generality of the invention as hereinbefore described and defined.

Protocol

It is first of all necessary to sequence the DNA coding for the heavy and light chain variable regions of the donor antibody, to determine their amino acid sequences. It is also necessary to choose appropriate acceptor heavy and light chain variable regions, of known amino acid sequence. The CDR-grafted chain is then designed

starting from the basis of the acceptor sequence. It will be appreciated that in some cases the donor and acceptor amino acid residues may be identical at a particular position and thus no change of acceptor framework residue is required.

1. As a first step donor residues are substituted for acceptor residues in the CDRs. For this purpose the CDRs are preferably defined as follows:

Heavy chain	-	CDR1:	residues 26-35
	-	CDR2:	residues 50-65
	-	CDR3:	residues 95-102
Light chain	-	CDR1:	residues 24-34
	-	CDR2:	residues 50-56
	-	CDR3:	residues 89-97

The positions at which donor residues are to be substituted for acceptor in the framework are then chosen as follows, first of all with respect to the heavy chain and subsequently with respect to the light chain.

2. Heavy Chain

- 2.1 Choose donor residues at all of positions 23, 24, 49, 71, 73 and 78 of the heavy chain or all of positions 23, 24 and 49 (71, 73 and 78 are always either all donor or all acceptor).
- 2.2 Check that the following have the same amino acid in donor and acceptor sequences, and if not preferably choose the donor: 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.

2.3 To further optimise affinity consider choosing donor residues at one, some or any of:

- i. 1, 3
- ii. 72, 76
- iii. If 48 is different between donor and acceptor sequences, consider 69
- iv. If at 48 the donor residue is chosen, consider 38 and 46
- v. If at 69 the donor residue is chosen, consider 80 and then 20
- vi. 67
- vii. If at 67 the donor residue is chosen, consider 82 and then 18
- viii. 91
- ix. 88
- x. 9, 11, 41, 87, 108, 110, 112

3. Light Chain

3.1 Choose donor at 46, 48, 58 and 71

3.2 Check that the following have the same amino acid in donor and acceptor sequences, if not preferably choose donor:

2, 4, 6, 35, 38, 44, 47, 49, 62, 64-69 inclusive, 85, 87, 98, 99, 101 and 102

3.3 To further optimise affinity consider choosing donor residues at one, some or any of:

- i. 1, 3
- ii. 63

- iii. 60, if 60 and 54 are able to form potential saltbridge
- iv. 70, if 70 and 24 are able to form potential saltbridge
- v. 73, and 21 if 47 is different between donor and acceptor
- vi. 37, and 45 if 47 is different between donor and acceptor
- vii. 10, 12, 40, 80, 103, 105

Rationale

In order to transfer the binding site of an antibody into a different acceptor framework, a number of factors need to be considered.

1. The extent of the CDRs

The CDRs (Complementary Determining Regions) were defined by Wu and Kabat (refs. 4 and 5) on the basis of an analysis of the variability of different regions of antibody variable regions. Three regions per domain were recognised. In the light chain the sequences are 24-34, 50-56, 89-97 (numbering according to Kabat (ref. 4), Eu Index) inclusive and in the heavy chain the sequences are 31-35, 50-65 and 95-102 inclusive.

When antibody structures became available it became apparent that these CDR regions corresponded in the main to loop regions which extended from the β barrel framework of the light and heavy variable domains. For H1 there was a discrepancy in that the loop was from 26 to 32 inclusive and for H2 the loop was 52 to 56 and for L2 from 50 to 53. However, with the exception of H1 the CDR regions encompassed the loop regions and extended into the β strand

frameworks. In H1 residue 26 tends to be a serine and 27 a phenylalanine or tyrosine, residue 29 is a phenylalanine in most cases. Residues 28 and 30 which are surface residues exposed to solvent might be involved in antigen-binding. A prudent definition of the H1 CDR therefore would include residues 26-35 to include both the loop region and the hypervariable residues 33-35.

It is of interest to note the example of Riechmann et al (ref. 3), who used the residue 31-35 choice for CDR-H1. In order to produce efficient antigen binding, residue 27 also needed to be recruited from the donor (rat) antibody.

2. Non-CDR residues which contribute to antigen binding

By examination of available X-ray structures we have identified a number of residues which may have an effect on net antigen binding and which can be demonstrated by experiment. These residues can be sub-divided into a number of groups.

2.1 Surface residues near CDR [all numbering as in Kabat et al (ref. 7)].

2.1.1. Heavy Chain - Key residues are 23, 71 and 73. Other residues which may contribute to a lesser extent are 1, 3 and 76. Finally 25 is usually conserved but the murine residue should be used if there is a difference.

2.1.2 Light Chain - Many residues close to the CDRs, e.g. 63, 65, 67 and 69 are conserved. If conserved none of the surface residues in the light chain are likely to have a major effect. However, if the murine residue at these positions

is unusual, then it would be of benefit to analyse the likely contribution more closely. Other residues which may also contribute to binding are 1 and 3, and also 60 and 70 if the residues at these positions and at 54 and 24 respectively are potentially able to form a salt bridge i.e. 60 + 54; 70 + 24.

2.2 Packing residues near the CDRs.

2.2.1. Heavy Chain - Key residues are 24, 49 and 78. Other key residues would be 36 if not a tryptophan, 94 if not an arginine, 104 and 106 if not glycines and 107 if not a threonine. Residues which may make a further contribution to stable packing of the heavy chain and hence improved affinity are 2, 4, 6, 38, 46, 67 and 69. 67 packs against the CDR residue 63 and this pair could be either both mouse or both human. Finally, residues which contribute to packing in this region but from a longer range are 18, 20, 80, 82 and 86. 82 packs against 67 and in turn 18 packs against 82. 80 packs against 69 and in turn 20 packs against 80. 86 forms an H bond network with 38 and 46. Many of the mouse-human differences appear minor e.g. Leu-Ile, but could have an minor impact on correct packing which could translate into altered positioning of the CDRs.

2.2.2. Light Chain - Key residues are 48, 58 and 71. Other key residues would be 6 if not glutamine, 35 if not tryptophan, 62 if not phenylalanine or tryosine, 64, 66, 68, 99 and 101 if not glycines and 102 if not a threonine. Residues which make a further contribution are 2, 4, 37, 45 and 47. Finally residues 73 and 21 and 19 may make long distance packing contributions of a minor nature.

- 2.3. Residues at the variable domain interface between heavy and light chains - In both the light and heavy chains most of the non-CDR interface residues are conserved. If a conserved residue is replaced by a residue of different character, e.g. size or charge, it should be considered for retention as the murine residue.
- 2.3.1. Heavy Chain - Residues which need to be considered are 37 if the residue is not a valine but is of larger side chain volume or has a charge or polarity. Other residues are 39 if not a glutamine, 45 if not a leucine, 47 if not a tryptophan, 91 if not a phenylalanine or tyrosine, 93 if not an alanine and 103 if not a tryptophan. Residue 89 is also at the interface but is not in a position where the side chain could be of great impact.
- 2.3.2. Light Chain - Residues which need to be considered are 36, if not a tyrosine, 38 if not a glutamine, 44 if not a proline, 46, 49 if not a tyrosine, residue 85, residue 87 if not a tyrosine and 98 if not a phenylalanine.
- 2.4. Variable-Constant region interface - The elbow angle between variable and constant regions may be affected by alterations in packing of key residues in the variable region against the constant region which may affect the position of V_L and V_H with respect to one another. Therefore it is worth noting the residues likely to be in contact with the constant region. In the heavy chain the surface residues potentially in contact with the variable region are conserved between mouse and human antibodies therefore the variable region contact residues may influence the V-C interaction. In the light chain the amino acids found at a number of the constant

region contact points vary, and the V & C regions are not in such close proximity as the heavy chain. Therefore the influences of the light chain V-C interface may be minor.

- 2.4.1. Heavy Chain - Contact residues are 7, 11, 41, 87, 108, 110, 112.
- 2.4.2. Light Chain - In the light chain potentially contacting residues are 10, 12, 40, 80, 83, 103 and 105.

The above analysis coupled with our considerable practical experimental experience in the CDR-grafting of a number of different antibodies have lead us to the protocol given above.

The present invention is now described, by way of example only, with reference to the accompanying Figures 1 - 13.

Brief Description of the Figures

- Figure 1 shows DNA and amino acid sequences of the OKT3 light chain;
- Figure 2 shows DNA and amino acid sequences of the OKT3 heavy chain;
- Figure 3 shows the alignment of the OKT3 light variable region amino acid sequence with that of the light variable region of the human antibody REI;
- Figure 4 shows the alignment of the OKT3 heavy variable region amino acid sequence with that of the heavy variable region of the human antibody KOL;
- Figure 5 shows the heavy variable region amino acid sequences of OKT3, KOL and various corresponding CDR grafts;
- Figure 6 shows the light variable region amino acid sequences of OKT3, REI and various corresponding CDR grafts;

- Figure 7 shows a graph of binding assay results for various grafted OKT3 antibodies'
- Figure 8 shows a graph of blocking assay results for various grafted OKT3 antibodies;
- Figure 9 shows a similar graph of blocking assay results;
- Figure 10 shows similar graphs for both binding assay and blocking assay results;
- Figure 11 shows further similar graphs for both binding assay and blocking assay results;
- Figure 12 shows a graph of competition assay results for a minimally grafted OKT3 antibody compared with the OKT3 murine reference standard, and
- Figure 13 shows a similar graph of competition assay results comparing a fully grafted OKT3 antibody with the murine reference standard.

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

EXAMPLE 1

CDR-GRAFTING OF OKT3

MATERIAL AND METHODS

1. INCOMING CELLS

Hybridoma cells producing antibody OKT3 were provided by Ortho (seedlot 4882.1) and were grown up in antibiotic free Dulbecco's Modified Eagles Medium (DMEM) supplemented with glutamine and 5% foetal calf serum, and divided to provide both an overgrown supernatant for evaluation and cells for extraction of RNA. The overgrown supernatant was shown to contain 250 ug/mL murine IgG2a/kappa antibody. The supernatant was negative for murine lambda light chain and IgG1, IgG2b, IgG3, IgA and IgM heavy chain. 20mL of supernatant was assayed to confirm that the antibody present was OKT3.

2. MOLECULAR BIOLOGY PROCEDURES

Basic molecular biology procedures were as described in Maniatis et al (ref. 9) with, in some cases, minor modifications. DNA sequencing was performed as described in Sanger et al (ref. 11) and the Amersham International Plc sequencing handbook. Site directed mutagenesis was as described in Kramer et al (ref. 12) and the Anglian Biotechnology Ltd. handbook. COS cell expression and metabolic labelling studies were as described in Whittle et al (ref. 13)

3. RESEARCH ASSAYS

3.1. ASSEMBLY ASSAYS

Assembly assays were performed on supernatants from transfected COS cells to determine the amount of intact IgG present.

3.1.1. COS CELLS TRANSFECTED WITH MOUSE OKT3 GENES

The assembly assay for intact mouse IgG in COS cell supernatants was an ELISA with the following format:

96 well microtitre plates were coated with F(ab')₂ goat anti-mouse IgG Fc. The plates were washed in water and samples added for 1 hour at room temperature. The plates were washed and F(ab')₂ goat anti-mouse IgG F(ab')₂ (HRPO conjugated) was then added. Substrate was added to reveal the reaction. UPC10, a mouse IgG2a myeloma, was used as a standard.

3.1.2. COS AND CHO CELLS TRANSFECTED WITH CHIMERIC OR CDR-GRAFTED OKT3 GENES

The assembly assay for chimeric or CDR-grafted antibody in COS cell supernatants was an ELISA with the following format:

96 well microtitre plates were coated with F(ab')₂ goat anti-human IgG Fc. The plates were washed and samples added and incubated for 1 hour at room temperature. The plates were washed and monoclonal mouse anti-human kappa chain was added for 1 hour at room temperature.

The plates were washed and F(ab')₂ goat anti-mouse IgG Fc (HRPO conjugated) was added. Enzyme substrate was added to reveal the reaction.

Chimeric B72.3 (IgG4) (ref. 13) was used as a standard. The use of a monoclonal anti-kappa chain in this assay allows grafted antibodies to be read from the chimeric standard.

3.2. ASSAY FOR ANTIGEN BINDING ACTIVITY

Material from COS cell supernatants was assayed for OKT3 antigen binding activity onto CD3 positive cells in a direct assay. The procedure was as follows:

HUT 78 cells (human T cell line, CD3 positive) were maintained in culture. Monolayers of HUT 78 cells were prepared onto 96 well ELISA plates using poly-L-lysine and glutaraldehyde. Samples were added to the monolayers for 1 hour at room temperature.

The plates were washed gently using PBS. F(ab')₂ goat anti-human IgG Fc (HRPO conjugated) or F(ab')₂ goat anti-mouse IgG Fc (HRPO conjugated) was added as appropriate for humanised or mouse samples.

Substrate was added to reveal the reaction.

The negative control for the cell-based assay was chimeric B72.3. The positive control was mouse Orthomune OKT3 or chimeric OKT3, when available.

This cell-based assay was difficult to perform, and an alternative assay was developed for CDR-grafted OKT3 which was more sensitive and easier to carry out.

In this system CDR-grafted OKT3 produced by COS cells was tested for its ability to bind to the CD3-positive HPB-ALL (human peripheral blood acute lymphocytic leukemia) cell line. It was also tested for its ability to block the binding of murine OKT3 to these cells. Binding was measured by the following procedure: HPB-ALL cells were harvested from tissue culture. Cells were incubated at 4°C for 1 hour with various dilutions of test antibody, positive control antibody, or negative control antibody. The cells were washed once and incubated at 4°C for 1 hour with an FITC-labelled goat anti-human IgG (Fc-

specific, mouse absorbed). The cells were washed twice and analysed by cytofluorography. Chimeric OKT3 was used as a positive control for direct binding. Cells incubated with mock-transfected COS cell supernatant, followed by the FITC-labelled goat anti-human IgG, provided the negative control. To test the ability of CDR-grafted OKT3 to block murine OKT3 binding, the HPB-ALL cells were incubated at 4°C for 1 hour with various dilutions of test antibody or control antibody. A fixed saturating amount of FITC OKT3 was added. The samples were incubated for 1 hour at 4°C, washed twice and analysed by cytofluorography. FITC-labelled OKT3 was used as a positive control to determine maximum binding. Unlabelled murine OKT3 served as a reference standard for blocking. Negative controls were unstained cells with or without mock-transfected cell supernatant. The ability of the CDR-grafted OKT3 light chain to bind CD3-positive cells and block the binding of murine OKT3 was initially tested in combination with the chimeric OKT3 heavy chain. The chimeric OKT3 heavy chain is composed of the murine OKT3 variable region and the human IgG4 constant region. The chimeric heavy chain gene is expressed in the same expression vector used for the CDR-grafted genes. The CDR-grafted light chain expression vector and the chimeric heavy chain expression vector were co-transfected into COS cells. The fully chimeric OKT3 antibody (chimeric light chain and chimeric heavy chain) was found to be fully capable of binding to CD3 positive cells and blocking the binding of murine OKT3 to these cells.

3.3 DETERMINATION OF RELATIVE BINDING AFFINITY

The relative binding affinities of CDR-grafted

anti-CD3 monoclonal antibodies were determined by competition binding (ref. 6) using the HPB-ALL human T cell line as a source of CD3 antigen, and fluorescein-conjugated murine OKT3 (Fl-OKT3) of known binding affinity as a tracer antibody. The binding affinity of Fl-OKT3 tracer antibody was determined by a direct binding assay in which increasing amounts of Fl-OKT3 were incubated with HPB-ALL (5×10^5) in PBS with 5% foetal calf serum for 60 min. at 4°C . Cells were washed, and the fluorescence intensity was determined on a FACScan flow cytometer calibrated with quantitative microbead standards (Flow Cytometry Standards, Research Triangle Park, NC). Fluorescence intensity per antibody molecule (F/P ratio) was determined by using microbeads which have a predetermined number of mouse IgG antibody binding sites (Simply Cellular beads, Flow Cytometry Standards). F/P equals the fluorescence intensity of beads saturated with Fl-OKT3 divided by the number of binding sites per bead. The amount of bound and free Fl-OKT3 was calculated from the mean fluorescence intensity per cell, and the ratio of bound/free was plotted against the number of moles of antibody bound. A linear fit was used to determine the affinity of binding (absolute value of the slope). For competitive binding, increasing amounts of competitor antibody were added to a sub-saturating dose of Fl-OKT3 and incubated with 5×10^5 HPB-ALL in 200 ml of PBS with 5% foetal calf serum, for 60 min at 4°C . The fluorescence intensities of the cells were measured on a FACScan flow cytometer calibrated with quantitative microbead standards. The concentrations of bound and free Fl-OKT3 were calculated. The affinities of competing anti-

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bodies were calculated from the equation $[X]-[OKT3] = (1/Kx) - (1/Ka)$, where Ka is the affinity of murine OKT3, Kx is the affinity of competitor X , $[]$ is the concentration of competitor antibody at which bound/free binding is $R/2$, and R is the maximal bound/free binding.

4. CDNA LIBRARY CONSTRUCTION

4.1. mRNA PREPARATION AND cDNA SYNTHESIS

OKT3 producing cells were grown as described above and 1.2×10^9 cells harvested and mRNA extracted using the guanidinium/LiCl extraction procedure. cDNA was prepared by priming from Oligo-dT to generate full length cDNA. The cDNA was methylated and EcoRI linkers added for cloning.

4.2. LIBRARY CONSTRUCTION

The cDNA library was ligated to pSP65 vector DNA which had been EcoRI cut and the 5' phosphate groups removed by calf intestinal phosphatase (EcoRI/CIP). The ligation was used to transform high transformation efficiency Escherichia coli (E.coli) HB101. A cDNA library was prepared. 3600 colonies were screened for the light chain and 10000 colonies were screened for the heavy chain.

5. SCREENING

E.coli colonies positive for either heavy or light chain probes were identified by oligonucleotide screening using the oligonucleotides:

5' TCCAGATGTAACTGCTCAC for the light chain, which is complementary to a sequence in the mouse kappa constant region, and 5' CAGGGGCCAGTGGATGGATAGAC for the heavy chain which is complementary to a sequence in the mouse IgG2a constant CH1 domain region. 12 light chain and 9 heavy chain clones

were identified and taken for second round screening. Positive clones from the second round of screening were grown up and DNA prepared. The sizes of the gene inserts were estimated by gel electrophoresis and inserts of a size capable of containing a full length cDNA were subcloned into M13 for DNA sequencing.

6. DNA SEQUENCING

Clones representing four size classes for both heavy and light chains were obtained in M13. DNA sequence for the 5' untranslated regions, signal sequences, variable regions and 3' untranslated regions of full length cDNAs [Figures 1(a) and 2(a)] were obtained and the corresponding amino acid sequences predicted [(Figures 1(b) and 2(b))]. In Figure 1(a) the untranslated DNA regions are shown in uppercase, and in both Figures 1 and 2 the signal sequences are underlined.

7. CONSTRUCTION OF cDNA EXPRESSION VECTORS

Celltech expression vectors are based on the plasmid pEE6hCMV (ref. 14). A polylinker for the insertion of genes to be expressed has been introduced after the major immediate early promoter/enhancer of the human Cytomegalovirus (hCMV). Marker genes for selection of the plasmid in transfected eukaryotic cells can be inserted as BamH1 cassettes in the unique BamH1 site of pEE6 hCMV; for instance, the neo marker to provide pEE6 hCMV neo. It is usual practice to insert the neo and gpt markers prior to insertion of the gene of interest, whereas the GS marker is inserted last because of the presence of internal EcoR1 sites in the cassette.

The selectable markers are expressed from the SV40 late promoter which also provides an origin of replication so that the vectors can be used for expression in the COS cell transient expression system.

The mouse sequences were excised from the M13 based vectors described above as EcoR1 fragments and cloned into either pEE6-hCMV-neo for the heavy chain and into EE6-hCMV-gpt for the light chain to yield vectors pJA136 and pJA135 respectively.

8. EXPRESSION OF cDNAS IN COS CELLS

Plasmids pJA135 and pJA136 were co-transfected into COS cells and supernatant from the transient expression experiment was shown to contain assembled antibody which bound to T-cell enriched lymphocytes. Metabolic labelling experiments using ³⁵S methionine showed expression and assembly of heavy and light chains.

9. CONSTRUCTION OF CHIMERIC GENES

Construction of chimeric genes followed a previously described strategy [Whittle et al (ref. 13)]. A restriction site near the 3' end of the variable domain sequence is identified and used to attach an oligonucleotide adapter coding for the remainder of the mouse variable region and a suitable restriction site for attachment to the constant region of choice.

9.1. LIGHT CHAIN GENE CONSTRUCTION

The mouse light chain cDNA sequence contains an Aval site near the 3' end of the variable region [Fig. 1(a)]. The majority of the sequence of the variable region was isolated as a 396 bp. EcoR1-Aval fragment. An oligonucleotide adapter was designed to replace the remainder of the 3'

region of the variable region from the Aval site and to include the 5' residues of the human constant region up to and including a unique NarI site which had been previously engineered into the constant region.

A HindIII site was introduced to act as a marker for insertion of the linker.

The linker was ligated to the VL fragment and the 413 bp EcoRI-NarI adapted fragment was purified from the ligation mixture.

The constant region was isolated as an NarI-BamHI fragment from an M13 clone NW361 and was ligated with the variable region DNA into an EcoRI/BamHI/CIP pSP65 treated vector in a three way reaction to yield plasmid JA143. Clones were isolated after transformation into E.coli and the linker and junction sequences were confirmed by the presence of the HindIII site and by DNA sequencing.

9.2 LIGHT CHAIN GENE CONSTRUCTION - VERSION 2

The construction of the first chimeric light chain gene produces a fusion of mouse and human amino acid sequences at the variable-constant region junction. In the case of the OKT3 light chain the amino acids at the chimera junction are:

.....Leu-Glu-Ile-Asn-Arg/ -/Thr-Val-Ala -Ala
 VARIABLE CONSTANT

This arrangement of sequence introduces a potential site for Asparagine (Asn) linked (N-linked) glycosylation at the V-C junction. Therefore, a second version of the chimeric light chain oligonucleotide adapter was designed in which the threonine (Thr), the first amino acid of the human constant region, was replaced with the equivalent amino acid from the mouse constant region, Alanine (Ala).

An internal HindIII site was not included in this adapter, to differentiate the two chimeric light chain genes.

The variable region fragment was isolated as a 376 bp EcoRI-AvaI fragment. The oligonucleotide linker was ligated to NarI cut pNW361 and then the adapted 396bp constant region was isolated after recutting the modified pNW361 with EcoRI. The variable region fragment and the modified constant region fragment were ligated directly into EcoRI/C1P treated pEE6hCMVneo to yield pJA137. Initially all clones examined had the insert in the incorrect orientation. Therefore, the insert was re-isolated and recloned to turn the insert round and yield plasmid pJA141. Several clones with the insert in the correct orientation were obtained and the adapter sequence of one was confirmed by DNA sequencing

9.3. HEAVY CHAIN GENE CONSTRUCTION

9.3.1. CHOICE OF HEAVY CHAIN GENE ISOTYPE

The constant region isotype chosen for the heavy chain was human IgG4.

9.3.2. GENE CONSTRUCTION

The heavy chain cDNA sequence showed a BanI site near the 3' end of the variable region [Fig. 2(a)]. The majority of the sequence of the variable region was isolated as a 426bp. EcoRI/C1P/BanI fragment. An oligonucleotide adapter was designated to replace the remainder of the 3' region of the variable region from the BanI site up to and including a unique HindIII site which had been previously engineered into the first two amino acids of the constant region.

The linker was ligated to the V_H fragment and the EcoRI-HindIII adapted fragment was purified from the ligation mixture.

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The variable region was ligated to the constant region by cutting pJA91 with EcoR1 and Hind111 removing the intron fragment and replacing it with the V_H to yield pJA142. Clones were isolated after transformation into E.coli JM101 and the linker and junction sequences were confirmed by DNA sequencing. (N.B. The Hind111 site is lost on cloning).

10. CONSTRUCTION OF CHIMERIC EXPRESSION VECTORS

10.1. neo AND gpt VECTORS

The chimeric light chain (version 1) was removed from pJA143 as an EcoR1 fragment and cloned into EcoR1/C1P treated pEE6hCMVneo expression vector to yield pJA145. Clones with the insert in the correct orientation were identified by restriction mapping.

The chimeric light chain (version 2) was constructed as described above.

The chimeric heavy chain gene was isolated from pJA142 as a 2.5Kbp EcoR1/BamH1 fragment and cloned into the EcoR1/Bcl1/C1P treated vector fragment of a derivative of pEE6hCMVgpt to yield plasmid pJA144.

10.2. GS SEPARATE VECTORS

GS versions of pJA141 and pJA144 were constructed by replacing the neo and gpt cassettes by a BamH1/Sal1/C1P treatment of the plasmids, isolation of the vector fragment and ligation to a GS-containing fragment from the plasmid pRO49 to yield the light chain vector pJA179 and the heavy chain vector pJA180.

10.3. GS SINGLE VECTOR CONSTRUCTION

Single vector constructions containing the cL (chimeric light), cH (chimeric heavy) and GS genes on one plasmid in the order cL-cH-GS, or cH-cL-GS

and with transcription of the genes being head to tail e.g. cL>cH>GS were constructed. These plasmids were made by treating pJA179 or pJA180 with BamH1/C1P and ligating in a Bgl111/Hind111 hCMV promoter cassette along with either the Hind111/BamH1 fragment from pJA141 into pJA180 to give the cH-cL-GS plasmid pJA182 or the Hind111/BamH1 fragment from pJA144 into pJA179 to give the cL-cH-GS plasmid pJA181.

11. EXPRESSION OF CHIMERIC GENES

11.1. EXPRESSION IN COS CELLS

The chimeric antibody plasmid pJA145 (cL) and pJA144 (cH) were co-transfected into COS cells and supernatant from the transient expression experiment was shown to contain assembled antibody which bound to the HUT 78 human T-cell line. Metabolic labelling experiments using ³⁵S methionine showed expression and assembly of heavy and light chains. However the light chain mobility seen on reduced gels suggested that the potential glycosylation site was being glycosylated. Expression in COS cells in the presence of tunicamycin showed a reduction in size of the light chain to that shown for control chimeric antibodies and the OKT3 mouse light chain. Therefore JA141 was constructed and expressed. In this case the light chain did not show an aberrant mobility or a size shift in the presence or absence of tunicamycin. This second version of the chimeric light chain, when expressed in association with chimeric heavy (cH) chain, produced antibody which showed good binding to HUT 78 cells. In both cases antigen binding was equivalent to that of the mouse antibody.

11.2 EXPRESSION IN CHINESE HAMSTER OVARY (CHO) CELLS
Stable cell lines have been prepared from plasmids PJA141/pJA144 and from pJA179/pJA180, pJA181 and pJA182 by transfection into CHO cells.

12. CDR-GRAFTING

The approach taken was to try to introduce sufficient mouse residues into a human variable region framework to generate antigen binding activity comparable to the mouse and chimeric antibodies.

12.1. VARIABLE REGION ANALYSIS

From an examination of a small database of structures of antibodies and antigen-antibody complexes it is clear that only a small number of antibody residues make direct contact with antigen. Other residues may contribute to antigen binding by positioning the contact residues in favourable configurations and also by inducing a stable packing of the individual variable domains and stable interaction of the light and heavy chain variable domains.

The residues chosen for transfer can be identified in a number of ways:

- (a) By examination of antibody X-ray crystal structures the antigen binding surface can be predominantly located on a series of loops, three per domain, which extend from the B-barrel framework.
- (b) By analysis of antibody variable domain sequences regions of hypervariability [termed the Complementarity Determining Regions (CDRs) by Wu and Kabat (ref. 5)] can be identified. In the most but not all cases these CDRs correspond to, but extend a short way beyond, the loop regions noted above.

(c) Residues not identified by (a) and (b) may contribute to antigen binding directly or indirectly by affecting antigen binding site topology, or by inducing a stable packing of the individual variable domains and stabilising the inter-variable domain interaction. These residues may be identified either by superimposing the sequences for a given antibody on a known structure and looking at key residues for their contribution, or by sequence alignment analysis and noting "idiosyncratic" residues followed by examination of their structural location and likely effects.

12.1.1. LIGHT CHAIN

Figure 3 shows an alignment of sequences for the human framework region RE1 and the OKT3 light variable region. The structural loops (LOOP) and CDRs (KABAT) believed to correspond to the antigen binding region are marked. Also marked are a number of other residues which may also contribute to antigen binding as described in 13.1(c).

Above the sequence in Figure 3 the residue type indicates the spatial location of each residue side chain, derived by examination of resolved structures from X-ray crystallography analysis. The key to this residue type designation is as follows:

- N - near to CDR (From X-ray Structures)
- P - Packing
- S - Surface
- I - Interface
- Packing/Part Exposed
- ? - Non-CDR Residues which may require to be left as Mouse sequence.
- B - Buried Non-Packing
- E - Exposed
- * - Interface

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Residues underlined in Figure 3 are amino acids. RE1 was chosen as the human framework because the light chain is a kappa chain and the kappa variable regions show higher homology with the mouse sequences than a lambda light variable region, e.g. KOL (see below). RE1 was chosen in preference to another kappa light chain because the X-ray structure of the light chain has been determined so that a structural examination of individual residues could be made.

12.1.2. HEAVY CHAIN

Similarly Figure 4 shows an alignment of sequences for the human framework region KOL and the OKT3 heavy variable region. The structural loops and CDRs believed to correspond to the antigen binding region are marked. Also marked are a number of other residues which may also contribute to antigen binding as described in 12.1(c). The residue type key and other indicators used in Figure 4 are the same as those used in Figure 3. KOL was chosen as the heavy chain framework because the X-ray structure has been determined to a better resolution than, for example, NEWM and also the sequence alignment of OKT3 heavy variable region showed a slightly better homology to KOL than to NEWM.

12.2. DESIGN OF VARIABLE GENES

The variable region domains were designed with mouse variable region optimal codon usage [Grantham and Perrin (ref. 15)] and used the B72.3 signal sequences [Whittle et al (ref. 13)]. The sequences were designed to be attached to the constant region in the same way as for the chimeric genes described above. Some constructs contained the "Kozak consensus sequence" [Kozak (ref. 16)] directly linked to the 5' of the signal

sequence in the gene. This sequence motif is believed to have a beneficial role in translation initiation in eukaryotes.

12.3. GENE CONSTRUCTION

To build the variable regions, various strategies are available. The sequence may be assembled by using oligonucleotides in a manner similar to Jones et al (ref. 17) or by simultaneously replacing all of the CDRs or loop regions by oligonucleotide directed site specific mutagenesis in a manner similar to Verhoeyen et al (ref. 2). Both strategies were used and a list of constructions is set out in Tables 1 and 2 and Figures 4 and 5. It was noted in several cases that the mutagenesis approach led to deletions and rearrangements in the gene being remodelled, while the success of the assembly approach was very sensitive to the quality of the oligonucleotides.

13. CONSTRUCTION OF EXPRESSION VECTORS

Genes were isolated from M13 or SP65 based intermediate vectors and cloned into pEE6hCMVneo for the light chains and pEE6hCMVgpt for the heavy chains in a manner similar to that for the chimeric genes as described above.

TABLE 1 CDR-GRAFTED GENE CONSTRUCTS

CODE	MOUSE SEQUENCE CONTENT	METHOD OF CONSTRUCTION	KOZAK SEQUENCE - +

LIGHT CHAIN	ALL HUMAN FRAMEWORK REL		
121	26-32, 50-56, 91-96 inclusive	SDM and gene assembly	+ n.d.
121A	26-32, 50-56, 91-96 inclusive +1, 3, 46, 47	Partial gene assembly	n.d. +
121B	26-32, 50-56, 91-96 inclusive + 46, 47	Partial gene assembly	n.d. +
221	24-24, 50-56, 91-96 inclusive	Partial gene assembly	+ +
221A	24-34, 50-56, 91-96 inclusive +1, 3, 46, 47	Partial gene assembly	+ +
221B	24-34, 50-56, 91-96 inclusive +1, 3	Partial gene assembly	+ +
221C	24-34, 50-56, 91-96 inclusive	Partial gene assembly	+ +
HEAVY CHAIN	ALL HUMAN FRAMEWORK KOL		
121	26-32, 50-56, 95-100B inclusive	Gene assembly	n.d. +
131	26-32, 50-58, 95-100B inclusive	Gene assembly	n.d. +
141	26-32, 50-65, 95-100B inclusive	Partial gene assembly	+ n.d.
321	26-35, 50-56, 95-100B inclusive	Partial gene assembly	+ n.d.
331	26-35, 50-58, 95-100B inclusive	Partial gene assembly	+ +
		Gene assembly	+ +
341	26-35, 50-65, 95-100B inclusive	SDM	+ +
		Partial gene assembly	+ +
341A	26-35, 50-65, 95-100B inclusive +6, 23, 24, 48, 49, 71, 73, 76, 78, 88, 91 (+63 = human)	Gene assembly	n.d. +
341B	26-35, 50-65, 95-100B inclusive + 48, 49, 71, 73, 76, 78, 88, 91 (+63 + human)	Gene assembly	n.d. +

KEY

n.d.	not done
SDM	Site directed mutagenesis
Gene assembly	Variable region assembled entirely from oligonucleotides
Partial gene assembly	Variable region assembled by combination of restriction fragments either from other genes originally created by SDM and gene assembly or by oligonucleotide assembly of part of the variable region and reconstruction with restriction fragments from other genes originally created by SDM and gene assembly

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14. EXPRESSION OF CDR-GRAFTED GENES

14.1. PRODUCTION OF ANTIBODY CONSISTING OF GRAFTED LIGHT (gL) CHAINS WITH MOUSE HEAVY (mH) OR CHIMERIC HEAVY (cH) CHAINS

All gL chains, in association with mH or cH produced reasonable amounts of antibody.

Insertion of the Kozak consensus sequence at a position 5' to the ATG (kgL constructs) however, led to a 2-5 fold improvement in net expression. Over an extended series of experiments expression levels were raised from approximately 200ng/ml to approximately 500 ng/ml for kgL/cH or kgL/mH combinations.

When direct binding to antigen on HUT 78 cells was measured, a construct designed to include mouse sequence based on loop length (gL121) did not lead to active antibody in association with mH or cH. A construct designed to include mouse sequence based on Kabat CDRs (gL221) demonstrated some weak binding in association with mH or cH. However, when framework residues 1, 3, 46, 47 were changed from the human to the murine OKT3 equivalents based on the arguments outlined in Section 12.1 antigen binding was demonstrated when both of the new constructs, which were termed 121A and 221A were co-expressed with cH. When the effects of these residues were examined in more detail, it appears that residues 1 and 3 are not major contributing residues as the product of the gL221B gene shows little detectable binding activity in association with cH. The light chain product of gL221C, in which mouse sequences are present at 46 and 47, shows good binding activity in association with cH.

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14.2 PRODUCTION OF ANTIBODY CONSISTING OF GRAFTED HEAVY (gH) CHAINS WITH MOUSE LIGHT (mL) OR CHIMERIC LIGHT (cL) CHAINS

Expression of the gH genes proved to be more difficult to achieve than for gL. First, inclusion of the Kozak sequence appeared to have no marked effect on expression of gH genes. Expression appears to be slightly improved but not to the same degree as seen for the grafted light chain.

Also, it proved difficult to demonstrate production of expected quantities of material when the loop choice (amino acid 26-32) for CDR1 is used, e.g. gH121, 131, 141 and no conclusions can be drawn about these constructs.

Moreover, co-expression of the gH341 gene with cL or mL has been variable and has tended to produce lower amounts of antibody than the cH/cL or mH/mL combinations. The alterations to gH341 to produce gH341A and gH341B lead to improved levels of expression.

This may be due either to a general increase in the fraction of mouse sequence in the variable region, or to the alteration at position 63 where the residue is returned to the human amino acid Valine (Val) from Phenylalanine (Phe) to avoid possible internal packing problems with the rest of the human framework. This arrangement also occurs in gH331 and gH321.

When gH321 or gH331 were expressed in association with cL, antibody was produced but antibody binding activity was not detected.

When the more conservative gH341 gene was used antigen binding could be detected in association with cL or mL, but the activity was only marginally above the background level.

When further mouse residues were substituted based on the arguments in 12.1, antigen binding could be clearly demonstrated for the antibody produced when kgH341A and kgH341B were expressed in association with cL.

14.3 PRODUCTION OF FULLY CDR-GRAFTED ANTIBODY

The kgL221A gene was co-expressed with kgH341, kgH341A or kgH341B. For the combination kgH221A/kgH341 very little material was produced in a normal COS cell expression.

For the combinations kgL221A/kgH341A or kgH221A/kgH341B amounts of antibody similar to gL/cH was produced.

In several experiments no antigen binding activity could be detected with kgH221A/gH341 or kgH221A/kgH341 combinations, although expression levels were very low.

Antigen binding was detected when kgL221A/kgH341A or kgH221A/kgH341B combinations were expressed.

In the case of the antibody produced from the kgL221A/kgH341A combination the antigen binding was very similar to that of the chimeric antibody.

An analysis of the above results is given below.

15. DISCUSSION OF CDR-GRAFTING RESULTS

In the design of the fully humanised antibody the aim was to transfer the minimum number of mouse amino acids that would confer antigen binding onto a human antibody framework.

15.1. LIGHT CHAIN

15.1.1. EXTENT OF THE CDRs

For the light chain the regions defining the loops known from structural studies of other antibodies to contain the antigen contacting residues, and

those hypervariable sequences defined by Kabat et al (refs. 4 and 5) as Complementarity Determining Regions (CDRs) are equivalent for CDR2. For CDR1 the hypervariable region extends from residues 24-34 inclusive while the structural loop extends from 26-32 inclusive. In the case of OKT3 there is only one amino acid difference between the two options, at amino acid 24, where the mouse sequence is a serine and the human framework RE1 has glutamine. For CDR3 the loop extends from residues 91-96 inclusive while the Kabat hypervariability extends from residues 89-97 inclusive. For OKT3 amino acids 89, 90 and 97 are the same between OKT3 and RE1 (Fig. 3). When constructs based on the loop choice for CDR1 (gL121) and the Kabat choice (gL221) were made and co-expressed with mH or cH no evidence for antigen binding activity could be found for gL121, but trace activity could be detected for the gL221, suggesting that a single extra mouse residue in the grafted variable region could have some detectable effect. Both gene constructs were reasonably well expressed in the transient expression system.

15.1.2. FRAMEWORK RESIDUES

The remaining framework residues were then further examined, in particular amino acids known from X-ray analysis of other antibodies to be close to the CDRs and also those amino acids which in OKT3 showed differences from the consensus framework for the mouse subgroup (subgroup VI) to which OKT3 shows most homology. Four positions 1, 3, 46 and 47 were identified and their possible contribution was examined by substituting the mouse amino acid for the human amino acid at each position. Therefore gL221A (gL221 + D1Q, Q3V, L46R, L47W,

see Figure 3 and Table 1) was made, cloned in EE6hCMVneo and co-expressed with cH (pJA144). The resultant antibody was well expressed and showed good binding activity. When the related genes gL221B (gL221 + D1Q, Q3V) and gL221C (gL221 + L46R, L47W) were made and similarly tested, while both genes produced antibody when co-expressed with cH, only the gL221C/cH combination showed good antigen binding. When the gL121A (gL121 + D1Q, Q3V, L46R, L47W) gene was made and co-expressed with cH, antibody was produced which also bound to antigen.

15.2. HEAVY CHAIN

15.2.1. EXTENT OF THE CDRs

For the heavy chain the loop and hypervariability analyses agree only in CDR3. For CDR1 the loop region extends from residues 26-32 inclusive whereas the Kabat CDR extends from residues 31-35 inclusive. For CDR2 the loop region is from 50-58 inclusive while the hypervariable region covers amino acids 50-65 inclusive. Therefore humanised heavy chains were constructed using the framework from antibody KOL and with various combinations of these CDR choices, including a shorter choice for CDR2 of 50-56 inclusive as there was some uncertainty as to the definition of the end point for the CDR2 loop around residues 56 to 58. The genes were co-expressed with mL or cL initially. In the case of the gH genes with loop choices for CDR1 e.g. gH121, gH131, gH141 very little antibody was produced in the culture supernatants. As no free light chain was detected it was presumed that the antibody was being made and assembled inside the cell but that the heavy chain was aberrant in some way, possibly incorrectly folded, and therefore the antibody was

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being degraded internally. In some experiments trace amounts of antibody could be detected in ³⁵S labelling studies.

As no net antibody was produced, analysis of these constructs was not pursued further.

When, however, a combination of the loop choice and the Kabat choice for CDR1 was tested (mouse amino acids 26-35 inclusive) and in which residues 31 (Ser to Arg), 33 (Ala to Thr), and 35 (Tyr to His) were changed from the human residues to the mouse residue and compared to the first series, antibody was produced for gH321, kgH331 and kgH341 when co-expressed with cL. Expression was generally low and could not be markedly improved by the insertion of the Kozak consensus sequence 5' to the ATG of the signal sequence of the gene, as distinct from the case of the gL genes where such insertion led to a 2-5 fold increase in net antibody production. However, only in the case of gH341/mL or kgH341/cL could marginal antigen binding activity be demonstrated. When the kgH341 gene was co-expressed with kgL221A, the net yield of antibody was too low to give a signal above the background level in the antigen binding assay.

15.2.2. FRAMEWORK RESIDUES

As in the case of the light chain the heavy chain frameworks were re-examined. Possibly because of the lower initial homology between the mouse and human heavy variable domains compared to the light chains, more amino acid positions proved to be of interest. Two genes kgH341A and kgH341B were constructed, with 11 or 8 human residues respectively substituted by mouse residues compared to gH341, and with the CDR2 residue 63 returned to the human amino acid potentially to

improve domain packing. Both showed antigen binding when combined with cL or kgL221A, the kgH341A gene with all 11 changes appearing to be the superior choice.

15.3 INTERIM CONCLUSIONS

It has been demonstrated, therefore, for OKT3 that to transfer antigen binding ability to the humanised antibody, mouse residues outside the CDR regions defined by the Kabat hypervariability or structural loop choices are required for both the light and heavy chains. Fewer extra residues are needed for the light chain, possibly due to the higher initial homology between the mouse and human kappa variable regions.

Of the changes seven (1 and 3 from the light chain and 6, 23, 71, 73 and 76 from the heavy chain) are predicted from a knowledge of other antibody structures to be either partly exposed or on the antibody surface. It has been shown here that residues 1 and 3 in the light chain are not absolutely required to be the mouse sequence; and for the heavy chain the gH341B heavy chain in combination with the 221A light chain generated only weak binding activity. Therefore the presence of the 6, 23 and 24 changes are important to maintain a binding affinity similar to that of the murine antibody. It was important, therefore, to further study the individual contribution of the other 8 mouse residues of the kgH341A gene compared to kgH341.

16. FURTHER CDR-GRAFTING EXPERIMENTS

Additional CDR-grafted heavy chain genes were prepared substantially as described above. With reference to Table 2 the further heavy chain genes were based upon the gh341 (plasmid pJA178) and

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gH341A (plasmid pJA185) with either mouse OKT3 or human KOL residues at 6, 23, 24, 48, 49, 63, 71, 73, 76, 78, 88 and 91, as indicated. The CDR-grafted light chain genes used in these further experiments were gL221, gL221A, gL221B and gL221C as described above.

TABLE 2

OKT3 HEAVY CHAIN CDR GRAFTS

1. gH341 and derivatives

RES NUM	6	23	24	48	49	63	71	73	76	78	88	91	
OKT3vh	<u>Q</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>F</u>	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>A</u>	<u>Y</u>	
gH341	E	S	S	V	A	F	R	N	N	L	G	F	JA178
gH341A	<u>Q</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>A</u>	<u>Y</u>	JA185
gH341E	<u>Q</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>G</u>	<u>G</u>	JA198
gH341*	<u>Q</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>N</u>	<u>A</u>	<u>G</u>	<u>F</u>	JA207
gH341*	<u>Q</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>R</u>	<u>N</u>	<u>N</u>	<u>A</u>	<u>G</u>	<u>F</u>	JA209
gH341D	<u>Q</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>N</u>	<u>L</u>	<u>G</u>	<u>F</u>	JA197
gH341*	<u>Q</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>R</u>	<u>N</u>	<u>N</u>	<u>L</u>	<u>G</u>	<u>F</u>	JA199
gH341C	<u>Q</u>	<u>K</u>	<u>A</u>	<u>V</u>	<u>A</u>	<u>F</u>	<u>R</u>	<u>N</u>	<u>N</u>	<u>L</u>	<u>G</u>	<u>F</u>	JA184
gH341*	<u>Q</u>	<u>S</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>A</u>	<u>Y</u>	JA203
gH341*	E	S	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>A</u>	<u>Y</u>	JA205
gH341B	E	S	S	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>A</u>	<u>Y</u>	JA183
gH341*	<u>Q</u>	<u>S</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>G</u>	<u>F</u>	JA204
gH341*	E	S	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>G</u>	<u>F</u>	JA206
gH341*	<u>Q</u>	<u>S</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>N</u>	<u>A</u>	<u>G</u>	<u>F</u>	JA208
KOL	E	S	S	V	A		R	N	N	L	G	F	

OKT3 LIGHT CHAIN CDR GRAFTS

2. gL221 and derivatives

RES NUM	1	3	46	47	
OKT3v1	<u>Q</u>	<u>V</u>	<u>R</u>	<u>W</u>	
GL221	D	Q	<u>L</u>	<u>L</u>	DA221
gL221A	<u>Q</u>	<u>V</u>	<u>R</u>	<u>W</u>	DA221A
gL221B	<u>Q</u>	<u>V</u>	L	L	DA221B
GL221C	D	Q	<u>R</u>	<u>W</u>	DA221C
RE1	D	Q	L	L	

MURINE RESIDUES ARE UNDERLINED

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The CDR-grafted heavy and light chain genes were co-expressed in COS cells either with one another in various combinations but also with the corresponding murine and chimeric heavy and light chain genes substantially as described above. The resultant antibody products were then assayed in binding and blocking assays with HPB-ALL cells as described above.

The results of the assays for various grafted heavy chains co-expressed with the gL221C light chain are given in Figures 7 and 8 (for the JA184, JA185, JA197 and JA198 constructs - see Table 2), in Figure 9 (for the JA183, JA184, JA185 and JA197 constructs) in Figure 10 (for the chimeric, JA185, JA199, JA204, JA205, JA207, JA208 and JA209 constructs) and in Figure 11 (for the JA183, JA184, JA185, JA198, JA203, JA205 and JA206 constructs).

The basic grafted product without any human to murine changes in the variable frameworks, i.e. gL221 co-expressed with gh341 (JA178), and also the "fully grafted" product, having most human to murine changes in the grafted heavy chain framework, i.e. gL221C co-expressed with gh341A (JA185), were assayed for relative binding affinity in a competition assay against murine OKT3 reference standard, using HPB-ALL cells. The assay used was as described above in section 3.3. The results obtained are given in Figure 12 for the basic grafted product and in Figure 13 for the fully grafted product. These results indicate that the basic grafted product has negligible binding ability as compared with the OKT3 murine reference standard; whereas the "fully grafted" product has a binding ability very similar to that of the OKT3 murine reference standard.

The binding and blocking assay results indicate the following:

The JA198 and JA207 constructs appear to have the best binding characteristics and similar binding abilities, both substantially the same as the chimeric and fully grafted gH341A products. This indicates that positions 88 and 91 and position 76 are not highly critical for maintaining the OKT3 binding ability; whereas at least some of positions 6, 23, 24, 48, 49, 71, 73 and 78 are more important.

This is borne out by the finding that the JA209 and JA199, although of similar binding ability to one another, are of lower binding ability than the JA198 and JA207 constructs. This indicates the importance of having mouse residues at positions 71, 73 and 78, which are either completely or partially human in the JA199 and JA209 constructs respectively.

Moreover, on comparing the results obtained for the JA205 and JA183 constructs it is seen that there is a decrease in binding going from the JA205 to the JA183 constructs. This indicates the importance of retaining a mouse residue at position 23, the only position changed between JA205 and JA183.

These and other results lead us to the conclusion that of the 11 mouse framework residues used in the gH341A (JA185) construct, it is important to retain mouse residues at all of positions 6, 23, 24, 48 and 49, and possibly for maximum binding affinity at 71, 73 and 78.

Similar Experiments were carried out to CDR-graft a number of the rodent antibodies including antibodies having specificity for CD4 (OKT4), ICAM-1 (R6-5), TAG72 (B72.3), and TNF α (61E71, 101.4, hTNF1, hTNF2 and hTNF3).

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EXAMPLE 2CDR-GRAFTING OF A MURINE ANTI-CD4 T CELL
RECEPTOR ANTIBODY, OKT4A

Anti OKT4A CDR-grafted heavy and light chain genes were prepared, expressed and tested substantially as described above in Example 1 for CDR-grafted OKT3. The CDR grafting of OKT4A is described in detail in Ortho patent application PCT/GB 90..... of even date herewith entitled "Humanised Antibodies". The disclosure of this Ortho patent application PCT/GB 90 is incorporated herein by reference. A number of CDR-grafted OKT4 antibodies have been prepared. Presently the CDR-grafted OKT4A of choice is the combination of the grafted light chain LCDR2 and the grafted heavy chain HCDR10.

THE LIGHT CHAIN

The human acceptor framework used for the grafted light chains was RE1. The preferred LCDR2 light chain has human to mouse changes at positions 33, 34, 38, 49 and 89 in addition to the structural loop CDRs. Of these changed positions, positions 33, 34 and 89 fall within the preferred extended CDRs of the present invention (positions 33 and 34 in CDR1 and position 89 in CDR3). The human to murine changes at positions 38 and 49 corresponds to positions at which the amino acid residues are preferably donor murine amino acid residues in accordance with the present invention. A comparison of the amino acid sequences of the donor murine light chain variable domain and the RE1 human acceptor light chain variable further reveals that the murine and human residues are identical at all of positions 46, 48 and 71 and at all of positions 2, 4, 6, 35, 36, 44, 47, 62, 64-69, 85, 87, 98, 99 and 101 and 102. However the amino acid residue at position 58 in LCDR2 is

the human RE1 framework residue not the mouse OKT4 residue as would be preferred in accordance with the present invention.

THE HEAVY CHAIN

The human acceptor framework used for the grafted heavy chains was KOL.

The preferred CDR graft HCDR10 heavy chain has human to mouse changes at positions 24, 35, 57, 58, 60, 88 and 91 in addition to the structural loop CDRs.

Of these positions, positions 35 (CDR1) and positions 57, 58 and 60 (CDR2) fall within the preferred extended CDRs of the present invention. Also the human to mouse change at position 24 corresponds to a position at which the amino acid residue is a donor murine residue in accordance with the present invention. Moreover, the human to mouse changes at positions 88 and 91 correspond to positions at which the amino acid residues are optionally donor murine residues.

Moreover, a comparison of the murine OKT4A and human KOL heavy chain variable amino acid sequences reveals that the murine and human residues are identical at all of positions 23, 49, 71, 73 and 78 and at all of positions 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.

Thus the OKT4A CDR-grafted heavy chain HCDR10 corresponds to a particularly preferred embodiment according to the present invention.

EXAMPLE 3CDR-GRAFTING OF AN ANTI-MUCIN SPECIFIC MURINE
ANTIBODY, B72.3

The cloning of the genes coding for the anti-mucin specific murine monoclonal antibody B72.3 and the preparation of B72.3 mouse-human chimeric antibodies has been described previously (ref. 13 and WO 89/01783).

CDR-grafted versions of B72.3 were prepared as follows.

(a) B72.3 Light Chain

CDR-grafting of this light chain was accomplished by direct transfer of the murine CDRs into the framework of the human light chain RE1.

The regions transferred were:

<u>CDR Number</u>	<u>Residues</u>
1	24-34
2	50-56
3	90-96

The activity of the resulting grafted light chain was assessed by co-expression in COS cells, of genes for the combinations:

B72.3 cH/B72.3 cL

and B72.3 cH/B72.3 gL

Supernatants were assayed for antibody concentration and for the ability to bind to microtitre plates coated with mucin. The results obtained indicated that, in combination with the B72.3 cH chain, B72.3 cL and B72.3 gL had similar binding properties.

Comparison of the murine B72.3 and RE1 light chain amino acid sequences reveals that the residues are identical at positions 46, 58 and 71 but are different at position 48.

Thus changing the human residue to the donor mouse residue at position 48 may further improve the binding characteristics of the CDR-grafted light chain, (B72.3 gL) in accordance with the present invention.

(b) B72.3 heavy chain

i. Choice of framework

At the outset it was necessary to make a choice of human framework. Simply put, the question was as follows: Was it necessary to use the framework regions from an antibody whose crystal structure was known or could the choice be made on some other criteria?

For B72.3 heavy chain, it was reasoned that, while knowledge of structure was important, transfer of the CDRs from mouse to human frameworks might be facilitated if the overall homology between the donor and receptor frameworks was maximised.

Comparison of the B72.3 heavy chain sequence with those in Kabat (ref. 4) for human heavy chains showed clearly that B72.3 had poor homology for KOL and NEWM (for which crystal structures are available) but was very homologous to the heavy chain for EU.

On this basis, EU was chosen for the CDR-grafting and the following residues transferred as CDRs.

<u>CDR Number</u>	<u>Residues</u>
1	27-36
2	50-63
3	93-102

Also it was noticed that the FR4 region of EU was unlike that of any other human (or mouse) antibody. Consequently, in the grafted heavy chain genes this was also changed to produce a "consensus" human sequence. (Preliminary experiments showed that grafted heavy chain genes containing the EU FR4 sequence expressed very poorly in transient expression systems.)

ii. Results with grafted heavy chain genes

Expression of grafted heavy chain genes containing all human framework regions with either gL or cL genes produced a grafted antibody with little ability to bind to mucin. The grafted antibody had about 1% the activity of the chimeric antibody.

In these experiments, however, it was noted that the activity of the grafted antibody could be increased to ~ 10% of B72.3 by exposure to pHs of 2-3.5.

This observation provided a clue as to how the activity of the grafted antibody could be improved without acid treatment. It was postulated that acid exposure brought about the protonation of an acidic residue (pKa of aspartic acid = 3.86 and of glutamine acid = 4.25) which in turn caused a change in structure of the CDR loops, or allowed better access of antigen.

From comparison of the sequences of B72.3 (ref. 13) and EU (refs. 4 and 5), it was clear that, in going from the mouse to human frameworks, only two positions had been changed in such a way that acidic residues had been introduced. These

positions are at residues 73 and 81, where K to E and Q to E changes had been made, respectively.

Which of these positions might be important was determined by examining the crystal structure of the KOL antibody. In KOL heavy chain, position 81 is far removed from either of the CDR loops.

Position 73, however, is close to both CDRs 1 and 3 of the heavy chain and, in this position it was possible to envisage that a K to E change in this region could have a detrimental effect on antigen binding.

iii. Framework changes in B72.3 gH gene

On the basis of the above analysis, E73 was mutated to a lysine (K). It was found that this change had a dramatic effect on the ability of the grafted Ab to bind to mucin. Further the ability of the grafted B72.3 produced by the mutated gH/gL combination to bind to mucin was similar to that of the B72.3 chimeric antibody.

iv. Other framework changes

In the course of the above experiments, other changes were made in the heavy chain framework regions. Within the accuracy of the assays used, none of the changes, either alone or together, appeared beneficial.

v. Other

All assays used measured the ability of the grafted Ab to bind to mucin and, as a whole, indicated that the single framework change at position 73 is sufficient to generate an antibody with similar binding properties to B72.3.

Comparison of the B72.3 murine and EU heavy chain sequences reveals that the mouse and human residues are identical at positions 23, 24, 71 and 78.

Thus the mutated CDR-grafted B72.3 heavy chain corresponds to a preferred embodiment of the present invention.

EXAMPLE 4

CDR-GRAFTING OF A MURINE ANTI-ICAM-1 MONOCLONAL ANTIBODY

A murine antibody, R6-5-D6 (EP 0314863) having specificity for Intercellular Adhesion Molecule 1 (ICAM-1) was CDR-grafted substantially as described above in previous examples. This work is described in greater detail in co-pending application, British Patent Application No. 9009549.8, the disclosure of which is incorporated herein by reference.

The human EU framework was used as the acceptor framework for both heavy and light chains. The CDR-grafted antibody currently of choice is provided by co-expression of grafted light chain gL221A and grafted heavy chain gH341D which has a binding affinity for ICAM 1 of about 75% of that of the corresponding mouse-human chimeric antibody.

LIGHT CHAIN

gL221A has murine CDRs at positions 24-34 (CDR1), 50-56 (CDR2) and 89-97 (CDR3). In addition several framework residues are also the murine amino acid. These residues were chosen after consideration of the possible contribution of these residues to domain packing and stability of the conformation of the antigen binding region. The residues which have been retained as mouse are at positions 2, 3, 48 (?), 60, 84, 85 and 87. Comparison of the murine anti-ICAM 1 and human EU light chain amino acid sequences reveals that the murine and human residues are identical at positions 46, 58 and 71.

HEAVY CHAIN

gH341D has murine CDRs at positions 26-35 (CDR1), 50-56 (CDR2) and 94-100B (CDR3). In addition murine residues were used in gH341D at positions 24, 48, 69, 71, 73, 80, 88 and 91. Comparison of the murine anti-ICAM 1 and human EU heavy chain amino acid sequences are identical at positions 23, 49 and 78.

EXAMPLE 5

CDR-Grafting of murine anti-TNF α antibodies

A number of murine anti-TNF α monoclonal antibodies were CDR-grafted substantially as described above in previous examples. These antibodies include the murine monoclonal antibodies designated 61 E71, hTNF1, hTNF3 and 101.4. A brief summary of the CDR-grafting of each of these antibodies is given below.

61E71

A similar analysis as described above (Example 1, Section 12.1.) was done for 61E71 and for the heavy chain 10 residues were identified at 23, 24, 48, 49, 68, 69, 71, 73, 75 and 88 as residues to potentially retain as murine. The human frameworks chosen for CDR-grafting of this antibody, and the hTNF3 and 101.4 antibodies were RE1 for the light chain and KOL for the heavy chain.

Three genes were built, the first of which contained 23, 24, 48, 49, 71 and 73 [gH341(6)] as murine residues. The second gene also had 75 and 88 as murine residues [gH341(8)] while the third gene additionally had 68, 69, 75 and 88 as murine residues [gH341(10)]. Each was co-expressed with gL221, the minimum grafted light chain (CDRs only). The gL221/gH341(6) and gL221/gH341(8) antibodies both bound as well to TNF as murine 61E71. The gL221/gH341(10) antibody did not express and this combination was not taken further.

Subsequently the gL221/gH341(6) antibody was assessed in an L929 cell competition assay in which the antibody competes against the TNF receptor on L929 cells for binding to TNF in solution. In this assay the gL221/gH341(6) antibody was approximately 10% as active as murine 61E71.

hTNF1

hTNF1 is a monoclonal antibody which recognises an epitope on human TNF- . The EU human framework was used for CDR-grafting of both the heavy and light variable domains.

Heavy Chain

In the CDR-grafted heavy chain (ghTNF1) mouse CDRs were used at positions 26-35 (CDR1), 50-65 (CDR2) and 95-102 (CDR3). Mouse residues were also used in the frameworks at positions 48, 67, 69, 71, 73, 76, 89, 91, 94 and 108. Comparison of the TNF1 mouse and EU human heavy chain residues reveals that these are identical at positions 23, 24, 29 and 78.

Light Chain

In the CDR-grafted light chain (gLhTNF1) mouse CDRs were used at positions 24-34 (CDR1), 50-56 (CDR2) and 89-97 (CDR3). In addition mouse residues were used in the frameworks at positions 3, 42, 48, 49, 83, 106 and 108. Comparison of the hTNF1 mouse and EU human light chain residues reveals that these are identical at positions 46, 58 and 71.

The grafted hTNF1 heavy chain was co-expressed with the chimeric light chain and the binding ability of the product compared with that of the chimeric light chain/chimeric heavy chain product in a TNF binding assay. The grafted heavy chain product appeared to have binding ability for TNF slightly better than the fully chimeric product.

Similarly, a grafted heavy chain/grafted light chain product was co-expressed and compared with the fully chimeric product and found to have closely similar binding properties to the latter product.

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hTNF3

hTNF3 recognises an epitope on human TNF- α . The sequence of hTNF3 shows only 21 differences compared to 61E71 in the light and heavy chain variable regions, 10 in the light chain (2 in the CDRs at positions 50, 96 and 8 in the framework at 1, 19, 40, 45, 46, 76, 103 and 106) and 11 in the heavy chain (3 in the CDR regions at positions 52, 60 and 95 and 8 in the framework at 1, 10, 38, 40, 67, 73, 87 and 105). The light and heavy chains of the 61E71 and hTNF3 chimeric antibodies can be exchanged without loss of activity in the direct binding assay. However 61E71 is an order of magnitude less able to compete with the TNF receptor on L929 cells for TNF- α compared to hTNF3. Based on the 61E71 CDR grafting data gL221 and gH341(+23, 24, 48, 49 71 and 73 as mouse) genes have been built for hTNF3 and tested and the resultant grafted antibody binds well to TNF- α , but competes very poorly in the L929 assay. It is possible that in this case also the framework residues identified for OKT3 programme may improve the competitive binding ability of this antibody.

101.4

101.4 is a further murine monoclonal antibody able to recognise human TNF- α . The heavy chain of this antibody shows good homology to KOL and so the CDR-grafting has been based on RE1 for the light chain and KOL for the heavy chain. Several grafted heavy chain genes have been constructed with conservative choices for the CDR's (gH341) and which have one or a small number of non-CDR residues at positions 73, 78 or 77-79 inclusive, as the mouse amino acids. These have been co-expressed with cL or gL221. In all cases binding to TNF equivalent to the chimeric antibody is seen and when co-expressed with cL the resultant antibodies are able to compete well in the L929 assay. However, with gL221 the resultant antibodies

are at least an order of magnitude less able to compete for TNF against the TNF receptor on L929 cells.

Mouse residues at other positions in the heavy chain, for example, at 23 and 24 together or at 76 have been demonstrated to provide no improvement to the competitive ability of the grafted antibody in the L929 assay.

A number of other antibodies including antibodies having specificity for interleukins e.g. IL1 and cancer markers such as carcinoembryonic antigen (CEA) e.g. the monoclonal antibody A5B7 (ref. 21), have been successfully CDR-grafted according to the present invention.

It will be appreciated that the foregoing examples are given by way of illustration only and are not intended to limit the scope of the claimed invention. Changes and modifications may be made to the methods described whilst still falling within the spirit and scope of the invention.

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CLAIMS

1. A CDR-grafted antibody heavy chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 6, 23 and/or 24, 48 and/or 49, 71 and/or 73, 75 and/or 76 and/or 78 and 88 and/or 91.
2. A CDR-grafted heavy chain according to Claim 1 comprising donor residues at positions 23, 24, 49, 71, 73 and 78, or at positions 23, 24 and 49.
3. A CDR-grafted heavy chain according to Claim 2 comprising donor residues at positions 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.
4. A CDR-grafted heavy chain according to Claim 2 or 3, comprising donor residues at one, some or all of positions:
1 and 3,
69 (if 48 is different between donor and acceptor),
38 and 46 (if 48 is the donor residue),
67,
82 and 18 (if 67 is the donor residue),
91, and
any one or more of 9, 11, 41, 87, 108, 110 and 112.
5. A CDR-grafted heavy chain according to any of the preceding comprising donor CDRs at positions 26-35, 50-65 and 95-100.
6. A CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 1 and/or 3 and 46 and/or 47.

7. A CDR-grafted light chain according to Claim 6 comprising donor residues at positions 46 and 47.
8. A CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 46, 48, 58 and 71.
9. A CDR-grafted light chain according to Claim 8 comprising donor residues at positions 46, 48, 58 and 71.
10. A CDR-grafted light chain according to Claim 8 or 9, comprising donor residues at positions 2, 4, 6, 35, 36, 38, 44, 47, 49, 62, 64-69, 85, 87, 98, 99, 101 and 102.
11. A CDR-grafted light chain according to Claim 9 or 10, comprising donor residues at one, some or all of positions:
1 and 3,
63,
60 (if 60 and 54 are able to form a potential saltbridge),
70 (if 70 and 24 are able to form a potential saltbridge),
73 and 21 (if 47 is different between donor and acceptor),
37 and 45 (if 47 if different between donor and acceptor), and
any one or more of 10, 12, 40, 83, 103 and 105.
12. A CDR-grafted light chain according to any one of Claims 6-11, comprising donor CDRs at positions 24-34, 50-56 and 89-97.

13. A CDR-grafted antibody molecule comprising at least one CDR-grafted heavy chain according to any one of Claims 1-5 and at least one CDR-grafted light chain according to any one of Claims 6-12.
14. A CDR-grafted antibody molecule according to Claim 13, which is a site-specific antibody molecule.
15. A CDR-grafted antibody molecule according to Claim 13 which has specificity for an interleukin, hormone or other biologically active compound or a receptor therefor.
16. A CDR-grafted antibody heavy or light chain or molecule according to any one of the preceding claims comprising human acceptor residues and non-human donor residues.
17. A DNA sequence which codes for a CDR-grafted heavy chain according to Claim 1 or a CDR-grafted light chain according to Claim 6 or Claim 8.
18. A cloning or expression vector containing a DNA sequence according to Claim 17.
19. A host cell transformed with a DNA sequence according to Claim 17.
20. A process for the production of a CDR-grafted antibody sequence according to Claim 17 in a transformed host cell.
21. A process for producing a CDR-grafted antibody product comprising:

- (a) producing in an expression vector an operon having a DNA sequence which encodes an antibody heavy chain according to Claim 1;
- and/or
- (b) producing in an expression vector an operon having a DNA sequence which encodes a complementary antibody light chain according to Claim 6 or Claim 8;
 - (c) transfecting a host cell with the or each vector;
- and
- (d) culturing the transfected cell line to produce the CDR-grafted antibody product.

22. A therapeutic or diagnostic composition comprising a CDR-grafted antibody heavy chain according to Claim 1, or a CDR-grafted light chain according to Claim 6 or Claim 8, or a CDR-grafted antibody molecule according to Claim 13 in combination with a pharmaceutically acceptable carrier, diluent or excipient.

23. A method of therapy or diagnosis comprising administering an effective amount of a CDR-grafted heavy chain according to Claim 1, or a CDR-grafted light chain according to Claim 6 or Claim 8, or a CDR-grafted antibody molecule according to Claim 13 to a human or animal subject.

1 GAATTC~~CCAA~~ AGACAAAatg gattttcaag tgcaqatttt cagcttcctg
 51 ctaatcagtg cctcagtc cat aatatccaga ggacaaattg ttctcaccca
 101 gtctccagca atcatgtctg catctccagg ggagaaggtc accatgacct
 151 gcagtgccag ctcaagtgt agttacatga actggtacca gcagaagtca
 201 ggcacctccc ccaaaagatg gatttatgac acatccaaac tggcttctgg
 251 agtccctgct cacttcaggg gcagtgggtc tgggacctct tactctctca
 301 caatcagcgg catggaggct gaagatgctg ccacttatta ctgccagcag
 351 tggagtagta acccattcac gttcggctcg gggacaaagt tggaaataaa
 401 ccgggctgat actgcaccaa ctgtatccat cttcccacca tccagtgagc
 451 agttaacatc tggaggtgcc tcagtcgtgt gcttcttgaa caacttctac
 501 cccaaagaca tcaatgtcaa gtggaagatt gatggcagtg aacgacaaaa
 551 tggcgtcctg aacagttgga ctgatcagga cagcaaagac agcacctaca
 601 gcatgagcag caccctcacg ttgaccaagg acgagtatga acgacataac
 651 agctatacct gtgaggccac tcacaagaca tcaacttcac ccattgtcaa
 701 gagcttcaac aggaatgagt gtTAGAGACA AAGGTCCTGA GACGCCACCA
 751 CCAGCTCCCA GCTCCATCCT. ATCTTCCCTT CTAAGGTCTT GGAGGCTTCC
 801 CCACAAGCGC tTACCACTGT TGCGGTGCTC tAAACCTCCT CCCACCTCCT
 851 TCTCCTCCTC CTCCTTTCC TTGGCTTTTA TCATGCTAAT ATTTGCAGAA
 901 AATATTCAAT AAAGTGAGTC TTTGCCTTGA AAAAAAAAAA AAA

Fig. 1(a)

1 MDFOVOIFSF LLISASVIIS RGOIVLTQSP AIMSASPGEK VTMTCSASSS
 51 VSYMNWYQQK SGTSPKRWIY DTSKLAGVVP AHFRGSGSGT SYSLTISGME
 101 AEDAATYYCQ QWSSNPFTFG SGTKLEINRA DTAPTVSIFP PSSEQLTSGG
 151 ASVVCFLNNF YPKDINVKWK IDGSERQNGV LNSWTDQDSK DSTYSMSSTL
 201 TLTKEDEYERH NSYTCEATHK TSTSPIVKSF NRNEC*

Fig. 1(b)

SUBSTITUTE SHEET

1 GAATCCCCCT CTCCACAGAC ACTGAAAAC TCGACTCAAC ATGGAAAGGC
 51 ACTGGATCTT TCTACTCCTG TTGTCAGTAA CTGCAGGTGT CCACTCCCAG
 101 GTCCAGCTGC AGCAGTCTGG GGCTGAACTG GCAAGACCTG GGGCCTCAGT
 151 GAAGATGTCC TGCAAGGCTT CTGGCTACAC CTTTACTAGG TACACGATGC
 201 ACTGGGTAAA ACAGAGGCCT GGACAGGGTC TGGAAATGGAT TGGATACATT
 251 AATCCTAGCC GTGGTTATAC TAATTACAAT CAGAAGTTCA AGGACAAGGC
 301 CACATTGACT ACAGACAAAT CCTCCAGCAC AGCCTACATG CAACTGAGCA
 351 GCCTGACATC TGAGGACTCT GCAGTCTATT ACTGTGCAAG ATATTATGAT
 401 GATCATTACT GCCTTGACTA CTGGGGCCAA GGCACCACTC TCACAGTCTC
 451 CTCAGCCAAA ACAACAGCCC CATCGGTCTA TCCACTGGCC CCTGTGTGTG
 501 GAGATAAAC TGGCTCCTCG GTGACTCTAG GATGCCTGGT CAAGGGTTAT
 551 TTCCCTGAGC CAGTGACCTT GACCTGGAAC TCTGGATCCC TGTCCAGTGG
 601 TGTGCACACC TTCCCAGCTG TCCTGCAGTC TGACCTCTAC ACCCTCAGCA
 651 GCTCAGTGAC TGTAACCTCG AGCACCTGGC CCAGCCAGTC CATCACCTGC
 701 AATGTGGCCC ACCCGGCAAG CAGCACCAAG GTGGACAAGA AAATTGAGCC
 751 CAGAGGGCCC ACAATCAAGC CCTGTCCTCC ATGCAAATGC CCAGCACCTA
 801 ACCTCTTGGG TGGACCATCC GTCTTCATCT TCCCTCCAAA GATCAAGGAT
 851 GTACTCATGA TCTCCCTGAG CCCCATAGTC ACATGTGTGG TGGTGGATGT
 901 GAGCGAGGAT GACCAGATG TCCAGATCAG CTGGTTTGTG AACAACGTGG
 951 AAGTACACAC AGCTCAGACA CAAACCCATA GAGAGGATTA CAACAGTACT
 1001 CTCCGGGTGG TCAGTGCCCT CCCCATCCAG CACCAGGACT GGATGAGTGG
 1051 CAAGGAGTTC AAATGCAAGG TCAACAACAA AGACCTCCCA GCGCCCATCG
 1101 AGAGAACCAT CTCAAAACCC AAAGGGTCAG TAAGAGCTCC ACAGGTATAT
 1151 GTCTTGCCCTC CACCAGAAGA AGAGATGACT AAGAAACAGG TCACTCTGAC
 1201 CTGCATGGTC ACAGACTTCA TGCCTGAAGA CATTTACGTG GAGTGGACCA
 1251 ACAACGGGAA AACAGAGCTA AACTACAAGA ACACTGAACC AGTCCTGGAC
 1301 TCTGATGGTT CTTACTTCAT GTACAGCAAG CTGAGAGTGG AAAAGAAGAA
 1351 CTGGGTGGAA AGAAATAGCT ACTCCTGTTC AGTGGTCCAC GAGGGTCTGC
 1401 ACAATCACCA CACGACTAAG AGCTTCTCCC GGACTCCGGG TAAATGAGCT
 1451 CAGCACCCAC AAAACTCTCA GGTCCAAAGA GACACCCACA CTCATCTCCA
 1501 TGCTTCCCTT GTATAAATAA AGCACCCAGC AATGCCTGGG ACCATGTAAA
 1551 AAAAAAAAAA AAAGGAATTC

Fig. 2(a)

OKT 3 HEAVY CHAIN PROTEIN SEQUENCE DEDUCED FROM DNA SEQUENCE

1 MERHWIFLLL LSVTAGVHSQ VQLQOSGAEL ARPGASVKMS CKASGYTFTR
 51 YTMHWVKQRP GQGLEWIGYI NPSRGYTNYN QKFKDKATLT TDKSSSTAYM
 101 QLSSLTSEDS AVYYCARYYD DHYCLDYWGQ GTTLTVSSAK TTAPSVYPLA
 151 PVCGDTTGSS VTLGCLVKGY FPEPVTLTWN SGLSSGVHT FPAVLQSDLY
 201 TLSSSVTVTS STWPSQSITC NVAHPASSTK VDKKIEPRGP TIKPCPPCKC
 251 PAPANLLGGPS VFIFPPKIKD VLMISLSPIV TCVVVDVSED DPDVQISWFW
 301 NNVEVHTAQT QTHREDYNST LRVVSALPIQ HQDWMSGKEF KCKVNNKDLF
 351 APIERTISKP KGSVRAPQVY VLPPPEEEMT KKQVTLTCMV TDFMPEDIYV
 401 EWTNNGKTEL NYKNTEPVLD SDGSYFMYSK LRVEKKNWVE RNSYSCSVVH
 451 EGLHNHHTTK SFSRTPGK*

Fig. 2(b)

	1		23		42
	NN	N	N	N	N
RES TYPE	SBspSPESsssBSbSsSssPSPSPsPSsse*s*p*Pi [^] ISsSe				
Okt3v1	QIVLTQSPA [^] IMASASPGEKVTMTCSASS.SVSYMNWYQQKSGT				
REI	DIQMTQSPSSLSASVGD [^] RVTTITCQASQDI [^] IKYLNWYQQTPGK				
	?	?			
	CDR1	(LOOP)		*****	
	CDR1	(KABAT)		*****	

		56		85
	N	NN		
RES TYPE	*IsiPpIeesesssSBesePsPSBSSEsPsp [^] sPseesSPePb			
Okt3v1	SPKRWIYDTSK [^] LASGVP [^] AHFRGSGSGT [^] SYSLTISGMEAE [^] DAAT			
REI	APKLLIYEASN [^] LQAGVPSR [^] FSGSGSGT [^] DYFT [^] ISSLQ [^] PEDIAT			
	?	??		? ?
	***** CDR2 (LOOP/KABAT)			

		102	108
RES TYPE	PiPIPIes**iPIIsPPSPSPSS		
Okt3v1	YYCQQWSSNPFTFGSGTKLEINR		
REIv1	YYCQQYQSLPYTFGQGTKLQITR		
		?	?

***** CDR3 (LOOP)
 ***** CRD3 (KABAT)

Fig. 3

4/15

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NN N                23 26      32 35 N39  43
RES TYPE  SESPs^SBssS^sSSsSpSpSPsPSEbSBssBePiIpiesss
Okt3h     QVQLQOSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPGQ
KOL       QVQLVESGGGVVQPGRSLRLSCSSSGFIFSSYAMYWVRQAPGK
           ?                ??

                               ***** CDR1 (LOOP)
                               ***** CDR1 (KABAT)

           52a      60 65      N N N      82abc      89
RES TYPE  IIeIppp^sssssss^ps^pSSsbSpseSsSseSp^pSpsSBsss^ePb
Okt3vh    GLEWIGYINPSRGYTNTNQKFKDKATLTTDKSSSTAYMQLSSLTSEDSAV
KOL       GLEWVAIIWDDGSDQHAYADSVKGRFTISRDNSKNTLFLQMDSLRPEDTGV
           ??                ? ? ? ?      ?

           ***** CDR2 (LOOP)
           ***** CDR2 (KABAT)

           92 N                107      113
RES TYPE  PiPIEissssiisssbibi*EIPiP*spSBSS
Okt3vh    YYCARYYDDHY.....CLDYWGQGTTLTVSS
KOL       YFCARDGGHGFCSSASCFGPDYWGQGTPTVTVSS
           ***** CRD3 (KABAT/LOOP)
    
```

Fig. 4

OKT 3 HEAVY CHAIN CDR GRAFTS

1. gh341 and derivatives

	1	26	35	39	43	
Okt3vh	QVQLQ	QSGAELARPGASVKMSCKASGYTFTRYTMHWVKQ	Q	RP	GP	
gh341	QVQLVESGGGVVQ	PGRSLRLSCSSSGYTFTRYTMHWVRQAPGK				JA178
gh341A	QVQLVQ	SGGGVVQ	PGRSLRLSCKASGYTFTRYTMHWVRQAPGK			JA185
gh341E	QVQLVQ	SGGGVVQ	PGRSLRLSCKASGYTFTRYTMHWVRQAPGK			JA198
gh341*	QVQLVQ	SGGGVVQ	PGRSLRLSCKASGYTFTRYTMHWVRQAPGK			JA207
gh341*	QVQLVQ	SGGGVVQ	PGRSLRLSCKASGYTFTRYTMHWVRQAPGK			JA209
gh341D	QVQLVQ	SGGGVVQ	PGRSLRLSCKASGYTFTRYTMHWVRQAPGK			JA197
gh341*	QVQLVQ	SGGGVVQ	PGRSLRLSCKASGYTFTRYTMHWVRQAPGK			JA199
gh341C	QVQLVQ	SGGGVVQ	PGRSLRLSCKASGYTFTRYTMHWVRQAPGK			JA184
gh341*	QVQLVQ	SGGGVVQ	PGRSLRLSCSASGYTFTRYTMHWVRQAPGK			JA203
gh341*	QVQLVESGGGVVQ	PGRSLRLSCSASGYTFTRYTMHWVRQAPGK				JA205
gh341B	QVQLVESGGGVVQ	PGRSLRLSCSSSGYTFTRYTMHWVRQAPGK				JA183
gh341*	QVQLVQ	SGGGVVQ	PGRSLRLSCSASGYTFTRYTMHWVRQAPGK			JA204
gh341*	QVQLVESGGGVVQ	PGRSLRLSCSASGYTFTRYTMHWVRQAPGK				JA206
gh341*	QVQLVQ	SGGGVVQ	PGRSLRLSCSASGYTFTRYTMHWVRQAPGK			JA208
KOL	QVQLVESGGGVVQ	PGRSLRLSCSSSGFIFSSYAMYWVRQAPGK				

Fig. 5(i)

	44	50	65	83
Okt3vh	GLEWIGYINPSRGYTNYNQFKDKATLTDDKSSSTAYMQLSSLT			
gH341	GLEWVAYINPSRGYTNYNQFKDRFTISRDN SKNTLFLQMSLR JA178			
gH341A	GLEWIGYINPSRGYTNYNQVKDRFTISTDKSKSTAFLQMSLR JA185			
gH341E	GLEWIGYINPSRGYTNYNQVKDRFTISTDKSKSTAFLQMSLR JA198			
gH341*	GLEWIGYINPSRGYTNYNQVKDRFTISTDKSKNTAFLQMSLR JA207			
gH341*	GLEWIGYINPSRGYTNYNQVKDRFTISRDN SKNTAFLQMSLR JA209			
gH341D	GLEWIGYINPSRGYTNYNQVKDRFTISTDKSKNTLFLQMSLR JA197			
gH341*	GLEWIGYINPSRGYTNYNQVKDRFTISRDN SKNTLFLQMSLR JA199			
gH341C	GLEWVAYINPSRGYTNYNQFKDRFTISRDN SKNTLFLQMSLR JA184			
gH341*	GLEWIGYINPSRGYTNYNQVKDRFTISTDKSKSTAFLQMSLR JA207			
gH341*	GLEWIGYINPSRGYTNYNQVKDRFTISTDKSKSTAFLQMSLR JA205			
gH341B	GLEWIGYINPSRGYTNYNQVKDRFTISTDKSKSTAFLQMSLR JA183			
gH341*	GLEWIGYINPSRGYTNYNQVKDRFTISTDKSKSTAFLQMSLR JA204			
gH341*	GLEWIGYINPSRGYTNYNQVKDRFTISTDKSKSTAFLQMSLR JA206			
gH341*	GLEWIGYINPSRGYTNYNQVKDRFTISTDKSKNTAFLQMSLR JA208			
KOL	GLEWVAIIWDDGSDQHYADSVKGRFTISRDN SKNTLFLQMSLR			

Fig. 5(ii)

	84	95	102	113	
Okt3vh	SEDSAVYYCARYYDDHY.....		CLDYWGQGTTLTVSS		
gH341	PEDTGVYFCARYYDDHY.....		CLDYWGQGTTLTVSS		JA178
gH341A	PEDT <u>AVYY</u> CARYYDDHY.....		CLDYWGQGTTLTVSS		JA185
gH341E	PEDTGVYFCARYYDDHY.....		CLDYWGQGTTLTVSS		JA198
gH341*	PEDTGVYFCARYYDDHY.....		CLDYWGQGTTLTVSS		JA207
gH341D	PEDTGVYFCARYYDDHY.....		CLDYWGQGTTLTVSS		JA197
gH341*	PEDTGVYFCARYYDDHY.....		CLDYWGQGTTLTVSS		JA209
gH341*	PEDTGVYFCARYYDDHY.....		CLDYWGQGTTLTVSS		JA199
gH341C	PEDTGVYFCARYYDDHY.....		CLDYWGQGTTLTVSS		JA184
gH341*	PEDT <u>AVYY</u> CARYYDDHY.....		CLDYWGQGTTLTVSS		JA203
gH341*	PEDT <u>AVYY</u> CARYYDDHY.....		CLDYWGQGTTLTVSS		JA205
gH341B	PEDT <u>AVYY</u> CARYYDDHY.....		CLDYWGQGTTLTVSS		JA183
gH341*	PEDTGVYFCARYYDDHY.....		CLDYWGQGTTLTVSS		JA204
gH341*	PEDTGVYFCARYYDDHY.....		CLDYWGQGTTLTVSS		JA206
gH341*	PEDTGVYFCARYYDDHY.....		CLDYWGQGTTLTVSS		JA208
KOL	PEDTGVYFCARDGGHGFCSASCFGPDYWGQGTPVTVSS				

Fig. 5(iii)

OKT3 LIGHT CHAIN CDR GRAFTING

1. gL221 and derivatives

	1	24	34	42
Okt3v1	QIVLTQSPAIMSASPGEKVTMTCSASS.SVSYMNWYQQKSGT			
gL221	DIQMTQSPSSLSASVGDRVTTITCSASS.SVSYMNWYQQTPGK			
gL221A	<u>QIV</u> MTQSPSSLSASVGDRVTTITCSASS.SVSYMNWYQQTPGK			
gL221B	<u>QIV</u> MTQSPSSLSASVGDRVTTITCSASS.SVSYMNWYQQTPGK			
gL221C	DIQMTQSPSSLSASVGDRVTTITCSASS.SVSYMNWYQQTPGK			
REI	DIQMTQSPSSLSASVGDRVTTITCQASQDIKYLNWYQQTPGK			
	43	50	56	85
Okt3v1	SPKRWIYDTSKLAGVPAHFRGSGSGTSYSLTISGMEAEDAAT			
gL221	APKLLIYDTSKLAGVPSRFSGSGSGTDYFTISSLQPEDIAT			
gL221A	APKRWIYDTSKLAGVPSRFSGSGSGTDYFTISSLQPEDIAT			
gL221B	APKRWIYDTSKLAGVPSRFSGSGSGTDYFTISSLQPEDIAT			
gL221C	APKRWIYDTSKLAGVPSRFSGSGSGTDYFTISSLQPEDIAT			
REI	APKLLIYEASNLOAGVPSRFSGSGSGTDYFTISSLQPEDIAT			
	86	91	96	108
Okt3v1	YYCQWSSNPFTFGSGTKLEINR			
gL221	YYCQWSSNPFTFGQGTKLQITR			
gL221A	YYCQWSSNPFTFGQGTKLQITR			
gL221B	YYCQWSSNPFTFGQGTKLQITR			
gL221C	YYCQWSSNPFTFGQGTKLQITR			
REI	YYCQQYQSLPYTFGQGTKLQITR			

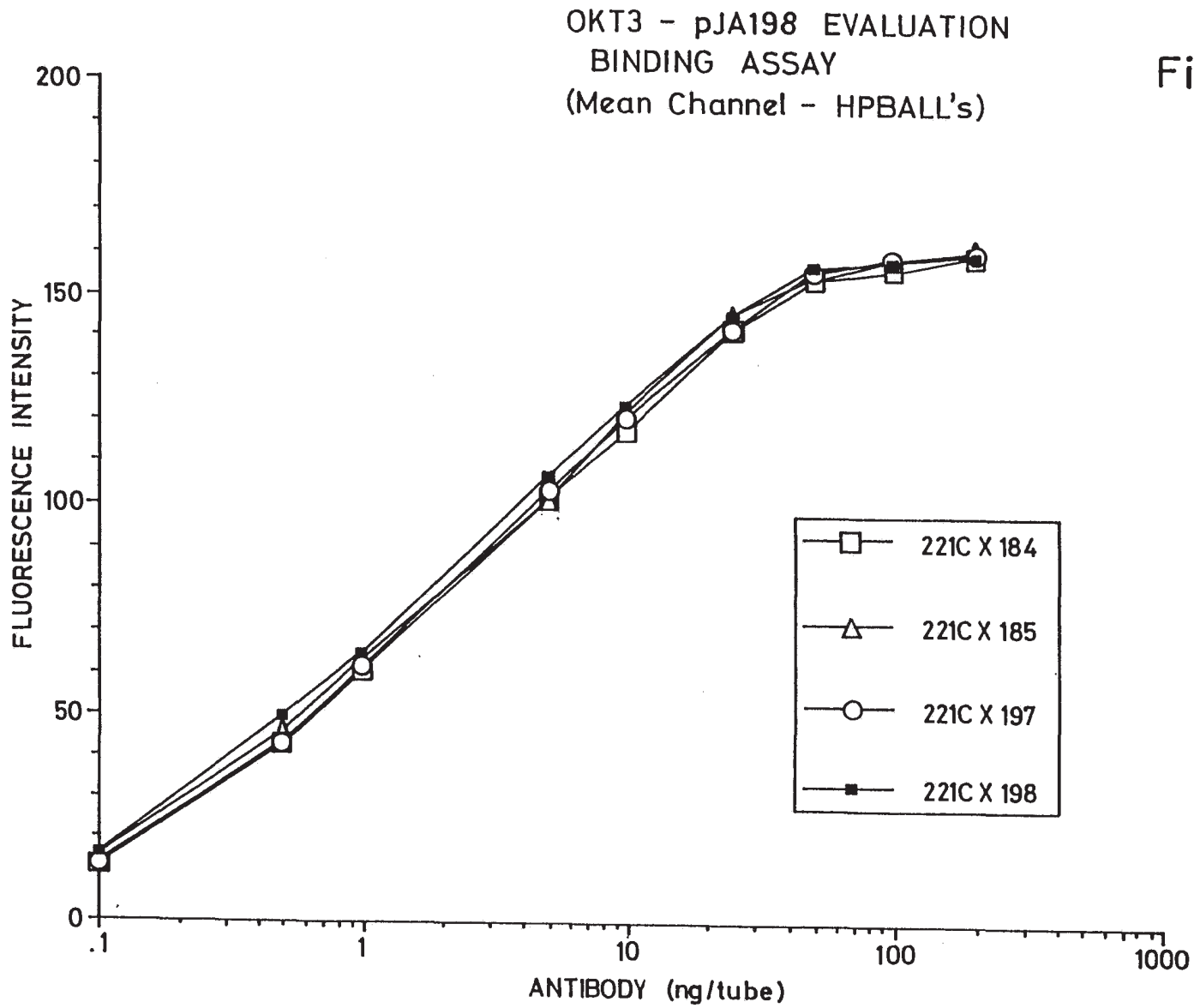
CDR'S ARE UNDERLINED

FRAMEWORK RESIDUES INCLUDED IN THE GENE ARE DOUBLE UNDERLINED

Fig. 6

SUBSTITUTE SHEET

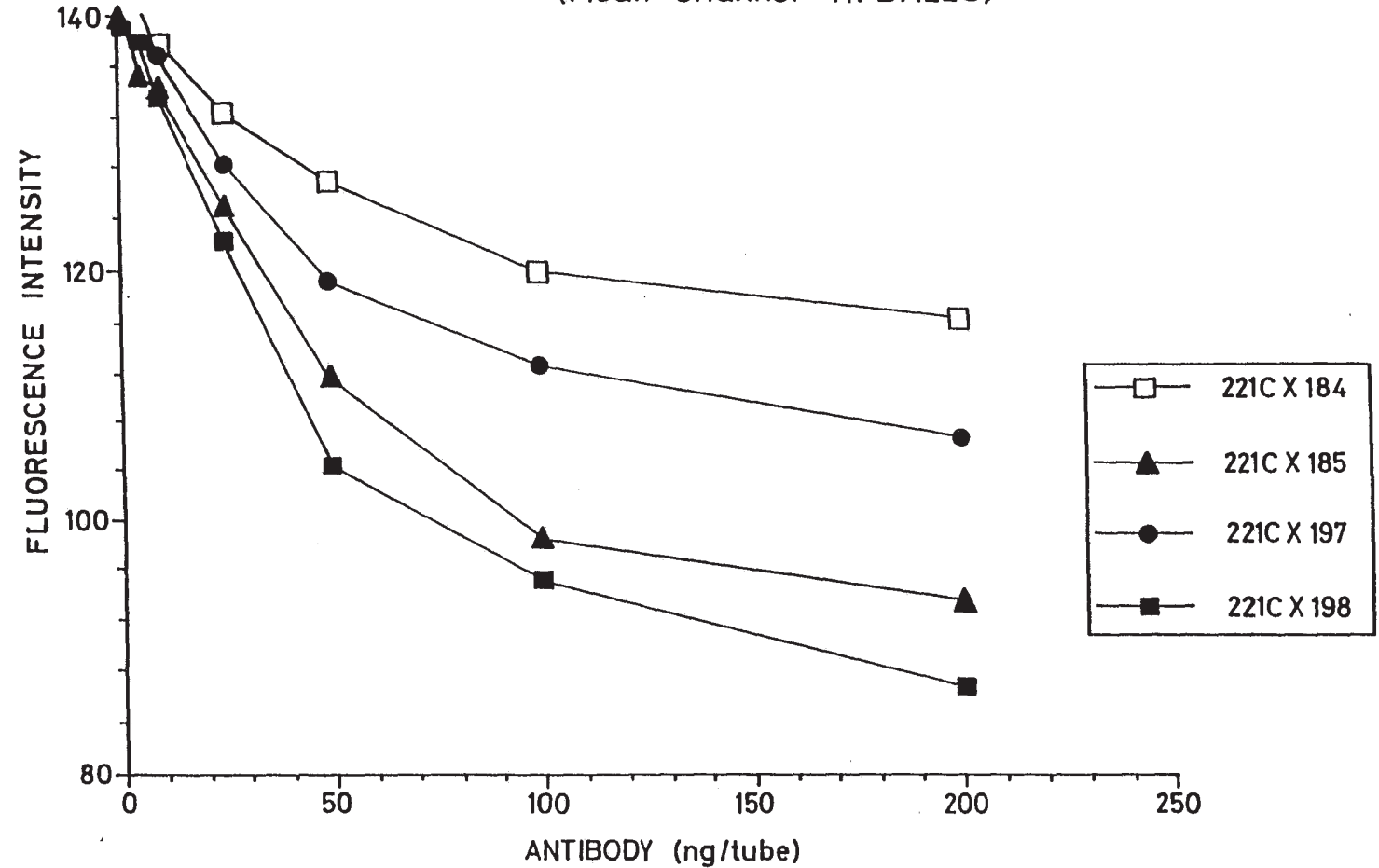
SUBSTITUTE SHEET



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OKT3 - pJA198 EVALUATION
BLOCKING ASSAY
(Mean Channel -HPBALL's)

Fig. 8



SUBSTITUTE SHEET

BLOCKING ASSAY
(Mean Channel - HPBALL's)

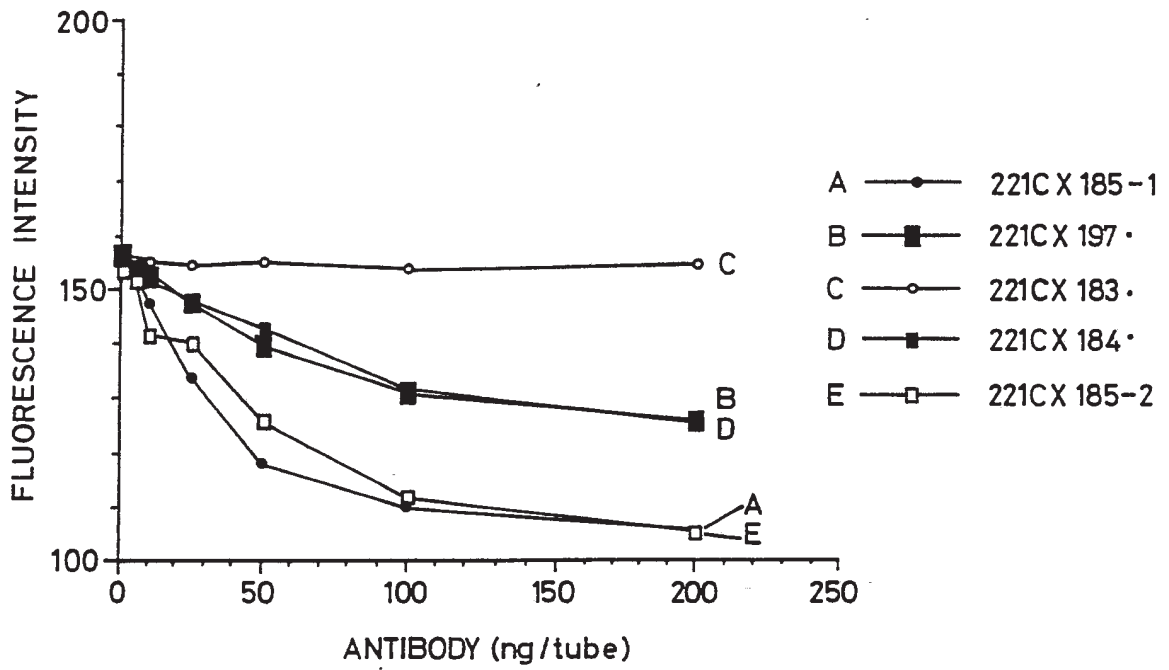
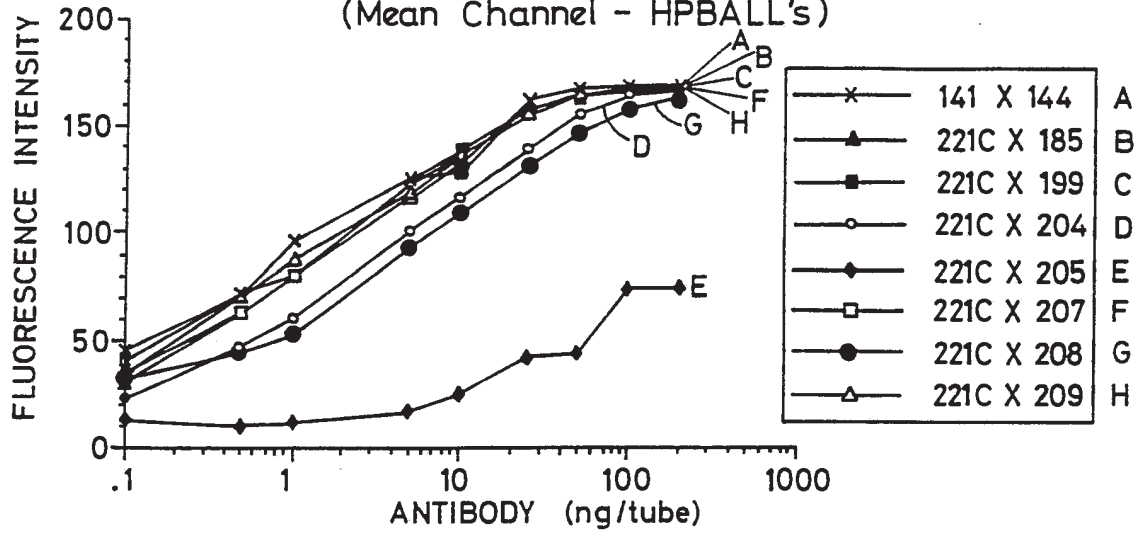


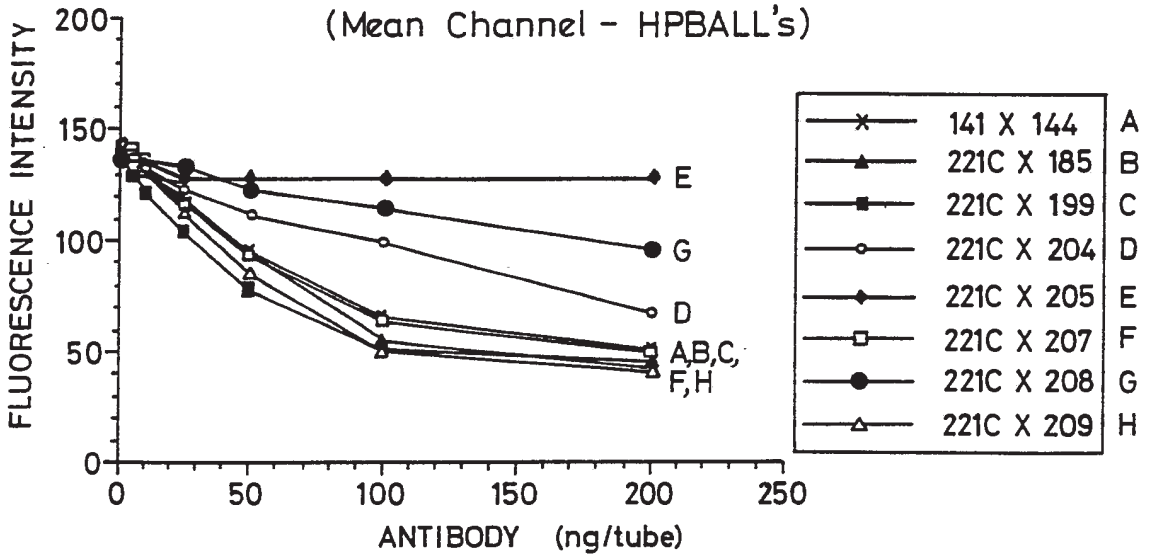
Fig. 9

Fig.10

OKT3 - GRAFTED HEAVY CHAINS
BINDING ASSAY
(Mean Channel - HPBALL's)



OKT3 - GRAFTED HEAVY CHAINS
BLOCKING ASSAY
(Mean Channel - HPBALL's)



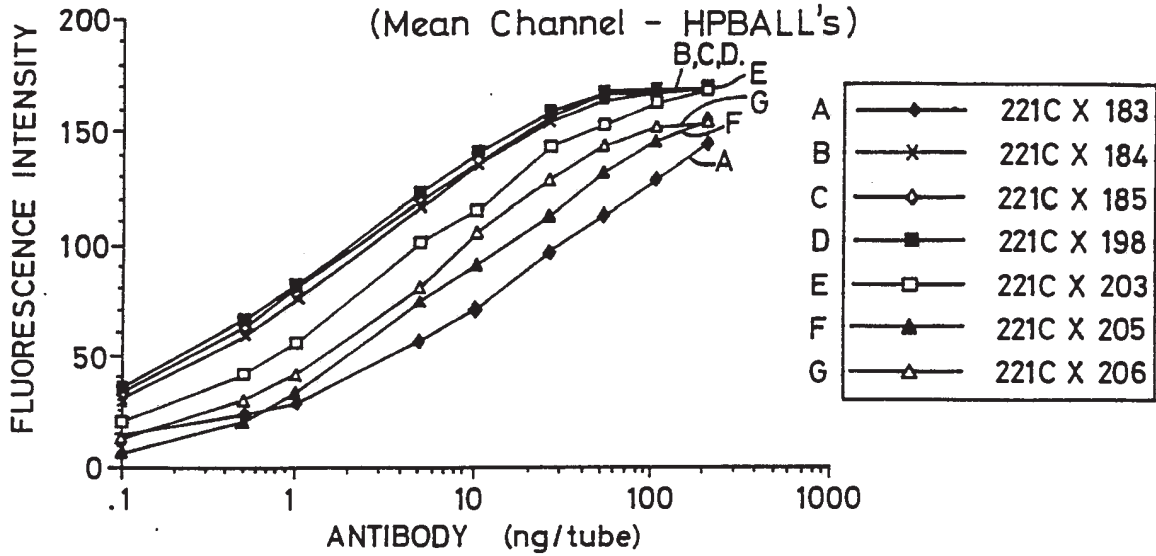
◆	(205)	6, 23, 24, 48, 49, 71, 73, 76, 78, 88, 91,
●	(208)	6, 23, 24, 48, 49, 71, 73, 76, 78, 88, 91,
○	(204)	6, 23, 24, 48, 49, 71, 73, 76, 78, 88, 91,
■	(199)	6, 23, 24, 48, 49, 71, 73, 76, 78, 88, 91,
□	(207)	6, 23, 24, 48, 49, 71, 73, 76, 78, 88, 91,
▲	(185)	6, 23, 24, 48, 49, 71, 73, 76, 78, 88, 91,
△	(209)	6, 23, 24, 48, 49, 71, 73, 76, 78, 88, 91,
*	141 X 144	6, 23, 24, 48, 49, 71, 73, 76, 78, 88, 91,

SUBSTITUTE SHEET

Fig. 11

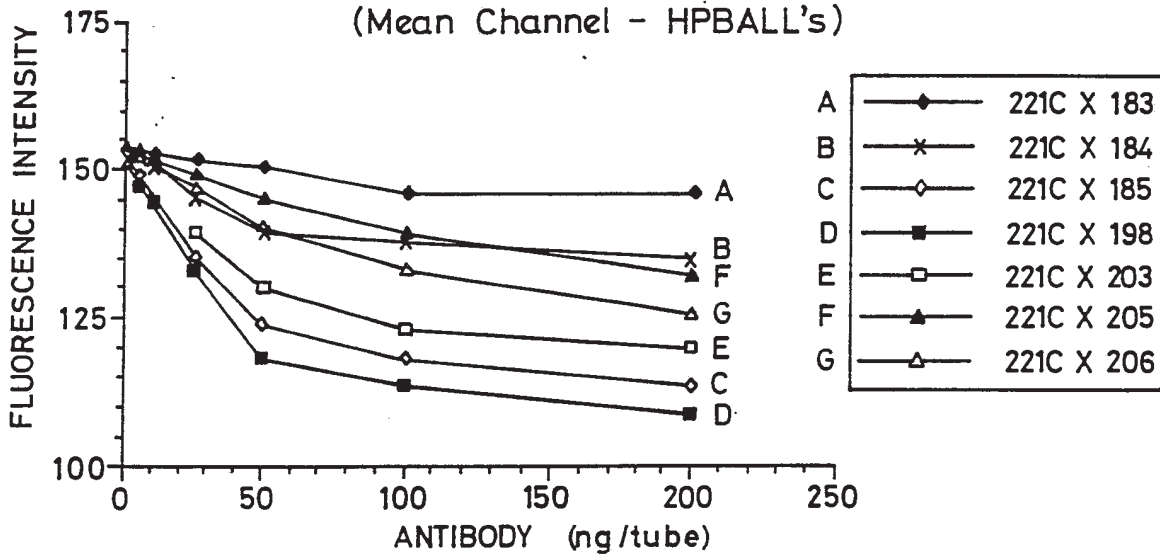
OKT3 - GRAFTED HEAVY CHAINS
BINDING ASSAY

(Mean Channel - HPBALL's)



OKT3 GRAFTED HEAVY CHAINS
BLOCKING ASSAY

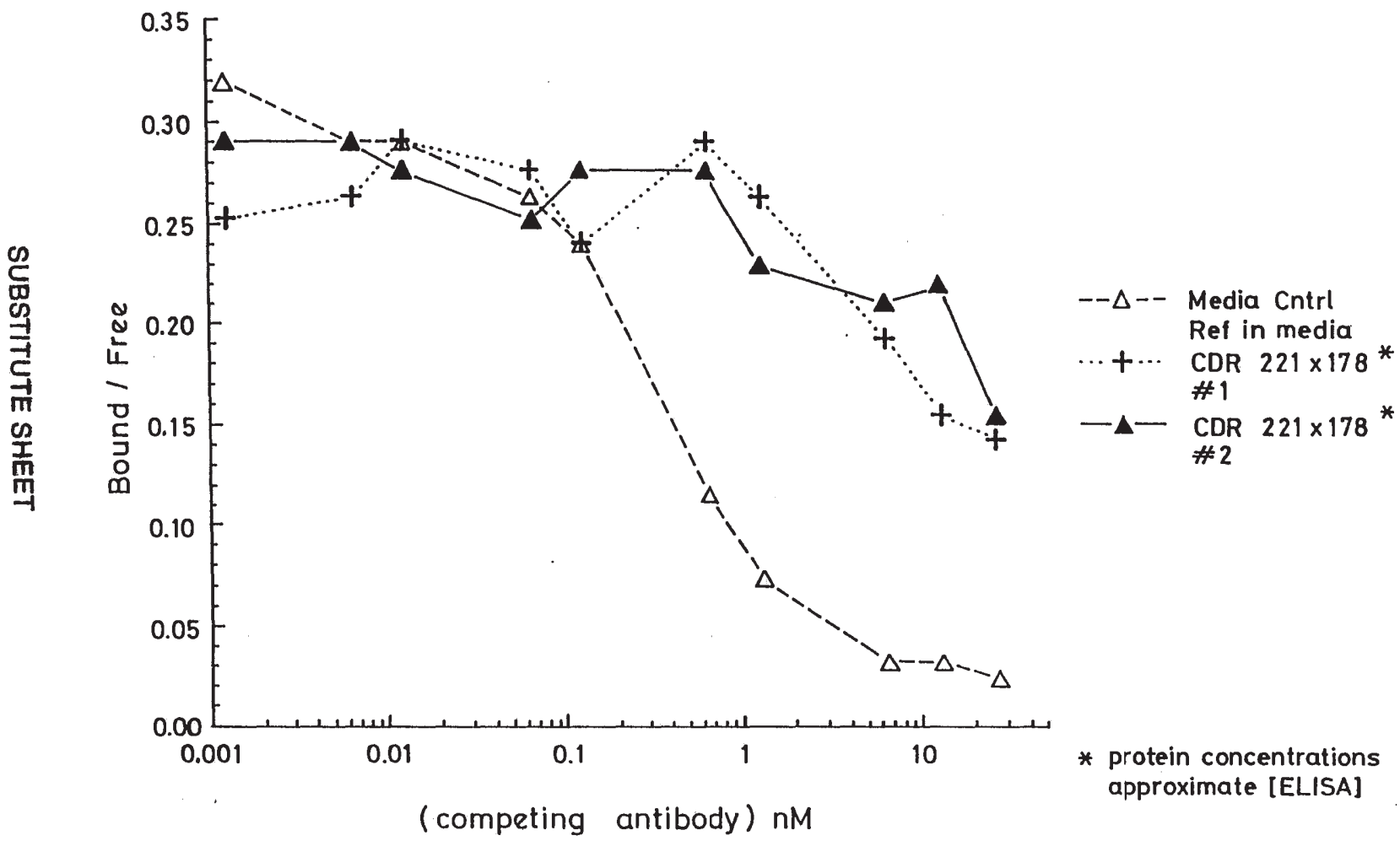
(Mean Channel - HPBALL's)



◆	(183),48,49,71,73,76,78,88,91,
▲	(205),24,48,49,71,73,76,78,88,91,
×	(184)	6,23,24,.....,.....
△	(206),24,48,49,71,73,76,78,.....,
□	(203)	6,.....,24,48,49,71,73,76,78,88,91,
◇	(185)	6,23,24,48,49,71,73,76,78,88,91,
■	(198)	6,23,24,48,49,71,73,76,78,.....,

OKT3 Competition Murine Ref Std vs. CDR Grafted OKT3

Fig. 12

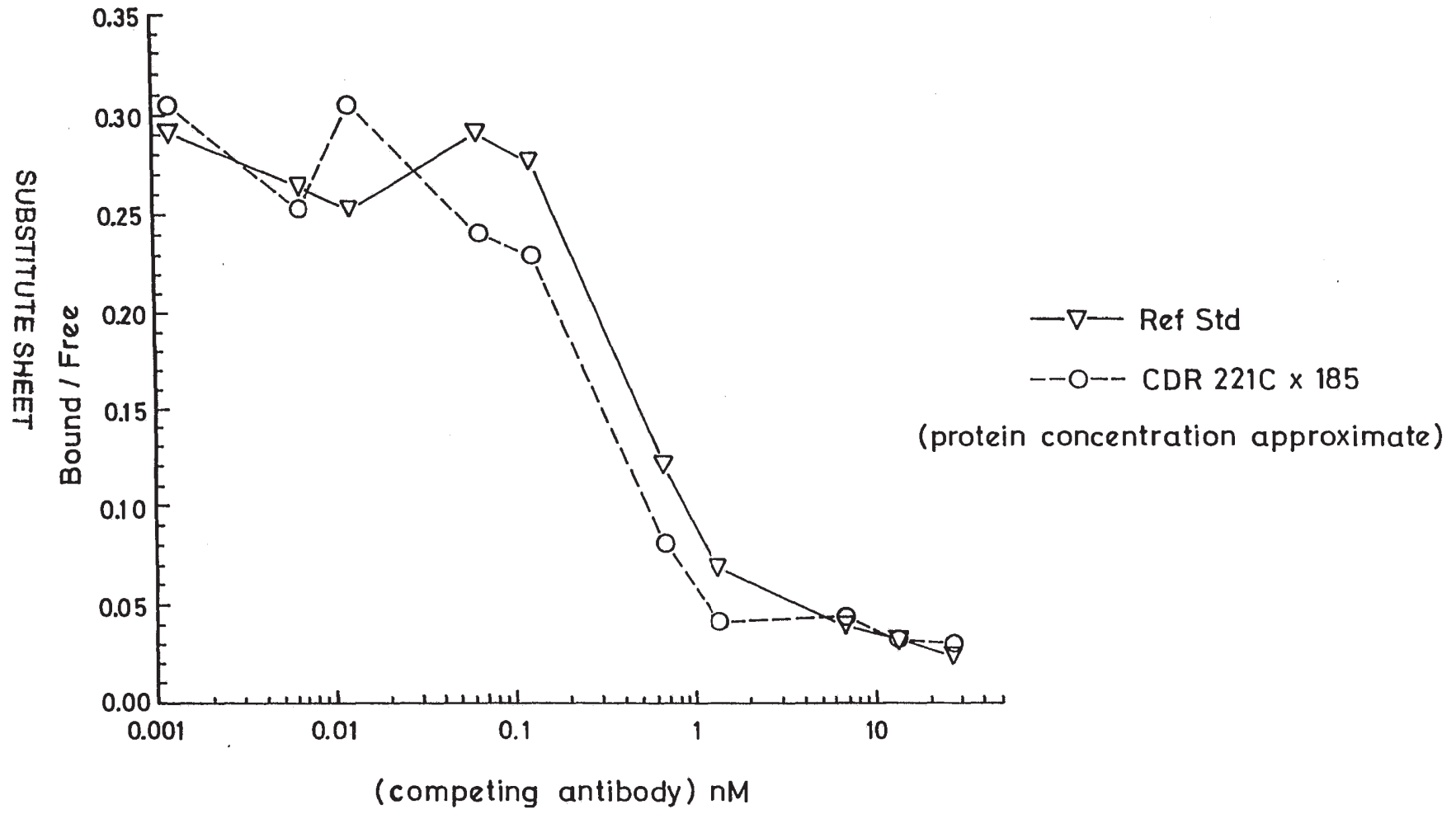


SUBSTITUTE SHEET

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OKT3 Competition Murine Ref Std vs. CDR Grafted OKT3

Fig. 13



INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 90/02017

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC5: C 12 P 21/08, C 12 N 15/13, A 61 K 39/395, C 07 K 15/06 C 12 N 5/10, 15/62		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC5	C 12 P; C 12 N; A 61 K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
P,X	EP, A1, 0403156 (GENZYME CORPORATION ET AL.) 19 December 1990, see examples 8-12 and corresponding tables --	1,6,8, 13,14- 22
Y	Proc. Natl. Acad. Sci. USA, vol. 86, December 1989, C. Queen et al.: "A humanized antibody that binds to the interleukin 2 receptor ", see page 10029- page 10033 see the whole document and in particular page 10031 right col. - page 10032; left col. and page 10033 left col. --	1,6,8, 13,14- 22
Y	EP, A1, 0328404 (MEDICAL RESEARCH COUNCIL ET AL.) 16 August 1989, see pages 1-3, page 9, lines 49-54 and the claims --	1,6,8, 13,14- 22
<p>* Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
11th April 1991	17.05.91	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	F.W. HECK	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Nature, vol. 332, March 1988, L. Riechmann et al.: "Reshaping human antibodies for therapy", see page 323 - page 327 see in particular page 327, right col. --	1,6,8, 13,144- 22
A	Nature, vol. 321, May 1986, P.T. Jones et al.: "Replacing the complementarity-determining regions in a human antibody with those from a mouse", see page 522 - page 525 see the whole document --	1-22
A	Nature, vol. 328, August 1987, S. Roberts et al.: "Generation of an antibody with enhanced affinity and specificity for its antigen by protein engineering", see page 731 - page 734 see the whole document --	1,6
A	Science, vol. 239, 1988, M. Verhoeyen et al.: "Reshaping Human Antibodies: Grafting an Antilysozyme Activity", see page 1534 - page 1536 see the whole document --	1,6
A	EP, A2, 0239400 (WINTER, GREGORY PAUL) 30 September 1987, see the whole document --	1,6,17- 22
A	EP, A1, 0323806 (CIBA-GEIGY AG) 12 July 1989, see pages 2-6 --	1,6,17- 22
A	Nature, vol. 341, October 1989, E.S. Ward et al.: "Binding activities of a repertoire of single immunoglobulin variable domains secreted from Escherichia coli", see page 544 - page 546 -- -----	1,6

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. PCT/GB 90/02017**

SA 43080

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 28/02/91. The European Patent office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A1- 0403156	19/12/90	NONE	
EP-A1- 0328404	16/08/89	AU-D- 3062689 GB-A- 2216126 WO-A- 89/07452	06/09/89 04/10/89 24/08/89
EP-A2- 0239400	30/09/87	GB-A-B- 2188638 JP-A- 62296890	07/10/87 24/12/87
EP-A1- 0323806	12/07/89	AU-D- 2759588 JP-A- 2154696	06/07/89 14/06/90

For more details about this annex : see Official Journal of the European patent Office, No. 12/82

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Inst 1

Field of the Invention

The present invention relates to humanised antibody molecules, to processes for their production using recombinant DNA technology, and to their therapeutic uses.

The term "humanised antibody molecule" is used to describe a molecule having an antigen binding site derived from an immunoglobulin from a non-human species, and remaining immunoglobulin-derived parts of the molecule being derived from a human immunoglobulin. The antigen binding site typically comprises complementarity determining regions (CDRs) which determine the binding specificity of the antibody molecule and which are carried on appropriate framework regions in the variable domains. There are 3 CDRs (CDR1, CDR2 and CDR3) in each of the heavy and light chain variable domains.

In the description, reference is made to a number of publications by number. The publications are listed in numerical order at the end of the description.

Background of the Invention

Natural immunoglobulins have been known for many years, as have the various fragments thereof, such as the Fab, (Fab')₂ and Fc fragments, which can be derived by enzymatic cleavage. Natural immunoglobulins comprise a generally Y-shaped molecule having an antigen-binding site towards the end of each upper arm. The remainder of the structure, and particularly the stem of the Y, mediates the effector functions associated with immunoglobulins.

Natural immunoglobulins have been used in assay, diagnosis and, to a more limited extent, therapy. However, such uses, especially in therapy, were hindered until recently by the polyclonal nature of natural immunoglobulins. A significant step towards the realisation of the potential

of immunoglobulins as therapeutic agents was the discovery of procedures for the production of monoclonal antibodies (MAbs) of defined specificity (1).

However, most MAbs are produced by hybridomas which are fusions of rodent spleen cells with rodent myeloma cells. They are therefore essentially rodent proteins. There are very few reports of the production of human MAbs.

Since most available MAbs are of rodent origin, they are naturally antigenic in humans and thus can give rise to an undesirable immune response termed the HAMA (Human Anti-Mouse Antibody) response. Therefore, the use of rodent MAbs as therapeutic agents in humans is inherently limited by the fact that the human subject will mount an immunological response to the MAb and will either remove it entirely or at least reduce its effectiveness. In practice, MAbs of rodent origin may not be used in patients for more than one or a few treatments as a HAMA response soon develops rendering the MAb ineffective as well as giving rise to undesirable reactions. For instance, OKT3 a mouse IgG2a/k MAb which recognises an antigen in the T-cell receptor-CD3 complex has been approved for use in many countries throughout the world as an immunosuppressant in the treatment of acute allograft rejection [Chatenoud et al (2) and Jeffers et al (3)]. However, in view of the rodent nature of this and other such MAbs, a significant HAMA response which may include a major anti-idiotypic component, may build up on use. Clearly, it would be highly desirable to diminish or abolish this undesirable HAMA response and thus enlarge the areas of use of these very useful antibodies.

Proposals have therefore been made to render non-human MAbs less antigenic in humans. Such techniques can be generically termed "humanisation" techniques. These

techniques typically involve the use of recombinant DNA technology to manipulate DNA sequences encoding the polypeptide chains of the antibody molecule.

Early methods for humanising MAbS involved production of chimeric antibodies in which an antigen binding site comprising the complete variable domains of one antibody is linked to constant domains derived from another antibody. Methods for carrying out such chimerisation procedures are described in EP0120694 (Celltech Limited), EP0125023 (Genentech Inc. and City of Hope), EP-A-0 171496 (Res. Dev. Corp. Japan), EP-A-0 173 494 (Stanford University), and WO 86/01533 (Celltech Limited). This latter Celltech application (WO 86/01533) discloses a process for preparing an antibody molecule having the variable domains from a mouse MAb and the constant domains from a human immunoglobulin. Such humanised chimeric antibodies, however, still contain a significant proportion of non-human amino acid sequence, i.e. the complete non-human variable domains, and thus may still elicit some HAMA response, particularly if administered over a prolonged period [Begent et al (ref. 4)].

In an alternative approach, described in EP-A-0239400 (Winter), the complementarity determining regions (CDRs) of a mouse MAb have been grafted onto the framework regions of the variable domains of a human immunoglobulin by site directed mutagenesis using long oligonucleotides. The present invention relates to humanised antibody molecules prepared according to this alternative approach, i.e. CDR-grafted humanised antibody molecules. Such CDR-grafted humanised antibodies are much less likely to give rise to a HAMA response than humanised chimeric antibodies in view of the much lower proportion of non-human amino acid sequence which they contain.

The earliest work on humanising MABs by CDR-grafting was carried out on MABs recognising synthetic antigens, such as the NP or NIP antigens. However, examples in which a mouse MAB recognising lysozyme and a rat MAB recognising an antigen on human T-cells were humanised by CDR-grafting have been described by Verhoeyen et al (5) and Riechmann et al (6) respectively. The preparation of CDR-grafted antibody to the antigen on human T cells is also described in WO 89/07452 (Medical Research Council).

In Riechmann et al/Medical Research Council it was found that transfer of the CDR regions alone [as defined by Kabat refs. (7) and (8)] was not sufficient to provide satisfactory antigen binding activity in the CDR-grafted product. Riechmann et al found that it was necessary to convert a serine residue at position 27 of the human sequence to the corresponding rat phenylalanine residue to obtain a CDR-grafted product having improved antigen binding activity. This residue at position 27 of the heavy chain is within the structural loop adjacent to CDR1. A further construct which additionally contained a human serine to rat tyrosine change at position 30 of the heavy chain did not have a significantly altered binding activity over the humanised antibody with the serine to phenylalanine change at position 27 alone. These results indicate that changes to residues of the human sequence outside the CDR regions, in particular in the structural loop adjacent to CDR1, may be necessary to obtain effective antigen binding activity for CDR-grafted antibodies which recognise more complex antigens. Even so the binding affinity of the best CDR-grafted antibodies obtained was still significantly less than the original MAB.

Very recently Queen et al (9) have described the preparation of a humanised antibody that binds to the

interleukin 2 receptor, by combining the CDRs of a murine MAb (anti-Tac) with human immunoglobulin framework and constant regions. The human framework regions were chosen to maximise homology with the anti-Tac MAb sequence. In addition computer modelling was used to identify framework amino acid residues which were likely to interact with the CDRs or antigen, and mouse amino acids were used at these positions in the humanised antibody.

In WO 90/07861 Queen et al propose four criteria for designing humanised immunoglobulins. The first criterion is to use as the human acceptor the framework from a particular human immunoglobulin that is unusually homologous to the non-human donor immunoglobulin to be humanised, or to use a consensus framework from many human antibodies. The second criterion is to use the donor amino acid rather than the acceptor if the human acceptor residue is unusual and the donor residue is typical for human sequences at a specific residue of the framework. The third criterion is to use the donor framework amino acid residue rather than the acceptor at positions immediately adjacent to the CDRs. The fourth criterion is to use the donor amino acid residue at framework positions at which the amino acid is predicted to have a side chain atom within about 3 Å of the CDRs in a three-dimensional immunoglobulin model and to be capable of interacting with the antigen or with the CDRs of the humanised immunoglobulin. It is proposed that criteria two, three or four may be applied in addition or alternatively to criterion one, and may be applied singly or in any combination.

WO 90/07861 describes in detail the preparation of a single CDR-grafted humanised antibody, a humanised antibody having specificity for the p55 Tac protein of the

K
IL-2 receptor. The combination of all four criteria, as above, were employed in designing this humanised antibody, the variable region frameworks of the human antibody Eu (7) being used as acceptor. In the resultant humanised antibody the donor CDRs were as defined by Kabat et al (7 and 8) and in addition the mouse donor residues were used in place of the human acceptor residues, at positions 27, 30, 48, 66, 67, 89, 91, 94, 103, 104, 105 and 107 in the heavy chain and at positions 48, 60 and 63 in the light chain, of the variable region frameworks. The humanised anti-Tac antibody obtained is reported to have an affinity for p55 of $3 \times 10^9 \text{ M}^{-1}$, about one-third of that of the murine MAb.

We have further investigated the preparation of CDR-grafted humanised antibody molecules and have identified a hierarchy of positions within the framework of the variable regions (i.e. outside both the Kabat CDRs and structural loops of the variable regions) at which the amino acid identities of the residues are important for obtaining CDR-grafted products with satisfactory binding affinity. This has enabled us to establish a protocol for obtaining satisfactory CDR-grafted products which may be applied very widely irrespective of the level of homology between the donor immunoglobulin and acceptor framework. The set of residues which we have identified as being of critical importance does not coincide with the residues identified by Queen et al (9).

Summary of the Invention

Accordingly, in a first aspect the invention provides a CDR-grafted antibody heavy chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 6, 23 and/or 24, 48 and/or 49, 71 and/or 73, 75 and/or 76 and/or 78 and 88 and/or 91.

In preferred embodiments, the heavy chain framework comprises donor residues at positions 23, 24, 49, 71, 73 and 78 or at positions 23, 24 and 49. The residues at positions 71, 73 and 78 of the heavy chain framework are preferably either all acceptor or all donor residues.

In particularly preferred embodiments the heavy chain framework additionally comprises donor residues at one, some or all of positions 6, 37, 48 and 94. Also it is particularly preferred that residues at positions of the heavy chain framework which are commonly conserved across species, i.e. positions 2, 4, 25, 36, 39, 47, 93, 103, 104, 106 and 107, if not conserved between donor and acceptor, additionally comprise donor residues. Most preferably the heavy chain framework additionally comprises donor residues at positions 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.

In addition the heavy chain framework optionally comprises donor residues at one, some or all of positions:

1 and 3,
72 and 76,
69 (if 48 is different between donor and acceptor),
38 and 46 (if 48 is the donor residue),
80 and 20 (if 69 is the donor residue),
67,
82 and 18 (if 67 is the donor residue),
91,
88, and
any one or more of 9, 11, 41, 87, 108, 110 and 112.

In the first and other aspects of the present invention reference is made to CDR-grafted antibody products comprising acceptor framework and donor antigen binding regions. It will be appreciated that the invention is widely applicable to the CDR-grafting of antibodies in

general. Thus, the donor and acceptor antibodies may be derived from animals of the same species and even same antibody class or sub-class. More usually, however, the donor and acceptor antibodies are derived from animals of different species. Typically the donor antibody is a non-human antibody, such as a rodent MAb, and the acceptor antibody is a human antibody.

In the first and other aspects of the present invention, the donor antigen binding region typically comprises at least one CDR from the donor antibody. Usually the donor antigen binding region comprises at least two and preferably all three CDRs of each of the heavy chain and/or light chain variable regions. The CDRs may comprise the Kabat CDRs, the structural loop CDRs or a composite of the Kabat and structural loop CDRs and any combination of any of these. Preferably, the antigen binding regions of the CDR-grafted heavy chain variable domain comprise CDRs corresponding to the Kabat CDRs at CDR2 (residues 50-65) and CDR3 (residues 95-100) and a composite of the Kabat and structural loop CDRs at CDR1 (residues 26-35).

The residue designations given above and elsewhere in the present application are numbered according to the Kabat numbering [refs. (7) and (8)]. Thus the residue designations do not always correspond directly with the linear numbering of the amino acid residues. The actual linear amino acid sequence may contain fewer or additional amino acids than in the strict Kabat numbering corresponding to a shortening of, or insertion into, a structural component, whether framework or CDR, of the basic variable domain structure. For example, the heavy chain variable region of the anti-Tac antibody described by Queen et al (9) contains a single amino acid insert (residue 52a) after residue 52 of CDR2 and a three amino

acid insert (residues 82a, 82b and 82c) after framework residue 82, in the Kabat numbering. The correct Kabat numbering of residues may be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence.

The invention also provides in a second aspect a CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 1 and/or 3 and 46 and/or 47. Preferably the CDR grafted light chain of the second aspect comprises donor residues at positions 46 and/or 47.

The invention also provides in a third aspect a CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 46, 48, 58 and 71.

In a preferred embodiment of the third aspect, the framework comprises donor residues at all of positions 46, 48, 58 and 71.

In particularly preferred embodiments of the second and third aspects, the framework additionally comprises donor residues at positions 36, 44, 47, 85 and 87. Similarly positions of the light chain framework which are commonly conserved across species, i.e. positions 2, 4, 6, 35, 49, 62, 64-69, 98, 99, 101 and 102, if not conserved between donor and acceptor, additionally comprise donor residues. Most preferably the light chain framework additionally comprises donor residues at positions 2, 4, 6, 35, 36, 38, 44, 47, 49, 62, 64-69, 85, 87, 98, 99, 101 and 102.

In addition the framework of the second or third aspects optionally comprises donor residues at one, some or all of positions:

1 and 3,

63,

60 (if 60 and 54 are able to form a potential saltbridge),

70 (if 70 and 24 are able to form a potential saltbridge),

73 and 21 (if 47 is different between donor and acceptor),

37 and 45 (if 47 is different between donor and acceptor),

and

any one or more of 10, 12, 40, 80, 103 and 105.

Preferably, the antigen binding regions of the CDR-grafted light chain variable domain comprise CDRs corresponding to the Kabat CDRs at CDR1 (residue 24-34), CDR2 (residues 50-56) and CDR3 (residues 89-97).

The invention further provides in a fourth aspect a CDR-grafted antibody molecule comprising at least one CDR-grafted heavy chain and at least one CDR-grafted light chain according to the first and second or first and third aspects of the invention.

The humanised antibody molecules and chains of the present invention may comprise: a complete antibody molecule, having full length heavy and light chains; a fragment thereof, such as a Fab, (Fab')₂ or FV fragment; a light chain or heavy chain monomer or dimer; or a single chain antibody, e.g. a single chain FV in which heavy and light chain variable regions are joined by a peptide linker; or any other CDR-grafted molecule with the same specificity as the original donor antibody. Similarly the CDR-grafted heavy and light chain variable region may be combined with other antibody domains as appropriate.

Also the heavy or light chains or humanised antibody molecules of the present invention may have attached to them an effector or reporter molecule. For instance, it may have a macrocycle, for chelating a heavy metal atom, or a toxin, such as ricin, attached to it by a covalent bridging structure. Alternatively, the procedures of recombinant DNA technology may be used to produce an immunoglobulin molecule in which the Fc fragment or CH3 domain of a complete immunoglobulin molecule has been replaced by, or has attached thereto by peptide linkage, a functional non-immunoglobulin protein, such as an enzyme or toxin molecule.

Any appropriate acceptor variable region framework sequences may be used having regard to class/type of the donor antibody from which the antigen binding regions are derived. Preferably, the type of acceptor framework used is of the same/similar class/type as the donor antibody. Conveniently, the framework may be chosen to maximise/optimize homology with the donor antibody sequence particularly at positions close or adjacent to the CDRs. However, a high level of homology between donor and acceptor sequences is not important for application of the present invention. The present invention identifies a hierarchy of framework residue positions at which donor residues may be important or desirable for obtaining a CDR-grafted antibody product having satisfactory binding properties. The CDR-grafted products usually have binding affinities of at least 10^5 M^{-1} , preferably at least about 10^8 M^{-1} , or especially in the range 10^8 - 10^{12} M^{-1} . In principle, the present invention is applicable to any combination of donor and acceptor antibodies irrespective of the level of homology between their sequences. A protocol for applying the invention to any particular donor-acceptor antibody pair is given hereinafter. Examples of human frameworks which may be

used are KOL, NEWM, REI, EU, LAY and POM (refs. 4 and 5) and the like; for instance KOL and NEWM for the heavy chain and REI for the light chain and EU, LAY and POM for both the heavy chain and the light chain.

Also the constant region domains of the products of the invention may be selected having regard to the proposed function of the antibody in particular the effector functions which may be required. For example, the constant region domains may be human IgA, IgE, IgG or IgM domains. In particular, IgG human constant region domains may be used, especially of the IgG1 and IgG3 isotypes, when the humanised antibody molecule is intended for therapeutic uses, and antibody effector functions are required. Alternatively, IgG2 and IgG4 isotypes may be used when the humanised antibody molecule is intended for therapeutic purposes and antibody effector functions are not required, e.g. for simple blocking of lymphokine activity.

However, the remainder of the antibody molecules need not comprise only protein sequences from immunoglobulins. For instance, a gene may be constructed in which a DNA sequence encoding part of a human immunoglobulin chain is fused to a DNA sequence encoding the amino acid sequence of a functional polypeptide such as an effector or reporter molecule.

Preferably the CDR-grafted antibody heavy and light chain and antibody molecule products are produced by recombinant DNA technology.

Thus in further aspects the invention also includes DNA sequences coding for the CDR-grafted heavy and light chains, cloning and expression vectors containing the DNA sequences, host cells transformed with the DNA sequences

and processes for producing the CDR-grafted chains and antibody molecules comprising expressing the DNA sequences in the transformed host cells.

The general methods by which the vectors may be constructed, transfection methods and culture methods are well known per se and form no part of the invention. Such methods are shown, for instance, in references 10 and 11.

The DNA sequences which encode the donor amino acid sequence may be obtained by methods well known in the art. For example the donor coding sequences may be obtained by genomic cloning, or cDNA cloning from suitable hybridoma cell lines. Positive clones may be screened using appropriate probes for the heavy and light chain genes in question. Also PCR cloning may be used.

DNA coding for acceptor, e.g. human acceptor, sequences may be obtained in any appropriate way. For example DNA sequences coding for preferred human acceptor frameworks such as KOL, REI, EU and NEWM, are widely available to workers in the art.

The standard techniques of molecular biology may be used to prepare DNA sequences coding for the CDR-grafted products. Desired DNA sequences may be synthesised completely or in part using oligonucleotide synthesis techniques. Site-directed mutagenesis and polymerase chain reaction (PCR) techniques may be used as appropriate. For example oligonucleotide directed synthesis as described by Jones et al (ref. 20) may be used. Also oligonucleotide directed mutagenesis of a pre-existing variable region as, for example, described by Verhoeyen et al (ref. 5) or Riechmann et al (ref. 6) may be used. Also enzymatic filling in of gapped

oligonucleotides using T₄ DNA polymerase as, for example, described by Queen et al (ref. 9) may be used.

Any suitable host cell/vector system may be used for expression of the DNA sequences coding for the CDR-grafted heavy and light chains. Bacterial e.g. E. coli, and other microbial systems may be used, in particular for expression of antibody fragments such as FAb and (Fab')₂ fragments, and especially FV fragments and single chain antibody fragments e.g. single chain FVs. Eucaryotic e.g. mammalian host cell expression systems may be used for production of larger CDR-grafted antibody products, including complete antibody molecules. Suitable mammalian host cells include CHO cells and myeloma or hybridoma cell lines.

Thus, in a further aspect the present invention provides a process for producing a CDR-grafted antibody product comprising:

- (a) producing in an expression vector an operon having a DNA sequence which encodes an antibody heavy chain according to the first aspect of the invention;

and/or

- (b) producing in an expression vector an operon having a DNA sequence which encodes a complementary antibody light chain according to the second or third aspect of the invention;
- (c) transfecting a host cell with the or each vector; and
- (d) culturing the transfected cell line to produce the CDR-grafted antibody product.

The CDR-grafted product may comprise only heavy or light chain derived polypeptide, in which case only a heavy chain or light chain polypeptide coding sequence is used to transfect the host cells.

For production of products comprising both heavy and light chains, the cell line may be transfected with two vectors, the first vector may contain an operon encoding a light chain-derived polypeptide and the second vector containing an operon encoding a heavy chain-derived polypeptide. Preferably, the vectors are identical, except in so far as the coding sequences and selectable markers are concerned, so as to ensure as far as possible that each polypeptide chain is equally expressed. Alternatively, a single vector may be used, the vector including the sequences encoding both light chain- and heavy chain-derived polypeptides.

The DNA in the coding sequences for the light and heavy chains may comprise cDNA or genomic DNA or both. However, it is preferred that the DNA sequence encoding the heavy or light chain comprises at least partially, genomic DNA, preferably a fusion of cDNA and genomic DNA.

The present invention is applicable to antibodies of any appropriate specificity. Advantageously, however, the invention may be applied to the humanisation of non-human antibodies which are used for in vivo therapy or diagnosis. Thus the antibodies may be site-specific antibodies such as tumour-specific or cell surface-specific antibodies, suitable for use in in vivo therapy or diagnosis, e.g. tumour imaging. Examples of cell surface-specific antibodies are anti-T cell antibodies, such as anti-CD3, and CD4 and adhesion molecules, such as CR3, ICAM and ELAM. The antibodies may have specificity for interleukins (including lymphokines, growth factors and stimulating factors), hormones and other biologically active compounds, and receptors for any of these. For

example, the antibodies may have specificity for any of the following: Interferons α , β , γ or δ , IL1, IL2, IL3, or IL4, etc., TNF, GCSF, GMCSF, EPO, hGH, or insulin, etc.

The the present invention also includes therapeutic and diagnostic compositions comprising the CDR-grafted products of the invention and uses of such compositions in therapy and diagnosis.

Accordingly in a further aspect the invention provides a therapeutic or diagnostic composition comprising a CDR-grafted antibody heavy or light chain or molecule according to previous aspects of the invention in combination with a pharmaceutically acceptable carrier, diluent or excipient.

Accordingly also the invention provides a method of therapy or diagnosis comprising administering an effective amount of a CDR-grafted antibody heavy or light chain or molecule according to previous aspects of the invention to a human or animal subject.

A preferred protocol for obtaining CDR-grafted antibody heavy and light chains in accordance with the present invention is set out below together with the rationale by which we have derived this protocol. This protocol and rationale are given without prejudice to the generality of the invention as hereinbefore described and defined.

Protocol

It is first of all necessary to sequence the DNA coding for the heavy and light chain variable regions of the donor antibody, to determine their amino acid sequences. It is also necessary to choose appropriate acceptor heavy and light chain variable regions, of known amino acid sequence. The CDR-grafted chain is then designed

starting from the basis of the acceptor sequence. It will be appreciated that in some cases the donor and acceptor amino acid residues may be identical at a particular position and thus no change of acceptor framework residue is required.

1. As a first step donor residues are substituted for acceptor residues in the CDRs. For this purpose the CDRs are preferably defined as follows:

Heavy chain - CDR1: residues 26-35
 - CDR2: residues 50-65
 - CDR3: residues 95-102
Light chain - CDR1: residues 24-34
 - CDR2: residues 50-56
 - CDR3: residues 89-97

The positions at which donor residues are to be substituted for acceptor in the framework are then chosen as follows, first of all with respect to the heavy chain and subsequently with respect to the light chain.

2. Heavy Chain

- 2.1 Choose donor residues at all of positions 23, 24, 49, 71, 73 and 78 of the heavy chain or all of positions 23, 24 and 49 (71, 73 and 78 are always either all donor or all acceptor).
- 2.2 Check that the following have the same amino acid in donor and acceptor sequences, and if not preferably choose the donor: 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.

2.3 To further optimise affinity consider choosing donor residues at one, some or any of:

- i. 1, 3
- ii. 72, 76
- iii. If 48 is different between donor and acceptor sequences, consider 69
- iv. If at 48 the donor residue is chosen, consider 38 and 46
- v. If at 69 the donor residue is chosen, consider 80 and then 20
- vi. 67
- vii. If at 67 the donor residue is chosen, consider 82 and then 18
- viii. 91
- ix. 88
- x. 9, 11, 41, 87, 108, 110, 112

3. Light Chain

3.1 Choose donor at 46, 48, 58 and 71

3.2 Check that the following have the same amino acid in donor and acceptor sequences, if not preferably choose donor:

2, 4, 6, 35, 38, 44, 47, 49, 62, 64-69 inclusive, 85, 87, 98, 99, 101 and 102

3.3 To further optimise affinity consider choosing donor residues at one, some or any of:

- i. 1, 3
- ii. 63

- iii. 60, if 60 and 54 are able to form potential saltbridge
- iv. 70, if 70 and 24 are able to form potential saltbridge
- v. 73, and 21 if 47 is different between donor and acceptor
- vi. 37, and 45 if 47 is different between donor and acceptor
- vii. 10, 12, 40, 80, 103, 105

Rationale

In order to transfer the binding site of an antibody into a different acceptor framework, a number of factors need to be considered.

1. The extent of the CDRs

The CDRs (Complementary Determining Regions) were defined by Wu and Kabat (refs. 4 and 5) on the basis of an analysis of the variability of different regions of antibody variable regions. Three regions per domain were recognised. In the light chain the sequences are 24-34, 50-56, 89-97 (numbering according to Kabat (ref. 4), Eu Index) inclusive and in the heavy chain the sequences are 31-35, 50-65 and 95-102 inclusive.

When antibody structures became available it became apparent that these CDR regions corresponded in the main to loop regions which extended from the β barrel framework of the light and heavy variable domains. For H1 there was a discrepancy in that the loop was from 26 to 32 inclusive and for H2 the loop was 52 to 56 and for L2 from 50 to 53. However, with the exception of H1 the CDR regions encompassed the loop regions and extended into the β strand

frameworks. In H1 residue 26 tends to be a serine and 27 a phenylalanine or tyrosine, residue 29 is a phenylalanine in most cases. Residues 28 and 30 which are surface residues exposed to solvent might be involved in antigen-binding. A prudent definition of the H1 CDR therefore would include residues 26-35 to include both the loop region and the hypervariable residues 33-35.

It is of interest to note the example of Riechmann et al (ref. 3), who used the residue 31-35 choice for CDR-H1. In order to produce efficient antigen binding, residue 27 also needed to be recruited from the donor (rat) antibody.

2. Non-CDR residues which contribute to antigen binding

By examination of available X-ray structures we have identified a number of residues which may have an effect on net antigen binding and which can be demonstrated by experiment. These residues can be sub-divided into a number of groups.

2.1 Surface residues near CDR [all numbering as in Kabat et al (ref. 7)].

2.1.1. Heavy Chain - Key residues are 23, 71 and 73. Other residues which may contribute to a lesser extent are 1, 3 and 76. Finally 25 is usually conserved but the murine residue should be used if there is a difference.

2.1.2 Light Chain - Many residues close to the CDRs, e.g. 63, 65, 67 and 69 are conserved. If conserved none of the surface residues in the light chain are likely to have a major effect. However, if the murine residue at these positions

is unusual, then it would be of benefit to analyse the likely contribution more closely. Other residues which may also contribute to binding are 1 and 3, and also 60 and 70 if the residues at these positions and at 54 and 24 respectively are potentially able to form a salt bridge i.e. 60 + 54; 70 + 24.

2.2 Packing residues near the CDRs.

2.2.1. Heavy Chain - Key residues are 24, 49 and 78. Other key residues would be 36 if not a tryptophan, 94 if not an arginine, 104 and 106 if not glycines and 107 if not a threonine. Residues which may make a further contribution to stable packing of the heavy chain and hence improved affinity are 2, 4, 6, 38, 46, 67 and 69. 67 packs against the CDR residue 63 and this pair could be either both mouse or both human. Finally, residues which contribute to packing in this region but from a longer range are 18, 20, 80, 82 and 86. 82 packs against 67 and in turn 18 packs against 82. 80 packs against 69 and in turn 20 packs against 80. 86 forms an H bond network with 38 and 46. Many of the mouse-human differences appear minor e.g. Leu-Ile, but could have an minor impact on correct packing which could translate into altered positioning of the CDRs.

2.2.2. Light Chain - Key residues are 48, 58 and 71. Other key residues would be 6 if not glutamine, 35 if not tryptophan, 62 if not phenylalanine or tryosine, 64, 66, 68, 99 and 101 if not glycines and 102 if not a threonine. Residues which make a further contribution are 2, 4, 37, 45 and 47. Finally residues 73 and 21 and 19 may make long distance packing contributions of a minor nature.

- 2.3. Residues at the variable domain interface between heavy and light chains - In both the light and heavy chains most of the non-CDR interface residues are conserved. If a conserved residue is replaced by a residue of different character, e.g. size or charge, it should be considered for retention as the murine residue.
- 2.3.1. Heavy Chain - Residues which need to be considered are 37 if the residue is not a valine but is of larger side chain volume or has a charge or polarity. Other residues are 39 if not a glutamine, 45 if not a leucine, 47 if not a tryptophan, 91 if not a phenylalanine or tyrosine, 93 if not an alanine and 103 if not a tryptophan. Residue 89 is also at the interface but is not in a position where the side chain could be of great impact.
- 2.3.2. Light Chain - Residues which need to be considered are 36, if not a tyrosine, 38 if not a glutamine, 44 if not a proline, 46, 49 if not a tyrosine, residue 85, residue 87 if not a tyrosine and 98 if not a phenylalanine.
- 2.4. Variable-Constant region interface - The elbow angle between variable and constant regions may be affected by alterations in packing of key residues in the variable region against the constant region which may affect the position of V_L and V_H with respect to one another. Therefore it is worth noting the residues likely to be in contact with the constant region. In the heavy chain the surface residues potentially in contact with the variable region are conserved between mouse and human antibodies therefore the variable region contact residues may influence the V-C interaction. In the light chain the amino acids found at a number of the constant

region contact points vary, and the V & C regions are not in such close proximity as the heavy chain. Therefore the influences of the light chain V-C interface may be minor.

2.4.1. Heavy Chain - Contact residues are 7, 11, 41, 87, 108, 110, 112.

2.4.2. Light Chain - In the light chain potentially contacting residues are 10, 12, 40, 80, 83, 103 and 105.

The above analysis coupled with our considerable practical experimental experience in the CDR-grafting of a number of different antibodies have lead us to the protocol given above.

The present invention is now described, by way of example only, with reference to the accompanying Figures 1 - 13.

Brief Description of the Figures

- Figure 1 shows DNA and amino acid sequences of the OKT3 light chain; (SEQ ID NO: 4 and 5)
- Figure 2 shows DNA and amino acid sequences of the OKT3 heavy chain; (SEQ ID NO: 6 and 7)
- Figure 3 shows the alignment of the OKT3 light variable region amino acid sequence with that of the light variable region of the human antibody REI; (SEQ ID NO: 29, 30, 8 and 9)
- Figure 4 shows the alignment of the OKT3 heavy variable region amino acid sequence with that of the heavy variable region of the human antibody KOL; (SEQ ID NO: 7 and 10)
- Figure 5 shows the heavy variable region amino acid sequences of OKT3, KOL and various corresponding CDR grafts; (SEQ ID NO: 7, 8, 9 and 10-24)
- Figure 6 shows the light variable region amino acid sequences of OKT3, REI and various corresponding CDR grafts; (SEQ ID NO: 18 and 25)

A J
A J S
A J
K S
A S
S

- Figure 7 shows a graph of binding assay results for various grafted OKT3 antibodies'
- Figure 8 shows a graph of blocking assay results for various grafted OKT3 antibodies;
- Figure 9 shows a similar graph of blocking assay results;
- Figure^s₁₀^{a and b} shows similar graphs for both binding assay and blocking assay results;
- Figure^s₁₁^{a and b} shows further similar graphs for both binding assay and blocking assay results;
- Figure 12 shows a graph of competition assay results for a minimally grafted OKT3 antibody compared with the OKT3 murine reference standard, and
- Figure 13 shows a similar graph of competition assay results comparing a fully grafted OKT3 antibody with the murine reference standard.

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

EXAMPLE 1

CDR-GRAFTING OF OKT3

MATERIAL AND METHODS

1. INCOMING CELLS

Hybridoma cells producing antibody OKT3 were provided by Ortho (seedlot 4882.1) and were grown up in antibiotic free Dulbecco's Modified Eagles Medium (DMEM) supplemented with glutamine and 5% foetal calf serum, and divided to provide both an overgrown supernatant for evaluation and cells for extraction of RNA. The overgrown supernatant was shown to contain 250 ug/mL murine IgG2a/kappa antibody. The supernatant was negative for murine lambda light chain and IgG1, IgG2b, IgG3, IgA and IgM heavy chain. 20mL of supernatant was assayed to confirm that the antibody present was OKT3.

2. MOLECULAR BIOLOGY PROCEDURES

Basic molecular biology procedures were as described in Maniatis et al (ref. 9) with, in some cases, minor modifications. DNA sequencing was performed as described in Sanger et al (ref. 11) and the Amersham International Plc sequencing handbook. Site directed mutagenesis was as described in Kramer et al (ref. 12) and the Anglian Biotechnology Ltd. handbook. COS cell expression and metabolic labelling studies were as described in Whittle et al (ref. 13)

3. RESEARCH ASSAYS

3.1. ASSEMBLY ASSAYS

Assembly assays were performed on supernatants from transfected COS cells to determine the amount of intact IgG present.

3.1.1. COS CELLS TRANSFECTED WITH MOUSE OKT3 GENES

The assembly assay for intact mouse IgG in COS cell supernatants was an ELISA with the following format:

96 well microtitre plates were coated with F(ab')₂ goat anti-mouse IgG Fc. The plates were washed in water and samples added for 1 hour at room temperature. The plates were washed and F(ab')₂ goat anti-mouse IgG F(ab')₂ (HRPO conjugated) was then added. Substrate was added to reveal the reaction. UPC10, a mouse IgG2a myeloma, was used as a standard.

3.1.2. COS AND CHO CELLS TRANSFECTED WITH CHIMERIC OR CDR-GRAFTED OKT3 GENES

The assembly assay for chimeric or CDR-grafted antibody in COS cell supernatants was an ELISA with the following format:

96 well microtitre plates were coated with F(ab')₂ goat anti-human IgG Fc. The plates were washed and samples added and incubated for 1 hour at room temperature. The plates were washed and monoclonal mouse anti-human kappa chain was added for 1 hour at room temperature.

The plates were washed and F(ab')₂ goat anti-mouse IgG Fc (HRPO conjugated) was added. Enzyme substrate was added to reveal the reaction.

Chimeric B72.3 (IgG4) (ref. 13) was used as a standard. The use of a monoclonal anti-kappa chain in this assay allows grafted antibodies to be read from the chimeric standard.

3.2. ASSAY FOR ANTIGEN BINDING ACTIVITY

Material from COS cell supernatants was assayed for OKT3 antigen binding activity onto CD3 positive cells in a direct assay. The procedure was as follows:

HUT 78 cells (human T cell line, CD3 positive) were maintained in culture. Monolayers of HUT 78 cells were prepared onto 96 well ELISA plates using poly-L-lysine and glutaraldehyde. Samples were added to the monolayers for 1 hour at room temperature.

The plates were washed gently using PBS. F(ab')₂ goat anti-human IgG Fc (HRPO conjugated) or F(ab')₂ goat anti-mouse IgG Fc (HRPO conjugated) was added as appropriate for humanised or mouse samples. Substrate was added to reveal the reaction.

The negative control for the cell-based assay was chimeric B72.3. The positive control was mouse Orthomune OKT3 or chimeric OKT3, when available. This cell-based assay was difficult to perform, and an alternative assay was developed for CDR-grafted OKT3 which was more sensitive and easier to carry out.

In this system CDR-grafted OKT3 produced by COS cells was tested for its ability to bind to the CD3-positive HPB-ALL (human peripheral blood acute lymphocytic leukemia) cell line. It was also tested for its ability to block the binding of murine OKT3 to these cells. Binding was measured by the following procedure: HPB-ALL cells were harvested from tissue culture. Cells were incubated at 4°C for 1 hour with various dilutions of test antibody, positive control antibody, or negative control antibody. The cells were washed once and incubated at 4°C for 1 hour with an FITC-labelled goat anti-human IgG (Fc-

specific, mouse absorbed). The cells were washed twice and analysed by cytofluorography. Chimeric OKT3 was used as a positive control for direct binding. Cells incubated with mock-transfected COS cell supernatant, followed by the FITC-labelled goat anti-human IgG, provided the negative control. To test the ability of CDR-grafted OKT3 to block murine OKT3 binding, the HPB-ALL cells were incubated at 4°C for 1 hour with various dilutions of test antibody or control antibody. A fixed saturating amount of FITC OKT3 was added. The samples were incubated for 1 hour at 4°C, washed twice and analysed by cytofluorography. FITC-labelled OKT3 was used as a positive control to determine maximum binding. Unlabelled murine OKT3 served as a reference standard for blocking. Negative controls were unstained cells with or without mock-transfected cell supernatant. The ability of the CDR-grafted OKT3 light chain to bind CD3-positive cells and block the binding of murine OKT3 was initially tested in combination with the chimeric OKT3 heavy chain. The chimeric OKT3 heavy chain is composed of the murine OKT3 variable region and the human IgG4 constant region. The chimeric heavy chain gene is expressed in the same expression vector used for the CDR-grafted genes. The CDR-grafted light chain expression vector and the chimeric heavy chain expression vector were co-transfected into COS cells. The fully chimeric OKT3 antibody (chimeric light chain and chimeric heavy chain) was found to be fully capable of binding to CD3 positive cells and blocking the binding of murine OKT3 to these cells.

3.3 DETERMINATION OF RELATIVE BINDING AFFINITY

The relative binding affinities of CDR-grafted

anti-CD3 monoclonal antibodies were determined by competition binding (ref. 6) using the HPB-ALL human T cell line as a source of CD3 antigen, and fluorescein-conjugated murine OKT3 (Fl-OKT3) of known binding affinity as a tracer antibody. The binding affinity of Fl-OKT3 tracer antibody was determined by a direct binding assay in which increasing amounts of Fl-OKT3 were incubated with HPB-ALL (5×10^5) in PBS with 5% foetal calf serum for 60 min. at 4°C. Cells were washed, and the fluorescence intensity was determined on a FACScan flow cytometer calibrated with quantitative microbead standards (Flow Cytometry Standards, Research Triangle Park, NC). Fluorescence intensity per antibody molecule (F/P ratio) was determined by using microbeads which have a predetermined number of mouse IgG antibody binding sites (Simply Cellular beads, Flow Cytometry Standards). F/P equals the fluorescence intensity of beads saturated with Fl-OKT3 divided by the number of binding sites per bead. The amount of bound and free Fl-OKT3 was calculated from the mean fluorescence intensity per cell, and the ratio of bound/free was plotted against the number of moles of antibody bound. A linear fit was used to determine the affinity of binding (absolute value of the slope).

For competitive binding, increasing amounts of competitor antibody were added to a sub-saturating dose of Fl-OKT3 and incubated with 5×10^5 HPB-ALL in 200 ml of PBS with 5% foetal calf serum, for 60 min at 4°C. The fluorescence intensities of the cells were measured on a FACScan flow cytometer calibrated with quantitative microbead standards. The concentrations of bound and free Fl-OKT3 were calculated. The affinities of competing anti-

bodies were calculated from the equation $[X]-[OKT3] = (1/Kx) - (1/Ka)$, where Ka is the affinity of murine OKT3, Kx is the affinity of competitor X, [] is the concentration of competitor antibody at which bound/free binding is R/2, and R is the maximal bound/free binding.

4. CDNA LIBRARY CONSTRUCTION

4.1. mRNA PREPARATION AND cDNA SYNTHESIS

OKT3 producing cells were grown as described above and 1.2×10^9 cells harvested and mRNA extracted using the guanidinium/LiCl extraction procedure. cDNA was prepared by priming from Oligo-dT to generate full length cDNA. The cDNA was methylated and EcoRI linkers added for cloning.

4.2. LIBRARY CONSTRUCTION

The cDNA library was ligated to pSP65 vector DNA which had been EcoRI cut and the 5' phosphate groups removed by calf intestinal phosphatase (EcoRI/CIP). The ligation was used to transform high transformation efficiency Escherichia coli (E.coli) HB101. A cDNA library was prepared. 3600 colonies were screened for the light chain and 10000 colonies were screened for the heavy chain.

5. SCREENING

E.coli colonies positive for either heavy or light chain probes were identified by oligonucleotide screening using the oligonucleotides:

A

5' TCCAGATGTTAACTGCTCAC^(SEQ ID NO:1) for the light chain, which is complementary to a sequence in the mouse kappa constant region, and 5' CAGGGGCCAGTGGATGGATAGAC^(SEQ ID NO:2)

A

for the heavy chain which is complementary to a sequence in the mouse IgG2a constant CH1 domain region. 12 light chain and 9 heavy chain clones

were identified and taken for second round screening. Positive clones from the second round of screening were grown up and DNA prepared. The sizes of the gene inserts were estimated by gel electrophoresis and inserts of a size capable of containing a full length cDNA were subcloned into M13 for DNA sequencing.

6. DNA SEQUENCING

Clones representing four size classes for both heavy and light chains were obtained in M13. DNA sequence for the 5' untranslated regions, signal sequences, variable regions and 3' untranslated regions of full length cDNAs [Figures 1(a) and 2(a)] were obtained and the corresponding amino acid sequences predicted [(Figures 1(b) and 2(b))]. In Figure 1(a) the untranslated DNA regions are shown in uppercase, and in both Figures 1 and 2 the signal sequences are underlined.

Handwritten notes: "A", "A", "A", "A", "DMS A" with arrows pointing to specific parts of the text.

(SEQ ID NO: 4)

(SEQ ID NO: 6)

(SEQ ID NO: 8)

(SEQ ID NO: 7)

(SEQ ID NO: 4 + 5)

A1

7. CONSTRUCTION OF cDNA EXPRESSION VECTORS

Celltech expression vectors are based on the plasmid pEE6hCMV (ref. 14). A polylinker for the insertion of genes to be expressed has been introduced after the major immediate early promoter/enhancer of the human Cytomegalovirus (hCMV). Marker genes for selection of the plasmid in transfected eukaryotic cells can be inserted as BamHI cassettes in the unique BamHI site of pEE6 hCMV; for instance, the neo marker to provide pEE6 hCMV neo. It is usual practice to insert the neo and gpt markers prior to insertion of the gene of interest, whereas the GS marker is inserted last because of the presence of internal EcoRI sites in the cassette.

The selectable markers are expressed from the SV40 late promoter which also provides an origin of replication so that the vectors can be used for expression in the COS cell transient expression system.

The mouse sequences were excised from the M13 based vectors described above as EcoR1 fragments and cloned into either pEE6-hCMV-neo for the heavy chain and into EE6-hCMV-gpt for the light chain to yield vectors pJA136 and pJA135 respectively.

8. EXPRESSION OF cDNAS IN COS CELLS

Plasmids pJA135 and pJA136 were co-transfected into COS cells and supernatant from the transient expression experiment was shown to contain assembled antibody which bound to T-cell enriched lymphocytes. Metabolic labelling experiments using ³⁵S methionine showed expression and assembly of heavy and light chains.

9. CONSTRUCTION OF CHIMERIC GENES

Construction of chimeric genes followed a previously described strategy [Whittle et al (ref. 13)]. A restriction site near the 3' end of the variable domain sequence is identified and used to attach an oligonucleotide adapter coding for the remainder of the mouse variable region and a suitable restriction site for attachment to the constant region of choice.

9.1. LIGHT CHAIN GENE CONSTRUCTION

The mouse light chain cDNA sequence contains an Aval site near the 3' end of the variable region [Fig. 1(a)]. ^(SEQ ID NO:4) The majority of the sequence of the variable region was isolated as a 396 bp. EcoR1-Aval fragment. An oligonucleotide adapter was designed to replace the remainder of the 3'

A

region of the variable region from the Aval site and to include the 5' residues of the human constant region up to and including a unique NarI site which had been previously engineered into the constant region.

A HindIII site was introduced to act as a marker for insertion of the linker.

The linker was ligated to the V_L fragment and the 413 bp EcoRI-NarI adapted fragment was purified from the ligation mixture.

The constant region was isolated as an NarI-BamHI fragment from an M13 clone NW361 and was ligated with the variable region DNA into an EcoRI/BamHI/ClP pSP65 treated vector in a three way reaction to yield plasmid JA143. Clones were isolated after transformation into E.coli and the linker and junction sequences were confirmed by the presence of the HindIII site and by DNA sequencing.

9.2 LIGHT CHAIN GENE CONSTRUCTION - VERSION 2

The construction of the first chimeric light chain gene produces a fusion of mouse and human amino acid sequences at the variable-constant region junction. In the case of the OKT3 light chain the amino acids at the chimera junction are:

.....Leu-Glu-Ile-Asn-Arg/ -/Thr-Val-Ala -Ala₁

VARIABLE CONSTANT

(SEQUENCE)

A
TE340X

This arrangement of sequence introduces a potential site for Asparagine (Asn) linked (N-linked) glycosylation at the V-C junction. Therefore, a second version of the chimeric light chain oligonucleotide adapter was designed in which the threonine (Thr), the first amino acid of the human constant region, was replaced with the equivalent amino acid from the mouse constant region, Alanine (Ala).

An internal HindIII site was not included in this adapter, to differentiate the two chimeric light chain genes.

The variable region fragment was isolated as a 376 bp EcoRI-AvaI fragment. The oligonucleotide linker was ligated to NarI cut pNW361 and then the adapted 396bp constant region was isolated after recutting the modified pNW361 with EcoRI. The variable region fragment and the modified constant region fragment were ligated directly into EcoRI/C1P treated pEE6hCMVneo to yield pJA137. Initially all clones examined had the insert in the incorrect orientation. Therefore, the insert was re-isolated and recloned to turn the insert round and yield plasmid pJA141. Several clones with the insert in the correct orientation were obtained and the adapter sequence of one was confirmed by DNA sequencing

9.3. HEAVY CHAIN GENE CONSTRUCTION

9.3.1. CHOICE OF HEAVY CHAIN GENE ISOTYPE

The constant region isotype chosen for the heavy chain was human IgG4.

9.3.2. GENE CONSTRUCTION

A The heavy chain cDNA sequence showed a BanI site ^(SEQ ID NO:6) near the 3' end of the variable region [Fig. 2(a)]. The majority of the sequence of the variable region was isolated as a 426bp. EcoRI/C1P/BanI fragment. An oligonucleotide adapter was designated to replace the remainder of the 3' region of the variable region from the BanI site up to and including a unique HindIII site which had been previously engineered into the first two amino acids of the constant region.

The linker was ligated to the V_H fragment and the EcoRI-HindIII adapted fragment was purified from the ligation mixture.

The variable region was ligated to the constant region by cutting pJA91 with EcoR1 and Hind111 removing the intron fragment and replacing it with the V_H to yield pJA142. Clones were isolated after transformation into E.coli JM101 and the linker and junction sequences were confirmed by DNA sequencing. (N.B. The Hind111 site is lost on cloning).

10. CONSTRUCTION OF CHIMERIC EXPRESSION VECTORS

10.1. neo AND gpt VECTORS

The chimeric light chain (version 1) was removed from pJA143 as an EcoR1 fragment and cloned into EcoR1/C1P treated pEE6hCMVneo expression vector to yield pJA145. Clones with the insert in the correct orientation were identified by restriction mapping.

The chimeric light chain (version 2) was constructed as described above.

The chimeric heavy chain gene was isolated from pJA142 as a 2.5Kbp EcoR1/BamH1 fragment and cloned into the EcoR1/Bcl1/C1P treated vector fragment of a derivative of pEE6hCMVgpt to yield plasmid pJA144.

10.2. GS SEPARATE VECTORS

GS versions of pJA141 and pJA144 were constructed by replacing the neo and gpt cassettes by a BamH1/Sall/C1P treatment of the plasmids, isolation of the vector fragment and ligation to a GS-containing fragment from the plasmid pR049 to yield the light chain vector pJA179 and the heavy chain vector pJA180.

10.3. GS SINGLE VECTOR CONSTRUCTION

Single vector constructions containing the cL (chimeric light), cH (chimeric heavy) and GS genes on one plasmid in the order cL-cH-GS, or cH-cL-GS

and with transcription of the genes being head to tail e.g. cL>CH>GS were constructed. These plasmids were made by treating pJA179 or pJA180 with BamH1/C1P and ligating in a Bgl111/Hind111 hCMV promoter cassette along with either the Hind111/BamH1 fragment from pJA141 into pJA180 to give the cH-cL-GS plasmid pJA182 or the Hind111/BamH1 fragment from pJA144 into pJA179 to give the cL-cH-GS plasmid pJA181.

11. EXPRESSION OF CHIMERIC GENES

11.1. EXPRESSION IN COS CELLS

The chimeric antibody plasmid pJA145 (cL) and pJA144 (cH) were co-transfected into COS cells and supernatant from the transient expression experiment was shown to contain assembled antibody which bound to the HUT 78 human T-cell line. Metabolic labelling experiments using ³⁵S methionine showed expression and assembly of heavy and light chains. However the light chain mobility seen on reduced gels suggested that the potential glycosylation site was being glycosylated. Expression in COS cells in the presence of tunicamycin showed a reduction in size of the light chain to that shown for control chimeric antibodies and the OKT3 mouse light chain. Therefore JA141 was constructed and expressed. In this case the light chain did not show an aberrant mobility or a size shift in the presence or absence of tunicamycin. This second version of the chimeric light chain, when expressed in association with chimeric heavy (cH) chain, produced antibody which showed good binding to HUT 78 cells. In both cases antigen binding was equivalent to that of the mouse antibody.

11.2 EXPRESSION IN CHINESE HAMSTER OVARY (CHO) CELLS
Stable cell lines have been prepared from plasmids PJA141/pJA144 and from pJA179/pJA180, pJA181 and pJA182 by transfection into CHO cells.

12. CDR-GRAFTING

The approach taken was to try to introduce sufficient mouse residues into a human variable region framework to generate antigen binding activity comparable to the mouse and chimeric antibodies.

12.1. VARIABLE REGION ANALYSIS

From an examination of a small database of structures of antibodies and antigen-antibody complexes it is clear that only a small number of antibody residues make direct contact with antigen. Other residues may contribute to antigen binding by positioning the contact residues in favourable configurations and also by inducing a stable packing of the individual variable domains and stable interaction of the light and heavy chain variable domains.

The residues chosen for transfer can be identified in a number of ways:

- (a) By examination of antibody X-ray crystal structures the antigen binding surface can be predominantly located on a series of loops, three per domain, which extend from the B-barrel framework.
- (b) By analysis of antibody variable domain sequences regions of hypervariability [termed the Complementarity Determining Regions (CDRs) by Wu and Kabat (ref. 5)] can be identified. In the most but not all cases these CDRs correspond to, but extend a short way beyond, the loop regions noted above.

(c) Residues not identified by (a) and (b) may contribute to antigen binding directly or indirectly by affecting antigen binding site topology, or by inducing a stable packing of the individual variable domains and stabilising the inter-variable domain interaction. These residues may be identified either by superimposing the sequences for a given antibody on a known structure and looking at key residues for their contribution, or by sequence alignment analysis and noting "idiosyncratic" residues followed by examination of their structural location and likely effects.

12.1.1. LIGHT CHAIN ²⁹
 (SEQ ID NO: 8 And 9)

Figure 3 shows an alignment of sequences for the human framework region RE1 and the OKT3 light variable region. ^(SEQ ID NO: 8 And 9) The structural loops (LOOP) and CDRs (KABAT) ^(SEQ ID NO: 8 And 9) believed to correspond to the antigen binding region are marked. Also marked are a number of other residues which may also contribute to antigen binding as described in 13.1(c).
 Above the sequence in Figure 3 ^(SEQ ID NO: 8 And 9) the residue type indicates the spatial location of each residue side chain, derived by examination of resolved structures from X-ray crystallography analysis. The key to this residue type designation is as follows:

- N - near to CDR (From X-ray Structures)
- P - Packing
- S - Surface
- I - Interface
- Packing/Part Exposed
- ? - Non-CDR Residues which may require to be left as Mouse sequence.
- B - Buried Non-Packing
- E - Exposed
- * - Interface

A S
 A A S
 A S

13964

29
(SEQ ID NO: 8 and 9)

A J
D

Residues underlined in Figure 3 are amino acids. (SEQ ID NO: 8 and 9)
RE1 was chosen as the human framework because the light chain is a kappa chain and the kappa variable regions show higher homology with the mouse sequences than a lambda light variable region, e.g. KOL (see below). (SEQ ID NO: 10) (SEQ ID NO: 8 and 9)
RE1 was chosen in preference to another kappa light chain because the X-ray structure of the light chain has been determined so that a structural examination of individual residues could be made.

A

12.1.2. HEAVY CHAIN

Similarly Figure 4 shows an alignment of sequences for the human framework region KOL and the OKT3 heavy variable region. (SEQ ID NO: 10) (SEQ ID NO: 7)
The structural loops and CDRs believed to correspond to the antigen binding region are marked. Also marked are a number of other residues which may also contribute to antigen binding as described in 12.1(c). The residue type key and other indicators used in Figure 4 are the same as those used in Figure 3.
KOL was chosen as the heavy chain framework because the X-ray structure has been determined to a better resolution than, for example, NEWM and also the sequence alignment of OKT3 heavy variable region showed a slightly better homology to KOL than to NEWM. (SEQ ID NO: 10) (SEQ ID NO: 10)

J A

A

A

12.2. DESIGN OF VARIABLE GENES

The variable region domains were designed with mouse variable region optimal codon usage [Grantham and Perrin (ref. 15)] and used the B72.3 signal sequences [Whittle et al (ref. 13)]. The sequences were designed to be attached to the constant region in the same way as for the chimeric genes described above. Some constructs contained the "Kozak consensus sequence" [Kozak (ref. 16)] directly linked to the 5' of the signal

sequence in the gene. This sequence motif is believed to have a beneficial role in translation initiation in eukaryotes.

12.3. GENE CONSTRUCTION

To build the variable regions, various strategies are available. The sequence may be assembled by using oligonucleotides in a manner similar to Jones et al (ref. 17) or by simultaneously replacing all of the CDRs or loop regions by oligonucleotide directed site specific mutagenesis in a manner similar to Verhoeyen et al (ref. 2). Both strategies were used and a list of constructions is set out in Tables 1 and 2 and Figures 4 and 5. ^{a-c} It was noted in several cases that the mutagenesis approach led to deletions and rearrangements in the gene being remodelled, while the success of the assembly approach was very sensitive to the quality of the oligonucleotides.

13. CONSTRUCTION OF EXPRESSION VECTORS

Genes were isolated from M13 or SP65 based intermediate vectors and cloned into pEE6hCMVneo for the light chains and pEE6hCMVgpt for the heavy chains in a manner similar to that for the chimeric genes as described above.

TABLE 1 CDR-GRAFTED GENE CONSTRUCTS

CODE	MOUSE SEQUENCE CONTENT	METHOD OF CONSTRUCTION	KOZAK SEQUENCE - +	
<hr/>				
LIGHT CHAIN ALL HUMAN FRAMEWORK REL				
121	26-32, 50-56, 91-96 inclusive	SDM and gene assembly	+	n.d.
121A	26-32, 50-56, 91-96 inclusive +1, 3, 46, 47	Partial gene assembly	n.d.	+
121B	26-32, 50-56, 91-96 inclusive + 46, 47	Partial gene assembly	n.d.	+
221	24-24, 50-56, 91-96 inclusive	Partial gene assembly	+	+
221A	24-34, 50-56, 91-96 inclusive +1, 3, 46, 47	Partial gene assembly	+	+
221B	24-34, 50-56, 91-96 inclusive +1, 3	Partial gene assembly	+	+
221C	24-34, 50-56, 91-96 inclusive	Partial gene assembly	+	+
HEAVY CHAIN ALL HUMAN FRAMEWORK KOL				
121	26-32, 50-56, 95-100B inclusive	Gene assembly	n.d.	+
131	26-32, 50-58, 95-100B inclusive	Gene assembly	n.d.	+
141	26-32, 50-65, 95-100B inclusive	Partial gene assembly	+	n.d.
321	26-35, 50-56, 95-100B inclusive	Partial gene assembly	+	n.d.
331	26-35, 50-58, 95-100B inclusive	Partial gene assembly	+	
		Gene assembly		+
341	26-35, 50-65, 95-100B inclusive	SDM	+	
		Partial gene assembly		+
341A	26-35, 50-65, 95-100B inclusive +6, 23, 24, 48, 49, 71, 73, 76,	Gene assembly	n.d.	+
341B	78-88, 91 (+63 = human) (SER 79 NO: 8-28) 26-35, 50-65, 95-100B inclusive + 48, 49, 71, 73, 76, 78, 88, 91 (+63 + human)	Gene assembly	n.d.	+

KEY

n.d. not done
SDM Site directed mutagenesis
Gene assembly Variable region assembled entirely from oligonucleotides
Partial gene assembly Variable region assembled by combination of restriction fragments either from other genes originally created by SDM and gene assembly or by oligonucleotide assembly of part of the variable region and reconstruction with restriction fragments from other genes originally created by SDM and gene assembly

14. EXPRESSION OF CDR-GRAFTED GENES

14.1. PRODUCTION OF ANTIBODY CONSISTING OF GRAFTED LIGHT (gL) CHAINS WITH MOUSE HEAVY (mH) OR CHIMERIC HEAVY (cH) CHAINS

All gL chains, in association with mH or cH produced reasonable amounts of antibody.

Insertion of the Kozak consensus sequence at a position 5' to the ATG (kgL constructs) however, led to a 2-5 fold improvement in net expression. Over an extended series of experiments expression levels were raised from approximately 200ng/ml to approximately 500 ng/ml for kgL/cH or kgL/mH combinations.

When direct binding to antigen on HUT 78 cells was measured, a construct designed to include mouse sequence based on loop length (gL121) did not lead to active antibody in association with mH or cH.

A construct designed to include mouse sequence based on Kabat CDRs (gL221) ^(SEQ ID NO: 20) demonstrated some weak binding in association with mH or cH. However, when framework residues 1, 3, 46, 47 were changed from the human to the murine OKT3 equivalents based on the arguments outlined in Section 12.1 antigen binding was demonstrated when both of the new constructs, which were termed 121A and 221A were co-expressed with cH. When the effects of these residues were examined in more detail, it appears that residues 1 and 3 are not major contributing residues as the product of the gL221B ^(SEQ ID NO: 27) gene shows little detectable binding activity in association with cH. The light chain product of gL221C, ^(SEQ ID NO: 28) in which mouse sequences are present at 46 and 47, shows good binding activity in association with cH.

14.2 PRODUCTION OF ANTIBODY CONSISTING OF GRAFTED HEAVY (gH) CHAINS WITH MOUSE LIGHT (mL) OR CHIMERIC LIGHT (cL) CHAINS

Expression of the gH genes proved to be more difficult to achieve than for gL. First, inclusion of the Kozak sequence appeared to have no marked effect on expression of gH genes. Expression appears to be slightly improved but not to the same degree as seen for the grafted light chain.

Also, it proved difficult to demonstrate production of expected quantities of material when the loop choice (amino acid 26-32) for CDR1 is used, e.g. gH121, 131, 141 and no conclusions can be drawn about these constructs.

Moreover, co-expression of the gH341 gene with cL or mL has been variable and has tended to produce lower amounts of antibody than the cH/cL or mH/mL combinations. The alterations to gH341 to produce gH341A and gH341B lead to improved levels of expression.

This may be due either to a general increase in the fraction of mouse sequence in the variable region, or to the alteration at position 63 where the residue is returned to the human amino acid Valine (Val) from Phenylalanine (Phe) to avoid possible internal packing problems with the rest of the human framework. This arrangement also occurs in gH331 and gH321.

When gH321 or gH331 were expressed in association with cL, antibody was produced but antibody binding activity was not detected.

When the more conservative gH341 gene was used antigen binding could be detected in association with cL or mL, but the activity was only marginally above the background level.

When further mouse residues were substituted based on the arguments in 12.1, antigen binding could be clearly demonstrated for the antibody produced when kgH341A and kgH341B were expressed in association with cL.

14.3 PRODUCTION OF FULLY CDR-GRAFTED ANTIBODY

The kgL221A gene was co-expressed with kgH341, kgH341A or kgH341B. For the combination kgH221A/kgH341 very little material was produced in a normal COS cell expression.

For the combinations kgL221A/kgH341A or kgH221A/kgH341B amounts of antibody similar to gL/CH was produced.

In several experiments no antigen binding activity could be detected with kgH221A/gH341 or kgH221A/kgH341 combinations, although expression levels were very low.

Antigen binding was detected when kgL221A/kgH341A or kgH221A/kgH341B combinations were expressed. In the case of the antibody produced from the kgL221A/kgH341A combination the antigen binding was very similar to that of the chimeric antibody.

An analysis of the above results is given below.

15. DISCUSSION OF CDR-GRAFTING RESULTS

In the design of the fully humanised antibody the aim was to transfer the minimum number of mouse amino acids that would confer antigen binding onto a human antibody framework.

15.1. LIGHT CHAIN

15.1.1. EXTENT OF THE CDRs

For the light chain the regions defining the loops known from structural studies of other antibodies to contain the antigen contacting residues, and

those hypervariable sequences defined by Kabat et al (refs. 4 and 5) as Complementarity Determining Regions (CDRs) are equivalent for CDR2. For CDR1 the hypervariable region extends from residues 24-34 inclusive while the structural loop extends from 26-32 inclusive. In the case of OKT3 ^(SEQ ID NO 5) there is only one amino acid difference between the two options, at amino acid 24, where the mouse sequence is a serine and the human framework RE1 has glutamine. For CDR3 the loop extends from residues 91-96 inclusive while the Kabat hypervariability extends from residues 89-97 inclusive. For OKT3 amino acids 89, 90 and 97 ²⁹ are the same between OKT3 and RE1 (Fig. 3) ^(SEQ ID NO 8 And 9). When constructs based on the loop choice for CDR1 (gL121) and the Kabat choice (gL221) were made and co-expressed with mH or cH no evidence for antigen binding activity could be found for gL121, but trace activity could be detected for the gL221, suggesting that a single extra mouse residue in the grafted variable region could have some detectable effect. Both gene constructs were reasonably well expressed in the transient expression system.

15.1.2. FRAMEWORK RESIDUES

The remaining framework residues were then further examined, in particular amino acids known from X-ray analysis of other antibodies to be close to the CDRs and also those amino acids which in OKT3 showed differences from the consensus framework for the mouse subgroup (subgroup VI) to which OKT3 shows most homology. Four positions 1, 3, 46 and 47 were identified and their possible contribution was examined by substituting the mouse amino acid for the human amino acid at each position. Therefore gL221A (gL221 + D1Q, Q3V, L46R, L47W,

see Figure 3 and Table 1) was made, cloned in EE6hCMVneo and co-expressed with cH (pJA144). The resultant antibody was well expressed and showed good binding activity. When the related genes gL221B^(SEQ ID NO:28) (gL221 + D1Q, Q3V) and gL221C (gL221 + L46R, L47W) were made and similarly tested, while both genes produced antibody when co-expressed with cH, only the gL221C/cH combination showed good antigen binding. When the gL121A (gL121 + D1Q, Q3V, L46R, L47W) gene was made and co-expressed with cH, antibody was produced which also bound to antigen.

15.2. HEAVY CHAIN

15.2.1. EXTENT OF THE CDRs

For the heavy chain the loop and hypervariability analyses agree only in CDR3. For CDR1 the loop region extends from residues 26-32 inclusive whereas the Kabat CDR extends from residues 31-35 inclusive. For CDR2 the loop region is from 50-58 inclusive while the hypervariable region covers amino acids 50-65 inclusive. Therefore humanised heavy chains were constructed using the framework from antibody KOL and with various combinations of these CDR choices, including a shorter choice for CDR2 of 50-56 inclusive as there was some uncertainty as to the definition of the end point for the CDR2 loop around residues 56 to 58. The genes were co-expressed with mL or cL initially. In the case of the gH genes with loop choices for CDR1 e.g. gH121, gH131, gH141 very little antibody was produced in the culture supernatants. As no free light chain was detected it was presumed that the antibody was being made and assembled inside the cell but that the heavy chain was aberrant in some way, possibly incorrectly folded, and therefore the antibody was

being degraded internally. In some experiments trace amounts of antibody could be detected in ³⁵S labelling studies.

As no net antibody was produced, analysis of these constructs was not pursued further.

When, however, a combination of the loop choice and the Kabat choice for CDR1 was tested (mouse amino acids 26-35 inclusive) and in which residues 31 (Ser to Arg), 33 (Ala to Thr), and 35 (Tyr to His) were changed from the human residues to the mouse residue and compared to the first series, antibody was produced for gH321, kgH331 and kgH341 when co-expressed with cL. Expression was generally low and could not be markedly improved by the insertion of the Kozak consensus sequence 5' to the ATG of the signal sequence of the gene, as distinct from the case of the gL genes where such insertion led to a 2-5 fold increase in net antibody production. However, only in the case of gH341/mL or kgH341/cL could marginal antigen binding activity be demonstrated. When the kgH341 gene was co-expressed with kgL221A, the net yield of antibody was too low to give a signal above the background level in the antigen binding assay. (SEQ ID NO: 26)

15.2.2. FRAMEWORK RESIDUES

As in the case of the light chain the heavy chain frameworks were re-examined. Possibly because of the lower initial homology between the mouse and human heavy variable domains compared to the light chains, more amino acid positions proved to be of interest. Two genes kgH341A and kgH341B were constructed, with 11 or 8 human residues respectively substituted by mouse residues compared to gH341, and with the CDR2 residue 63 returned to the human amino acid potentially to

improve domain packing. Both showed antigen binding when combined with cL or kgL221A, the kgH341A gene with all 11 changes appearing to be the superior choice.

15.3 INTERIM CONCLUSIONS

It has been demonstrated, therefore, for OKT3 that to transfer antigen binding ability to the humanised antibody, mouse residues outside the CDR regions defined by the Kabat hypervariability or structural loop choices are required for both the light and heavy chains. Fewer extra residues are needed for the light chain, possibly due to the higher initial homology between the mouse and human kappa variable regions.

Of the changes seven (1 and 3 from the light chain and 6, 23, 71, 73 and 76 from the heavy chain) are predicted from a knowledge of other antibody structures to be either partly exposed or on the antibody surface. It has been shown here that residues 1 and 3 in the light chain are not absolutely required to be the mouse sequence; and for the heavy chain the gH341B heavy chain in combination with the 221A light chain generated only weak binding activity. Therefore the presence of the 6, 23 and 24 changes are important to maintain a binding affinity similar to that of the murine antibody. It was important, therefore, to further study the individual contribution of the other 8 mouse residues of the kgH341A gene compared to kgH341.

16. FURTHER CDR-GRAFTING EXPERIMENTS

Additional CDR-grafted heavy chain genes were prepared substantially as described above. With reference to Table 2 the further heavy chain genes were based upon the gh341 (plasmid pJA178) and

(SEQ ID NO: 12)

gH341A (plasmid pJA185) with either mouse OKT3 or human KOL residues at 6, 23, 24, 48, 49, 63, 71, 73, 76, 78, 88 and 91, as indicated. The CDR-

grafted light chain genes used in these further experiments were gL221_λ, gL221A_λ, gL221B_λ and gL221C_λ as described above.

(SEQ ID NO: 15) (SEQ ID NO: 26) (SEQ ID NO: 27) (SEQ ID NO: 28)

TABLE 2

OKT3 HEAVY CHAIN CDR GRAFTS

1. gH341 and derivatives

RES NUM	6	23	24	48	49	63	71	73	76	78	88	91	
OKT3vh	<u>Q</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>F</u>	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>A</u>	<u>Y</u>	
gH341	E	S	S	V	A	F	R	N	N	L	G	F	JA178
gH341A	<u>Q</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>A</u>	<u>Y</u>	JA185
gH341E	<u>Q</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>G</u>	<u>G</u>	JA198
gH341*	<u>Q</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>N</u>	<u>A</u>	<u>G</u>	<u>F</u>	JA207
gH341*	<u>Q</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>R</u>	<u>N</u>	<u>N</u>	<u>A</u>	<u>G</u>	<u>F</u>	JA209
gH341D	<u>Q</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>N</u>	<u>L</u>	<u>G</u>	<u>F</u>	JA197
gH341*	<u>Q</u>	<u>K</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>R</u>	<u>N</u>	<u>N</u>	<u>L</u>	<u>G</u>	<u>F</u>	JA199
gH341C	<u>Q</u>	<u>K</u>	<u>A</u>	<u>V</u>	<u>A</u>	<u>F</u>	<u>R</u>	<u>N</u>	<u>N</u>	<u>L</u>	<u>G</u>	<u>F</u>	JA184
gH341*	<u>Q</u>	<u>S</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>A</u>	<u>Y</u>	JA203
gH341*	E	S	<u>A</u>	<u>I</u>	<u>G</u>	V	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>A</u>	<u>Y</u>	JA205
gH341B	E	S	S	<u>I</u>	<u>G</u>	V	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>A</u>	<u>Y</u>	JA183
gH341*	<u>Q</u>	<u>S</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>G</u>	<u>F</u>	JA204
gH341*	E	S	<u>A</u>	<u>I</u>	<u>G</u>	V	<u>T</u>	<u>K</u>	<u>S</u>	<u>A</u>	<u>G</u>	<u>F</u>	JA206
gH341*	<u>Q</u>	<u>S</u>	<u>A</u>	<u>I</u>	<u>G</u>	<u>V</u>	<u>T</u>	<u>K</u>	<u>N</u>	<u>A</u>	<u>G</u>	<u>F</u>	JA208
KOL	E	S	S	V	A		R	N	N	L	G	F	

AS (SEQ ID NO: ³⁰7, 10 and 11-24)
OKT3 LIGHT CHAIN CDR GRAFTS

2. gL221 and derivatives

RES NUM	1	3	46	47	
OKT3v1	<u>Q</u>	<u>V</u>	<u>R</u>	<u>W</u>	
GL221	D	Q	L	L	DA221
gL221A	<u>Q</u>	<u>V</u>	<u>R</u>	<u>W</u>	DA221A
gL221B	<u>Q</u>	<u>V</u>	L	L	DA221B
GL221C	D	Q	<u>R</u>	<u>W</u>	DA221C
RE1	D	Q ²⁹	L	L	

AS (SEQ ID NO: 5, 8, 9 and 25-28)
 MURINE RESIDUES ARE UNDERLINED

The CDR-grafted heavy and light chain genes were co-expressed in COS cells either with one another in various combinations but also with the corresponding murine and chimeric heavy and light chain genes substantially as described above. The resultant antibody products were then assayed in binding and blocking assays with HPB-ALL cells as described above.

A
S
S
The results of the assays for various grafted heavy chains co-expressed with the gL221C light chain ^(SEQ ID NO: 28) are given in Figures 7 and 8 (for the JA184, JA185, JA197 and JA198 constructs - see Table 2), in Figure 9 (for the JA183, JA184, JA185 and JA197 constructs) in Figure 10 ^{a and b} (for the chimeric, JA185, JA199, JA204, JA205, JA207, JA208 and JA209 constructs) and in Figure 11 ^{a and b} (for the JA183, JA184, JA185, JA198, JA203, JA205 and JA206 constructs).

A
A
A
The basic grafted product without any human to murine changes in the variable frameworks, i.e. gL221 ^(SEQ ID NO: 25) co-expressed with gh341 (JA178) ^(SEQ ID NO: 11) and also the "fully grafted" product, having most human to murine changes in the grafted heavy chain framework, i.e. gL221C ^(SEQ ID NO: 28) co-expressed with gh341A (JA185) ^(SEQ ID NO: 12) were assayed for relative binding affinity in a competition assay against murine OKT3 reference standard, using HPB-ALL cells. The assay used was as described above in section 3.3. The results obtained are given in Figure 12 for the basic grafted product and in Figure 13 for the fully grafted product. These results indicate that the basic grafted product has negligible binding ability as compared with the OKT3 murine reference standard; whereas the "fully grafted" product has a binding ability very similar to that of the OKT3 murine reference standard.

The binding and blocking assay results indicate the following:

The JA198 and JA207 constructs appear to have the best binding characteristics and similar binding abilities, both substantially the same as the chimeric and fully grafted gH341A products. This indicates that positions 88 and 91 and position 76 are not highly critical for maintaining the OKT3 binding ability; whereas at least some of positions 6, 23, 24, 48, 49, 71, 73 and 78 are more important.

This is borne out by the finding that the JA209 and JA199, although of similar binding ability to one another, are of lower binding ability than the JA198 and JA207 constructs. This indicates the importance of having mouse residues at positions 71, 73 and 78, which are either completely or partially human in the JA199 and JA209 constructs respectively.

Moreover, on comparing the results obtained for the JA205 and JA183 constructs it is seen that there is a decrease in binding going from the JA205 to the JA183 constructs. This indicates the importance of retaining a mouse residue at position 23, the only position changed between JA205 and JA183.

These and other results lead us to the conclusion that of the 11 mouse framework residues used in the gH341A (JA185) construct, it is important to retain mouse residues at all of positions 6, 23, 24, 48 and 49, and possibly for maximum binding affinity at 71, 73 and 78.

Similar Experiments were carried out to CDR-graft a number of the rodent antibodies including antibodies having specificity for CD4 (OKT4), ICAM-1 (R6-5), TAG72 (B72.3), and TNF α (61E71, 101.4, hTNF1, hTNF2 and hTNF3).

EXAMPLE 2

CDR-GRAFTING OF A MURINE ANTI-CD4 T CELL
RECEPTOR ANTIBODY, OKT4A

Anti OKT4A CDR-grafted heavy and light chain genes were prepared, expressed and tested substantially as described above in Example 1 for CDR-grafted OKT3. The CDR grafting of OKT4A is described in detail in Ortho patent application PCT/GB 90..... of even date herewith entitled "Humanised Antibodies". The disclosure of this Ortho patent application PCT/GB 90..... is incorporated herein by reference. A number of CDR-grafted OKT4 antibodies have been prepared. Presently the CDR-grafted OKT4A of choice is the combination of the grafted light chain LCDR2 and the grafted heavy chain HCDR10.

THE LIGHT CHAIN

5 The human acceptor framework used for the grafted light chains was REL. ^(see ID NO: 8 and 9) The preferred LCDR2 light chain has human to mouse changes at positions 33, 34, 38, 49 and 89 in addition to the structural loop CDRs. Of these changed positions, positions 33, 34 and 89 fall within the preferred extended CDRs of the present invention (positions 33 and 34 in CDR1 and position 89 in CDR3). The human to murine changes at positions 38 and 49 corresponds to positions at which the amino acid residues are preferably donor murine amino acid residues in accordance with the present invention. A comparison of the amino acid sequences of the donor murine light chain variable domain and the RE1 human acceptor light chain variable further reveals that the murine and human residues are identical at all of positions 46, 48 and 71 and at all of positions 2, 4, 6, 35, 36, 44, 47, 62, 64-69, 85, 87, 98, 99 and 101 and 102. However the amino acid residue at position 58 in LCDR2 is

the human RE1 framework residue not the mouse OKT4 residue as would be preferred in accordance with the present invention.

THE HEAVY CHAIN

A The human acceptor framework used for the grafted heavy chains was KOL^(SEQ ID NO. 10).

The preferred CDR graft HCDR10 heavy chain has human to mouse changes at positions 24, 35, 57, 58, 60, 88 and 91 in addition to the structural loop CDRs.

Of these positions, positions 35 (CDR1) and positions 57, 58 and 60 (CDR2) fall within the preferred extended CDRs of the present invention. Also the human to mouse change at position 24 corresponds to a position at which the amino acid residue is a donor murine residue in accordance with the present invention. Moreover, the human to mouse changes at positions 88 and 91 correspond to positions at which the amino acid residues are optionally donor murine residues.

Moreover, a comparison of the murine OKT4A and human KOL heavy chain variable amino acid sequences reveals that the murine and human residues are identical at all of positions 23, 49, 71, 73 and 78 and at all of positions 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.

Thus the OKT4A CDR-grafted heavy chain HCDR10 corresponds to a particularly preferred embodiment according to the present invention.

EXAMPLE 3

CDR-GRAFTING OF AN ANTI-MUCIN SPECIFIC MURINE ANTIBODY, B72.3

The cloning of the genes coding for the anti-mucin specific murine monoclonal antibody B72.3 and the preparation of B72.3 mouse-human chimeric antibodies has been described previously (ref. 13 and WO 89/01783). CDR-grafted versions of B72.3 were prepared as follows.

(a) B72.3 Light Chain

CDR-grafting of this light chain was accomplished by direct transfer of the murine CDRs into the framework of the human light chain RE1. The regions transferred were:

<u>CDR Number</u>	<u>Residues</u>
1	24-34
2	50-56
3	90-96

The activity of the resulting grafted light chain was assessed by co-expression in COS cells, of genes for the combinations:

- B72.3 cH/B72.3 cL
- and B72.3 cH/B72.3 gL

Supernatants were assayed for antibody concentration and for the ability to bind to microtitre plates coated with mucin. The results obtained indicated that, in combination with the B72.3 cH chain, B72.3 cL and B72.3 gL had similar binding properties.

Comparison of the murine B72.3 and RE1 light chain amino acid sequences reveals that the residues are identical at positions 46, 58 and 71 but are different at position 48.

*and 9
(SEQ ID NO: 8)*

A 5

Thus changing the human residue to the donor mouse residue at position 48 may further improve the binding characteristics of the CDR-grafted light chain, (B72.3 gL) in accordance with the present invention.

(b) B72.3 heavy chain

i. Choice of framework

At the outset it was necessary to make a choice of human framework. Simply put, the question was as follows: Was it necessary to use the framework regions from an antibody whose crystal structure was known or could the choice be made on some other criteria?

For B72.3 heavy chain, it was reasoned that, while knowledge of structure was important, transfer of the CDRs from mouse to human frameworks might be facilitated if the overall homology between the donor and receptor frameworks was maximised.

Comparison of the B72.3 heavy chain sequence with those in Kabat (ref. 4) for human heavy chains showed clearly that B72.3 had poor homology for KOL and NEWM (for which crystal structures are available) but was very homologous to the heavy chain for EU. ^(SEQ ID NO:10)

On this basis, EU was chosen for the CDR-grafting and the following residues transferred as CDRs.

<u>CDR Number</u>	<u>Residues</u>
1	27-36
2	50-63
3	93-102

Also it was noticed that the FR4 region of EU was unlike that of any other human (or mouse) antibody. Consequently, in the grafted heavy chain genes this was also changed to produce a "consensus" human sequence. (Preliminary experiments showed that grafted heavy chain genes containing the EU FR4 sequence expressed very poorly in transient expression systems.)

- ii. Results with grafted heavy chain genes
Expression of grafted heavy chain genes containing all human framework regions with either gL or cL genes produced a grafted antibody with little ability to bind to mucin. The grafted antibody had about 1% the activity of the chimeric antibody. In these experiments, however, it was noted that the activity of the grafted antibody could be increased to ~10% of B72.3 by exposure to pHs of 2-3.5. This observation provided a clue as to how the activity of the grafted antibody could be improved without acid treatment. It was postulated that acid exposure brought about the protonation of an acidic residue (pKa of aspartic acid = 3.86 and of glutamine acid = 4.25) which in turn caused a change in structure of the CDR loops, or allowed better access of antigen. From comparison of the sequences of B72.3 (ref. 13) and EU (refs. 4 and 5), it was clear that, in going from the mouse to human frameworks, only two positions had been changed in such a way that acidic residues had been introduced. These

positions are at residues 73 and 81, where K to E and Q to E changes had been made, respectively.

Which of these positions might be important was determined by examining the crystal structure of the KOL antibody. In KOL heavy chain, ^(SEQ ID NO: 10) position 81 is far removed from either of the CDR loops.

Position 73, however, is close to both CDRs 1 and 3 of the heavy chain and, in this position it was possible to envisage that a K to E change in this region could have a detrimental effect on antigen binding.

iii. Framework changes in B72.3 gH gene

On the basis of the above analysis, E73 was mutated to a lysine (K). It was found that this change had a dramatic effect on the ability of the grafted Ab to bind to mucin. Further the ability of the grafted B72.3 produced by the mutated gH/gL combination to bind to mucin was similar to that of the B72.3 chimeric antibody.

iv. Other framework changes

In the course of the above experiments, other changes were made in the heavy chain framework regions. Within the accuracy of the assays used, none of the changes, either alone or together, appeared beneficial.

v. Other

All assays used measured the ability of the grafted Ab to bind to mucin and, as a whole, indicated that the single framework change at position 73 is sufficient to generate an antibody with similar binding properties to B72.3.

Comparison of the B72.3 murine and EU heavy chain sequences reveals that the mouse and human residues are identical at positions 23, 24, 71 and 78.

Thus the mutated CDR-grafted B72.3 heavy chain corresponds to a preferred embodiment of the present invention.

EXAMPLE 4

CDR-GRAFTING OF A MURINE ANTI-ICAM-1 MONOCLONAL ANTIBODY

A murine antibody, R6-5-D6 (EP 0314863) having specificity for Intercellular Adhesion Molecule 1 (ICAM-1) was CDR-grafted substantially as described above in previous examples. This work is described in greater detail in co-pending application, British Patent Application No. 9009549.8, the disclosure of which is incorporated herein by reference.

The human EU framework was used as the acceptor framework for both heavy and light chains. The CDR-grafted antibody currently of choice is provided by co-expression of grafted light chain gL221A (SEQ ID NO: 26) and grafted heavy chain gH341D (SEQ ID NO: 16) which has a binding affinity for ICAM 1 of about 75% of that of the corresponding mouse-human chimeric antibody.

LIGHT CHAIN

gL221A has murine CDRs at positions 24-34 (CDR1), 50-56 (CDR2) and 89-97 (CDR3). In addition several framework residues are also the murine amino acid. These residues were chosen after consideration of the possible contribution of these residues to domain packing and stability of the conformation of the antigen binding region. The residues which have been retained as mouse are at positions 2, 3, 48 (?), 60, 84, 85 and 87. Comparison of the murine anti-ICAM 1 and human EU light chain amino acid sequences reveals that the murine and human residues are identical at positions 46, 58 and 71.

HEAVY CHAIN

gH341D has murine CDRs at positions 26-35 (CDR1), 50-56 (CDR2) and 94-100B (CDR3). In addition murine residues were used in gH341D at positions 24, 48, 69, 71, 73, 80, 88 and 91. Comparison of the murine anti-ICAM 1 and human EU heavy chain amino acid sequences are identical at positions 23, 49 and 78.

EXAMPLE 5

CDR-Grafting of murine anti-TNF α antibodies

A number of murine anti-TNF α monoclonal antibodies were CDR-grafted substantially as described above in previous examples. These antibodies include the murine monoclonal antibodies designated 61 E71, hTNF1, hTNF3 and 101.4. A brief summary of the CDR-grafting of each of these antibodies is given below.

61E71

A similar analysis as described above (Example 1, Section 12.1.) was done for 61E71 and for the heavy chain 10 residues were identified at 23, 24, 48, 49, 68, 69, 71, 73, 75 and 88 as residues to potentially retain as murine. The human frameworks chosen for CDR-grafting of this antibody, and the hTNF3 and 101.4 antibodies were RE1 for the light chain and KOL for the heavy chain.

Three genes were built, the first of which contained 23, 24, 48, 49, 71 and 73 [gH341(6)] as murine residues. The second gene also had 75 and 88 as murine residues [gH341(8)] while the third gene additionally had 68, 69, 75 and 88 as murine residues [gH341(10)]. Each was co-expressed with gL221, the minimum grafted light chain (CDRs only). The gL221/gH341(6) and gL221/gH341(8) antibodies both bound as well to TNF as murine 61E71. The gL221/gH341(10) antibody did not express and this combination was not taken further.

Subsequently the gL221/gH341(6) antibody was assessed in an L929 cell competition assay in which the antibody competes against the TNF receptor on L929 cells for binding to TNF in solution. In this assay the gL221/gH341(6) antibody was approximately 10% as active as murine 61E71.

hTNF1

hTNF1 is a monoclonal antibody which recognises an epitope on human TNF- . The EU human framework was used for CDR-grafting of both the heavy and light variable domains.

Heavy Chain

In the CDR-grafted heavy chain (ghTNF1) mouse CDRs were used at positions 26-35 (CDR1), 50-65 (CDR2) and 95-102 (CDR3). Mouse residues were also used in the frameworks at positions 48, 67, 69, 71, 73, 76, 89, 91, 94 and 108. Comparison of the TNF1 mouse and EU human heavy chain residues reveals that these are identical at positions 23, 24, 29 and 78.

Light Chain

In the CDR-grafted light chain (gLhTNF1) mouse CDRs were used at positions 24-34 (CDR1), 50-56 (CDR2) and 89-97 (CDR3). In addition mouse residues were used in the frameworks at positions 3, 42, 48, 49, 83, 106 and 108. Comparison of the hTNF1 mouse and EU human light chain residues reveals that these are identical at positions 46, 58 and 71.

The grafted hTNF1 heavy chain was co-expressed with the chimeric light chain and the binding ability of the product compared with that of the chimeric light chain/chimeric heavy chain product in a TNF binding assay. The grafted heavy chain product appeared to have binding ability for TNF slightly better than the fully chimeric product.

Similarly, a grafted heavy chain/grafted light chain product was co-expressed and compared with the fully chimeric product and found to have closely similar binding properties to the latter product.

hTNF3

hTNF3 recognises an epitope on human TNF- α . The sequence of hTNF3 shows only 21 differences compared to 61E71 in the light and heavy chain variable regions, 10 in the light chain (2 in the CDRs at positions 50, 96 and 8 in the framework at 1, 19, 40, 45, 46, 76, 103 and 106) and 11 in the heavy chain (3 in the CDR regions at positions 52, 60 and 95 and 8 in the framework at 1, 10, 38, 40, 67, 73, 87 and 105). The light and heavy chains of the 61E71 and hTNF3 chimeric antibodies can be exchanged without loss of activity in the direct binding assay. However 61E71 is an order of magnitude less able to compete with the TNF receptor on L929 cells for TNF-a compared to hTNF3. Based on the 61E71 CDR grafting data gL221 and gH341(+23, 24, 48, 49 71 and 73 as mouse) genes have been built for hTNF3 and tested and the resultant grafted antibody binds well to TNF-a, but competes very poorly in the L929 assay. It is possible that in this case also the framework residues identified for OKT3 programme may improve the competitive binding ability of this antibody.

101.4

101.4 is a further murine monoclonal antibody able to recognise human TNF-a. The heavy chain of this antibody shows good homology to KOL and so the CDR-grafting has been based on RE1 for the light chain and KOL for the heavy chain. Several grafted heavy chain genes have been constructed with conservative choices for the CDR's (gH341)^(SEQ ID NO:11) and which have one or a small number of non-CDR residues at positions 73, 78 or 77-79 inclusive, as the mouse amino acids. These have been co-expressed with cL or gL221. In all cases binding to TNF equivalent to the chimeric antibody is seen and when co-expressed with cL the resultant antibodies are able to compete well in the L929 assay. However, with gL221 the resultant antibodies

are at least an order of magnitude less able to compete for TNF against the TNF receptor on L929 cells.

Mouse residues at other positions in the heavy chain, for example, at 23 and 24 together or at 76 have been demonstrated to provide no improvement to the competitive ability of the grafted antibody in the L929 assay.

A number of other antibodies including antibodies having specificity for interleukins e.g. IL1 and cancer markers such as carcinoembryonic antigen (CEA) e.g. the monoclonal antibody A5B7 (ref. 21), have been successfully CDR-grafted according to the present invention.

It will be appreciated that the foregoing examples are given by way of illustration only and are not intended to limit the scope of the claimed invention. Changes and modifications may be made to the methods described whilst still falling within the spirit and scope of the invention.

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INS
AA
4 5+1

CLAIMS

1. A CDR-grafted antibody heavy chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 6, 23 and/or 24, 48 and/or 49, 71 and/or 73, 75 and/or 76 and/or 78 and 88 and/or 91.
2. A CDR-grafted heavy chain according to Claim 1 comprising donor residues at positions 23, 24, 49, 71, 73 and 78, or at positions 23, 24 and 49.
3. A CDR-grafted heavy chain according to Claim 2 comprising donor residues at positions 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.
4. A CDR-grafted heavy chain according to Claim 2 or 3, comprising donor residues at one, some or all of positions:
 1 and 3,
 69 (if 48 is different between donor and acceptor),
 38 and 46 (if 48 is the donor residue),
 67,
 82 and 18 (if 67 is the donor residue),
 91, and
 any one or more of 9, 11, 41, 87, 108, 110 and 112.
5. A CDR-grafted heavy chain according to any of the preceding comprising donor CDRs at positions 26-35, 50-65 and 95-100.
6. A CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 1 and/or 3 and 46 and/or 47.

7. A CDR-grafted light chain according to Claim 6 comprising donor residues at positions 46 and 47.
8. A CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 46, 48, 58 and 71.
9. A CDR-grafted light chain according to Claim 8 comprising donor residues at positions 46, 48, 58 and 71.
10. A CDR-grafted light chain according to Claim 8 or 9, comprising donor residues at positions 2, 4, 6, 35, 36, 38, 44, 47, 49, 52, 64-69, 85, 87, 98, 99, 101 and 102.
11. A CDR-grafted light chain according to Claim 9 or 10, comprising donor residues at one, some or all of positions:
1 and 3,
63,
60 (if 60 and 54 are able to form a potential saltbridge),
70 (if 70 and 24 are able to form a potential saltbridge),
73 and 21 (if 47 is different between donor and acceptor),
37 and 45 (if 47 if different between donor and acceptor), and
any one or more of 10, 12, 40, 83, 103 and 105.
12. A CDR-grafted light chain according to any one of Claims 6-11, comprising donor CDRs at positions 24-34, 50-56 and 89-97.

13. A CDR-grafted antibody molecule comprising at least one CDR-grafted heavy chain according to any one of Claims 1-5 and at least one CDR-grafted light chain according to any one of Claims 6-12.
14. A CDR-grafted antibody molecule according to Claim 13, which is a site-specific antibody molecule.
15. A CDR-grafted antibody molecule according to Claim 13 which has specificity for an interleukin, hormone or other biologically active compound or a receptor therefor.
16. A CDR-grafted antibody heavy or light chain or molecule according to any one of the preceding claims comprising human acceptor residues and non-human donor residues.
17. A DNA sequence which codes for a CDR-grafted heavy chain according to Claim 1 or a CDR-grafted light chain according to Claim 6 or Claim 8.
18. A cloning or expression vector containing a DNA sequence according to Claim 17.
19. A host cell transformed with a DNA sequence according to Claim 17.
20. A process for the production of a CDR-grafted antibody sequence according to Claim 17 in a transformed host cell.
21. A process for producing a CDR-grafted antibody product comprising:

sub
B1

add B1 cont.

- (a) producing in an expression vector an operon having a DNA sequence which encodes an antibody heavy chain according to Claim 1;
and/or
- (b) producing in an expression vector an operon having a DNA sequence which encodes a complementary antibody light chain according to Claim 6 or Claim 8;
- (c) transfecting a host cell with the or each vector;
and
- (d) culturing the transfected cell line to produce the CDR-grafted antibody product.

22. A therapeutic or diagnostic composition comprising a CDR-grafted antibody heavy chain according to Claim 1, or a CDR-grafted light chain according to Claim 6 or Claim 8, or a CDR-grafted antibody molecule according to Claim 13 in combination with a pharmaceutically acceptable carrier, diluent or excipient.

23. A method of therapy or diagnosis comprising administering an effective amount of a CDR-grafted heavy chain according to Claim 1, or a CDR-grafted light chain according to Claim 6 or Claim 8, or a CDR-grafted antibody molecule according to Claim 13 to a human or animal subject.

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ABSTRACT

CDR-grafted antibody heavy and light chains
comprise acceptor framework and donor antigen binding
regions, the heavy chains comprising donor residues at at
5 least one of positions (6, 23) and/or (24, 48) and/or (49,
71) and/or (73, 75) and/or (76) and/or (78) and (88) and/or
(91). The CDR-grafted light chains comprise donor residues
at at least one of positions (1) and/or (3) and (46) and/or
(47) or at at least one of positions (46, 48, 58) and (71).
10 The CDR-grafted antibodies are preferably humanised
antibodies, having non human, e.g. rodent, donor and human
acceptor frameworks, and may be used for *in vivo* therapy and
diagnosis. A generally applicable protocol is disclosed for
obtaining CDR-grafted antibodies.

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1 GAATTC~~CCAA~~ AGACAAAatg gattttcaag tgcagatttt cagcttctg
51 ctaatcagtg cctcagtcat aatatccaga ggacaaattg ttctcacc~~ca~~
101 gtctccagca atcatgtctg catctccagg ggagaaggte accatgacct
151 gcagtgccag ctcaagtgta agttacatga actggtagca gcagaagtca
201 ggcacctccc ccaaaagatg gatttatgac acatccaaac tggcttctgg
251 agtccctgct cacttcaggg gcagtg~~gg~~tc tgggacctct tactctctca
301 caatcagcgg catggaggct gaagatgctg ccaattatta ctgccagcag
351 tggagtagta acccattcac gttcggctcg gggacaaagt tggaaataaa
401 ccgggctgat actgcaccaa ctgtatccat cttcccacca tccagtgagc
451 agttaacatc tggaggtgcc tcagtcgtgt gcttcttgaa caacttctac
501 cccaaagaca tcaatgtcaa gtggaagatt gatggcagtg aacgacaaaa
551 tggcgtcctg aacagttgga ctgatcagga cagcaaagac agcacctaca
601 gcatgagcag caccctcacg ttgaccaagg acgagtatga acgacataac
651 agctatacct gtgaggccac tcacaagaca tcaacttcac ccattgtcaa
701 gagcttcaac aggaatgagt gtTAGAGACA AAGGTCCTGA GACGCCACCA
751 CCAGCTCCCA GCTCCATCCT ATCTTCCTT CTAAGGTCTT GGAGGCTTCC
801 CCACAAGCGC tTACCACTGT TGCGGTGCTC tAAACCTCCT CCCACCTCCT
851 TCTCCTCCTC CTCCTTTCC TTGGCTTTTA TCATGCTAAT ATTTGCAGAA
901 AATATTCAAT AAAGTGAGTC TTTGCCTTGA AAAAAAAAAA AAA

Fig. 1(a)

1 MDFOVOIFSF LLISASVIIS RGQIVLTQSP AIMSASPGEK VTMTCSASSS
51 VSYMNWYQQK SGTSPKRWIY DTSKLAGVP AHFRGSGSGT SYSLTISGME
101 AEDAATYYCQ QWSSNPFTFG SGTKLEINRA DTAPTVSIFP PSSEQLTSGG
151 ASVVCFLNNF YPKDINVKWK IDGSERQNGV LNSWTDQDSK DSTYSMSSTL
201 TLTKEYERH NSYTCEATHK TSTSPIVKSF NRNEC*

Fig. 1(b)

SUBSTITUTE SHEET

1 GAATTCCCCT CTCCACAGAC ACTGAAAACCT CTGACTCAAC ATGGAAAGGC
 51 ACTGGATCTT TCTACTCCTG TTGTCAGTAA CTGCAGGTGT CCACTCCCAG
 101 GTCCAGCTGC AGCAGTCTGG GGCTGAACTG GCAAGACCTG GGGCCTCAGT
 151 GAAGATGTCC TGCAAGGCTT CTGGCTACAC CTTTACTAGG TACACGATGC
 201 ACTGGGTAAA ACAGAGGCCT GGACAGGGTC TGGAAATGGAT TGGATAACATT
 251 AATCCTAGCC GTGGTTATAC TAATTACAAT CAGAAGTTCA AGGACAAGGC
 301 CACATTGACT ACAGACAAAT CCTCCAGCAC AGCCTACATG CAACTGAGCA
 351 GCCTGACATC TGAGGACTCT GCAGTCTATT ACTGTGCAAG ATATTATGAT
 401 GATCATTACT GCCTTGACTA CTGGGGCCAA GGCACCACTC TCACAGTCTC
 451 CTCAGCCAAA ACAACAGCCC CATCGGTCTA TCCACTGGCC CCTGTGTGTG
 501 GAGATACAAC TGGCTCCTCG GTGACTCTAG GATGCCTGGT CAAGGGTTAT
 551 TTCCCTGAGC CAGTGACCTT GACCTGGAAC TCTGGATCCC TGTCCAGTGG
 601 TGTGCACACC TTCCCAGCTG TCCTGCAGTC TGACCTCTAC ACCCTCAGCA
 651 GCTCAGTGAC TGTAACCTCG AGCACCTGGC CCAGCCAGTC CATCACCTGC
 701 AATGTGGCCC ACCCGGCAAG CAGCACCAAG GTGGACAAGA AAATTGAGCC
 751 CAGAGGGCCC ACAATCAAGC CCTGTCTCTC ATGCAAATGC CCAGCACCTA
 801 ACCTCTTGGG TGGACCATCC GTCTTCATCT TCCCTCCAAA GATCAAGGAT
 851 GTACTCATGA TCTCCCTGAG CCCCATAGTC ACATGTGTGG TGGTGGATGT
 901 GAGCGAGGAT GACCCAGATG TCCAGATCAG CTGGTTTGTG AACAACTGGG
 951 AAGTACACAC AGCTCAGACA CAAACCCATA GAGAGGATTA CAACAGTACT
 1001 CTCCGGGTGG TCAGTGCCCT CCCCATCCAG CACCAGGACT GGATGAGTGG
 1051 CAAGGAGTTC AAATGCAAGG TCAACAACAA AGACCTCCCA GCGCCCATCG
 1101 AGAGAACCAT CTCAAACCC AAAGGGTCAG TAAGAGCTCC ACAGGTATAT
 1151 GTCTTGCCTC CACCAGAAGA AGAGATGACT AAGAAACAGG TCACTCTGAC
 1201 CTGCATGGTC ACAGACTTCA TGCCTGAAGA CATTTACGTG GAGTGGACCA
 1251 ACAACGGGAA AACAGAGCTA AACTACAAGA AACTGAACC AGTCCTGGAC
 1301 TCTGATGGTT CTTACTTCAT GTACAGCAAG CTGAGAGTGG AAAAGAAGAA
 1351 CTGGGTGGAA AGAAATAGCT ACTCCTGTTC AGTGGTCCAC GAGGGTCTGC
 1401 ACAATCACCA CACGACTAAG AGCTTCTCCC GGACTCCGGG TAAATGAGCT
 1451 CAGCACCCAC AAAACTCTCA GGTCCAAAGA GACACCCACA CTCATCTCCA
 1501 TGCTTCCCTT GTATAAATAA AGCACCCAGC AATGCCTGGG ACCATGTAAA
 1551 AAAAAAAAAA AAAGGAATTC

Fig. 2(a)

SUBSTITUTE SHEET

OKT 3 HEAVY CHAIN PROTEIN SEQUENCE DEDUCED FROM DNA SEQUENCE

1 MERHWIFLLL LSVTAGVHSQ VQLQQSGAEL ARPGASVKMS CKASGYTFTR
 51 YTMHWVKQRP GQGLEWIGYI NPSRGYTNYN QKFKDKATLT TDKSSSTAYM
 101 QLSSLTSEDS AVYICARYYD DHYCLDYWGQ GTTLTVSSAK TTAPSVYPLA
 151 PVCGDTTGSS VTLGCLVKGY FPEPVTLTWN SGSLSSGVHT FPAVLQSDLY
 201 TLSSSVTVTS STWPSQSITC NVAHPASSTK VDKKIEPRGP TIKPCPPCKC
 251 PAPNLLGGPS VFIFPPKIKD VLMISLSPIV TCVVVDVSED DPDVQISWV
 301 NNVEVHTAQT QTHREDYNST LRVVSALPIQ HQDWMGKKEF KCKVNNKDLP
 351 APIERTISKP KGSVRAPQVY VLPPPEEEMT KKQVTLTCMV TDFMPEDIYV
 401 EWTNNGKTEL NYKNTEPVL SDGSYFMYSK LRVEKKNWVE RNSYSCSVVH
 451 EGLHNHHTTK SFSRTPGK*

Fig. 2(b)

	1		23		42
	NN	N	N	N	N
RES TYPE	SBspSPESsSBSbSsSsPSPSPsPSsse*s*p*Pi`ISsSe				
Okt3v1	QIVLTQSPAIMASAPGEXVTMTCSASS.SVSYM N WYQKSGT				
REI	DIQMTQSPSSLASVGDVRTITCQASQDIIKYLNWYQQT P GK				
	? ?				
	CDR1	(LOOP)	*****		
	CDR1	(KABAT)	*****		

		56		85
	N	NN		
RES TYPE	*IsiPpIeesesssSBesePsPSBSSEsPspPsseesSPePb			
Okt3v1	SPKRWIYDTSKLAGVPAHFRGSGSGTYSYSLTISGMEAEADAAT			
REI	APKLLIYEASN L QAGVPSRFSGSGSGTDY T P T ISSLQPED I A T			
	? ?? ? ?			
	***** CDR2 (LOOP/KABAT)			

		102	108
RES TYPE	PiPIPIes**iPIIsPPSPSPSS		
Okt3v1	YYCQQWSSNPFTFG G TKLEI N R		
REIv1	YYCQQYQSLPYTFGQGT K LOI T R		
	? ?		
	*****	CDR3 (LOOP)	
	*****	CRD3 (KABAT)	

Fig. 3

SUBSTITUTE SHEET

NN N 23 26 32 35 N39 43
 RES TYPE SESPs`SBssS`sSSsSpSpSPsPSEbSBssBePiPiesss
 Okt3h QVQLQQGGAELARPGASVKMSCKASGYTFTRYTMHWVKORPGQ
 KOL QVQLVESGGGVQPGRSLRLSCSSGFISSYAMYWVRQAPGK
 ? .??
 ***** CDR1 (LOOP)
 ***** CDR1 (KABAT)

52a 60 65 N N N 82abc 89
 RES TYPE IIeIppp`ssssssps`pSSsbSpseSsSseSp`pSpSbSSs`epb
 Okt3vh GLEWIGYINPSRGYTNTNQFKDKATLITDKSSSTAYMQLSSLTSESAV
 KOL GLEWVAIIWDDGSDQHYADSVKGRFTISRDNKNTLFLQMDSLRPEDTGV
 ?? ? ? ? ? ?
 ***** CDR2 (LOOP)
 ***** CDR2 (KABAT)

92 N 107 113
 RES TYPE PiPIEiSSSSiiSSsbibi*EIPiP*spSBSS
 Okt3vh YYCARYYDDHY.....CLDYWGQGTTLTVSS
 KOL YFCARDGGHGFCSASCFGPDYWGQGTPEVTVSS
 ***** CRD3 (KABAT/LOOP)

Fig. 4

SUBSTITUTE SHEET

OKT 3 HEAVY CHAIN CDR GRAFTS

1. gh341 and derivatives

	1	26	35	39	43	
Okt3vh	QVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPGQ					
gh341	QVQLVESGGGVVQPGRSLRLSCSSSGYTFTRYTMHWVRQAPGK					JA178
gh341A	QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK					JA185
gh341E	QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK					JA198
gh341*	QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK					JA207
gh341*	QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK					JA209
gh341D	QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK					JA197
gh341*	QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK					JA199
gh341C	QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK					JA184
gh341*	QVQLVQSGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK					JA203
gh341*	QVQLVESGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK					JA205
gh341B	QVQLVESGGGVVQPGRSLRLSCSSSGYTFTRYTMHWVRQAPGK					JA183
gh341*	QVQLVQSGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK					JA204
gh341*	QVQLVESGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK					JA206
gh341*	QVQLVQSGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK					JA208
KOL	QVQLVESGGGVVQPGRSLRLSCSSSGFIFSSYAMYWVRQAPGK					

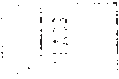
Fig. 5(i)

SUBSTITUTE SHEET

	44	50	65	83	
Okt3vh	GLEW	IGYIN	PSRGY	TNYN	QKFKDKATLTTDKSSSTAYMQLSSLT
gH341	GLEW	VAYIN	PSRGY	TNYN	QKFKDRFTISRDN
gH341A	GLEW	IGYIN	PSRGY	TNYN	QKVKDRFTISTDKSKSTAFLQMDSL
gH341E	GLEW	IGYIN	PSRGY	TNYN	QKVKDRFTISTDKSKSTAFLQMDSL
gH341*	GLEW	IGYIN	PSRGY	TNYN	QKVKDRFTISTDKSKNTAFLQMDSL
gH341*	GLEW	IGYIN	PSRGY	TNYN	QKVKDRFTISRDN
gH341D	GLEW	IGYIN	PSRGY	TNYN	QKVKDRFTISTDKSKNTLFLQMDSL
gH341*	GLEW	IGYIN	PSRGY	TNYN	QKVKDRFTISRDN
gH341C	GLEW	VAYIN	PSRGY	TNYN	QKFKDRFTISRDN
gH341*	GLEW	IGYIN	PSRGY	TNYN	QKVKDRFTISTDKSKSTAFLQMDSL
gH341*	GLEW	IGYIN	PSRGY	TNYN	QKVKDRFTISTDKSKSTAFLQMDSL
gH341B	GLEW	IGYIN	PSRGY	TNYN	QKVKDRFTISTDKSKSTAFLQMDSL
gH341*	GLEW	IGYIN	PSRGY	TNYN	QKVKDRFTISTDKSKSTAFLQMDSL
gH341*	GLEW	IGYIN	PSRGY	TNYN	QKVKDRFTISTDKSKSTAFLQMDSL
gH341*	GLEW	IGYIN	PSRGY	TNYN	QKVKDRFTISTDKSKNTAFLQMDSL
KOL	GLEW	VAI	IWDDG	SDQHY	ADSVKGRFTISRDN

Fig. 5(ii)

SUBSTITUTE SHEET



	84	95	102	113	
Okt3vh	SEDSAVYYCARYYDDHY.....		CLDYWGQGTTLTVSS		
gH341	PEDTGVYFCARYYDDHY.....		CLDYWGQGTTLTVSS		JA178
gH341A	PEDTAVYYCARYYDDHY.....		CLDYWGQGTTLTVSS		JA185
gH341E	PEDTGVYFCARYYDDHY.....		CLDYWGQGTTLTVSS		JA198
gH341*	PEDTGVYFCARYYDDHY.....		CLDYWGQGTTLTVSS		JA207
gH341D	PEDTGVYFCARYYDDHY.....		CLDYWGQGTTLTVSS		JA197
gH341*	PEDTGVYFCARYYDDHY.....		CLDYWGQGTTLTVSS		JA209
gH341*	PEDTGVYFCARYYDDHY.....		CLDYWGQGTTLTVSS		JA199
gH341C	PEDTGVYFCARYYDDHY.....		CLDYWGQGTTLTVSS		JA184
gH341*	PEDTAVYYCARYYDDHY.....		CLDYWGQGTTLTVSS		JA203
gH341*	PEDTAVYYCARYYDDHY.....		CLDYWGQGTTLTVSS		JA205
gH341B	PEDTAVYYCARYYDDHY.....		CLDYWGQGTTLTVSS		JA183
gH341*	PEDTGVYFCARYYDDHY.....		CLDYWGQGTTLTVSS		JA204
gH341*	PEDTGVYFCARYYDDHY.....		CLDYWGQGTTLTVSS		JA206
gH341*	PEDTGVYFCARYYDDHY.....		CLDYWGQGTTLTVSS		JA208
KOL	PEDTGVYFCARDGGHGFCSASCFGPDYWGQGTPTVSS				

Fig. 5(iii)

SUBSTITUTE SHEET

OKT3 LIGHT CHAIN CDR GRAFTING

1. gL221 and derivatives

	1	24	34	42
Okt3v1	QIVLTQSPAIMSASPGEKVTMTCSASS.SVSYMNWYQQKSGT			
gL221	DIQMTQSPSSLSASVGDRTITCSASS.SVSYMNWYQQTTPGK			
gL221A	<u>QIV</u> MTQSPSSLSASVGDRTITCSASS.SVSYMNWYQQTTPGK			
gL221B	<u>QIV</u> MTQSPSSLSASVGDRTITCSASS.SVSYMNWYQQTTPGK			
gL221C	DIQMTQSPSSLSASVGDRTITCSASS.SVSYMNWYQQTTPGK			
REI	DIQMTQSPSSLSASVGDRTITCQASQDIIKYLNWYQQTTPGK			
	43	50	56	85
Okt3v1	SPKRWIYDTSKLAGVPAHFRGSGSGTYSYSLTISGMEAEDAAT			
gL221	APKLLIYDTSKLAGVPSRFSGSGSDYFTFTISSLPEDIAT			
gL221A	APKRWIYDTSKLAGVPSRFSGSGSDYFTFTISSLPEDIAT			
gL221B	APKRWIYDTSKLAGVPSRFSGSGSDYFTFTISSLPEDIAT			
gL221C	APKRWIYDTSKLAGVPSRFSGSGSDYFTFTISSLPEDIAT			
REI	APKLLIYEASNLAQAVPSRFSGSGSDYFTFTISSLPEDIAT			
	86	91	96	108
Okt3v1	YVCQWSSNPFTFGSGTKLEINR			
gL221	<u>YVCQWSSNPFTFGQ</u> GTKLQITR			
gL221A	<u>YVCQWSSNPFTFGQ</u> GTKLQITR			
gL221B	<u>YVCQWSSNPFTFGQ</u> GTKLQITR			
gL221C	<u>YVCQWSSNPFTFGQ</u> GTKLQITR			
REI	YVCQQYQSLPYTFGQGTKLQITR			

CDR'S ARE UNDERLINED

FRAMEWORK RESIDUES INCLUDED IN THE GENE ARE DOUBLE UNDERLINED

Fig. 6

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18 RES'D BCLN10 17 JUL 1997

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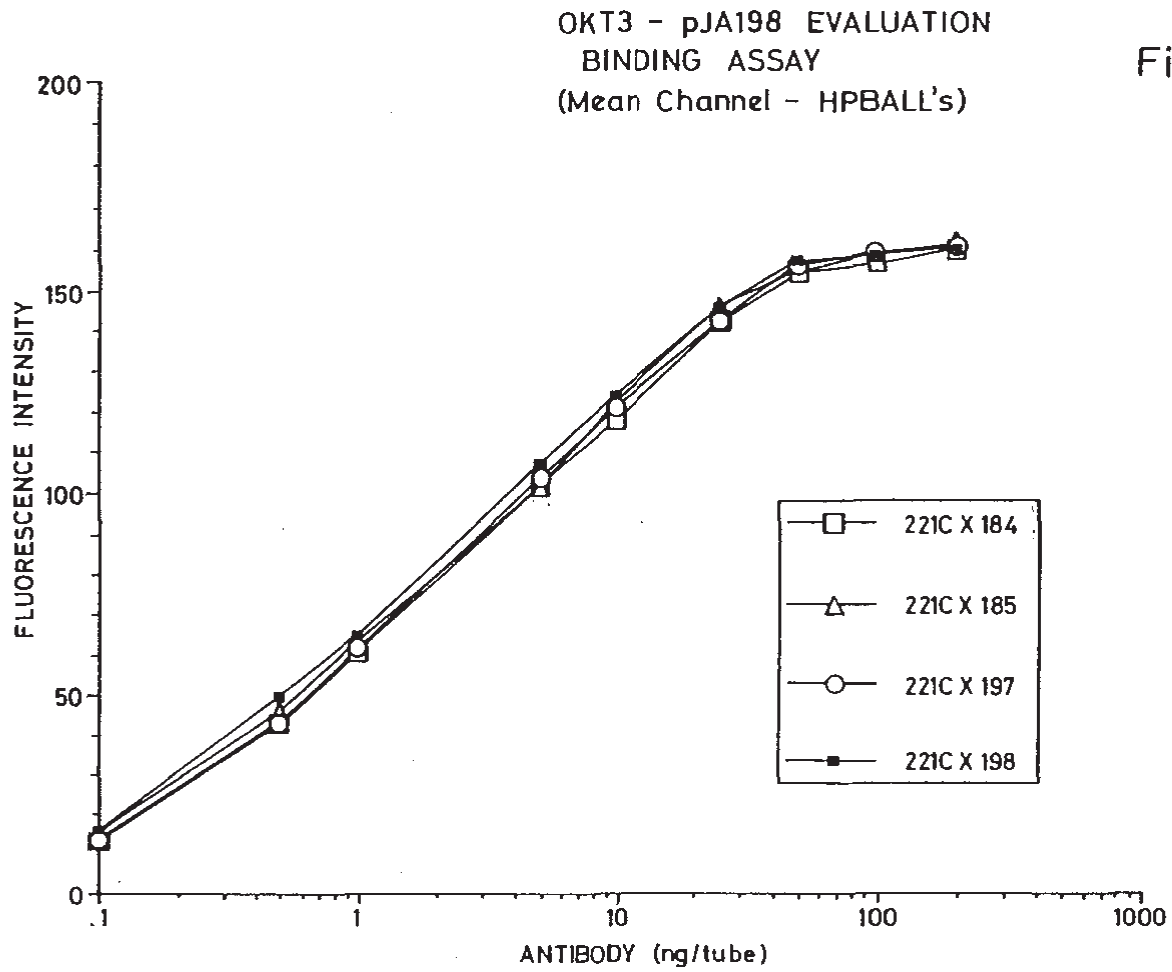
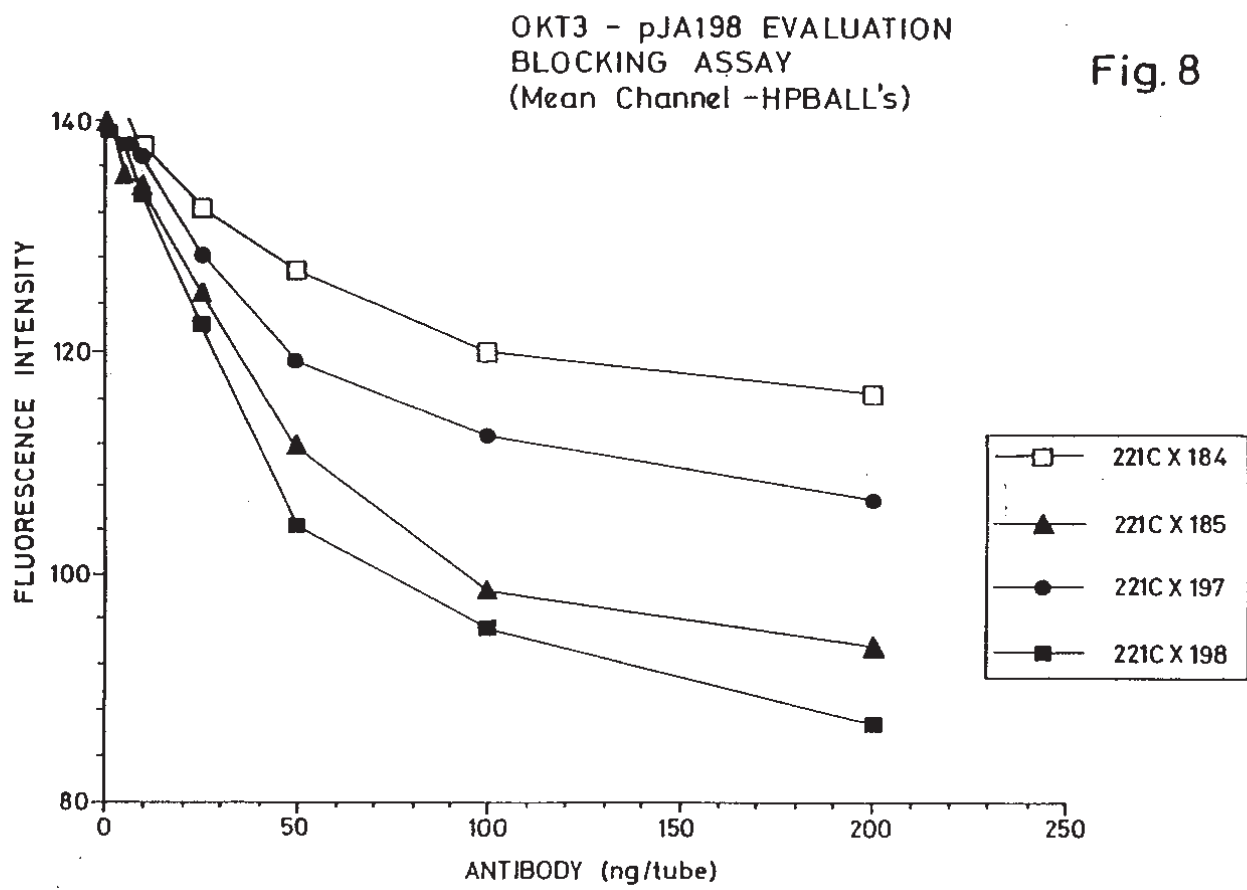


Fig. 7

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08/30/3569

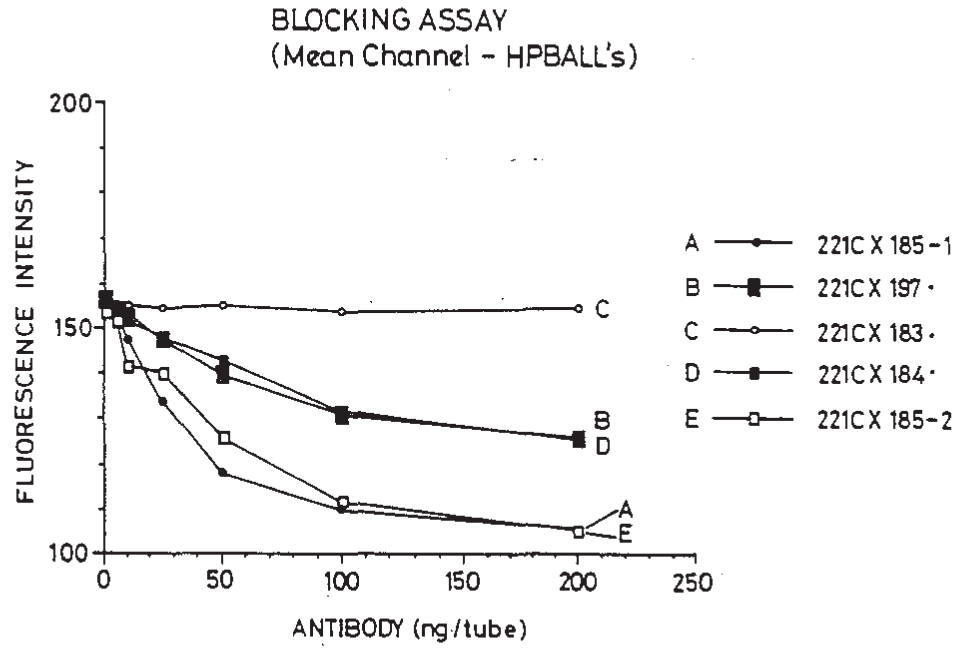
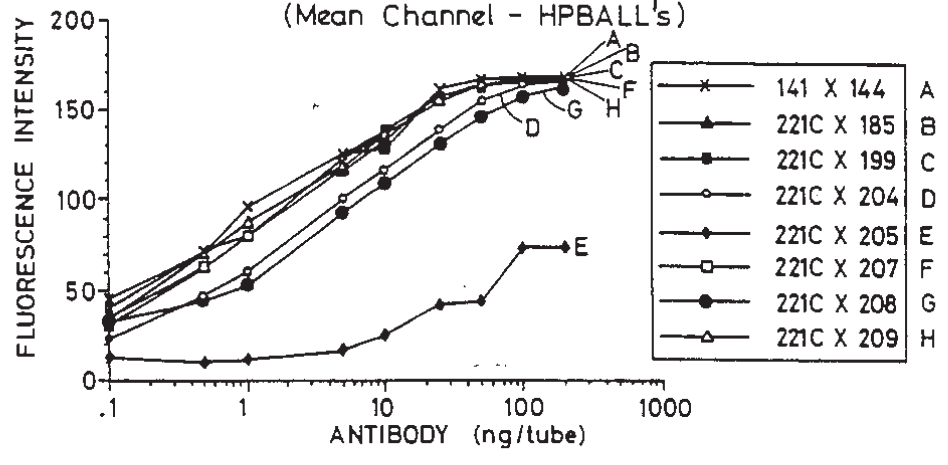


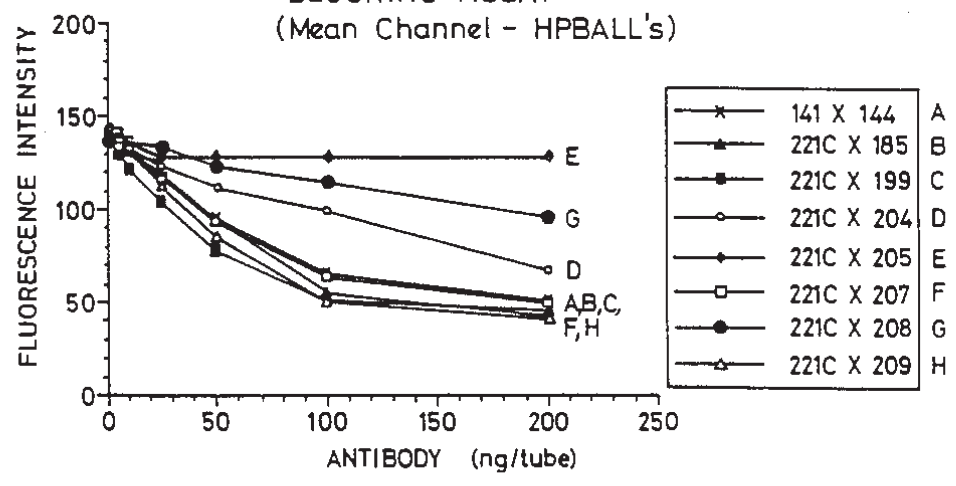
Fig. 9

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Fig.10 OKT3 - GRAFTED HEAVY CHAINS
BINDING ASSAY
(Mean Channel - HPBALL's)



OKT3 - GRAFTED HEAVY CHAINS
BLOCKING ASSAY
(Mean Channel - HPBALL's)



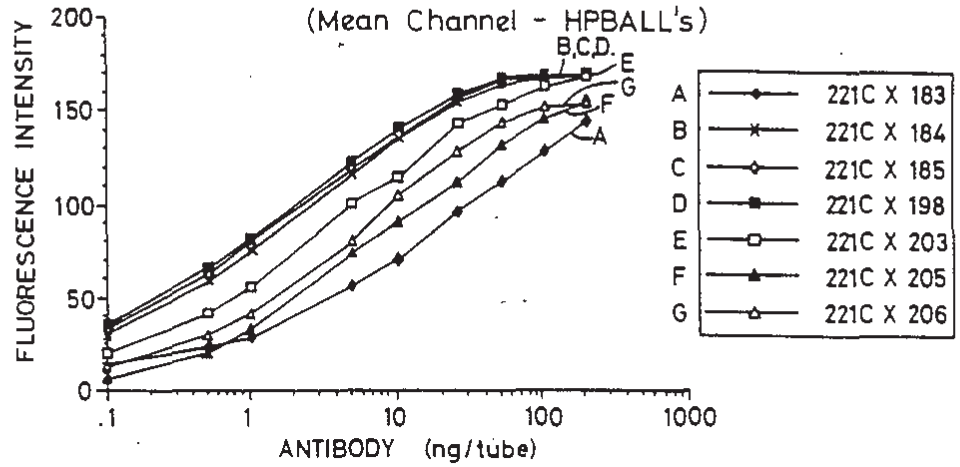
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● (208)	6, 23, 24, 48, 49, 71, 73, 76, 78, 88, 91,
○ (204)	6, 23, 24, 48, 49, 71, 73, 76, 78, 88, 91,
■ (199)	6, 23, 24, 48, 49, 71, 73, 76, 78, 88, 91,
□ (207)	6, 23, 24, 48, 49, 71, 73, 76, 78, 88, 91,
▲ (185)	6, 23, 24, 48, 49, 71, 73, 76, 78, 88, 91,
△ (209)	6, 23, 24, 48, 49, 71, 73, 76, 78, 88, 91,
× 141 X 144	6, 23, 24, 48, 49, 71, 73, 76, 78, 88, 91,

SUBSTITUTE SHEET

Fig. 11

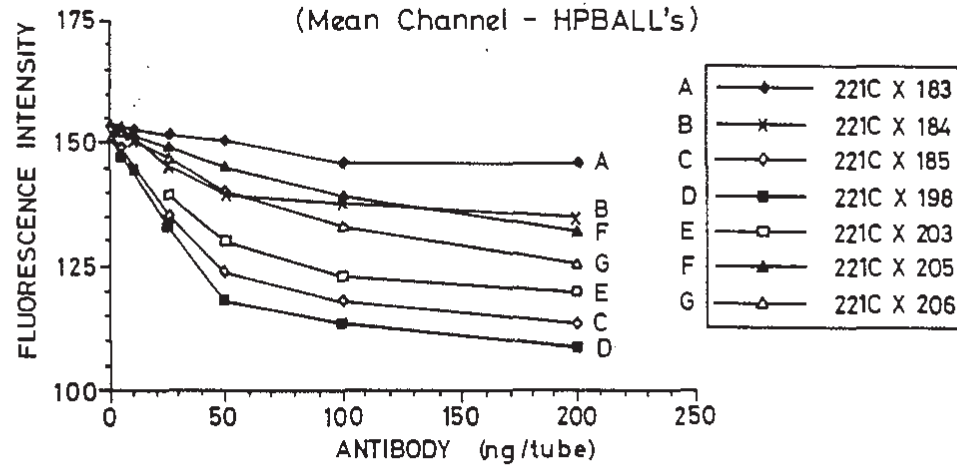
OKT3 - GRAFTED HEAVY CHAINS
BINDING ASSAY

(Mean Channel - HPBALL's)



OKT3 GRAFTED HEAVY CHAINS
BLOCKING ASSAY

(Mean Channel - HPBALL's)

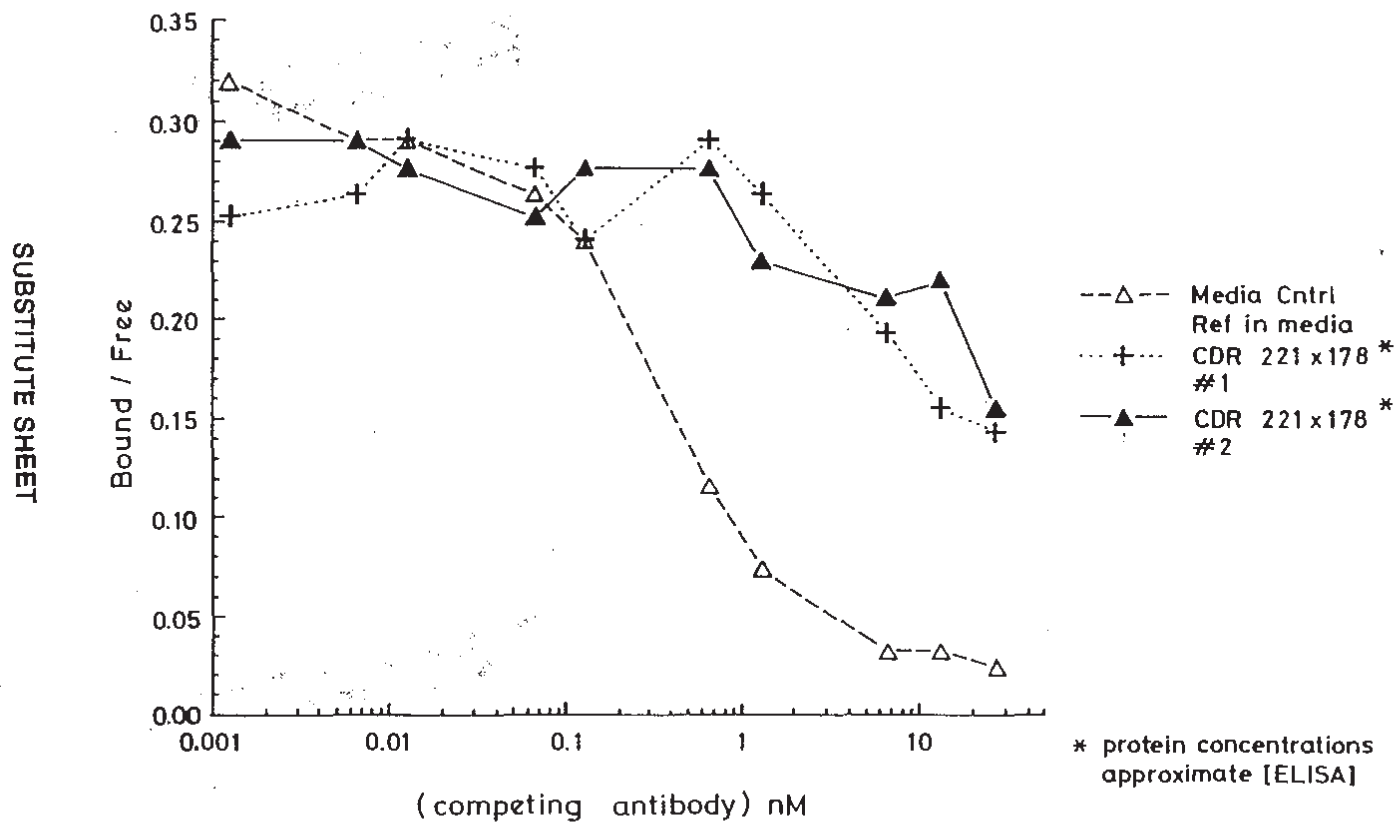


◆	(183)	-----,48,49,71,73,76,78,88,91,
▲	(205)	-----,24,48,49,71,73,76,78,88,91,
×	(184)	6,23,24,-----,-----,
△	(206)	-----,24,48,49,71,73,76,78,-----,
□	(203)	6,-----,24,48,49,71,73,76,78,88,91,
◇	(185)	6,23,24,48,49,71,73,76,78,88,91,
■	(198)	6,23,24,48,49,71,73,76,78,-----,

SUBSTITUTE SHEET

OKT3 Competition
 Murine Ref Std vs. CDR Grafted OKT3

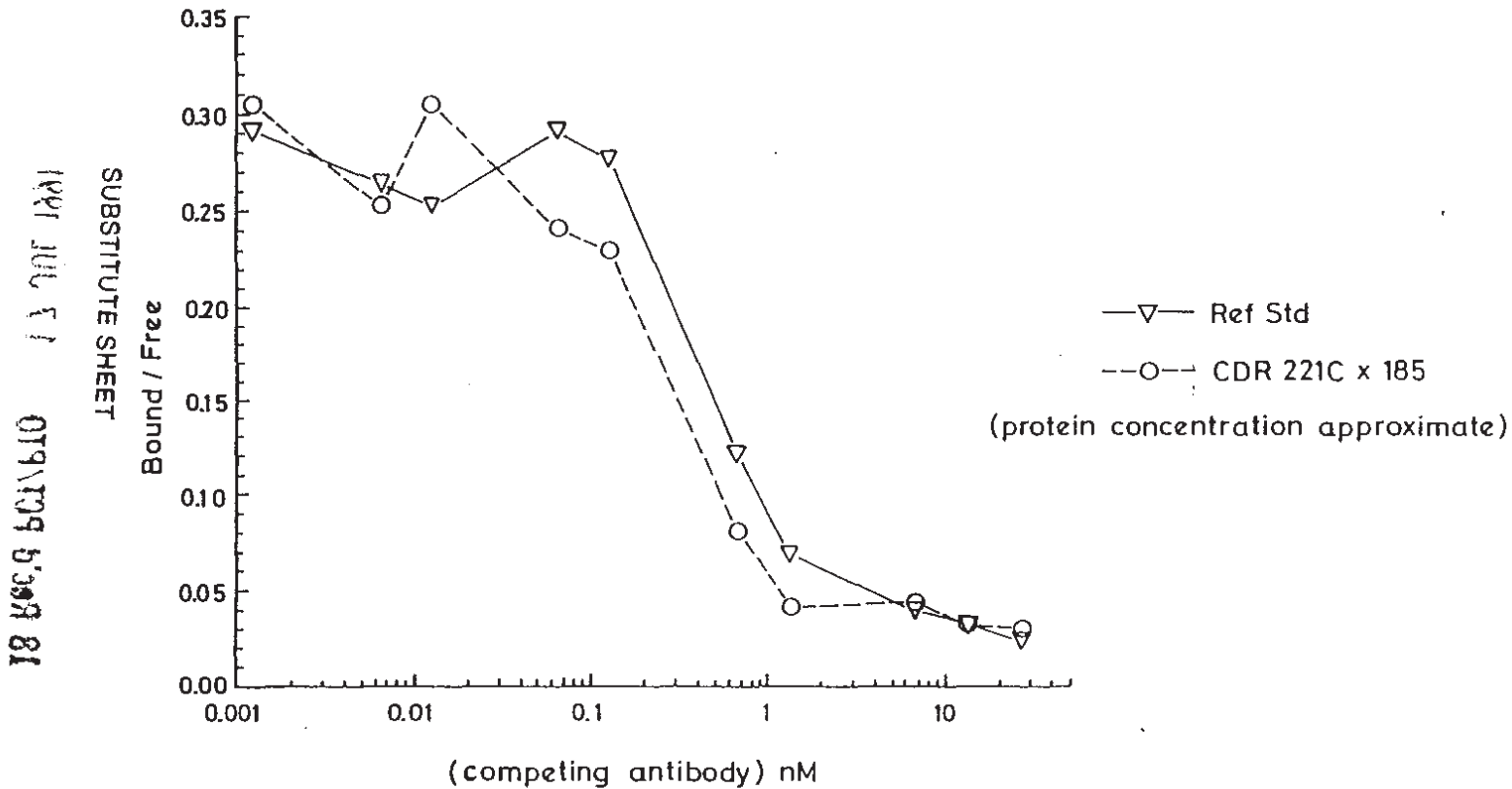
Fig. 12



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OKT3 Competition
Murine Ref Std vs. CDR Grafted OKT3

Fig. 13



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#4

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

CARP-0009

INTERNATIONAL APPLICATION NO. PCT/GB90/02017

INTERNATIONAL FILING DATE 21 December 1990

PRIORITY DATE CLAIMED 21 December 1989

TITLE OF INVENTION HUMANISED ANTIBODIES

APPLICANT(S) FOR DO/EO/US ADAIR, John, Robert, ATHWAL, Diljeet, Singh, and EMTAGE, John, Spencer

Applicant herewith submits to the United States Designated/ Elected Office (DO/EO/US) the following items under 35 U.S.C. 371:

- 1. This express request to immediately begin national examination procedures (35 U.S.C. 371(f)).
- 2. The U.S. National Fee (35 U.S.C. 371(c)(1)) and other fees as follows:

CLAIMS	(1) FOR	(2) NUMBER FILED	(3) NUMBER EXTRA	(4) RATE	(5) CALCULATIONS
TOTAL CLAIMS		-20-		X\$ 20.00	\$
INDEPENDENT CLAIMS		-3-		X\$ 60.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)				+\$ 200.00	
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(4)):					
<input type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482) \$ 330					
<input type="checkbox"/> No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445 (a)(2)) \$ 370					
<input type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$ 500					
<input type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2) to (4) \$ 50					
Surcharge of \$120 for furnishing the National fee or oath or declaration later than <input checked="" type="checkbox"/> 20 <input type="checkbox"/> 30 mos. from the earliest claimed priority date (37 CFR 1.492(e)).					\$120.00
TOTAL OF ABOVE CALCULATIONS					-\$120.00
Reduction by 1/2 for filing by small entity, if applicable. Affidavit must be filed also. (Note 37 CFR 1.9, 1.27, 1.28.)					\$60.00
SUBTOTAL					+\$60.00
Processing fee of \$30 for furnishing the English Translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 mos. from the earliest claimed priority date (37 CFR 1.492(f)).					
TOTAL NATIONAL FEE					\$ 60.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)).					+
TOTAL FEES ENCLOSED					\$ 60.00

- a. A check in the amount of \$ 60.00 to cover the above fees is enclosed.
- b. Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.
- c. The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 23-3050. A duplicate copy of this sheet is enclosed.

10/16/91X060 * 00236-00257*

01143329

25-9

EXPRESS MAIL Mailing List No. RB567260177

Date of Deposit September 17, 1991

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Commissioner of Patents and Trademarks, Washington, D. C. 20591

Diane M. Kushner

Diane M. Kushner

- 3. A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. is not required, as the application was filed in the United States Receiving Office (RO/US).
 - c. has been transmitted by the International Bureau.
- 4. A translation of the International Application into English (35 U.S.C. 371 (c)(2)).
- 5. Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. have been transmitted by the International Bureau.
- 6. A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
- 7. An oath or declaration of the inventor (35 U.S.C. 371(c)(4)).
- 8. A translation of the Annexes to the International Preliminary Examination Report under PCT Article 36(35 U.S.C. 371(c)(5)).

Other document(s) or information included:

- 9. An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
- 10. An assignment document for recording.
Please mail the recorded assignment document to:
 - a. the person whose signature, name & address appears at the bottom of this page.
 - b. the following:

- 11. The above checked items are being transmitted
 - a. before the 18th month publication.
 - b. after publication and the Article 20 communication but before 20 months from the priority date.
 - c. after 20 months but before 22 months (surcharge and/or processing fee included).
 - d. after 22 months (surcharge and/or processing fee included).
Note: Petition to revive (37 CFR 1.137(a) or (b)) is necessary if 35 U.S.C. 371 requirements submitted after 22 months and no proper demand for International Preliminary Examination was made by 19 months from the earliest claimed priority date.
 - e. by 30 months and a proper demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
 - f. after 30 months but before 32 months and a proper demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date (surcharge and/or processing fee included).
 - g. after 32 months (surcharge and/or processing fee included).
Note: Petition to revive (37 CFR 1.137(a) or (b)) is necessary if 35 U.S.C. 371 requirements submitted after 32 months and a proper demand for International Preliminary Examination was made by 19 months from the earliest claimed priority date.
- 12. At the time of transmittal, the time limit for amending claims under Article 19
 - a. has expired and no amendments were made.
 - b. has not yet expired.
- 13. Certain requirements under 35 U.S.C. 371 were previously submitted by the applicant on _____, namely:
date

14. Submission of Verified Statement Claiming Small Entity Status and Request for Refund

Francis A. Paintin

NAME

Woodcock Washburn Kurtz Mackiewicz & Norris

ADDRESS

One Liberty Place - 46th Floor

Philadelphia, PA 19103

SIGNATURE

REGISTRATION NUMBER

19,386

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name; and

I verily believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: HUMANISED ANTIBODIES the specification of which:

_____ is attached hereto.

was filed on 21 December 1990 as International Application Serial No. PCT/GB90/02017 and was amended on _____ (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with 37 CFR § 1.56(a).

I hereby claim foreign priority benefits under 35 U.S.C. § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of any application on which priority is claimed:

Country	Number	Date Filed	Priority Claimed
<u>U.K.</u>	<u>8928874.0</u>	<u>21.12.89</u>	<u>yes</u>
_____	_____	_____	_____
_____	_____	_____	_____

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17 SEP 1991

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose material information as defined in 37 CFR § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.	Filing Date	Status (patented, pending)
_____	_____	_____
_____	_____	_____
_____	_____	_____

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:
Francis A. Paintin

Registration Nos. 19,386
of the firm of **WOODCOCK WASHBURN KURTZ MACKIEWICZ & NORRIS**, One Liberty Place - 46th Floor, Philadelphia, Pennsylvania 19103, and

Address all telephone calls and correspondence to:
Francis A. Paintin

WOODCOCK WASHBURN KURTZ MACKIEWICZ & NORRIS
One Liberty Place - 46th Floor
Philadelphia, PA 19103
Telephone No. 215-568-3100.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the

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17 SEP 1991

United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00 1	Full Name JOHN ROBERT ADAIR	Inventor's Signature <i>John Robert Adair</i>	Date 13/8/91
	Residence 23 George Road, Stokenchurch High Wycombe, Buckinghamshire HP14 3RN, U.K. GBX		Citizenship U.K.
	Post Office Address 23 George Road, Stokenchurch, High Wycombe, Buckinghamshire HP14 3RN, U.K.		
2-00 2	Full Name DILJEET SINGH ATHWAL	Inventor's Signature <i>[Signature]</i>	Date 13/8/91
	Residence Flat 35, Knollys House, Tavistock Square, London WC1, U.K. GBX		Citizenship U.K.
	Post Office Address Flat 35, Knollys House, Tavistock Square, London WC1, U.K.		
3-00 3	Full Name JOHN SPENCER EMTAGE	Inventor's Signature <i>John Spencer Emtage</i>	Date 13/8/91
	Residence 49 Temple Mill Island, Temple Marlow, Buckinghamshire, SL7 1SQ, U.K. GBX		Citizenship U.K.
	Post Office Address 49 Temple Mill Island, Temple, Marlow, Buckinghamshire SL7 1SQ, U.K.		
4	Full Name	Inventor's Signature	Date
	Residence		Citizenship
	Post Office Address		
5	Full Name	Inventor's Signature	Date
	Residence		Citizenship
	Post Office Address		

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17 SEP 1991

#15

DOCKET NO.: CARP-0009

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

John R. Adair et al.

Serial No.: 07/743,329

Group No.: 1807

Filed: August 16, 1991

Examiner: L. Bennett

For: HUMANISED ANTIBODIES

Certificate of Facsimile Transmission

I hereby certify that the paper is being facsimile transmitted to the Patent and Trademark Office on the date shown below.

On January 19, 1993

D. McMahon

Dawn L. McMahon

Commissioner of Patents & Trademarks
Washington, DC 20231

Sir:

RESPONSE TO OFFICE ACTION

This is in response to the Office Action of November 18, 1992, the time for a response to which is set to expire on January 18, 1993. January 18, 1993 is a Federal Holiday in the District of Columbia (observance of Martin Luther King, Jr.'s birthday). This response is timely filed under 37 CFR §1.7 since it is filed on January 19, 1993, the next succeeding day that is not a Saturday, Sunday or Federal Holiday within the District of Columbia. Please amend the above-identified patent application as follows.

In the claims:

21. (amended) A process for producing [a CDR-grafted antibody product] ~~an antigen-binding molecule~~ comprising:

B1

Carter Exhibit 2007
Carter v. Adair
Interference No. 105,744

(a) producing in an expression vector an operon having a DNA sequence which encodes a heavy chain according to [Claim 1] Claim 24 or Claim 25;

[and/or]

B1
Cmt.

(b) producing in an expression vector an operon having a DNA sequence which encodes a complementary light chain according to Claim [6] 42 or Claim [8] 43;

(c) transfecting a host cell with the [or each vector] expression vectors of steps (a) and (b) to form a transfected cell containing said expression vectors;

and

(d) culturing [the] said transfected cell [line] to produce [the CDR-grafted antibody product] said antigen binding molecule.

Please cancel claims 1-20, 22 and 23 without prejudice and substitute therefor the following new claims.

24. An antigen-binding molecule having affinity for a predetermined antigen, comprising a heavy chain and a complementary light chain, said heavy chain having a variable domain comprising framework regions from an acceptor antibody heavy chain and antigen binding regions from the heavy chain of a donor antibody which has affinity for said predetermined antigen, wherein amino acid residues 23, 24, 31 to 35, 49 to 58 and 95 to

B2

102, according to the Kabat numbering system, of said heavy chain are donor antibody residues.

25. The antigen binding molecule of claim 24 wherein residues 71, 73 and 78, according to the Kabat numbering system, in the heavy chain are additionally donor antibody residues.

26. The antigen-binding molecule of claim 24, wherein residues 26 to 30, according to the Kabat numbering system, in the heavy chain are additionally donor antibody residues.

B2 cont.
27. The antigen-binding molecule of claim 25, wherein residues 26 to 30, according to the Kabat numbering system, in the heavy chain are additionally donor antibody residues.

28. The antigen-binding molecule of claim 24, wherein residues 59 to 65, according to the Kabat numbering system, in the heavy chain are additionally donor antibody residues.

29. The antigen-binding molecule of claim 25, wherein residues 59 to 65, according to the Kabat numbering system, in the heavy chain are additionally donor antibody residues.

30. The antigen-binding molecule of claim 24, wherein residues 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107, according to the Kabat numbering system, in the heavy chain are additionally donor antibody residues.

31. The antigen-binding molecule of claim 25, wherein residues 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106

and 107, according to the Kabat numbering system, in the heavy chain are additionally donor antibody residues.

32. The antigen-binding molecule of claim 30, wherein, according to the Kabat numbering system, if residue 48 in the donor antibody heavy chain is different from residue 48 in the acceptor antibody heavy chain, then residue 69 in the heavy chain is additionally a donor antibody residue.

33. The antigen-binding molecule of claim 31, wherein, according to the Kabat numbering system, if residue 48 in the donor antibody heavy chain is different from residue 48 in the acceptor antibody heavy chain, then residue 69 in the heavy chain is additionally a donor antibody residue.

34. The antigen-binding molecule of claim 30, wherein residues 38 and 46, according to the Kabat numbering system, in the heavy chain are additionally donor antibody residues.

35. The antigen-binding molecule of claim 31, wherein residues 38 and 46, according to the Kabat numbering system, in the heavy chain are additionally donor antibody residues.

36. The antigen-binding molecule of claim 32, wherein residues 20 and 80, according to the Kabat numbering system, in the heavy chain are additionally donor antibody residues.

37. The antigen-binding molecule of claim 33, wherein residues 20 and 80, according to the Kabat numbering system, in the heavy chain are donor antibody residues.

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cont.*

38. The antigen-binding molecule of claim 30, wherein residues 18, 67 and 82, according to the Kabat numbering system, in the heavy chain are donor antibody residues.

39. The antigen-binding molecule of claim 31, wherein residues 18, 67 and 82, according to the Kabat numbering system, in the heavy chain are donor antibody residues.

40. The antigen-binding molecule of claim 30, wherein residues 1, 3, 9, 11, 41, 72, 76, 87, 88, 91, 108, 110 and 112, according to the Kabat numbering system, in the heavy chain are donor antibody residues.

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cont.*

41. The antigen-binding molecule of claim 31, wherein residues 1, 3, 9, 11, 41, 72, 76, 87, 88, 91, 108, 110 and 112, according to the Kabat numbering system, in the heavy chain are donor antibody residues.

42. An antigen-binding molecule having affinity for a predetermined antigen, comprising a light chain and a complementary heavy chain, the light chain having a variable domain comprising framework regions from an acceptor antibody light chain and antigen binding regions from the light chain of a donor antibody which has affinity for said predetermined antigen, wherein amino acid residues 24 to 34, 46, 48, 50 to 56, 58, 71 and 89 to 97, according to the Kabat numbering system, are donor antibody residues.

43. The antigen-binding molecule of claim 42, wherein residues 2, 4, 6, 35, 38, 44, 47, 49, 64 to 69, 85, 87, 98, 99, 101 and 102, according to the Kabat numbering system, in the heavy chain are donor antibody residues.

44. The antigen-binding molecule of claim 42, wherein, according to the Kabat numbering system, if residue 60 in the light chain can form a salt bridge with residue 54 in the light chain, then residue 60 in the light chain is a donor antibody residue.

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cont.*

45. The antigen-binding molecule of claim 42, wherein, according to the Kabat numbering system, if residue 70 in the light chain can form a salt bridge with residue 24 in the light chain, then residue 70 in the light chain is a donor antibody residue.

46. The antigen-binding molecule of claim 43, wherein, according to the Kabat numbering system, residues 21 and 73 in the light chain are additionally donor antibody residues.

47. The antigen-binding molecule of claim 43, wherein, according to the Kabat numbering system, residues 37 and 45 in the light chain are additionally donor antibody residues.

48. The antigen-binding molecule of claim 43, wherein residues 1, 3, 10, 12, 40, 63, 80, 103 and 105, according to the Kabat numbering system, in the light chain are additionally donor antibody residues.

49. An antigen-binding molecule having affinity for a predetermined antigen, comprising a heavy chain and a complementary light chain:

the heavy chain having a variable domain comprising framework regions from an acceptor antibody heavy chain and antigen binding regions from the heavy chain of a donor antibody which has affinity for said predetermined antigen, wherein amino acid residues 23, 24, 31 to 35, 49 to 58 and 95 to 102, accordingly to the Kabat numbering system, are donor antibody residues; and

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cont.*
the complementary light chain having a variable domain comprising framework regions from an acceptor antibody light chain and antigen binding regions from said donor antibody light chain wherein amino acid residues 24 to 34, 46, 48, 50 to 56, 58, 71 and 89 to 97 according to the Kabat numbering system are donor antibody residues.

50. The antigen-binding molecule of claim 49 wherein residues 71, 73, 78 and 95 to 102, according to the Kabat numbering system, of said heavy chain are additionally donor antibody residues.

51. The antigen-binding molecule of claim 49 or claim 50, which is a site specific antibody molecule.

52. The antigen-binding molecule of claim 49 or claim 50, which has specificity for an antigen selected from the group

consisting of interleukins, hormones, biologically active compounds and receptors therefor.

53. A DNA molecule which encodes an antigen-binding molecule heavy chain having a variable domain comprising framework regions from the heavy chain of a donor antibody which has affinity for said predetermined antigen wherein amino acid residues 23, 24, 31 to 35, 49 to 58 and 95 to 102 of said heavy chain, according to the Kabat numbering system, are donor antibody residues.

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Cmt.* 54. The DNA molecule of claim 53 wherein residues 71, 73 and 78, according to the Kabat numbering system, of said heavy chain are additionally donor antibody residues.

55. A DNA molecule which encodes an antigen-binding molecule light chain having a variable domain comprising framework regions from an acceptor antibody light chain and antigen binding regions from the light chain of an antibody having affinity for a predetermined antigen wherein amino acid residues 24 to 34, 46, 48, 50 to 56, 58, 71 and 89 to 97, according to the Kabat numbering system, of said light are donor antibody residues.

56. A cloning vector comprising a DNA molecule according to claims 53, 54 or 55.

57. An expression vector comprising a DNA molecule according to Claims 53, 54 or 55.

58. A host cell transformed with a DNA molecule according to claims 53, 54 or 55.

59. A therapeutic composition comprising a therapeutically effective amount of an antigen-binding molecule according to claims 24, 25 or 42 in combination with a pharmaceutically acceptable carrier.

60. A therapeutic composition comprising a therapeutically effective amount of an antigen-binding molecule according to claim 49 or claim 50 in combination with a pharmaceutically acceptable carrier.

B2 cont.
61. A diagnostic composition comprising a diagnostically effective amount of an antigen-binding molecule according to claims 24, 25 or 42 in combination with a diagnostically acceptable carrier.

62. A diagnostic composition comprising a diagnostically effective amount of an antigen-binding molecule according to claim 49 or claim 50 in combination with a diagnostically acceptable carrier.

63. A method of therapy comprising administering to a human or animal subject an antigen-binding molecule according to claims 24, 25 or 42.

64. A method of diagnosis comprising administering to a human or animal subject a composition according to claims 24, 25 or 42.

65. A method for producing an antigen-binding molecule having affinity for a predetermined antigen comprising the steps of:

(1) providing a heavy chain for an antigen-binding molecule, said heavy chain having acceptor framework regions and donor antigen binding regions wherein amino acid residues 23, 24, 31 to 35, 49 to 58 and 95 to 102 according to the Kabat numbering system are donor antibody residues;

(2) associating the heavy chain of step (1) with a complementary light chain to form an antigen-binding molecule;

(3) determining the affinity of the antigen-binding molecule formed in step (2) for said predetermined antigen;

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Cont.
(4) if the affinity determined in step (3) is not equivalent to that of the donor antibody, providing a heavy chain as described in step (1) in which amino acid residues 71, 73 and 78, according to the Kabat numbering system, are additionally donor antibody residues;

(5) associating the heavy chain of step (4) with a complementary light chain to form an antigen-binding molecule;

(6) determining the affinity of the antigen-binding molecule formed in step (5) for said predetermined antigen;

(7) if the affinity determined in step (6) is not equivalent to that of the donor antibody, providing a heavy chain as described in step (4) in which amino acid residues 26 to 30,

according to the Kabat numbering system, are additionally donor antibody residues;

(8) associating the heavy chain of step (7) with a complementary light chain to form an antigen-binding molecule;

(9) determining the affinity of the antigen-binding molecule formed in step (8) for said predetermined antigen;

(10) if the affinity determined in step (9) is not equivalent to that of the donor antibody, providing a heavy chain as described in step (7) in which amino acid residues 59 to 65, according to the Kabat numbering system, are additionally donor antibody residues;

B2 Cont.
(11) associating the heavy chain of step (10) with a complementary light chain to form an antigen-binding molecule;

(12) determining the affinity of the antigen-binding molecule formed in step (11) for said predetermined antigen;

(13) if the affinity determined in step (12) is not equivalent to that of the donor antibody, providing a heavy chain as described in step (10) in which amino acid residues 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107, according to the Kabat numbering system, are additionally donor antibody residues; and

(14) associating the heavy chain produced in step (13) with a complementary light chain to form an antigen-binding molecule.

66. A method for producing an antigen-binding molecule having affinity for a predetermined antigen comprising the steps of:

(1) providing a light chain for an antigen-binding molecule, said light chain having acceptor framework regions and donor antigen binding regions wherein amino acid residues 24 to 34, 46, 48, 50 to 56, 58, 71 and 89 to 97 according to the Kabat numbering system are donor antibody residues;

(2) associating the light chain of step (1) with a complementary heavy chain to form an antigen-binding molecule;

(3) determining the affinity of the antigen-binding molecule formed in step (2) for said predetermined antigen;

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CMT.*
(4) if the affinity determined in step (3) is not equivalent to that of the donor antibody, providing a light chain as described in step (1) in which amino acid residues 2, 4, 6, 35, 38, 44, 47, 49, 64 to 69, 85, 87, 98, 99, 101 and 102, according to the Kabat numbering system, are additionally donor antibody residues;

(5) associating the light chain of step (4) with a complementary heavy chain to form an antigen-binding molecule;

(6) determining the affinity of the antigen-binding molecule formed in step (5) for said predetermined antigen;

(7) if the affinity determined in step (6) is not equivalent to that of the donor antibody, providing a light chain

as described in step (4) in which amino acid residues 21, 37, 45, 60, 70 and 73, accordingly to the Kabat numbering system, are additionally donor antibody residues;

(8) associating the light chain of step (7) with a complementary heavy chain to form an antigen-binding molecule;

(9) determining the affinity of the antigen-binding molecule formed in step (8) for said predetermined antigen;

(10) if the affinity determined in step (9) is not equivalent to that of the donor antibody, providing a light chain as described in step (7) in which amino acid residues 1, 3, 10, 12, 40, 63, 80, 103 and 105, according to Kabat numbering system, are additionally donor antibody residues; and

(11) associating the light chain of step (10) with a complementary heavy chain to form an antigen-binding molecule.

REMARKS

The Office Action of November 18, 1992 has been carefully considered and this response prepared. Claims 1-20, 22 and 23 have been cancelled without prejudice and replaced with new claims 24-66. Claim 21 has been amended to more clearly point out aspects of the claimed method. Support for the newly submitted claims can be found throughout the specification, particularly at pages 17 and 18, page 19, line 23, page 41, Table 1 (each relating to the heavy chain), pages 17-19 (relating to

the light chain), and pages 17-23 (relating to methods for production of antigen-binding molecules).

At paragraph 15 of the Office Action, the Examiner objected to the disclosure, stating that various words in the specification are misspelled.

It is respectfully submitted that the spellings referred to by the Examiner are not incorrect. The use of an "s" instead of a "z" in such words as "recognise" is acceptable English spelling. In fact, the use of "s" is the original English spelling and the use of "z" has only more recently been recognized as being acceptable. In this respect, the Examiner is referred to the enclosed page from Chambers 20th Century Dictionary (1973 Edition) which illustrates this point. Therefore, no amendments have been made. Withdrawal of this objection to the specification is respectfully requested.

At paragraph 16 of the Office Action, the Examiner objected to claims 5, 11-16, 22 and 23 under 37 CFR §1.75(c) as being in improper form because a multiple dependent claims cannot depend from any other multiple dependent claim.

Claims 5, 11-16, 22 and 23 have been cancelled without prejudice and substituted with new claims having proper dependent form. Withdrawal of this objection to the specification is respectfully requested.

At paragraph 17 of the Office Action, the Examiner objected to claims 1-23 stating that the term "CDR-grafted" is improper because abbreviations and acronyms are not appropriate in claim language.

The term CDR-grafted has been deleted from the claims. Withdrawal of this objection to the claims is respectfully requested.

At paragraph 18 of the Office Action, the Examiner rejected claims 1-12 under 35 USC §101. This rejection is divided into four parts which will be answered separately.

In Part A of this section 101 rejection, claims 1-12 were rejected as being inoperative and thus lacking utility. It was stated that the claims are drawn to single heavy or light chains and that there is no evidence in the specification indicating that heavy and light chains alone have activity.

Applicants respectfully traverse this rejection.

It is respectfully submitted that there is no reason to show that the isolated antibody heavy and light chains of the present invention have utility in binding to antigen. As the Examiner has correctly pointed out, the assembled antibodies of the invention have been shown to be able to bind to antigen. What has also been shown is that the isolated heavy and light chains have utility in that they can be used to form the antigen-binding antibody. Thus, the isolated chains have patentable

utility as intermediates for use in CDR-grafted antibodies. There is no requirement that the claimed invention be the ultimate product.

Nonetheless, in order to reduce the number of issues to be dealt with, claims 1-12 have been cancelled without prejudice and new claims have been submitted that are drawn to antibody-binding molecules having heavy and light chains. However, the Applicants reserve the right to file a divisional application relating to the isolated chains.

In Part B of this section 101 rejection, claim 17 was rejected as being drawn to non-statutory subject matter. The Examiner stated that claim 17 is drawn to a DNA sequence coding for a CDR-grafted heavy or light chain, but that "DNA sequences" are not patentable because they are algorithms. The Examiner suggested amending the claims to recite "DNA molecule" instead of "DNA sequence".

Applicants respectfully traverse this rejection. An algorithm is a procedure for solving a given type of mathematical problem. DNA sequences are chemical compounds. Thus the prohibition of section 101 against granting patents on mathematical algorithms does not apply to DNA sequences.

Applicants respectfully submit that the term DNA sequence is proper claim language. This language appears in the claims of issued patents as well as the rules issued by the

Patent and Trademark Office for applications containing nucleic acid and/or amino acid sequences (see 37 CFR §§1.821 - 1.825). In order to advance prosecution of this application, however, the term "DNA molecule" has been substituted for "DNA sequence" in the claims.

In part C of this section 101 rejection, the Examiner rejected claims 22-23 as inoperative and therefor lacking utility. The Examiner stated that the specification fails to establish the utility of the claim method and pharmaceutical composition using a CDR-grafted antibody in humans or any other animal. The Examiner further stated that the specification does not present any *in vivo* or *in vitro* data to support the claims, and that pharmaceutical therapy with reshaped monoclonal antibodies is unpredictable in the absence of *in vivo* clinical data.

Applicants respectfully traverse this rejection. The Examiner asserts that because the application does not contain any *in vivo* data, it is not possible for a person of ordinary skill in the art to use the humanized antibodies shown in the application in human therapy and diagnosis *in vivo*. It is submitted that at the priority date of the application a person of ordinary skill, having read the application, would have had every reason to expect that a humanized antibody, for instance

one directed against the CD3 antigen, would be useful diagnostically or therapeutically *in vivo*.

For any antibody to be useful *in vivo* in therapy or diagnosis, there are two main factors to consider. The first is the affinity of the antibody for the antigen. The second is the bioavailability of the antibody after administration. As regards the affinity, clearly the antibody would be of no use if its affinity were so low that it did not bind to the antigen, however much of the antibody was available to be bound. Similarly, if the antibody can never reach the antigen, for instance because it is degraded too fast, it will be of no use, however strongly it binds to the antigen.

In the present case, it is clearly shown that the humanized antibodies according to the invention have equivalent binding affinity to that of the prototype murine antibody such as OKT3, OKT4, 61E71 or B72.3. It is therefore plain to anyone with reasonable knowledge in this field that, if the humanized antibody can reach its site of action, it will be able to bind effectively to the appropriate antigen. The *in vitro* data given in the application clearly show that the humanized antibody would be expected to be equally effective as the prototype antibody once it reaches the relevant site.

As to bioavailability, it is plain to anyone with reasonable knowledge of the art that the humanized antibodies of

the present invention are predominantly human in appearance to the human immune system. The only part which may appear foreign is the antigen binding site. However, in this respect, the humanized antibody is no different from a natural human antibody. The antigen binding site in a natural human antibody is also, eventually, regarded as being foreign and the body mounts an immune response against it. This is called an anti-idiotypic response and may be involved in the regulation of the immune system.

In view of the close, overall similarity between the humanized antibodies of the present invention and a natural human antibody, anyone with reasonable knowledge in the field would expect that the humanized antibody would have about the same bioavailability as a normal human antibody. The knowledgeable person would therefore expect that the humanized antibody would have very good bioavailability.

The skilled person would certainly expect that the humanized antibody would have better bioavailability than the prototype murine antibody. The prototype antibody is entirely of mouse origin and would therefore be regarded as being foreign by a human immune system. The human immune system would therefore

set up a response against the murine antibody which would lead to its more rapid clearance from the body.

The knowledgeable person would therefore have expected the humanized antibody of the present invention to have good bioavailability and would have seen from the *in vitro* data in the application that it has acceptable affinity. There would therefore have been every reason to expect that the humanized antibody would have good therapeutic and diagnostic uses *in vivo*.

The skilled person would certainly have known that OKT3, even though it is a totally murine antibody, is a highly effective therapeutic agent, especially in the treatment of acute organ transplant rejection episodes. This is very well documented in the prior art. On the basis of the data given in the present application, it would be expected that the humanized antibody could be used in the same way as is OKT3 to treat the same conditions.

The skilled person would also have known that OKT4, 61E71 and B72.3, while not approved for general use, have been used therapeutically and diagnostically in certain limited circumstances, demonstrating their utility. It would again be expected that this same utility could be demonstrated by the humanized versions of these murine antibodies.

The knowledgeable person would not need any data correlating the *in vitro* activity with *in vivo* use to come to

this conclusion. There is nothing in the art which would lead him in any way to expect that the *in vitro* data did not give a reasonable basis for a confident prediction of *in vivo* utility. Certainly, the Applicants are not aware of any studies which show that *in vitro* results with antibodies cannot be extrapolated to *in vivo* results.

The Examiner raises various possible reasons why the humanized antibodies might not work. However, the Examiner has provided no documentary evidence to support these suggestions. In the absence of such documentary evidence, it is respectfully submitted that these reasons represent no more than speculation. In any event, the speculations are unfounded. As to (1), there is no reason why a humanized antibody should be any more subject to proteolytic degradation than the murine prototype antibodies or normal human antibodies. Both murine and normal human antibodies are subjected to such degradation in the human body, in order to clear unwanted antibodies from the system. However, since the rate of clearance is generally of the order of a week or two, such degradation will not prevent their efficacious use. As to (2), it has been shown that murine antibodies reach the required target site. Why should a humanized antibody not reach the target site also? As to (3), the data in the specification show that acceptable affinity can be obtained. The specification also shows how the choice of the heavy chain constant domains

leads to desired effector functions. Thus, there is no basis for the Examiner's assertions. As to (4), all antibodies are eventually recognized as foreign and cleared from circulation. There is no reason to expect a rejection reaction, as discussed above, since the antibodies are predominantly human in appearance.

It is therefore submitted that there is no force in any of the arguments put forward by the Examiner. Accordingly, one skilled in the art is adequately taught by the specification how to practice the claimed invention. Withdrawal of this 35 USC §101 rejection is respectfully requested.

At paragraph 19 of the Office Action, the Examiner objected to the specification and rejected claims 1-12 under 37 USC §112, first paragraph as failing to adequately teach how to make and use the claimed invention. The Examiner stated that isolated heavy and light chains appear to be inoperable for binding antigen and as a consequence undue experimentation would be required of the skilled artisan in order to practice the invention.

It is respectfully submitted that the points made in response to the section 101 rejection above also apply here. The isolated chains have patent utility as intermediates in the preparation of the antibodies which have been demonstrated as having end use utility. The point is now moot, however, in view

of the cancellation of claims 1 through 12 and the submission of new claims drawn to antigen-binding molecule having both heavy and light chains. Withdrawal of this 35 USC §112, first paragraph rejection is respectfully requested.

At paragraph 20 of the Office Action, the Examiner objected to the specification and rejected claims 22 and 23 under 35 USC §112, first paragraph as failing to adequately teach how to make and use the claimed invention. The Examiner stated that the claimed invention appears to be inoperable without supporting *in vivo* data for the reasons discussed in the rejection made under 35 USC §101, and therefore undue experimentation would be required of the skilled artisan in order to practice the claimed invention.

Applicants respectfully traverse this rejection.

A person of ordinary skill in the art could, without the need for undue experimentation, practice the invention disclosed in the present application. It should be borne in mind that the use of murine monoclonal antibody OKT3 for therapy and murine monoclonal antibodies OKT3, OKT4, 61E71 and B72.3 for diagnosis has been reported in the prior art. Thus, therapeutic treatment regimes, amounts used per treatment, intervals between treatments, routes of administration, etc. for OKT3 and diagnostic regimes for all four antibodies are well known in the art. Since the *in vitro* results show that the humanized

antibodies of the present invention have the same affinity as the prototype antibodies and since it can reasonably be predicted that the humanized antibodies will have as good, if not better, bioavailability as the prototype antibodies, the knowledgeable person will confidently adopt the same sort of treatment regimes as are adopted for the prototype antibodies.

It is therefore submitted that the invention claimed in the present application can readily be put in to effect by the skilled person. Withdrawal of this 35 USC §112, first paragraph rejection is respectfully requested.

At paragraph 21 of the Office Action, the Examiner rejected claims 13-16 under 35 USC §112, first paragraph stating that the disclosure is enabling only for claims limited to specific CDR-grafted antibodies disclosed in the specification as having effective binding affinities for their specific antibody, i.e. which are similar to the non-humanized donor antibodies.

Applicants respectfully traverse this rejection. The Examiner contends that the present specification is only enabling in respect of the exemplified humanized antibodies because, in the Examiner's view, it would require undue experimentation to adapt the teaching in the present application to other antibodies. This view appears to be based on an analysis of the prior art rather than an analysis of the present invention.

It is indeed true that in the prior art, there is very little guidance as to how to go about producing a recombinant antibody which has the same antigen binding affinity as the prototype antibody. The most guidance is provided by Queen et al. (PNAS-USA, 86, 10029-10033, 1989). However, the amount of guidance provided is small. Although it is not explicitly stated in Queen et al., it is clear that it is necessary before beginning the process described therein to determine the amino acid sequence of the antibody chain to be recombinant. The skilled person reading Queen et al. is told firstly to select a human chain which is as closely comparable to the murine chain as possible. It is then necessary to carry out computer modelling of the chain to determine which residues outside the Kabat CDRs may be important for antigen binding or retaining the appropriate shape in the antigen binding region. This computer modelling is by no means trivial as it requires the modeler to make a number of critical choices of parameters will lead to a different model and may well lead to a different determination of non-CDR residues.

By carrying out this procedure, Queen et al. identified a number of residues outside the CDR which are altered in order to improve the affinity of the recombinant antibody. However, Queen et al. provides no guidance as to which residues are critical for improving affinity in the particular case referred

to by Queen et al. (the anti-TAC antibody). Moreover, there is no indication that it might be possible to change the same residues in a different antibody in the expectation of achieving good affinity.

It is also to be noted that Queen et al. does not use the Kabat numbering for the amino acid residues. The sequences are merely numbered in a linear fashion. It is thus not possible readily to determine which residues according to the Kabat numbering system were altered. This makes the teaching in Queen et al. even more specific to the particular antibody shown by Queen et al.

Thus, the teaching of Queen et al. is that, in order to reshape any antibody, it is necessary to treat each antibody individually and to carry out for each antibody the steps of sequence determination, acceptor sequence selection and computer modelling. Clearly, this requires undue experimentation to apply the teaching of Queen et al. to other antibodies.

In contrast, the teaching in the present application can be applied without any undue experimentation to any antibody. All that is required is experimentation following a protocol which is clearly set out in the description, in particular at page 16, line 30 to page 19, line 9. In order to follow this protocol, as a first step, it is necessary to determine the amino acid sequence of the donor chain. The sequence of the acceptor

chain will already be known, for instance from a sequence data base.

There is then no need to carry out computer modelling to determine which donor residues to substitute into the acceptor sequence. The protocol in the present application provides the teaching directly. It instructs the skilled person to compare the two sequences and change certain specified residues in the acceptor sequence to donor residues.

Moreover, the present application provides a hierarchical structure of residues which can be considered. Thus, if changing the residues identified at the top of the structure does not provide adequate affinity, then a lower level of residues are considered, and so on until acceptable affinity is obtained.

The manipulation of an acceptor sequence using recombinant DNA technology is a matter of routine. For instance, a known sequence can be altered using site directed mutagenesis. Alternatively, a complete sequence can be synthesized from isolated nucleotides. The chain having the altered amino acid sequence can be tested for affinity using assays of the type described in the application. Thus, producing recombinant chains and testing them for affinity merely involves routine experimentation following a protocol which is clearly defined in the application.

It is submitted that this identifies where the present invention makes a significant departure from the prior art. The prior art indicates that each antibody has to be treated individually. In contrast, the present invention teaches that, by following the protocol set forth in the present application, it is possible to reshape any antibody.

The Examiner pointed to the fact that the present application refers to only three antibodies (OKT3, OKT4 and anti-ICAM) and contends that this is no basis for predicting that the protocol is generally application. It is respectfully submitted that the Examiner is incorrect. In the first place, the application refers to nine different antibodies (OKT3, OKT4, RG-5, B72.3, 61R71, 101.4, hTNF1, hTNF2 and hTNF3) (see page 52, last paragraph) which have been humanized successfully using the protocol set out in the application. It therefore cannot be seen how it can be contended that the concept underlying the present claims has not been fully developed. Since it has proved possible to humanize all nine antibodies, it is submitted that a reasonably skilled person would readily predict that the concept is applicable to other antibodies.

The Examiner refers to the absence from the application of binding affinity values and alleges that the description of binding affinity is only qualitative. It is submitted that this is not the case. Figures 7 to 13 clearly show data. On page 60,

second paragraph, the binding affinity of the humanized anti-ICAM antibody is given a quantitative value. On page 62, quantitative statements regarding binding activity are also given. Thus, the application does contain quantitative data showing that the concept is applicable to produce a useful antigen-binding molecule.

It is therefore submitted that the description is fully enabling without the need for undue experimentation. Withdrawal of this 35 USC §112 rejection is therefore respectfully requested.

At paragraph 22 of the Office Action, the Examiner rejected claims 1-23 under 35 USC §112, second paragraph as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention. This rejection is in several parts which will be answered separately.

In Part A of the rejection, the Examiner stated that claims 1-5 are indefinite due to the language of the claims relating to donor positions and because the position numbers are arbitrary.

Claims 1-5 have been cancelled without prejudice. The newly submitted claims do not contain the language found objectionable by the Examiner. The residue numbers in the newly submitted claims refers to the numbering system devised by Kabat.

This is a standard numbering system used to enable comparisons of antibody chain amino acid sequences to be made.

The Kabat numbering system was developed by studying the amino acid sequences of a large number of heavy and light chains. It was discovered that, within the variable domains, there were some residues which are highly conserved, some areas in which sequence variation is low and some areas in which the sequence variation is high. On the basis of the highly conserved residues and the low variability areas, it proved to be possible to assign numbers to residues in all heavy and light chains.

In some cases, it was found that there had been deletions in the sequence. In other cases, there were additional residues. Generally, these additions or deletions occurred in the highly variable areas. In order to allow for this, in some sequences the numbering has a jump in it to account for a deletion and in other sequences there are a number of residues with the same number, followed by a, b, c, etc., to account for insertions. Nonetheless, it is still readily possible to align antibody chain sequences on the basis of the Kabat numbering. This is a system which is widely used and commonly recognized in the art. In this respect, reference can be made to Riechmann et al., page 325, first sentence after "Strategy".

It is therefore believed that the language now used in the claims is in accordance with the requirements of section 112.

In Part B of the rejection, the Examiner stated that claims 6-12 are also indefinite due to the language of the claims relating to donor positions and because the position numbers are arbitrary.

Claims 6-12 have been cancelled without prejudice. The newly submitted claims do not contain the language found objectionable by the Examiner. The position numbers of the donor residues refer to the numbering system devised by Kabat, as discussed in the response to Part A of this rejection.

In Part C of the rejection, the Examiner stated that claims 4 and 11 are indefinite and confusing because they are in an improper Markush listing.

Claims 4 and 11 have been cancelled without prejudice. This rejection is now moot.

In Part D of the rejection, the Examiner stated that claim 21 is indefinite. Claim 21 has been amended to correct an inadvertent clerical in step (c), so that step (c) now recites "transfecting a host cell with the expression vectors of steps (a) and (b) to form a transfected cell containing said expression vectors". Step (c) indicates that the expression vectors are transfected into one host cell. The amendment to step (c) also provides an antecedent basis for "the transfected cell line" in step (d).

The use of the term "complementary" in claim 21 does not render the claim indefinite. It is submitted that this is clear to the person skilled in the art. It is well known that it is possible for almost any heavy chain to associate to some extent with almost any light chain. However, randomly associated heavy/light chain dimers will generally not have any antigen binding activity. Antigen binding activity will only be obtainable if the heavy and light chains are of such sequence and configuration as to provide antigen binding regions which fit together to form a site which binds to a desired antigen. It is well known to the art that a heavy and a light chain which, when associated, form a binding site for a desired antigen are said to be complementary. Thus, in the present claims, "complementary" is used in the way it is normally understood by those skilled in the art and does not introduce any lack of clarity in the claims. Withdrawal of this entire 35 USC §112, second paragraph rejection is respectfully requested.

At paragraph 23 of the Office Action the Examiner rejected the claims under 35 USC §102(b). This rejection is divided into two parts with will be answered separately.

In Part A of this rejection, claims 1, 5, 6-8, and 12-22 were rejected as anticipated by Riechmann et al. The Examiner stated that claim 1 and claim 6 were interpreted to mean that the framework has donor residues in at least one of any of positions

6, 23, 24, 48, 49, 71, 73, 75, 76, 78, 88, or 91 in the heavy chain and (1, 3, 46, or 47) or 46, 48, 58, or 71) in the light chain, and thus, the teachings of Riechmann et al. anticipate the invention as claimed.

The Examiner contends that the original claims lacked novelty over Riechmann et al. Claims 1, 5, 6-8, 12 and 22 have been cancelled without prejudice and submitted as new claims that more distinctly point out certain aspects of the present invention.

In present claims 24 and 25, it is specified that residues 23 and 24 in the heavy chain should be donor residues. However, as can be seen from Fig. 1, panel (a) in Riechmann et al., in the recombinant antibody shown there, residues 23 and 24 are acceptor residues.

In the present claim 42, it is specified that residue 58 in the light chain should be a donor residue. However, as can be seen from Fig. 1, panel (b) in Riechmann et al., in the recombinant antibody shown there, residue 58 is an acceptor residue. Applicants' claimed antigen-binding molecules are thus not anticipated by Riechmann et al.

In Part B of the rejection, the Examiner rejected claims 1-6 and 12-22 as anticipated by Queen et al.

Claims 1-6, 12-20 and 22 have been cancelled without prejudice and submitted as new claims that more distinctly point out certain aspects of the present invention.

In present claims 24 and 25, it is specified that residues 48, 66, 67, 68, 93, 103 to 108 and 110 should all be acceptor residues. However, in Queen et al., as can be seen from Fig. 2B, in these positions Queen et al. uses donor, rather than acceptor, residues. It should again be borne in mind that Queen et al. does not use the Kabat numbering and it is therefore necessary to look carefully at the disclosure in Queen et al. before it is possible to come to any final conclusion.

In present claim 38, it is specified that residue 71 should be a donor residue. However, as can be seen from Fig. 2A of Queen et al., in that position Queen et al. uses an acceptor, rather than a donor residue.

Applicants' claimed antigen-binding molecules are thus not anticipated by Queen et al. Withdrawal of this entire 35 USC §102(b) rejection is respectfully requested.

At paragraph 24 of the Office Action, the Examiner rejected claims 1-21 under 35 USC §103 as being obvious over Riechmann et al. in view of Queen et al. The Examiner states

It would have been *prima facie* obvious to one of ordinary skill in the art in the art at the time the invention was made to use the guidelines taught by Riechmann et al. and Queen et al. to reshape any given antibody to

"humanize" that antibody by making changes in the framework regions of the human acceptor to the donor residue when those residues are close to the CDR's and when those amino acids affect the conformation of the CDR's.

Applicants respectfully traverse this rejection.

The Examiner contended that all the previous claims lacked an inventive step over either Riechmann et al. or Queen et al. It is submitted, for the following reasons, that the Examiner's contention is unfounded.

With respect to Riechmann et al., it is submitted that this document does not go much beyond the original idea of Winter et al. (see WO-A) 89/07452 referred to on page 4 of the present application) of transferring only the CDRs to a human framework. Thus, Riechmann et al. shows transferring the hypervariable regions identified according to Kabat (i.e. the Kabat CDRs) to a human framework (see Riechmann et al., page 325, after "Strategy"). This basic concept was to a certain extent modified for the heavy chain on the basis of the difference between Kabat CDR1 (as defined by sequence) and CDR1 as defined by structural studies.

On the basis of specific sequence differences in structural CDR1 between the rat (donor) and human (acceptor) sequences, Riechmann et al. decided to change residues 27 and 30 in the acceptor sequence to the equivalent residues in the donor sequence. It is made clear that these sequence changes were made

because the rat (donor) sequence was unusual. It is also to be noted that Riechmann et al. made no other changes outside the CDRs in the heavy chain.

As regards the light chain, all that Riechmann et al. did was to transfer the rat CDRs (as determined by Kabat) to the human acceptor framework. No residue changes outside the CDRs were made.

It can thus be seen that, at best, Riechmann et al. teaches the skilled person to transfer the six CDRs and to look at the possibility of transferring a composite CDR1 comprising a combination of the Kabat and structural CDR1, but only if there are any unusual residues in the area of the structural CDR1 not covered by the Kabat CDR1. Even this teaching, however, is specific to the particular antibody considered by Riechmann et al. There is nothing in Riechmann et al. to suggest that this teaching is generally applicable to other antibodies.

In any event, Riechmann et al. does not in any way suggest that altering residues remote from the CDRs might be effective in improving affinity. Certainly, Riechmann et al. in no way suggest that there might be a hierarchy of residues which should be considered if improvements in affinity are to be sought. Still less does Riechmann et al. provide any teaching which would allow the skilled person to identify the hierarchy of residues as set forth in the present application.

It is therefore submitted that the subject matter of present claims is in no way suggested by Riechmann et al. Thus, the present invention is not at all obvious over Riechmann et al.

As regards Queen et al., as the Examiner has noted, the teaching there is quite different from that in the present application. Queen et al. teaches that the amino acid sequence of the antibody chain in question (donor) should be determined and then compared to that of known acceptor chains. An acceptor chain should then be chosen which is as homologous as possible to the donor chain.

The next step is to carry out a computer modelling exercise to determine which residues might be involved in antigen binding or in ensuring that the antigen binding site adopts the correct conformation. It is again to be pointed out that computer modelling can lead to different results, depending on the parameter choices made. Thus, following Queen et al. may not always lead to the same results. That this is the case can be seen from page 10031, paragraph 3, lines 13 to 15, which shows that an earlier model differed from a later model.

It is also to be pointed out that the criteria for making the choices are indeterminate. All that Queen et al. indicates is that the residues need to be "close enough" to the CDR's (see page 10031, paragraph 3). There is no indication as to how close is "close enough". It is therefore difficult for

the skilled person to follow the teaching of Queen et al. without having to make critical decisions on which no guidance is given.

Once these exercises have been carried out, the acceptor sequence is altered so that not only the Kabat CDR's but also all the other residues outside the CDRs identified by the modelling procedure are changed to donor residues.

The fact that the donor sequence is compared to a number of possible acceptor sequences and that a computer model of the donor antibody must be made shows that the whole procedure in Queen et al. is specific to one antibody at a time. Underlying these facts is the assumption that each antibody is different from other antibodies and that it is not possible to predict from work carried on one antibody how to deal with another antibody.

It is no doubt true that implication of Queen et al. is that carrying out the procedure disclosed therein will enable one to produce a recombinant antibody having at least some of the affinity of the prototype antibody. (In the case shown in Queen et al. the affinity of the recombinant antibody was only one third that of the prototype antibody.) However, there is no suggestion in Queen et al. that changing the same residues as were changed when reshaping the anti-TAC antibody could be expected to provide acceptable binding affinity in another recombinant antibody.

Even if (which is denied) a skilled person did try to change residues in accordance with the numbers determined by Queen et al., this still does not lead to the present invention. As has been discussed above, Queen et al. changes residues which, according to the present invention, need not be changed or leaves as acceptor residues ones which should be changed. Thus, following the Queen et al. procedure cannot lead to the production of an antibody as now claimed.

The Queen et al. procedure can be contrasted with the protocol set forth in the present application. In the case of the present invention, there is no need to compare the donor sequence with a number of possible acceptor sequences. Any acceptor sequence can be used. Moreover, there is no need to carry out any computer modelling. All that is required is for the skilled person to go through the hierarchy of residue changes set forth in the application, beginning at page 16, and to make the minimum number of changes required to obtain acceptable activity.

It is submitted that there is nothing in Queen et al. which in any way suggests that this simple hierarchical protocol can be adopted in the expectation of being able to reshape any desired antibody. Queen et al. teaches that each antibody requires its own reshaping procedure. The present invention

teaches that one reshaping procedure can be applied to any antibody.

In order to establish a *prima facie* case of obviousness, it must be shown that the prior art would have suggested to those of ordinary skill in the art that they should make the claimed composition or device, or carry out the claimed process; and that the prior art would also have revealed that in so making or carrying out, those of ordinary skill would have a reasonable expectation of success. Both the suggestion and the reasonable expectation of success must be founded in the prior art, not in the Applicants' disclosure.

As discussed above, at best, Riechmann et al. teaches the skilled person to transfer the six CDRs and to look at the possibility of transferring a composite CDR1 comprising of the Kabat and structural CDR1, but only if there are any unusual residues in the area of the structural CDR1 not covered by the Kabat CDR1. Riechmann et al. does not in any way suggest that altering residues remote from the CDR's might be effective in improving affinity of the CDR-grafted antibody. Additionally, Riechmann et al. in no way suggests that there might be a hierarchy of residues which should be considered if improvements in affinity are to be sought. Still less does Riechmann et al. provide any teaching which would allow the skilled person to

identify the hierarchy of residues as set forth in the present application.

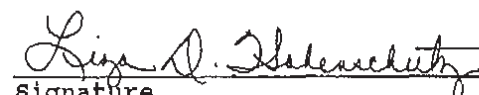
Regarding Queen et al., the fact that the donor sequence is compared to a number of possible acceptor sequences and that a computer model of the donor antibody must be made shows that the whole procedure in Queen et al. is specific to one antibody at a time. Underlying these facts is the assumption that it is not possible to predict from work carried out on one antibody how to deal with another antibody. There is no suggestion in Queen et al. that changing the same residues as were changed when reshaping the anti-TAC antibody could be expected to provide acceptable binding affinity in another recombinant antibody. Queen et al. thus teaches that each antibody requires its own reshaping procedure thereby teaching away from the present invention.

In summary, there is neither a suggestion in the prior art to make the claimed CDR-grafted antibodies, nor does the prior art show a reasonable expectation of success of making the claimed CDR-grafted antibodies. Applicants' claims are thus not *prima facie* obvious over Riechmann et al. and Queen et al. Withdrawal of this 35 USC §103 rejection is respectfully requested.

In view of the above, the present application is believed to be in a condition ready for allowance. Reconsideration of the application is respectfully requested and an early Notice of Allowance is earnestly solicited.

Respectfully submitted,

Date: January 19, 1993


Signature
Liza D. Hohenschutz
Registration No. 33,712

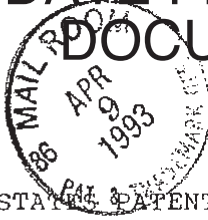
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DOCUMENT NO: 40

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PATENT



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B. White
4-21-93

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: John R. ADAIR et al.
Serial No.: 07/743 329
Group Art Unit: 1807
Filed: 17th September 1991
Examiner: L. Bennett
For: Humanised Antibodies

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GROUP 180

Dear Sir,

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AMENDMENT

1. This amendment is filed following the interview with
Examiners Bennett and Chambers on 27th January 1993.

2. Amendments

Please cancel all the present claims and replace them with the new
claims as follows.

67. An antibody molecule having affinity for a predetermined
antigen and comprising a "composite" heavy chain and a complementary
light chain, said composite heavy chain having a variable domain
comprising human acceptor antibody heavy chain framework residues and
donor antibody heavy chain antigen-binding residues, said donor
antibody having affinity for said predetermined antigen, wherein,
according to the Kabat numbering system, in said composite heavy
chain, amino acid residues 5, 8, 10, 12 to 17, 19, 21, 22, 40, 42 to
44, 66, 68, 70, 74, 77, 79, 81, 83 to 85, 90, 92, 105, 109, 111 and
113 at least are acceptor residues and amino acid residues (23, 24,

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Carter v. Adair
Interference No. 105,744

31 to 35, 49 to 58, 71, 73, 78 and 95 to 102 at least are donor residues.

68. The antibody molecule of claim 67, wherein amino acid residues 26 to 30 and 59 to 65 in said composite heavy chain are additionally donor residues.

69. The antibody molecule of claim 67, wherein at least one of amino acid residues 1, 3, and 76 in said composite heavy chain are additionally donor residues.

70. The antibody molecule of claim 67, wherein at least one of amino acid residues 36, 94, 104, 106 and 107 in said composite heavy chain are additionally donor residues.

71. The antibody molecule of claim 70, wherein at least one of amino acid residues 2, 4, 6, 38, 48, 67 and 69 in said composite heavy chain are additionally donor residues.

72. The antibody molecule of claim 67, wherein amino acid residues 7, 9, 11, 18, 20, 25, 37, 39, 41, 45, 47, 48, 72, 75, 80, 82, 86 to 89, 91, 93, 103, 108, 110 and 112 in said composite heavy chain are additionally acceptor residues.

73. A tumour-specific antibody molecule having affinity for a predetermined tumour antigen and comprising a composite heavy chain and a complementary light chain, said composite heavy chain having a variable domain comprising human acceptor antibody heavy chain framework residues and donor antibody heavy chain antigen-binding residues, said donor antibody having affinity for said predetermined tumour antigen, wherein, according to the Kabat numbering system, in said composite heavy chain, amino acid residues 5, 8, 10, 12 to 17, 19, 21, 22, 40, 42 to 44, 66, 68, 70, 74, 77, 79, 81, 83 to 85, 90, 92, 105, 109, 111 and 113 at least are acceptor residues and amino acid residues 23, 24, 31 to 35, 49 to 58 and 95 to 102 at least are donor residues.

74. The antibody molecule of claim 73, wherein amino acid residues 71, 73 and 78 in said composite heavy chain are additionally donor residues.

75. The antibody molecule of claim 73, wherein amino acid residues 26 to 30 and 59 to 65 in said composite heavy chain are additionally donor residues.

76. The antibody molecule of claim 73, wherein at least one of amino acid residues 1, 3, and 76 in said composite heavy chain are additionally donor residues.

77. The antibody molecule of claim 73, wherein at least one of amino acid residues 36, 94, 104, 106 and 107 in said composite heavy chain are additionally donor residues.

78. The antibody molecule of claim 77, wherein at least one of amino acid residues 2, 4, 6, 38, 48, 67 and 69 in said composite heavy chain are additionally donor residues.

79. The antibody molecule of claim 73, wherein amino acid residues 7, 9, 11, 18, 20, 25, 37, 39, 41, 45, 47, 48, 72, 75, 80, 82, 86 to 89, 91, 93, 103, 108, 110 and 112 in said composite heavy chain are additionally acceptor residues.

80. An interleukin-specific antibody molecule having affinity for a predetermined interleukin and comprising a composite heavy chain and a complementary light chain, said composite heavy chain having a variable domain comprising human acceptor antibody heavy chain framework residues and donor antibody heavy chain antigen-binding residues, said donor antibody having affinity for said predetermined interleukin wherein, according to the Kabat numbering system, in said composite heavy chain, amino acid residues 5, 8, 10, 12 to 17, 19, 21, 22, 40, 42 to 44, 66, 68, 70, 74, 77, 79, 81, 83 to 85, 90, 92, 105, 109, 111 and 113 at least are acceptor residues and amino acid residues 23, 24, 31 to 35, 49 to 58 and 95 to 102 at least are donor residues.

81. The antibody molecule of claim 80, wherein amino acid residues 71, 73 and 78 in said composite heavy chain are additionally donor residues.

82. The antibody molecule of claim 80, wherein amino acid residues 26 to 30 and 59 to 65 in said composite heavy chain are additionally donor residues.

83. The antibody molecule of claim 80, wherein at least one of amino acid residues 1, 3 and 76 in said composite heavy chain are additionally donor residues.

84. The antibody molecule of claim 80, wherein at least one of amino acid residues 36, 94, 104, 106 and 107 in said composite heavy chain are additionally donor residues.

85. The antibody molecule of claim 84, wherein at least one of amino acid residues 2, 4, 6, 38, 48, 67 and 69 in said composite heavy chain are additionally donor residues.

86. The antibody molecule of claim 80, wherein amino acid residues 7, 9, 11, 18, 20, 25, 37, 39, 41, 45, 47, 48, 72, 75, 80, 82, 86 to 89, 91, 93, 103, 108, 110 and 112 in said composite heavy chain are additionally acceptor residues.

87. An anti-CD3 antibody molecule having affinity for the CD3 antigen comprising a composite heavy chain and a complementary light chain, said composite heavy chain having a variable domain comprising human acceptor antibody heavy chain framework residues and donor antibody heavy chain antigen-binding residues from a donor antibody heavy chain, said donor antibody having affinity for said CD3 antigen, wherein, according to the Kabat numbering system, in said composite heavy chain, amino acid residues 5, 8, 10, 12 to 17, 19, 21, 22, 40, 42 to 44, 66, 68, 70, 74, 77, 79, 81, 83 to 85, 90, 92, 105, 109, 111 and 113 at least are acceptor residues and amino acid residues 23, 24, 31 to 35, 49 to 58 and 95 to 102 at least are donor residues.

88. The antibody molecule of claim 87, wherein amino acid residues 71, 73 and 78 in said composite heavy chain are additionally donor residues.

89. The antibody molecule of claim 87, wherein amino acid residues 26 to 30 and 59 to 65 in said composite heavy chain are additionally donor residues.

90. The antibody molecule of claim 87, wherein at least one of amino acid residues 1, 3, and 76 in said composite heavy chain are additionally donor residues.

91. The antibody molecule of claim 87, wherein at least one of amino acid residues 36, 94, 104, 106 and 107 in said composite heavy chain are additionally donor residues.

92. The antibody molecule of claim 91, wherein at least one of amino acid residues 2, 4, 6, 38, 48, 67 and 69 in said composite heavy chain are additionally donor residues.

93. The antibody molecule of claim 87, wherein amino acid residues 7, 9, 11, 18, 20, 25, 37, 39, 41, 45, 47, 48, 72, 75, 80, 82, 86 to 89, 91, 93, 103, 108, 110 and 112 in said composite heavy chain are additionally acceptor residues.

94. An anti-CD4 antibody molecule having affinity for the CD4 antigen and comprising a composite heavy chain and a complementary light chain, said composite heavy chain having a variable domain comprising human acceptor antibody heavy chain framework residues and donor antibody heavy chain antigen-binding residues, said donor antibody having affinity for said CD4 antigen, wherein, according to the Kabat numbering system, in said composite heavy chain, amino acid residues 5, 8, 10, 12 to 17, 19, 21, 22, 40, 42 to 44, 66, 68, 70, 74, 77, 79, 81, 83 to 85, 90, 92, 105, 109, 111 and 113 at least are acceptor residues and amino acid residues 23, 24, 31 to 35, 49 to 58 and 95 to 102 at least are donor residues.

95. The antibody molecule of claim 94, wherein amino acid residues 71, 73 and 78 in said composite heavy chain are additionally donor residues.

96. The antibody molecule of claim 94, wherein amino acid residues 26 to 30 and 59 to 65 in said composite heavy chain are additionally donor residues.

97. The antibody molecule of claim 94, wherein at least one of amino acid residues 1, 3, and 76 in said composite heavy chain are additionally donor residues.

98. The antibody molecule of claim 94, wherein at least one of amino acid residues 36, 94, 104, 106 and 107 in said composite heavy chain are additionally donor residues.

99. The antibody molecule of claim 98, wherein at least one of amino acid residues 2, 4, 6, 38, 48, 67 and 69 in said composite heavy chain are additionally donor residues.

100. The antibody molecule of claim 94, wherein amino acid residues 7, 9, 11, 18, 20, 25, 37, 39, 41, 45, 47, 48, 72, 75, 80, 82, 86 to 89, 91, 93, 103, 108, 110 and 112 in said composite heavy chain are additionally acceptor residues.

101. An anti-adhesion molecule antibody molecule having affinity for an adhesion molecule and comprising a composite heavy chain and a complementary light chain, said composite heavy chain having a variable domain comprising human acceptor antibody heavy chain framework residues and donor antibody heavy chain antigen-binding residues from a donor antibody heavy chain, said donor antibody having affinity for said adhesion molecule wherein, according to the Kabat numbering system, in said composite heavy chain, amino acid residues 5, 8, 10, 12 to 17, 19, 21, 22, 40, 42 to 44, 66, 68, 70, 74, 77, 79, 81, 83 to 85, 90, 92, 105, 109, 111 and 113 at least are acceptor residues and amino acid residues 23, 24, 31 to 35, 49 to 58 and 95 to 102 at least are donor residues.

102. The antibody molecule of claim 101, wherein amino acid residues 71, 73 and 78 in said composite heavy chain are additionally donor residues.

103. The antibody molecule of claim 101, wherein amino acid residues 26 to 30 and 59 to 65 in said composite heavy chain are additionally donor residues.

104. The antibody molecule of claim 101, wherein at least one of amino acid residues 1, 3, and 76 in said composite heavy chain are additionally donor residues.

105. The antibody molecule of claim 101, wherein at least one of amino acid residues 36, 94, 104, 106 and 107 in said composite heavy chain are additionally donor residues.

106. The antibody molecule of claim 105, wherein at least one of amino acid residues 2, 4, 6, 38, 48, 67 and 69 in said composite heavy chain are additionally donor residues.

107. The antibody molecule of claim 101, wherein amino acid residues 7, 9, 11, 18, 20, 25, 37, 39, 41, 45, 47, 48, 72, 75, 80, 82, 86 to 89, 91, 93, 103, 108, 110 and 112 in said composite heavy chain are additionally acceptor residues.

108. The antibody molecule of claim 67, wherein said complementary light chain is a composite light chain having a variable domain comprising human acceptor antibody light chain framework residues and donor antibody light chain antigen-binding residues, said donor antibody having affinity for said predetermined antigen, wherein, according to the Kabat numbering system, in said composite light chain, amino acid residues 5, 7 to 9, 11, 13 to 18, 20, 22, 23, 39, 41 to 43, 57, 59, 61, 72, 74 to 79, 81, 82, 84, 86, 88, 100, 104, 106 and 107 at least are acceptor residues and amino acid residues 24 to 34, 46, 48, 50 to 56, 58, 71 and 89 to 97 at least are donor residues.

109. The antibody molecule of claim 73, wherein said complementary light chain is a composite light chain having a variable domain comprising human acceptor antibody light chain framework residues and donor antibody light chain antigen-binding residues, said donor antibody having affinity for said predetermined antigen, wherein, according to the Kabat numbering system, in said composite light chain, amino acid residues 5, 7 to 9, 11, 13 to 18, 20, 22, 23, 39, 41 to 43, 57, 59, 61, 72, 74 to 79, 81, 82, 84, 86, 88, 100, 104, 106 and 107 at least are acceptor residues and amino acid residues 24 to 34, 46, 48, 50 to 56, 58, 71 and 89 to 97 at least are donor residues.

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110. The antibody molecule of claim 80, wherein said complementary light chain is a composite light chain having a variable domain comprising human acceptor antibody light chain framework residues and donor antibody light chain antigen-binding residues, said donor antibody having affinity for said predetermined antigen, wherein, according to the Kabat numbering system, in said composite light chain, amino acid residues 5, 7 to 9, 11, 13 to 18, 20, 22, 23, 39, 41 to 43, 57, 59, 61, 72, 74 to 79, 81, 82, 84, 86, 88, 100, 104, 106 and 107 at least are acceptor residues and amino acid residues 24 to 34, 46, 48, 50 to 56, 58, 71 and 89 to 97 at least are donor residues.

111. The antibody molecule of claim 87, wherein said complementary light chain is a composite light chain having a variable domain comprising human acceptor antibody light chain framework residues and donor antibody light chain antigen-binding residues, said donor antibody having affinity for said predetermined antigen, wherein, according to the Kabat numbering system, in said composite light chain, amino acid residues 5, 7 to 9, 11, 13 to 18, 20, 22, 23, 39, 41 to 43, 57, 59, 61, 72, 74 to 79, 81, 82, 84, 86, 88, 100, 104, 106 and 107 at least are acceptor residues and amino acid residues 24 to 34, 46, 48, 50 to 56, 58, 71 and 89 to 97 at least are donor residues.

112. The antibody molecule of claim 94, wherein said complementary light chain is a composite light chain having a variable domain comprising human acceptor antibody light chain framework residues and donor antibody light chain antigen-binding residues, said donor antibody having affinity for said predetermined antigen, wherein, according to the Kabat numbering system, in said composite light chain, amino acid residues 5, 7 to 9, 11, 13 to 18, 20, 22, 23, 39, 41 to 43, 57, 59, 61, 72, 74 to 79, 81, 82, 84, 86, 88, 100, 104, 106 and 107 at least are acceptor residues and amino acid residues 24 to 34, 46, 48, 50 to 56, 58, 71 and 89 to 97 at least are donor residues.

113. The antibody molecule of claim 101, wherein said complementary light chain is a composite light chain having a variable domain comprising human acceptor antibody light chain framework residues and donor antibody light chain antigen-binding residues, said donor antibody having affinity for said predetermined antigen, wherein, according to the Kabat numbering system, in said composite light chain, amino acid residues 5, 7 to 9, 11, 13 to 18, 20, 22, 23, 39, 41 to 43, 57, 59, 61, 72, 74 to 79, 81, 82, 84, 86, 88, 100, 104, 106 and 107 at least are acceptor residues and amino acid residues 24 to 34, 46, 48, 50 to 56, 58, 71 and 89 to 97 at least are donor residues.

114. The antibody molecule of claim 108, wherein amino acid residues 1, 3 and 47 in said composite light chain are additionally donor residues.

115. The antibody molecule of claim 108, wherein amino acid residues 36, 44, 47, 85 and 87 in said composite light chain are additionally donor residues.

116. The antibody molecule of claim 108, wherein at least one of amino acid residues 2, 4, 6, 49, 62, 64 to 69, 98, 99, 101 and 102 in said composite light chain are additionally donor residues.

117. The antibody molecule of claim 108, wherein at least one of amino acid residues 1, 3, 10, 12, 21, 40, 60, 63, 70, 73, 80, 103 and 105 in said composite light chain are additionally donor residues.

118. A method for producing a recombinant antigen binding molecule having affinity for a predetermined antigen comprising the steps of:

[1] determining the amino acid sequence of the variable domain of the heavy chain of a donor antibody which has affinity for said predetermined antigen;

[2] determining the amino acid sequence of the variable domain of the heavy chain of a non-specific acceptor antibody;

[3] providing a composite heavy chain for an antibody molecule, said composite heavy chain having acceptor framework residues and donor antigen binding residues wherein, according to the Kabat numbering system, amino acid residues 5, 8, 10, 12 to 17, 19, 21, 22, 40, 42 to 44, 66, 68, 70, 74, 77, 79, 81, 83 to 85, 90, 92, 105, 109, 111 and 113 at least are acceptor residues and amino acid residues 23, 24, 31 to 35, 49 to 58 and 95 to 102 at least are donor residues;

[4] associating the heavy chain produced in step [3] with a complementary light chain to form an antibody molecule;

[5] determining the affinity of the antibody molecule formed in step [4] for said predetermined antigen;

[6] if the affinity determined in step [5] is not equivalent to that of the donor antibody, providing a heavy chain as described in [3] above but in which amino acid residues 71, 73 and 78 are additionally donor residues;

[7] associating the heavy chain produced in step [6] with a complementary light chain to form an antibody molecule;

[8] determining the affinity of the antibody molecule formed in step [7] for said predetermined antigen;

[9] if the affinity determined in step [8] is not equivalent to that of the donor antibody, providing a heavy chain as described in [6] above but in which amino acid residues 26 to 30 are additionally donor residues;

[10] associating the heavy chain produced in step [9] with a complementary light chain to form an antibody molecule;

[11] determining the affinity of the antibody molecule formed in step [10] for said predetermined antigen;

[12] if the affinity determined in step [11] is not equivalent to that of the donor antibody, providing a heavy chain as described in [9] above but in which at least one of amino acid residues 1, 3, and 76 are additionally donor residues;

[13] associating the heavy chain produced in step [12] with a complementary light chain to form an antibody molecule;

[14] determining the affinity of the antibody molecule formed in step [13] for said predetermined antigen;

[15] if the affinity determined in step [14] is not equivalent to that of the donor antibody, providing a heavy chain as described in [12] above but in which at least one of amino acid residues 36, 94, 104, 106, 107 are additionally donor residues;

[16] associating the heavy chain produced in step [15] with a complementary light chain to form an antibody molecule.

[17] determining the affinity of the antibody molecule formed in step [16] for said predetermined antigen;

[18] if the affinity determined in step [17] is not equivalent to that of the donor antibody, providing a heavy chain as described in [15] above but in which at least one of amino acid residues 2, 4, 6, 38, 48, 67 and 69 are additionally donor residues; and

[19] associating the heavy chain produced in step [18] with a complementary light chain to form an antibody molecule.

119. The method of claim 118, further comprising the steps of:

[1] determining the amino acid sequence of the variable domain of the light chain of said donor antibody which has affinity for said predetermined antigen;

[2] determining the amino acid sequence of the variable domain of the light chain of a non-specific acceptor antibody;

[3] providing a composite light chain for an antibody molecule, said composite light chain having acceptor framework residues and donor antigen binding residues wherein, according to the Kabat numbering system, amino acid residues 5, 7 to 9, 11, 13 to 18,

20, 22, 23, 39, 41 to 43, 57, 59, 61, 72, 74 to 79 to 79, 81, 82, 84, 86, 88, 100, 104 and 106 to 109 at least are acceptor residues and amino acid residues 24 to 34, 46, 48, 50 to 56, 58, 71 and 89 to 97 at least are donor residues;

[4] associating the light chain produced in step [3] with a complementary heavy chain to form an antibody molecule;

[5] determining the affinity of the antibody molecule formed in step [4] for said predetermined antigen;

[6] if the affinity determined in step [5] is not equivalent to that of the donor antibody, providing a light chain as described in [3] above but in which amino acid residues 1, 2, 3 and 47 are additionally donor residues;

[7] associating the light chain produced in step [6] with a complementary heavy chain to form an antigen-binding molecule;

[8] determining the affinity of the antigen-binding molecule formed in step [7] for said predetermined antigen;

[9] if the affinity determined in step [8] is not equivalent to that of the donor antibody, providing a light chain as described in [6] above but in which amino acid residues 36, 44, 47, 85 and 87 are additionally donor residues;

[10] associating the light chain produced in step [9] with a complementary heavy chain to form an antibody molecule;

[11] determining the affinity of the antibody molecule formed in step [10] for said predetermined antigen;

[12] if the affinity determined in step [11] is not equivalent to that of the donor antibody, providing a light chain as described in [9] above but in which at least one of amino acid residues 2, 4, 6, 49, 62, 64 to 69, 98, 99, 101 are additionally donor residues; and

[13] associating the light chain produced in step [9] with a complementary heavy chain to form an antibody molecule.

3. Remarks

The undersigned would like to thank Examiners Lisa Bennett and Scott Chambers for the very cordial and helpful interview on January 27, 1993 relating to this application. The amendments to the claims reflect the discussions of the interview as recorded in the Examiner

Interview Summary Record.

Having considered the Examiner's concerns that the language of the claims might be indefinite, because it was not clear whether the specified residues were the only or the minimum number of residues to be donor residues, the Applicants have amended the claims. In all the claims it is made clear that there is a minimum number of residues which have to be donor residues and a minimum number which have to be acceptor residues. Those residues which are not specified in the claims may be either donor or acceptor.

The claims have been amended to delete "antigen-binding molecule" and recite instead "antibody molecule". In order to reduce issues, the claims have also been amended recite that the acceptor antibody heavy or light chain is a human acceptor antibody heavy or light chain. These amendments to the claims address the Examiner's concern relating to utility and enablement of the claims. In view of the great conservation of structure between antibodies of different species, however, Applicants believe the acceptor and donor antibodies can come from any species.

In order to reduce the issues further, the claims have been limited to antibodies in which the heavy chain has been "superhumanised". Claims referring to the light chain have been made dependent on the claims referring to the heavy chain.

Regarding claim 67, it can be seen from the description (see page 6 line 29 to page 7, line 28; page 17, lines 9 to 11, Sections 2.1 to 2.3 bridging pages 17 and 18; Section 1 bridging pages 19 and 20; Sections 2.1.1, 2.2.1, 2.3.1 and 2.4.1 in the passage bridging pages 20 to 23; and Section 15.2.1 on pages 46 and 47) that a number of residues are mentioned which can be considered for changing from acceptor to donor residues. It follows that if a residue has not been considered for changing, it must remain as in the acceptor chain. In order to make this clear in the claim, it has been specified in claim 67 that all the unmentioned residues must be acceptor residues.

As regards the recitation of the donor residues in claim 67, this comprises a combination of the minimum residues need to define the CDR loops or the Kabat CDRs. For CDR1, this comprises residues 31 to 35 (see page 46, line 18) and for CDR2 this comprises residues 50 to 58 (see page 46, line 20). For CDR3, the Kabat definition of residues 95 to 102 was used (see page 17, line 11).

In addition claim 67 recites as donor residues those identified on page 20, line 25 and page 21, line 9 as being key residues outside the CDRs.

In claim 67, it has been specified that residues 71, 73 and 78 are all donor residues in order to ensure that claim 67 is novel over the anti-TAC antibody disclosed by Queen. This anti-TAC antibody has an acceptor residue at residue 73. However, as can be seen from page 7, lines 1 to 5, the Applicant considers that in general, residues 71, 73 and 78 can be either all donor or all acceptor.

Claim 68 increases the sizes of CDR1 and CDR2 to the prudent definition given on page 17, line 9 and page 20, lines 6 to 9.

Claim 69 is based on the passage at page 20, lines 26 and 27. Claim 70 is based on the passage at page 21, lines 10 to 12. Claim 71 is based on the passage on page 21, lines 13 to 16.

Claim 72 is derived by taking all the donor residues mentioned in claims 67 to 71 and specifying that all apart from these residues are acceptor residues.

Claim 73 is based on page 15, line 27 and pages 55 to 59 of the description. Claim 80 is based on page 15, line 33 of the description. Claim 87 is based on page 15, line 31 and pages 25 to 52 of the description. Claim 94 is based on page 15, line 31 and pages 53 and 54 of the description. Claim 101 is based on page 15, line 32 and pages 60 and 61 of the description.

None of the prior art CDR-grafted antibodies has specificity for a tumour-specific antigen, an interleukin, the CD3 or CD4 antigen, or an adhesion molecule. It is therefore submitted that claims 73, 80, 87, 94 and 101 are all novel.

It is stated on page 7, lines 1 to 5 that residues 71, 73 and 78 should all be either acceptor or donor. Claims 73, 80, 87, 94 and 101 cover the first alternative and claims 74, 81, 88, 95 and 102 cover the second alternative.

Claims 75 to 90, 82 to 86, 89 to 93, 96 to 100 and 103 to 107 are equivalent to claims 68 to 72.

Claims 108 to 113 are based on previously filed claim 42 and is derived in the same way that present claim 67 was derived. The residues which can be donor residues are listed on page 9, line 7 to page 10, line 15; page 17, lines 12 to 14; Section 3 bridging pages 18 and 19; Section 2.1.2 on page 20; Section 2.2.2 on page 21; Section 2.3.2 on page 22 and Section 2.4.2 on page 23.

Claims 114 to 117 are based on the passage from page 9, line 7 to page 10, line 11.

Claims 118 and 119 are based on previous claims 65 and 66 but with amendments to make them consistent with claims 67 onwards.

It is therefore believed that all the claims are fully supported by the description.

Although all the previous claims have been cancelled, the applicants reserve the right to file divisional applications relating to the deleted subject matter.

At the interview, the Examiner expressed concerns that the claims may lack novelty and that the method disclosed in the application might not be universally applicable. In an effort to resolve these questions, the applicants send herewith three Tables relating to a

number of antibodies which have been "superhumanised" by the method of the invention. The first Table relates to the heavy chain and the second Table relates to the light chain. On the third Table is set out the degree of affinity recovered as a percentage of the affinity of the original monoclonal antibody. There is also provided on the Tables a comparison with antibodies which have been produced by prior art proposals.

On each of the Tables, the top line shows the residue numbering according to the Kabat scheme. It can be seen that this includes, for the heavy chain, residues 52a-52c, 82a-82c and 100a to 100i.

Underneath the residue numbering is set out schematically the residue assignment for each of the antibodies referred to. This is schematic in that it does not give the actual residue but instead indicates whether it is a donor (D), acceptor (A) or common (c) residue. "Common" means that the same residue was present in both the donor and the acceptor sequences. Some of the common residues are also highly or completely conserved, but this has not been marked on the Tables.

In the heavy chain Table, the first five residues of 39D10 are marked by a question mark. This is because, due to the cloning method used, the first five residues in the donor chain were not determined. Thus, no comparison can be made.

At the end of each line is given the name of the acceptor sequence used to produce the chain.

It should be noted that for the antibodies 61E71 and hTNF3, the projects were terminated before the method had been fully applied. The applicants are confident that, had they had the funds to complete the work on these antibodies, good recovery of affinity would have been obtained.

In the Tables, the B1.8, D1.3, CAMPATH and anti-TAC entries represent prior art antibodies. There is no entry in the light chain Table for

B1.8 because the B1.8 light chain was never CDR-grafted.

B1.8 recognises an artificial antigen, the nitrophenyl hapten. Its heavy chain has acceptor residues at positions 23, 24, 73 and 78.

D1.3 recognises lysosyme. Its heavy chain has acceptor residues at positions 71, 73 and 78. Its light chain has acceptor residues at positions 48 and 71.

CAMPATH recognises the CD52 differentiation antigen found on various leukocytes. Its heavy chain has acceptor residues at positions 23, 24, 71, 73 and 78. Its light chain has an acceptor residue at position 58.

Anti-TAC is the antibody described by Queen. It recognises an epitope on a surface receptor for IL-2. (It does not recognise IL-2 itself). Its heavy chain has an acceptor residue at position 73. Its light chain has an acceptor residue at position 71.

Looking at the heavy chain Table, it can be seen that in all the successfully "superhumanised" antibodies produced by the applicants, in the heavy chain residues 23, 24, 31 to 35, 49 to 58 and 95 to 102 are all donor residues. Also in each case residues 26 to 30 are donor residues. Moreover, in most cases, residues 59 to 65 are also donor residues.

In one chain, OKT3 209, residues 71 and 73 were left as acceptor. For this particular antibody, it was shown that as long as residue 78 is donor, it does not make much difference whether residues 71 and 73 are donor or acceptor. This seems to be a peculiarity of this antibody, but which highlights the importance of residue 78.

In the heavy chains for 61E71 and hTNF3, residue 78 remained as acceptor. In both cases, the donor residue is alanine and the acceptor residue is leucine. Since leucine is more bulky than alanine, this may explain why the affinity of these chains was lower than desirable.

In hTNF1 a number of residues (12, 66 and 83) are donor residues whereas claim 67 specifies them as being acceptor residues. This antibody was prepared as the first in a series and the aim was to ensure that it worked. Therefore, any residues which were in the slightest unusual were changed to donor residues, even though it was suspected that some of the changes were unnecessary. As can be seen from, for instance 101/4, a later antibody in the series, if residues 12, 66 and 83 remain as acceptor, reasonable affinity is recovered. It is submitted that this is evidence that hTNF1 was overengineered.

In P67.6, residue 44 is a donor residue whereas claim 67 specifies that it should be an acceptor residue. In the donor antibody in this case, residue 44 was a very unusual amino acid for that position and it was therefore felt that, in that particular case, the unusual amino acid should be adopted.

At residue 77, which is specified as an acceptor residue, 101/4 and 39D10 have the donor residue. The change here in both cases is of a surface residue and is from leucine to valine. These are both hydrophobic residues and it is unlikely that this change will significantly affect the recovery of activity.

In 101/4 residue 79 is donor whereas claim 67 specifies it as acceptor. The donor residue is tyrosine and the acceptor is phenylalanine. This is a very conservative change and it is highly unlikely that this change will significantly affect the recovery of affinity.

For residue 105, which should be acceptor, B1RRO001, CTM01 and P67.6 all have donor residues. B1RRO001 gave very low recovery of affinity and it is believed that retaining the acceptor sequence at residue 105 would have improve the recovery of affinity. For the other two antibodies, the change is asparagine for glutamine, a very conservative change. It is highly unlikely that this change will have had any significant effect on the recovery of affinity.

Heavy chain residue 78 is specified as donor. In 61E71 and hTNF3, it is acceptor. However, these antibodies have low recovery of affinity. The projects on these antibodies were terminated at an early stage. If residue 78 had been changed to donor, it is believed that better recovery of affinity would have been obtained.

It is submitted that the data given in the three Tables amply demonstrate that successful "superhumanisation" can be achieved by following the method of the invention.

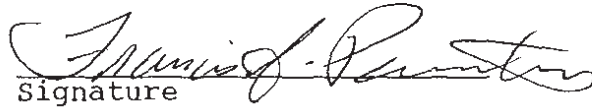
The Examiners expressed some concern that the language of the claims could read on naturally-occurring antibody heavy or light chains. The claims recite a "donor" antibody and an "acceptor" antibody as sources of residues in the heavy or light chains. Such an antibody, as claimed, cannot exist in nature. Therefore, the claims cannot read on a naturally-occurring antibody.

It is submitted that the present claims define inventive subject matter, for the reasons set forth in the Amendment mailed 19th January 1993.

In view of the above amendments and discussion, the present application is believed to be in a condition ready for allowance. Reconsideration of the application is respectfully requested and an early Notice of Allowance is respectfully requested.

Respectfully submitted,

Date: *April 7, 1993*


Signature
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LIGHT CHAIN

	1	2	3	4	5	6	7	8	9	0	1	1
	123456789012345678901234567890	123456789012345678901234567890	123456789012345678901234567890	123456789012345678901234567890	123456789012345678901234567890	123456789012345678901234567890	123456789012345678901234567890	123456789012345678901234567890	123456789012345678901234567890	123456789012345678901234567890	123456789012345678901234567890	ACCEPTOR
B72.3	cccccccc	AcccAccc	AAcccc	DDDDDD	DDDDcccc	AAccAc	AccAc	DDDDDD	cccccccc	cccccccc	REI
OKT3	AcAAcccc	AAcccc	AcAAcc	AccDDDD	DDDDcccc	AAcAAcc	DDcc	DDDDDD	ccccAAc	Acccccc	REI
OKT4a	cccccccc	cccccc	AcAAcc	AcDDDD	DDDDcccc	DAccAc	Accc	DDDDDD	AcAcccccc	Acccccc	REI
B1RR001	cDDcccc	AAcAAc	AAcAAc	AcDDDD	DDDDcccc	AccAc	Acccccc	DcDDDD	ccccDcc	Acccccc	EU
A5B7	DDDDAccc	AAcAc	AcAAcc	AccDDDD	DDDDcccc	cccc	AAcc	DDDDDD	cccc	Acccccc	LAY
CTM01	ccAccc	AAAAAA	AAcAAc	AcDDDD	DDDDcccc	DAcAc	AAcAc	DDDDDD	ccccAcc	Dcccccc	EU
P67.6	ccADcccc	AAcAAc	AcAAc	AcDDDD	DDDDcccc	DccAc	Acccccc	DcDDDD	ccccAcc	AcAAcc	EU
A33	ccAccc	AAAA	Acccccc	Accc	DDDDcccc	cccc	AAcc	DDDDDD	cccc	Acccccc	LAY
61E71	AcAccc	AcAAc	AcAcccccc	DDDDDD	DDDDcccc	AAcAAc	Accc	DDDDDD	cccc	Acccccc	REI
hTNF3	AcAccc	AcAAc	AcAcccccc	DDDDDD	DDDDcccc	AcAAc	Accc	DDDDDD	cccc	Acccccc	REI
101/4	DcDDcccc	AAccc	AcAAcc	AccDDDD	DDDDcccc	DccAAc	Accc	DDDDDD	cccc	Acccccc	REI
hTNF1	ccDc	Accc	AcAAccc	AAcAAc	DDDDcccc	DAccc	DDDDDD	cccc	Acccccc	Acccccc	REI
39D10	ccccccc	Accccc	AcAAAA	AcDDDD	DDDDcccc	cccc	AAc	DDDDDD	cccccccc	cccc	LAY
L243	ccccccc	Accc	Accc	AAcccc	DDDDcccc	AccAc	AcDcc	ADDDDD	cccccccc	cccc	SGI consensus
HLT29	ccccccc	cccc	Acccccc	DDDDDD	DDDDcccc	DccAc	AcDcc	DDDDDD	cccccccc	cccc	SGI consensus
D1.3	ccccccc	Acccccc	AAcccc	DDDDDD	DDDDcccc	AccAc	Accc	DDDDDD	cccccccc	AAAAc	REI (LMB)
CAMPATH-1	ccAccccc	Acccccc	cccc	AAc	DDDDcccc	AcAAccc	cccc	DDDDDD	AcAcccccc	Acccccc	REI (LMB)
anti-TAC	AcAAcccc	AAAAc	AcAAccc	cccc	DDDDcccc	AcAccc	Acc	DDDDDD	cccccccc	AAAAc	EU (PDL)

c CONSERVED BETWEEN DONOR AND ACCEPTOR
D DONOR RESIDUE IN HUMANIZED FORM
A ACCEPTOR RESIDUE IN HUMANIZED FORM

SGI consensus means the acceptor sequence was the consensus sequence of the human antibodies in subgroup I of the Kabat et al 1987 compendium
REI(LMB) is a modified form of the REI sequence in which certain residues have been altered to make the sequence more homologous to the consensus sequence for the subgroup
EU(PDL) is a modified form of the EU sequence in which certain residues have been altered to make the sequence more homologous to the consensus sequence for the subgroup.

ANTIBODY	SPECIFICITY	POTENCY ¹ RELATIVE TO RODENT ANTIBODY %
ANTI-TUMOUR		
B72.3	Tumour associated glycoprotein 72 (TAG72)	30
P67.6	CD33	85
CTM01	Polymorphic epithelial mucin (PEM)	> 100
A5B7	Carcinoembryonic antigen (CEA)	50
A33	Epithelial antigen	75
ANTI-CYTOKINE		
HTNF1	Tumour necrosis factor (TNF) α	100 ²
101/4	TNF α	30 ²
61E71	TNF α	100; < 1 ²
HTNF3	TNF α	100; < 1 ²
HLT29	Lymphotoxin (LT)	> 70 ²
39D10	Interleukin-5	> 80
ANTI-LYMPHOCYTE		
OKT3	CD3	100
OKT4	CD4	68
L243	MHC CLASS II	60
ANTI-ADHESION MOLECULE		
BIRR-001	ICAM-1	25

1. POTENCY IS MEASURED AS RELATIVE ABILITY TO COMPETE WITH THE RODENT ANTIBODY FOR BINDING TO ANTIGEN
2. CYTOKINE NEUTRALIZATION ASSAY. ANTIBODY BINDS TO CYTOKINE AND THE COMPLEX IS TESTED FOR ABILITY TO AFFECT THE GROWTH OF L929 CELLS.

TOTAL P.02

DATE FILED: 03/20/2010
DOCUMENT NO: 41
CARPMAELS & RANSFORD

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European Patent Office,
Munich.

→ 21747

9th September 1993

YOUR REF

OUR REF

P07856EP/CPM/EK

Dear Sirs,

Re: European Patent Application No. 91901433.2
Celltech Limited

2116

1. Accelerated Prosecution

1.1. It is hereby requested that the above-referenced application be subjected to accelerated prosecution. The Examination Fee was paid on 23rd May 1991. It is requested that the first communication on Substantive Examination be issued as soon as possible.

1.2. The present application relates to "humanised" antibody molecules and to methods for producing them. In order to "humanise" an antibody, a donor antibody, generally a mouse monoclonal antibody, having a desired specificity is produced. The sequence of the variable domain in each of the heavy and light chains of the donor antibody is then determined. An acceptor antibody, usually a human antibody, is taken and the sequences of its variable domains are changed so that the complementarity determining regions (CDRs) and a number of the framework residues in the original acceptor antibody correspond to the equivalent residues in the donor antibody. In this way, the antibody thus produced, which is a hybrid of donor and acceptor, has donor antigen-binding properties but mainly acceptor non-antigen-binding functions.

1.3. The technique of humanising antibodies is growing in importance in the medical field. It is anticipated that the market for humanized antibodies for use in medical treatments will grow considerably over the next few years.

1.4. The Applicant believes that the present application covers a pioneering invention in this field as it allows any combination of donor and acceptor antibody to be used to produce the hybrid antibody. Moreover, it allows the production of a hybrid antibody having about the same (or in some cases better) affinity for the target antigen as the donor antibody. It is believed that these possibilities have not previously been available.

1-5. 09. 931*

1.5. The Applicant is aware that there are a number of companies which are offering antibody humanization services. It is likely that the antibodies they produce will infringe valid claims to be granted on the present application. As evidence of the existence of such companies, I enclose copies of three extracts from SCRIP (of: 9/8/91, page 14; 19/2/92, page 15; and 12/2/93, page 11). These relate to one company which, the Applicant believes, is intending to commercialize at least one of its humanized antibodies in the near future.

1.6. The Applicant would like to be in a position to take action against the company mentioned in SCRIP, and any other similar company, as soon as possible in order to prevent its legitimate rights from being infringed. For the above reasons, it is submitted that the request for Accelerated Prosecution is justified.

2. Amendments

2.1. In order to assist in accelerating the prosecution of this application, it is requested that the present claims be deleted and that new claims 1 to 20 (on new pages 67 to 74) (enclosed in triplicate) be used as the basis for substantive examination.

2.2. At present, no amendments to the description are offered. However, it is appreciated that it will be necessary to amend the description. It is suggested that it would be more efficient to agree on an allowable set of claims. Thereafter, the description can be amended to bring it into conformity with the agreed claims.

2.3. On review of the Application, it was felt that the language of the claims might be imprecise, because it was not clear whether the specified residues were the only or the minimum number of residues to be donor residues. The Applicants have therefore amended the claims to make it clear that there is a minimum number of residues which have to be donor residues and a minimum number which have to be acceptor residues. Those residues which are not specified in the claims may be either donor or acceptor.

2.4. In order to limit the issues raised in this case, the main independent claims have been limited to antibodies comprising both a heavy chain and a complementary light chain. Claims to the separate chains have been cancelled. Also, claims relating to changes in the light chain have been made dependent on the main independent claims.

2.5. Further, claims to DNA sequences, vectors, transformed host cells and methods of culturing such cells have been cancelled. Claim 23 has also been cancelled, in view of Article 52(4) EPC.

2.6. Since claims have been cancelled in order to limit the issues in the present application, the Applicant reserves the right to file divisional applications relating to the deleted subject matter.

2.7. Regarding new claim 1, it can be seen from the description (see page 6, line 29 to page 7, line 28; page 17, lines 9 to 11,

Sections 2.1 to 2.3 bridging pages 17 and 18; Section 1 bridging pages 19 and 20; Sections 2.1.1, 2.2.1, 2.3.1 and 2.4.1 in the passage bridging pages 20 to 23; and Section 15.2.1 on pages 46 and 47) that a number of residues are mentioned which can be considered for changing from acceptor to donor residues. It follows that if a residue has not been considered for changing, it must remain as in the acceptor chain. In order to make this clear, it has been specified in new claim 1 that all the unmentioned residues must be acceptor residues.

2.8. As regards the recitation of the donor residues in new claim 1, this comprises a combination of the minimum residues need to define the CDR loops or the Kabat CDRs. For CDR1, this comprises residues 31 to 35 (see page 46, line 18) and for CDR2 this comprises residues 50 to 58 (see page 46, line 20). For CDR3, the Kabat definition of residues 95 to 102 was used (see page 17, line 11).

2.9. In addition, new claim 1 recites as donor residues those identified on page 20, line 25 and page 21, line 9 as being key residues outside the CDRs.

2.10. In new claim 1, it has been specified that residues 71, 73 and 78 are all donor residues in order to ensure that new claim 1 is novel over the anti-TAC antibody disclosed in PNAS-USA, 86, 10029-10033, 1989 (Queen) (cited in the International Search Report). This anti-TAC antibody has an acceptor residue at residue 73. However, as can be seen from page 7, lines 1 to 5, the Applicant considers that in general, residues 71, 73 and 78 can be either all donor or all acceptor.

2.11. Claim 2 is based on page 15, line 27 and pages 55 to 59 of the description. Claim 3 is based on page 15, line 33 of the description. Claim 4 is based on page 15, line 31 and pages 25 to 52 of the description. Claim 5 is based on page 15, line 31 and pages 53 and 54 of the description. Claim 6 is based on page 15, line 32 and pages 60 and 61 of the description.

2.12. It is stated on page 7, lines 1 to 5 that residues 71, 73 and 78 should all be either acceptor or donor. Claims 2 to 6 cover the first alternative and claim 7 covers the second alternative.

2.13. Claim 8 increases the sizes of CDR1 and CDR2 to the prudent definition given on page 17, line 9 and page 20, lines 6 to 9.

2.14. Claim 9 is based on the passage at page 20, lines 26 and 27. Claim 10 is based on the passage at page 21, lines 10 to 12. Claim 11 is based on the passage on page 21, lines 13 to 16.

2.15. Claim 12 is derived by taking all the donor residues mentioned in claims 1 and 7 to 11 and specifying that all apart from these residues are acceptor residues.

2.16. Claim 13 is derived in the same way that present claim 1 was derived. The residues which can be donor residues are listed on page 9, line 7 to page 10, line 15; page 17, lines 12 to 14; Section 3 bridging pages 18 and 19; Section 2.1.2 on page 20; Section 2.2.2 on

page 21; Section 2.3.2 on page 22 and Section 2.4.2 on page 23.

2.17. Claims 14 to 17 are based on the passage from page 9, line 7 to page 10, line 11.

2.18. Claim 18 is equivalent to original claim 22.

2.19. Claims 19 and 20 put into claim format the Protocol set forth on pages 16 to 23 of the description in a way which is consistent with new claims 1 to 17.

2.20. It is therefore believed that all the claims are fully supported by the description.

3. The Prior art

3.1. In the International Search Report, the first cited document is EP-A-0 403 156 (Genzyme). The present application claims a priority date of 21st December 1989. The Genzyme application was published on 19th December 1990 and therefore cannot be used as a citation under Article 54(2) EPC. It can be used as a citation under Article 54(3) EPC, but only as long as the relevant disclosures in the citation are entitled to a priority date earlier than that of the present application.

3.2. The Genzyme application claims priority from US Patent Applications Nos. 362 549 and 529 979. The '549 application was filed before the priority date of the present application but the '979 application was not. Therefore, only subject matter which is supported by the '549 application can be cited against the present application. We enclose a copy of the '549 application.

3.3. Reference is made to Examples 8 to 12 of the Genzyme application. Examples 8 to 11, at least as far as page 7, line 55 of the Genzyme application, are equivalent to Examples 8 to 11 in the '549 application. However, these Examples relate solely to the production of chimeric antibodies, that is an antibody in which the whole variable domain is derived from a donor antibody. There is no disclosure of any antibody in which the variable domains comprise a mixture of donor and acceptor residues. Thus, these Examples do not deprive the present claims of novelty.

3.4. Example 12 and Tables 6A, 6B, 7A and 7B of the Genzyme application do not appear in the '549 application and thus cannot be cited against the present claims.

3.5. It is therefore believed that the present claims are novel over the parts of the Genzyme application which are entitled to the date of the '549 application.

3.6. Regarding the Queen article referred to above, this discloses a CDR-grafted antibody which has an acceptor residue at position 73 in the heavy chain whereas new claim 1 requires a donor residue at this position. Thus, it is submitted that new claim 1 is novel over Queen.

3.7. The antibody disclosed in the Queen article is directed against the p55 chain of the human interleukin 2 receptor. It does not have specificity for a tumour-specific antigen, an interleukin, the CD3 or CD4 antigen, or an adhesion molecule. It is therefore not specific for any of the types of antigen specified in claims 2 to 6. It is therefore submitted that all these claims are novel over Queen.

3.8. Since claims 7 to 18 are dependent on claims 1 to 6, it is submitted that all these claims are novel over Queen.

3.9. The Queen article discloses a method for "humanising" antibodies which involves molecular modelling and selection of residues to be changed to donor residues on the basis of comparisons of molecular models. This is an entirely different process from the one set forth in claims 19 and 20. Thus, these claims are novel over Queen.

3.10. In fact, there is nothing in the Queen paper which even suggests that it might be possible to determine a hierarchy of residues to be changed to donor which will work for any antibody with any framework. The disclosure in Queen suggests that each donor antibody needs to be treated as an individual case in comparison with the acceptor antibody. There is no suggestion that results obtained with one pair of donor and acceptor antibodies could be applied successfully to a different pair of antibodies. In contrast, the present invention shows that it is possible to apply general rules to any pair of antibodies in the expectation of success.

MIC
JCC
Lund's
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"

3.11. It is therefore submitted that the present claims are both novel and inventive over Queen.

3.12. EP-A-0 239 400 (Winter) is also cited in the International Search Report. The basic disclosure in the Winter application is limited to grafting only the CDRs from one antibody to another. All the Examples in the Winter application relate to this. There is a passage in the Winter application which indicates that it may be necessary to alter some framework residues. However, there is no guidance at all as to which residues should be changed or as to how such residues should be selected. There is certainly no suggestion that there is a hierarchy of residues which should be considered. Thus, it is submitted that the present claims are both novel and inventive over the Winter application.

3.13. It is believed that none of the other documents cited in the International Search Report is more relevant to the present claims than the three documents referred to above. Therefore, no discussion of any of these documents is provided. However, the Applicant would be pleased to address any concern the Examiner may have in this respect.

4. Procedure

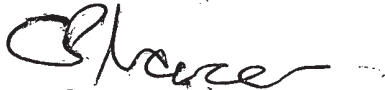
4.1. It is suggested that, once the Examiner has had an opportunity to study the file and familiarize himself with the case, it would be useful to hold an interview to address any concerns that the Examiner may have. If, after consideration of the file, the Examiner agrees

with this suggestion, it is requested that the Examiner sends out a Communication identifying any areas of concern and any documents which need to be discussed so that the Applicant can address those points fully.

4.2. If the Examiner feels that prosecution could be expedited in any other way, he is requested to telephone the undersigned to discuss the matter.

4.3. As a precautionary measure, the Applicant hereby requests Oral Proceedings, in the event that the Examining Division should feel minded to refuse the application.

Your faithfully,



MERCER, CHRISTOPHER PAUL

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CLAIMS

1. An antibody molecule having affinity for a predetermined antigen and comprising a composite heavy chain and a complementary light chain, said composite heavy chain having a variable domain comprising acceptor antibody heavy chain framework residues and donor antibody heavy chain antigen-binding residues, said donor antibody having affinity for said predetermined antigen, wherein, according to the Kabat numbering system, in said composite heavy chain, amino acid residues 5, 8, 10, 12 to 17, 19, 21, 22, 40, 42 to 44, 66, 68, 70, 74, 77, 79, 81, 83 to 85, 90, 92, 105, 109, 111 and 113 at least are acceptor residues and amino acid residues 23, 24, 31 to 35, 49 to 58, 71, 73, 78 and 95 to 102 at least are donor residues.

2. A tumour-specific antibody molecule having affinity for a predetermined tumour antigen and comprising a composite heavy chain and a complementary light chain, said composite heavy chain having a variable domain comprising acceptor antibody heavy chain framework residues and donor antibody heavy chain antigen-binding residues, said donor antibody having affinity for said predetermined tumour antigen, wherein, according to the Kabat numbering system, in said composite heavy chain, amino acid residues 5, 8, 10, 12 to 17, 19, 21, 22, 40, 42 to 44, 66, 68, 70, 74, 77, 79, 81, 83 to 85, 90, 92, 105, 109, 111 and 113 at least are acceptor residues and amino acid residues 23, 24, 31 to 35, 49 to 58 and 95 to 102 at least are donor residues.

3. An interleukin-specific antibody molecule having affinity for a predetermined interleukin and comprising a composite heavy chain and a complementary light chain, said composite heavy chain having a variable domain comprising acceptor antibody heavy chain framework residues and donor antibody heavy chain antigen-binding residues, said donor antibody having affinity for said predetermined interleukin wherein, according to the Kabat numbering system, in said