Office Action Summary

Application No.	Applicant(s)
09/146,206	
Examiner	Group Art Unit
	1642

—The MAILING DATE of this communication appears on the cover sheet beneath the correspondence address—

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, such period shall, by default, expire SIX (6) MONTHS from the mailing date of this communication .
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).

Status

☒ Responsive to communication(s) filed on 8/30/99.

☐ This action is **FINAL.**

☐ Since this application is in condition for allowance except for formal matters, **prosecution as to the merits is closed** in accordance with the practice under *Ex parte Quayle,* 1935 C.D. 1 1; 453 O.G. 213.

Disposition of Claims

☒ Claim(s) 43-105, 113-128 is/are pending in the application.

Of the above claim(s)_____ is/are withdrawn from consideration.

☐ Claim(s)_____ is/are allowed.

☒ Claim(s) 43-105, 113-128 is/are rejected.

☐ Claim(s)_____ is/are objected to.

☐ Claim(s)_____ are subject to restriction or election requirement.

Application Papers

☐ See the attached Notice of Draftsperson's Patent Drawing Review, PTO-948.

☐ The proposed drawing correction, filed on_____ is ☐ approved ☐ disapproved.

☐ The drawing(s) filed on_____ is/are objected to by the Examiner.

☐ The specification is objected to by the Examiner.

☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. § 119 (a)-(d)

☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 11 9(a)-(d).

☐ All ☐ Some* ☐ None of the CERTIFIED copies of the priority documents have been

☐ received.

☐ received in Application No. (Series Code/Serial Number)_____.

☐ received in this national stage application from the International Bureau (PCT Rule 1 7.2(a)).

*Certified copies not received:_____.

Attachment(s)

☐ Information Disclosure Statement(s), PTO-1449, Paper No(s)._____

☒ Notice of Reference(s) Cited, PTO-892

☐ Notice of Draftsperson's Patent Drawing Review, PTO-948

☐ Interview Summary, PTO-413

☐ Notice of Informal Patent Application, PTO-152

☐ Other_____

Office Action Summary

Effective February 7, 1998, the Group Art Unit location has been changed, and the examiner of the application has been changed. To aid in correlating any papers for this application, all further correspondence regarding this application should be directed to Minh-Tam Davis, Group Art Unit 1642.

Since this application is eligible for the transitional procedure of 37 CFR 1.129(a), and the fee set forth in 37 CFR 1.17(r) has been timely paid, the finality of the previous office action has been withdrawn pursuant to 37 CFR 1.129(a). Applicant's amendment filed on 08/26/98 has been entered.

The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

Applicant cancels claims 106-112, and adds new claims 115-128, which are related to claims 43-105, and are not new matter.

Accordingly, claims 43-105, 113-128 are being examined.

The following are the remaining rejections.

REJECTION UNDER 35 USC 112 FIRST PARAGRAPH, SCOPE, NEW REJECTION

Claims 43-105, 113-128 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for humanized antibody muMAb4D5, and an anti-CD3 antibody, or variable domains thereof, comprising CDR amino acids which bind specifically to p185, or CD3, does not reasonably provide enablement for any humanized antibody , or variable

domain thereof, comprising CDR amino acids which binds non-specifically to any antigen, wherein the framework region amino acids are substituted 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, or of 24H, 73H, 76H, 78H and 93H, for treating any chronic diseases. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims.

Claims 43-105, 113-128 are drawn to a humanized antibody , or variable domain thereof, comprising CDR amino acids which bind an antigen, or which bind $p185^{HER2}$. The framework region amino acids of said antibody or variable domain are substituted 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, or of 24H, 73H, 76H, 78H and 93H. Claim 105 is further drawn to a humanized antibody which lacks immunogenicity upon repeated administration for treating a chronic disease, and wherein its non-human CDR amino acids bind an antigen.

The specification discloses examples of humanized antibody muMAb4D5, anti-CD3, and anti-CD18 antibody, or variable domain thereof, comprising CDR amino acids which bind specifically to p185, CD3, and CD18, respectively. The substituted framework residues for the heavy chain of antibody muMAb4D5 are amino acids number 71, 73, 78, 93, and for the light chains are amino acid number 66 (table 3, and p.68). Only one humanized antibody, huMab4D5-8,

with all of the above five substitutions in the framework region binds to p185 3-fold more tightly than the murine counterpart. The humanized antibodies, huMab4D5-2 and huMab4D5-3, with one and four substitutions in the framework region, respectively, are, however, at least 10-fold less potent than the murine counterpart, having a K_d of 4.7nM and 4.4nM, respectively, as compared to a K_d value of 0.30nM of the murine counterpart. The substituted framework residues for the heavy chain of antibody anti-CD3 are amino acids number 75 and 76. Although the specification discloses that humanized anti-CD3 antibody enhances the cytotoxic effects of cytotoxic T cells 4-fold against tumor cells expressing $p185^{HER2}$, there is no disclosure in the specification concerning the binding affinity of the humanized anti-CD3 or anti-CD18 as compared to the murine counterpart. The claims however encompass any humanized antibody, without any specificity, binding to $p185^{HER2}$ or any antigen, with just any one of 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, of 24H, 73H, 76H, 78H and 93H. The claims further encompass any humanized antibody for treating any chronic disease.

One cannot extrapolate from humanizing one antibody, which binds to $p185^{HER2}$ 3-fold more tightly than the murine counterpart, to humanizing any antibody, wherein its affinity would be up to 3-fold or at least 3-fold more tightly than the murine counterpart, or wherein its affinity would be still intact for therapeutic purposes. In addition, one cannot extrapolate from humanizing an anti-p185 antibody by substitution at all five framework amino acids number H71, H73, H78, H93 and L66 in an anti-p185 antibody, or from humanizing an anti-CD3 antibody by

substitution at both framework amino acids number H75 and H76 in an anti-CD3 antibody, with humanizing any antibody by substitution at only any one amino acid 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, or of 24H, 73H, 76H, 78H and 93H. Patent '101 teach that different antibodies require different combinations of different substitutions in the light chain and heavy chain (table 1). Even the specification discloses that only one variant, huMab4D5-8, wherein all five framework amino acids number H71, H73, H78, H93 and L66 are substituted, binds to p185 3-fold more tightly than the murine counterpart. Other variants, with only one or even four substitutions have much less binding affinity than the murine counterpart(table 3). Thus it is unpredictable that substitution at only one framework amino acid in any antibody, or any kind of combination of framework amino acid substitutions would result in a humanized antibody that binds to its antigen 3-fold more tightly than its murine counterpart, or retains adequate affinity for therapeutic purposes. The specification does not disclose whether subtitution at only one of the claimed amino acid positions would produce a humanized antibody that has 3-fold more in affinity as the murine counterpart, or retains adequate affinity for therapeutic purposes. The specification does not disclose which combination of what substituted framework amino acids, other than H71, H73, H78, H93 and L66 for anti-p185 antibody, and H75 and H76 in anti-CD3 antibody would produce a humanized antibody that has 3-fold more in affinity as the murine counterpart, or retains adequate affinity for therapeutic purposes. It is well known in the art that not any substitution at any amino acids would produce a humanized

antibody having an affinity similar to the murine counterpart, unless it is tested by binding assays. The specification provides insufficient guidance with regard to the issues raised above and provides no working examples which would provide guidance to one skilled in the art and no evidence has been provided which would allow one of skill in the art to make the claimed humanized antibodies with a reasonable expectation of success. In view of the above, one of skill in the art would be forced into undue experimentation to practice the claimed invention.

Moreover, a humanized antibody that does not have a specificity for a particular antigen is of little practical use for treating a chronic disease, because said antibody would not target to the target tissues. In addition, although the specification discloses that murine anti-$p185^{HER2}$ antibody has been suceessfully used in treating tumor cell growth in culture (p.5), $p185^{HER2}$ and CD-3 are not specific for any tissues responsible for chronic disease, e.g. chronic headache, chronic lung inflammation, or chronic kidney disease. The specification does not disclose how to treat any chronic disease using the claimed humanized antibody. In the absence of a teaching of a method of treating any chronic disease, using the claimed humanized antibody, one of skill in the art would be forced into undue experimentation to practice the claimed invention.

REJECTION UNDER 35 USC 102, NEW REJECTION

1. New claims 115-117, 123, 127 are rejected under 35 USC 102(e) or 102(b) pertaining to anticipation by PN=5,530,101 or Queen et al, 1989, PNAS, USA, 86: 10029-10033.

Claims 115-117, 123, 127 are drawn to a humanized antibody or its heavy chain variable domain comprising non-human CDR amino acids, and a framework region amino acid wherein amino acid position 93H is substituted, utilizing the numbering system of Kabat, and wherein the substituted residue is the residue found in the corresponding location of the non-human antibody.

PN=5,530,101, teach humanized anti-Tac antibody, wherein amino acid 93 is substituted in heavy chain, using the aligned Kabat Eu sequence to provide the framework for the humanized antibody (column 45).

Queen et al, PNAS, teach humanized anti-Tac antibody, wherein amino acid 93 is substituted in heavy chain, using the aligned Kabat Eu sequence to provide the framework for the humanized antibody (figure 2).

Since anti-Tac antibody is a mouse antibody, its inherent heavy chain variable domain would comprise non-human CDR amino acids. Thus the humanized antibody and its heavy chain variable domain taught by patent '101 or Queen et al is the same as the claimed invention.

2. Claims 43, 44, 48, 55, 67, 71, 105, 115-117, 120, 127 are rejected under 35 USC 102(e) pertaining to anticipation by PN=5,530,101.

It is noted that PN=5,530,101 is filed on Sept, 1990, which is within a year before the claimed filing date of 06/14/91.

Claims 43, 44, 48, 55, 67, 71, 105, 115-117, 120, 127 are drawn to a humanized antibody or its heavy chain variable domain comprising non-human CDR amino acids, and a framework region amino acid wherein amino acid position 38L, 67L, 69H, 73H or 93H is substituted,

utilizing the numbering system of Kabat, and wherein the substituted residue is the residue found in the corresponding location of the non-human antibody. Claim 105 is further drawn to said humanized antibody which lacks immunogenicity compared to a non-human parent antibody upon repeated administration to a human patient.

PN=5,530,101 teaches humanized antibodies, wherein amino acid 38 or 67 are substituted in light chain (table 1, antibody Fd79 and M195, respectively), and amino acid 69, 73 or 93 is substituted in heavy chain (table 1, antibody CMV5, mik-beta-1, and Fd138-80, respectively), using the aligned Kabat Eu sequence to provide the framework for the humanized antibody. The humanized antibodies in table 1 would comprise non-human CDR amino acids (Summary). Patent '101 further teaches that the humanized antibodies will be substantially non-immunogenic in humans (Abstract). Thus the humanized antibody taught by patent '101 and its variable domain is the same as the claimed invention.

REJECTION UNDER 35 USC 102

1. Claim 128 is rejected under 35 USC 102(e) as being anticipated by PN=5,530,101, for the same reasons set forth in paper No.27 for the rejection of previous claims 23-24.

Applicant amends the claim 128 to read that the humanized antibody binds the antigen up to about 3-fold more tightly than the parent antibody. The language "up to" 3-fold reads on anything below 3-fold. Thus the structure and binding affinity of the claimed humanized antibody is the same as that of the humanized antibody taught by '101.

2. Claim 113 is rejected under 35 USC 102(e) as being anticipated by PN=5,693,762, for the same reasons set forth in paper No.27 for the rejection of previous claims 22-25, 38 and 39.

Applicant argues that the "consensus sequence" in '762 is the most homologous sequence from a single human immunoglobulin, and is thus different from the consensus sequence of the claimed invention.

Applicant's arguments set forth in paper No. 39 have been considered but are not deemed to be persuasive for the following reasons:

Although '762 uses the most homologous sequence from a single human immunoglobulin as an example, '762 also teach that as a principle, a framework is used from either a human immunoglobulin which is unusually homologous to the donor immunoglobulin, **or** a consensus framework from many human antibodies is used (column 13, first paragraph, lines 4-7). Thus the consensus sequence taught by '762 is the same as the claimed consensus sequence, as defined by the specification, i.e. the most frequently occurring amino acids, based on immunoglobulin of a particular species (p.14).

REJECTION UNDER 35 USC 103

Claims 113, 115-118, 123, 127-128 are rejected under 35 USC 103 as being unpatentable over US PN=5,693,762 in view of Kabat et al, for the same reasons set forth in paper No:27, for the rejection of previous claims 26-36 and 40-41.

Applicant argues as follows:

The rejection is made using hindsight reconstruction of the present invention. Patent '762 actually teaches away from the invention. The term "consensus framework" from '762 patent was not intended to refer to a sequence representing the most frequently occurring amino acids in the present invention. Furthermore, Kabat et al do not use the term "consensus", but rather "occurrences of most common amino acid". Thus there is no motivation to combine "consensus framework" from '762 patent with "occurrences of most common amino acid", especially the term "consensus framework" from '762 patent was not intended to refer to a sequence representing the most frequently occurring amino acids. Moreover, the present invention produces humanized antibodies with unexpected results, such as 1) lack of significant immunogenecity, as disclosed in the Declaration by Dr. Shak, 2) higher increase in binding affinity as compared to that of humanized antibodies known in the art, and 3) the same consensus sequence could be used to generate many different strong affinity humanized antibodies.

Applicant's arguments set forth in paper No. 39 have been considered but are not deemed to be persuasive for the following reasons:

Although '762 uses the most homologous sequence from a single human immunoglobulin as an example, '762 also teach that **as a principle**, a framework is used from either a human immunoglobulin which is unusually homologous to the donor immunoglobulin, **or** use a consensus framework **from many human antibodies** is used (column 13, first paragraph, lines 4-7). Thus the consensus sequence taught by '762 is the same as the claimed consensus sequence, as defined by the specification, i.e. the most frequently occurring amino acids, based on immunoglobulin of a

particular species (p.14). It is only Applicant's interpretation that the term "consensus framework" from '762 patent was not intended to refer to a sequence representing the most frequently occurring amino acids in the present invention. Furthermore, although Kabat et al do not use the term "consensus", but rather "occurrences of most common amino acid", one of ordinary skill in the art would readily understand that " a consensus sequence" from many antibodies is a sequence that occurs most frequently.

In addition, .In re Kerkhoven (205 USPQ 1069, CCPA 1980) summarizes:

"It is prima facie obvious to combine two compositions each of which is taught by prior art to be useful for same purpose in order to form third composition that is to be used for very same purpose: idea of combining them flows logically from their having been individually taught in prior art."

Applicant asserts that the claimed humanized antibodies are not obvious in view of the cited references because the cited prior art does not suggest such a combination. However, the instant situation is amenable to the type of analysis set forth in In re Kerkhoven,205 USPQ 1069 (CCPA 1980) wherein the court held that it is prima facie obvious to combine two compositions each of which is taught by the prior art to be useful for the same purpose in order to for a third composition that is to be used for the very same purpose since the idea of combining them flows logically from their having been individually taught in the prior art. Applying the same logic to the instant claims, given the teaching of the prior art that as a principle, a framework is used from either a human immunoglobulin which is unusually

homologous to the donor immunoglobulin, **or** a consensus framework from many human antibodies is used, and the structures of sequences that are most commonly occurred among many antibodies, it would have been obvious to humanize antibodies as taught by patent '762, using the most commonly occurred sequences taught by Kabat et al, because the idea of doing so would have logically followed from their having been individually taught in the prior art, and because patent '762 teaches the use of "consensus sequence", for the same purpose of producing humanized monoclonal antibodies for therapeutic purposes. One of ordinary skill in the art would have motivated to make humanized antibodies using the methods taught by '762 and the sequences taught by Kabat et al with a reasonable expectation of success. In addition, the arguments that the claimed invention is unexpected are not applicable, because the claims are broad, and drawn to any antibodies, and not specifically the claimed antibodies, wherein their specific target antigens, and their binding properties are not disclosed in the claims.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Minh-Tam B. Davis whose telephone number is (703) 305-2008. The examiner can normally be reached on Monday-Friday from 9:30am to 3:30pm, except on Wesnesday.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Tony Caputa, can be reached on (703) 308-3995. The fax phone number for this Group is (703) 308-4227.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the Group receptionist whose telephone number is (703) 308-0916.

Minh-Tam B. Davis

October 13, 2000

SUSAN UNGAR, PH.D
PRIMARY EXAMINER

Notice of References Cited

Application No.	Applicant(s)		
08/146,206	Carter et al.		
Examiner	Group Art Unit	Page 1 of 1	
M. T. Davis	1642		

U.S. PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	NAME	CLASS	SUBCLASS
	A					
	B					
	C					
	D					
	E					
	F					
	G					
	H					
	I					
	J					
	K					
	L					
	M					

FOREIGN PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	COUNTRY	NAME	CLASS	SUBCLASS
	N						
	O						
	P						
	Q						
	R						
	S						
	T						

NON-PATENT DOCUMENTS

*		DOCUMENT (Including Author, Title, Source, and Pertinent Pages)	DATE
	U	~~Queen et al. PNAS, USA, 86: 10029 - 10033~~ Dupl.	~~1989~~
	V		
	W		
	X		

* A copy of this reference is not being funished with this Office action.
(See Manual of Patent Examining Procedure, Section 707.05(a).)

U.S. Patent and Trademark Office
PTO-892 (Rev. 9-96)

Part of Paper No. 57

U.S. GPO: 1996-420-311/40176

1642

Patent Docket P0709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Paul J. Carter et al. Serial No.: 08/146,206 Filed: November 17, 1993 For: METHOD FOR MAKING HUMANIZED ANTIBODIES	Group Art Unit: 1642 Examiner: M. Davis **CERTIFICATE OF MAILING** I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner of Patents, Washington, D.C. 20231 on April 25, 2001 Wendy M. Lee

59/60 5-2-01

AMENDMENT UNDER 37 C.F.R. §1.111

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Responsive to the Office Action dated 10/25/00, reconsideration of the present application is respectfully requested in view of the following amendments and remarks. A request for a 3 month extension of time and the requisite fee accompany this amendment.

IN THE CLAIMS:

Please amend claims 113 and 114 as follows:

Sub 01

113. (Amended) A humanized variant of a non-human parent antibody which binds an antigen and comprises a consensus human variable domain 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

N'

respect to one another.

114. (Amended) The humanized variant of claim ~~128~~ which binds the antigen ~~about~~ up to 3-fold more ~~tightly~~ in the binding affinity than the parent antibody binds antigen.

REMARKS

Claims 43-105 and 113-128 are in the application. Claims 113 and 114 have been amended. Attached hereto is a marked-up version of the changes made to the claims by the current amendment. The attached page is captioned "Version with Markings to Show Changes Made".

Claim 113 no longer requires that the humanized variant bind antigen with better affinity than the parent antibody, up to about 3-fold tighter binding than the parent antibody. Hence, claim 114 has been amended herein to depend on claim 128, which claim requires that the humanized variant bind antigen more tightly than the parent antibody.

Prosecution History of the Present Application

Applicants first wish to express their concern about the undue prejudice to them due to the repeated transfer of this case from patent examiner to patent examiner, and to explain that this is a case which has thrice previously been indicated to be in condition for allowance.

The case was originally with Examiner Adams, then was transferred to Examiner Nolan. In the 8/13/98 interview, Examiner Nolan indicated that unexpected results would overcome the 103 rejection based on Queen Patent 5,693,762 (hereinafter "the '762 patent"). An amendment was filed 8/24/98 presenting the unexpected results. Shortly thereafter, the case was transferred to the present Examiner. Pending claims 43-114 were discussed in an interview on 10/16/98 between the undersigned, the present Examiner and Examiner Feisee at which time the only outstanding issue in the case related to the clarity of the terms "binding of CDR" and "significant immunogenicity". An amendment was filed 11/6/98 addressing those issues. The case was then transferred to Examiner Reeves, who issued a restriction requirement 3/29/99 at that late stage in prosecution. In an 8/23/99 interview, Examiners Reeves/Burke and Feisee indicated that the case would be in order for allowance with the filing of a terminal disclaimer for claim 111 and addition of an upper limit to affinity in claims 113 and 128. Claims 113 and 128 were amended as suggested by the Examiners and claim 111 was canceled to avoid the

obviousness-type double patenting rejection (see 8/30/99 amendment). Now the case has been transferred yet again to the present Examiner and prosecution has been re-opened on a case that was indicated to be in condition for allowance three times previously.

To the extent that any issues remain following entry of this amendment, Applicants specifically request an interview with the present Examiner and her supervisor to discuss this case so as to ensure speedy resolution of the issues and allowance of the application. It is noted that this is a pre-GATT case and two 129(a) responses have previously been filed.

Section 112, first paragraph, Scope, New Rejection

Claims 43-105 and 113-128 are rejected under 35 USC Section 112, first paragraph on the basis that the specification, while being enabling for humanized antibody muMAb4D5 and an anti-CD3 antibody, or variable domains thereof, "does not reasonably provide enablement for any humanized antibody, or variable domain thereof, comprising CDR amino acids which binds non-specifically to any antigen, wherein the framework region amino acids are substituted 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, or of 24H, 73H, 76H, 78H and 93H, for treating any chronic disease."

The Examiner contends that the specification discloses examples of humanized muMAb4D5, anti-CD3 and anti-CD18 antibodies or variable domains thereof; that the substituted FR residues for muMAb4D5 are 71H, 73H, 78H, 93H and 66L; and that only one humanized antibody (huMAb4D5-8) with all the above five substitutions binds to p185 3-fold more tightly than the murine counterpart. The Examiner further contends that the substituted framework residues for the heavy chain of antibody anti-CD3 are FR residues 75 and 76, and that there is no disclosure concerning the binding affinity of the humanized anti-CD3 or anti-CD18 as compared to the murine counterpart. The Examiner contends that one cannot extrapolate from humanizing one antibody, which binds to $p185^{HER2}$ 3-fold more tightly than the murine counterpart, to humanizing any antibody,

wherein its affinity would be up to 3-fold or at least 3-fold tighter than the murine counterpart, or wherein its affinity would still be intact for therapeutic purposes. The Examiner further argues that one cannot extrapolate from humanizing an anti-p185 antibody by substitution of all five FR residues at positions 71H, 73H, 78H, 93H and 66L in an anti-p185 antibody, or from humanizing an anti-CD3 antibody by substitution at both framework residues 75H and 76H, with humanizing any antibody by substitution at only one amino acid residue 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, or of 24H, 73H, 76H, 78H and 93H. The Examiner opines that the specification does not disclose whether substitution at only one of the claimed amino acid positions would produce a humanized antibody that has 3-fold more affinity, or which combination of what substituted FR residues (other than 71H, 73H, 78H, 93H and 66L for an anti-p185 antibody or 75H and 76H in an anti-CD3 antibody) would produce a humanized antibody that has 3-fold more affinity than the murine counterpart, or retains adequate affinity for therapeutic purposes. The Examiner contends that a humanized antibody that does not have specificity for a particular antigen is of little practical use for treating a chronic disease and that the specification does not disclose how to treat any chronic disease using the claimed humanized antibody.

Applicants submit that claims 43-105 and 113-128 are enabled by the present application.

First, Applicants point out that none of the claims (other than claim 114) require that the humanized antibody bind antigen about 3-fold more tightly than the parent antibody binds antigen, as the Office Action seems to imply. The independent claims herein merely recite that the humanized antibody variable domain comprises CDR residues which bind an antigen (claims 43, 104 and 115); the antibody comprising the humanized antibody variable domain binds $p185^{HER2}$ (claim 72); the humanized antibody comprises CDR residues which bind an antigen (claim 105); the humanized variant bind antigen (claim 113 herein); or the humanized variant bind

antigen more tightly than the parent antibody - up to about 3-fold more tightly than the parent antibody (claim 128).

Second, Applicants submit that the claims herein encompass the humanized variable domain or antibody having at least one of the FR substitutions specified, but optionally having further FR substitution(s) in order to improve affinity to a level at which an antibody comprising the variable domain is able to bind antigen.

Finally, Applicants wish to clarify some issues concerning the Office's characterization of the working examples. First, it is noted that Example 1 actually describes several humanized anti-p185^{HER2} variants with FR substitution(s) as set forth in the claims herein: huMAb4D5-2, huMAb4D5-3, huMAb4D5-4, huMAb4D5-5, huMAb4D5-6, huMAb4D5-7, huMAb4D5-8 (Table 3 on page 72). Thus, it is clear that this example teaches humanized variants which do not include substitution of all of FR residues 71H, 73H, 78H, 93H and 66L. Each of these FR substitution variants bound antigen with better affinity than the initial antibody (huMAb4D5-1) comprising non-human CDR amino acid residues, but lacking any FR substitution(s). Two of the humanized anti-p185^{HER2} variants surprisingly bound antigen better than the murine parent antibody muMAb4D5, *i.e.* huMAb4D5-6 and huMAb4D5-8. With regard to Example 3 concerning the humanized anti-CD3 variants, aside from the 75H and 76H FR substitutions noted by the Office, this Example further teaches the following FR substitutions: L71, 71H, 73H and 78H. See, *e.g.*, Fig. 5 which aligns the murine anti-CD3 "muxCD3" sequences, the humanized variant "huxCD3v1" sequences, and the human sequences, "huκI" and "huIII".

The specification clearly teaches how to make humanized antibody variable domains and antibodies comprising such domains, and identifies FR residues that can be substituted to improve the binding affinity of an antibody comprising the humanized variable domain. See, *e.g.* pages 12-13, 20-26 and 28-29; Example 1 on pages 63-74; Example 3 on pages 79-88; and Example 4 on page 89. The specification teaches FR substitution(s)

individually or in combination. Based on the disclosure of the present application, one is able to make an antibody comprising a humanized antibody variable domain which binds antigen. The Office has provided no evidence that the humanized antibody variable domains or humanized antibodies comprising the FR substitution(s) claimed herein would not be functional, beyond speculating that the affinity might not be about 3-fold better than the parent antibody (and, as noted above, the claims other than claim 114 do not require this improvement in affinity). Hence, Applicants submit that the presently claimed variable domains and antibodies are enabled by the specification.

Reconsideration and withdrawal of the enablement rejection is respectfully requested in view of the above.

Section 102 - Claims 115-117, 123 and 127

Claims 115-117, 123 and 127 are rejected under 35 USC Section 102(e) or 102(b) as anticipated by US Patent No. 5,530,101 (hereinafter "the '101 patent") or Queen *et al. PNAS (USA)* 86:10029-10033 (1989) (hereinafter "Queen *et al.*"). The Examiner contends that the '101 patent and Queen *et al.* teach a humanized anti-Tac antibody wherein amino acid 93 is substituted in the heavy chain, using the aligned Kabat Eu sequence to provide the framework for the humanized antibody.

Applicants point out that - as explained earlier in prosecution - the substituted 93 FR residue in the cited references is not 93H "utilizing the numbering system set forth in Kabat" (see page 13, line 33 through to line 22 on page 14 of the present application) as required by claims 115-117, 123 and 127 of the present application. In particular, as noted on page 6 of the amendment hand carried to the Office on 10/7/97, residue no. 93 in the heavy chain of the anti-Tac antibody in the cited references, is actually 89H utilizing the numbering system set forth in Kabat. The cited references use a sequential numbering system, rather than the Kabat numbering system claimed herein.

Reconsideration of the 102(e) and 102(b) rejections based on the '101

patent and Queen *et al.* is respectfully requested in view of the above.

Section 102 - Claims 43, 44, 48, 55, 67, 71, 105, 115-117, 120 and 127

Claims 43, 44, 48, 55, 67, 71, 105, 115-117, 120 and 127 are rejected under 35 USC Section 102(e) as being anticipated by the '101 patent. The Examiner urges that FR substitutions 38L, 67L, 69H, 73H and 93H are taught by the '101 patent. Specifically, the Examiner contends that amino acids 38 or 67 are substituted in the light chain of the Fd79 and M195 antibodies, respectively, and amino acids 69, 73 or 93 are substituted in the heavy chains of the CMV5, mik-β1 and Fd138-80 antibodies, respectively. The '101 patent is further alleged to teach (in the abstract thereof) that the humanized antibodies therein will be substantially non-immunogenic in humans.

Applicants submit that the presently claimed FR 38L, 67L, 69H and 93H substitutions are different from those in the '101 patent to which the Examiner refers, since the numbering of the presently claimed FR substitutions utilizes the numbering system set forth in Kabat, whereas the '101 patent uses sequential numbering for the residues. In particular, VL residue 38 of Fd79 is a CDR residue, as opposed to a FR residue (note Table 1 in column 43 of the '101 patent which states that residue 38 is in "Category 1" and therefore is a CDR residue; see lines 66-67 in column 13 of the '101 patent); VL residue 67 of M195 is FR residue 63L utilizing the numbering system set forth in Kabat (see page 8 of Applicants' 10/7/97 amendment); VH residue 69 of CMV5 is 68H utilizing the numbering system set forth in Kabat (see page 9 of the 10/7/97 amendment); and VH residue 93 of Fd138-80 is FR residue 89H utilizing the numbering system set forth in Kabat (see page 7 of the 10/7/97 amendment).

As to the FR 73H substitution (utilizing the numbering system set forth in Kabat) claimed herein, Applicants submit that the disclosure of the humanized mik-β1 antibody is too late to qualify as Section 102 prior art to claim 115 which recites that substitution. See page 11, first full paragraph of Applicants' 1/15/99 amendment.

Finally, as to the recitation in claim 105 herein that the humanized antibody "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", Applicants have shown that antibodies humanized according to one preferred embodiment of the present invention possess this property. See the Shak Declaration filed 8/24/99. The '101 patent merely states that the humanized antibodies will be "substantially non-immunogenic" in humans, but fails to disclose that the humanized antibodies lack substantial immunogenicity upon repeated administration to a human patient in order to treat a chronic disease in that patient.

Reconsideration and withdrawal of the Section 102(e) rejection is respectfully requested in view of the above.

Section 102(e) - Claim 128

Claim 128 is rejected under 35 USC Section 102(e) as being anticipated by the '101 patent. The Examiner states that the language "up to" 3-fold reads on anything below 3-fold.

Claim 128 pertains to a humanized antibody which binds antigen more tightly than the parent antibody (up to about 3-fold more tightly). The Queen patents state that the humanized antibodies therein bind the target antigen with the same affinity, or bind less tightly, than the parent antibody. See pages 21-22 of Applicants' amendment filed 8/24/98. While humanized M195 was later discovered to bind antigen up to about 3-fold more tightly than the parent antibody bound antigen (see paragraph 2 on page 2 of the 8/30/99 amendment), this property of the humanized M195 antibody is not described in the '101 patent (see lines 28-29 in column 60 of the '101 patent).

Reconsideration and withdrawal of the Section 102(e) rejection of claim 128 is respectfully requested.

Section 102(e) - Claim 113

Claim 113 is rejected under 35 USC Section 102(e) as being anticipated

by US Patent 5,693,762 ("the '762 patent") for the same reasons set forth in paper No. 27 for the rejection of previous claims 22-25, 38 and 39.

The Examiner contends that the '762 patent teaches "as a principle, a framework is used from either a human immunoglobulin which is unusually homologous to the donor immunoglobulin, or a consensus framework from many human antibodies is used".

Applicants submit that this disclosure in the '762 patent simply fails to anticipate the presently claimed "consensus human variable domain" in claim 113 as defined by the present specification. See the discussion of the '762 patent on pages 13-14 of the 8/24/98 amendment. The Examiner states on page 11 of the above Office Action that it 'is only Applicant's interpretation that the term "consensus framework" from '762 patent was not intended to refer to a sequence representing the most frequently occurring amino acids in the present invention'. Applicants respectfully disagree. Indeed the Office initially suggested the alternative interpretation for the term "consensus framework" as it was used by Queen *et al.* See page 4 of the Office Action dated 12/23/96 in which Examiner Nolan stated:

> "Regarding the consensus sequence, the combination of references teach the human framework regions having a significantly high degree of sequence homology (conservative regions). Queen et al. in particular point to Kabat as demonstrating that this was known in the art well in advance of applicant's filing date, see reference 38, cited by Queen et al." (Emphasis added).

The Queen *PNAS* paper to which Examiner Nolan referred, was concerned with using a human framework region from a human immunoglobulin which was unusually homologous to the donor immunoglobulin, and failed to mention a consensus human variable domain as that expression is used in the present application. Hence, the Office has previously used the expression "consensus sequence" to describe the highly homologous approach taught by Queen *et al.*

Nothwithstanding this, Applicants note that in order to anticipate a claimed invention, the reference alone much teach each and every element of the claim. Even if it were the case that the "consensus framework" in the '762 patent was intended to refer to an amino acid sequence which comprises the most frequently occurring amino acid residues at each location in all human immunoglobulins (see page 14, lines 29-31 of the present application), which is denied, the Office has not shown that the '762 patent unambiguously disclosed the selection invention recited in claim 113 herein pertaining to a "consensus human variable domain of a human heavy chain immunoglobulin subgroup". The Office has combined the '762 patent with Kabat *et al.* (see Section 103 discussion below) in an attempt to show that this particular consensus sequence had been disclosed previously. Hence, Applicants submit that claim 113 is novel over the '762 patent. Applicants will demonstrate in the following section how the invention set forth in claim 113 is also nonobvious over the '762 patent, due to the unexpected results attributable thereto.

Reconsideration and withdrawal of the Section 102 rejection based on the '762 patent is respectfully requested in view of the above.

Section 103

Claims 113, 115-118, 123 and 127-128 are rejected under 35 USC Section 103 as being unpatentable over the '762 patent in view of Kabat *et al.*

First, it is noted that the Examiner relies on the rejection based on the '762 patent in view of Kabat *et al.* for the same reasons as set forth in paper no. 27 (Applicants assume paper no. 34 - Examiner Nolan's Office Action dated 12/23/97 is intended). Examiner Nolan previously indicated that the unexpected results would overcome the 103 rejection based on the '762 patent combined with Kabat *et al.* (see Paper no. 37; 8/13/98 Interview Summary).

Applicants rely on the unexpected results attributable to the consensus human variable domain of a human heavy chain immunoglobulin subgroup as demonstrating that the presently claimed antibodies are not obvious over

the '762 patent combined with Kabat *et al.* See pages 18-23 of the 8/24/98 amendment and the Shak declaration attached thereto.

The Examiner urges that "the arguments that the claimed invention is unexpected are not applicable, because the claims are broad, and drawn to any antibodies, and not specifically the claimed antibodies, wherein their specific target antigens, and their binding properties are not disclosed in the claims."

Applicants submit that the Examiner's basis for ignoring the evidence of unexpected results is legally flawed - at least with respect to (1) the lack of significant immunogenicity of the claimed humanized antibodies upon repeated administration to a human patient, *e.g.* to treat a chronic disease in that patient and (2) the ability to make many strong affinity antibodies, thus avoiding tailoring each human framework to each non-human antibody to be humanized. Those unexpected results provide objective evidence of non-obviousness. *Specialty Composites v. Cabot Corp.*, 845 F. 2d 981, 6 USPQ 2d 1601 (Fed. Cir. 1988).

As to unexpected result (1), Applicants have demonstrated that antibodies humanized using a consensus human variable domain of a human heavy chain immunoglobulin subgroup as set forth in claim 113 herein lack significant immunogenicity upon repeated administration to a human patient in order to treat a chronic disease in that patient. This was shown in the Shak Declaration for humanized anti-HER2, anti-IgE, anti-VEGF and anti-CD11a antibodies. See pages 18-21 of the 8/24/98 amendment and the Shak Declaration attached thereto. Hence, this unexpected property is not linked to certain antibodies or specific target antigens, but is generally applicable and the claims are commensurate in scope with the unexpected result relied upon.

Turning now to unexpected result (2), Applicants have shown that a consensus human variable domain of a human heavy chain immunoglobulin subgroup as set forth in claim 113 can be used to generate many different strong affinity humanized antibodies, including anti-HER2, anti-CD3,

anti-CD18, anti-IgE, anti-CD11a and anti-VEGF humanized antibodies (see pages 22-23 of the 8/24/98 amendment). Again, this further unexpected property is not dependent on the antibody or target antigen, and hence should be considered with respect to the non-obviousness of the presently claimed antibodies.

Hence, Applicants submit that claim 113 directed to a humanized variant comprising a consensus human variable domain of a human heavy chain immunoglobulin subgroup is non-obvious over the cited art, because of unexpected results (1) and (2) noted above.

As to the other rejected claims, Applicants point out that claim 115 recites FR substitutions at one or more of positions 24H, 73H, 76H, 78H and 93H, utilizing the numbering system set forth in Kabat. The Office has not shown how the cited art disclosed or suggested substitution of FR residues 24H, 76H, 78H and 93H, utilizing the numbering system set forth in Kabat; and, as noted above, the disclosure concerning substitution of 73H in the mik-β1 antibody is too late to qualify as Section 102 prior art to the invention set forth in claim 115 herein. With regard to claim 117, the Office fails to teach a humanized antibody with FR substitution(s) limited to positions 24H, 73H, 76H, 78H and 93H, utilizing the numbering system set forth in Kabat. As to claim 118, the Office has not demonstrated how the art would have taught combining the listed FR substitution(s) in claim 115 with a consensus human variable domain. With regard to claim 123, as noted previously, substituted 93 FR residue in Queen's anti-Tac or Fd138-80 antibodies is not the same as FR substitution 93H "utilizing the numbering system set forth in Kabat." Finally, with respect to claim 128, as noted above, the Queen patents state that the humanized antibodies therein bind the target antigen with the same affinity, or bind <u>less tightly</u>, than the parent antibody. See pages 21-22 of Applicants' amendment filed 8/24/98. While humanized M195 was later discovered to bind antigen up to about 3-fold more tightly than the parent antibody bound antigen (see paragraph 2 on page 2 of the 8/30/99 amendment), this property of the humanized M195 antibody is not described in the '101 patent (see lines 28-29 in column 60 of the '101

patent). The ability to bind antigen more tightly than the parent antibody was a further unexpected result observed with respect to certain humanized antibodies of the present application.

Reconsideration and withdrawal of the Section 103 rejection of claims 113, 115-118, 123 and 127-128 is respectfully requested in view of the above.

Respectfully submitted,

GENENTECH, INC.

Date: April 25, 2001

By:
Wendy M. Lee
Reg. No. 40,378
Telephone: (650) 225-1994

VERSION WITH MARKINGS TO SHOW CHANGES MADE

Claims 113 and 114 have been amended as follows:

113. (Three Times Amended) A humanized variant of a non-human parent antibody which binds an antigen [with better affinity than the parent antibody] and comprises a consensus human variable domain 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 [, wherein the humanized variant binds antigen up to about 3-fold more tightly than the parent antibody binds antigen].

114. (Amended) The humanized variant of claim [113] 128 which binds the antigen about 3-fold more tightly than the parent antibody binds antigen.

Patent Docket P0709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Paul J. Carter et al. Serial No.: 08/146,206 Filed: November 17, 1993 For: METHOD FOR MAKING HUMANIZED ANTIBODIES	Group Art Unit: 1642 Examiner: M. Davis **CERTIFICATE OF MAILING** I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner of Patents, Washington, D.C. 20231 on April 25, 2001 Wendy M. Lee

PETITION AND FEE FOR THREE MONTH EXTENSION OF TIME
(37 CFR 1.136(a))

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Applicants petition the Commissioner of Patents and Trademarks to extend the time for response to the Office Action dated October 25, 2000 for three months from January 25, 2001 to April 25, 2001. The extended time for response does not exceed the statutory period.

Please charge Deposit Account No. 07-0630 in the amount of $890.00 to cover the cost of the extension. Any deficiency or overpayment should be charged or credited to this deposit account. A duplicate of this sheet is enclosed.

Respectfully submitted,

GENENTECH, INC.

Date: April 25, 2001

By: _____
Wendy M. Lee
Reg. No. 40,378
Telephone No. (650) 225-1994

08146206 05/02/2001 KDOWNING 00000001 07-0630
01 FC:117 890.00 CH

09157
PATENT TRADEMARK OFFICE

Interview Summary	Application No. 08/146,206	Applicant(s) Carter et al
	Examiner Minh-Tam Davis	Group Art Unit 1642

All participants (applicant, applicant's representative, PTO personnel):

(1) *Minh-Tam Davis* (3) _____

(2) *Ewndy Lee* (4) _____

Date of Interview *Apr 26, 2001*

Type: a)☒ Telephonic b)☐ Video Conference
c)☐ Personal [copy is given to 1)☐ applicant 2)☐ applicant's representative]

Exhibit shown or demonstration conducted: d)☐ Yes e)☒ No. If yes, brief description:

Claim(s) discussed: _____

Identification of prior art discussed:

Agreement with respect to the claims f)☐ was reached. g)☐ was not reached. h)☐ N/A.

Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments:

Applicant requests an iinterview if the case is not ready for allowance following entry of the amendment to be filed today.

(A fuller description, if necessary, and a copy of the amendments which the examiner agreed would render the claims allowable, if available, must be attached. Also, where no copy of the amendments that would render the claims allowable is available, a summary thereof must be attached.)

i)☒ It is not necessary for applicant to provide a separate record of the substance of the interview (if box is checked).

Unless the paragraph above has been checked, THE FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP section 713.04). If a reply to the last Office action has already been filed, APPLICANT IS GIVEN ONE MONTH FROM THIS INTERVIEW DATE TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW. See Summary of Record of Interview requirements on reverse side or on attached

Examiner Note: You must sign this form unless it is an Attachment to a signed Office action.

7/13/01

#6

7/13/01

Patent Docket P0709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Paul J. Carter et al. Serial No.: 08/146,206 Filed: November 17, 1993 For: METHOD FOR MAKING HUMANIZED ANTIBODIES	Group Art Unit: 1642 Examiner: M. Davis **Certificate of Facsimile Transmission Under 37 CFR § 1.8** In accordance with CFR § 1.6(d), this correspondence addressed to Examiner Minh-Tam Davis, The Patent and Trademark Office, Washington, DC 20231 is being transmitted to facsimile No. (703) 308-4426 on July 13, 2001 Wendy M. Lee

AMENDMENT TRANSMITTAL

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Transmitted herewith is an amendment in the above-identified application.

The fee has been calculated as shown below.

	Claims Remaining After Amendment		Highest No. Previously Paid For	Present Extra	Rate	Additional Fees
Total	82	-	86	0	18	$0.00
Independent	8	-	9	0	80	$0.00
__ 0Multiple dependent claim(s), if any					270	$0.00
					Total Fee Calculation	$0.00

X
X

No additional fee is required.
The reference O'Connor *et al. Protein Engineering* 11(4):321-328 (1998) is attached.

The Commissioner is hereby authorized to charge any additional fees required under 37 CFR 1.16 and 1.17, or credit overpayment to Deposit Account No. 07-0630.

Respectfully submitted,
GENENTECH, INC.

Date: July 13, 2001

By: _____
Wendy M. Lee
Reg. No. 40,378
Telephone No. (650) 225-1994

09157
PATENT TRADEMARK OFFICE

61/0 61

Patent Docket P0709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE 9/13/01

In re Application of Paul J. Carter et al. Serial No.: 08/146,206 Filed: November 17, 1993 For: METHOD FOR MAKING HUMANIZED ANTIBODIES	Group Art Unit: 1642 Examiner: M. Davis **Certificate of Facsimile Transmission Under 37 CFR § 1.8** In accordance with CFR § 1.6(d), this correspondence addressed to Examiner Minh-Tam Davis, The Patent and Trademark Office, Washington, DC 20231 is being transmitted to facsimile No. (703) 308-4426 on July 13, 2001 Wendy M. Lee

SUPPLEMENTAL AMENDMENT

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

IN THE CLAIMS:

Please amend claims 113 and 128 as indicated below:

64 113. (Three times amended) 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.

01

D

128. (Twice Amended) 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 a Framework Region (FR) 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 humanized variant binds the antigen more tightly than and up to about 3-fold more tightly than the parent antibody binds antigen.

Please add the following claims:

--129. 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.

130. The humanized variable domain of claim 129 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.

131. The humanized variable domain of claim 129 wherein no human Framework Region (FR) residue other than those set forth in the group has been substituted.--

REMARKS

Applicants wish to thank Examiners Davis and Caputa for granting an interview to the representatives of Applicants on July 3, 2001. It is noted that the interview was terminated before its completion due to a fire alarm and evacuation of the building. The response herein reflects points raised by the Office during the interview. To the extent that issues remain in the case following entry of this and the previous amendment, Applicants respectfully request a further interview given the protracted prosecution of the case as discussed in the interview.

The pending claims

In the above-noted interview Examiner Caputa asked how the framework in claim 113 differed from the "consensus framework from many human antibodies" as in column 13 of the cited Queen '762 patent. In the interests of expediting prosecution, Applicants have amended claim 113 herein to recite the language found on page 14, lines 29-31 of the present application. The differences between the disclosure of the '762 patent and the invention set forth in claim 113 will be discussed below.

As discussed in the interview, claim 128 is amended herein to emphasize that the humanized antibody of this claim is one with better affinity than the non-human parent. This amendment obviates the §102 rejection over the disclosure of the '101 patent.

Claims 129-131 have been added herein. Claim 129 represents a combination of claims 43 and 115 and includes the FR substitution language from claim 128. Claims 130-131 employ language from claims 44 and 45, respectively.

Attached hereto is a marked-up version of the changes made to the claims by the current amendment. The attached page is captioned "**Version with markings to show changes made.**" Applicants submit that the amendments do not introduce new matter and therefore should be entered. Following entry of this amendment, claims 43-105 and 113-131 will be pending in the present application.

As was pointed out in the interview, the present application contains three different types of independent claims: (1) claims 43, 72, 104, 105 and 115 encompassing humanized antibody variable domains or antibodies comprising FR substitution(s) including one or more FR substitutions from a specified selection of FR positions; (2) claim 128 directed to a humanized variant which binds the antigen more tightly than the parent antibody binds antigen (up to about 3-fold more tightly); and (3) claim 113 directed to a humanized antibody comprising non-human CDR and FR residue(s) incorporated into 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.

Section 102

A comprehensive reply to the outstanding Section 102 rejections can be found in the amendment dated April 25, 2001. As discussed in the interview, it is believed that the Section 102 rejections should be withdrawn.

With respect to claims 43, 72, 104, 105 and 115, Applicants pointed out that Queen used sequential numbering, rather than Kabat numbering, for the FR residues, such that the 93H, 38H, 67L and 69H FR substitutions according to Kabat herein were not disclosed by Queen. As to the 73H FR substitution claimed herein, Applicants will submit shortly a swearing behind declaration showing completion of the invention of a humanized variable domain or antibody comprising that FR substitution, prior to cited Queen patent.

As to claim 128, Applicants pointed out that Queen did not describe humanized antibodies with improved affinity - affinity was either about the same or worse than the rodent antibody. The amendment herein clarifies that claim 128 pertains to antibodies with better affinity than the non-human parent antibody.

Finally, Applicants submit that recitation of "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" in claim 113 renders the humanized antibody therein novel over the cited Queen '762 patent. The Section 103 rejection will be addressed below.

Withdrawal of the outstanding Section 102 rejections is respectfully requested.

Section 112, first paragraph, scope

A full and complete response to the outstanding rejection of claims 43-105 and 113-128 may be found in the communication to the Office dated April 25, 2001.

In the outstanding Office Action, the Examiner maintains that each of the claims presented is not enabled by the disclosure. The basis for the assertion of the Examiner is that she believes the practice of the invention as reflected in each of the claims presented would constitute undue experimentation. Based on the points raised by the Examiner in the July 3 interview and the outstanding Office Action, Applicants believe this conclusion is based on misunderstandings of the law governing enablement, particularly as it pertains to the issue of undue experimentation, and a mischaracterization of the claims at issue and the disclosure. Moreover, Applicants will summarize hereinbelow relevant evidence which demonstrates the reproducibility of the methods disclosed in the present application for generating useful humanized antibody variable domains and antibodies encompassed by the claims herein.

Enablement must be measured in relation to the claims, the disclosure and what is known to a person skilled in the art. See, *United States v. Telectronics, Inc.*, 857 F.2d 778, 785, 8 USPQ2d 1217, 1223 (Fed. Cir. 1988) ("The test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation."). Undue experimentation, in turn, is a conclusion based on a number of discrete factual determinations. *In re Wands*, 858

F.2d 731, 737 (wherein the court listed eight factors that must be considered as a group when determining an issue of undue experimentation). In the present rejection, the only factors that apparently have been considered by the Examiner are the breadth of the claims and unpredictability in the art.

With respect to the scope of the claims, it is respectfully submitted that the Examiner has not accurately construed the claim scope, either in the rejections set forth in the outstanding Office Action or as characterized during the interview of July 3.

First, as has been noted in previous communications, only one claim (claim 114) specifically requires a three-fold increase in affinity of the humanized antibody relative to the non-human parent antibody. Claim 128, as amended, requires a binding affinity greater than the parent antibody, up to about three times the parent antibody affinity. Claims 43 to 105, 113 and 115 to 127 each contain no reference to minimum binding affinity relative to the parent antibody. Assertions that it would not have been possible to produce a humanized antibody subject to these claims having a three-fold increase in binding affinity are simply irrelevant to all but one claim.

Second, a requirement in each claim presented is that the variable domain retain the functional capacity to bind the antigen bound by the parent antibody. Thus, claims are not directed to single amino acid substitutions in an abstract sense that result in polypeptides that are inoperative as antibody binding domains. Instead, each of the claims presented requires the resulting humanized antibody variable domain or antibody to retain the antibody binding specificity of the parent antibody, and certain of the claims require the binding affinity to be greater than the parent antibody. Omitting the antibody binding limitation present in each claim improperly changes the scope of the claim.

Third, each of the independent claims is further limited in respect of one or more specific and objectively defined physical attributes of the resulting humanized antibody variable domain or antibody. For example, claim 43 identifies -- and thereby limits the claimed invention to -- a finite number of species of antibody binding domains which comprise amino acid substitutions in said binding domain selected from a finite range of substitutions in the framework region. If this physical characteristic of the humanized antibody variable domain is not present, it is outside the scope of this claim. Similarly, the claims do not encompass alterations of a human antibody variable domain that do not result in antibodies that bind to the antigen bound by the parent antibody.

Thus, it is respectfully submitted that the specific physical and functional characteristics of the claimed antibody variable regions must be given weight in determining the scope of the claims. The failure of the Examiner to do so has resulted in an improper characterization of the claimed invention, which is fundamental to the determination of enablement.

The second issue upon which the Examiner has not given sufficient weight are the extensive teachings in the disclosure, in view of what was known in the art as of the time of filing of the present application. The present disclosure provides more than ample direction to a person skilled in the art to rely upon in producing the variable domains and antibodies falling within the scope of the present claims. In particular, the present disclosure provides specific guidance to a person skilled in the art to produce, alter and select variants falling within the scope of the claims without the exercise of undue experimentation.

For example, the disclosure at pages 10-16, 20-29 and in the working examples recites a summary of the process to be used to produce the claimed humanized antibody domains and antibodies. As noted therein, steps for identifying and producing the variant sequences are described,

as are a variety of physical attributes of the resulting variants that are to be selected for through the process described therein (*e.g.*, the substituted FR residue interacts with a CDR, non-covalently binds antigen directly or participates in the V_L-V_H interface). A person reasonably skilled in this field would face no difficulty in taking any parent antibody having a particular binding specificity and, following the explicit and comprehensive teachings of the present disclosure, construct and select humanized antibody domains and antibodies as defined in the claims.

The third basis of the Examiner's rejection appears to be the belief that the claims cannot be practiced without undue experimentation. Undue experimentation is a conclusion that must be reached after considering a number of discrete factors. Two of these, claim scope and the teachings of the disclosure, have been addressed above and in the earlier response to the outstanding Office Action. In addition, the Examiner appears have relied on an assumption that there is an abnormally high level of unpredictability in the field of the invention. In particular, the Examiner is apparently asserting that there is such an inherent degree of unpredictability in the art that no claim to a humanized antibody could ever issue if it were not limited to a specifically defined amino acid sequence associated with a specific antibody specificity. This is an inaccurate characterization of the level of unpredictability in the field of the invention at the time the present application was filed, and is used in an improper manner by the Examiner in light of law governing lack of enablement due to undue experimentation.

Unpredictability in the art, standing alone, is not a conclusion that can support a rejection on the basis of lack of enablement. *In re Wands*, 858 F.2d 731, 737 (Fed. Cir. 1988). Instead, it is a factor whose significance must be assessed in making the legal determination of whether practice of the claimed invention would involve undue experimentation. Moreover, the fact that an art has unpredictability

associated with it does not condemn any claim that goes beyond a specific working example. As §2164.03 of the MPEP provides:

> The "predictability or lack thereof" in the art refers to the ability of one skilled in the art to extrapolate the disclosed or known results to the claimed invention. If one skilled in the art can readily anticipate the effect of a change within the subject matter to which the claimed invention pertains, then there is predictability in the art. On the other hand, if one skilled in the art cannot readily anticipate the effect of a change within the subject matter to which that claimed invention pertains, then there is lack of predictability in the art.

In the present case, neither the Examiner's characterization of unpredictability nor the assessment of the significance of unpredictability in light of the present disclosure is accurate.

As to the former issue, and as noted in the earlier response to the outstanding Office Action, the number of examples of successful modifications (*i.e.*, modifications resulting in functional humanized antibody binding domains) made according to the teachings of the present disclosure far exceeds the number suggested by the Examiner. For example, for one target antigen (HER2), eight successful variants were constructed using the procedures of the present invention. Each of these variants preserved binding affinity of a nature to make it a useful humanized binding domain.

Examiner Davis explained in the interview her opinion that variants (*e.g.* huMAb4D5-2 and huMAb4D5-3) without all 5 FR substitutions of the huMAb4D5-8 variant were not able to bind antigen with appropriate affinity.

With respect to the huMAb4D5-2 variant in Table 3, it was acknowledged that the variant with the single FR substitution did not appear to have growth inhibitory activity in the SK-BR-3 assay used. However, the

undersigned explained that even the 4.7nM Kd of this variant rendered it useful, *e.g.*, for diagnostic uses (see pages 55-57), as an immunotoxin (see pages 58-59), and/or for killing cells *in vivo* via Antibody Dependent Cellular Cytotoxicity (ADCC, see pages 59-60). Indeed, the affinity of the huMAb4D5-2 variant significantly surpasses the affinity of the murine and humanized anti-gD antibodies in column 45 of the cited Queen '762 patent, for instance. There is nothing in the art to indicate that 4.7nM is not a useful Kd. The other variant relied on by the Examiner as supporting her view that the claims were not enabled (huMAb4D5-3 in Table 3 with 4.4nM Kd) would also have the above-noted uses in addition to its ability to inhibit the proliferation of breast cancer cells as assessed by the SK-BR-3 assay. Hence, it was emphasized that the antibodies of the present invention need not have superior binding affinities in order to be useful.

Examiner Caputa asked what evidence was available to demonstrate that the teachings of the present application could be applied to other useful humanized antibodies.

Applicants are able to demonstrate that humanized antibody variants that bind at least seven distinct antigens have been made based on the teachings in the above patent application. For each antigen, several humanized antibody variants with the claimed FR substitution(s) could be made. In particular:

1. Example 1 on pages 63-74 describes several humanized variants which bound HER2 comprising the presently claimed FR substitution(s). Each of those variants was able to bind HER2 antigen (see Table 3 on page 72).

2. Example 3 on pages 79-88 describes eight humanized anti-CD3 antibody variants ($BsF(ab')_2v1$ as well as variants v6-12) which comprised the presently claimed FR substitutions. That example describes the $BsF(ab')_2v1$ variant (see huxCD3v1 in Fig. 5) and the other variants which were useful for retargeting the cytotoxic activity of human CD3+

CTL against HER2 overexpressing breast cancer cells (see, page 79, first paragraph, and Shalaby *et al. J. Exp. Med.* 175:217-225 (1992), of record). The FR substitutions in the BsF(ab')$_2$v1 variant (71L, 71H, 73H and 78H) were those which (a) non-covalently bound antigen directly; (b) interacted with a CDR; or (c) participated in the V_L-V_H interface, such FR substitutions being described and enabled by the present specification. Example 3 describes how the affinity of the humanized antibody BsF(ab')$_2$v1 was further improved by incorporating additional rodent CDR amino acid residues in the humanized antibody to generate BsF(ab')$_2$v9. In addition, that example describes variants with further FR substitutions at positions 75H and/or 76H.

3. Example 4 on page 89 describes yet a further example of the presently claimed humanized antibody variable domains/antibodies. The humanized anti-CD18 antibody included the presently claimed FR substitutions that (a) non-covalently bound antigen directly; (b) interacted with a CDR; or (c) participated in the V_L-V_H interface, and were identified using molecular modeling as taught in the present application.

4. Presta *et al. Cancer Research* 57:4593-4599 (1997) (of record) describes nine humanized anti-VEGF variants that were generated following the enabling disclosure of the present application.

5. Various humanized anti-Protein C variants are described in O'Connor *et al. Protein Engineering* 11(4):321-328 (1998) (copy attached), those variants being enabled by the present application.

6. Humanized antibody variants which bind the IgE antigen covered by certain claims herein have also been made (see Presta *et al. J. Immunol.* 151(5): 2623-2632 (1993) (of record)).

7. Werther *et al. J. Immunol.* 157(11): 4986-4995 (1996) (of record) is concerned with the humanization of anti-LFA-1 antibodies and describes several humanized antibody variants encompassed by the present claims.

These facts suggest that the "unpredictability" in the art is in fact much lower than suggested by the Examiner. When this actual level of unpredictability is then considered in view of the claim scope and the breadth of the disclosure, it becomes clear that unpredictability in the present application is not a factor that can support an assertion of undue experimentation. Indeed, through the teachings of the present disclosure, the moderate degree of unpredictability that exists in the art does not operate as a barrier to practice of the claimed invention, particularly in light of the teachings of the disclosure as to how to produce, identify and select variants falling within the scope of the claims.

As a consequence, it is respectfully submitted that the basis of the Examiner's belief that there is a lack of enablement due to undue experimentation is misplaced and should be withdrawn. Moreover, it is respectfully submitted that unless the Examiner can provide specific evidence demonstrating that the procedures disclosed in the present application will not yield success in producing humanized antibody variable domains as claimed, to counter the evidence provided in the specification and the specific responses, the maintenance of this rejection is improper. *In re Wright*, 999 F.2d 1557, 1562 (Fed. Cir. 1993); *In re Marzocchi*, 439 F.2d 220, 224 (CCPA 1971). Accordingly, Applicants respectfully request that the Examiner reconsider and withdraw the rejections based on lack of enablement.

Section 103 rejection

Claims 113, 115-118, 123 and 127-128 are rejected under Section 103 as being unpatentable over the Queen '762 patent in view of Kabat *et al.* Applicants responded to the rejection in the amendment dated April 25, 2001 and that response is supplemented hereinbelow.

At the outset, it is noted that the 103 rejection as to 115-118, 123, 127-128 should fall with the withdrawal of the Section 102 rejections of these claims, since the Office has not advanced any reason why one would substitute the presently recited FR residues, or why one would

have thought it would be possible to make a humanized antibody with improved affinity compared to the rodent antibody based on the cited art.

With regard to claim 113, now reciting "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", Applicants pointed out that it is believed that a *prima facie* case for obviousness of this invention has not been established; and even if it had, unexpected results provide objective evidence as to the patentability of the presently claimed invention.

Applicants' representatives explained in the interview that the term "consensus framework from many human antibodies" was used in the Queen patent synonymously with "a framework from a particular human immunoglobulin that is unusually homologous to the donor immunoglobulin to be humanized" - the position also taken by a former Patent Examiner (see page 10 of the amendment dated April 25, 2001). This is abundantly clear from a reading of the relied upon reference to a "consensus framework from many human antibodies" in the '762 patent. Immediately after this phrase in column 13, first full paragraph, the '762 patent states "For example, comparison of the sequence of a mouse heavy (or light) chain variable region against human heavy (or light) variable regions in a data bank (for example, the National Biomedical Research Foundation Protein Identification Resource) shows that the extent of homology to different human regions varies greatly, typically from about 40% to about 60-70%. By choosing as the acceptor immunoglobulin one of the human heavy (respectively light) chain variable regions that is most homologous to the heavy (respectively light) chain variable region of the donor immunoglobulin, fewer amino acids will be changed in going from the donor immunoglobulin to the humanized immunoglobulin. Thus, it is clear from the '762 patent that what it intended by the "consensus framework from many human antibodies" was indeed the "most homologous" human framework region as selected in the quoted paragraph of the '762 patent above. Thus, Applicants submit that the rejection based on the

combination of the '762 patent and Kabat *et al.* has been made with the benefit of hindsight of the present invention, which is impermissible.

Aside from the lack of teaching or motivation in the '762 patent to use 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, the '762 patent teaches away from this approach. Indeed, Queen taught the importance of selecting an unusually homologous human framework in order to avoid distorting the CDRs (column 13, lines 19-27). Applicants have shown previously how antibodies humanized with the human variable domain in claim 113 lack the unusually high homology to the non-human variable domain (paragraph bridging pages 17-18 of the amendment filed August 24, 1998), but nonetheless bind antigen extremely well. For instance, Applicants referenced the humanized anti-CD18 antibody with only 53% homology between the rodent and human framework sequences; humanized anti-IgE antibody with only 58% homology; humanized anti-CD11a with only 57% homology etc. These homologies were much lower that the homologies considered by Queen to be critical to avoid distorting the CDRs and for retaining affinity. The present application goes beyond the Queen method and discloses the benefits of using 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 for humanizing many different antibodies. This was not possible based on Queen's work which required that the human variable domain be tailored to each new rodent variable domain sequence to be humanized.

Applicants believe that the above arguments make out a strong case for patentability of the presently claimed invention over the cited combination of the '762 patent and Kabat *et al.* Moreover, Applicants are able to demonstrate that the presently claimed invention is patentable over the cited art due to the unexpected results attributable thereto. In particular, Applicants have demonstrated through the Shak declaration that antibodies directed against four different antigens humanized with

the presently claimed human variable domain in claim 113 display the unexpected property of lack of significant immunogenicity upon repeated administration to a human patient. This was not predictable in view of art such as Isaacs *et al. The Lancet* 340:748-752 (1992) (of record) in which 3/4 patients developed inhibitory antiglobulins upon repeated administration of the prior art humanized antibody thereto.

The Examiner had indicated that the unexpected results are not applicable because "the claims are broad, and drawn to any antibodies, and not specifically the claimed antibodies, wherein their specific target antigens, and their binding properties are not disclosed in the claims". Applicants submit that the Shak declaration filed demonstrates that the unexpected result applies regardless of the antigen or binding properties of the antibodies; the unexpected result was shown for humanized anti-HER2, anti-IgE, anti-CD11a and anti-VEGF antibodies. Hence, Applicants submit that the unexpected results are commensurate in scope with the invention recited in claim 113.

Thus, Applicants submit that the presently claimed invention is patentable over the cited art.

Applicants believe that this application is now in order for allowance and look forward to early notification to that effect.

Respectfully submitted,

GENENTECH, INC.

Date: July 13, 2001

By: _____

Wendy M. Lee
Reg. No. 40,378
Telephone: (650) 225-1994

Part / #61

7/13/01

Serial No.: 08/146,206

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the claims:
Please amend claims 113 and 128 as follows:

113. (Three times amended) A humanized variant of a non-human parent antibody which binds an antigen and comprises a [consensus] 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.

19

128. (Twice Amended) 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 a Framework Region (FR) 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 humanized variant binds the antigen more tightly than and up to about 3-fold more tightly than the parent antibody binds antigen.

Please add the following claims:

129. 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.

130. The humanized variable domain of claim 129 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.

131. The humanized variable domain of claim 129 wherein no human Framework Region (FR) residue other than those set forth in the group has been substituted.

Genentech, Inc.
Genentech, Inc.
Genentech, Inc.
Genentech, Inc.
Genentech, Inc.

1 DNA WAY
South San Francisco, CA 94080
(650) 225-1994
Facsimile: **(650) 952-9881**

#62

FACSIMILE TRANSMITTAL

DATE: July 30, 2001

Please deliver the following Amendment to:

NAME: Examiner Minh-Tam Davis
U.S. Patent and Trademark office
Washington, DC 20231

Fax No.:(703) 308-4426

FROM: Wendy M. Lee
Registration No.: 40,378

RE: U.S. Serial No.: 08/146,206
Our Docket No.: P0709P1

Number of Pages including this cover sheet - 13

Certificate of Facsimile Transmission Under 37 CFR § 1.8

In accordance with CFR § 1.6(d), this Amendment and Zenapax product information is addressed to Examiner Minh-Tam Davis, The Patent and Trademark Office, Washington, DC 20231 and is being transmitted to facsimile No. (703) 308-4426.

If you do not receive all pages, please notify Wendy Lee at (650) 225-1994.

Patent Docket P0709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Paul J. Carter et al. Serial No.: 08/146,206 Filed: November 17, 1993 For: METHOD FOR MAKING HUMANIZED ANTIBODIES	Group Art Unit: 1642 Examiner: Minh-Tam Davis

Certificate of Facsimile Transmission Under 37 CFR § 1.8

In accordance with CFR § 1.6(d), this correspondence addressed to Examiner Minh-Tam Davis, The Patent and Trademark Office, Washington, DC 20231 is being transmitted to facsimile No. (703) 308-4426 on

July 30, 2001

Wendy M. Lee

SUPPLEMENTAL AMENDMENT

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

IN THE CLAIMS:

Please amend claim 128 as follows:

128. (Three Times Amended) 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.

REMARKS

Applicants confirm having discussed the above application with Examiner Davis in the telephonic interview of July 25, 2001. In that interview, Examiner Davis indicated that on reconsideration the Section 112, first paragraph rejection would be withdrawn except with respect to claim 128. The Examiner considers the claim to antibodies with improved affinity to be enabled only for variants with FR substitutions at all the positions

for the exemplified better-binding variants. Applicants strongly disagree with the rejection of claim 128 for all the reasons previously elaborated. Nonetheless, in order to expedite allowance of the case, claim 128 is amended herein to recite the FR substitutions in the heavy chain variable region of the huMAb4D5-8 and huMAb4D5-6 variants which bound antigen more tightly than the parent antibody. Support for the claim language can be found on page 72, for instance. Due to the recitation of the FR substitutions, the functional language concerning the improved binding has been removed from the claim. The Examiner indicated that such an amendment would address the maintained Section 112 rejection.

The Examiner further stated in the above interview that the previous Section 103 rejection of claims 113, 115-118, 123 and 127-128 would be maintained unless Applicants could demonstrate the unexpected results through a side-by-side comparison of the antibody described in the cited Queen prior art and the antibodies of the present application. Applicants are now able to provide that comparison. In particular, Applicants attach the Physicians' Desk Reference ® (PDR) entry for the humanized anti-Tac antibody (ZENAPAX®) of the Queen prior art. Applicants note that the other humanized antibodies added to the Queen patents by way of CIP applications are not prior art to Applicants' invention set forth in the rejected claims herein.

As noted in section entitled "PRECAUTIONS" in the PDR entry for ZENAPAX®, when patients received multiple doses of that humanized antibody, anti-idiotype antibodies to ZENAPAX® were detected in patients with an overall incidence of 8.4%. The presently disclosed antibodies produce unexpectedly lower immunogenicity compared to that of the Queen antibody. This is demonstrated in the Shak declaration previously submitted which explained that patients receiving multiple doses of the humanized anti-HER2 antibody (HERCEPTIN®) exemplified in the present application only developed an antibody response 0.1% of the time (1 of the 885 patients evaluated; see paragraph 4 of the Shak declaration); and 0% of the patients treated with an anti-IgE antibody humanized according to the

teachings of the above patent specification developed a HAHA response (paragraph 7 of the Shak declaration). The total lack of an immune response in the patients treated with the humanized anti-IgE antibody is particularly unexpected, given that the allergic rhinitis and asthma patients treated therewith were hyper-sensitive to foreign antigens. Likewise, no significant immunogenicity upon repeated administration was observed for the anti-VEGF and anti-CD11a antibodies humanized according to the teachings in the present application. Paragraphs 8 and 9 of the Shak declaration. Applicants submit that this side-by-side comparison shows that the antibodies of the present application possess unexpected properties with respect to minimal or no immunogenicity upon repeated administration to human patients.

Reconsideration and withdrawal of the Section 103 rejection is respectfully requested in view of the above.

Applicants believe that this case is now in order for allowance and look forward to early notification of same.

Respectfully submitted,
GENENTECH, INC.

Date: July 30, 2001

By: _____
Wendy M. Lee
Reg. No. 40,378
Telephone: (650) 225-1994

Serial No.: 08/146,206

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the claims:

Please amend claim 128 as follows:

128. (Three Times Amended) 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 [a] Framework Region (FR) substitutions at heavy chain positions 71H, 73H, 78H and 93H, utilizing the numbering system set forth in Kabat [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 humanized variant binds the antigen more tightly than and up to about 3-fold more tightly than the parent antibody binds antigen].

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

APPLICATION NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.
09/146,206			

EXAMINER	
ART UNIT	PAPER NUMBER

DATE MAILED: 8/29/01

INTERVIEW SUMMARY

All participants (applicant, applicant's representative, PTO personnel):

(1) Minh-Tam Davis (3) Wendy Lee

(2) Anthony Caputa (4)

Date of Interview 08/29/01

Type: ☐ Telephonic ☐ Personal (copy is given to ☐ applicant ☒ applicant's representative).

Exhibit shown or demonstration conducted: ☐ Yes ☐ No If yes, brief description:

Agreement ☐ was reached. ☐ was not reached.

Claim(s) discussed:

Identification of prior art discussed:

Description of the general nature of what was agreed to if an agreement was reached, or any other comments: Applicant representative discusses prior art of record & pending claims

(A fuller description, if necessary, and a copy of the amendments, if available, which the examiner agreed would render the claims allowable must be attached. Also, where no copy of the amendments which would render the claims allowable is available, a summary thereof must be attached.)

1. ☐ It is not necessary for applicant to provide a separate record of the substance of the interview.

Unless the paragraph above has been checked to indicate to the contrary. A FORMAL WRITTEN RESPONSE TO THE LAST OFFICE ACTION IS NOT WAIVED AND MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a response to the last Office action has are ready been filed, APPLICANT IS GIVEN ONE MONTH FROM THIS INTERVIEW DATE TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW.

2. ☐ Since the Examiner's interview summary above (including any attachments) reflects a complete response to each of the objections, rejections and requirements that may be present in the last Office action, and since the claims are now allowable, this completed form is considered to fulfill the response requirements of the last Office action. Applicant is not relieved from providing a separate record of the interview unless box 1 above is also checked.

Examiner Note: You must sign this form unless it is an attachment to another form.

FORM **PTOL-413** (REV.1-96)

A complete written statement as to the substance of any face-to-face or telephone interview with regard to an application must be made of record in the application, whether or not an agreement with the examiner was reached at the interview.

§1.133 Interviews

* * * * *

(b) In every instance where reconsideration is requested in view of an interview with an examiner, a complete written statement of the reasons presented at the interview as warranting favorable action must be filed by the applicant. An interview does not remove the necessity for response to Office action as specified in §§ 1.111,1.135. (35 U.S.C.132)

§ 1.2. Business to be transacted in writing. All business with the Patent or Trademark Office should be transacted in writing. The personal attendance of applicants or their attorneys or agents at the Patent and Trademark Office is unnecessary. The action of the Patent and Trademark Office will be based exclusively on the written record in the Office. No attention will be paid to any alleged oral promise, stipulation, or understanding in relation to which there is disagreement or doubt.

The action of the Patent and Trademark Office cannot be based exclusively on the written record in the Office if that record is itself incomplete through the failure to record the substance of interviews.

It is the responsibility of the applicant or the attorney or agent to make the substance of an interview of record in the application file, unless the examiner indicates he or she will do so. It is the examiner's responsibility to see that such a record is made and to correct material inaccuracies which bear directly on the question of patentability.

Examiners must complete a two-sheet carbon interleaf Interview Summary Form for each interview held after January 1, 1978 where a matter of substance has been discussed during the interview by checking the appropriate boxes and filling in the blanks in neat handwritten form using a ball point pen. Discussions regarding only procedural matters, directed solely to restriction requirements for which interview recordation is otherwise provided for in Section 812.01 of the Manual of Patent Examining Procedure, or pointing out typographical errors or unreadable script in Office actions or the like, are excluded from the interview recordation procedures below.

The Interview Summary Form shall be given an appropriate paper number, placed in the right hand portion of the file, and listed on the "Contents" list on the file wrapper. The docket and serial register cards need not be updated to reflect interviews. In a personal interview, the duplicate copy of the Form is removed and given to the applicant (or attorney or agent) at the conclusion of the interview. In the case of a telephonic interview, the copy is mailed to the applicant's correspondence address either with or prior to the next official communication. If additional correspondence from the examiner is not likely before an allowance or if other circumstances dictate, the Form should be mailed promptly after the telephonic interview rather than with the next official communication.

The Form provides for recordation of the following information:

- Serial Number of the application
- Name of applicant
- Name of examiner
- Date of interview
- Type of interview (personal or telephonic)
- Name of participant(s)) (applicant, attorney or agent, etc.)
- An indication whether or not an exhibit was shown or a demonstration conducted
- An identification of the claims discussed
- An identification of the specific prior art discussed
- An indication whether an agreement was reached and if so, a description of the general nature of the agreement (may be by attachment of a copy of amendments or claims agreed as being allowable). (Agreements as to allowability are tentative and do not restrict further action by the examiner to the contrary.)
- The signature of the examiner who conducted the interview
- Names of other Patent and Trademark Office personnel present.

The Form also contains a statement reminding the applicant of his responsibility to record the substance of the interview.

It is desireable that the examiner orally remind the applicant of his obligation to record the substance of the interview in each case unless both applicant and examiner agree that the examiner will record same. Where the examiner agrees to record the substance of the interview, or when it is adequately recorded on the Form or in an attachment to the Form, the examiner should check a box at the bottom of the Form informing the applicant that he need not supplement the Form by submitting a separate record of the substance of the interview.

It should be noted, however, that the Interview Summary Form will not normally be considered a complete and proper recordation of the interview unless it includes, or is supplemented by the applicant or the examiner to include, all of the applicable items required below concerning the substance of the interview:

A complete and proper recordation of the substance of any interview should include at least the following applicable items:

1) A brief description of the nature of any exhibit shown or any demonstration conducted,
2) an identification of the claims discussed,
3) an identification of specific prior art discussed,
4) an identification of the principal proposed amendments of a substantive nature discussed, unless these are already described on the Interview Summary Form completed by the examiner,
5) a brief identification of the general thrust of the principal arguments presented to the examiner. The identification of arguments need not be lengthy or elaborate. A verbatim or highly detailed description of the arguments is not required. The identification of the arguments is sufficient if the general nature or thrust of the principal arguments made to the examiner can be understood in the context of the application file. Of course, the applicant may desire to emphasize and fully describe those arguments which he feels were or might be persuasive to the examiner,
6) a general indication of any other pertinent matters discussed, and
7) if appropriate, the general results or outcome of the interview unless already described in the Interview Stimmary Form completed by the examiner.

Examiners are expected to carefully review the applicant's record of the substance of an interview. If the record is not complete or accurate, the examiner will give the applicant one month from the date of the notifying letter or the remainder of any period for response, whichever is longer, to complete the response and thereby avoid abandonment of the application (37 CFR 1.135(c)).

Examiner to Check for Accuracy

Applicant's summary of what took place at the interview should be carefully checked to determine the accuracy of any argument or statement attributed to the examiner during the interview. If there is an inaccuracy and it bears directly on the question of patentability, it should be pointed out in the next Office letter. If the claims are allowable for other reasons of record, the examiner should send a letter setting forth his or her version of the statement attributed to him. If the record is complete and accurate, the examiner should place the indication "Interview record OK" on the paper recording the substance of the interview along with the date and the examiner's initials.

1642

Patent Docket P0709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Paul J. Carter et al. Serial No.: 08/146,206 Filed: November 17, 1993 For: METHOD FOR MAKING HUMANIZED ANTIBODIES	Group Art Unit: 1642 Examiner: Minh-Tam Davis CERTIFICATE OF MAILING I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner of Patents, Washington, D.C. 20231 on August 30, 2001 Anna Kan Anna Kan

#6 KO 10/10/01

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Applicants submit herewith patents, publications or other information (attached hereto and listed on the attached revised Form PTO-1449) of which they are aware, which they believe may be material to the examination of this application and in respect of which there may be a duty to disclose in accordance with 37 CFR §1.56.

This Information Disclosure Statement is filed in accordance with the provisions of:

[] **37 CFR §1.97(b)**
- within three months of the filing date of the application other than a continued prosecution application under 37 CFR §1.53(d); **or**
- within three months of the date of entry of the national stage of a PCT application as set forth in 37 CFR§1.491, **or**
- before the mailing of the first Office action on the merits; **or**
- before the mailing of the first Office action after the filing of a request for a continued examination under 37 CFR §1.114.

[X] **37 CFR §1.97(c)**
- by the applicant after the period specified in 37 CFR §1.97(b), but prior to the mailing date of any of a final action under 37 CFR §1.113, or a notice of allowance under 37 CFR §1.311, or an action that otherwise closes prosecution in the application, and is accompanied by either the fee set forth in 37 CFR §1.17(p) **or** a statement as specified in 37 CFR §1.97(e), as checked below.

[] **37 CFR §1.97(d)**
- after the period specified in CFR §1.97(c), and is accompanied by the fee set

09/06/2001 MGEBREN1 00000111 070336 09618206
01 FC:122 180.00 OP

forth in 37 CFR §1.17(p) **and** a statement as specified in 37 CFR §1.97(e), as checked below.

[If either of boxes 37 CFR §1.97(c) or 37 CFR §1.97(d) is checked above, the following statement under 37 CFR §1.97(e) may need to be completed.]

[] **37 CFR §1.97(e)**Each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of this information disclosure statement.

[] **37 CFR §1.704(d)** Each item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application and the communication was not received by any individual designated in §1.56(c) more than thirty days prior to the filing of this information disclosure statement. Therefore, in accordance with the provisions of 37 CFR §1.704(d), the filing of this information disclosure statement will not be considered a failure to engage in reasonable efforts to conclude prosecution under 37 CFR §1.704.

[X] The U.S. Patent and Trademark Office is hereby authorized to charge Deposit Account No. 07-0630 in the amount of $180.00 to cover the cost of this Information Disclosure Statement under 37 CFR §1.17(p). Any deficiency or overpayment should be charged or credited to this deposit account.

A list of the patent(s) or publication(s) is set forth on the attached revised Form PTO-1449 (Modified).

A copy of the items on PTO-1449 is supplied herewith.

Those patent(s) or publication(s) which are marked with an asterisk (*) in the attached PTO-1449 form are not supplied because they were previously cited by or submitted to the Office in a prior application Serial No. 07/715,272, filed 14 June 1991 and relied upon in this application for an earlier filing date under 35 USC §120.

A concise explanation of relevance of the items listed on PTO-1449 is:

[X] not given

[] given for each listed item

[] given for only non-English language listed item(s) [Required]

[] in the form of an English language copy of a Search Report from a foreign patent office, issued in a counterpart application, which refers to the relevant portions of the references.

In accordance with 37 CFR §1.97(g), the filing of this information disclosure statement shall not be construed as a representation that a search has been made.

In accordance with 37 CFR §1.97(h), the filing of this information disclosure statement shall not be construed to be an admission that the information cited in the statement is, or is considered to be, material to patentability as defined in 37 CFR § 1.56(b).

In the event that the Office determines a fee to be due where none is specifically authorized in this paper, the U.S. Patent and Trademark Office is hereby authorized to charge Deposit Account No. 07-0630 in the amount of $180.00 to cover the cost of this Information Disclosure Statement under 37 CFR §1.17(p).

Respectfully submitted,

GENENTECH, INC.

Date: August 30, 2001

By: _____

By: Steven X. Cui Reg. No.. 44,637
for Wendy M. Lee Reg. No. 40,378
Telephone No. (650) 225-1994

09157

PATENT TRADEMARK OFFICE

9/4/01

File History Report

□ **Paper number(s) _____ is/are missing from the United States Patent and Trademark Office's original copy of the file history.**

☑ **The following page(s) 1 of paper number 64 is/are missing from the United States Patent and Trademark Office's original copy of the file history.**

□ **The following checked item(s) of paper number _____ is/are missing from the United States Patent and Trademark Office's original copy of the file history.**

□ **PTO-892 Form** □ **PTO-1449 Form**

□ **PTO-948 Form** □ **Other_____**

to this declaration which represent excerpts from our laboratory notebooks with dates obscured.

4. Exhibit A provides the amino acid sequences of humanized 4D5 (anti-HER2) antibody variable domain sequences. A humanized antibody (Hu4D5 Fab) comprising the Hum4D5a V_L and Hum4D5a V_H sequences from Exhibit A (the variable domain sequences of the variant called "huMAb4D5-5" in the above application) was recombinantly produced and found to bind the HER2 antigen as evidenced by the laboratory notebook entries in Exhibit B attached hereto. Hu4D5 Fab comprised a heavy chain variable domain comprising non-human CDR amino acid residues which bound antigen incorporated into a human antibody variable domain, and further comprised a FR amino acid substitution at site 73H. The experimental work in Exhibits A and B was completed prior to September 28, 1990.

We declare further that all statements made herein of our 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 United States Code and that willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: _____

_____
Paul J. Carter

Date: Sept. 4, 2001

Leonard G. Presta
Leonard G. Presta

#63

Genentech, Inc.
Genentech, Inc.
Genentech, Inc.
Genentech, Inc.
Genentech, Inc.

1 DNA WAY
South San Francisco, CA 94080
(650) 225-1994
Facsimile: **(650) 952-9881**

FACSIMILE TRANSMITTAL

DATE: October 2, 2001

Please deliver the following Supplemental Amendment, Vincenti et al. reference, and Declaration under 37 CFR §1.131 with attached Exhibits A and B to:

NAME: Examiner Minh-Tam Davis - Group 1642
U.S. Patent and Trademark office
Washington, DC 20231

Fax No.: (703) 308-4426

FROM: Wendy M. Lee
Registration No.: 40,378

RE: U.S. Serial No.: 08/146,206
Our Docket No.: P0709P1

Number of Pages including this cover sheet - 20

Certificate of Facsimile Transmission Under 37 CFR § 1.8

In accordance with CFR § 1.6(d), this correspondence addressed to The Patent and Trademark Office, Box: Assignments, Washington, DC 20231 is being transmitted to facsimile No. (703) 308-4426

If you do not receive all pages, please notify Wendy Lee at (650) 225-1994.

Patent Docket P0709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Paul J. Carter et al. Serial No.: 08/146,206 Filed: November 17, 1993 For: METHOD FOR MAKING HUMANIZED ANTIBODIES	Group Art Unit: 1642 Examiner: Minh-Tam Davis Certificate of Facsimile Transmission Under 37 CFR § 1.8 In accordance with CFR § 1.8(d), this correspondence addressed to Examiner Minh-Tam Davis at the Patent and Trademark Office, Washington, DC 20231 is being transmitted to facsimile No. (703) 308-4426 October 2, 2001 Wendy M. Lee

#63 10-10-01

SUPPLEMENTAL SUBMISSION

Official Fax 10/04/01

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

The undersigned confirms having discussed the present application with Examiners Caputa and Davis in the interview on August 29, 2001. Based on and responsive to that discussion, Applicants wish to provide the following additional observations and information.

Status of Previous Rejections

During the most recent interviews, Examiner Davis indicated that the Section 112 and 102 rejections would likely be withdrawn, but that certain of the claims may continue to be rejected under Section 103. The following comments address the 103 rejection.

Additional Information on ZENAPAX®

Examiner Caputa requested that evidence be presented to demonstrate that ZENAPAX® - for which Applicants provided the side-by-side comparison in the July 30, 2001 amendment - was the same as the antibody in the cited Queen references. To confirm that ZENAPAX® (Daclizumab) is the humanized anti-IL2 receptor antibody described in the cited Queen patents and Queen, *PNAS* (1989) paper, Applicants direct the Examiner's attention to the attached copy of Vincenti *et al. N. Engl. J. Med.* 338:161-165 (1998). Vincenti *et al.* refers to Daclizumab (the generic

name for the ZENAPAX® antibody - see PDR entry attached to the July 30, 2001 amendment) and states in column 2 on page 161 that it is a molecularly engineered human IgG1 incorporating the antigen-binding regions of the parent, murine monoclonal antibody. There, Vincenti cross-references the Queen *et al. PNAS* (1989) paper (ref. no. 14 in Vincenti *et al.*) as describing Daclizumab. Hence, Applicants submit that ZENAPAX®/Daclizumab is the humanized anti-IL2 receptor antibody described in the cited Queen references.

Rejection of Claim 113 under 35 USC 103 based on Queen in view of Kabat

The Office Action dated October 25, 2000 (hereinafter, "Action") includes a rejection of claims 113, 115-118, 123, and 127-128 made under 35 USC 103 as being obvious over Queen in view of Kabat. Applicants submit this response to supplement and clarify their previous remarks.

Applicants have previously explained why the Action's conclusions of obviousness made against claim 113 are formed through improper use of hindsight in interpreting the words of the disclosure of Queen. Applicants have also pointed out functional attributes of the humanized antibodies of claim 113 of the present invention that reflect unexpected results, thus providing a distinct and separate basis for overcoming the rejection imposed under §103. Through this supplemental amendment, Applicants respond to points made by Examiner in the Action, and as suggested in personal and telephonic interviews conducted earlier this year. On the basis of each of these points, Applicants respectfully submit that the Examiner has not presented and cannot sustain a *prima facie* showing of obviousness of the claimed inventions. In particular, the Queen disclosure fails to disclose the requisite motivation to combine it with Kabat to set forth a *prima facie* case of obviousness of claim 113.

It is well established that in order for a combination of references to render an invention obvious, there must be a clear motivation in the references that their teachings can be combined. *In re Avery*, 518 F.2d 1228 (1975, CCPA). The mere fact that references address issues within the same field of the invention does not render the resultant combination obvious unless the prior art also suggests the desirability of the combination. *ACS Hospital Systems Inc. v. Montefiore Hospital*, 732 F.2d 1572 (Fed. Cir. 1984). In fact, "[t]he references, viewed by themselves and not in retrospect must suggest doing what applicant has done" *In re Skoll*, 523 F.2d 1392 (1975 CCPA). Furthermore, the Federal Circuit and the PTO have made it clear that where a modification must be made to the

prior art to reject or invalidate a claim under 35 USC §103, there must be a showing of proper motivation to do so. In order to establish obviousness, there must be suggestion or motivation in the references. *In re Gordon*, 733 F.2d 900 (Fed. Cir. 1984).

The Action asserts that combining the references to provide the advantages of the present invention would be obvious. However, it identifies nothing within the applied references that would suggest combining those references to arrive at the claimed invention. Rather, the Action improperly cites the findings of *In re Kerkhoven*, 626 F.2d 846 (C.C.P.A. 1980) to support the conclusion of obviousness. Specifically, the Action states that combining the references "would have logically flowed from their having been individually taught in the prior art, and because patent '762 teaches the use of 'consensus sequence', for the same purpose of producing humanized monoclonal antibodies for therapeutic purposes." Applicants contend, however, that the use of *Kerkhoven* in the present case to support a finding of obviousness is improper as the facts of that case are distinguishable from those at hand.

In *Kerkhoven*, the Appellant's claimed a process for producing a detergent containing a mixture of anionic and nonionic detergent materials. In that method, the Appellant's combined two compositions, each taught by the prior art to be useful for the same purpose, in order to form a third composition that was also useful for the same purpose. The patent examiner rejected the method as obvious in light of the prior art under *35 U.S.C. §103*. The Court of Patent Appeals affirmed the rejection and stated that the idea of combining two compositions taught by the prior art to be useful for the same purpose in order to form a third composition to be used for same purpose as the individual components is *prima facie* obvious. *Id* at 850.

The holding in *Kerkhoven* cannot be applied to the instant situation. Most significantly, the disclosure of Queen does not teach the usefulness of a sequence "comprising the most frequently occurring amino acid residues at each location in all human immunoglobulins of a human heavy chain immunoglobulin subgroup" for the purpose of humanizing antibodies, which concept is disclosed and claimed in the present application. In contrast, the Queen patent merely refers to using a "consensus framework from many human antibodies" for humanizing antibodies (column 13, line 7). One of skill in the art interpreting the phrase "many human antibodies" as recited in Queen would construe the phrase to refer to an arbitrarily selected group of human antibodies, with the specification

guiding that such an arbitrarily selected group should consist of sequences that are "unusually homologous to the donor immunoglobulin to be humanized" (column 13, line 6).

There is no specific teaching, suggestion or motivation found in the Queen disclosure that would direct a person of ordinary skill to select sets of consensus sequences that correspond to what is disclosed and claimed in the present application. Specifically, in contrast to Queen, the term "consensus" is used in the present application to refer to the relationship among a well-defined group of human antibody subgroups. See, page 14, lines 29 to 35 and page 15, lines 1-25 of disclosure.

The lack of any specific teaching or motivation in Queen is not cured by the disclosure of Kabat. The Action's analysis of Kabat does not provide any suggestion that the frequency of occurrence of amino acid residues in the immunoglobulin chains can be exploited or used for any particular purpose related to humanizing antibodies.

Indeed, nothing in the '762 patent or in Kabat teaches that 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 is useful for producing humanized monoclonal antibodies for therapeutic purposes. Therefore, regardless of what usefulness may be ascribed to the "consensus framework from many human antibodies" taught in the '762 patent, the sequences taught by Kabat could not have been, and were not, identified in the cited art as being useful for producing humanized monoclonal antibodies for therapeutic purposes. Because the prior art had not equated the potential use of the "consensus framework from many human antibodies" taught in the '762 patent with the potential use of the sequences taught by Kabat, the cited art does not provide motivation to substitute the sequences identified by Kabat for the sequences referred to in the '762 patent.

In summary, in *Kerkhoven*, both components had been taught by the prior art to be useful for the same purpose, and, in addition, the resulting component was also useful for the same purpose. However, in the instant situation only one of the prior art components, namely the "consensus framework from many human antibodies" as recited in the '762 patent, had been referred to for "producing humanized monoclonal antibodies for therapeutic purposes." Therefore, *Kerkhoven* does not control the facts of the present application, and a *prima facie* case of

obviousness on the basis of Queen in view of Kabat is improper because there is no suggestion or motivation to combine the cited references.

Applicants respectfully request that the rejection of claim 113 on the basis of Queen in view of Kabat be withdrawn.

Rejection of Claims 115-118, 123 and 127-128 under 35 USC 103 on the basis of Queen in view of Kabat

Claims 115-118, 123 and 127-128 have also been rejected under 35 USC 103 on the basis of Queen in view of Kabat Since the rationale for this rejection and the facts that control its disposition are distinct from those related to claim 113, Applicants are separately addressing the basis of the rejection of these claims.

Each of the rejected claims recite substitutions at specific FR positions. Applicants have explained that the Queen '762 patent relied on in the Section 103 rejection did not describe a humanized antibody having these specific FR substitution(s), except for antibodies comprising a 73H FR substitution as claimed herein. With respect to the 73H substitution, Applicants provide herewith a swearing behind declaration showing a completion of that invention by the inventors of the present application prior to September 28, 1990 - the 2nd Queen CIP filing date, after which time the disclosure concerning the 73H substitution was added.

The Office has not advanced any reasons why substituting the specifically identified FR positions recited in the claims would have been obvious in view of Queen. The previous 103 rejection was based on the sequential numbering of the FR residues, rather than the Kabat numbering as presently claimed - see the April 25, 2001 amendment which clarifies this distinction at pages 8 and 13. In this regard, Examiner Caputa asked that Applicants emphasize the selection invention claimed herein by contrasting the specifically recited FR substitutions to the disclosure in the Queen patent. Aside from the specific FR substitutions for the exemplified humanized antibodies, Queen refers to FR substitutions in Categories 2-4 (columns 13-15 of the '762 patent). Thus, according to Queen, any one of the approximately 80 V_L FR residues or approximately 87 V_H FR residues can be substituted according to those criteria. This would not provide a specific teaching as to the selection invention set forth in claims herein which recite specific FR positions to be substituted.

In considering the appropriateness of the rejection of these claims on the basis of Queen in view of Kabat, the Examiner's attention is directed to the Federal Circuit decision of *In re Baird*, 16 F.3d 380. In *Baird* the court held that a reference, which discloses a generic formula that encompasses a species claimed by applicant did not render the species obvious because there was no motivation provided to select the particular species that applicant claimed. Moreover, the vast number of species encompassed by the reference's generic disclosure, and the fact that the preferred species of the reference were different from the applicant's species led the court to conclude that the reference did not fairly suggest the selection of the particular species claimed by applicants.

Baird controls the question of non-obviousness of claims 115-118, 123 and 127 in the present situation. As Applicants have previously indicated, the Queen disclosure reveals a genus that encompasses a vast number of species. According to Queen, any one of the approximately 80 V_L FR residues or approximately 87 V_H FR residues can be substituted according to their criteria. This would not provide a specific teaching as to the selection invention set forth in claims herein which recite specifically identified substitutions in FR positions. Further, as explained at the interview, the present case is entitled to a 1991 filing date and, as such, represents one of the early disclosures concerning humanized antibodies. Applicants submit that this should be taken into account when reconsidering the patentability of the present invention over the prior art.

For these reasons, Applicants respectfully request that the rejection of claims 115-118, 123 and 127-128 be withdrawn.

Conclusions

In light of the above and previous amendments and remarks, Applicants respectfully submit that all pending claims as currently presented are in condition for allowance.

Applicants believe that is application is now in condition for allowance, and look forward to early notification that effect. If however, there are outstanding issues, the Examiner is invited to call the undersigned to discuss those.

Respectfully submitted,
GENENTECH, INC.

Date: October 2, 2001

By: _____
Wendy M. Lee
Reg. No. 40,378
Telephone: (650) 225-1994

Patent Docket P0709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Paul J. Carter et al. Serial No.: 08/146,206 Filed: November 17, 1993 For: Method for Making Humanized Antibodies	Group Art Unit: 1642 Examiner: Minh-Tam Davis

DECLARATION UNDER 37 CFR §1.131

Assistant Commissioner of Patents
Washington, D.C. 20231

10/04/01

Sir:

We, Paul J. Carter and Leonard G. Presta, do hereby declare and say as follows:

1. We are inventors of the subject matter of the above-identified patent application. All work described hereinafter was performed by us or on our behalf in the Unites States of America.

2. Prior to September 28, 1990, we conceived of and reduced to practice 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 site 73H, utilizing the numbering system set forth in Kabat, as well as an antibody comprising that humanized variable domain.

3. Evidence of the reduction to practice of the claimed invention is set forth in the exhibits attached

to this declaration which represent excerpts from our laboratory notebooks with dates obscured.

4. Exhibit A provides the amino acid sequences of humanized 4D5 (anti-HER2) antibody variable domain sequences. A humanized antibody (Hu4D5 Fab) comprising the Hum4D5a V_L and Hum4D5a V_H sequences from Exhibit A (the variable domain sequences of the variant called "huMAb4D5-5" in the above application) was recombinantly produced and found to bind the HER2 antigen as evidenced by the laboratory notebook entries in Exhibit B attached hereto. Hu4D5 Fab comprised a heavy chain variable domain comprising non-human CDR amino acid residues which bound antigen incorporated into a human antibody variable domain, and further comprised a FR amino acid substitution at site 73H. The experimental work in Exhibits A and B was completed prior to September 28, 1990.

We declare further that all statements made herein of our 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 United States Code and that willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 9/5/01 Paul J. Carter

Paul J. Carter

Date: _____

_____

Leonard G. Presta

Project No.:_____

22 Book No._____ TITLE Humanized 4D5

From Page No.__

Dennis and Paul,
here are the human consensus sequences and the proposed humanized 4D5 sequences.

##########
VARIABLE LIGHT
HuLys has 83% similarity to human kappa subgroup I even when the CDR sequences are included. The sequences inside the CDR boxes (Chothia/Lesk definition) can be taken from HuLys or humkapI -- those outside the boxes should be taken from humkapI.

(Kabat/Wu CDR in capital letters; Chothia/Lesk CDR in boxes)

```
                1                                                  10
humkapI  asp ile gln met thr gln ser pro ser ser leu ser ala ser val gly
HuLys

                          20                                                  30
humkapI  asp arg val thr ile thr cys ARG ALA | SER GLN ASP ILE SER SER TYR |
HuLys                                        |     GLY ASN     HIS ASN    |

                                                  40
humkapI  LEU ASN trp tyr gln gln lys pro gly lys ala pro lys leu leu ile
HuLys        ALA

                  50                                                  60
humkapI  tyr | ALA ALA SER | SER LEU GLU SER gly val pro ser arg phe ser gly
HuLys        | TYR THR THR | THR     ALA ASP
                  70                                                  80
humkapI  ser gly ser gly thr asp phe thr leu thr ile ser ser leu gln pro
HuLys                                      phe

                                                 90
humkapI  glu asp phe ala thr tyr tyr cys GLN GLN | TYR ASN SER LEU PRO TYR |
HuLys            ile                       HIS | PHE TRP     THR     ARG |

                        100
humkapI  THR phe gly gln gly thr lys val glu ile lys arg thr
HuLys
```

##########
VARIABLE HEAVY
The sequences inside the CDR boxes (Chothia/Lesk definition) can be taken from KOL or humIII -- those outside the boxes should be taken from humIII.

(Kabat/Wu CDR in capital letters; Chothia/Lesk CDR between bars)

```
                1                                                  10
humiii   glu val gln leu val glu ser gly gly gly leu val gln pro gly gly
kol                        gln                 val                 arg

                          20                                                  30
humiii   ser leu arg leu ser cys ala ala ser | GLY PHE THR PHE SER ASP TYR |
kol                              scr scr     |     ILE     SER            |

                                                  40
humiii   ALA MET | SER trp val arg gln ala pro gly lys gly leu glu trp val
kol              | TYR
                  50          52 | 52a  53                          60
humiii   ala VAL ILE SER | GLU ASN GLY SER | ASP THR TYR TYR ALA ASP SER VAL
kol          ILE     TRP | ASP ASP         |     GLN HIS
```

To Page No. 23

Witnessed & Understood by me,	Date	Invented by	Date
		Recorded by Leonard Presta	EXHIBIT A

TITLE Humanized 4D5 Project No._____ Book No._____ 23

From Page No. 22

```
                                                    70
humiii  LYS GLY arg phe thr ile ser arg asp asp ser lys asn thr leu tyr
kol                                      asn                     phe

         60      62 62a 62b 62c  63                              90
humiii  leu gln met asn ser leu arg ala glu asp thr ala val tyr tyr cys
kol              asp               pro              gly         phe

                                                  100  a   b   c   d   e   f   g   h
humiii  ala arg ASP |ARG GLY GLY ALA VAL SER TYR CLY PHE PHE GLY TYR GLY
kol                 |GLY     HIS GLY PHE CYS SER SER ALA SER CYS PHE GLY

         i   j   k  101|                                          110
humiii  GLY PHE PHE ASP|VAL trp gly gln gly thr leu val thr val ser ser
kol     PRO --- ---    |TYR                     pro
                       ----------

################################################################################
The following are proposed humanized 4D5 sequences; changes in Hum4D5b
and Hum4D5c from Hum4D5a are followed by an asterisk

          1                                        10
humkapI asp ile gln met thr gln ser pro ser ser leu ser ala ser val gly
Hum4D5a
Hum4D5b
Hum4D5c

                    20                                                  30
humkapI asp arg val thr ile thr cys ARG ALA|SER GLN ASP ILE SER SER TYR|
Hum4D5a                                   |         VAL ASN THR ALA|
Hum4D5b                                   |         VAL ASN THR ALA|
Hum4D5c                                   |         VAL ASN THR ALA|

                                        40
humkapI LEU ASN trp tyr gln gln lys pro gly lys ala pro lys leu leu ile
Hum4D5a VAL ALA
Hum4D5b VAL ALA
Hum4D5c VAL ALA

          50                                                60
humkapI tyr|ALA ALA SER|SER LEU GLU SER gly val pro ser arg phe ser gly
Hum4D5a    |SER        |PHE
Hum4D5b    |SER        |PHE
Hum4D5c    |SER        |PHE     TYR*

                              70                                        80
humkapI ser gly ser gly thr asp phe thr leu thr ile ser ser leu gln pro
Hum4D5a     arg
Hum4D5b     gly*
Hum4D5c     arg

                                                  90|
humkapI glu asp phe ala thr tyr tyr cys GLN GLN|TYR ASN SER LEU PRO TYR|
Hum4D5a                                        |HIS TYR THR THR     PRO|
Hum4D5b                                        |HIS TYR THR THR     PRO|
Hum4D5c                                        |HIS TYR THR THR     PRO|

                    100
humkapI THR phe gly gln gly thr lys val glu ile lys arg thr
Hum4D5a
Hum4D5b
Hum4D5c
```

To Page No. 24

Witnessed & Understood by me,	Date	Invented by	Date
		Recorded by	

Project No.

24 Book No. TITLE Humanized 4D5

From Page No. 23

V_H

```
                1                                                  10
humiii   glu val gln leu val glu ser gly gly gly leu val gln pro gly gly
Hum4D5a
Hum4D5b
Hum4D5c

                        20                                                  30
humiii   ser leu arg leu ser cys ala ala ser|GLY PHE THR PHE SER ASP TYR
Hum4D5a                                     |      ASN ILE LYS      THR
Hum4D5b                                     |      ASN ILE LYS      THR
Hum4D5c                                     |      ASN ILE LYS      THR

                                        40
humiii   ALA MET|SER trp val arg gln ala pro gly lys gly leu glu trp val
Hum4D5a  TYR ILE|HIS
Hum4D5b  TYR ILE|HIS
Hum4D5c  TYR ILE|HIS

                50      52|52a  53                          60
humiii   ala VAL ILE SER|GLU ASN GLY SER|ASP THR TYR TYR ALA ASP SER VAL
Hum4D5a      ARG     TYR|PRO THR ASN GLY|TYR     ARG
Hum4D5b      ARG     TYR|PRO THR ASN GLY|TYR     ARG
Hum4D5c      ARG     TYR|PRO THR ASN GLY|TYR     ARG

                                70
humiii   LYS GLY arg phe thr ile ser arg asp asp ser lys asn thr leu tyr
Hum4D5a                           ala     thr                     ala
Hum4D5b                           ala     thr                     ala
Hum4D5c                           ala     thr                     ala

         80      82 82a 82b 82c  83                          90
humiii   leu gln met asn ser leu arg ala glu asp thr ala val tyr tyr cys
Hum4D5a
Hum4D5b
Hum4D5c

                                    100  a   b   c  101|
humiii   ala arg ASP|ARG GLY GLY ALA VAL SER TYR GLY ASP|VAL trp gly gln
Hum4D5a  ser     TRP|GLY GLY ASP GLY PHE TYR ALA MET ASP|
Hum4D5b  ser     TRP|GLY GLY ASP GLY PHE TYR ALA MET ASP|
Hum4D5c  ser     TRP|GLY GLY ASP GLY PHE TYR ALA MET ASP|TYR

humiii   gly thr leu val thr val ser ser
Hum4D5a
Hum4D5b
Hum4D5c
```

To Page No. 25

Witnessed & Understood by me, Date

Invented by Leonard Presta Date

Recorded by Leonard Presta

Her2 binding

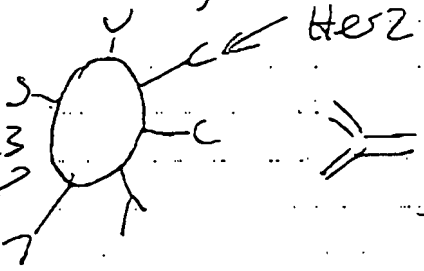
TITLE #313 Activity of Hu4D5 Fab Project No. Book No. 63

From Page No.

AIM: To investigate the activity of Hu4D5 for inhibiting the binding of 4D5 to the cell line SKBR3 which over-expresses Her2

PROTOCOL

Assay performed ~~per~~ by Jay Saxup + Kathy King



[125I]-4D5 (0.1nM).

+ Varying amounts of Hu4D5 Fab (2/2A shockate)

V_{assay} = 400λ

		$\bar{x}$	$\bar{x}$ corrected for non-specific binding (447cpm)	
Total counts	11665, 11567, 11603	11612	11365	
Test buffer	10862, 11473	11168	10921	
(200mM THS-HCl, pH8.0; 80mM Glycine)				0.1nM
Hu4D5 (200λ)		2482	2235	$[^{125}I]$-4D5
Hu4D5 (100λ)		4052	3805	

Test buffer	12746, 13147	12947	12700
Hu4D5 (200λ)	9044, 9583	9314	9067
Hu4D5 (100λ)	10938, 9918	10428	10181

0.1nM $[^{125}I]$-6E9

another mab which binds her2

Interpretation

- Hu4D5 specifically inhib the interaction of 4D5 with the her2 expressing cell line SKBR3, but does not interfere with the interaction between this cell line and the antibody 6E9
- Orders of Magnitude estimate of Kd (10-100 fold) above 4D5 From cell (opposite) - estimate that sample contains ~1.6ng/λ Hu4D5 Fab

To Page No.

Witnessed & Understood by me, Date Invented by Date ,

Recorded by

EXHIBIT B

INTERLEUKIN-2–RECEPTOR BLOCKADE WITH DACLIZUMAB TO PREVENT ACUTE REJECTION IN RENAL TRANSPLANTATION

FLAVIO VINCENTI, M.D., ROBERT KIRKMAN, M.D., SUSAN LIGHT, M.D., GINNY BUMGARDNER, M.D., PH.D., MARK PESCOVITZ, M.D., PHILIP HALLORAN, M.D., PH.D., JOHN NEYLAN, M.D., ALAN WILKINSON, M.D., HENRIK EKBERG, M.D., PH.D., ROBERT GASTON, M.D., LARS BACKMAN, M.D., PH.D., AND JAMES BURDICK, M.D., FOR THE DACLIZUMAB TRIPLE THERAPY STUDY GROUP*

ABSTRACT

Background Monoclonal antibodies that block the high-affinity interleukin-2 receptor expressed on alloantigen-reactive T lymphocytes may cause selective immunosuppression. Daclizumab is a genetically engineered human IgG1 monoclonal antibody that binds specifically to the α chain of the interleukin-2 receptor and may thus reduce the risk of rejection after renal transplantation.

Methods We administered daclizumab (1.0 mg per kilogram of body weight) or placebo intravenously before transplantation and once every other week afterward, for a total of five doses, to 260 patients receiving first cadaveric kidney grafts and immunosuppressive therapy with cyclosporine, azathioprine, and prednisone. The patients were followed at regular intervals for 12 months. The primary end point was the incidence of biopsy-confirmed acute rejection within six months after transplantation.

Results Of the 126 patients given daclizumab, 28 (22 percent) had biopsy-confirmed episodes of acute rejection, as compared with 47 of the 134 patients (35 percent) who received placebo ($P=0.03$). Graft survival at 12 months was 95 percent in the daclizumab-treated patients, as compared with 90 percent in the patients given placebo ($P=0.08$). The patients given daclizumab did not have any adverse reactions to the drug, and at six months, there were no significant differences between the two groups with respect to infectious complications or cancers. The serum half-life of daclizumab was 20 days, and its administration resulted in prolonged saturation of interleukin-2α receptors on circulating lymphocytes.

Conclusions Daclizumab reduces the frequency of acute rejection in kidney-transplant recipients. (N Engl J Med 1998;338:161-5.)

ACUTE rejection is a strong risk factor for chronic rejection in recipients of renal grafts from cadaveric donors.[1] This fact has prompted the development of new immunosuppressive agents designed to reduce the incidence and severity of acute rejection.[2-6] All these agents, however, achieve reductions in the frequency and severity of acute rejection at the price of generalized immunosuppression, with its attendant risks of opportunistic infection and cancer.

One potential target for more specific immunosuppressive therapy with monoclonal antibodies is the interleukin-2 receptor.[7] The high-affinity interleukin-2 receptor is composed of three noncovalently bound chains: a 55-kd α chain (also referred to as CD25 or Tac), a 75-kd β chain, and a 64-kd γ chain.[7] This receptor is present on nearly all activated T cells but not on resting T cells. The interaction of interleukin-2 with this high-affinity receptor is required for the clonal expansion and continued viability of activated T cells. A variety of rodent monoclonal antibodies directed against the α chain of the receptor have been used in animals and humans to achieve selective immunosuppression by targeting only T-cell clones responding to the allograft.[8-13] Daclizumab, a molecularly engineered human IgG1 incorporating the antigen-binding regions of the parent murine monoclonal antibody, offers the potential for greater therapeutic use of interleukin-2–receptor blockade.[14-17] We compared the efficacy of daclizumab with placebo for the prevention of acute rejection in renal-transplant recipients.

METHODS

Study Design

We performed a randomized, double-blind, placebo-controlled trial at 11 transplantation centers in the United States, 3 in Canada, and 3 in Sweden. Adults receiving first renal allografts from cadaveric donors were eligible for the study. Patients were excluded if they were receiving multiple organ transplants or had a positive crossmatch for T-cell lymphocytes. The protocol was approved by the institutional review board or ethics committee at each participating center, and all patients gave written informed consent.

Immunosuppressive Treatment

All patients received cyclosporine, azathioprine, and prednisone. The first dose of cyclosporine was given during the period from 12 hours before to 24 hours after transplantation.

Daclizumab (Zenapax, Hoffmann-LaRoche) or placebo was

From the University of California, San Francisco (F.V.); Brigham and Women's Hospital, Boston (R.K.); Hoffmann-LaRoche, Nutley, N.J. (S.L.); Ohio State University, Columbus (G.B.); Indiana University, Indianapolis (M.P.); the University of Alberta, Edmonton, Alta., Canada (P.H.); Emory University, Atlanta (J.N.); the University of California, Los Angeles (A.W.); Malmö University Hospital, Malmö, Sweden (H.E.); the University of Alabama, Birmingham (R.G.); Sahlgrenska Hospital, Gothenburg, Sweden (L.B.); and Johns Hopkins University, Baltimore (J.B.). Address reprint requests to Dr. Vincenti at the Transplant Service, University of California, San Francisco, 505 Parnassus Ave., Rm. M884, Box 0116, San Francisco, CA 94143-0116.

*Other members of the Daclizumab Triple Therapy Study Group are listed in the Appendix.

administered intravenously over a period of 15 minutes. Each patient received five doses of either daclizumab (1 mg per kilogram of body weight, to a maximum of 100 mg per dose) or placebo (0.2 mg of polysorbate 80 per milliliter in 67 mM phosphate buffer). The first dose was administered within 24 hours before transplantation, with subsequent doses given two, four, six, and eight weeks after transplantation.

Primary and Secondary End Points

The primary end point of the study was the incidence of biopsy-confirmed acute rejection within the first six months after transplantation. All patients with an unexplained rise in the serum creatinine concentration or one or more symptoms of acute rejection (fever, pain over the graft, or a decrease in urinary volume) were required to undergo a renal biopsy within 24 hours after the initiation of antirejection therapy, which consisted initially of intravenous methylprednisolone (7 mg per kilogram per day) for three days. The histologic diagnosis of rejection was based on the presence of acute tubulitis or vasculitis and was made by the pathologist at each institution. Patients were considered to have presumptive rejection if they received a course of antirejection therapy in the absence of histologic confirmation of rejection. The diagnosis of any subsequent episodes of rejection in patients presenting with renal dysfunction was based on clinical criteria, such as the absence of evidence of nephrotoxicity or of urinary tract obstruction or infection, with a biopsy for confirmation performed at the investigator's discretion.

Secondary end points included patient survival and graft survival at one year, the time to the first episode of acute rejection, the number of acute rejection episodes per patient, the need for antilymphocyte therapy (OKT3 or polyclonal antithymocyte globulin) because of glucocorticoid-resistant rejection (defined as the absence of a response to intravenous methylprednisolone pulse therapy), graft function (as indicated by the serum creatinine concentration and glomerular filtration rate), and the cumulative dose of prednisone in the first six months after transplantation.

Pharmacokinetic Measurements

Blood samples were collected immediately before and after (for trough and peak concentrations, respectively) the first and fifth infusions of daclizumab or placebo and on days 70 and 84 after transplantation. A sandwich enzyme-linked immunosorbent assay was used to measure daclizumab in serum.[18]

In 20 consecutive patients at one U.S. center (University of California, San Francisco), lymphocyte analysis was performed to determine the saturation of the interleukin-2–receptor α chain, with the use of methods reported previously.[17]

Glomerular Filtration Rate

The glomerular filtration rate was measured in all patients with functioning grafts six months after transplantation. Measurements were based on iohexol, radioisotope, or inulin clearance.

Statistical Analysis

Differences in categorical variables between the two groups were determined with the use of the Mantel–Haenszel test (with stratification according to center). Differences in the time to the first biopsy-confirmed episode of rejection were determined with the use of the log-rank test (with stratification according to center). The log-rank test was also used to analyze the time to graft failure (or death with a functioning graft) because of the small number of events reported. Kaplan–Meier estimates of the probability of patient survival and graft survival and the cumulative probability of biopsy-confirmed rejection were plotted over time. Differences in the number of presumptive or biopsy-confirmed rejection episodes per patient in the first six months were analyzed with a normal regression model. The serum creatinine concentrations, glomerular filtration rates, and cumulative doses of prednisone administered during the first six months after transplantation in the two groups were compared with the use of the Wilcoxon rank-sum test. Logistic-regression analysis was used to determine the effects of various factors on the probability of biopsy-confirmed rejection. Proportional-hazards analysis was used to determine the effects of various factors on the time to biopsy-confirmed rejection. The results of lymphocyte and interleukin-2–receptor assays were compared with the use of Student's t-test. All statistical tests were two-sided.

All patients randomly assigned to a treatment group were included in the primary analyses of efficacy and safety, according to the intention-to-treat principle. Values are reported as means ±SD.

RESULTS

A total of 260 patients were enrolled in the study: 134 patients were assigned to the placebo group, and 126 to the daclizumab group. The two groups were similar with respect to age, sex, race, cause of end-stage renal disease, presence or absence of panel-reactive anti-HLA antibodies, number of HLA-DR mismatches between donor and recipient, and duration of cold ischemia for the graft (Table 1).

All patients received at least one dose of the study drug, and 107 of the patients in the placebo group (80 percent) and 107 of those in the daclizumab group (85 percent) received all five doses. Graft function was delayed in 39 patients in the placebo group (29 percent) and 27 patients in the daclizumab group (21 percent). The early use of prophylactic antilymphocyte therapy for delayed graft function led to the discontinuation of the study drug in nine patients in the placebo group (7 percent) and nine in the daclizumab group (7 percent).

Efficacy

Daclizumab prophylaxis resulted in a significant reduction in the incidence of biopsy-documented acute rejection during the first six months after transplantation (22 percent, vs. 35 percent in the placebo group; $P=0.03$; odds ratio, 0.5; 95 percent confidence interval, 0.3 to 0.9) (Table 2). The proportion of patients with presumptive or biopsy-confirmed acute rejection and the number of rejection episodes per patient were also lower in the daclizumab group, and the time to the first rejection was longer. There was a trend toward a reduction in the number of patients with two or more rejection episodes and the number receiving antilymphocyte preparations for severe rejection in the daclizumab group. The beneficial effect of daclizumab was not influenced by delayed graft function, initial use of other antilymphocyte therapies, or exclusion of patients who did not receive all five infusions of the study drug (data not shown).

The patient-survival rates at one year were 98 percent in the daclizumab group and 96 percent in the placebo group (Table 3). The graft-survival rates in the daclizumab and placebo groups were 95 and 90 percent, respectively. None of the patients in the daclizumab group but three of those in the placebo group died of infections: one each of aspergillosis,

TABLE 1. BASE-LINE CHARACTERISTICS OF RENAL-ALLOGRAFT RECIPIENTS.*

CHARACTERISTIC	PLACEBO (N=134)	DACLIZUMAB (N=126)
Age — yr	47±13	47±13
Sex — no. of patients (%)		
Male	81 (60)	74 (59)
Female	53 (40)	52 (41)
Race or ethnic group — no. of patients (%)		
White	81 (60)	84 (67)
Black	27 (20)	24 (19)
Other	26 (19)	18 (14)
Cause of renal failure — no. of patients (%)		
Glomerulonephritis	40 (30)	33 (26)
Diabetes mellitus	29 (22)	32 (25)
Hereditary or polycystic kidney disease	20 (15)	24 (19)
Hypertension	19 (14)	18 (14)
Other	26 (19)	19 (15)
Panel-reactive serum antibodies — no. of patients (%)†		
0–10%	121 (90)	113 (89)
11–49%	10 (7)	12 (10)
50–100%	3 (2)	1 (1)
No. of HLA-DR mismatches — no. of patients (%)‡		
0	22 (16)	19 (15)
1	62 (46)	49 (39)
2	40 (30)	50 (40)
Graft cold-ischemia time — hr	21±9	22±8

*Plus–minus values are means ±SD. Percentages may not sum to 100 because of rounding.

†Panel-reactive antibodies are anti-HLA antibodies that have a cytotoxic effect on lymphocytes obtained from a panel of donors from the general population.

‡Data were missing for some patients.

TABLE 2. ACUTE REJECTION EPISODES IN THE FIRST SIX MONTHS AFTER RENAL TRANSPLANTATION IN THE PLACEBO AND DACLIZUMAB GROUPS.

REJECTION	PLACEBO (N=134)	DACLIZUMAB (N=126)	P VALUE
One or more biopsy-confirmed episodes — no. of patients (%)	47 (35)	28 (22)	0.03
One or more biopsy-confirmed or presumptive episodes — no. of patients (%)	52 (39)	32 (25)	0.04
Two or more biopsy-confirmed or presumptive episodes — no. of patients (%)	18 (13)	9 (7)	0.08
Mean no. of episodes/patient	0.6	0.3	0.01
Time to first episode — days*	30±27	75±59	0.008
Episode requiring antilymphocyte therapy — no. of patients (%)†	19 (14)	10 (8)	0.09

*Plus–minus values are means ±SD.

†Antilymphocyte therapy consisted of OKT3 or polyclonal antithymocyte globulin.

TABLE 3. CAUSES OF DEATH AND RENAL-GRAFT FAILURE AT ONE YEAR IN THE PLACEBO AND DACLIZUMAB GROUPS.

CAUSE	PLACEBO (N=134)	DACLIZUMAB (N=126)
	no. of patients (%)	
Death	5 (4)	3 (2)
Infection or lymphoma	3 (2)	1 (1)
Cardiovascular cause	1 (1)	0
Pulmonary embolism	1 (1)	0
Intracerebral bleeding	0	1 (1)
Suicide	0	1 (1)
Graft failure	13 (10)	6 (5)
Death	5 (4)	3 (2)
Rejection	3 (2)	1 (1)
Technical cause	4 (3)	2 (2)
Primary nonfunction	1 (1)	0

coccidioidomycosis, and pseudomonas sepsis. One patient in the daclizumab group died of lymphoma.

The mean serum creatinine concentrations six months after transplantation were the same in the two groups (1.7±0.7 mg per deciliter [150±60 μmol per liter]). The mean glomerular filtration rate was 55±23 ml per minute in the daclizumab group and 52±22 ml per minute in the placebo group. The average daily doses of prednisone and cyclosporine did not differ between the groups at any time during the study, nor was there a difference in the mean trough whole-blood cyclosporine concentrations at any time.

Adverse Events

The administration of daclizumab was not associated with any immediate side effects. There was no significant difference in reported adverse events between the two groups (Table 4). One patient in the placebo group and two patients in the daclizumab group had lymphoma during the first year after transplantation.

Pharmacokinetic Data

Pharmacokinetic data were available for 92 patients in the daclizumab group. The mean serum half-life of daclizumab was 20 days.

Circulating Peripheral-Blood Lymphocytes and Interleukin-2 α-Chain Receptor

There were no differences in absolute lymphocyte numbers between the placebo and daclizumab groups before transplantation or for six months afterward. Circulating CD3+ cell concentrations and T-cell subgroups were not measured, because they were not affected by daclizumab therapy in an earlier study.[17] There was a significant decrease in the percentage of circulating lymphocytes that stained with anti-

TABLE 4. ADVERSE EVENTS AT SIX MONTHS IN THE PLACEBO AND DACLIZUMAB GROUPS.

ADVERSE EVENTS	PLACEBO (N = 134)	DACLIZUMAB (N = 126)
	no. of patients (%)	
Serious event*	13 (10)	6 (5)
Fever	16 (12)	11 (9)
Sepsis and bacteremia	9 (7)	4 (3)
Pneumonia	4 (3)	3 (2)
Fungal infection	27 (20)	21 (17)
Fungemia	2 (1)	0
Local infection	25 (19)	21 (17)
Local infection†	70 (52)	59 (47)
Cellulitis and wound infection	4 (3)	7 (6)
Urinary tract infection	44 (33)	34 (27)
Other	38 (28)	36 (29)
Any viral infection†	32 (24)	29 (23)
Viremia	12 (9)	12 (10)
Local infection	21 (16)	20 (16)
Cytomegalovirus infection	14 (10)	15 (12)
Viremia	10 (7)	12 (10)
Tissue infection	4 (3)	3 (2)

*Serious adverse events were defined as complications other than death or rejection that prolonged or required hospitalization and were possibly or probably related to the study drug.

†Some patients had more than one type of infection.

CD25 antibody starting 10 hours after transplantation and lasting up to four months in the daclizumab group (data not shown). Similarly, there was a significant decrease in the percentage of circulating lymphocytes that stained with the fluorescein-conjugated antibody 7g7, which binds to an interleukin-2 α-chain–receptor epitope distinct from the epitope recognized by daclizumab and reflects total interleukin-2α–receptor expression (data not shown).

DISCUSSION

We found that the patients receiving daclizumab in addition to maintenance therapy with three immunosuppressive agents had a lower frequency of biopsy-confirmed acute rejection in the first six months after transplantation than the patients receiving placebo with the three immunosuppressive agents. In addition, the time to the first episode of acute rejection was significantly prolonged, and the mean number of episodes per patient significantly reduced in the daclizumab group. These results were obtained without a concomitant increase in infectious complications or cancers. The efficacy of daclizumab is probably related to its selective target, the α-chain component of the high-affinity interleukin-2 receptor, which is present almost exclusively on activated T cells. Use of the drug thus spares other immunocompetent cells.[7]

Only 10 percent of daclizumab is composed of murine sequences, which are from the antigen-binding regions of the parent antibody. These sequences are inserted into human immunoglobulin with the use of molecular biologic techniques.[14] Our study highlights the advantages of this type of antibody, including its prolonged serum half-life, approaching that of human IgG, and the absence of functional immunogenicity associated with its use.[15,16,19,20]

The exact mechanism or mechanisms of action of daclizumab are not known. A likely mechanism is that it binds to circulating lymphocytes with interleukin-2 α-chain receptors but does not activate the receptors, and the cells therefore have no free interleukin-2 α-chain receptors available for activation by interleukin-2. In addition, the decline in the percentage of circulating lymphocytes expressing CD25 (measured by staining with 7g7 antibody) without an accompanying decrease in the absolute number of lymphocytes suggests that the expression of interleukin-2 receptors is down-regulated or the shedding of the daclizumab-bound interleukin-2 α chain is increased.

In conclusion, when added to therapy with cyclosporine, azathioprine, and prednisone, daclizumab reduces the frequency of acute rejection and improves short-term graft survival in renal-transplant recipients.

Supported by a grant from Hoffmann–LaRoche.

We are indebted to Dr. Thomas A. Waldmann for his contribution to the development of daclizumab, and to Ms. Peggy Millar for her assistance in the preparation of the manuscript.

APPENDIX

In addition to the authors, the following investigators participated in the Daclizumab Triple Therapy Study Group: *Victoria General Hospital, Halifax, N.S., Canada* — B. Kibert; *Huddinge Hospital, Huddinge, Sweden* — G. Tyden; *University of Minnesota, Minneapolis* — A. Matas; *Beth Israel Deaconess Medical Center, Boston* — M. Shapiro; *Tampa General Hospital, Tampa, Fla.* — G. Chan; *Vancouver General Hospital, Vancouver, B.C., Canada* — P. Keown; *University of California, San Francisco* — M. Lantz; *University of Alberta, Edmonton, Alta., Canada* — K. Solez; and *Hoffmann–LaRoche, Nutley, N.J.* — A. Lin, I. Patel, K. Nieforth, A. Wolitzky, and J. Hakimi.

REFERENCES

1. Ferguson R. Acute rejection episodes — best predictor of long-term primary cadaveric renal transplant survival. Clin Transplant 1994;8:328-31.
2. Sollinger HW. Mycophenolate mofetil for the prevention of acute rejection in primary cadaveric renal allograft recipients. Transplantation 1995; 60:225-32.
3. Vincenti F, Laskow DA, Neylan JF, Mendez R, Matas AJ. One-year follow-up from an open-label trial of FK506 in primary kidney transplantation: a report of the U.S. Multicenter FK506 Kidney Transplantation Group. Transplantation 1996;61:1576-81.
4. Morris RE. Mechanisms of action of new immunosuppressive drugs. Kidney Int Suppl 1996;53:S-26–S-38.
5. Kahan BD. Sirolimus: a new agent for clinical renal transplantation. Transplant Proc 1997;29:48-50.
6. Gruber SA, Chan GLC, Canafax DM, Matas AJ. Immunosuppression in renal transplantation. II. Corticosteroids, antilymphocyte globulin, and OKT3. Clin Transplant 1991;5:219-32.

63

7. Taniguchi T, Minami Y. The IL-2/IL-2 receptor system: a current overview. Cell 1993;73:5-8.
8. Kupiec-Weglinski JW, Diamantstein T, Tilney NL, Strom TB. Therapy with monoclonal antibody to interleukin 2 receptor spares suppressor T cells and prevents or reverses acute allograft rejection in rats. Proc Natl Acad Sci U S A 1986;83:2624-7.
9. Reed MH, Shapiro ME, Strom TB, et al. Prolongation of primate renal allograft survival by anti-Tac, an anti-human IL-2 receptor monoclonal antibody. Transplantation 1989;47:55-9.
10. Soulillou J-P, Cantarovich D, Le Mauff B, et al. Randomized controlled trial of a monoclonal antibody against the interleukin-2 receptor (33B3.1) as compared with rabbit antithymocyte globulin for prophylaxis against rejection of renal allografts. N Engl J Med 1990;322:1175-82.
11. Kirkman RL, Shapiro ME, Carpenter CB, et al. A randomized prospective trial of anti-Tac monoclonal antibody in human renal transplantation. Transplantation 1991;51:107-13.
12. van Gelder T, Zietse R, Mulder AH, et al. A double-blind, placebo-controlled study of monoclonal anti-interleukin-2 receptor antibody (BT563) administration to prevent acute rejection after kidney transplantation. Transplantation 1995;60:248-52.
13. Reding R, Vraux H, de Ville de Goyet J, et al. Monoclonal antibodies in prophylactic immunosuppression after liver transplantation: a randomized controlled trial comparing OKT3 and anti-IL-2 receptor monoclonal antibody LO-Tact-1. Transplantation 1993;55:534-41.
14. Queen C, Schneider WP, Selick HE, et al. A humanized antibody that binds to the interleukin 2 receptor. Proc Natl Acad Sci U S A 1989;86:10029-33.
15. Hakimi J, Mould D, Waldmann TA, Queen C, Anasetti C, Light S. Development of Zenapax: a humanized anti-Tac antibody. In: Harris WJ, Adair JR, eds. Antibody therapeutics. New York: CRC Press, 1997:277-300.
16. Hakimi J, Chizzonite R, Luke DR, et al. Reduced immunogenicity and improved pharmacokinetics of humanized anti-Tac in cynomolgus monkeys. J Immunol 1991;147:1352-9.
17. Vincenti F, Lantz M, Birnbaum J, et al. A phase I trial of humanized anti-interleukin 2 receptor antibody in renal transplant recipients. Transplantation 1997;63:33-8.
18. Fayer BE, Soni PP, Binger MH, Mould DR, Satoh H. Determination of humanized anti-Tac in human serum by a sandwich enzyme linked immunosorbent assay. J Immunol Methods 1995;186:47-54.
19. Brown PS Jr, Pareuteau GL, Dirbas FM, et al. Anti-Tac-H, a humanized antibody to the interleukin 2 receptor, prolongs primate cardiac allograft survival. Proc Natl Acad Sci U S A 1991;88:2663-7.
20. Anasetti C, Hansen JA, Waldmann TA, et al. Treatment of acute graft-versus-host disease with humanized anti-Tac: an antibody that binds to the interleukin-2 receptor. Blood 1994;84:1320-7.

Interview Summary

Application No.	Applicant(s)
08/146,206	Carter et al
Examiner	**Group Art Unit**
MINH TAM DAVIS	1642

All participants (applicant, applicant's representative, PTO personnel):

(1) *MINH TAM DAVIS* (3) _____

(2) *Wendy Lee* (4) _____

Date of Interview *Dec 11, 2001*

Type: a)☒ Telephonic b)☐ Video Conference
c)☐ Personal [copy is given to 1)☐ applicant 2)☐ applicant's representative]

Exhibit shown or demonstration conducted: d)☐ Yes e)☒ No. If yes, brief description:

Claim(s) discussed: _____

Identification of prior art discussed:

Agreement with respect to the claims f)☐ was reached. g)☐ was not reached. h)☐ N/A.

Substance of Interview including description of the general nature of what was agreed to if an agreement was reached, or any other comments:

Pending claims 43-105, 113-131 are allowable.

(A fuller description, if necessary, and a copy of the amendments which the examiner agreed would render the claims allowable, if available, must be attached. Also, where no copy of the amendments that would render the claims allowable is available, a summary thereof must be attached.)

i)☒ It is not necessary for applicant to provide a separate record of the substance of the interview (if box is checked).

Unless the paragraph above has been checked, THE FORMAL WRITTEN REPLY TO THE LAST OFFICE ACTION MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP section 713.04). If a reply to the last Office action has already been filed, APPLICANT IS GIVEN ONE MONTH FROM THIS INTERVIEW DATE TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW. See Summary of Record of Interview requirements on reverse side or on attached

Examiner Note: You must sign this form unless it is an Attachment to a signed Office action.

M-T DAVIS Re-Pier

1600

RAW SEQUENCE LISTING DATE: 12/11/2001
PATENT APPLICATION: US/08/146,206C TIME: 13:58:59

Input Set : A:\p0709pl.txt
Output Set: N:\CRF3\12112001\H146206C.raw

#
67
CS
12-14-01

SEQUENCE LISTING

```
W--> 3 SEQUENCE LISTING
     5 (1) GENERAL INFORMATION:
     7      (i) APPLICANT: Carter, Paul J.
     8                     Presta, Leonard G.
    10     (ii) TITLE OF INVENTION: Method for Making Humanized Antibodies
    12    (iii) NUMBER OF SEQUENCES: 26
    14     (iv) CORRESPONDENCE ADDRESS:
    15           (A) ADDRESSEE: Genentech, Inc.
    16           (B) STREET: 1 DNA Way
    17           (C) CITY: South San Francisco
    18           (D) STATE: California
    19           (E) COUNTRY: USA
    20           (F) ZIP: 94080
    22      (v) COMPUTER READABLE FORM:
    23           (A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk
    24           (B) COMPUTER: IBM PC compatible
    25           (C) OPERATING SYSTEM: PC-DOS/MS-DOS
    26           (D) SOFTWARE: WinPatin (Genentech)
    28     (vi) CURRENT APPLICATION DATA:
C--> 29           (A) APPLICATION NUMBER: US/08/146,206C
C--> 30           (B) FILING DATE: 17-Nov-1993
    31           (C) CLASSIFICATION:
    33    (vii) PRIOR APPLICATION DATA:
    34           (A) APPLICATION NUMBER: 07/715272
    35           (B) FILING DATE: 14-JUN-1991
    37   (viii) ATTORNEY/AGENT INFORMATION:
    38           (A) NAME: Lee, Wendy M.
    39           (B) REGISTRATION NUMBER: 40,378
    40           (C) REFERENCE/DOCKET NUMBER: P0709P1
    42     (ix) TELECOMMUNICATION INFORMATION:
    43           (A) TELEPHONE: 650/225-1994
    44           (B) TELEFAX: 650/952-9881
    45 (2) INFORMATION FOR SEQ ID NO: 1:
    47      (i) SEQUENCE CHARACTERISTICS:
    48           (A) LENGTH: 109 amino acids
    49           (B) TYPE: Amino Acid
    50           (D) TOPOLOGY: Linear
    52     (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:
    54  Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
    55    1               5                  10                  15
    57  Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val Asn
    58                  20                  25                  30
    60  Thr Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
    61                  35                  40                  45
    63  Leu Leu Ile Tyr Ser Ala Ser Phe Leu Glu Ser Gly Val Pro Ser
    64                  50                  55                  60
```

ENTERED

RAW SEQUENCE LISTING DATE: 12/11/2001
PATENT APPLICATION: US/08/146,206C TIME: 13:58:59

Input Set : A:\p0709pl.txt
Output Set: N:\CRF3\12112001\H146206C.raw

```
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                 100                 105
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
 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                 100                 105
 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
```

RAW SEQUENCE LISTING DATE: 12/11/2001
PATENT APPLICATION: US/08/146,206C TIME: 13:58:59

Input Set : A:\p0709pl.txt
Output Set: N:\CRF3\12112001\H146206C.raw

```
                    95                  100                 105
 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                 100                 105
 Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
                  110                 115                 120
(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                 100                 105
 Ile Lys Arg Ala
             109
(2) INFORMATION FOR SEQ ID NO: 6:
     (i) SEQUENCE CHARACTERISTICS:
```

RAW SEQUENCE LISTING DATE: 12/11/2001
PATENT APPLICATION: US/08/146,206C TIME: 13:58:59

Input Set : A:\p0709pl.txt
Output Set: N:\CRF3\12112001\H146206C.raw

(A) LENGTH: 120 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

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

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
95 100 105
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 27

(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

```
288           (D) TOPOLOGY: Linear
290      (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:
293  TGAGGAGACG GTGACCGTGG TCCCTTGGCC CCAG 34
295 (2) INFORMATION FOR SEQ ID NO: 11:
297       (i) SEQUENCE CHARACTERISTICS:
298            (A) LENGTH: 36 base pairs
299            (B) TYPE: Nucleic Acid
300            (C) STRANDEDNESS: Single
301            (D) TOPOLOGY: Linear
303      (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:
306  GTAGATAAAT CCTCTAACAC AGCCTATCTG CAAATG 36
308 (2) INFORMATION FOR SEQ ID NO: 12:
310       (i) SEQUENCE CHARACTERISTICS:
311            (A) LENGTH: 36 base pairs
312            (B) TYPE: Nucleic Acid
313            (C) STRANDEDNESS: Single
314            (D) TOPOLOGY: Linear
316      (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:
319  GTAGATAAAT CCAAATCTAC AGCCTATCTG CAAATG 36
321 (2) INFORMATION FOR SEQ ID NO: 13:
323       (i) SEQUENCE CHARACTERISTICS:
324            (A) LENGTH: 36 base pairs
325            (B) TYPE: Nucleic Acid
326            (C) STRANDEDNESS: Single
327            (D) TOPOLOGY: Linear
329      (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:
332  GTAGATAAAT CCTCTTCTAC AGCCTATCTG CAAATG 36
334 (2) INFORMATION FOR SEQ ID NO: 14:
336       (i) SEQUENCE CHARACTERISTICS:
337            (A) LENGTH: 68 base pairs
338            (B) TYPE: Nucleic Acid
339            (C) STRANDEDNESS: Single
340            (D) TOPOLOGY: Linear
342      (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:
345  CTTATAAAGG TGTTTCCACC TATAACCAGA AATTCAAGGA TCGTTTCACG 50
347  ATATCCGTAG ATAAATCC 68
349 (2) INFORMATION FOR SEQ ID NO: 15:
351       (i) SEQUENCE CHARACTERISTICS:
352            (A) LENGTH: 30 base pairs
353            (B) TYPE: Nucleic Acid
354            (C) STRANDEDNESS: Single
355            (D) TOPOLOGY: Linear
357      (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:
360  CTATACCTCC CGTCTGCATT CTGGAGTCCC 30
362 (2) INFORMATION FOR SEQ ID NO: 16:
364       (i) SEQUENCE CHARACTERISTICS:
365            (A) LENGTH: 107 amino acids
366            (B) TYPE: Amino Acid
367            (D) TOPOLOGY: Linear
```

VERIFICATION SUMMARY DATE: 12/11/2001
PATENT APPLICATION: US/08/146,206C TIME: 13:59:00

Input Set : A:\p0709pl.txt
Output Set: N:\CRF3\12112001\H146206C.raw

L:3 M:244 W: Invalid beginning of sequence listing, Data=[SEQUENCE LISTING], Duplicate Sequence Listing Title!
L:29 M:220 C: Keyword misspelled or invalid format, [(A) APPLICATION NUMBER:]
L:30 M:220 C: Keyword misspelled or invalid format, [(B) FILING DATE:]

Genentech, Inc.

Anna S. Kan
Legal Department

(650) 225-2830
Fax (650) 952-9881
kan@gene.com

To: Examiner Minh-Tam Davis
From: Wendy Lee

This is the priority document for 08/146,206.

12/12/2001

Genentech Legal Department

1 DNA Way
South San Francisco, CA 94080
650-225-2830
Fax: 650-952-9881/9882

FAX TRANSMISSION COVER SHEET

Date: *December 12, 2001*

To: *Examiner Minh-Tam Davis*
Group Art 1642

Fax: *(703) 746-7145*

Re: *U.S. Ser. No 09/146,206*
filed August 1, 1995
Attorney Docket No.: P0709P1

Sender: *Anna Kan for Wendy Lee*

YOU SHOULD RECEIVE 12 PAGE(S), INCLUDING THIS COVER SHEET. IF YOU DO NOT RECEIVE ALL THE PAGES, PLEASE CALL 650-225-2830.

Dear Examiner Davis,

Pursuant to your request, attached are courtesy copies of the IDS Transmittals and PTO-1449 Forms filed on August 1, 1995 and February 1, 1999. We understand that you have the cited references but, if not, let us know and we will be happy to provide further copies.

Kindly send us initialed copies of the PTO-1449 Forms for the IDSs filed on the following dates. The reference nos. are noted below in parentheses.

09/02/97 (refs. 100-207)
08/24/98 (refs. 215-224)
02/01/99 (refs. 225-262)
03/09/99 (ref. 263)
08/30/01 (ref. 264-265)

Very truly yours,
Genentech, Inc.

Anna Kan

Anna Kan for Wendy Lee

WL/LS

Patent Docket P0709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Paul J. Carter et al. Serial No.: 08/146,206 Filed: November 17, 1993 For: METHOD FOR MAKING HUMANIZED ANTIBODIES	Group Art Unit: 1642 Examiner: J. Reeves CERTIFICATE OF HAND DELIVERY I hereby certify that this correspondence is being hand delivered in an envelope addressed to: Assistant Commissioner of Patents, Washington, D.C. 20231 on February 1, 1999 R. H. Mitchell

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

Official

12/13/01

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Applicants submit herewith patents, publications or other information (attached hereto and listed on the attached Form PTO-1449) of which they are aware, which they believe may be material to the examination of this application and in respect of which there may be a duty to disclose in accordance with 37 CFR §1.56.

This Information Disclosure Statement:

(a) 0 accompanies the new patent application submitted herewith. 37 CFR §1.97(a).

(b) 0 is filed within three months after the filing date of the application or within three months after the date of entry of the national stage of a PCT application as set forth in 37 CFR§1.491.

(c) 0 as far as is known to the undersigned, is filed before the mailing date of a first Office action on the merits.

(d) 0 is filed after the first Office Action and more than three months after the application's filing date or PCT national stage date of entry filing but, as far as is known to the undersigned, prior to the mailing date of either a final rejection or a notice of allowance, whichever occurs first, and is accompanied by either the fee ($240) set forth in 37 CFR §1.17(p) or a statement as specified in 37 CFR §1.97(e), as checked below. Should any fee be due, the U.S. Patent and Trademark Office is hereby authorized to charge Deposit Account No. 07-0630 in the amount of $240.00 to cover the cost of this Information Disclosure Statement. Any deficiency or overpayment

should be charged or credited to this deposit account. A duplicate of this sheet is enclosed.

(e) 0 is filed after the mailing date of either a final rejection or a notice of allowance, whichever occurred first, and is accompanied by the fee ($130) set forth in 37 CFR §1.17(i) and a statement as specified in 37 CFR §1.97(e), as checked below. This document is to be considered as a petition requesting consideration of the information disclosure statement. The U.S. Patent and Trademark Office is hereby authorized to charge Deposit Account No. 07-0630 in the amount of $130.00 to cover the cost of this Information Disclosure Statement. Any deficiency or overpayment should be charged or credited to this deposit account. A duplicate of this sheet is enclosed.

(f) (x) is filed after the mailing date of a final rejection, but a request to withdraw the finality thereof under 37 CFR § 1.129(a) was submitted on August 24, 1998. The U.S. Patent and Trademark Office is hereby authorized to charge Deposit Account No. 07-0630 to cover the cost of this Information Disclosure Statement in the event that any fees are due. A duplicate of this sheet is enclosed.

(If either of boxes (d) or (e) is checked above, the following statement under 37 CFR §1.97(e) may need to be completed.) The undersigned states that:

0 Each item of information contained in the information disclosure statement was cited in a communication mailed from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of this information disclosure statement.

0 No item of information contained in this information disclosure statement was cited in a communication mailed from a foreign patent office in a counterpart foreign application and, to the knowledge of the undersigned after making reasonable inquiry, was known to any individual designated in 37 CFR §1.56(c) more than three months prior to the filing of this information disclosure statement.

A list of the patent(s) or publication(s) is set forth on the attached Form PTO-1449 (Modified).

A copy of the items on PTO-1449 is supplied herewith:

(x) each 0 none 0 only those listed below:

A concise explanation of relevance of the items listed on PTO-1449 is:

(x) not given

0 given for each listed item

0 given for only non-English language listed item(s) (Required)

0 In the form of an English language copy of a Search Report from a foreign patent

office, issued in a counterpart application, which refers to the relevant portions of the references.

The Examiner is reminded that a "concise explanation of the relevance" of the submitted prior art "may be nothing more than identification of the particular figure or paragraph of the patent or publication which has some relation to the claimed invention," MPEP §609.

While the information and references disclosed in this Information Disclosure Statement may be "material" pursuant to 37 CFR §1.56, it is not intended to constitute an admission that any patent, publication or other information referred to therein is "prior art" for this invention unless specifically designated as such.

In accordance with 37 CFR §1.97(g), the filing of this Information Disclosure Statement shall not be construed to mean that a search has been made or that no other material information as defined in 37 CFR §1.56(a) exists. It is submitted that the Information Disclosure Statement is in compliance with 37 CFR §1.98 and MPEP §609 and the Examiner is respectfully requested to consider the listed references.

Respectfully submitted,
GENENTECH, INC.

Date: January 29, 1999 By: _____
Wendy M. Lee
Reg. No. 40,378

1 DNA Way
So. San Francisco, CA 94080-4990
Phone: (650) 225-1994
Fax: (650) 952-9881

In re Application of: Paul J. Carter et al.
Serial No.: 08/146,206
Filed On: 17 November 1993
Mailed On: 1 August 1995

Docket No.: 709P1
By: Wendy M. Lee
Reg. No.:

The following has been received in the U.S. Patent Office on the date stamped:

___ Amendment/Response
___ Extension of Time Request (dup)
___ Communication/Transmittal Letter (dup)
___ Notice of Appeal (dup)
___ Issue Fee Transmittal Form
X Information Disclosure Statement (Supplemental)
X Form 1449 with 3 References
X Certificate of Mailing
___ Certificate of Express Mailing
Express Mail Label No.:
X Other: Limited Recognition

___ U.S. Patent Application
___ Rule 60 ___ Rule 62
___ Declaration/Power of Atty
___ Assignment
___ Recordation Form/Fee
___ Drawings: __ Sheets
___ Informal ___ Formal
___ Sequence Listing & Diskette
___ PCT Patent Application

In re Application of: Paul J. Carter et al.
Serial No.: 08/146,206
Filed On: 17 November 1993
Mailed On: 1 August 1995

Docket No.: 709P1
By: Wendy M. Lee
Reg. No.:

The following has been received in the U.S. Patent Office on the date stamped:

___ Amendment/Response
___ Extension of Time Request (dup)
___ Communication/Transmittal Letter (dup)
___ Notice of Appeal (dup)
___ Issue Fee Transmittal Form
X Information Disclosure Statement (Supplemental)
X Form 1449 with 3 References
X Certificate of Mailing
___ Certificate of Express Mailing
Express Mail Label No.:
X Other: Limited Recognition

___ U.S. Patent Application
___ Rule 60 ___ Rule 62
___ Declaration/Power of Atty
___ Assignment
___ Recordation Form/Fee
___ Drawings: __ Sheets
___ Informal ___ Formal
___ Sequence Listing & Diskette
___ PCT Patent Application

MAIL ROOM 74 AUG 3 1995 PAT. & TRADEMARK OFF.

PATENT DOCKET 709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of

Paul J. Carter et al.

Serial No. 08/146,206

Filed: 17 November 1993

For: METHOD FOR MAKING HUMANIZED ANTIBODIES

Group Art Unit: 1806

Examiner: D. Adams

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner of Patents and Trademarks, Washington, D.C. 20231 on

8/1/95

(Date of Deposit)

Duane Alexander Vick

Name of Depositing Party

Duane Alexander Vick

Signature of Depositing Party

8/1/95

Date of Signature

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT

Official
TDis
12/13/01

BOX DD
Honorable Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

Applicants submit herewith patents, publications or other information (attached hereto and listed on the attached Form PTO-1449) of which they are aware, which they believe may be material to the examination of this application and in respect of which there may be a duty to disclose in accordance with 37 CFR §1.56.

This Information Disclosure Statement:

(a) [] accompanies the new patent application submitted herewith. 37 CFR §1.97(a).

(b) [] is filed within three months after the filing date of the application or within three months after the date of entry of the national stage of a PCT application as set forth in 37 CFR§1.491.

(c) [] as far as is known to the undersigned, is filed before the mailing date of a first Office action on the merits.

(d) [X] is filed after the first Office Action and more than three months after the application's filing date or PCT national stage date of entry filing but, as far as is known to the undersigned, prior to the mailing date of either a final rejection or a notice of allowance, whichever occurs first, and is accompanied by either the fee ($210) set forth in 37 CFR §1.17(p) or a certification as specified in 37 CFR §1.97(e), as checked below. Should any fee be due, the U.S. Patent and Trademark Office is hereby authorized to charge Deposit Account No. 07-0630 in the amount of $210.00 to cover the cost of this

Information Disclosure Statement. Any deficiency or overpayment should be charged or credited to this deposit account. **A duplicate of this sheet is enclosed.**

(e) [] is filed after the mailing date of either a final rejection or a notice of allowance, whichever occurred first, and is accompanied by the fee ($130) set forth in 37 CFR §1.17(i)(1) and a certification as specified in 37 CFR §1.97(e), as checked below. **This document is to be considered as a petition requesting consideration of the information disclosure statement.** The U.S. Patent and Trademark Office is hereby authorized to charge Deposit Account No. 07-0630 in the amount of $130.00 to cover the cost of this Information Disclosure Statement. Any deficiency or overpayment should be charged or credited to this deposit account. **A duplicate of this sheet is enclosed.**

[If either of boxes (d) or (e) is checked above, the following "certification" under 37 CFR §1.97(e) may need to be completed.] The undersigned certifies that:

[] Each item of information contained in the information disclosure statement was cited in a communication mailed from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of this information disclosure statement.

[] No item of information contained in this information disclosure statement was cited in a communication mailed from a foreign patent office in a counterpart foreign application or, to the knowledge of the undersigned after making reasonable inquiry, was known to any individual designated in 37 CFR §1.56(c) more than three months prior to the filing of this information disclosure statement.

A list of the patent(s) or publication(s) is set forth on the attached Form PTO-1449 (Modified).

A copy of the items on PTO-1449 is supplied herewith:

[X] each [] none [] only those listed below:

Those patent(s) or publication(s) which are marked with an asterisk (*) in the attached PTO-1449 form are not supplied because they were previously cited by or submitted to the Office in a prior application Serial No. , filed and relied upon in this application for an earlier filing date under 35 USC §120.

A concise explanation of relevance of the items listed on PTO-1449 is:

[X] not given

[] given for each listed item

[] given for only non-English language listed item(s) [Required]

[] in the form of an English language copy of a Search Report from a foreign patent office, issued in a counterpart application, which refers to the relevant portions of the references.

The Examiner is reminded that a "concise explanation of the relevance" of the submitted prior art "may be nothing more than identification of the particular figure or paragraph of the patent or publication which has some relation to the claimed invention," MPEP §609.

While the information and references disclosed in this Information Disclosure Statement may be "material" pursuant to 37 CFR §1.56, it is not intended to constitute an admission that any patent, publication or other information referred to therein is "prior art" for this invention unless specifically designated as such.

In accordance with 37 CFR §1.97(g), the filing of this Information Disclosure Statement shall not be construed to mean that a search has been made or that no other material information as defined in 37 CFR §1.56(a) exists. It is submitted that the Information Disclosure Statement is in compliance with 37 CFR §1.98 and MPEP §609 and the Examiner is respectfully requested to consider the listed references.

A copy of a document pursuant to 37 C.F.R. § 10.9(b) is attached as proof of the authorization of the undersigned to prosecute the above-mentioned application. The original of this document is on file in the Office of Enrollment and Discipline.

Respectfully submitted,
GENENTECH, INC.

Date: August 1, 1995

By: _____
Wendy M. Lee

460 Pt. San Bruno Blvd.
So. San Francisco, CA 94080-4990
Phone: (415) 225-1994
Fax: (415) 952-9881

In re Application of: Paul J. Carter et al.
Serial No.: 08/146,206
Filed On: November 17, 1993
Hand Delivered On: ___ February 1999

Docket No.: P0709P1
By: Wendy M. Lee
Reg. No.: 40,378

The following has been received in the U.S. Patent Office on the date stamped:

_x_ Information Disclosure Statement
_x_ Form 1449 with 38 References
_x_ Communication with Exhibit A and two priority documents
_x_ Certificate of Hand Delivery

In re Application of: Paul J. Carter et al.
Serial No.: 08/146,206
Filed On: November 17, 1993
Hand Delivered On: ___ February 1999

Docket No.: P0709P1
By: Wendy M. Lee
Reg. No.: 40,378

The following has been received in the U.S. Patent Office on the date stamped:

_x_ Information Disclosure Statement
_x_ Form 1449 with 38 References
_x_ Communication with Exhibit A and two priority documents
_x_ Certificate of Hand Delivery

99 FEB -1 PM 12:21

RECEIVED
TECH. CENTER 1800/2900

FORM PTO-1449	U.S. Dept. of Commerce Patent and Trademark Office	Atty Docket No. P0709P1	Serial No. 08/146,206
LIST OF DISCLOSURES CITED BY APPLICANT (Use several sheets if necessary)		Applicant Carter et al.	
		Filing Date 17 Nov 1993	Group 1806

U.S. PATENT DOCUMENTS

Examiner Initials		Document Number	Date	Name	Class	Subclass	Filing Date
	225	5,714,350	03.02.98	Co et al.			13.01.95
	226	5,821,337	13.10.98	Carter et al.			

FOREIGN PATENT DOCUMENTS

Examiner Initials		Document Number	Date	Country	Class	Subclass	Translation Yes	Translation No
	227	0 460 167 B1	11.12.91	EPO				
	228	0 519 596 A1	23.12.92	EPO				
	229	0 592 106 A1	13.04.94	EPO				
	230	120,694	03.10.84	EPO				
	231	125,023 A1	14.11.84	EPO				
	232	368,684	16.05.90	EPO				
	233	94/11509	26.05.94	PCT				
	234	WO 89/09622		PCT				
	235	WO 92/11383	09.07.92	PCT				
	236	2 188941	14.10.87	UNITED KINGDOM				

OTHER DISCLOSURES (Including Author, Title, Date, Pertinent Pages, etc.)

	237	"Biosym Technologies" in New Products, Chemical Design Automation 3" (December 1988)
	238	"Polygen Corporation" in New Products, Chemical Design Automation 3" (November 1988)
	239	Adair et al., "Humanization of the murine anti-human CD3 monoclonal antibody OKT3" Hum. Antibod. Hybridomas 5:41-47 (1994)
	240	Chothia et al., "Principles of protein-protein recognition" Nature 256:705-708 (1975)
	241	Chothia et al., "Transmission of conformational change in insulin" Nature 302:500-505 (1983)
	242	Corti et al., "Idiotope Determining Regions of a Mouse Monoclonal Antibody and Its Humanized Versions" J. Mol. Biol. 235:53-60 (1994)
	243	Couto et al., "Anti-BA46 Monoclonal Antibody Mc3 Humanization Using a Novel Positional Consensus and in Vivo and in Vitro Characterization" Cancer Research Supplement 55:1717-1722 (1995)
	244	Couto et al., "Humanization of KC4G3, an Anti-Human Carcinoma Antibody" Hybridoma 13:215-219 (1994)
	245	Ellis et al., "Engineered Anti-CD38 Monoclonal Antibodies for Immunotherapy of Multiple Myeloma" The Journal of Immunology pps. 925-937 (1995)
	246	Hieter et al., "Evolution of Human Immunoglobulin K J Region Genes" The Journal of Biological Chemistry 257:1516-1522 (1982)
	247	Lesk, Arthur M., "How Different Amino Acid Sequences Determine Similar Protein Structures: The Structure and Evolutionary Dynamics of the Globins" J. Mol. Biol. 136:225-270 (1980)

Examiner	Date Considered

*Examiner: Initial if reference considered, whether or not citation is in conformance with MPEP 609; draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

USCOMM-DC 80-398.

FORM PTO-1449 U.S. Dept. of Commerce Patent and Trademark Office

Atty Docket No.	Serial No.
P0709P1	08/146,206
Applicant: Carter et al.	
Filing Date: 17 Nov 1993	Group: 1806

LIST OF DISCLOSURES CITED BY APPLICANT

(Use several sheets if necessary)

OTHER DISCLOSURES (Including Author, Title, Date, Pertinent Pages, etc.)

	248	Matsumura et al., "Hydrophobic stabilization in T4 lysozyme determined directly by multiple substitutions of Ile 3" Nature 334:406-410 (1988)
	249	Morrison, S. L., "Transfectomas Provide Novel Chimeric Antibodies" Science 229:1202-1207 (September 20, 1985)
	250	Nakatani et al., "Humanization of mouse anti-human IL-2 receptor antibody 8-B10" Protein Engineering 7:435-443 (1994)
	251	Ohtomo et al., "Humanization of Mouse ONS-M21 Antibody with the Aid of Hybrid Variable Regions" Molecular Immunology 32:407-416 (1995)
	252	Padlan et al., "Model-Building Studies of Antigen-Binding Sites: The Hapten-Binding Site of MOPC-315" C.S. Harbor Symp. Quant. Biol. 41:627-637 (1977)
	253	Rodrigues et al., "Engineering a humanized bispecific F(ab')$_2$ fragment for improved binding to T cells" Int. J. Cancer (Suppl.) 7:45-50 (1992)
	254	Sha et al., "A Heavy-Chain Grafted Antibody that Recognizes the Tumor-Associated TAG72 Antigen" Cancer Biotherapy 9:341-349 (1994)
	255	Tempest et al., "Identification of framework residues required to restore antigen binding during reshaping of a monoclonal antibody against the glycoprotein gB of human cytomegalovirus" Int. J. Biol. Macromol. 17:37-42 (1995)
	256	Tramontano, "Structural Determinants of the Conformations of Medium-Sized Loops in Proteins" Proteins 6:382-394 (1989)
	257	Uchiyama et al., "A Monoclonal Antibody (ANTI-Tac) Reactive with Activated and Functionally Mature Human T Cells" Journal of Immunology 126:1393-1397 (1981)
	258	Vincenti et al., "Interleukin-2-Receptor Blockade with Daclizumab to Prevent Acute Rejection in Renal Transplantation" New Engl. J. Med. 338:161-165 (1998)
	259	Vitetta et al., "Redesigning Nature's Poisons to Create Anti-Tumor Reagents" Science 238:1098-1104 (1987)
	260	Waldmann et al., "Interleukin 2 Receptor (Tac Antigen) Expression in HTLV-1-associated Adult T-Cell Leukemia" Cancer Research 45:4559s-4562s (1985)
	261	Waldmann, Thomas A., "The Structure, Function, and Expression of Interleukin-2 Receptors on Normal and Malignant Lymphocytes" Science 232:727-732 (1986)
	262	Wu et al., "An Analysis of the Sequences of the Variable Regions of Bence Jones Proteins and Myeloma Light Chains and Their Implications for Antibody Complementarity" Journal of Experimental Medicine 132:211-250 (1970)

Examiner	Date Considered

*Examiner: Initial if reference considered, whether or not citation is in conformance with MPEP 609; draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

USCOMM-DC 80-398.

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231
www.uspto.gov

NOTICE OF ALLOWANCE AND FEE(S) DUE

7590 12/18/2001

GENENTECH, INC.
1 DNA WAY
SOUTH SAN FRANCISCO, CA 940804990

EXAMINER	
DAVIS, MINH TAM B	
ART UNIT	CLASS-SUBCLASS
1642	530-387300

DATE MAILED: 12/18/2001

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
08/146,206	11/17/1993	PAUL J. CARTER	709P1	3992

TITLE OF INVENTION: METHOD FOR MAKING HUMANIZED ANTIBODIES

TOTAL CLAIMS	APPLN. TYPE	SMALL ENTITY	ISSUE FEE	PUBLICATION FEE	TOTAL FEE(S) DUE	DATE DUE
82	nonprovisional	NO	$1280	$0	$1280	03/18/2002

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT.

PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151.

HOW TO REPLY TO THIS NOTICE:

I. Review the SMALL ENTITY status shown above. If the SMALL ENTITY is shown as YES, verify your current SMALL ENTITY status:

A. If the status is changed, pay the PUBLICATION FEE (if required) and twice the amount of the ISSUE FEE shown above and notify the United States Patent and Trademark Office of the change in status, or

B. If the status is the same, pay the TOTAL FEE(S) DUE shown above.

If the SMALL ENTITY is shown as NO:

A. Pay TOTAL FEE(S) DUE shown above, or

B. If applicant claimed SMALL ENTITY status before, or is now claiming SMALL ENTITY status, check the box below and enclose the PUBLICATION FEE and 1/2 the ISSUE FEE shown above.

☐ Applicant claims SMALL ENTITY status. See 37 CFR 1.27.

II. PART B - FEE(S) TRANSMITTAL should be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). Even if the fee(s) have already been paid, Part B - Fee(s) Transmittal should be completed and returned. If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Box ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

PTOL-85 (REV. 07-01) Approved for use through 01/31/2004.

PART B - FEE(S) TRANSMITTAL

Complete and mail this form, together with applicable fee(s), to:

Box ISSUE FEE
Assistant Commissioner for Patents
Washington, D.C. 20231

MAILING INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 4 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Legibly mark-up with any corrections or use Block 1)

7590 12/18/2001

GENENTECH, INC.
1 DNA WAY
SOUTH SAN FRANCISCO, CA 940804990

Note: The certificate of mailing below can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing.

Certificate of Mailing

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Box Issue Fee address above on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
08/146,206	11/17/1993	PAUL J. CARTER	709P1	3992

TITLE OF INVENTION: METHOD FOR MAKING HUMANIZED ANTIBODIES

TOTAL CLAIMS	APPLN. TYPE	SMALL ENTITY	ISSUE FEE	PUBLICATION FEE	TOTAL FEE(S) DUE	DATE DUE
82	nonprovisional	NO	$1280	$0	$1280	03/18/2002

EXAMINER	ART UNIT	CLASS-SUBCLASS
DAVIS, MINH TAM B	1642	530-387300

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363). Use of PTO form(s) and Customer Number are recommended, but not required.

❑ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

❑ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47) attached.

2. For printing on the patent front page, list (1) the names of up to 3 registered patent attorneys or agents OR, alternatively, (2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____

2 _____

3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. Inclusion of assignee data is only appropriate when an assignment has been previously submitted to the USPTO or is being submitted under separate cover. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE (B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) ❑ individual ❑ corporation or other private group entity ❑ government

4a. The following fee(s) are enclosed:

❑ Issue Fee

❑ Publication Fee

❑ Advance Order - # of Copies _____

4b. Payment of Fee(s):

❑ A check in the amount of the fee(s) is enclosed.

❑ Payment by credit card. Form PTO-2038 is attached.

❑ The Commissioner is hereby authorized by charge the required fee(s), or credit any overpayment, to Deposit Account Number _____(enclose an extra copy of this form).

The COMMISSIONER OF PATENTS AND TRADEMARKS is requested to apply the Issue Fee and Publication Fee (if any) to the application identified above.

(Authorized Signature) (Date)

NOTE; The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

Burden Hour Statement: This form is estimated to take 0.2 hours to complete. Time will vary depending on the needs of the individual case. Any comments on the amount of time required to complete this form should be sent to the Chief Information Officer, United States Patent and Trademark Office, Washington, D.C. 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND FEES AND THIS FORM TO: Box Issue Fee, Assistant Commissioner for Patents, Washington, D.C. 20231

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

TRANSMIT THIS FORM WITH FEE(S)

PTOL-85 (REV. 07-01) Approved for use through 01/31/2004. OMB 0651-0033 U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
08/146,206	11/17/1993	PAUL J. CARTER	709P1	3992

7590 12/18/2001

GENENTECH, INC.
1 DNA WAY
SOUTH SAN FRANCISCO, CA940804990

EXAMINER	
DAVIS, MINH TAM B	
ART UNIT	**PAPER NUMBER**
1642	

DATE MAILED: 12/18/2001

Determination of Patent Term Extension or Adjustment under 35 U.S.C. 154 (b)
(application filed prior to June 8, 1995)

This patent application was filed prior to June 8, 1995, thus no Patent Term Extension or Adjustment applies.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) system. (http://pair.uspto.gov)

PTOL-85 (REV. 07-01) Approved for use through 01/31/2004.

Notice of Allowability

Application No.	Applicant(s)
08/146,206	Carter et al
Examiner	**Art Unit**
MINH TAM DAVIS	1642

--The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance and Issue Fee Due or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *interview on 12/11/01*.

2. ☒ The allowed claim(s) is/are *43-105, 113-128, renumbered as 1-82*.

3. ☐ The drawings filed on _____ are acceptable as formal drawings.

4. ☐ Acknowledgement is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d).

   a) ☐ All b) ☐ Some* c) ☐ None of the:

   1. ☐ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____.
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   *Certified copies not received: _____.

5. ☐ Acknowledgement is made of a claim for domestic priority under 35 U.S.C. § 119(e).

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application. **THIS THREE-MONTH PERIOD IS NOT EXTENDABLE FOR SUBMITTING NEW FORMAL DRAWINGS, OR A SUBSTITUTE OATH OR DECLARATION.** ~~**This three-month period for complying with the REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL is extendable under 37 CFR 1.136(a).**~~

6. ☐ Note the attached EXAMINER'S AMENDMENT or NOTICE OF INFORMAL APPLICATION (PTO-152) which gives reason(s) why the oath or declaration is deficient. A SUBSTITUTE OATH OR DECLARATION IS REQUIRED.

7. ☒ Applicant MUST submit NEW FORMAL DRAWINGS

   (a) ☒ including changes required by the Notice of Draftsperson's Patent Drawing Review (PTO-948) attached

   1) ☐ hereto or 2) ☒ to Paper No. *12*.

   (b) ☐ including changes required by the proposed drawing correction filed _____, which has been approved by the examiner.

   (c) ☐ including changes required by the attached Examiner's Amendment/Comment or in the Office action of Paper No. _____.

   Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings. The drawings should be filed as a separate paper with a transmittal letter addressed to the Official Draftsperson.

8. ☐ Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Any reply to this letter should include, in the upper right hand corner, the APPLICATION NUMBER (SERIES CODE/SERIAL NUMBER). If applicant has received a Notice of Allowance and Issue Fee Due, the ISSUE BATCH NUMBER and DATE of the NOTICE OF ALLOWANCE should also be included.

Attachment(s)

1 ☐ Notice of References Cited (PTO-892)
2 ☐ Notice of Informal Patent Application (PTO-152)
3 ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
4 ☐ Interview Summary (PTO-413), Paper No. _____.
5 ☒ Information Disclosure Statement(s) (PTO-1449), Paper No(s). *10 sheets*
6 ☐ Examiner's Amendment/Comment
7 ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
8 ☐ Examiner's Statement of Reasons for Allowance
9 ☐ Other

EXAMINER'S AMENDMENT

An examiner's amendment to the record appears below. Should the changes and/or additions be unacceptable to applicant, an amendment may be filed as provided by 37 CFR 1.312. To ensure consideration of such an amendment, it MUST be submitted no later than the payment of the issue fee.

Authorization for this examiner's amendment was given in a telephone interview with Wendy Lee on 12/13/01.

The application has been amended as follows:

In the claims:

Claim 114. Delete "about", and replace it with --- up to ---

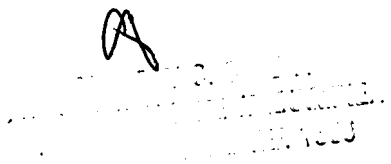
Delete "tightly", and replace it with --- in the binding affinity ---

Any inquiry concerning this communication or earlier communications from the examiner should be directed to MINH-TAM DAVIS whose telephone number is 703-305-2008. The examiner can normally be reached on 9:30AM-4:00PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, ANTHONY CAPUTA can be reached on 703-308-3995. The fax phone numbers for the organization where this application or proceeding is assigned are 703-308-4426 for regular communications and 703-308-4426 for After Final communications.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist whose telephone number is 703-308-0916.

MINH TAM DAVIS
December 14, 2001



Attachment #64

FORM PTO-1449

U.S. Dept. of Commerce
Patent and Trademark Office

LIST OF DISCLOSURES CITED BY APPLICANT

(Use several sheets if necessary)

Atty Docket No.	Serial No.
P0709P1	08/146,206
Applicant: Carter et al.	
Filing Date: 17 Nov 1993	Group: 1642

U.S. PATENT DOCUMENTS

Examiner Initials		Document Number	Date	Name	Class	Subclass	Filing Date
MTS	264	5,834,598	10/11/98	Lowman et al.	530	399	

OTHER DISCLOSURES (Including Author, Title, Date, Pertinent Pages, etc.)

MTS	265	Rhodes, P., "Recombinant antibodies from CHO cells" ABSTR PAP AM CHEM SOC (Abstract No. 60 from the 199th American Chemical Society National Meeting held in Boston, MA April 22-27, 1990) 199(1-2):BIOT 60 (Apr 1990)

Examiner: M. T. DAVIS

Date Considered: 12/05/01

*Examiner: Initial if reference considered, whether or not citation is in conformance with MPEP 609; draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

USCOMM-DC 80-398.

Attachment to Paper #47

Sheet 1 of 1

FORM PTO-1449 U.S. Dept. of Commerce Patent and Trademark Office

LIST OF DISCLOSURES CITED BY APPLICANT

(Use several sheets if necessary)

Atty Docket No.	Serial No.
P0709P1	08/146,206
Applicant: Carter et al.	
Filing Date: 17 Nov 1993	Group: 1642

U.S. PATENT DOCUMENTS

Examiner Initials		Document Number	Date	Name	Class	Subclass	Filing Date
MJR	263	5,859,205	12.01.99	Adair et al.	530	387.3	07.09.94

Examiner: Julie Reeves, PhD Date Considered: 3/25/99

*Examiner: Initial if reference considered, whether or not citation is in conformance with MPEP 609; draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

M. T. Davis 12/05/01

USCOMM-DC 80-398.

#46 Attach

FORM PTO-1449 U.S. Dept. of Commerce Patent and Trademark Office

LIST OF DISCLOSURES CITED BY APPLICANT

(Use several sheets if necessary)

Atty Docket No.	Serial No.
P0709P1	08/146,206
Applicant	
Carter et al.	
Filing Date	**Group**
17 Nov 1993	1806

U.S. PATENT DOCUMENTS

Examiner Initials		Document Number	Date	Name	Class	Subclass	Filing Date
TDS	225	5,714,350	03.02.98	Co et al.	435	69.6	13.01.95
	226	5,821,337	13.10.98	Carter et al.	530	387.3	

FOREIGN PATENT DOCUMENTS

Examiner Initials		Document Number	Date	Country	Class	Subclass	Translation Yes	Translation No
TDS	227	0 460 167 B1	11.12.91	EPO				
	228	0 519 596 A1	23.12.92	EPO				
	229	0 592 106 A1	13.04.94	EPO				
	230	120,694	03.10.84	EPO				
	231	125,023 A1	14.11.84	EPO				
	232	368,684	16.05.90	EPO				
	233	94/11509	26.05.94	PCT				
	234	WO 89/09622	10/1989	PCT				
	235	WO 92/11383	09.07.92	PCT				
	236	2 188941	14.10.87	UNITED KINGDOM				

OTHER DISCLOSURES (Including Author, Title, Date, Pertinent Pages, etc.)

TDS	237	"Biosym Technologies" in New Products, Chemical Design Automation 3" (December 1988)
	238	"Polygen Corporation" in New Products, Chemical Design Automation 3" (November 1988)
	239	Adair et al., "Humanization of the murine anti-human CD3 monoclonal antibody OKT3" Hum. Antibod. Hybridomas 5:41-47 (1994)
	240	Chothia et al., "Principles of protein-protein recognition" Nature 256:705-708 (1975)
	241	Chothia et al., "Transmission of conformational change in insulin" Nature 302:500-505 (1983)
	242	Corti et al., "Idiotope Determining Regions of a Mouse Monoclonal Antibody and Its Humanized Versions" J. Mol. Biol. 235:53-60 (1994)
	243	Couto et al., "Anti-BA46 Monoclonal Antibody Mc3 Humanization Using a Novel Positional Consensus and in Vivo and in Vitro Characterization" Cancer Research Supplement 55:1717-1722 (1995)
	244	Couto et al., "Humanization of KC4G3, an Anti-Human Carcinoma Antibody" Hybridoma 13:215-219 (1994)
	245	Ellis et al., "Engineered Anti-CD38 Monoclonal Antibodies for Immunotherapy of Multiple Myeloma" The Journal of Immunology pps. 925-937 (1995)
	246	Hieter et al., "Evolution of Human Immunoglobulin K J Region Genes" The Journal of Biological Chemistry 257:1516-1522 (1982)
	247	Lesk, Arthur M., "How Different Amino Acid Sequences Determine Similar Protein Structures: The Structure and Evolutionary Dynamics of the Globins" J. Mol. Biol. 136:225-270 (1980)

Examiner	Date Considered
M. T. DAVIS	12/05/01

*Examiner: Initial if reference considered, whether or not citation is in conformance with MPEP 609; draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

FORM PTO-1449 | U.S. Dept. of Commerce, Patent and Trademark Office

Atty Docket No.	Serial No.
P0709P1	08/146,206
Applicant: Carter et al.	
Filing Date: 17 Nov 1993	Group: 1806

LIST OF DISCLOSURES CITED BY APPLICANT

(Use several sheets if necessary)

OTHER DISCLOSURES (Including Author, Title, Date, Pertinent Pages, etc.)

	248	Matsumura et al., "Hydrophobic stabilization in T4 lysozyme determined directly by multiple substitutions of Ile 3" Nature 334:406-410 (1988)
	249	Morrison, S. L., "Transfectomas Provide Novel Chimeric Antibodies" Science 229:1202-1207 (September 20, 1985)
	250	Nakatani et al., "Humanization of mouse anti-human IL-2 receptor antibody B-B10" Protein Engineering 7:435-443 (1994)
	251	Ohtomo et al., "Humanization of Mouse ONS-M21 Antibody with the Aid of Hybrid Variable Regions" Molecular Immunology 32:407-416 (1995)
	252	~~Padlan et al., "Model-Building Studies of Antigen-Binding Sites: The Hapten-Binding Site of MOPC-315" C.S. Harbor Symp. Quant. Biol. 41:627-637 (1977)~~ Dupl.
	253	Rodrigues et al., "Engineering a humanized bispecific F(ab')$_2$ fragment for improved binding to T cells" Int. J. Cancer (Suppl.) 7:45-50 (1992)
	254	Sha et al., "A Heavy-Chain Grafted Antibody that Recognizes the Tumor-Associated TAG72 Antigen" Cancer Biotherapy 9:341-349 (1994)
	255	Tempest et al., "Identification of framework residues required to restore antigen binding during reshaping of a monoclonal antibody against the glycoprotein gB of human cytomegalovirus" Int. J. Biol. Macromol. 17:37-42 (1995)
	256	Tramontano, "Structural Determinants of the Conformations of Medium-Sized Loops in Proteins" Proteins 6:382-394 (1989)
	257	Uchiyama et al., "A Monoclonal Antibody (ANTI-Tac) Reactive with Activated and Functionally Mature Human T Cells" Journal of Immunology 126:1393-1397 (1981)
	258	Vincenti et al., "Interleukin-2-Receptor Blockade with Daclizumab to Prevent Acute Rejection in Renal Transplantation" New Engl. J. Med. 338:161-165 (1998)
	259	Vitetta et al., "Redesigning Nature's Poisons to Create Anti-Tumor Reagents" Science 238:1098-1104 (1987)
	260	Waldmann et al., "Interleukin 2 Receptor (Tac Antigen) Expression in HTLV-1-associated Adult T-Cell Leukemia" Cancer Research 45:4559s-4562s (1985)
	261	Waldmann, Thomas A., "The Structure, Function, and Expression of Interleukin-2 Receptors on Normal and Malignant Lymphocytes" Science 232:727-732 (1986)
✓	262	Wu et al., "An Analysis of the Sequences of the Variable Regions of Bence Jones Proteins and Myeloma Light Chains and Their Implications for Antibody Complementarity" Journal of Experimental Medicine 132:211-250 (1970)

Examiner: M.T. Davis | Date Considered: 12/06/01

*Examiner: Initial if reference considered, whether or not citation is in conformance with MPEP 609; draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

USCOMM-DC 80-398.

copy. Attachment # 15 (17)

Sheet 1 of 1

FORM PTO-1449 U.S. Dept. of Commerce Patent and Trademark Office

Atty Docket No.	Serial No.
P0709P1	08/146,206
Applicant: Carter et al.	
Filing Date: 17 Nov 1993	Group: 1806

LIST OF DISCLOSURES CITED BY APPLICANT

(Use several sheets if necessary)

FOREIGN PATENT DOCUMENTS

Examiner Initials		Document Number	Date	Country	Class	Subclass	Translation Yes	Translation No
TDB	79	WO 92/04381	19.03.92	PCT				
	80	WO 92/05274	02.04.92	PCT				
	81	WO 92/15683	17.09.92	PCT				

Examiner M. T. DAVIS Date Considered 12/05/01

*Examiner: Initial if reference considered, whether or not citation is in conformance with MPEP 609; draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

USCOMM-DC 80-398.

copy

FORM PTO-1449 — U.S. Dept. of Commerce, Patent and Trademark Office

Atty Docket No.	Serial No.
P0709P1	08/146,206
Applicant: Carter et al.	
Filing Date: 17 Nov 1993	Group: 1806

LIST OF DISCLOSURES CITED BY APPLICANT

(Use several sheets if necessary)

U.S. PATENT DOCUMENTS

Examiner Initials		Document Number	Date	Name	Class	Subclass	Filing Date
PN	81	5,225,539 7-6-93	~~06.07.93~~	Winter, G.	530/387.3	~~C07K~~	~~25.10.91~~ 10/25/91
PN	82	5,530,101 6-25-96	~~25.06.96~~	Queen et al.	530/387.3	~~A61K~~ 39/395	~~19.12.90~~, 2/19/90

FOREIGN PATENT DOCUMENTS

Examiner Initials		Document Number	Date	Country	Class	Subclass	Translation Yes	Translation No
PN	83	85058/91 3-30-92	~~30.03.92~~	AUSTRALIA	C07K	15/12		
	84	328,404 A1 8-16-89	~~16.08.89~~	EPO	A61K	39/395		
	85	451,216 B1 1-24-96	~~24.01.96~~	EPO	C12P	21/08		
	86	WO 91/09966 7-11-91	~~11.07.91~~	PCT	C12P	21/08		
	87	WO 91/09968 7-11-91	~~11.07.91~~	PCT	C12P	21/08		
PN	88	WO 92/11018 9-7-92	~~07.09.92~~	PCT	A61K	35/14		

OTHER DISCLOSURES (Including Author, Title, Date, Pertinent Pages, etc.)

PN	89	Carter et al., "High level escherichia coli expression and production of a bivalent humanized antibody fragment" Bio/Technology 10:163-167 (1992)
	90	Foote et al., "Antibody Framework Residues Affecting the Conformation of the Hypervariable Loops" J. Mol. Biol. 224:487-499 (1992)
	91	Foote, J., "Humanized Antibodies" Nova acta Leopoldina 61(269):103-110 (1989)
	92	Kabat et al., "Sequences of Proteins of Immunological Interest", Bethesda, MD:National Institute of Health pps. 14-32 (1983)
	93	Kettleborough et al., "Humanization of a Mouse Monoclonal Antibody by CDR-grafting: the Importance of Framework Residues on Loop Conformation" Protein Engineering 4(7):773-783 (1991)
	94	Maeda et al., "Construction of Reshaped Human Antibodies with HIV-neutralizing Activity" Hum. Antibod. Hybridomas 2:124-134 (July 1991)
	95	Riechmann et al, "Expression of an Antibody Fv Fragment in Myeloma Cells" J. Mol. Biol. 203:825-828 (1988)
	96	Routledge et al., "A Humanized Monovalent CD3 Antibody which Can Activate Homologous Complement" European Journal of Immunology 21:2717-2725 (1991)
	97	Shearman et al., "Construction, Expression and Characterization of Humanized Antibodies Directed Against the Human α/β T Cell Receptor" J. Immunol. 147(12):4366-4373 (December 15, 1991)
PN	98	Tempest et al., "Reshaping a Human Monoclonal Antibody to Inhibit Human Respiratory Syncytial Virus Infection In Vivo" Bio/Technology 9:266-271 (March 1991)

Examiner: Patrick J. Nolan — Date Considered: 12-16-96

*Examiner: Initial if reference considered, whether or not citation is in conformance with MPEP 609; draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

M.T. Davis 12/05/01

USCOMM-DC 80-398.

#40 Attach

FORM PTO-1449

U.S. Dept. of Commerce
Patent and Trademark Office

Attorney Docket No.	Serial No.
P0709P1	08/146,206
Applicant: Carter et al.	
Filing Date: 17 Nov 1993	Group: 1806

LIST OF DISCLOSURES CITED BY APPLICANT

(Use several sheets if necessary)

U.S. PATENT DOCUMENTS

Examiner Initials		Document Number	Date	Name	Class	Subclass	Filing Date
	*215	~~07/934,373~~		~~Carter et al.~~			~~21.08.92~~
	*216	~~08/437,642~~		~~Carter et al.~~			~~09.05.95~~
	*217	~~09/115,800~~		~~Carter et al.~~			~~15.07.98~~
MT D	218	5,677,171	14.10.97	Hudziak et al.	435	7.23	05.08.94
MT D	219	5,772,997	30.06.98	Hudziak et al.	424	130.1	23.05.95

OTHER DISCLOSURES (Including Author, Title, Date, Pertinent Pages, etc.)

MT D	220	Brown, Jr. et al., "Anti-Tac-H, a humanized antibody to the interleukin 2 receptor, prolongs primate cardiac allograft survival" Proc. Natl. Acad. Sci. USA 88:2663-2667 (1991)
	221	Casale et al., "Use of an anti-IgE humanized monoclonal antibody in ragweed-induced allergic rhinitis" J. Allergy Clin. Immunol. 100:110-121 (1997)
	222	Fahy et al., "The Effect of an Anti-IgE Monoclonal Antibody on the Early- and Late-Phase Responses to Allergen Inhalation in Asthmatic Subjects" Am J. Respir. Crit. Care Med 155:1828-1834 (1997)
	223	Mathieson et al., "Monoclonal-Antibody Therapy in Systemic Vasculitis" New England J. of Medicine pps. 250-254 (July 1990)
	224	Presta et al., "Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders" Cancer Research 57(20):4593-4599 (Oct 15, 1997)

Examiner	Date Considered
M.T. DAVIS	12/05/01

*Examiner: Initial if reference considered, whether or not citation is in conformance with MPEP 609; draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

USCOMM-DC 80-398.

Attachment

FORM PTO-1449 U.S. Dept. of Commerce Patent and Trademark Office

LIST OF DISCLOSURES CITED BY APPLICANT

(Use several sheets if necessary)

Atty Docket No.	Serial No.
P0709P1	08/146,206
Applicant: Carter et al.	
Filing Date: 17 Nov 1993	Group: ~~1806~~ 1816

FOREIGN PATENT DOCUMENTS

Examiner Initials		Document Number	Date	Country	Class	Subclass	Translation Yes	Translation No
TMS PN	208	403,156 A1	19.12.90	EPO				
	209	438,310 A2	24.07.91	EPO				
	210	438,312 A2	24.07.91	EPO				
	211	440,351 A2	07.08.91	EPO				
	212	WO 91/07492	30.05.91	PCT				
PN	213	WO 92/16562*	01.10.92	PCT				
	214	WO 94/12214	09.06.94	PCT				

Examiner: Patricia J. NOLL Date Considered: 12/22/97

*Examiner: Initial if reference considered, whether or not citation is in conformance with MPEP 609; draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

M. T. DAVIS 12/05/01

USCOMM-DC 80-398.

03-19-02

95

Patent Docket P0709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Paul J. Carter et al. Serial No.: 08/146,206 Filed: November 17, 1993 For: METHOD FOR MAKING HUMANIZED ANTIBODIES	Group Art Unit: 1642 Examiner: Minh-Tam Davis **Date of Mailing of PTOL 85 entitled "Notice of Allowance and Issue Fee Due" December 18, 2001**

#69M

CERTIFICATE OF MAILING
I hereby certify that this correspondence 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 below and is addressed to: U.S. Patent and Trademark Office, Washington, D.C. 20231-9999

Express Mail Label No. EL 889 330 529 US

March 18, 2002

Wendy M. Lee

TRANSMITTAL OF NEW DRAWINGS TO CORRECT INFORMALITIES WITHIN THREE MONTH PERIOD OF RESPONSE SET IN NOTICE OF ALLOWABILITY (PTOL 37)

BOX ISSUE FEE
Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

1. To correct the informalities in the drawings as noted in the Draftsman's objections on PTO-948 applicant submits herewith new drawings for this application. Number of sheets of drawings submitted: 9.

2. The three month period of response set in the Notice of Allowability (PTOL 37) expires on March 18, 2002 and this submission is on or before this expiry date.

Respectfully submitted,
GENENTECH, INC.

Date: March 18, 2002 By: _____
Wendy M. Lee
Reg. No. 40,378
Telephone No. (650) 225-1994

09157
PATENT TRADEMARK OFFICE

FIG. 1A

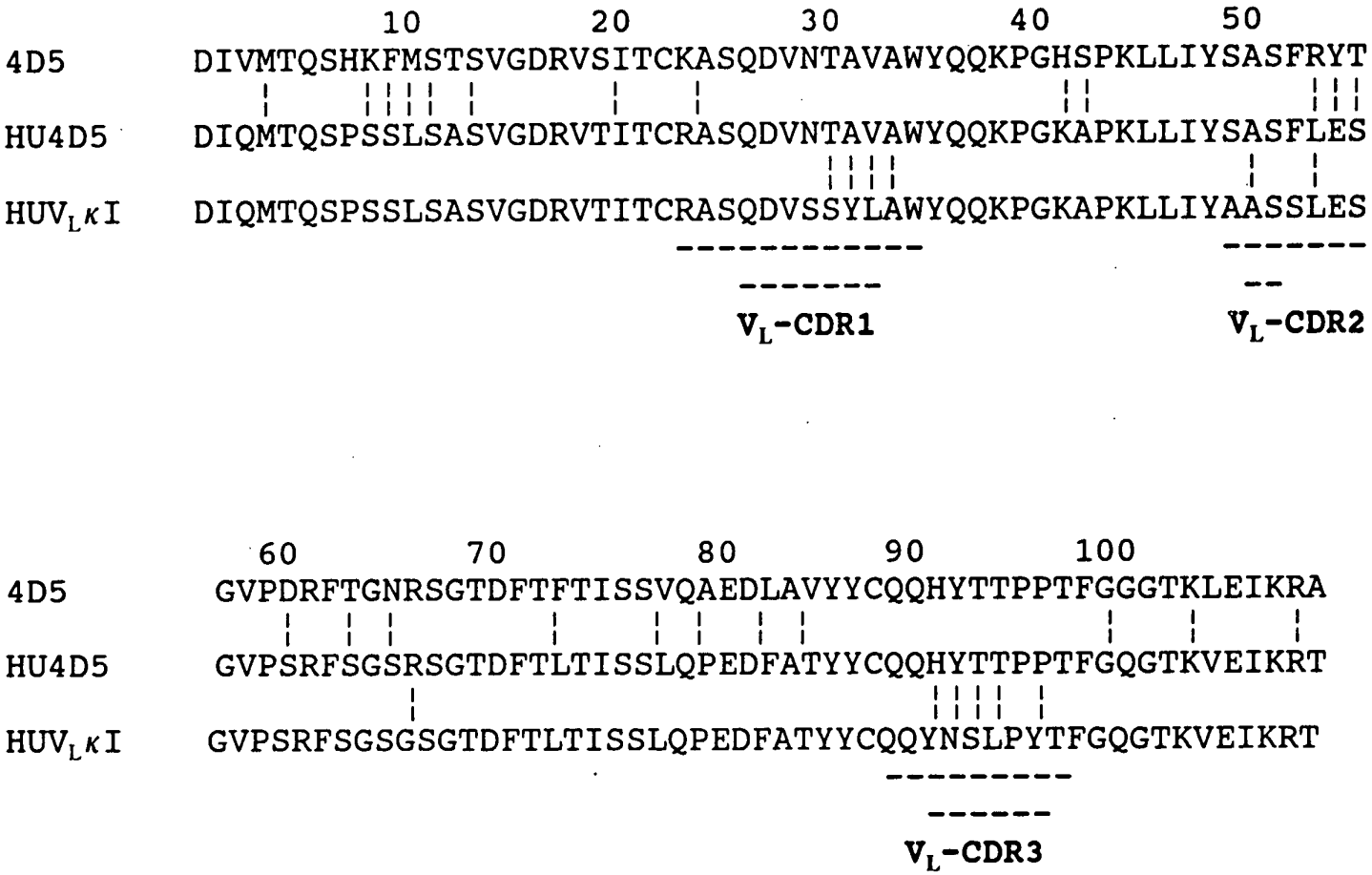
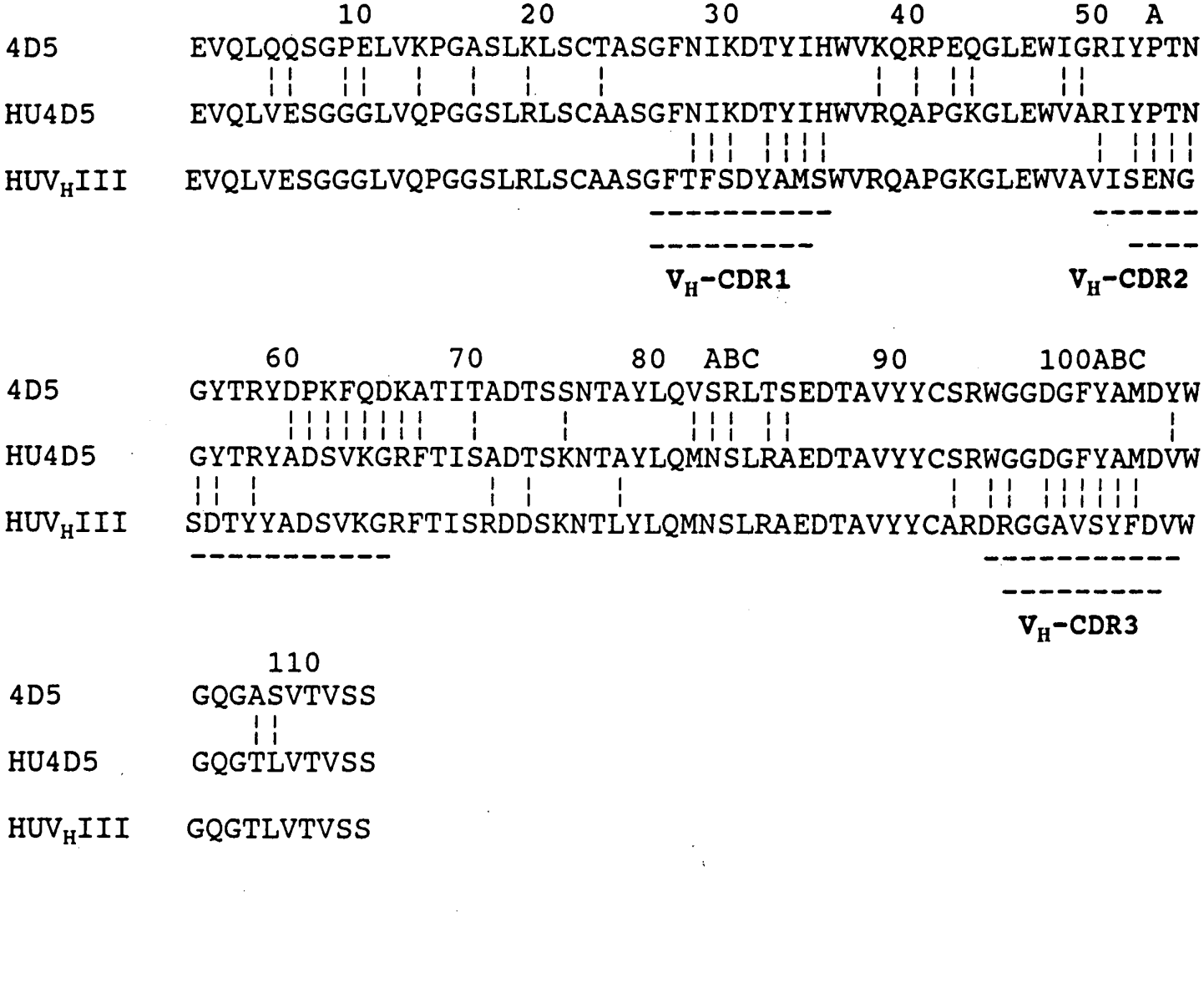

10 20 30 40 50
4D5 DIVMTQSHKFMSTSVGDRVSITCKASQDVNTAVAWYQQKPGHSPKLLIYSASFRYT
HU4D5 DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLES
HUV$_L$κI DIQMTQSPSSLSASVGDRVTITCRASQDVSSYLAWYQQKPGKAPKLLIYAASSLES
V$_L$-CDR1
V$_L$-CDR2
60 70 80 90 100
4D5 GVPDRFTGNRSGTDFTFTISSVQAEDLAVYYCQQHYTTPPTFGGGTKLEIKRA
HU4D5 GVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRT
HUV$_L$κI GVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYNSLPYTFGQGTKVEIKRT
V$_L$-CDR3

FIG. 1B



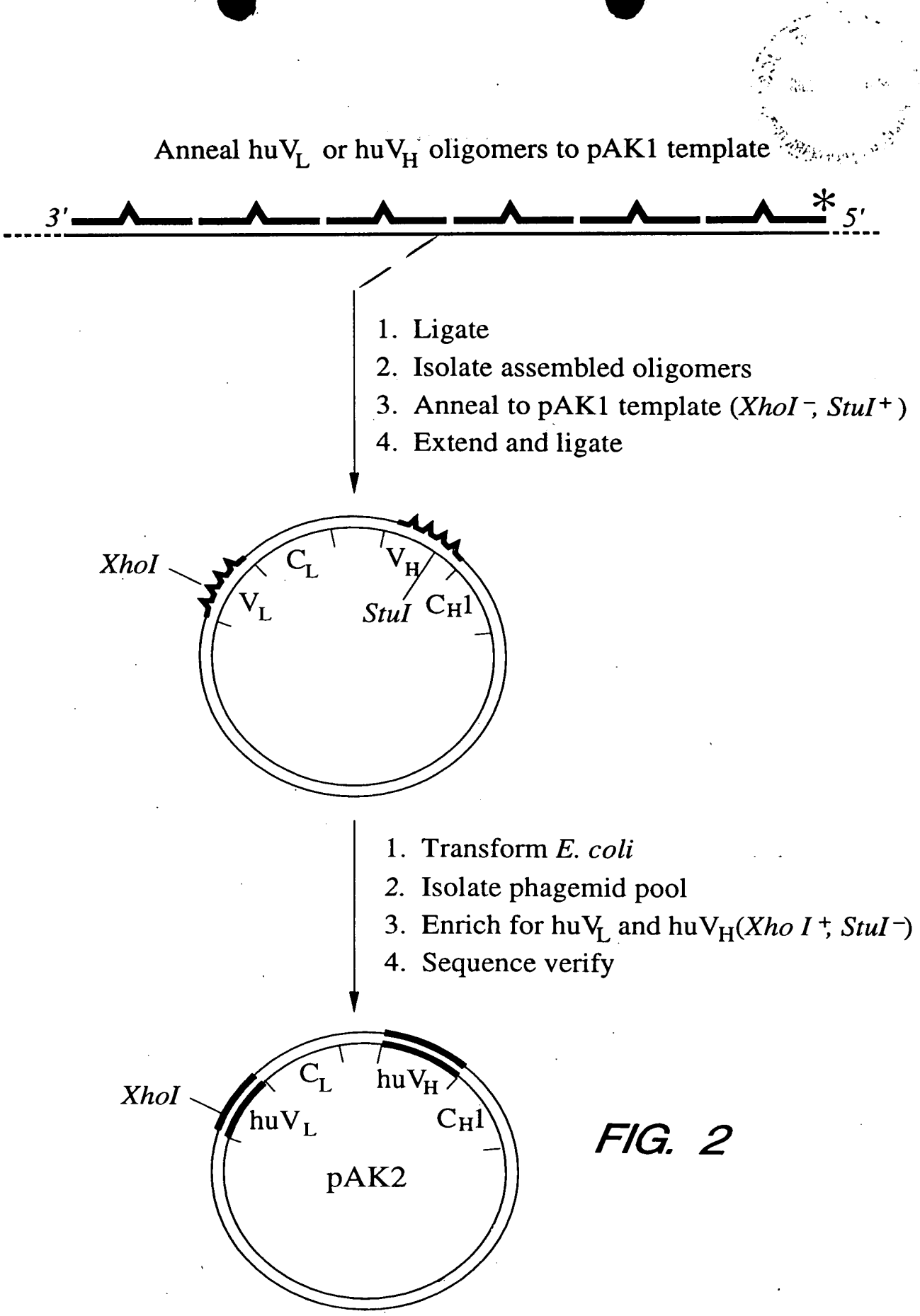

Anneal huV$_L$ or huV$_H$ oligomers to pAK1 template
3'
5'
*
1. Ligate
2. Isolate assembled oligomers
3. Anneal to pAK1 template (*XhoI* $^-$, *StuI* $^+$)
4. Extend and ligate
XhoI
C$_L$
V$_H$
V$_L$
StuI
C$_H$1
1. Transform *E. coli*
2. Isolate phagemid pool
3. Enrich for huV$_L$ and huV$_H$(*Xho I* $^+$, *StuI* $^-$)
4. Sequence verify
XhoI
C$_L$
huV$_H$
huV$_L$
C$_H$1
pAK2

FIG. 2

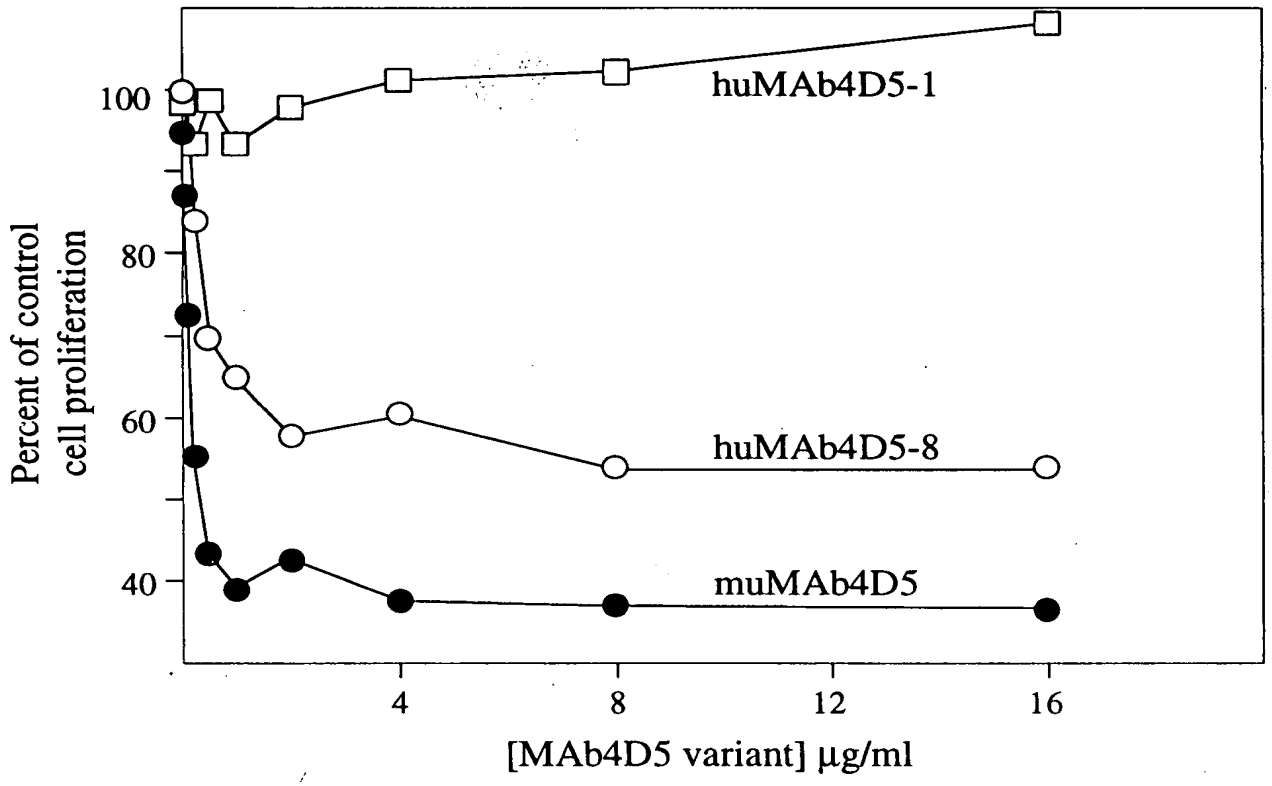


FIG. 3

FIG. 4

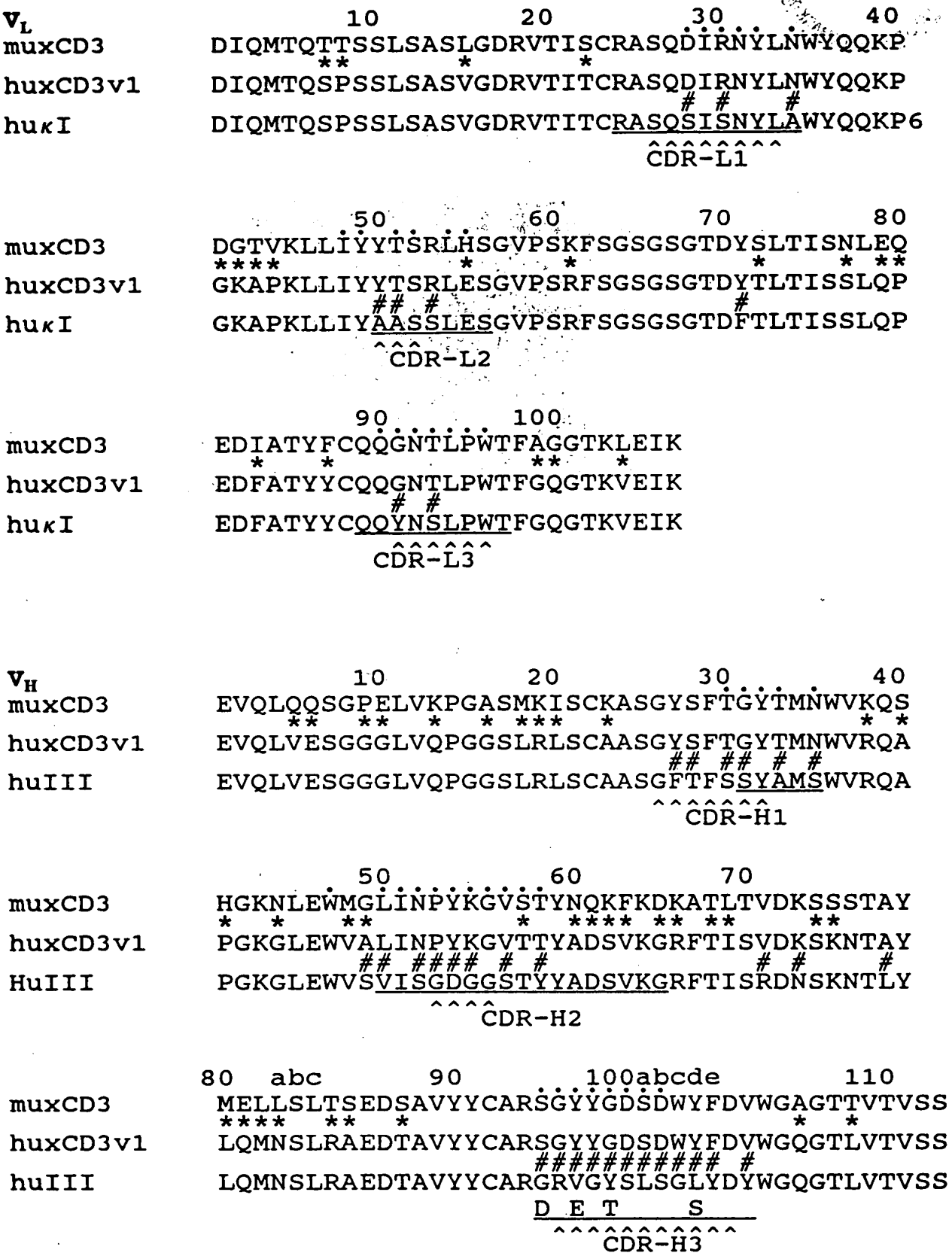

V_L
muxCD3 DIQMTQTTSSLSASLGDRVTISCRASQDIRNYLNWYQQKP
huxCD3v1 DIQMTQSPSSLSASVGDRVTITCRASQDIRNYLNWYQQKP
huκI DIQMTQSPSSLSASVGDRVTITCRASQSISNYLAWYQQKP
CDR-L1
muxCD3 DGTVKLLIYYTSRLHSGVPSKFSGSGSGTDYSLTISNLEQ
huxCD3v1 GKAPKLLIYYTSRLESGVPSRFSGSGSGTDYTLTISSLQP
huκI GKAPKLLIYAASSLESGVPSRFSGSGSGTDFTLTISSLQP
CDR-L2
muxCD3 EDIATYFCQQGNTLPWTFAGGTKLEIK
huxCD3v1 EDFATYYCQQGNTLPWTFGQGTKVEIK
huκI EDFATYYCQQYNSLPWTFGQGTKVEIK
CDR-L3
V_H
muxCD3 EVQLQQSGPELVKPGASMKISCKASGYSFTGYTMNWVKQS
huxCD3v1 EVQLVESGGGLVQPGGSLRLSCAASGYSFTGYTMNWVRQA
huIII EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQA
CDR-H1
muxCD3 HGKNLEWMGLINPYKGVSTYNQKFKDKATLTVDKSSSTAY
huxCD3v1 PGKGLEWVALINPYKGVTTYADSVKGRFTISVDKSKNTAY
HuIII PGKGLEWVSVISGDGGSTYYADSVKGRFTISRDNSKNTLY
CDR-H2
muxCD3 MELLSLTSEDSAVYYCARSGYYGDSDWYFDVWGAGTTVTVSS
huxCD3v1 LQMNSLRAEDTAVYYCARSGYYGDSDWYFDVWGQGTLVTVSS
huIII LQMNSLRAEDTAVYYCARGRVGYSLSGLYDYWGQGTLVTVSS
D E T S
CDR-H3

FIG. 5

FIG. 6A-1

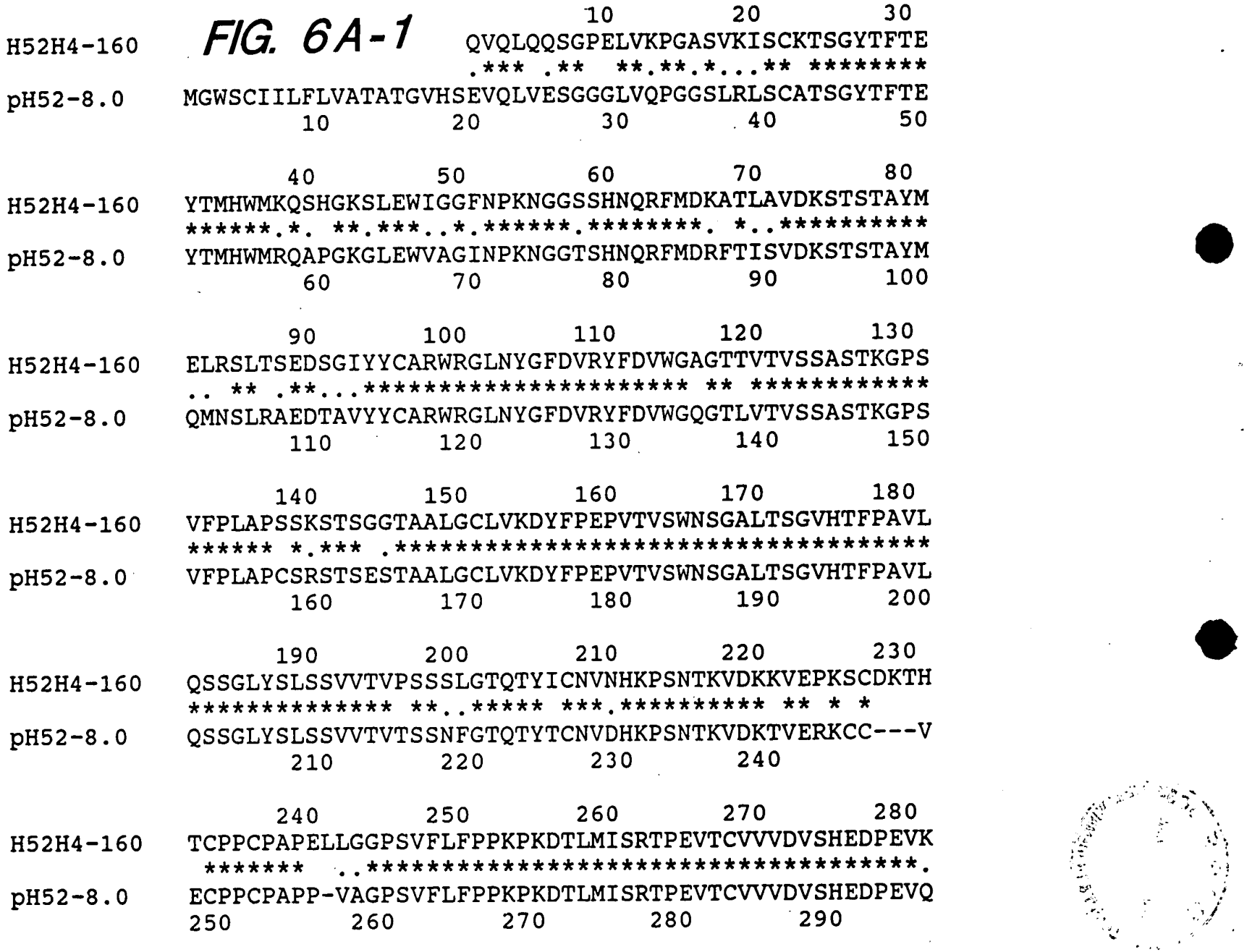


FIG. 6A-2

```
                290        300        310        320        330
H52H4-160       FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVS
                *******.*************.***.********.***************
pH52-8.0        FNWYVDGMEVHNAKTKPREEQFNSTFRVVSVLTVVHQDWLNGKEYKCKVS
                 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    MGWSCIILFLVATATGVHSDIQMTQSPSSLSASVGDRVTITCRASQDINN
                    10        20        30        40        50

                    40        50        60        70        80
H52L6-158   YLNWYQQKPNGTVKLLIYYTSTLHSGVPSRFSGSGSGTDYSLTISNLDQE
            *********  . ****************************.****.*. *
pH52-9.0    YLNWYQQKPGKAPKLLIYYTSTLHSGVPSRFSGSGSGTDYTLTISSLQPE
                    60        70        80        90        100

                   90       100       110       120       130
H52L6-158   DIATYFCQQGNTLPPTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTAS
            *.***.*********** ********************************
pH52-9.0    DFATYYCQQGNTLPPTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTAS
                    110       120       130       140       150

                  140       150       160       170       180
H52L6-158   VVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTL
            **************************************************
pH52-9.0    VVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTL
                    160       170       180       190       200

                  190       200       210
H52L6-158    SKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
             ********************************
pH52-9.0     SKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
                    210       220       230
```

03-19-02

PART B - FEE(S) TRANSMITTAL

Complete and mail this form together with applicable fee(s), to: **Box ISSUE FEE**
Assistant Commissioner for Patents
Washington, D.C. 20231

MAILING INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 4 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Legibly mark-up with any corrections or use Block 1)

7590 12/18/2001

GENENTECH, INC.
1 DNA WAY
SOUTH SAN FRANCISCO, CA 940804990

Note: The certificate of mailing below can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing.

Certificate of Mailing

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Box Issue Fee address above on the date indicated below. [illegible]

Wendy Maria Lee	(Typed or printed name)
[signature]	(Signature)
3/18/02	(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
08/146,206	11/17/1993	PAUL J. CARTER	7090.1	3992

TITLE OF INVENTION: METHOD FOR MAKING HUMANIZED ANTIBODIES

TOTAL CLAIMS	APPLN. TYPE	SMALL ENTITY	ISSUE FEE	PUBLICATION FEE	TOTAL FEE(S) DUE	DATE DUE
82	nonprovisional	NO	$1280	$0	$1280	03/18/2002

EXAMINER	ART UNIT	CLASS-SUBCLASS
DAVIS, MINH TAM B	1642	530-387300

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363). Use of PTO form(s) and Customer Number are recommended, but not required.

☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47) attached.

2. For printing on the patent front page, list (1) the names of up to 3 registered patent attorneys or agents OR, alternatively, (2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 Wendy M. Lee
2 _____
3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. Inclusion of assignee data is only appropriate when an assignment has been previously submitted to the USPTO or is being submitted under separate cover. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE: Genentech, Inc.

(B) RESIDENCE: (CITY and STATE OR COUNTRY): South San Francisco, California

Please check the appropriate assignee category or categories (will not be printed on the patent) ☐ individual ☒ corporation or other private group entity ☐ government

4a. The following fee(s) are enclosed:

☒ Issue Fee
☐ Publication Fee
☒ Advance Order - # of Copies 4

4b. Payment of Fee(s):

☐ A check in the amount of the fee(s) is enclosed.
☐ Payment by credit card. Form PTO-2038 is attached.
☒ The Commissioner is hereby authorized by charge the required fee(s), or credit any overpayment, to Deposit Account Number 07-0630 (enclose an extra copy of this form).

The COMMISSIONER OF PATENTS AND TRADEMARKS is requested to apply the Issue Fee and Publication Fee (if any) to the application identified above.

(Authorized Signature) [signature] (Date) 3/18/02

Wendy M. Lee - Reg. No. 40,378

NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

03/20/2002 SDENBER 00000076 070630 08146206
01 FC:142 1280.00 CH
02 FC:1463 12.00 CH

Burden Hour Statement: This form is estimated to take 0.2 hours to complete. Time will vary depending on the needs of the individual case. Any comments on the amount of time required to complete this form should be sent to the Chief Information Officer, United States Patent and Trademark Office, Washington, D.C. 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND FEES AND THIS FORM TO: Box Issue Fee, Assistant Commissioner for Patents, Washington, D.C. 20231

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

TRANSMIT THIS FORM WITH FEE(S)

C7C
#70
DGO
R
9-25-02

Patent Docket P0709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Paul J. Carter et al. U.S. Patent No.: 6,407,213 B1 Issued: June 18, 2002 For: METHOD FOR MAKING HUMANIZED ANTIBODIES	CERTIFICATE OF MAILING I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner of Patents, Washington, D.C. 20231 on August 12, 2002 Wendy M. Lee

REQUEST FOR CERTIFICATE OF CORRECTION UNDER 37 CFR 1.322

Certificate

AUG 27 2002

of Correction

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Enclosed is a Certificate of Correction for the above-referenced patent. Because the mistake occurred in the printing of the patent, it is not believed that any fee is required. However, if this is not the case, the Commissioner is hereby authorized to charge the required fee to Deposit Account No. 07-0630. Acceptance of this Certificate of Correction is respectfully requested.

Respectfully submitted,
GENENTECH, INC.

Date: August 12, 2002

By: _____
Wendy M. Lee
Reg. No. 40,378
Telephone No. (650) 225-1994

09157
PATENT TRADEMARK OFFICE

AUG 27 2002

UNITED ST[illegible] AND TRADEMA[illegible] OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : U.S. 6,407,213 B1

DATED : June 18, 2002

INVENTOR(S) : Carter et al.

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

In column 88, claim 65, line 63, please delete "63" and insert therefor --79--.

MAILING ADDRESS OF SENDER:

Wendy M. Lee

09157

PATENT TRADEMARK OFFICE

PATENT NO. U.S. 6,407,213

Form PTO1050(amended)

NOTICE RE: CERTIFICATES OF CORRECTION

DATE : 9-25-02

Paper No.: 71

TO : Supervisor, Art Unit 1642

SUBJECT : Certificate of Correction Request in Patent No.: 6,407,213

A response to the following question is requested with respect to the accompanying request for a certific of correction.

With respect to the change(s) requested, correcting Office and/or Applicant's errors, should the patent read as shown in the certificate of correction? No new matter should be introduced, nor should the scope or meaning of the claims be changed.

See red tags.

D. G. Olson

PLEASE COMPLETE THIS FORM AND
RETURN WITH FILE, WITHIN 7 DAYS,
TO CERTIFICATES OF CORRECTION BRANCH - PK 3-915/922
PALM LOCATION 7580 - TEL. NO. 305-8309

THANK YOU FOR YOUR ASSISTANCE!

Note your decision, regarding the changes requested in the Request for Certificate of Correction, placing a check mark (+) in the box that reflects your decision, which corresponds to the question checke above.

☒ YES ☐ NO ☐ Comments below

☐ Comments: Please enter the corrections Ex: Mioph -TAH DAVIS

ANTHONY C. CAPUTA
SUPERVISORY PATENT EXAMINER
TECHNOLOGY

Supervisor

AU 1642

Art Unit

PTOL-306 (REV. 2/02) U.S. DEPARTMENT OF COMMERCE Patent and Trademark O

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

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

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

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

DAC

#45

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent of: Paul J. Carter *et al.* -- § 156

Patent No.: 6,407,213

Issued: June 18, 2002

Application No: 08/146,206

For: METHOD FOR MAKING HUMANIZED ANTIBODIES – Application for § 156 Patent Term Extension

Docket No: 22338-80060

Assignee: Genentech, Inc.

Unit: OPLA

CERTIFICATE OF MAILING - 37 C.F.R. § 1.10
EXPRESS MAIL LABEL NO. ER 736919973 US

I hereby certify this correspondence is being deposited with the U.S. Postal Service with sufficient postage as "Express Mail – Post Office to Addressee" addressed to: Mail Stop Patent Ext., Commissioner for Patents, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450, on the date shown below.

Signature	David Devereux	Aug. 25, 2006
	Printed Name	Date

Mail Stop Patent Ext.
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

APPLICATION FOR EXTENSION OF PATENT TERM UNDER 35 U.S.C. § 156

Dear Sir:

Applicant, Genentech, Inc., hereby submits this application for extension of the term of United States Letters Patent 6,407,213 under 35 U.S.C. § 156 by providing the following information in accordance with the requirements specified in 37 C.F.R. § 1.740.

Applicant represents that it is the assignee of the entire interest in and to United States Letters Patent No. 6,407,213, granted to Paul J. Carter and Leonard G. Presta (Carter *et al.*) by virtue of an assignment of such patent to Genentech, Inc., recorded June 28, 1994, at Reel 7035, Frame 0272.

1. Identification of the Approved Product [§ 1.740(a)(1)]

The name of the approved product is LUCENTIS™. The name of the active ingredient of LUCENTIS™ is ranibizumab. Ranibizumab is a recombinant humanized monoclonal IgG_1 antibody antigen-binding fragment (Fab) based on a humanized framework with complementarity-determining regions (CDRs) derived from a murine monoclonal antibody that binds to human Vascular Endothelial Growth Factor (VEGF).

2. Federal Statute Governing Regulatory Approval of the Approved Product [§ 1.740(a)(2)]

The approved product was subject to regulatory review under, *inter alia*, the Public Health Service Act (42 U.S.C. § 201 *et seq.*) and the Federal Food, Drug and Cosmetic Act (21 U.S.C. § 355 *et seq.*).

3. Date of Approval for Commercial Marketing [§ 1.740(a)(3)]

LUCENTIS™ was approved for commercial marketing or use under § 351 of the Public Heath Service Act on **June 30, 2006**.

4. Identification of Active Ingredient and Certifications Related to Commercial Marketing of Approved Product [§ 1.740(a)(4)]

(a) The active ingredient of LUCENTIS™ is ranibizumab. Ranibizumab is a humanized monoclonal IgG_1 antibody antigen-binding fragment produced by an *E. coli* expression system. It contains human framework regions (FRs) and the complementarity-determining regions (CDRs) derived from a murine antibody that binds to VEGF.

(b) Applicant certifies that ranibizumab had not been approved for commercial marketing or use under the Federal Food, Drug and Cosmetic Act, the Public Health Service Act or the Virus-Serum-Toxin Act prior to the approval granted on June 30, 2006 to the present Applicant.

(c) Ranibizumab has been approved for the treatment of patients with neovascular (wet) age-related macular degeneration. *See* LUCENTIS™ product label, provided as Attachment A.

(d) LUCENTIS™ was approved for commercial marketing pursuant to § 351 of the Public Health Service Act (42 U.S.C. § 262) under Genentech's existing Department of Health and Human Services (DHHS) U.S. License No. 1048. *See* LUCENTIS™ approval letter, provided as Attachment B.

5. Statement Regarding Timeliness of Submission of Patent Term Extension Request [§ 1.740(a)(5)]

Applicant certifies that this application for patent term extension is being timely submitted within the sixty (60) day period permitted for submission specified in 35 U.S.C. § 156(d)(1) and 37 C.F.R. § 1.720(f). The last date on which this application may be submitted is August 28, 2006.

6. Complete Identification of the Patent for Which Extension Is Being Sought [§ 1.740(a)(6)]

The complete identification of the patent for which an extension is being sought is as follows:

(a) Names of the inventors: Paul J. Carter and Leonard G. Presta.

(b) Patent Number: 6,407,213 ("the '213 patent")

(c) Date of Issue: June 18, 2002

(d) Date of Expiration: June 18, 2019

7. Copy of the Patent for Which an Extension is Being Sought [§ 1.740(a)(7)]

A copy of U.S. Patent No. 6,407,213 is provided as Attachment C to the present application.

8. Copies of Disclaimers, Certificates of Correction, Receipt of Maintenance Fee Payment, or Reexamination Certificate [§ 1.740(a)(8)]

(a) U.S. Patent No. 6,407,213 is not subject to a terminal disclaimer.

(b) A Certificate of Correction was issued for U.S. Patent No. 6,407,213 on December 3, 2002. A copy of the Certificate of Correction is provided in Attachment D to the present application.

(c) The first maintenance fee for U.S. Patent No. 6,407,213 has been paid and there are no maintenance fees currently due, as provided in Attachment E.

(d) U.S. Patent No. 6,407,213 has not been the subject of a reexamination proceeding.

9. Statement Regarding Patent Claims Relative to Approved Product [§ 1.740(a)(9)]

The statements below are made solely to comply with the requirements of 37 C.F.R. § 1.740(a)(9). Applicant notes that, as the M.P.E.P. acknowledges, § 1.740(a)(9) does not require an applicant to show whether or how the listed claims would be infringed, and that this question cannot be answered without specific knowledge concerning acts performed by third parties. As such, these comments are not an assertion or an admission of Applicant as to the scope of the listed claims, or whether or how any of the listed claims would be infringed, literally or under the doctrine of equivalents, by the manufacture, use, sale, offer for sale or the importation of any product.

(a) At least claims 1-2, 4-5, 25, 29, 62-64, 66-67, 69, 71-73, 75-78, and 80-81 of U.S. Patent No. 6,407,213 claim the active pharmaceutical ingredient in the approved product or a method that may be used to make or use that ingredient.

(b) Pursuant to M.P.E.P. § 2753 and 37 C.F.R. § 1.740(a)(9), the following explanation is provided which shows how at least one of the above-listed claims of the '213 patent claim the approved product.

(1) Description of the approved product

The approved product is described in Section 11 of the approved label for LUCENTIS™ as follows, a copy of which is provided as Attachment A.

> LUCENTIS™ (ranibizumab injection) is a recombinant humanized IgG1 kappa isotype monoclonal antibody fragment designed for intraocular use. Ranibizumab binds to and inhibits the biologic activity of human vascular endothelial growth factor A (VEGF-A). Ranibizumab has a molecular weight of approximately 48 kilodaltons and is produced by an *E. coli* expression system in a nutrient medium containing the antibiotic tetracycline. Tetracycline is not detectable in the final product.
>
> LUCENTIS™ is a sterile, colorless to pale yellow solution in a single-use glass vial. LUCENTIS™ is supplied as a preservative-free, sterile solution in a single-use glass vial designed to deliver 0.05 mL of 10 mg/mL LUCENTIS™ aqueous solution with 10 mM histidine HCL, 10% α, α-trehalose dihydrate, 0.01% polysorbate 20, pH 5.5.

Ranibizumab is further characterized in a scientific reference, Chen *et al.* published in 1999 in the Journal of Molecular Biology (JMB) entitled "Selection and Analysis of an Optimized Anti-VEGF Antibody: Crystal Structure of an Affinity-matured Fab in Complex with Antigen."[1] The Chen *et al.* article discusses the lineage of the ranibizumab antibody fragment. In this respect, the article states that "[a] murine monoclonal antibody, A.4.6.1, was found to block VEGF-dependent cell proliferation *in vitro* and to antagonize tumor growth *in vivo.* [Citation omitted]. The murine mAb was previously humanized in Fab form to yield a variant known as Fab-12." [Citation omitted] *See* p. 866, left col., ¶1. The abstract explains that the authors affinity-matured Fab-12 and obtained Fab fragment Y0317, now known as ranibizumab. According to the article, ranibizumab was derived from the humanization and affinity-maturation of a non-human, murine monoclonal antibody that binds to VEGF. The Chen *et al.* article also describes the humanized structure of ranibizumab. *See, e.g.,* Figure 1.

---

[1] 293:865-881 (1999) (Attachment F)

WO 98/45331 (Figures 1A, 1B, 10A, 10B, provided as Attachment G) also provides sequence data for the heavy and light chain variable domains of Y0317, together with the heavy and light chain variable domains of murine A.4.6.1, the heavy and light chain variable domains of humanized variant Fab-12, and the Kabat human consensus framework, humIII. WO 98/45331 confirms that, in addition to non-human CDRs derived from the sequence of the murine antibody, ranibizumab comprises framework substitutions in the variable domains at positions 4 and 46 in the light chain (V_L) and positions 49, 69, 71, 73, 76, 78, and 94 in the heavy chain (V_H).[2]

(2) Explanation Regarding Claim 29 of the '213 Patent Relative to Ranibizumab

As explained below, the active pharmaceutical ingredient of the approved product, ranibizumab, is a humanized Fab fragment that is covered by at least claim 29.

Claim 29 of the '213 patent reads as follows:

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

Claim 29 depends from claim 1, which reads as follows:

> 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.

The term "antibody," as defined in the '213 patent specification includes, in addition to full-length antibodies, antibody fragments such as Fab, Fab', $F(ab)_2$ and Fv so long as those fragments retain the desired biological activity, *i.e.*, binding to VEGF (*See, e.g.,* '213 at col. 8, lines 11-17; col. 24, lines 13-18). As recited in the '213 specification – "FAb fragments with specificity for the antigen are specifically encompassed within the term 'antibody' as it is defined, discussed, and claimed herein." '213 at col. 24, lines 13-18. Ranibizumab, being

---

[2] Compare sequence data for the heavy and light chain variable domains of Y0317 (Figs. 10A-10B), A.4.6.1 (Figs. 1A-1B) and humIII (Figs. 1A-1B) as set forth in WO 98/45331, provided as Attachment G.

a Fab fragment that binds VEGF, falls within the scope of the term "antibody" as used in Claims 1 and 29.

The amino acid sequences of the V_L and V_H domains of ranibizumab include human framework substitutions at positions 4L, 46L, 49H, 69H, 71H, 73H, 76H, 78H and 94H.[3] Of these, substitutions at positions 4L and 69H are among those recited in the Markush group of claim 1. Figures 1A-1B of WO 98/45331, provided as Attachment G, show the heavy and light chain variable domains of sequences of the same import antibody ("A4.6.1") used to design ranibizumab on the lines above the variable domains of the Fab-12 sequence and the Kabat consensus sequences ("humIII").[4] The A4.6.1 antibody is a murine monoclonal antibody; its sequence is therefore "non-human." *See, e.g.,* Chen *et al.* Figures 10A-10B of WO 98/45331, provided as Attachment G (and Figure 1 of Chen *et al.*), show the variable domains of the Y0317 sequence. When the heavy and light chain variable domains of A4.6.1, Y0317 and humIII are aligned, the framework substitutions noted above are apparent utilizing the Kabat numbering system.

In each of the V_L and V_H domains of ranibizumab, "substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species" (*i.e.*, the murine antibody A4.6.1). *See, e.g.,* '213 at col. 2, lines 27-31. Ranibizumab is therefore "humanized" within the meaning of claims 1 and 29 of the '213 patent.

As also required in claim 1, ranibizumab includes non-human amino acid residues in its CDRs. The CDRs in ranibizumab are also functional to "bind an antigen" – here, the VEGF protein. *See* LUCENTIS™ label, provided as Attachment A.

Ranibizumab thus meets the limitations of dependent claim 29.

---

[3] *See* WO 98/45331 at Figures 1A-1B (humIII) and 10A-10B (Y0317).

[4] The residues in a human Ig sequence that are substituted with residues from an "import antibody" are identified according to standard numbering conventions published by Kabat. *See* '213 at col. 10, line 45 through col. 11, line 26. The Kabat sequences represent consensus amino acid sequences for various human antibodies in each subclass. *See id.*

10. Relevant Dates Under 35 U.S.C. § 156 for Determination of Applicable Regulatory Review Period [§ 1.740(a)(10)]

(a) Patent Issue Date

U.S. Patent No. 6,407,213 was issued on June 18, 2002.

(b) IND Effective Date [35 U.S.C. § 156(g)(1)(B)(i); 37 C.F.R. § 1.740(a)(10)(i)(A)]

The date that an exemption under § 505(i) of the Federal Food, Drug and Cosmetic Act became effective (*i.e.*, the date that an investigational new drug application ("IND") became effective) for LUCENTIS ™ (referred to as "Humanized Monoclonal Antibody Fragment (rhuFab V2)(E. coli, Genentech) to Vascular Endothelial Growth Factor (VEGF), Intravitreal) was October 7, 1999. The IND was assigned number BB-IND # 8633. A copy of the letter from the FDA reflecting the effective date of the IND is provided in Attachment H. The application date for this IND was October 6, 1999.

(c) BLA Submission Date [35 U.S.C. § 156(g)(1)(B)(i); 37 C.F.R. § 1.740(a)(10)(i)(B)]

The BLA was submitted by Genentech to the FDA on December 29, 2005. The BLA was assigned number BL# 125156/0. A copy of the letter from the FDA acknowledging receipt of the BLA and reflecting the BLA submission date is provided in Attachment I.

(d) BLA Issue Date [35 U.S.C. § 156(g)(1)(B)(ii); 37 C.F.R. § 1.740(a)(10)(i)(C)]

The FDA approved biologic license application 125156/0 authorizing the marketing of LUCENTIS ™ on June 30, 2006. LUCENTIS ™ was approved under Department of Health and Human Services (DHHS) U.S. License No. 1048. A copy of the approval letter from the FDA is provided as Attachment B.

11. Summary of Significant Events During Regulatory Review Period [§ 1.740(a)(11)]

Pursuant to 37 C.F.R. § 1.740(a)(11), the following provides a brief description of the activities of Genentech, Inc. before the FDA in relation to the regulatory review of LUCENTIS™. The brief description lists the significant events that occurred during the regulatory review period for the approved product. In several instances, communications to or from the FDA are referenced. Pursuant to 37 C.F.R. § 1.740(a)(11), 21 C.F.R. § 60.20(a), and M.P.E.P. § 2753, copies of all such communications are not provided in this application, but can be obtained from records maintained by the FDA.

- On October 6, 1999, Genentech submitted to FDA (See Attachment H) an investigational new drug application for a recombinant humanized monoclonal antibody fragment (rhuFab V2, now known as Ranibizumab) against Vascular Endothelial Growth Factor (VEGF). The antibody was developed as a potential new therapeutic in treating patients with the exudative (wet or neovascular) form of age-related macular degeneration (AMD).

- On October 7, 1999 FDA made BB-IND #8633 effective via a communication mailed to Genentech on October 13, 1999 (*See* Attachment H). According to the FDA, initiation of trials could begin 30 days after October 7, 1999.

- The first human clinical trial (Phase I) was initiated on February 8, 2000 followed by Phase II human trials and Phase III human trials, some of which remain ongoing at the time of this application.

- On February 5, 2002, representatives of Genentech and the FDA (CBER and CDER) participated in a Type C meeting to discuss the proposed clinical development plan for ranibizumab in AMD.

- On October 31, 2002 representatives of Genentech and FDA (CBER and CDER) participated in an Type B End-of-Phase II meeting.

- Beginning in approximately March 2003, and continuing at the time of this application, Phase III studies have been conducted. The three Phase III trials forming the basis of the Biologics License Application (BLA), FVF2598g, FVF2587g, and FVF3192g are studies of two year duration with primary endpoints of one year. FVF2587g and FVF3192g, along with extension study FVF3426g and safety study FVF3689g, remain ongoing at the time of this application.

- On September 21, 2005 representatives of Genentech and CDER participated in a Type B Pre-BLA submission meeting to discuss information requirements for the BLA.

- Genentech submitted a BLA for ranibizumab for the treatment of patients with wet AMD on December 29, 2005 (*See* Attachment I).

- FDA acknowledged receipt of the BLA for ranibizumab via a communication mailed to Genentech dated January 27, 2006. The letter indicated that FDA had assigned the Submission Tracking Number (STN) of BL #125156/0 to the BLA (*See* Attachment I).

- By way of a communication mailed to Genentech on March 14, 2006 FDA made Genentech aware that the BLA for ranibizumab was filed on February 28, 2006 and that FDA had assigned a user fee goal date of June 30, 2006 (*See* Attachment J).

- On June 30, 2006 FDA approved BLA 125156/0, issuing marketing authorization for LUCENTIS™ (*See* Attachment B).

12. Statement Concerning Eligibility for and Duration of Extension Sought Under 35 U.S.C. § 156 [37 C.F.R. § 1.740(a)(12)]

(a) In the opinion of the Applicant, U.S. Patent No. 6,407,213 is eligible for an extension under § 156 because:

(i) one or more claims of the '213 patent claim the approved product or a method of making or using the approved product;

(ii) the term of the '213 patent has not been previously extended on the basis of § 156;

(iii) the '213 patent has not expired;

(iv) no other patent has been extended pursuant to § 156 on the basis of the regulatory review process associated with the approved product, LUCENTIS™;

(v) there is an eligible period of regulatory review by which the patent may be extended pursuant to § 156;

(vi) the applicant for marketing approval exercised due diligence within the meaning of § 156(d)(3) during the period of regulatory review;

(vii) the present application has been submitted within the 60-day period following the approval date of the approved product, pursuant to § 156(c); and

(viii) this application otherwise complies with all requirements of 35 U.S.C. § 156 and applicable rules and procedures.

(b) The period by which the term of the '213 patent is requested by Applicant to be extended is **378 days.**

(c) The requested period of extension of term for the '213 patent corresponds to the regulatory review period that is eligible for extension pursuant to § 156, based on the facts and circumstances of the regulatory review associated with the approved product LUCENTIS™ and the issuance of the '213 patent. The period was determined as follows.

(i) The relevant dates for calculating the regulatory review period, based on the events discussed in the section above, are the following.

Exemption under FDCA § 505(i) became effective	October 7, 1999
Patent was granted	June 18, 2002
Biologics License Application (BLA) under PHSA § 351 was filed	December 29, 2005
BLA was approved	June 30, 2006

(ii) The '213 patent was granted during the period specified in § 156(g)(1)(B)(i) (*i.e.*, the period from the date of the grant of the exemption under § 505(i) of the FDCA until the date of submission of the BLA). Pursuant to § 156(b) and (c)(2), the calculated regulatory review period therefore includes a component of time between when the patent was granted and when the BLA was submitted (1/2 of 1289 days or 644 days).

(iii) The patent was granted prior to the start of the period specified in § 156(g)(1)(B)(ii) (*i.e.*, the period from the date of submission of the BLA until the date of approval). The regulatory review period under § 156(b) therefore includes a component equal to the total number of days in that period that are after the BLA was submitted (184 days).

(iv) The period determined according to § 156(b), (c)(2), and (g)(1) for the approved product (*i.e.*, the number of days following the date of issue of the patent between the dates of submission and of approval of the BLA for LUCENTIS™) is 828 days.

(v) The '213 patent will expire on June 18, 2019.

(vi) The date of approval of the approved product is June 30, 2006.

(vii) The date that is fourteen years from the date of approval of the approved product is June 30, 2020.

(viii) The period measured from the date the patent expires (*i.e.*, June 18, 2019) until the end of the fourteen-year period specified in §156 (c)(3) (*i.e.*, June 30, 2020) is approximately 1 year and 13 days or 378 days.

(ix) The number of days in the regulatory review period determined pursuant to § 156(g)(1)(B)(ii) (i.e., 828 days) exceeds the number of days that the

patent may be extended pursuant to §156(c)(3). As such, the period by which the patent may be extended is limited by the fourteen-year rule of §156(c)(3) to **378 days**.

(x) The '213 patent issued after the effective date of Public Law No. 98-417. As such, the two- or three-year limit of 35 U.S.C. § 156(g)(6)(C) does not apply.

13. Statement Pursuant to 37 C.F.R. § 1.740(a)(13)

Pursuant to 37 C.F.R. § 1.740(a)(13), Applicant acknowledges its duty to disclose to the Director of the PTO and to the Secretary of Health and Human Services any information which is material to the determination of entitlement to the extension sought, particularly as that duty is defined in 37 C.F.R. § 1.765.

14. Applicable Fee [§ 1.740(a)(14)]

Our check in payment of the fee prescribed in 37 C.F.R. § 1.20(j) for a patent term extension application under 35 U.S.C. § 156 accompanies this application. Please deduct any additional required fees from, or credit any overpayments to our deposit account no. 18-1260.

15. Name and Address for Correspondence [§ 1.740(a)(14)]

Please direct all inquiries, questions, and communications regarding this application for term extension to:

Jeffrey P. Kushan
SIDLEY AUSTIN LLP
1501 K Street, N.W.
Washington, D.C. 20005
Phone: 202-736-8914
Fax: 202-736-8111
email: jkushan@sidley.com

The correspondence address for U.S. Patent No. 6,407,213 is unchanged for all other purposes. A Power of Attorney granted to the undersigned by the patent assignee, a copy of which is included with this application as Attachment K, accompanies this communication.

Two additional copies of this application are enclosed, in compliance with 37 C.F.R. § 1.740(b). Applicant also provides herewith two further copies of the application for the convenience of the Office, pursuant to M.P.E.P. § 2763.

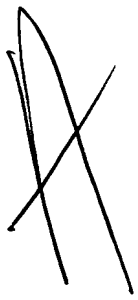
Sincerely,

Jeffrey P. Kushan
Attorney for Applicant
Registration No. 43,401

Sidley Austin LLP
1501 K Street, N.W.
Washington, D.C. 20005

Dated: August 25, 2006

INDEX OF ATTACHMENTS



HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use LUCENTIS safely and effectively. See full prescribing information for LUCENTIS.

LUCENTIS™ (ranibizumab injection)

Initial U.S. Approval: 2006

----------INDICATIONS AND USAGE----------

LUCENTIS is indicated for the treatment of patients with neovascular (wet) age-related macular degeneration (1).

----------DOSAGE AND ADMINISTRATION----------

- FOR OPHTHALMIC INTRAVITREAL INJECTION ONLY (2.1)
- LUCENTIS 0.5 mg (0.05 mL) is recommended to be administered by intravitreal injection once a month (2.2).
- Although less effective, treatment may be reduced to one injection every three months after the first four injections if monthly injections are not feasible. Compared to continued monthly dosing, dosing every 3 months will lead to an approximate 5-letter (1-line) loss of visual acuity benefit, on average, over the following 9 months. Patients should be evaluated regularly (2.2).

----------DOSAGE FORMS AND STRENGTHS----------

- 10 mg/mL single-use vial (3)

----------CONTRAINDICATIONS----------

- Ocular or periocular infections (4.1)
- Hypersensitivity (4.2)

----------WARNINGS AND PRECAUTIONS----------

- Endophthalmitis and retinal detachments may occur following intravitreal injections. Patients should be monitored during the week following the injection (5.1).
- Increases in intraocular pressure have been noted within 60 minutes of intravitreal injection (5.2).

----------ADVERSE REACTIONS----------

The most common adverse reactions (reported ≥ 6% higher in LUCENTIS-treated subjects than control subjects) are conjunctival hemorrhage, eye pain, vitreous floaters, increased intraocular pressure, and intraocular inflammation (6.2).

To report SUSPECTED ADVERSE REACTIONS, contact Genentech at 1-888-835-2555 or FDA at 1-800-FDA-1088 or www.fda.gov/medwatch.

See Section 17 for PATIENT COUNSELING INFORMATION.

FULL PRESCRIBING INFORMATION: CONTENTS*

* Sections or subsections omitted from the Full Prescribing Information are not listed.

FULL PRESCRIBING INFORMATION

1 INDICATIONS AND USAGE

LUCENTIS is indicated for the treatment of patients with neovascular (wet) age-related macular degeneration.

2 DOSAGE AND ADMINISTRATION

2.1 General Dosing Information

FOR OPHTHALMIC INTRAVITREAL INJECTION ONLY.

2.2 Dosing

LUCENTIS 0.5 mg (0.05 mL) is recommended to be administered by intravitreal injection once a month.

Although less effective, treatment may be reduced to one injection every three months after the first four injections if monthly injections are not feasible. Compared to continued monthly dosing, dosing every 3 months will lead to an approximate 5-letter (1-line) loss of visual acuity benefit, on average, over the following 9 months. Patients should be evaluated regularly [*see Clinical Studies (14.2)*].

2.3 Preparation for Administration

Using aseptic technique, all (0.2 mL) of the LUCENTIS vial contents are withdrawn through a 5-micron 19-gauge filter needle attached to a 1-cc tuberculin syringe. The filter needle should be discarded after withdrawal of the vial contents and should not be used for intravitreal injection. The filter needle should be replaced with a sterile 30-gauge × 1/2-inch needle for the intravitreal injection. The contents should be expelled until the plunger tip is aligned with the line that marks 0.05 mL on the syringe.

2.4 Administration

The intravitreal injection procedure should be carried out under controlled aseptic conditions, which include the use of sterile gloves, a sterile drape, and a sterile eyelid speculum (or equivalent). Adequate anesthesia and a broad-spectrum microbicide should be given prior to the injection.

Following the intravitreal injection, patients should be monitored for elevation in intraocular pressure and for endophthalmitis. Monitoring may consist of a check for perfusion of the optic nerve head immediately after the injection, tonometry within 30 minutes following the injection, and biomicroscopy between two and seven days following the injection. Patients should be instructed to report any symptoms suggestive of endophthalmitis without delay.

Each vial should only be used for the treatment of a single eye. If the contralateral eye requires treatment, a new vial should be used and the sterile field, syringe, gloves, drapes, eyelid speculum, filter, and injection needles should be changed before LUCENTIS is administered to the other eye.

No special dosage modification is required for any of the populations that have been studied (e.g., gender, elderly).

2.5 Stability and Storage

LUCENTIS should be refrigerated at 2°-8°C (36°-46°F). DO NOT FREEZE. Do not use beyond the date stamped on the label. LUCENTIS vials should be protected from light. Store in the original carton until time of use.

3 DOSAGE FORMS AND STRENGTHS

Single-use glass vial designed to deliver 0.05 mL of 10 mg/mL.

4 CONTRAINDICATIONS

4.1 Ocular or Periocular Infections

LUCENTIS is contraindicated in patients with ocular or periocular infections.

4.2 Hypersensitivity

LUCENTIS is contraindicated in patients with known hypersensitivity to ranibizumab or any of the excipients in LUCENTIS.

5 WARNINGS AND PRECAUTIONS

5.1 Endophthalmitis and Retinal Detachments

Intravitreal injections, including those with LUCENTIS, have been associated with endophthalmitis and retinal detachments. Proper aseptic injection technique should always be used when administering LUCENTIS. In addition, patients should be monitored during the week following the injection to permit early treatment should an infection occur [*see Dosage and Administration (2.3, 2.4) and Patient Counseling Information (17)*].

5.2 Increases in Intraocular Pressure

Increases in intraocular pressure have been noted within 60 minutes of intravitreal injection with LUCENTIS. Therefore, intraocular pressure as well as the perfusion of the optic nerve head should be monitored and managed appropriately [*see Dosage and Administration (2.4)*].

5.3 Thromboembolic Events

Although there was a low rate (<4%) of arterial thromboembolic events observed in the LUCENTIS clinical trials, there is a theoretical risk of arterial thromboembolic events following intravitreal use of inhibitors of VEGF [*see Adverse Reactions (6.3)*].

6 ADVERSE REACTIONS

6.1 Injection Procedure

Serious adverse events related to the injection procedure have occurred in < 0.1% of intravitreal injections, including endophthalmitis [*see Warnings and Precautions (5.1)*], rhegmatogenous retinal detachments, and iatrogenic traumatic cataracts.

6.2 Clinical Trials Experience – Ocular Events

Other serious ocular adverse events observed among LUCENTIS-treated patients occurring in <2% of patients

included intraocular inflammation and increased intraocular pressure [*see Warnings and Precautions (5.1, 5.2)*].

The available safety data include exposure to LUCENTIS in 874 patients with neovascular age-related macular degeneration in three double-masked, controlled studies with dosage regimens of 0.3 mg (375 patients) or 0.5 mg (379 patients) administered monthly by intravitreal injection (Studies 1 and 2) [*see Clinical Studies (14.1)*] and dosage regimens of 0.3 mg (59 patients) or 0.5 mg (61 patients) administered once a month for 3 consecutive doses followed by a dose administered once every 3 months (Study 3) [*see Clinical Studies (14.2)*].

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in one clinical trial of a drug cannot be directly compared with rates in the clinical trials of the same or another drug and may not reflect the rates observed in practice.

Table 1 shows the most frequently reported ocular adverse events that were reported with LUCENTIS treatment. The ranges represent the maximum and minimum rates across all three studies for control, and across all three studies and both dose groups for LUCENTIS.

Table 1

Adverse Event	LUCENTIS	Control
Conjunctival hemorrhage	77%-43%	66%-29%
Eye pain	37%-17%	33%-11%
Vitreous floaters	32%-3%	10%-3%
Retinal hemorrhage	26%-15%	56%-37%
Intraocular pressure increased	24%-8%	7%-3%
Vitreous detachment	22%-7%	18%-13%
Intraocular inflammation	18%-5%	11%-3%
Eye irritation	19%-4%	20%-6%
Cataract	16%-5%	16%-6%
Foreign body sensation in eyes	19%-6%	14%-6%
Lacrimation increased	17%-3%	16%-0%
Eye pruritis	13%-0%	12%-3%
Visual disturbance	14%-0%	9%-2%
Blepharitis	13%-3%	9%-4%
Subretinal fibrosis	13%-0%	19%-10%
Ocular hyperemia	10%-5%	10%-1%
Maculopathy	10%-3%	11%-3%
Visual acuity blurred/decreased	17%-4%	24%-10%
Detachment of the retinal pigment epithelium	11%-1%	15%-3%
Dry eye	10%-3%	8%-5%
Ocular discomfort	8%-0%	5%-0%
Conjunctival hyperemia	9%-0%	7%-0%
Posterior capsule opacification	8%-0%	5%-0%
Retinal exudates	9%-1%	11%-3%

6.3 Clinical Trials Experience – Non-Ocular Events

Table 2 shows the most frequently reported non-ocular adverse events with LUCENTIS treatment. The ranges represent the maximum and minimum rates across all three studies for control, and across all three studies and both dose groups for LUCENTIS.

Table 2

Adverse Event	LUCENTIS	Control
Hypertension/elevated blood pressure	23%-5%	23%-8%
Nasopharyngitis	16%-5%	13%-5%
Arthralgia	11%-3%	9%-0%
Headache	15%-2%	10%-3%
Bronchitis	10%-3%	8%-2%
Cough	10%-3%	7%-2%
Anemia	8%-3%	8%-0%
Nausea	9%-2%	6%-4%
Sinusitis	8%-2%	6%-4%
Upper respiratory tract infection	15%-2%	10%-4%
Back pain	10%-1%	9%-0%
Urinary tract infection	9%-4%	8%-5%
Influenza	10%-2%	5%-1%
Arthritis	8%-0%	8%-2%
Dizziness	8%-2%	10%-2%
Constipation	7%-3%	8%-2%

The rate of arterial thromboembolic events in the three studies in the first year was 2.1% of patients (18 out of 874) in the combined group of patients treated with 0.3 mg or 0.5 mg LUCENTIS compared with 1.1% of patients (5 out of 441) in the control arms of the studies. In the second year of Study 1, the rate of arterial thromboembolic events was 3.0% of patients (14 out of 466) in the combined group of patients treated with 0.3 mg or 0.5 mg LUCENTIS compared with 3.2% of patients (7 out of 216) in the control arm [*see Warnings and Precautions (5.3)*].

6.4 Immunogenicity

The pre-treatment incidence of immunoreactivity to LUCENTIS was 0%-3% across treatment groups. After monthly dosing with LUCENTIS for 12 to 24 months, low titers of antibodies to LUCENTIS were detected in approximately 1%-6% of patients. The immunogenicity data reflect the percentage of patients whose test results were considered positive for antibodies to LUCENTIS in an electrochemiluminescence assay and are highly dependent on the sensitivity and specificity of the assay. The clinical significance of immunoreactivity to LUCENTIS is unclear at this time, although some patients with the highest levels of immunoreactivity were noted to have iritis or vitritis.

7 DRUG INTERACTIONS

Drug interaction studies have not been conducted with LUCENTIS.

LUCENTIS intravitreal injection has been used adjunctively with verteporfin photodynamic therapy (PDT). Twelve of 105 (11%) patients developed serious intraocular inflammation; in 10 of the 12 patients, this occurred when LUCENTIS was administered 7 days (± 2 days) after verteporfin PDT.

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy
Pregnancy Category C. Animal reproduction studies have not been conducted with ranibizumab. It is also not known whether ranibizumab can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. LUCENTIS should be given to a pregnant woman only if clearly needed.

8.3 Nursing Mothers
It is not known whether ranibizumab is excreted in human milk. Because many drugs are excreted in human milk, and because the potential for absorption and harm to infant growth and development exists, caution should be exercised when LUCENTIS is administered to a nursing woman.

8.4 Pediatric Use
The safety and effectiveness of LUCENTIS in pediatric patients has not been established.

8.5 Geriatric Use
In the controlled clinical studies, approximately 94% (822/879) of the patients randomized to treatment with LUCENTIS were ≥ 65 years of age and approximately 68% (601/879) were ≥ 75 years of age. No notable difference in treatment effect was seen with increasing age in any of the studies. Age did not have a significant effect on systemic exposure in a population pharmacokinetic analysis after correcting for creatinine clearance.

8.6 Patients with Renal Impairment
No formal studies have been conducted to examine the pharmacokinetics of ranibizumab in patients with renal impairment. Sixty-eight percent of patients (136 of 200) in the population pharmacokinetic analysis had renal impairment (46.5% mild, 20% moderate, and 1.5% severe). Reduction in ranibizumab clearance is minimal in patients with renal impairment and is considered clinically insignificant. Dose adjustment is not expected to be needed for patients with renal impairment.

8.7 Patients with Hepatic Dysfunction
No formal studies have been conducted to examine the pharmacokinetics of ranibizumab in patients with hepatic impairment. Dose adjustment is not expected to be needed for patients with hepatic dysfunction.

10 OVERDOSAGE
Planned initial single doses of ranibizumab injection 1.0 mg were associated with clinically significant intraocular inflammation in 2 of 2 patients injected. With an escalating regimen of doses beginning with initial doses of ranibizumab injection 0.3 mg, doses as high as 2.0 mg were tolerated in 15 of 20 patients.

11 DESCRIPTION
LUCENTIS™ (ranibizumab injection) is a recombinant humanized IgG1 kappa isotype monoclonal antibody fragment designed for intraocular use. Ranibizumab binds to and inhibits the biologic activity of human vascular endothelial growth factor A (VEGF-A). Ranibizumab has a molecular weight of approximately 48 kilodaltons and is produced by an *E. coli* expression system in a nutrient medium containing the antibiotic tetracycline. Tetracycline is not detectable in the final product.

LUCENTIS is a sterile, colorless to pale yellow solution in a single-use glass vial. LUCENTIS is supplied as a preservative-free, sterile solution in a single-use glass vial designed to deliver 0.05 mL of 10 mg/mL LUCENTIS aqueous solution with 10 mM histidine HCl, 10% α, α-trehalose dihydrate, 0.01% polysorbate 20, pH 5.5.

12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action
Ranibizumab binds to the receptor binding site of active forms of VEGF-A, including the biologically active, cleaved form of this molecule, $VEGF_{110}$. VEGF-A has been shown to cause neovascularization and leakage in models of ocular angiogenesis and is thought to contribute to the progression of the neovascular form of age-related macular degeneration (AMD). The binding of ranibizumab to VEGF-A prevents the interaction of VEGF-A with its receptors (VEGFR1 and VEGFR2) on the surface of endothelial cells, reducing endothelial cell proliferation, vascular leakage, and new blood vessel formation.

12.2 Pharmacodynamics
Neovascular AMD is associated with foveal retinal thickening as assessed by optical coherence tomography (OCT) and leakage from CNV as assessed by fluorescein angiography.

In Study 3, foveal retinal thickness was assessed by OCT in 118/184 patients. OCT measurements were collected at baseline, Months 1, 2, 3, 5, 8, and 12. In patients treated with LUCENTIS, foveal retinal thickness decreased, on average, more than the sham group from baseline through Month 12. Retinal thickness decreased by Month 1 and decreased further at Month 3, on average. Foveal retinal thickness data did not provide information useful in influencing treatment decisions [*see Clinical Studies (14.2)*].

In patients treated with LUCENTIS, the area of vascular leakage, on average, decreased by Month 3 as assessed by fluorescein angiography. The area of vascular leakage for an individual patient was not correlated with visual acuity.

12.3 Pharmacokinetics
In animal studies, following intravitreal injection, ranibizumab was cleared from the vitreous with a half-life of approximately 3 days. After reaching a maximum at approximately 1 day,

the serum concentration of ranibizumab declined in parallel with the vitreous concentration. In these animal studies, systemic exposure of ranibizumab is more than 2000-fold lower than in the vitreous.

In patients with neovascular AMD, following monthly intravitreal administration, maximum ranibizumab serum concentrations were low (0.3 ng/mL to 2.36 ng/mL). These levels were below the concentration of ranibizumab (11 ng/mL to 27 ng/mL) thought to be necessary to inhibit the biological activity of VEGF-A by 50%, as measured in an in vitro cellular proliferation assay. The maximum observed serum concentration was dose proportional over the dose range of 0.05 to 1.0 mg/eye. Based on a population pharmacokinetic analysis, maximum serum concentrations of 1.5 ng/mL are predicted to be reached at approximately 1 day after monthly intravitreal administration of LUCENTIS 0.5 mg/eye. Based on the disappearance of ranibizumab from serum, the estimated average vitreous elimination half-life was approximately 9 days. Steady-state minimum concentration is predicted to be 0.22 ng/mL with a monthly dosing regimen. In humans, serum ranibizumab concentrations are predicted to be approximately 90,000-fold lower than vitreal concentrations.

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

No carcinogenicity or mutagenicity data are available for ranibizumab injection in animals or humans.

No studies on the effects of ranibizumab on fertility have been conducted.

14 CLINICAL STUDIES

The safety and efficacy of LUCENTIS were assessed in three randomized, double-masked, sham- or active-controlled studies in patients with neovascular AMD. A total of 1323 patients (LUCENTIS 879, Control 444) were enrolled in the three studies.

14.1 Study 1 and Study 2

In Study 1, patients with minimally classic or occult (without classic) CNV lesions received monthly LUCENTIS 0.3 mg or 0.5 mg intravitreal injections or monthly sham injections. Data are available through Month 24. Patients treated with LUCENTIS in Study 1 received a mean of 22 total treatments out of a possible 24 from Day 0 to Month 24.

In Study 2, patients with predominantly classic CNV lesions received one of the following: 1) monthly LUCENTIS 0.3 mg intravitreal injections and sham PDT; 2) monthly LUCENTIS 0.5 mg intravitreal injections and sham PDT; or 3) sham intravitreal injections and active verteporfin PDT. Sham PDT (or active verteporfin PDT) was given with the initial LUCENTIS (or sham) intravitreal injection and every 3 months thereafter if fluorescein angiography showed persistence or recurrence of leakage. Data are available through Month 12. Patients treated with LUCENTIS in Study 2 received a mean of 12 total treatments out of a possible 13 from Day 0 through Month 12.

In both studies, the primary efficacy endpoint was the proportion of patients who maintained vision, defined as losing fewer than 15 letters of visual acuity at 12 months compared with baseline. Almost all LUCENTIS-treated patients (approximately 95%) maintained their visual acuity. 34%-40% of LUCENTIS-treated patients experienced a clinically significant improvement in vision, defined as gaining 15 or more letters at 12 months. The size of the lesion did not significantly affect the results. Detailed results are shown in the tables below.

Table 3
Outcomes at Month 12 and Month 24 in Study 1

Outcome Measure	Month	Sham n = 238	LUCENTIS 0.5 mg n = 240	Estimated Difference (95% CI)[a]
Loss of < 15 letters in visual acuity (%)[b]	Month 12	62%	95%	32% (26%, 39%)
	Month 24	53%	90%	37% (29%, 44%)
Gain of ≥ 15 letters in visual acuity (%)[b]	Month 12	5%	34%	29% (22%,-35%)
	Month 24	4%	33%	29% (23%, 35%)
Mean change in visual acuity (letters) (SD)[b]	Month 12	-10.5 (16.6)	+7.2 (14.4)	17.5 (14.8, 20.2)
	Month 24	-14.9 (18.7)	+6.6 (16.5)	21.1 (18.1, 24.2)

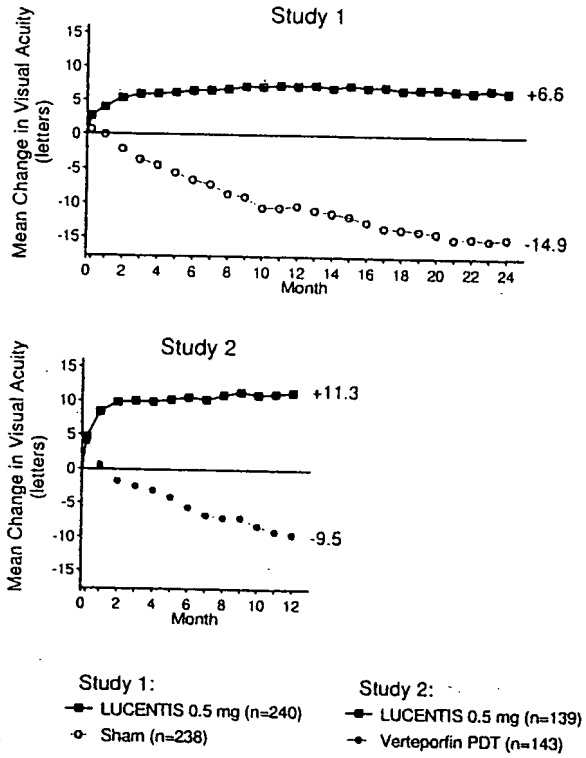
[a] Adjusted estimate based on the stratified model.
[b] $p<0.01$.

Table 4
Outcomes at Month 12 in Study 2

Outcome Measure	Verteporfin PDT n = 143	LUCENTIS 0.5 mg n = 140	Estimated Difference (95% CI)[a]
Loss of < 15 letters in visual acuity (%)[b]	64%	96%	33% (25%, 41%)
Gain of ≥ 15 letters in visual acuity (%)[b]	6%	40%	35% (26%, 44%)
Mean change in visual acuity (letters) (SD)[b]	-9.5 (16.4)	+11.3 (14.6)	21.1 (17.5, 24.6)

[a] Adjusted estimate based on the stratified model.
[b] $p<0.01$.

Figure 1
Mean Change in Visual Acuity from Baseline to Month 24 in Study 1 and to Month 12 in Study 2



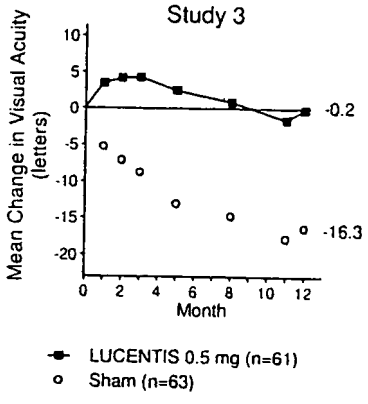
Patients in the group treated with LUCENTIS had minimal observable CNV lesion growth, on average. At Month 12, the mean change in the total area of the CNV lesion was 0.1-0.3 DA for LUCENTIS versus 2.3-2.6 DA for the control arms.

The use of LUCENTIS beyond 24 months has not been studied.

14.2 Study 3
Study 3 was a randomized, double-masked, sham-controlled, two-year study designed to assess the safety and efficacy of LUCENTIS in patients with neovascular AMD (with or without a classic CNV component). Data are available through Month 12. Patients received LUCENTIS 0.3 mg or 0.5 mg intravitreal injections or sham injections once a month for 3 consecutive doses, followed by a dose administered once every 3 months. A total of 184 patients were enrolled in this study (LUCENTIS 0.3 mg, 60; LUCENTIS 0.5 mg, 61; sham, 63); 171 (93%) completed 12 months of this study. Patients treated with LUCENTIS in Study 3 received a mean of 6 total treatments out of possible 6 from Day 0 through Month 12.

In Study 3, the primary efficacy endpoint was mean change in visual acuity at 12 months compared with baseline (see Figure 2). After an initial increase in visual acuity (following monthly dosing), on average, patients dosed once every three months with LUCENTIS lost visual acuity, returning to baseline at Month 12. In Study 3, almost all LUCENTIS-treated patients (90%) maintained their visual acuity at Month 12.

Figure 2
Mean Change in Visual Acuity from Baseline to Month 12 in Study 3



16 HOW SUPPLIED/STORAGE AND HANDLING
Each LUCENTIS carton, NDC 50242-080-01, contains one 2-cc glass vial of ranibizumab, one 5-micron, 19-gauge × 1-1/2-inch filter needle for withdrawal of the vial contents, one 30-gauge × 1/2-inch injection needle for the intravitreal injection, and one package insert [*see Dosage and*

Administration (2.4)]. VIALS ARE FOR SINGLE EYE USE ONLY.

17 PATIENT COUNSELING INFORMATION

In the days following LUCENTIS administration, patients are at risk of developing endophthalmitis. If the eye becomes red, sensitive to light, painful, or develops a change in vision, the patient should seek immediate care from an ophthalmologist [*see Warnings and Precautions (5.1)*].

LUCENTIS™ [ranibizumab injection]

Manufactured by:
Genentech, Inc.
1 DNA Way
South San Francisco, CA 94080-4990

8277700
LL1404
4833801
FDA Approval Date:
June 2006

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Rockville, MD 20852

BLA 125156

Genentech, Inc.
Attention: Robert L. Garnick, Ph.D.
Senior Vice President, Regulatory Affairs, Quality & Compliance
1 DNA Way
South San Francisco, California 94080-4990

Dear Dr. Garnick:

We have approved your biologics' license application for Lucentis (ranibizumab injection) effective this date. You are hereby authorized to introduce or deliver for introduction into interstate commerce, ranibizumab injection under your existing Department of Health and Human Services U.S. License No. 1048. Lucentis (ranibizumab injection) is indicated for the treatment of patients with neovascular (wet) age-related macular degeneration.

Under this license, you are approved to manufacture ranibizumab drug substance at Genentech, Inc., South San Francisco, California; fill the final formulated product at [redacted]; and label and package filled vials at Genentech, Inc., South San Francisco, California. You may label your product with the proprietary name Lucentis and market it in 10 mg/mL single use glass vials.

We acknowledge receipt of your submissions dated December 29, 2005, and January 31, February 10, 17, 21, and 24, March 17, 23, and 31, April 10, and 28, May 5, 10, 25 (2), 26 (2), and 31, and June 1, 5 (2), 6, 9, 13, 16, 23, 26, 27, 28 (3), and 29, 2006.

The final printed labeling (FPL) must be identical in content to the enclosed labeling text for the package insert, submitted June 28, 2006; the immediate vial container submitted March 31, 2006; and the carton labels submitted June 5, 2006. The statement "No U.S. standard of potency" should be added with the next printing of carton labels. Marketing this product with FPL that is not identical in content to the approved labeling text may render the product misbranded and an unapproved new drug.

The dating period for formulated drug product shall be 18 months from the date of manufacture when stored at 2°-8°C (36°-46°F). The date of manufacture shall be defined as the date of final sterile filtration of the formulated drug product. The dating period for ranibizumab drug substance shall be [redacted] when stored at -20 °C.

You currently are not required to submit samples of future lots of Lucentis to the Center for Drug Evaluation and Research (CDER) for release by the Director, CDER, under 21 CFR 610.2. We will continue to monitor compliance with 21 CFR 610.1 requiring completion of tests for conformity with standards applicable to each product prior to release of each lot.

You must submit information to your biologics license application for our review and written approval under 21 CFR 601.12 for any changes in the manufacturing, testing, packaging or labeling of Lucentis, or in the manufacturing facilities.

All applications for new active ingredients, new dosage forms, new indications, new routes of administration, and new dosing regimens are required to contain an assessment of the safety and effectiveness of the product in pediatric patients unless this requirement is waived or deferred. We are waiving the pediatric study requirement for this application.

The following are Postmarketing Studies that are subject to reporting requirements of 21 CFR 601.70:

1. Submit the final Clinical Study Report from Study FVF3689g by June 30, 2008.

2. Provide safety and efficacy data from a 2-year adequate and well-controlled clinical trial of a mutually acceptable design exploring multiple dosing frequencies of Lucentis.

   Date of submission of protocol: November 14, 2008.

   Date of start of study: September 21, 2009.

   Date of final clinical study report: April 1, 2013.

3. To detect and characterize immune responses to ranibizumab:

   a. Develop and validate a confirmatory assay capable of detecting both IgG and IgM isotype responses.

   b. Develop and validate an assay to detect neutralizing anti-ranibizumab antibodies.

   The assay methodology and validation reports: September 28, 2007.

4. To characterize further the immune response to ranibizumab, serum samples collected in studies FVF2587g, FVF2598g, FVF3192g will be assayed using the validated methods described above in Postmarketing Commitment #3. The data obtained will be analyzed to discover and evaluate any association between immunoreactivity and dosing frequency as well as any potential impact of immunoreactivity on efficacy or safety outcomes.

   The need for an additional clinical study will be determined based on the results from the analysis described above.

   Date of submission of protocol and statistical analysis plan: February 28, 2007.

   Date of submission of final study report: September 30, 2008.

The following are Postmarketing Studies that are not subject to reporting requirements of 21 CFR 601.70:

5. To revise release specifications, shelf-life specifications and in-process limits for ranibizumab drug substance and drug product after [redacted] commercial manufacturing runs to reflect increased manufacturing experience.

   These revisions to the Quality control system, the corresponding data from the [redacted] commercial manufacturing runs and the analysis plan used to create the revisions will be submitted as a supplement on or before June 30, 2008.

6. To perform additional Lucentis stability studies at 40°C using Ion Exchange Chromatography (IEC) to demonstrate that the corrective actions taken at [redacted] to address the atypical accelerated stability profile observed in the Lucentis 2005 qualification campaign have been sufficient.

   Specifically, a one time stability study consisting of [redacted] centis Drug Product launch lots are placed at 40°C and tested by IEC at [redacted] months. These [redacted] Lucentis Drug Product lots are derived from the following:

   - [redacted] of these Lucentis Drug Product lots are manufactured from distinct lots of [redacted].
   - At least [redacted] these [redacted] lots are aliquoted and used to manufacture [redacted] ucentis drug product lots.

   Data will be submitted as a supplement on or before March 31, 2007.

We request that you submit clinical protocols to your IND, with a cross-reference letter to this biologics license application. Submit nonclinical and chemistry, manufacturing, and controls protocols and all study final reports to this application. Please use the following designators to label prominently all submissions, including supplements, relating to these postmarketing study commitments as appropriate:

- **Postmarketing Study Protocol**
- **Postmarketing Study Final Report**
- **Postmarketing Study Correspondence**
- **Annual Report on Postmarketing Studies**

For each postmarketing study subject to the reporting requirements of 21 CFR 601.70, you must describe the status in an annual report on postmarketing studies for this product. The status report for each study should include:

- information to identify and describe the postmarketing commitment,
- the original schedule for the commitment,
- the status of the commitment (i.e. pending, ongoing, delayed, terminated, or submitted),

- an explanation of the status including, for clinical studies, the patient accrual rate (i.e. number enrolled to date and the total planned enrollment), and
- a revised schedule if the study schedule has changed and an explanation of the basis for the revision.

As described in 21 CFR 601.70(e), we may publicly disclose information regarding these postmarketing studies on our Web site (http://www.fda.gov/cder/pmc/default.htm). Please refer to the April 2001 Draft Guidance for Industry: Reports on the Status of Postmarketing Studies – Implementation of Section 130 of the Food and Drug Administration Modernization Act of 1997 (see http://www.fda.gov/cber/gdlns/post040401.htm) for further information.

You must submit adverse experience reports under the adverse experience reporting requirements for licensed biological products (21 CFR 600.80). You should submit postmarketing adverse experience reports to the Central Document Room, Center for Drug Evaluation and Research, Food and Drug Administration, 5901-B Ammendale Road, Beltsville, MD 20705-1266. Prominently identify all adverse experience reports as described in 21 CFR 600.80.

The MedWatch-to-Manufacturer Program provides manufacturers with copies of serious adverse event reports that are received directly by the FDA. New molecular entities and important new biologics qualify for inclusion for three years after approval. Your firm is eligible to receive copies of reports for this product. To participate in the program, please see the enrollment instructions and program description details at www.fda.gov/medwatch/report/mmp.htm.

You must submit distribution reports under the distribution reporting requirements for licensed biological products (21 CFR 600.81).

You must submit reports of biological product deviations under 21 CFR 600.14. You should promptly identify and investigate all manufacturing deviations, including those associated with processing, testing, packing, labeling, storage, holding and distribution. If the deviation involves a distributed product, may affect the safety, purity, or potency of the product, and meets the other criteria in the regulation, you must submit a report on Form FDA-3486 to the Division of Compliance Risk Management and Surveillance (HFD-330), Center for Drug Evaluation and Research, Food and Drug Administration, 5600 Fishers Lane, Rockville, MD 20857. Biological product deviations sent by courier or overnight mail should be addressed to Food and Drug Administration, CDER, Office of Compliance, Division of Compliance Risk Management and Surveillance, HFD-330, Montrose Metro 2, 11919 Rockville Pike, Rockville, MD 20852.

Please submit all FPL at the time of use and include implementation information on FDA Form 356h. Please provide a PDF-format electronic copy as well as original paper copies (ten for circulars and five for other labels). In addition, you may wish to submit draft copies of the proposed introductory advertising and promotional labeling with a cover letter requesting advisory comments to the Food and Drug Administration, Center for Drug Evaluation and Research, Division of Drug Marketing, Advertising and Communication, 5901-B Ammendale Road, Beltsville, MD 20705-1266. Final printed advertising and promotional labeling should be submitted at the time of initial dissemination, accompanied by a FDA Form 2253.

All promotional claims must be consistent with and not contrary to approved labeling. You should not make a comparative promotional claim or claim of superiority over other products unless you have substantial evidence to support that claim.

Please refer to http://www.fda.gov/cder/biologics/default.htm for important information regarding therapeutic biological products, including the addresses for submissions.

If you have any questions, call Lori M. Gorski, Project Manager, at (301) 796-0722.

Sincerely,

Mark J. Goldberger, M.D., M.P.H.
Director
Office of Antimicrobial Products
Center for Drug Evaluation and Research

Enclosure

C

US006407213B1

(12) **United States Patent**
Carter et al.

(10) **Patent No.: US 6,407,213 B1**
(45) **Date of Patent: Jun. 18, 2002**

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

(75) Inventors: **Paul J. Carter; Leonard G. Presta,** both of San Francisco, CA (US)

(73) Assignee: **Genentech, Inc.,** South San Francisco, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **08/146,206**

(22) PCT Filed: **Jun. 15, 1992**

(86) PCT No.: **PCT/US92/05126**

§ 371 (c)(1),
(2), (4) Date: **Nov. 17, 1993**

Related U.S. Application Data

(63) Continuation-in-part of application No. 07/715,272, filed on Jun. 14, 1991, now abandoned.

(51) **Int. Cl.**[7] **C07K 16/00**
(52) **U.S. Cl.** **530/387.3**; 435/69.6; 435/69.7; 435/70.21; 435/91; 536/23.53; 424/133.1
(58) **Field of Search** 435/69.6, 69.7, 435/70.21, 91, 172.2, 240.1, 240.27, 252.3, 320.1, 328; 536/23.53; 424/133.1; 530/387.3

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,816,567	A	3/1989	Cabilly et al.	
4,845,198	A	7/1989	Urdal et al.	530/388.22
5,132,405	A	7/1992	Huston et al.	530/387.3
5,225,539	A	7/1993	Winter	530/389.3
5,530,101	A	6/1996	Queen et al.	530/387.3
5,558,864	A	9/1996	Bendig et al.	424/133.1
5,585,089	A	12/1996	Queen et al.	424/133.1
5,677,171	A	10/1997	Hudziak et al.	435/7.23
5,693,762	A	* 12/1997	Queen et al.	530/387.2
5,714,350	A	2/1998	Co et al.	435/69.6
5,772,997	A	6/1998	Hudziak et al.	424/130.1
5,821,337	A	10/1998	Carter et al.	530/387.3
5,834,598	A	11/1998	Lowman et al.	530/399
5,859,205	A	1/1999	Adair et al.	530/387.3

FOREIGN PATENT DOCUMENTS

AU	85058/91	3/1992	C07K/15/12
EP	120694	10/1984	
EP	125023 A1	11/1984	
EP	0 239 400	* 9/1987	C12N/15/00
EP	323806 A1	7/1989	
EP	328404 A1	8/1989	A61K/39/395
EP	338745 A1	10/1989	
EP	365209 A2	4/1990	
EP	365997 A2	5/1990	
EP	368684	5/1990	
EP	403156 A1	12/1990	
EP	438310 A2	7/1991	
EP	438312 A2	7/1991	
EP	440351 A2	8/1991	
EP	0 460 167 B1	12/1991	
EP	0 519 596 A1	12/1992	
EP	0 592 106 A1	4/1994	
EP	0 620 276	10/1994	
EP	682040 A1	11/1995	
EP	451216 B1	1/1996	C12P/21/08
EP	432249 B1	9/1996	
GB	2 188941	10/1987	
WO	WO 87/02671	5/1987	
WO	WO 88/09344	12/1988	
WO	WO 89/01783	3/1989	
WO	WO 89/06692	7/1989	
WO	WO 89/09622	10/1989	
WO	WO 90/07861	7/1990	
WO	90/07861	* 7/1990	C12P/21/00
WO	WO 91/07492	5/1991	
WO	WO 91/07500	5/1991	
WO	WO 91/09966	7/1991	C12P/21/08
WO	WO 91/09968	7/1991	C12P/21/08
WO	WO 91/09967	11/1991	
WO	WO 92/01047	1/1992	
WO	WO 92/04380	3/1992	
WO	WO 92/04381	3/1992	
WO	WO 92/05274	4/1992	
WO	WO 92/11383	7/1992	
WO	WO 92/11018	9/1992	A61K/35/14
WO	WO 92/15683	9/1992	
WO	WO 92/16562	10/1992	
WO	WO 92/22653	12/1992	
WO	WO 93/02191	2/1993	
WO	94/11509	5/1994	
WO	WO 94/12214	6/1994	

OTHER PUBLICATIONS

Riechmann et al. [Nature 332:323–327 (1988)].*
Queen et al. [Proc. Natl. Acad. Sci. 86:10029–10033 (1989)].*
Roitt [*Immunology,* published 1985, by Gower Medical Publishing Ltd. (London, England) p. 5.5].*
Tramontano et al. [J. Mol. Biol. 215:175–182 (1990)].*
"Biosym Technologies" in New Products, Chemical Design Automation 3 (Dec. 1988).
"Polygen Corporation" in New Products, Chemical Design Automation 3 (Nov. 1988).
Adair et al., "Humanization of the murine anti-human CD3 monoclonal antibody OKT3" *Hum. Antibod. Hybridomas* 5:41–47 (1994).
Chothia et al., "Principles of protein–protein recognition" *Nature* 256:705–708 (1975).
Chothia et al., "Transmission of conformational change in insulin" *Nature* 302:500–505 (1983).
Corti et al., "Idiotope Determining Regions of a Mouse Monoclonal Antibody and Its Humanized Versions" *J. Mol. Biol.* 235:53–60 (1994).

(List continued on next page.)

Primary Examiner—Anthony C. Caputa
Assistant Examiner—Minh-Tam Davis
(74) *Attorney, Agent, or Firm*—Wendy M. Lee

(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

OTHER PUBLICATIONS

Couto et al., "Anti-BA46 Monoclonal Antibody Mc3 Humanization Using a Novel Positional Consensus and in Vivo and in Vitro Characterization" *Cancer Research Supplement* 55:1717–1722 (1995).

Couto et al., "Humanization of KC4G3, an Anti-Human Carcinoma Antibody" *Hybridoma* 13:215–219 (1994).

Ellis et al., "Engineered Anti-CD38 Monoclonal Antibodies for Immunotherapy of Multiple Myeloma" *The Journal of Immunology* pp. 925–937 (1995).

Hieter et al., "Evolution of Human Immunoglobulin K J Region Genes" *The Journal of Biological Chemistry* 257:1516–1522 (1982).

Lesk, Arthur M., "How Different Amino Acid Sequences Determine Similar Protein Structures: The Structure and Evolutionary Dynamics of the Globins" *J. Mol. Biol.* 135:225–270 (1980).

Matsumura et al., "Hydrophobic stabilization in T4 lysozyme determined directly by multiple substitutions of Ile 3" *Nature* 334:406–410 (1988).

Morrison, S. L., "Transfectomas Provide Novel Chimeric Antibodies" *Science* 229:1202–1207 (Sep. 20, 1985).

Nakatani et al., "Humanization of mouse anti-human IL-2 receptor antibody B-B10" *Protein Engineering* 7:435–443 (1994).

Ohtomo et al., "Humanization of Mouse ONS-M21 Antibody with the Aid of Hybrid Variable Regions" *Molecular Immunology* 32:407–416 (1995).

Rodrigues et al., "Engineering a humanized bispecific $F(ab')_2$ fragment for improved binding to T cells" *Int. J. Cancer* (Suppl.) 7:45–50 (1992).

Sha et al., "A Heavy-Chain Grafted Antibody that Recognizes the Tumor-Associated TAG72 Antigen" *Cancer Biotherapy* 9:341–349 (1994).

Tempest et al., "Identification of framework residues required to restore antigen binding during reshaping of a monoclonal antibody against the glycoprotein gB of human cytomegalovirus" *Int. J. Biol. Macromol.* 17:37–42 (1995).

Tramontano, "Structural Determinants of the Conformations of Medium-Sized Loops in Proteins" *Proteins* 6:382–394 (1989).

Uchiyama et al., "A Monoclonal Antibody (Anti-Tac) Reactive with Activated and Functionally Mature Human T Cells" *Journal of Immunology* 126:1393–1397 (1981).

Vincenti et al., "Interleukin-2-Receptor Blockade with Daclizumab to Prevent Acute Rejection in Renal Transplantation" *New Engl. J. Med.* 338:161–165 (1998).

Vitetta et al., "Redesigning Nature's Poisons to Create Anti-Tumor Reagents" *Science* 238:1098–1104 (1987).

Waldmann et al., "Interleukin 2 Receptor (Tac Antigen) Expression in HTLV-1-associated Adult T-Cell Leukemia" *Cancer Research* 45:4559s–4562s (1985).

Waldmann, Thomas A., "The Structure, Function, and Expression of Interleukin-2 Receptors on Normal and Malignant Lymphocytes" *Science* 232:727–732 (1986).

Wu et al., "An Analysis of the Sequences of the Variable Regions of Bence Jones Proteins and Myeloma Light Chains and Their Implications for Antibody Complementarity" *Journal of Experimental Medicine* 132:211–250 (1970).

Rhodes, P., "Recombinant antibodies from CHO cells" *Abstr Pap Am Chem Soc* (Abstract No. 60 from the 199th American Chemical Society National Meeting held in Boston, MA Apr. 22–27, 1990) 199(1–2):BIOT 60 (Apr. 1990).

Amzel and Poljak, "Three-dimensional structure of immunoglobulins" *Ann. Rev. Biochem.* 48:961–967 (1979).

Bindon et al., "Human monoclonal IgG isotypes differ in complement activating function at the level of C4 as well as C1q" *Journal of Experimental Medicine* 168(1):127–142 (Jul. 1988).

Boulianne, G. L. et al., "Production of functional chimaeric mouse/human antibody" *Nature* 312(5995):643–646 (Dec. 1984).

Brown et al., "Anti-Tac-H, a humanized antibody to the interleukin 2 receptor, prolongs primate cardiac allograft survival" *Proc. Natl. Acad. Sci. USA* 88:2663–2667 (1991).

Bruccoleri, "Structure of antibody hypervariable loops reproduced by a conformational search algorithm" *Nature* (erratum to article in Nature 335(6190):564–568 and) 336:266 (1988).

Bruggemann, M. et al., "Comparison of the effector functions of human immunoglobulins using a matched set of chimeric antibodies" *Journal of Experimental Medicine* 166:1351–1361 (1987).

Burgess et al., "Possible Dissociation of the Heparin-binding and Mitogenic Activities of Heparin-binding (Acidic Fibroblast) Growth Factor-1 from Its Receptor-binding Activities by Site-directed Mutagenesis of a Single Lysine Residue" *Journal of Cell Biology* 111:2129–2138 (1990).

Carter et al., "Humanization of an anti-$p185^{HER2}$ antibody for human cancer therapy" *Proc. Natl. Acad. Sci.* 89:4285–4289 (1992).

Cheetham, J., "Reshaping the antibody combining site by CDR replacement-tailoting or tinkering to fit?" *Protein Engineering* 2(3):170–172 (1988).

Chothia and Lesk, "Canonical Structures for the Hypervariable Regions" *J. Mol. Biol.* 196:901–917 (1987).

Chothia et al., "The predicted structure of immunoglobulin D1.3 and its comparison with the crystal structure" *Science* 233:755–758 (Aug. 15, 1986).

Chothia, C. et al., "Conformations of immunoglobulin hypervariable regions" *Nature* 342(6252):877–883 (1989).

Chothia, Cyrus, "Domain association in immunoglobulin molecules: The packing of variable domains" *J. Mol. Biol.* 186:651–663 (1985).

Clark et al., "The improved lytic function and in vivo efficacy of monovalent monoclonal CD3 antibodies" *European Journal of Immunology* 19:381–388 (1989).

Co et al., "Humanized antibodies for antiviral therapy" *Proc. Natl. Acad. Sci. USA* 88:2869–2873 (1991).

Coussens et al., "Tyrosine Kinase Receptor with Extensive Homology to EGF Receptor Shares Chromosomal Location with neu Oncogene" *Science* 230:1132–1139 (1985).

Daugherty, BL et al., "Polymerase chain reaction facilitates the cloning, CDR-grafting, and rapid expression of a murine monoclonal antibody directed against the CD18 component of leukocyte integrins" *Nucleic Acids Research* 19(9):2471–2476 (May 11, 1991).

Davies, D. R. et al., "Antibody-Antigen Complexes" *Ann. Rev. Biochem.* 59:439–473 (1990).

Epp et al., "The molecular structure of a dimer composed of the variable portions of the Bence-Jones protein REI refined at 2.0-A resolution" *Biochemistry* 14(22):4943–4952 (1975).

Fendly et al., "Characterization of murine monoclonal antibodies reactive to either the human epidermal growth factor receptor or HER2/neu gene product" *Cancer Research* 50:1550–1558 (1990).

Furey et al., "Structure of a novel Bence–Jones protein (Rhe) fragment at 1.6 A resolution" *J. Mol. Biol.* 167(3):661–692 (Jul. 5, 1983).

Gorman, SD et al., "Reshaping a therapeutic CD4 antibody" *Proc. Natl. Acad. Sci. USA* 88(10):4181–4185 (May 15, 1991).

Gregory et al., "The solution conformations of the subclasses of human IgG deduced from sedimentation and small angle X–ray scattering studies" *Molecular Immunology* 24(8):821–829 (Aug. 1987).

Hale et al., "Remission induction in non–hodgkin lymphoma with reshaped human monoclonal antibody campath–1H" *Lancet* 1:1394–1399 (1988).

Harris and Emery, "Therapeutic antibodies—the coming of age" *Tibtech* 11:42–44 (Feb. 1993).

Huber et al., "Crystallographic structure studies of an IgG molecule and an Fc fragment" *Nature* 264:415–420 (Dec. 2, 1976).

Hudziak et al., "p185^{HER2} Monoclonal Antibody Has Antiproliferative Effects In Vitro and Sensitizes Human Breast Tumor Cells to Tumor Necrosis Factor" *Molecular & Cellular Biology* 9(3):1165–1172 (1989).

Jaffers, G. J. et al., "Monoclonal antibody therapy. Anti–idiotypic and non–anti–idiotypic antibodies to OKT3 arising despite intense immunosuppression" *Transplantation* 41(5):572–578 (May 1986).

Jones, P. T. et al., "Replacing the complementarity–determining regions in a human antibody with those from a mouse" *Nature* 321(6069):522–525 (1986).

Junghans et al., "Anti–Tac–H, a humanized antibody to the interleukin 2 receptor with new features for immunotherapy in malignant and immune disorders" *Cancer Research* 50(5):1495–1502 (Mar. 1, 1990).

Kabat et al. *Sequences of Proteins of Immunological Interest,* Bethesda, MD:National Institutes of Health pp. iii–xxvii, 41–176 (1987).

King et al., "Amplification of a Novel v–erbB–Related Gene in a Human Mammary Carcinoma" *Science* 229:974–976 (1985).

Lazar et al., "Transforming Growth Factor α: Mutation of Aspartic Acid 47 and Leucine 48 Results in Different Biological Activities" *Molecular & Cellular Biology* 8(3):1247–1252 (1988).

Love et al, "Recombinant antibodies possessing novel effector functions" *Methods in Enzymology* 178:515–527 (1989).

Lupu et al., "Direct interaction of a ligand for the erbB2 oncogene product with the EGF receptor and p185^{erbB2}" *Science* 249:1552–1555 (1990).

Margni RA and Binaghi RA, "Nonprecipitating asymmetric antibodies" *Ann. Rev. Immunol.* 6:535–554 (1988).

Margolies et al., "Diversity of light chain variable region sequences among rabbit antibodies elicited by the same antigens." *Proc. Natl. Acad. Sci. USA* 72:2180–84 (Jun. 1975).

Marquart et al., "Crystallographic refinement and atomic models of the intact immunoglobulin molecule Kol and its antigen–binding fragment at 3.0 A and 1.0 A resolution" *J. Mol. Biol.* 141(4):369–391 (Aug. 25, 1980).

Mian, IS et al., "Structure, function and properties of antibody binding sites" *J. Mol. Biol.* 217(1):133–151 (Jan. 5, 1991).

Miller, R. et al., "Monoclonal antibody therapeutic trials in seven patients with T–cell lymphoma" *Blood* 62:988–995 (1983).

Morrison, S. L. et al., "Chimeric human antibody molecules: mouse antigen–binding domains with human constant region domains" *Proc. Natl. Acad. Sci. USA* 81(21):6851–6855 (Nov. 1984).

Neuberger et al., "Recombinant antibodies possessing novel effector functions" *Nature* 312(5995):604–608 (Dec. 1984).

Neuberger, M. S. et al., "A hapten–specific chimaeric IgE antibody with human physiological effector function" *Nature* 314(6008):268–270 (Mar. 1985).

Novotny and Haber, "Structural invariants of antigen binding: comparison of immunoglobulin V_L–V_H and V_L–V_L domain dimers" *Proc. Natl. Acad. Sci. USA* 82(14):4592–4596 (Jul. 1985).

Pluckthun, Andreas, "Antibody engineering: advances from the use of *Escherichia coli* expression systems" *Biotechnology* 9:545–51 (1991).

Queen, M. et al., "A humanized antibody that binds to the interleukin 2 receptor" *Proc. Natl. Acad. Sci. USA* 86:10029–10033 (1989).

Riechmann, L. et al., "Reshaping human antibodies for therapy" *Nature* 332:323–327 (1988).

Roitt et al. *Immunology* (Gower Medical Publishing Ltd., London, England) pp. 5.5 (1985).

Saul et al., "Preliminary refinement and structural analysis of the Fab fragment from human immunoglobulin new at 2.0 A resolution" *Journal of Biological Chemistry* 253(2):585–597 (Jan. 25, 1978).

Schroff, R. et al., "Human anti–murine immunoglobulin responses in patients receiving monoclonal antibody therapy" *Cancer Research* 45:879–885 (1985).

Segal et al., "The three–dimensional structure of a phosphorylcholine–binding mouse immunoglobulin Fab and the nature of the antigen binding site" *Proc. Natl. Acad. Sci. USA* 71(11):4298–4302 (Nov. 1974).

Shalaby et al., "Development of humanized bispecific antibodies reactive with cytotoxic lymphocytes and tumor cells overexpressing the HER2 protooncogene" *Journal of Experimental Medicine* 175(1):217–225 (Jan. 1, 1992).

Shepard and Lewis, "Resistance of tumor cells to tumor necrosis factor" *J. Clin. Immunol.* 8(5):333–395 (1988).

Sheriff et al., "Three–dimensional structure of an antibody–antigen complex" *Proc. Natl. Acad. Sci. USA* 84(22):8075–8079 (Nov. 1987).

Sherman et al., "Haloperidol binding to monoclonal antibodies" *Journal of Biological Chemistry* 263:4064–4074 (1988).

Silverton et al., "Three–dimensional structure of an intact human immunoglobulin" *Proc. Natl. Acad. Sci. USA* 74:5140–5144 (1977).

Slamon et al., "Human Breast Cancer: Correlation of Relapse and Survival with Amplification of the HER–2/neu Oncogene" *Science* 235:177–182 (1987).

Slamon et al., "Studies of the HER–2/neu proto–oncogene in human breast and ovarian cancer" *Science* 244:707–712 (1989).

Snow and Amzel, "Calculating three–dimensional changes in protein structure due to amino–acid substitutions: the variable region of immunoglobulins" *Protein: Structure, Function, and Genetics,* Alan R. Liss, Inc. vol. 1:267–279 (1986).

Sox et al., "Attachment of carbohydrate to the variable region of myeloma immunoglubulin light chains" *Proc. Natl. Acad. Sci. USA* 66:975–82 (Jul. 1970).

Spiegelberg et al., "Localization of the carbohydrate within the variable region of light and heavy chains of human γG myeloma proteins" *Biochemistry* 9:4217–23 (Oct. 1970).
Takeda et al., "Construction of chimaeric processed immunoglobulin genes containing mouse variable and human constant region sequences" *Nature* 314(6010):452–454 (Apr. 1985).
Tao et al., "Role of Carbohydrate in the Structure and Effector Functions Mediated by the H uman IgG Constant Region" *J. Immunol.* 143(8):2595–2601 (1989).
Tramontano et al., "Framework residue 71 is a major determinant of the position and conformation of the second hypervariable region in the VH domains of immunoglobulins" *J–Mol–Biol* 215(1):175–182 (Sep. 5, 1990).
Verhoeyen, M. et al., "Reshaping human antibodies: grafting an antilysozyme activity" *Science* 239(4847):1534–1536 (Mar. 25, 1988).
Waldmann, T., "Monoclonal antibodies in diagnosis and therapy" *Science* 252:1657–1662 (1991).
Wallick et al., "Glycosylation of a VH residue of a monoclonal antibody against alpha (1——6) dextran increases its affinity for antigen" *Journal of Experimental Medicine* 168(3):1099–1109 (Sep. 1988).
Winter and Milstein, "Man–made antibodies" *Nature* 349(6307):293–299 (Jan. 24, 1991).
Yamamoto et al., "Similarity of protein encoded by the human c–erb–B–2 gene to epidermal growth factor receptor" *Nature* 319:230–34 (1986).
Carter et al., "High level *escherichia coli* expression and production of a bivalent humanized antibody fragment" *Bio/Technology* 10:163–167 (1992).
Foote et al., "Antibody Framework Residues Affecting the Conformation of the Hypervariable Loops" *J. Mol. Biol.* 224:487–499 (1992).
Foote, J., "Humanized Antibodies" *Nova acta Leopoldina* 61(269):103–110 (1989).
Kabat et al., "Sequences of Proteins of Immunological Interest", Bethesda, MD:National Institute of Health pp. 14–32 (1983).
Kettleborough et al., "Humanization of a Mouse Monoclonal Antibody by CDR–grafting: the Importance of Framework Residues on Loop Conformation" *Protein Engineering* 4(7):773–783 (1991).
Maeda et al., "Construction of Reshaped Human Antibodies with HIV–neutralizing Activity" *Hum. Antibod. Hybridomas* 2:124–134 (Jul. 1991).
Riechmann et al, "Expression of an Antibody Fv Fragment in Myeloma Cells" *J. Mol. Biol.* 203:825–828 (1988).
Routledge et al., "A Humanized Monovalent CD3 Antibody which Can Activate Homologous Complement" *European Journal of Immunology* 21:2717–2725 (1991).
Shearman et al., "Construction, Expression and Characterization of Humanized Antibodies Directed Against the Human α/β T Cell Receptor" *J. Immunol.* 147(12):4366–4373 (Dec. 15, 1991).
Tempest et al., "Reshaping a Human Monoclonal Antibody to Inhibit Human Respiratory Syncytial Virus Infection In Vivo" *Bio/Technology* 9:266–271 (Mar. 1991).
Brown, Jr. et al., "Anti–Tac–H, a humanized antibody to the interleukin 2 receptor, prolongs primate cardiac allograft survival" *Proc. Natl. Acad. Sci. USA* 88:2663–2667 (1991).
Casale et al., "Use of an anti–IgE humanized monoclonal antibody in ragweed–induced allergic rhinitis" *J. Allergy Clin. Immunol.* 100:110–121 (1997).
Fahy et al., "The Effect of an Anti–IgE Monoclonal Antibody on the Early– and Late–Phase Responses to Allergen Inhalation in Asthmatic Subjects" *Am J. Respir. Crit. Care Med* 155:1828–1834 (1997).
Mathieson et al., "Monoclonal–Antibody Therapy in Systemic Vasculitis" *New England J. of Medicine* pp. 250–254 (Jul. 1990).
Presta et al., "Humanization of an anti–vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders" *Cancer Research* 57(20):4593–4599 (Oct. 15, 1997).
Amit et al., "Three–Dimensional Structure of an Antigen–Antibody Complex at 2.8 A Resolution" *Science* 233:747–753 (Aug. 1986).
Amzel et al., "The Three Dimensional Structure of a Combining Region–Ligand Complex of Immunglobulin New at 3.5–A Resolution" *Proc. Natl. Acad. Sci. USA* 71(4):1427–1430 (Apr. 1974).
Baselga et al., "Phase II Study of Weekly Intravenous Recombinant Humanized Anti–p185/HER2 Monoclonal Antibody in Patients With HER2/neu–Overexpressing Metastatic Breast Cancer" *J. Clin. Oncol.* 14(3):737–744 (1996).
Beverley & Callard, "Distinctive functional charcteristics of human "T" lymphocytes defined by E rosetting or a monoclonal anti–T cell antibody" *European Journal of Immunology* 11:329–334 (1981).
Bird et al., "Single–chain antigen–binding proteins" *Science* 242:423–426 (Oct. 1988).
Brennan et al., "Preparation of bispecific antibodies by chemical recombination of monoclonal immunoglobulin G_1 fragments" *Science* 229:81–83 (Jul. 1985).
Bruccoleri et al., "Structure of antibody hypervariable loops reproduced by a conformational search algorithm" *Nature* 335:564–568 (Oct. 1988).
Caron et al., "Biological and Immunological Features of Humanized M195 (Anti–CD33) Monoclonal Antibodies" *Cancer Research* 52:6761–6767 (Dec. 1992).
Chothia & Lesk, "The relation between the divergence of sequence and structure in proteins" *EMBO Journal* 5(4):823–826 (1986).
Co & Queen, "Humanized antibodies for therapy" *Nature* 351:501–502 (Jun. 1991).
Co et al., "Chimeric and Humanized Antibodies with Specificity for the CD33 Antigen" *J. of Immunology* 148(4):1149–1154 (Feb. 1992).
Co et al., "Humanized Anti–Lewis Y Antibodies: In Vitro Properties and Pharmacokinetics in Rhesus Monkeys" *Cancer Research* 56:1118–1125 (Mar. 1996).
Colman et al., "Crystal and Molecular Structure of the Dimer of Variable Domains of the Bence–Jones Protein ROY" *J. Mol. Biol.* 116:73–79 (1977).
Colman et al., "Three–dimensional structure of a complex of antibody with influenza virus neuraminidase" *Nature* 326:358–363 (Mar. 1987).
Cook et al., "A map of the human immunoglbulin V_H locus completed by analysis of the telometric region of chromosome 14q" *Nature Genetics* 7:162–168 (Jun. 1994).
Darsley & Rees, "Nucleotide sequences of five anti–lysozyme monoclonal antibodies" *EMBO Journal* 4(2):393–398 (1985).
Davies & Metzger, "Structural Basis of Antibody Function" *Ann. Rev. Immunol.* 1:87–117 (1983).

Davies et al., "Antibody–Antigen Complexes" *Journal of Biological Chemistry* 263(22):10541–10544 (Aug. 1988).

Eigenbrot et al., "X–Ray Structures of Fragments From Binding and Nonbinding Versions of a Humanized Anti–CD18 Antibody: Structural Indications of the Key Role of V_H Residues 59 to 65" *Proteins* 18:49–62 (1994).

Eigenbrot et al., "X–ray structures of the antigen–binding domains from three variants of humanized anti–p185HER2 antibody 4D5 and comparison with molecular modeling" *J. Mol. Biol.* 229:969–995 (1993).

Ellison et al., "The nucleotide sequence of a human immunoglobulin $C_{\gamma 1}$ gene" *Nucleic Acids Research* 10(13):4071–4079 (1982).

Emery & Adair, "Humanised monoclonal antibodies for therapeutic applications" *Exp. Opin. Invest. Drugs* 3(3):241–251 (1994).

Epp et al., "Crystal and Molecular Structure of a Dimer Composed of the Variable Portions of the Bence–Jones Protein REI" *European Journal of Biochemistry* 45:513–524 (1974).

Fanger et al., "Bispecific antibodies and targeted cellular cytotoxicity" *Immunology Today* 12(2):51–54 (1991).

Fanger et al., "Cytotoxicity mediated by human Fc receptors for IgG" *Immunology Today* 10(3):92–99 (1989).

Feldmann et al., "A Hypothetical Space–Filling Model of the V–Regions of the Galactan–Binding Myeloma Immunoglobulin J539" *Molecular Immunology* 18(8):683–698 (1981).

Fendley et al., "The Extracellular Domain of HER2/neu Is a Potential Immunogen for Active Specific Immunotherapy of Breast Cancer" *J. Biol. Resp. Mod.* 9:449–455 (1990).

Glennie et al., "Preparation and Performance of Bispecific F(ab'γ)$_2$ Antibody Containing Thioether–Linked Fab'γ Fragments" *J. Immunol.* 139(7):2367–2375 (Oct. 1, 1987).

Gonzalez et al., "Humanization of Murine 6G425:An Anti–IL8 Monoclonal Antibody Which Blocks Binding of IL8 to Human Neutrophils" *1996 Keystone Symposia on Exploring and Exploiting Antibody and Ig Superfamily Combining Sites* (Poster) pp. 1–21 (Feb. 1996).

Gussow & Seemann, "Humanization of Monoclonal Antibodies" *Meth. Enzymology,* Academic Press, Inc. vol. 203:99–121 (1991).

Hieter et al., "Cloned human and mouse kappa immunoglobulin constant and J region genes conserve homology in functional segments" *Cell* 22 (Part 1):197–207 (1980).

Houghton, A., "Building a better monoclonal antibody" *Immunology Today* 9(9):265–267 (1988).

Huston et al., "Protein engineering of antibody binding sites: Recovery of specific activity in an anti–digoxin single–chain Fv analogue produced in *Escherichia coli*" *Proc. Natl. Acad. Sci. USA* 85:5879–5883 (Aug. 1988).

Isaacs et al., "Humanised Monoclonal Antibody Therapy for Rheumatoid Arthritis" *Lancet* 340:748–752 (Sep. 26, 1992).

Johnson et al., "Biological and Molecular Modeling Studies Comparing Murine Monoclonal Antibodies with Their Engineered Chimeric and Humanized Counterparts" *J. Cell. Biochem. Suppl 0 (13 Part A)* (18th Ann. UCLA Symp on Mol. & Cell. Biol., Park City, UT Jan. 17–22, 1989) pp. 87 (1989).

Kabat E., "Origins of Antibody Complementarity and Specificity—Hypervariable Regions and the Minigenen Hypothesis" *J. of Immunology* 125(3):961–969 (Sep. 1980).

Kabat et al. *Sequences of Proteins of Immunological Interest,* U.S. Dept. of Health and Human Services, NIH, 5th edition vol. 1:103–108, 324–331 (1991).

Kindt & Capra *The Antibody Enigma,* New York:Plenum Press pp. 79–86 (1984).

Lesk & Chothia, "Evolution of Proteins Formed by β–Sheets" *J. Mol. Biol.* 160:325–342 (1982).

Lesk & Chothia, "The response of protein structures to amino–acid sequence changes" *Phil. Trans. R. Soc. Lond.* A 317:345–356 (1986).

Mariuzza et al., "The Structure Basis of Antigen–Antibody Recognition" *Ann. Rev. Biophys. Biophys. Chem.* 16:139–159 (1987).

Nadler et al., "Immunogenicity of Humanized and Human Monoclonal Antibodies" *Clin. Pharmacology & Therapeutics* pp. 180 (Feb. 1994).

Nelson, H., "Targeted Cellular Immunotherapy with Bifunctional Antibodies" *Cancer Cells* 3:163–172 (1991).

Neuberger et al., "Antibody Engineering" *Proceedings 8th Intl. Biotech. Symp.,* Paris II:792–799 (1988).

Newmark, P., "Making Chimeric Antibodies Even More Human" *Bio/Technology* 6:468 (May 1988).

Nishimura et al., "Human c–erbB–2 Proto–Oncogene Product as a Target for Bispecific–Antibody–Directed Adoptive Tumor Immunotherapy" *Int. J. Cancer* 50:800–804 (1992).

Nitta et al., "Preliminary trial of specific targeting therapy against malignant glioma" *Lancet* 335(8686):368–371 (Feb. 17, 1990).

Nitta, T. et al., "Bispecific F(ab')$_2$ monomer prepared with anti–CD3 and anti–tumor monoclonal antibodies is most potent in induction of cytolysis of human T cells" *European Journal of Immunology* 19:1437–1441 (1989).

Nolan et al., "Bifunctional antibodies: concept, production and applications" *Biochimica et Biophysica Acta* 1040:1–11 (1990).

O'Connor et al., "Calcium Dependence of an Anti–Protein C Humanized Antibody Involves Framework Residues" (manuscript).

Orlandi et al., "Cloning Immunoglobulin Variable Domains for Expression by the Polymerase Chain Reaction" *Proc. Natl. Acad. Sci. USA* 86:3833–3837 (May 1989).

Orlandi et al., "Cloning of cDNA Corresponding to Heavy and Light Chain Immunoglobulin Variable Domains" *Protein and Pharmaceutical Engineering* pp. 90 (1989).

Ostberg & Queen, "Human and humanized monoclonal antibodies: preclinical studies and clinical experience" *Biochem. Soc. Transactions* pp. 1038–1043 (1995).

Pedlan et al., "Model–building Studies of Antigen–binding Sites:The Hapten–binding Site of MOPC–315" *Cold Springs Harbor Symposia On Quantitative Biology* XLI:627–637 (1977).

Padlan, E., "Anatomy of the Antibody Molecule" *Molecular Immunology* 31(3):169–217 (1994).

Padlan, E., "Evaluation of the Structural Variation Among Light Chain Variable Domains" *Molecular Immunology* 16:287–296 (1979).

Palm & Hilschmann, "Primary structure of a crystalline monoclonal immunoglobulin K–type L–chain, subgroup I (Bence–Jones preotin Rei); isolation & characterization of the tryptic peptides: . . . " *Hoppes–Seyler's Z. Physiol. Chem.* 356:167–191 (Feb. 1975).

Palm & Hilschmann, "The primary structure of a crystalline, monoclonal immunoglobulin–L–chain of the x–type, subgroup I (Bence–Jones Protein Rei): a contribution to the elucidation of the three–dimensional structure of the immunoglobulins" *Hoppe–Seyler's Z. Physiol. Chem.* 354:1651–1654 (Dec. 1973).

Panka et al., "Variable region framework differences result in decreased or increased affinity of variant anti–digoxin antibodies" *Proc. Natl. Acad. Sci. USA* 85:3080–3084 (May 1988).

Presta et al., "Humanization of an Antibody Directed Against IgE" *J. Immunol.* 151(5):2623–2632 (Sep. 1, 1993).

Preval & Fougereau, "Specific Interaction between V_H and V_L Regions of Human Monoclonal Immunoglobulins" *J. Mol. Biol.* 102:657–678 (1976).

Queen et al., "Construction of Humanized Antibodies and Testing in Primates" *J. Cell. Biochem. Suppl. 15 (Part E)* (20th Ann. Mtg. Keystone Symp. Denver, CO Mar. 10–16, 1991) pp. 137 (1991).

Queen et al., "Humanised antibodies to the IL–2 receptor" *Protein Eng. Antibody Mol. Prophyl. Ther. Appl. Man,* Clark, M., Nottingham, UK:Academic Titles pp. 159–170 (1993).

Rhodes & Birch, "Large–Scale Production of Proteins from Mammalian Cells" *Bio/Technology* 6:518, 521, 523 (May 1988).

Riechmann, "Humanizing of Recombinant Antibodies" (Intl. Symp. on Clin. Appl. of Monoclonal Antibodies, Guildford, England) pp. 33–34 (Sep. 1987).

Riechmann & Winter, "Recombinant Antibodies" (U. of London Royal Postgraduate Medical School, Wolfson Institute, Abstract) (May 1987).

Riechmann et al. *Alignment of VL Sequences* (1988).

Roberts & Rees, "Generation of an antibody with enhanced affinity and specificity for its antigen by protein engineering" *Nature* 328:731–734 (Aug. 1987).

Rostapshov et al., "Effective method for obtaining long nucleotide chains on partially complementary templates" *FEBS Letters* 249(2):379–382 (Jun. 1989).

Schneider et al., "The Anti–Idiotypic Response by Cynomolgus Mondkeys to Humanized Anti–Tac Is Primarily Directed to Complementarity–Determining Regions H1, H2, and L3" *J. of Immunology* 150:3086–3090 (Apr. 1993).

Sedlacek et al., "Monoclonal Antibodies in Tumor Therapy", Karger pp. 119–126, 133–179 (1988).

Shields et al., "Inhibition of Allergic Reactions with Antibodies to IgE" *International Archives of Allergy and Immunology* 107(1–3):308–312 (May 1995).

Sims et al., "A Humanized CD18 Antibody Can Block Function Without Cell Destruction" *The Journal of Immunology* 151(4):2296–2308 (Aug. 1993).

Smith–Gill et al., "A Three–dimensional Model of an Anti–lysozyme Antibody" *Mol. Biol.* 194:713–724 (1987).

Songsivilai et al., "Bispecific antibody: a tool for diagnosis and treatment of disease" *Clin. Exp. Immunol.* 79:315–321 (1990).

Stanford, "A Predictive Method for Determining Possible Three–dimensional Foldings of Immunoglobulin Backbones Around Antibody Combining Sites" *Theor. Biol.* 88:421–439 (1981).

Stickney et al., "Bifunctional Antibody: ZCE/CHA ^{III}Indium BLEDTA–IV Clinical Imaging in Colorectal Carcinoma" *Antibody, Immuno Radiopharm* 2:1–13 (1989).

Tighe et al., "Delayed Allograft Rejection in Primates Treated with Anti–IL–2 Receptor Monoclonal antibody Campath–6" *Transplantation* 45(1):226–228 (Jan. 1988).

Verhoeyen & Riechmann, "Engineering of Antibodies" *BioEssays* 8(2):74–78 (Feb./Mar. 1988).

Verhoeyen et al., "Grafting Hypervariable Regions in Antibodies" *Protein Structure, Folding, and Design 2* (Proc. DuPont–UCLA Symp. Streamboat Springs, CO, Apr. 4–11, 1987), Dale L. Oxender, New York:Alan R. Liss, Inc. pp. 501–502 (1987).

Verhoeyen et al., "Humanising Mouse Antibodies: A Protein Engineering Approach" *Soc. for Analytical Cytology* (XIIth Intl. Mtg. for the Soc. for Analytical Cytology, Cambridge, UK) pp. 22 and slide presented at mtg.

Verhoeyen et al., "Re–shaped human anti–PLAP antibodies" *Monoclonal Antibodies Applications in clinical oncology,* Epenetos, 1st edition, Chapman & Hall Medical pp. 37–43 (1991).

Ward et al., "Expression and Secretion of Repertoires of VH Domains in *Escherichia coli:* Isolation of Antigen Binding Activites" *Progress in Immunology* (7th Intl. Congress Immunol. Berlin, W. Germany), F. Melchers vol. VII:1144–1151 (1989).

Ward, E.S. et al., "Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli" Nature* 341:544–546 (1989).

Werther et al., "Humanization of an Anti–Lymphocyte Function–Associated Antigen (LFA)–1 Monoclonal Antibody and Reengineering of the Humanized Antibody for Binding to Rhesus LFA–1" *J. of Immunology* pp. 4986–4995 (1996).

Whittle et al., "Construction and Expression of A CDR–Grafted Anti–TNF Antibody" *J. Cell Biochem. Suppl. 0* (Symp. on Protein and Pharm. Eng. Mol. and Cell. Biol. Park City, Utah)13 Part A:96 (1989).

Winter & Neuberger, "Restructuring Enzymes and Antibodies" *Investigation and Exploitation of Antibody Combining Sites,* Eric Reid, Plenum Press pp. 139–140 (1985).

Winter et al., "Protein Engineering by Site Directed Mutagenesis" *Chemical Synthesis in Molecular Biology,* H. Blocker et al., VCH pp. 189–197 (1987).

Winter G., "Antibody Engineering" *Phil. Trans. R. Soc. Lond. B* 324:99–109 (1989).

Woodle et al., "Humanized OKT3 Antibodies: Successful Transfer of Immune Modulating Properties and Idiotype Expression" *J. of Immunology* 148(9):2756–2763 (May 1992).

* cited by examiner

FIG. 1A

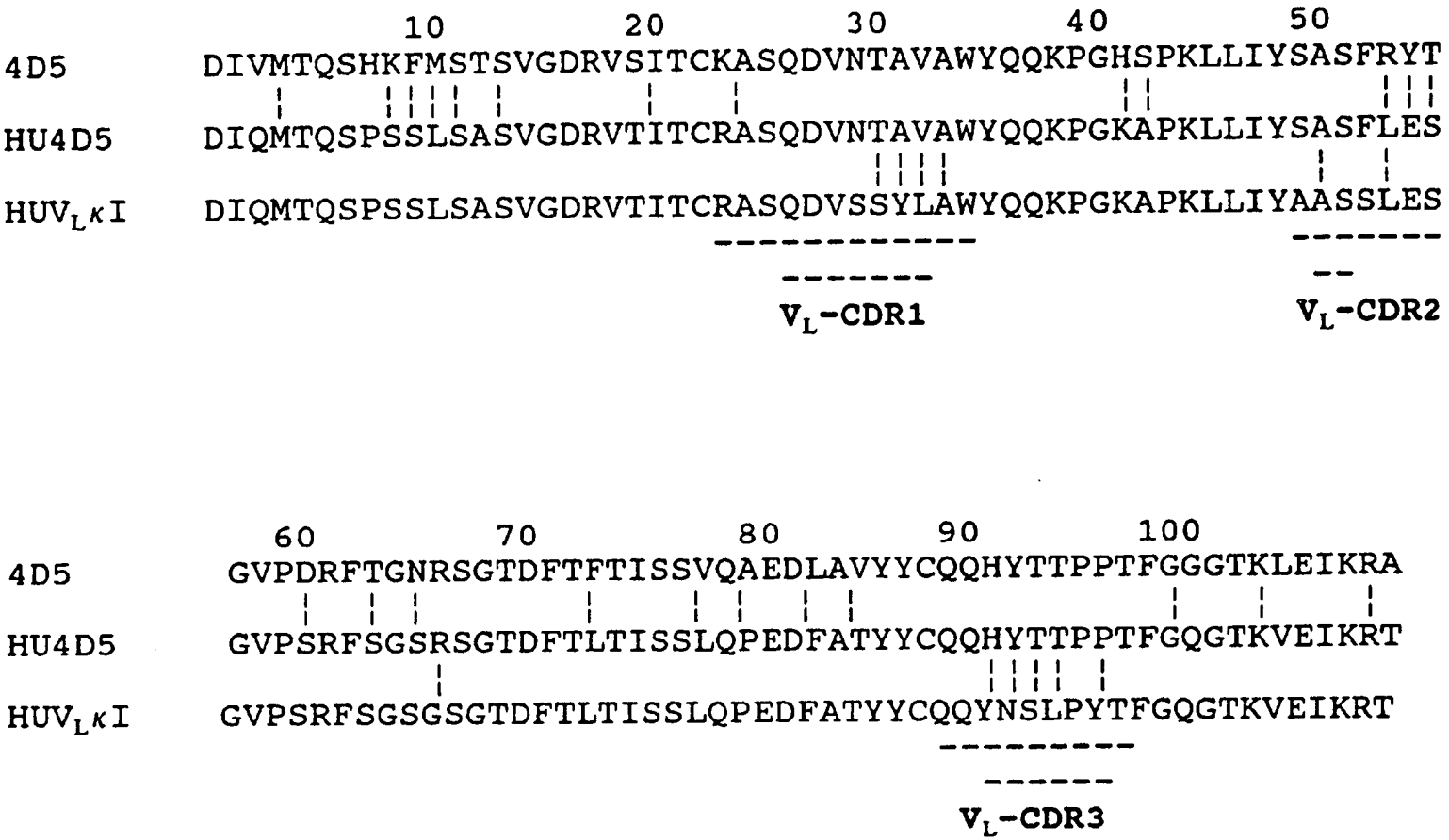
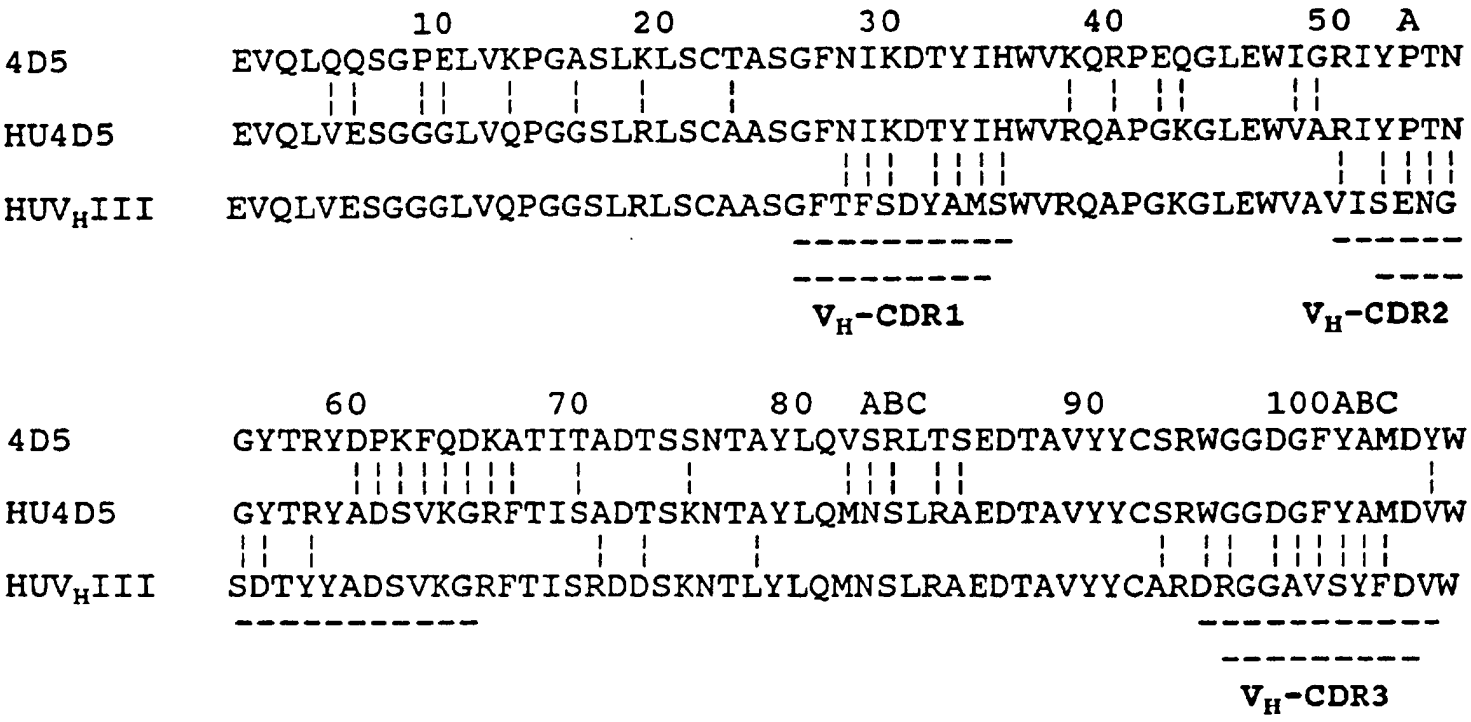


FIG. 1B



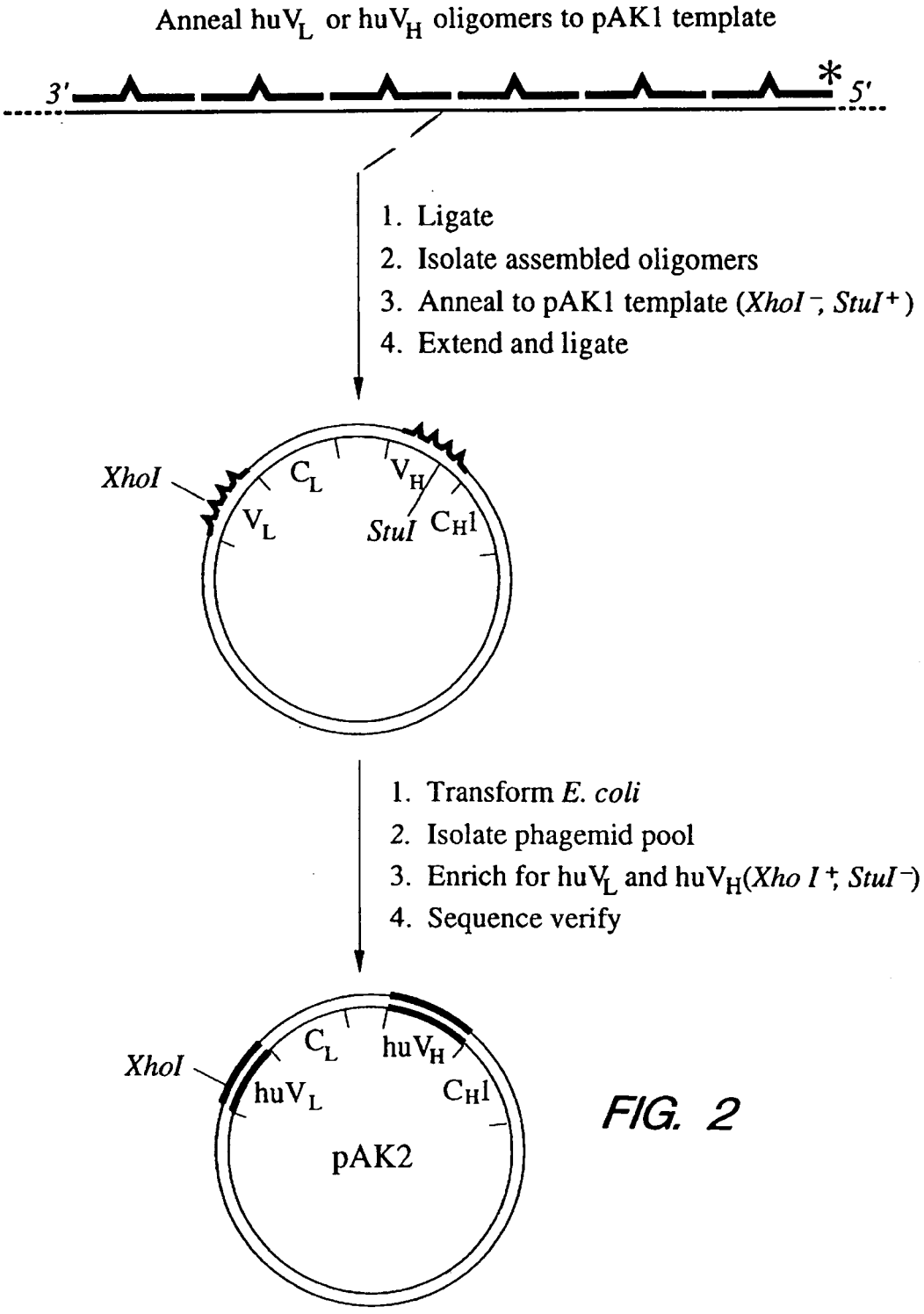

Anneal huV$_L$ or huV$_H$ oligomers to pAK1 template
3'
*
5'
1. Ligate
2. Isolate assembled oligomers
3. Anneal to pAK1 template (*XhoI*$^-$, *StuI*$^+$)
4. Extend and ligate
XhoI
C$_L$
V$_H$
V$_L$
StuI
C$_H$1
1. Transform *E. coli*
2. Isolate phagemid pool
3. Enrich for huV$_L$ and huV$_H$(*Xho I*$^+$, *StuI*$^-$)
4. Sequence verify
XhoI
C$_L$
huV$_H$
huV$_L$
C$_H$1
pAK2

FIG. 2

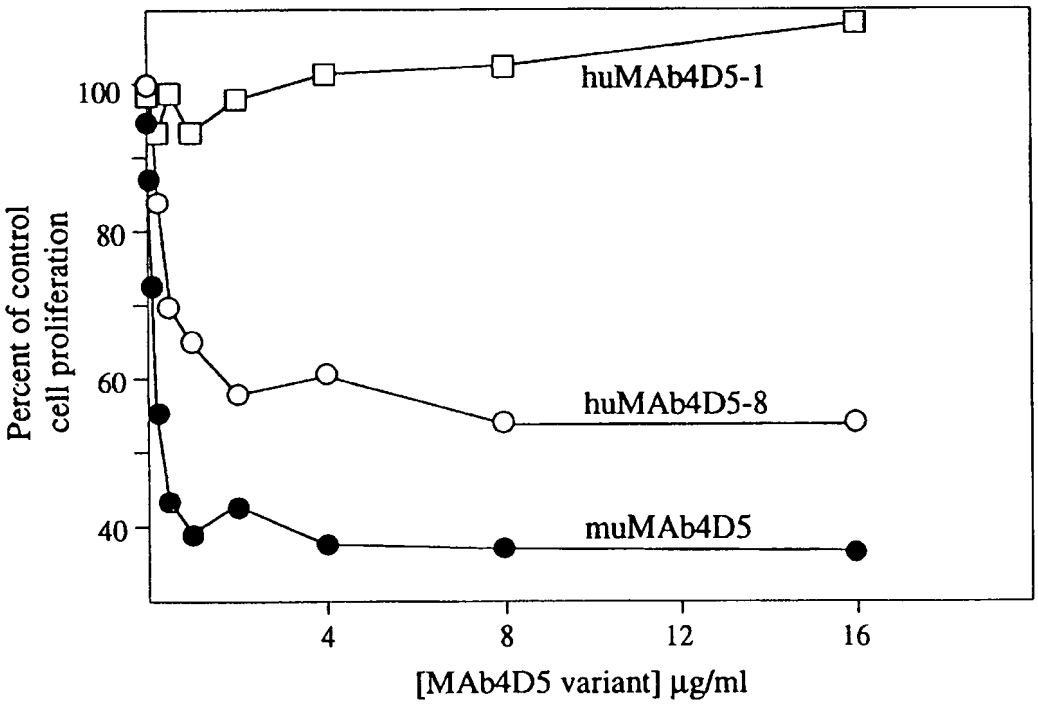

huMAb4D5-1
huMAb4D5-8
muMAb4D5
100
80
60
40
Percent of control cell proliferation
4
8
12
16
[MAb4D5 variant] µg/ml

FIG. 3

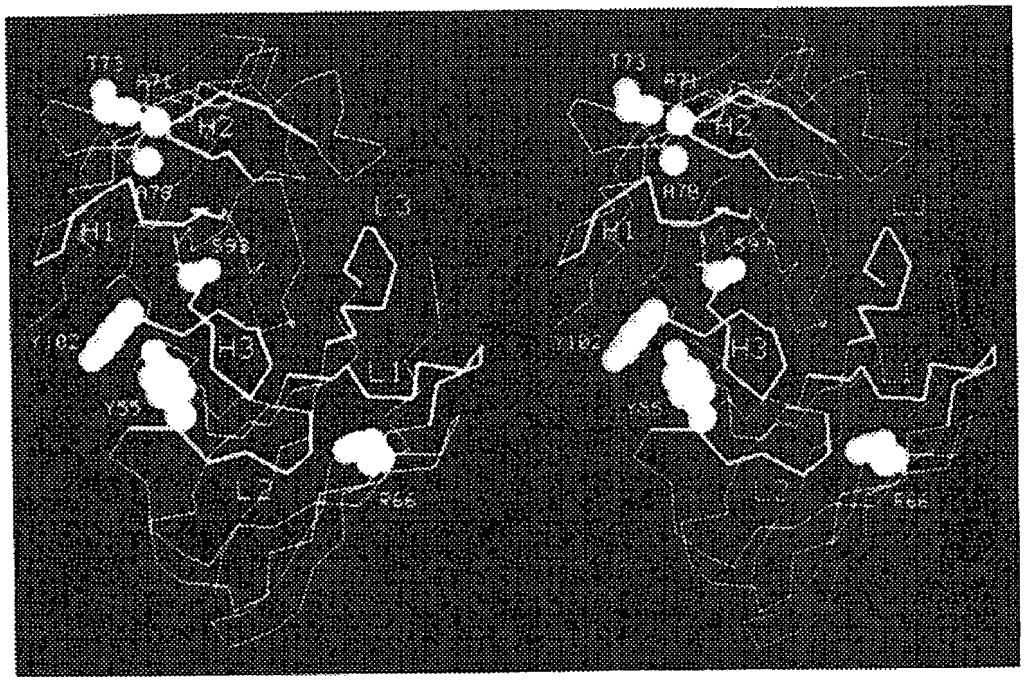


FIG. 4

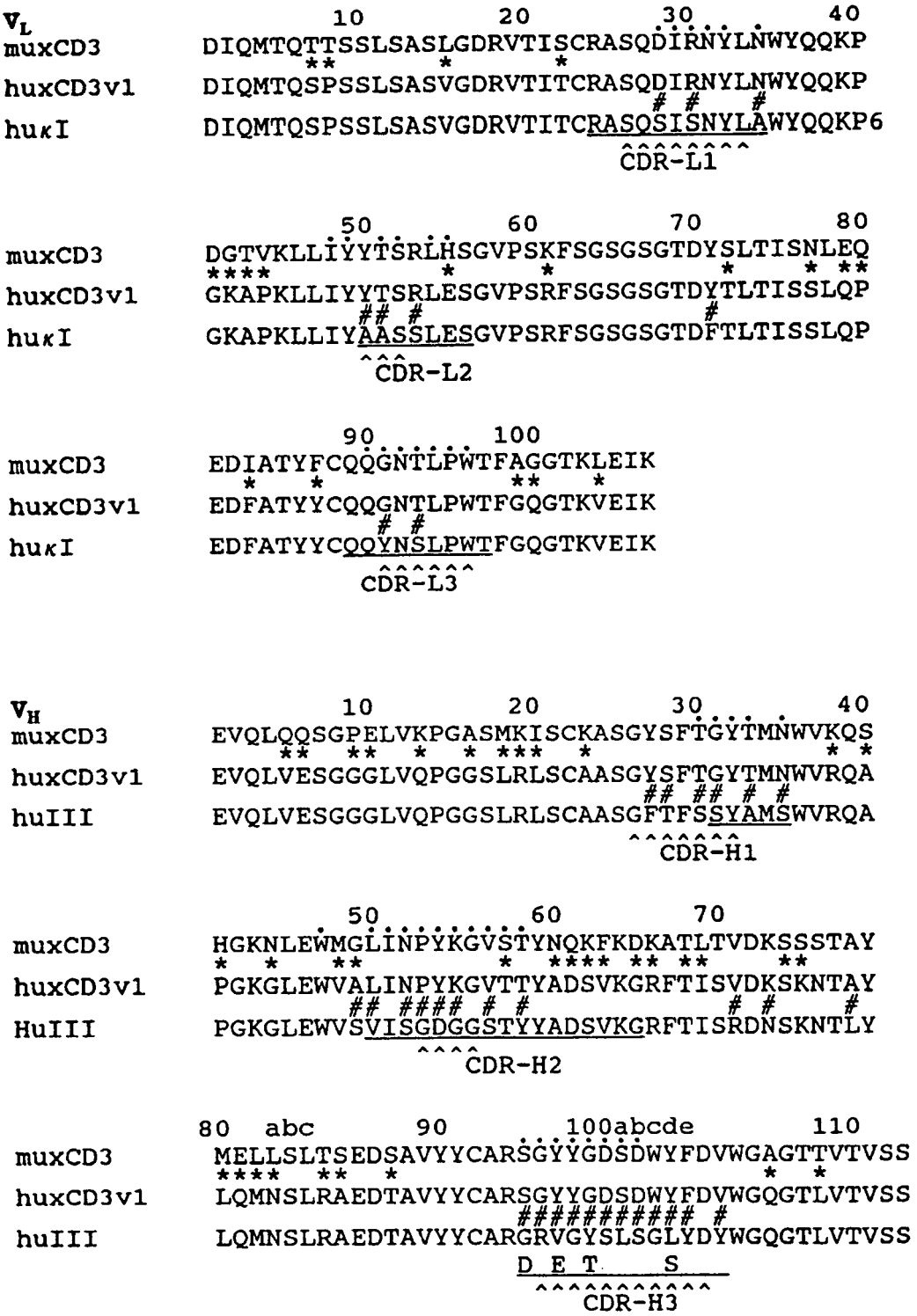

VL
muxCD3
huxCD3v1
huκI
CDR-L1
CDR-L2
muxCD3
huxCD3v1
huκI
CDR-L3
VH
muxCD3
huxCD3v1
huIII
CDR-H1
muxCD3
huxCD3v1
HuIII
CDR-H2
muxCD3
huxCD3v1
huIII
CDR-H3

FIG. 5

FIG. 6A-1

```
                                        10        20        30
H52H4-160                       QVQLQQSGPELVKPGASVKISCKTSGYTFTE
                                .*** .**  **.**.*...** ********
pH52-8.0    MGWSCIILFLVATATGVHSEVQLVESGGGLVQPGGSLRLSCATSGYTFTE
                    10        20        30        40        50

                   40        50        60        70        80
H52H4-160   YTMHWMKQSHGKSLEWIGGFNPKNGGSSHNQRFMDKATLAVDKSTSTAYM
            ******.*. **.***..*.******.********. *..**********
pH52-8.0    YTMHWMRQAPGKGLEWVAGINPKNGGTSHNQRFMDRFTISVDKSTSTAYM
                    60        70        80        90        100

                  90       100       110       120       130
H52H4-160   ELRSLTSEDSGIYYCARWRGLNYGFDVRYFDVWGAGTTVTVSSASTKGPS
            .. ** .**...********************** ** ************
pH52-8.0    QMNSLRAEDTAVYYCARWRGLNYGFDVRYFDVWGQGTLVTVSSASTKGPS
                    110       120       130       140       150

                 140       150       160       170       180
H52H4-160   VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL
            ****** *.*** .************************************
pH52-8.0    VFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL
                    160       170       180       190       200

                 190       200       210       220       230
H52H4-160   QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH
            ************** **..***** ***.********** ** * *
pH52-8.0    QSSGLYSLSSVVTVTSSNFGTQTYTCNVDHKPSNTKVDKTVERKCC---V
                    210       220       230       240

                 240       250       260       270       280
H52H4-160   TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVK
             *******  ..************************************.
pH52-8.0    ECPPCPAPP-VAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQ
            250       260       270       280       290
```

FIG. 6A-2

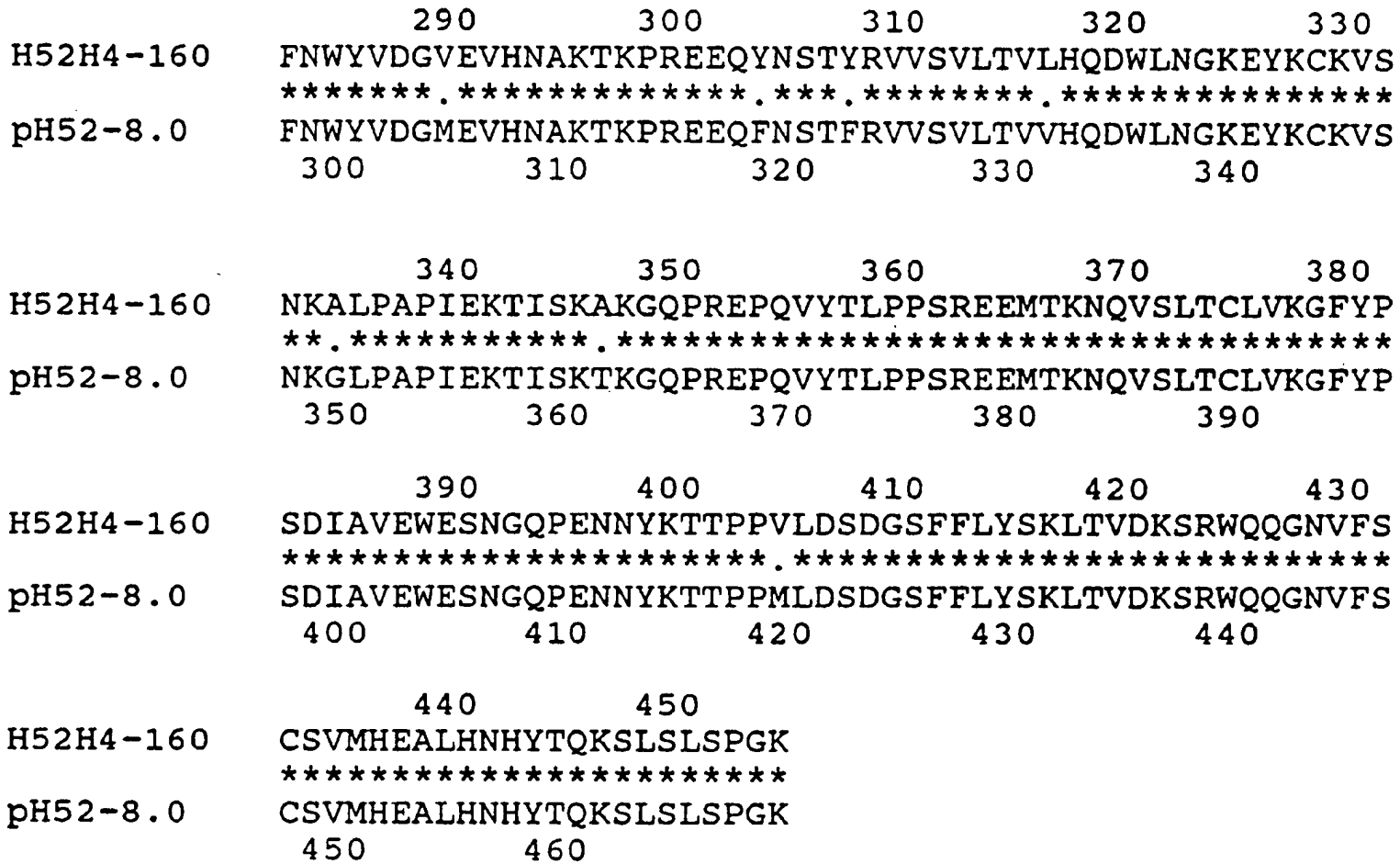


FIG. 6B

```
                                        10        20        30
H52L6-158                             DVQMTQTTSSLSASLGDRVTINCRASQDINN
                                      *.****. ******.****** *********
pH52-9.0    MGWSCIILFLVATATGVHSDIQMTQSPSSLSASVGDRVTITCRASQDINN
                    10        20        30        40        50

                   40        50        60        70        80
H52L6-158   YLNWYQQKPNGTVKLLIYYTSTLHSGVPSRFSGSGSGTDYSLTISNLDQE
            *********  . ****************************.****.*. *
pH52-9.0    YLNWYQQKPGKAPKLLIYYTSTLHSGVPSRFSGSGSGTDYTLTISSLQPE
                    60        70        80        90        100

                   90       100       110       120       130
H52L6-158   DIATYFCQQGNTLPPTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTAS
            *.***.************ ******************************
pH52-9.0    DFATYYCQQGNTLPPTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTAS
                    110       120       130       140       150

                  140       150       160       170       180
H52L6-158   VVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTL
            **************************************************
pH52-9.0    VVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTL
                    160       170       180       190       200

                  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 a., *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 CORs 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 a., *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); Verhoeyen, 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); Verhoeyen, 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. Bol.* 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); Silverton 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 is 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:
DIQMTQSPSSLSASVGDRVTITCRASQD-
VNTAVAWYQQKPGKAPKLLIYSASFLES-
GVPSRFSGSRSGTDFTLTISSLQPEDFA-
TYYCQQHYTTPPTFGQGTKVEIKRT

2. SEQ. ID NO. 2, which is the heavy chain variable domain of a humanized version of muMAb4D5):
EVQLVESGGGLVQPGGSLRLSCAASGFNIK
DTYIHWVRQAPGKGLEWVARIYPTNGYTRY
ADSVKGRFTISADTSKNTAYLQMNSLRAED
TAVYYCSRWGGDGFYAMDVWGQGTLVTVSS

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): DDIQMTQSPSSLSAS-
VGDRVTITCRASQDVSSYLAWYQQKPGKAPKLL
IYAASSLESGVPSRFSGSGSGTDFTLTISSLQP
EDFATYYCQQYNSLPYTFGQGTKVEIKRT, and

SEQ. ID NO. 4 (heavy chain): EVQLVESGGGLVQPG
GSLRLSCAASGFTFSDYAMSWVRQAPGKGL
EWVAVISENGGYTRYADSVKGRFTISADTSKNT
AYLQMNSLRAEDTAWYCSRWGGDGFYAMD
VWGQGTLVTVSS

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 IgG_1. Where such cytotoxic activity is not desirable, the constant domain may be of the IgG_2 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, Cβ) 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 setforth 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	$\gamma1$, $\gamma2$, $\gamma3$, $\gamma4$	κ or λ	$(\gamma_2\kappa_2)$, $(\gamma_2\lambda_2)$
IgA	α	$\alpha1$, $\alpha2$	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 IgGγl 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:

DIQMTQSPSSLSASVGDRVTITCRASQD-VSSYLAWYQQKPGKAPKLLIYAASSLES-GVPSRFSGSGSGTDFTLTISSLQPEDFA-TYYCQQYNSLPYTFGQGTKVEIKRT (SEQ. ID NO. 3);

the V_H consensus domain has the amino acid sequence:

EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYAMSW VRQAPGKGLEWVAVISENGGYTRYADSVKGRFT ISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGD GFYAMDVWGQGTLVTVSS (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:

DIQMTQSPSSLSASVGDRVTITCRASODVNTAVAWY QQKPGKAPKLLIYSASFLESGVPSRFSGSRSGT DFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTK VEIKRT (SEQ. ID NO. 1, which is the light chain variable domain of huMAb4D5); or

EVQLVESGGGLVOPGGSLRLSCAASGFNIKDTYIHW VRQAPGKGLEWVARIYPTNGYTRYADSVKGRFT ISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGD GFYAMDVWGQGTLVTVSS (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 covalent 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% $NaDodSO_4$ at 50° C.; (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serumalbumin/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 1 REI which are human structures, and 2MCP, 1 FBJ, 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]
				$V_L\kappa$ domain				
								2–11
	18–24	18–24	19–25	18–24	19–25	19–25	19–25	16–27
	32–37	34–39	39–44	32–37	32–37	32–37	33–38	33–39
								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	
				V_H domain				
								3–8
	18–25		18–25	18–25	18–25	18–25		17–23
	34–39		34–39	34–39	34–39	34–39		33–41
	46–52		46–52	46–52	46–52	46–52		45–51
	57–61		59–63	56–60	57–61	57–61		57–61
	68–71		70–73	67–70	68–71	68–71		66–71
	78–84		80–86	77–83	78–84	78–84		75–82
	92–99		94–101	91–98	92–99	92–99		88–94
								102–108

TABLE I-continued

Immunoglobulin Residues Used in Superpositioning and Those Included in the Consensus Structure								
Ig[a]	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			

[a]Four-letter code for Protein Data Bank file.
[b]Residue 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.
[c]Root-mean-square deviation in Å for (N, Cα, C) atoms superimposed on 2FB4.
[d]Root-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 Ca 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

	V_LK before (Å)	V_LK after (Å)	V_H before (Å)	V_H after (Å)	Standard Geometry (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 Bol.* 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. fin 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 is anhydride, $SOCl_2$, or $R^1N{=}C{=}NR$, 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 μg 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 ⅕ to 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 antibodiesare 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 mutagenesism" 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, lb) 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 utilized 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 μg) 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 50μl. The reaction mixture is overlayed with 35 μl mineral oil. The reaction is denatured for 5 minutes at 100° C., placed briefly on ice, and then 1 μl *Thermus aquaticus* (Taq) DNA polymerase (5 units/μl, 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 II 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 2μ 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 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 ate 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 AATAAA 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 β1 gene in cultured mouse and rabbit cells, and Gorman et al., *Proc. Natl. Aced. 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 transformants 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 pSVI6B.

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/Technologvy* 6: 47–55 (1988); Miller et al., in *Genetic Engineering* Setlow, J. K. et a., 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 cutures 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, $CaPO_4$ 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 ^{32}P. 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 absorb 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.

Cysteinyl residues most commonly are reacted with α-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α-bromo-β-(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides,3-nitro-2-pyridyl disulfide, methyl2-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. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1M sodium cacodylate at pH 6.0.

Lysinyl 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 lysinyl residues. Other suitable reagents for derivatizing α-amino-containing residues include imidoesters such as methyl picolinimidate; 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-acetylimidizole 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]propioimidate 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, phophorylation of hydroxyl groups of seryl or threonyl resides, 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 amidaatioon 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 glucosylatuion 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.

Gylcosylation of polypeptides is typically either N-linked or O-linked refers to the attachment of the carbonhydrate moiety to the side chain of an asparagine reisdue. The tri-peptide sequences asparagine-X-resine and asparagine-X-threonine, where X is any aminoe acid except proline, are the recoginition 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-acetylgactosamine, 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 hte 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 theonine resides to the native target polypeptide sequence (for O-linked glycosylation sites). For ease, the target polypeptide amino acid sequences is preferably altered through changes at the DNA level, particularly by mutating the DNA encoding the target polypeptide at preselected bases such that condons 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-[methylmethacylate]microcapsules, respectively), in colloidal drug deliverysystems (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, ^{3}H, and ^{131}I, fluorophores such as rare earth chelates or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, luceriferases, 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, biotintavidin, spin labels, bacteriophage labels, stable free radicals, and the like.

Conventional methods are available to bind these labels covalenily 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 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, curcin, crotin, 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 (Uananue 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 lgG2a 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 lgG2a 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 preservatives. 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., Mauassas, 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 Cα to the analogous Cα in each of the superimposed structures was calculated for each residue position. If all (or nearly all) Cα—Cα 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, Cα, C, O and Cβ 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 Cα 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 notas 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 ihe 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 γ1 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 γ1 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 (Brüggemann, 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 V_{104}L and T109A (variants are denoted by the amino acid residue and number followed by the replacement amino acid). The human γ1 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-^{32}P-ATP (Carter, P. *Methods Enzymol.* 154: 382–403 (1987)) and separately annealed with 3.7 pmol of pAK1 template in 40 μl 10 mM Tris-HCl (pH 8.0) and 10 mM $MgCl_2$ 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 μl 5 mM ATP and 2 μl 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 oligonucleotides (~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 μl 40 mM Tris-HCl (pH 7.5) and 16 mM $MgCl_2$ 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 huV_L by restriction purification using XhoI and then for huV_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 huV_L and huV_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 oligonucle-

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 (Kd_d=0.1 μM) and its human IgG_1 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 ($K_d \leq 1$ 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 (Brcüggemann, 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 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 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

	V_H Residue*					V_L Residue*			
MAb4D5 cell Variant proliferation‡	71 FR3	73 FR3	78 FR3	93 FR3	102 CDR3	55 CDR2	56 FR3	K_d† nM	Relative
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 ≦ ± 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 μg/ml. Data are all taken from the same experiment with an estimated standard error of ≦ ± 15%.

TABLE 4

Selectivity of antibody dependent tumor cell cytotoxicity mediated by huMAb4D5-8

Effector:Target ratio†	WI-38* muMAb4D5	WI-38* huMAb4D5-8	SK-BR-3 muMAb4D5	SK-BR-3 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 ^{51}Cr release. Estimated standard error in these quadruplicate determinations was ≦±10%.
‡Monoclonal antibody concentrations used were 0.1 μg/ml (A) and 0.1 μg/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 be 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 Bisnecific $F(ab')_2$ 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 $(ab')_2$v1. In contrast $BsF(ab')_2$ v1 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, Ss $F(ab')_2$v9, was created by replacing six residues in the second hypervariable loop of the anti-CD3 heavy chain variable domain of $BsF(ab')_2$v1 with their counterparts from the murine parent anti-CD3 antibody. $BsF(ab')_2$v9 binds to T cells (Jurkat) much more efficiently than does $BsF(ab')_2$v1 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 Songsivilai, 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. Aced. 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, $BsF(ab')_2$v1 enhanced the cytotoxic effects of activated human CTL 4-fold against SK-SR-3 tumor cells overexpressing $p185^{HER2}$. The example descries 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-CTAtCTGCAAATG 3' (SEQ.ID. NO. 11) V_HK75S, v6;

HX12, 5' GTAGATAAATCCAAAtctACAGC-CTAtCTGCAAATG 3' (SEQ.ID. NO. 12) V_H N76S, v7;

HX13, 5' GTAGATAAATCCtcttctACAGC-CTAtCTGCAAATG 3' (SEQ.ID. NO. 13) V_H K75S:N76S, v8;

X14, 5' CTTATAAAGGTGTTtCcACCTATaaCcA-gAaatTCAAGGatCGTTTCACgATAtc-CGTAGATAAATCC 3' (SEO.ID.NO. 14) V_H T57S:A60N:D61Q:S62K:V63F:G65D, v9;

LX6, 5' CTATACCTCCCGTCTgcatTCTGGAGTCCC 3' (SEQ.ID. NO. 15) V_L E55H, v11.

Oliconucleotides 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, supre. 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_H1gene 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 OD_{550} 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, suora). 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-phenylenedimalemide (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 centriprep-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 10^6 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 (8×10^3) 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 v_1 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_HK75S:N76S) and v12 (V_HK75S: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_HK75S (v6), V_HN76S (v7), V_HK75S:N76S (V8), V_LE55H (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-$p185^{HEE2}$ $F(ab')_2$ 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.

Example 4

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
```

-continued

```
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:

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 27

-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 CCAG 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 TGTTTCCACC TATAACCAGA AATTCAAGGA 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 Ser
122

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 454 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly
1 5 10 15

Ala Ser Val Lys Ile Ser Cys Lys Thr Ser Gly Tyr Thr Phe Thr
20 25 30

Glu Tyr Thr Met His Trp Met Lys Gln Ser His Gly Lys Ser Leu
35 40 45

Glu Trp Ile Gly Gly Phe Asn Pro Lys Asn Gly Gly Ser Ser His
50 55 60

Asn Gln Arg Phe Met Asp Lys Ala Thr Leu Ala Val Asp Lys Ser
65 70 75

Thr Ser Thr Ala Tyr Met Glu Leu Arg Ser Leu Thr Ser Glu Asp
80 85 90

Ser Gly Ile Tyr Tyr Cys Ala Arg Trp Arg Gly Leu Asn Tyr Gly
95 1 00 1 05

Phe Asp Val Arg Tyr Phe Asp Val Trp Gly Ala Gly Thr Thr Val
110 115 120

Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu
125 130 135

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

Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp
155 160 165

Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
170 175 180

Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val
185 190 195

Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
200 205 210

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys
215 220 225

Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu
230 235 240

Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys
245 250 255

Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val
260 265 270

Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr
275 280 285

Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
290 295 300

Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val
305 310 315

Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val
320 325 330

-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
```

-continued

```
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
```

(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
```

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.

* * * * *

D

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

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

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

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

Return To:

USPTO Home Page

Finance Online Shopping Page

United States Patent and Trademark Office

Maintenance Fee Statement **08/24/2006 03:59 PM EDT**

Patent Number: 6407213

Customer Number: 000000

GENENTECH, INC.
1 DNA WAY
SOUTH SAN FRANCISC CA 94080-4990

The data shown below is from the records of the U.S. Patent and Trademark Office. If the maintenance fee and any necessary surcharge have been timely paid for the patent listed below, the notation "PAID" will appear in the "STAT" column.

If the statement of small entity status is defective the reason will be indicated below in the "Small Entity" status column. THE STATEMENT OF SMALL ENTITY STATUS WILL BE ENTERED UPON RECEIPT OF ACCEPTABLE CORRECTION.

PATENT NUMBER	FEE AMT	SUR-CHARGE	U.S. APPLICATION NUMBER	PATENT ISSUE DATE	APPL. FILING DATE	PAYMENT YEAR	SMALL ENTITY?	STAT	ATTY DKT NUMBER
6,407,213	$900.00	$0.00	08/146,206	06/18/02	11/17/93	04	NO	PAID	709P1

Direct any questions about this notice to:
Mail Stop: M. Correspondence
Director of the United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450

Click here to obtain your Maintenance Fee Statement as a PDF.
Please note that in order to view your Maintenance Fee Statement as a PDF, you must have Adobe's Acrobat Reader installed. If you do not have it, download it for FREE!

Get Acrobat Reader

Need Help? | USPTO Home Page | Finance Online Shopping Page

Return To:

USPTO Home Page

Finance Online Shopping Page

United States Patent and Trademark Office

Patent Maintenance Fees		08/24/2006 03:59 PM EDT	
Patent Number:	6407213	**Application Number:**	08146206
Issue Date:	06/18/2002	**Filing Date:**	11/17/1993
Window Opens:	06/18/2009	**Surcharge Date:**	12/21/2009
Window Closes:	06/18/2010	**Payment Year:**	
Entity Status:	LARGE		
Customer Number:	000000		
Street Address:	GENENTECH, INC. 1 DNA WAY		
City:	SOUTH SAN FRANCISCO		
State:	CA		
Zip Code:	940804990		
Phone Number:	(000) 000-0000		
Currently there are no fees due.			

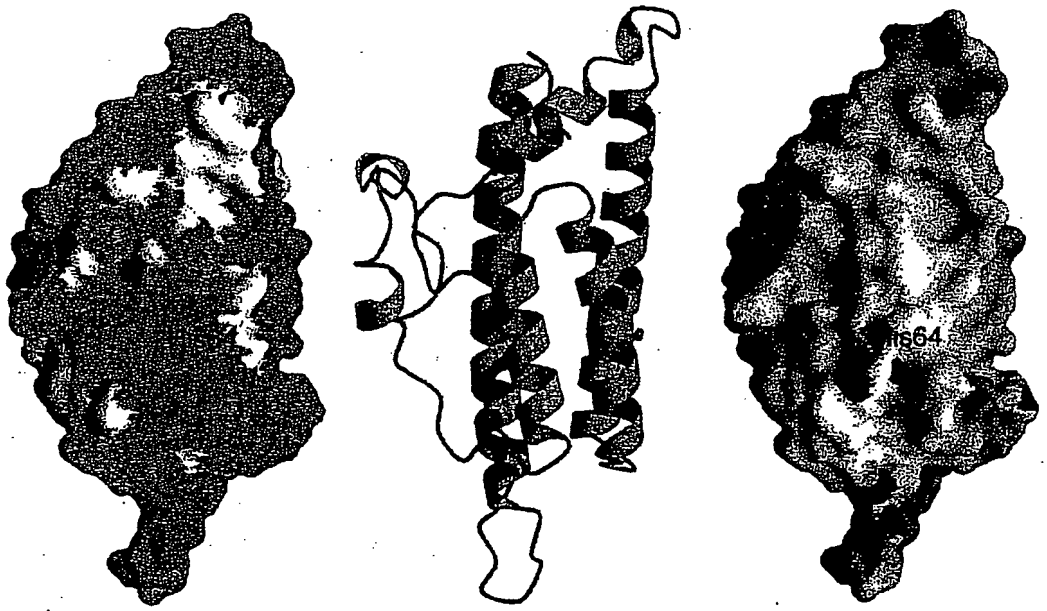
Need Help? | USPTO Home Page | Finance Online Shopping Page

Volume 293
Number 4
5 November 1999

CELEBRATING 40 YEARS 1959–1999 JMB

JMB

JOURNAL OF MOLECULAR BIOLOGY



IDEAL

ACADEMIC PRESS

Article No. jmbi.1999.3192 available online at http://www.idealibrary.com on IDEAL® J. Mol. Biol. (1999) 293, 865–881

JMB

Selection and Analysis of an Optimized Anti-VEGF Antibody: Crystal Structure of an Affinity-matured Fab in Complex with Antigen

Yvonne Chen[1], Christian Wiesmann[1], Germaine Fuh[1], Bing Li[1], Hans W. Christinger[1], Patrick McKay[2], Abraham M. de Vos[1] and Henry B. Lowman[1]*

[1]*Department of Protein Engineering, Genentech, Inc. 1 DNA Way, South San Francisco, CA 94080, USA*

[2]*Department of Process Sciences, Genentech, Inc. 1 DNA Way, South San Francisco, CA 94080, USA*

**Corresponding author*

The Fab portion of a humanized antibody (Fab-12; IgG form known as rhuMAb VEGF) to vascular endothelial growth factor (VEGF) has been affinity-matured through complementarity-determining region (CDR) mutation, followed by affinity selection using monovalent phage display. After stringent binding selections at 37 °C, with dissociation (off-rate) selection periods of several days, high affinity variants were isolated from CDR-H1, H2, and H3 libraries. Mutations were combined to obtain cumulatively tighter-binding variants. The final variant identified here, Y0317, contained six mutations from the parental antibody. *In vitro* cell-based assays show that four mutations yielded an improvement of about 100-fold in potency for inhibition of VEGF-dependent cell proliferation by this variant, consistent with the equilibrium binding constant determined from kinetics experiments at 37 °C. Using X-ray crystallography, we determined a high-resolution structure of the complex between VEGF and the affinity-matured Fab fragment. The overall features of the binding interface seen previously with wild-type are preserved, and many contact residues are maintained in precise alignment in the superimposed structures. However, locally, we see evidence for improved contacts between antibody and antigen, and two mutations result in increased van der Waals contact and improved hydrogen bonding. Site-directed mutants confirm that the most favorable improvements as judged by examination of the complex structure, in fact, have the greatest impact on free energy of binding. In general, the final antibody has improved affinity for several VEGF variants as compared with the parental antibody; however, some contact residues on VEGF differ in their contribution to the energetics of Fab binding. The results show that small changes even in a large protein-protein binding interface can have significant effects on the energetics of interaction.

Keywords: angiogenesis; humanized antibody-antigen complex; affinity maturation; phage display; X-ray crystallography

Introduction

Angiogenic factors (Folkman & Klagsbrun, 1987), which stimulate endothelial cells leading to new vascularization, have roles in such disease states as cancer, rheumatoid arthritis, and macular degeneration (reviewed by Ferrara, 1995; Folkman, 1995; Iruela-Arispe & Dvorak, 1997). Vascular endothelial growth factor (VEGF), a heparin-binding protein initially identified from pituitary cells (Ferrara & Henzel, 1989), is clearly a key angio-

Abbreviations used: CDR, complementarity-determining region; FR, framework region; HuVEC, human umbilical vein endothelial cell; $K_d^{25°}$, equilibrium dissociation constant determined at 25 °C; mAb, IgG form of monoclonal antibody; PBS, phosphate-buffered saline; SPR, surface plasmon resonance; VEGF, vascular endothelial growth factor; VEGF(109), receptor-binding fragment of VEGF with residues 8-109; VEGF(165), VEGF form with residues 1-165.

E-mail address of the corresponding author: hbl@gene.com

0022-2836/99/440865–17 $30.00/0

genic factor in development as well as in certain disease states, including the growth of solid tumors (reviewed by Ferrara, 1999). A murine monoclonal antibody, A.4.6.1, was found to block VEGF-dependent cell proliferation *in vitro* and to antagonize tumor growth *in vivo* (Kim *et al.*, 1993). The murine mAb was previously humanized in Fab form to yield a variant known as Fab-12 (Presta *et al.*, 1997). Both chimeric and humanized antibodies retained high affinity binding to VEGF, with an apparent equilibrium dissociation constant, $K_d^{25°}$, of 0.9 to 3 nM (Presta *et al.*, 1997; Baca *et al.*, 1997; Muller *et al.*, 1998a). The corresponding full-length IgG form of this antibody, rhumAb VEGF, is being developed as a possible therapeutic agent for the treatment of human solid tumors (Mordenti *et al.*, 1999).

We became interested in obtaining higher affinity variants of Fab-12 in order to test whether affinity improvements of this antibody might improve its potency and efficacy. Phage display of randomized libraries of antibodies and other proteins has been extensively used to engineer proteins with improved affinity and specificity (Lowman *et al.*, 1991; reviewed by Kay & Hoess, 1996; Rader & Barbas, 1997; Griffiths & Duncan, 1998). In particular, a phage-based *in vitro* affinity maturation process has been successful in improving the binding affinity of antibodies previously identified from traditional monoclonal or naive-library sources (e.g. Hawkins *et al.*, 1992; Marks *et al.*, 1992; Barbas *et al.*, 1994; Yang *et al.*, 1995; Schier *et al.*, 1996; Thompson *et al.*, 1996).

In previous work, the humanized anti-VEGF antibody Fab-12 was adapted for improved monovalent phage display through selection of a CDR-L1 variant, designated Y0192 (Muller *et al.*, 1998a). To select target residues for randomization and affinity optimization, we also previously screened all CDR residues, as defined by a combination of the hypervariable (Kabat *et al.*, 1987) and structurally defined (Chothia & Lesk, 1987) CDR residues. Fab variants of Y0192 generated by alanine scanning were analyzed for side-chain contributions to antigen binding (Muller *et al.*, 1998a). In addition, a crystal structure of Fab-12 in complex with the receptor-binding domain of VEGF, VEGF(109), was determined (Muller *et al.*, 1998a). The results of these studies showed that the antigen binding site is almost entirely composed of residues from the heavy chain CDRs, CDR-H1, H2, and H3. Therefore, these CDRs appeared most likely to provide the opportunity for improved binding interactions with antigen.

Here, we describe the selection of an affinity-improved anti-VEGF antibody by phage display and off-rate selection. We show that the affinity-matured antibody binds VEGF with at least 20-fold improved affinity and inhibits VEGF-induced cell proliferation with enhanced potency in a cell-based assay. We also report the crystal structure of an affinity-optimized antibody in complex with VEGF, to our knowledge, representing the first reported structure of an *in vitro* affinity-matured antibody:antigen complex. The structure, together with mutational analysis, shows that subtle changes in the antibody-antigen interface account for improved affinity.

Results

Library design

We used the results of an alanine-scanning analysis, combined with a crystal structure of the wild-type Fab fragment in complex with VEGF (Muller *et al.*, 1998a), to design targeted libraries within the antibody CDRs for random mutagenesis and affinity selection. This strategy enabled us to construct theoretically complete libraries with a small number of residues randomized in each CDR. Although sites remote from the antigen-combining region or buried within the protein could modulate antigen binding affinity indirectly and have in fact been exploited for affinity improvement (Hawkins *et al.*, 1993), clearly residues shown to be important by alanine scanning are useful starting points for binding-affinity optimization (Lowman *et al.*, 1991; Lowman & Wells, 1993). Furthermore, we reasoned that by making mutations at residues of the antibody CDRs which were known to affect antigen binding and were located at or near points of contact in the bound complex, we could minimize the possibility of other indirect effects which might alter stability, immunogenicity, or other properties of the antibody.

Both Ala-scanning and crystallography (Muller *et al.*, 1998a) identified CDR-H3 as the predominant contact segment for VEGF, consistent with the general observation that CDR-H3 is often key to antigen binding (Chothia & Lesk, 1987). Within CDR-H3, residues Y95, P96, H97, Y98, Y99, S100b, H100c, W100d, Y100e, and F100f (numbering is as described by Kabat *et al.* (1987)), all showed effects on binding over a range of twofold to >150-fold when mutated to Ala, and Ala substitution at S100a caused a slight improvement in binding. However, H100c, Y100e, and F100f were found to have little or no direct contact with VEGF and presumed to have indirect effects on binding. On the other hand, Y95 and W100d have significant contacts with VEGF, and Ala substitutions resulted in no detectable binding to VEGF. Therefore, these residues were excluded from optimization. Inspection of the complex structure suggested that substitutions at P96 and Y98 could be disruptive to the antibody structure, while G100, where Ala mutation had little effect, might tolerate further substitutions. We therefore constructed a library (YC81) which fully randomized positions H97, Y99, G100, S100a, and S100b, within CDR-H3.

Significant effects of Ala substitution were also found in CDR-H2. Here, W50, I51, N52, T52a, Y53, T54, T58 alanine mutants all showed >twofold loss in binding affinity, with the greatest residue surface area buried at positions W50, I51, Y53, and

T58 (Muller *et al.*, 1998a). Indeed, W50 along with other aromatic side-chains was observed to form a deep pocket into which a loop of VEGF inserts in the complex, and was excluded from further optimization. Residue I51, on the other hand, showed no direct contact with VEGF and was also excluded. Residue T58 had multiple interactions within the interface, including contacts with VEGF and with the critical W50 of the CDR pocket. Although E56 showed no contact with VEGF and little effect (<twofold) upon alanine substitution, its side-chain lies at the periphery of the interface, near several hydrophobic residues of VEGF. We reasoned that these might be exploited for additional binding interactions. Two CDR-H2 libraries were constructed: YC266, randomizing positions T52a, Y53, T54, and E56; and YC103, randomizing positions N52, T52a, Y53, and T54.

In CDR-H1 G26, Y27, F29, N31, Y32, G33, M34, and N35 were implicated by alanine mutagenesis as important for binding VEGF; however, only N31, Y32, and G33 had significant direct contacts with VEGF. Since Ala substitution of G33 showed reduced binding, larger side-chains seemed less desirable; for this reason, this position was not randomized. Residues 27 (buried in the antibody structure) and T28 and T30 (which are mutually contacting) were included at the end of the H1 loop as possible indirect determinants of binding. Residues 27, 28, and 30-32 were randomized in a library designated YC265.

Framework residues, especially heavy chain residues 71 and 93, normally outside the region of contact with antigen, have also been found to affect antibody binding affinity (Tramontano *et al.*, 1990; Foote & Winter, 1992; Hawkins *et al.*, 1993; Xiang *et al.*, 1995), and sometimes participate in antigen contacts (reviewed by Nezlin, 1998). Therefore, an additional region of the anti-VEGF Fab, within FR-H3 and including position 71, was also targeted for randomization. Since the residue 71-76 region has contacts with CDR-H1 (at F29) and CDR-H2 (at I51 and T52a), these represented potential sites for affinity improvement through secondary effects on the interface residues. Residues L71, T73, and S76 were randomized in this FR-H3 library.

Phage selections

Fab libraries were constructed using a fusion to the g3p minor coat protein in a monovalent phage display (phagemid) vector (Bass *et al.*, 1990; Lowman *et al.*, 1991). For each library, stop codons were introduced by mutagenesis into the Y0192 phage template (Muller *et al.*, 1998a) at each residue position to be randomized. Each stop-codon construct was then used for construction of a fully randomized (using NNS codons) library as described in Materials and Methods. Phage were precipitated from overnight *Escherichia coli* shake-flask cultures and applied to VEGF-coated immunosorbant plates for binding selections. Cycles of selection followed by amplification were carried out essentially as described (Lowman, 1998).

We used an off-rate selection process (see Materials and Methods) similar to previously described procedures (Hawkins *et al.*, 1992; Yang *et al.*, 1995), modified by gradually increasing the selective pressure for binding to antigen during successive cycles of enrichment. The enrichment factor (ratio of displaying phage to non-displaying phage eluted *versus* applied) was used to monitor the stringency of selection at each step (Table 1). As a control, and to obtain a relative measure of affinity improvement, Y0192-phage were subjected to the same procedure at each cycle.

Fab-phage clones were sequenced from several phage-binding selection rounds that showed enrichment for Fab-phage over non-displaying phage. From round 6 of the CDR-H1 library selections, a dominant clone, Y0243-1 was found, having wild-type residues at Y27, T30, and Y32, and substitutions T28D and N31H (Table 2). Additional clones had related sequences, with N31H found in all selectants; Asp or Glu substituting for T28; and Thr, Ser, Gln, or Gly found at position T30.

Table 1. Enrichment factors from phage-displayed Fab libraries

Round	Wash time (hours)	CDR-H1 YC265	CDR-H2 YC266	CDR-H2 YC103	CDR-H3 YC81	FR-H3 YC101	Control Y0192
1	0	8.2	1.7	1.3	3.3	4	1.5
2	1	1.6	25	0.7	10	110	90
3	2	340	880	100	570	2300	22000
4	18	6800	880	5200	3700	600	2700
5	37[a]	210	900	920	1300	480	32
6	47[a]	130	80	100	3500	30	20
7	63[a]	1	1	>3	>25	1	>8

Libraries are designated by CDR region and oligonucleotide label (see the text for details). Library Fab-phage (ampicillin-resistant) were mixed with non-displaying control phage (chloramphenicol-resistant) in each starting pool, and subjected to VEGF binding selection, washing, and elution as described in the text.

The enrichment factor for each library is reported here as the ratio of Amp/Cam colony-forming units in the eluted pool, divided by the ratio of Amp/Cam colony-forming units in the starting pool. Starting phage concentrations were about 10^{12}/ml, except 10^{13}/ml in round 1. The wild-type Fab-phage, Y0192 was included at each round for comparison of enrichment under the particular conditions used.

[a] In some cases, the wash-step included incubation at 37 °C.

Table 2. Anti-VEGF Fab variants selected from a CDR-H1 library (HL-265)

Variant	*n*	Y 27	T 28	T 30	N 31	Y 32	I 34[a]	K_d(Y0192)/ K_d(variant)
Round 6 (HCl)								
Y0243-1	5	Y	D	T	H	Y	M	3.1
Y0243-2	1	Y	E	Q	H	Y	M	
Y0243-3	1	Y	E	T	H	Y	M	
Y0243-4	1	Y	D	G	H	Y	M	
Y0243-5	1	Y	D	S	H	Y	M	
Y0243-6	1	Y	E	S	H	Y	M	
Consensus:		Y	D	T	H	Y	M	3.1

All variants are in the background of Y0192 (Muller *et al.*, 1998a). *n* indicates the number of clones found with identical DNA sequence. The wild-type (Y0192) residue is shown at the top of each column, and the sequence position number is indicated according to Kabat *et al.* (1987).

[a] Position 34 was not randomized, but was changed to Met (as in Fab-12) in this library. The consensus reported here, equivalent to clone Y0243-1, represents the most abundant amino acid residue at each position (including clones with multiple representation ($n > 1$)). K_d(Y0192)/K_d (variant) indicates the fold increase in binding affinity *versus* the wild-type humanized antibody Y0192 (see Table 6).

Clones from two independently constructed CDR-H2 libraries were remarkable in that all sequenced library clones conserved wild-type residues at virtually all positions mutated, except at position Y53, where all clones contained a Trp substitution (Table 3).

Because of the strong enrichment observed from the CDR-H3 library, a number of clones were sequenced from rounds 5 and 7 (Table 4). Of 39 sequenced clones, 37 retained the wild-type residue S100b, and all contained the mutation H97Y. The remaining positions showed greater diversity, even after seven cycles of selection. The dominant clone at round 7, Y0238-3, contained the mutation S100aT (in addition to H97Y), with wild-type residues Y99 and G100. Other substitutions observed included Lys or Arg for Y99 (in 18 of 39 clones), G100N (11 of 39 clones), and a variety of substitutions including Arg, Glu, Gln, and Asn at S100a. In this library, the consensus sequence is represented by the dominant clone, Y0238-1 (Table 4).

Clones from round 6 of the FR-H3 library (Table 5) showed conservation of wild-type residue S76, with wild-type residues or various substitutions at the remaining positions: Val or Ile substituting for L71, and Val or Lys substitutions at T73.

Binding affinity of selected variants

For measurements of binding affinity, we made use of an amber stop codon placed between the genes for the Fab heavy chain and the g3p C-terminal domain, and expressed soluble Fab variants from *E. coli* shake-flask or fermentation cultures. Fab variants purified from protein-G affinity chromatography were characterized for binding affinity using an SPR-based assay on a BIAcore™-2000 instrument. The binding-kinetics assay has been described (Muller *et al.*, 1998a).

Association kinetics (k_{on}) for the wild-type antibody binding to immobilized VEGF are slow (Presta *et al.*, 1997; Baca *et al.*, 1997; Muller *et al.*, 1998a), and none of the variants tested had significantly improved on-rates. On the other hand, dissociation kinetics varied over a range of 10^{-4} s^{-1} to $\leqslant 4 \times 10^{-6}$ s^{-1} at 25 °C (Table 6). Based on measurements of instrumental drift, we could not accurately measure k_{off} (and consequently K_d)

Table 3. Anti-VEGF Fab variants selected from CDR-H2 libraries (HL-266, YC103)

Variant	*n*	N 52[a]	T 52a	Y 53	T 54	G 55[a,b]	E 56[a]	K_d(Y0192)/ K_d(variant)
Round 6 (HCl)								
HL266-A[b]	6	N	T	W	T	G	E	1.3
HL266-E	1	N	T	W	T	G	T	
HL266-I	1	N	T	W	T	G	Q	
YC103-A[b]	7	N	T	W	T	G	E	1.3
YC103-C	1	N	T	W	D	G	E	
Consensus		N	T	W	T	G	E	1.3

All variants are in the background of Y0192 (Muller *et al.*, 1998a). *n* indicates the number of clones found with identical DNA sequence. The wild-type (Y0192) residue is shown at the top of each column, and the sequence position number is indicated according to Kabat *et al.* (1987). The consensus reported here, equivalent to clones HL266A and YC103A, represents the most abundant amino acid at each position (including clones with multiple representation; i.e. $n > 1$). K_d(Y0192)/K_d(variant) indicates the fold increase in binding affinity *versus* the wild-type humanized antibody Y0192 (see Table 6).

[a] Constant positions were position 52 in the HL-266 library and position 56 in the YC103 library.

[b] Equivalent clones are assumed to have equal affinity.

Table 4. Anti-VEGF Fab variants selected from a CDR-H3 library (YC81)

Variant	*n*	H 97	Y 99	G 100	S 100a	S 100b	K_d(Y0192)/K_d(variant)
Round 5 (VEGF)							
Y0228-21	1	Y	R	N	A	S	
Y0228-22	1	Y	T	T	R	S	
Y0228-23	1	Y	E	G	S	S	
Y0228-24	1	Y	R	Q	R	G	
Y0228-26	1	Y	T	G	R	S	
Y0228-27	1	Y	T	N	T	S	
Y0228-28	1	Y	R	K	G	S	
Y0228-29	1	Y	T	G	S	S	
Y0228-30	1	Y	R	S	G	S	
Round 5 (HCl)							
Y0229-20	1	Y	T	N	R	S	
Y0229-21	1	Y	R	N	S	S	
Y0229-22	1	Y	K	E	S	S	
Y0229-23	1	Y	R	D	A	S	
Y0229-24	1	Y	R	Q	K	G	
Y0229-25	1	Y	K	G	G	S	
Y0229-26	1	Y	Y	G	A	S	
Y0229-27	1	Y	R	G	E	S	
Y0229-28	1	Y	R	S	T	S	
Y0238-10[a]	1	Y	R	N	T	S	3.8
Round 7 (HCl)							
Y0238-3	6	Y	Y	G	T	S	⩾9.4
Y0238-1	2	Y	R	G	T	S	7.3
Y0238-2	2	Y	I	N	K	S	
Y0238-10[a]	2	Y	R	N	T	S	3.8
Y0238-4	1	Y	Y	N	Q	S	
Y0238-5	1	Y	I	A	K	S	2.1
Y0238-6	1	Y	R	D	N	S	⩾5.4
Y0238-7	1	Y	W	G	T	S	
Y0238-8	1	Y	R	Q	N	S	
Y0238-9	1	Y	R	Q	S	S	
Y0238-11	1	Y	K	N	T	S	
Y0238-12	1	Y	I	E	R	S	
Consensus		Y	R	G	T	S	7.3

All variants are in the background of Y0192 (Muller *et al.*, 1998a). The clones are grouped according to the round of selection (5 or 7) and the type of elution (VEGF competition or HCl elution) used for recovery of bound phage. *n*, indicates the number of clones found with identical DNA sequence within each group. The wild-type (Fab-12, or Y0192) residue is shown at the top of each column, and the sequence position number is indicated according to Kabat *et al.* (1987). The consensus reported here, equivalent to clone Y0238-1, represents the most abundant amino acid at each position (including clones with multiple representation (n > 1)). K_d(Y0192)/K_d(variant) indicates the fold increase in binding affinity *versus* the wild-type humanized antibody Y0192 (see Table 6).

[a] One clone was identified at both rounds 5 and 7. Equivalent clones are assumed to have equal affinity.

under these conditions, but instead used the kinetics data to place an upper limit on K_d.

The phage-derived Fab variants tested showed a range of small (within experimental error of about twofold) to significant (>fivefold) improvements in binding affinity over the wild-type (parental phage) antibody Y0192 (Table 6). From the CDR-H1 library, the dominant clone (Y0243-1) showed threefold improved affinity. Variant Y0242-1, the dominant clone in each of three CDR-H2 libraries, showed an affinity equivalent to wild-type within experimental error, and two clones derived from the FR-H3 library (Y0244-1 and Y0244-4) were equivalent or slightly weaker in affinity. Small

Table 5. Anti-VEGF Fab variants selected from a FR-H3 library

Variant	*n*	L 71	T 73	S 76	K_d(Y0192)/K_d(variant)
Round 6 (HCl)					
Y0244-1	1	V	V	S	0.3
Y0244-2	1	L	K	S	
Y0244-3[a]	1	L	V	S	
Y0244-4	1	I	K	S	0.9

All variants are in the background of Y0192 (Muller *et al.*, 1998a). *n*, indicates the number of clones found with identical DNA sequence. The wild-type (Fab-12, or Y0192) residue is shown at the top of each column, and the sequence position number is indicated according to Kabat *et al.* (1987). K_d(Y0192)/K_d(variant) indicates the fold increase in binding affinity *versus* the wild-type humanized antibody Y0192 (see Table 6).

[a] One variant contained a spontaneous mutations, S74W.

Table 6. Binding kinetics of anti-VEGF Fab variants at 25 °C

Variant	$k_{on}/10^4$ (M^{-1} s^{-1})	$k_{off}/10^{-4}$ (s^{-1})	K_d (nM)	K_d(Y0192)/K_d(variant)
Y0192[a]	4.1	1.2	2.9	1
A. *Library-derived*				
Y0238-1	2.6	0.09	0.4	7.3
Y0238-3	1.3	≤0.04[b]	≤0.3[b]	≥9.4[b]
Y0238-5	0.57	0.08	1.4	2.1
Y0238-7	1.1	≤0.06[b]	≤0.5[b]	≥5.4[b]
Y0238-10	1.2	0.09	0.8	3.8
Y0242-1	3.8	0.86	2.3	1.3
Y0243-1	4.8	0.45	0.9	3.1
Y0244-1	3.0	2.7	9.0	0.3
Y0244-4	5.2	1.7	3.3	0.9
B. *Engineered*				
Y0268-1	4.0	0.15	0.38	7.6
Y0313-1	3.5	≤0.05[b]	≤0.15[b]	≥20[b]
Y0192(T28D)	6.8	1.4	2.0	1.4
Y0192(N31H)	4.8	0.37	0.8	3.6
Y0192(H97Y)	2.5	0.045	0.2	14
Y0192(S100aT)	6.8	1.0	1.5	1.9
Y0317	3.6	≤0.05[b]	≤0.14[b]	≥20[b]

Kinetic constants were determined from measurements using a BIAcore™-2000 instrument with a biosensor chip containing immobilized VEGF(109). Measurements were performed at 25 °C. Fab concentrations were calculated from quantitative amino acid analysis. The equilibrium dissociation constant, K_d, is calculated form the ratio of the rate constants, k_{off}/k_{on}. The relative affinity, reported as K_d(Y0192)/K_d(variant) indicates the fold increase in binding affinity versus the wild-type humanized antibody Y0192. Errors in K_d were approximately ±25%. Variant Y0242-1 corresponds to the point mutations Y53W in CDR-H2 of Fab Y0192; for descriptions of the other variants, see Tables 2, 3, 4, 5, and 8.

[a] Data for Y0192 is from Muller *et al.* (1998a,).

[b] In some cases, the dissociation rate constant observed was at or near the limit of detection; therefore, the reported k_{off} and K_d are upper limits, and the relative affinities are an upper limit.

improvements were seen in CDR-H3 variants Y0238-5 and Y0238-10. However, larger improvements (exceeding the limits of measurement (>fivefold to >ninefold)) were observed for the CDR-H3 variants Y0238-1, Y0238-3, and Y0238-7.

All tested variants (in fact all sequenced clones) from the CDR-H3 library contained the mutation H97Y. In the higher affinity group, Gly was conserved at position 100, while the lower affinity variant contained Ala (known to cause 1.8-fold reduction in Y0192 binding; Muller *et al.*, 1998a) or Asn (Table 4). The S100a position, while quite varied among sequenced clones, was changed to Thr in the higher affinity CDR-H3 variants, and Thr or Lys in the lower affinity ones. Substitutions at Y99, though mostly confined to basic or aromatic residues, apparently had little effect since Y0238-1 (representing the consensus CDR-H3 sequence with Y99R) was not significantly different in affinity from Y0238-3, which retained Y99.

Affinity improvements from combinations of CDR mutations

To improve affinity further, several combinations of the phage-selected CDR-H1, H2, and H3 mutations were made by site-directed mutagenesis (Table 7). Among these, the highest affinity was obtained with pY0313-1 (i.e. pY0192 with mutations CDR-H1 (T28D/N31H/I34M) and CDR-H3 (H97Y/S100aT); note I34M is a reversion to Fab-12 wild-type). From BIAcore™ kinetics measurements carried out at 25 °C, this Fab variant had ≥20-fold higher affinity than Y0192 (Table 6).

Addition of the Y53W mutation, which alone produced little or no improvement over Y0192, to Y0313-1 (producing variant Y0268-1) actually reduced binding affinity by >twofold (Table 6).

The final Fab version was constructed by removing the phage-expression enhancing mutations in CDR-L1 from pY0313-1 by site-directed mutagen-

Table 7. Anti-VEGF CDR combination variants

	CDR-L1					CDR-H1			CDR-H2	CDR-H3	
Y0192: Variant	R 24	N 26	E 27	Q 28	L 29	T 28	N 31	I 34	Y 53	H 97	S 100a
Y0313-1	-	-	-	-	-	D	H	M	-	Y	T
Y0268-1	-	-	-	-	-	D	H	M	W	Y	T
Y0317	S	S	Q	D	I	D	H	M	-	Y	T
Fab-12	S	S	Q	D	I	-	-	-	-	-	-

Substitutions are shown relative to Y0192. Fab-12 also contains T221 in the heavy chain. Dashes (-) indicate no substitution. Numbering is according to Kabat *et al.* (1987) for both the light chain (CDR-L1) and heavy chain (CDR-H1, H2, H3).

esis. The M4L substitution was identified during phage-humanization experiments (Baca *et al.*, 1997), and the Leu residue was retained so as to preclude possible oxidation of the Met side-chain. The first libraries were constructed from a Fab-12 phagemid derivative, pY0101, which contained a buried framework mutation, V_L(M4L), as well as a mutation (T221L) at the junction to g3p. Thus the final version, Y0317 (Table 7 and Figure 1) differs from Fab-12 by the following six mutations: V_L(M4L), V_H(T28D/N31H/H97Y/S100aT/T221L).

Each of the CDR mutations in H1 and H3 was tested for its effect on VEGF binding affinity by introducing the corresponding point mutation into the parental Y0192 Fab and measuring binding kinetics. The results (Table 6) show a 14-fold and 3.6-fold improvement with substitution of H97Y or N31H, respectively, into the parental Fab. However, T28D or S100aT had identical affinity to Y0192, within experimental error.

We compared Fab-12 and Y0317 Fab affinities in a solution binding assay, using VEGF competition with [^{125}I]VEGF for binding to Fab. The results showed Fab-12 having $K_d^{25°}$ = 433 pM and Y0317 Fab having $K_d^{25°}$ = 20 pM, a 22-fold improvement in binding affinity (Figure 2).

Because dissociation kinetics in surface plasmon resonance (SPR) experiments exceeded instrumental capabilities at 25 °C, and in order to assess binding affinity under more physiological conditions, we compared binding affinities of the original humanized antibody Fab-12 with the final variant Y0317 in kinetics experiments at 37 °C. k_{on} and k_{off} were faster for both antibodies than at 25 °C, and k_{off} was clearly measurable above background. Using either immobilized VEGF(109) or immobilized VEGF(165), Y0317 was 120-fold to 140-fold improved in affinity over Fab-12, with a $K_d^{37°}$ of 80-190 pM (Table 8).

VEGF Ala-scan of the Y0317 binding epitope

In order to understand how mutations in the Fab affected binding affinity to VEGF, we also tested VEGF variants for binding to the affinity-improved antibody. For these experiments, we made use of the full-length IgG forms of Fab-12 (known as rhuMab VEGF) and Y0317 (termed Y0317-IgG) produced in CHO cells (V. Chisholm, unpublished results). These VEGF variants were previously used for mapping the parental antibody's binding site on VEGF (Muller *et al.*, 1998a).

In this assay, carried out at 37 °C, VEGF competed with biotin-VEGF with an IC_{50} of 9 nM in binding rhuMab VEGF, compared with an IC_{50} of 1 nM for Y0317-IgG (Table 9). SPR measurements have shown similar affinity improvement of Y0317-IgG over rhuMAb VEGF (H. Lowman, unpublished results).

Alanine mutations of VEGF that affected rhuMab VEGF binding also affected Y0317-IgG. For example, M81A, G88A, and G92A all caused large (100 to >500-fold) losses in binding affinity. And smaller reductions (3 to 30-fold) in binding affinity for both antibodies were seen for I80A, K84A, I91A, E93A, and M94A.

However, significant differences in the magnitude of the effect were observed at certain sites, including Y45A, fourfold reduced in affinity for rhuMAb VEGF *versus* 26-fold for Y0317-IgG; Q89A, 19-fold *versus* sixfold; and M94A, 11-fold versus 25-fold. Most surprisingly, two mutations that led to loss of detectable binding affinity for rhumAb VEGF (>500-fold) had only modest effects (four- to ninefold) on binding to Y0317-IgG. These differences might suggest a shift in the binding epitope of the antibody, and this possibility was addressed with receptor-inhibition assays and structural analysis, both described below.

Inhibition of VEGF activity

Cell-proliferation assays have been described (Fairbrother *et al.*, 1998) for the measurement of VEGF mitogenic activity on human umbilical vein endothelial cells. Here, we compared the potency of Fab-12 and the affinity-improved variants Y0238-3 and Y0313-1.

The results (Figure 3) show both variants Y0238-3 and Y0313-1 inhibit VEGF activity more potently than Y0192 Fab. Comparing the Fab forms, variant Y0313-1 appeared at least 30-fold to 100-fold more potent than the wild-type Fab. In additional experiments, Y0317 activity was similar to that of Y0313-1 (data not shown). It should be noted that the amount of VEGF (0.2 nM) used in this assay is potentially limiting for determination of an accurate IC_{50} for the mutant. For example, if the bind-

Table 8. Binding kinetics of anti-VEGF Fab variants at 37 °C

Variant	Immobilized	$k_{on}/10^4$ (M^{-1} s^{-1})	$k_{off}/10^{-4}$ (s^{-1})	K_d (nM)	K_d(Fab-12)/K_d(variant)
Fab-12	VEGF(109)	5.1	6.6	13 ± 2.2	1
Y0317	VEGF(109)	5.4	0.059	0.11 ± 0.02	120
Fab-12	VEGF(165)	5.5	11	20 ± 3.8	1
Y0317	VEGF(165)	5.3	0.074	0.14 ± 0.05	140

Kinetic constants were determined by injecting Fab solutions onto a BIAcore™-2000 instrument with a biosensor chip containing approximately 190 RU of immobilized VEGF(109) or VEGF(165), as indicated. The equilibrium dissociation constant, K_d, is calculated from the ratio of the rate constants, k_{off}/k_{on}. The relative affinity, reported as K_d(Fab-12)/K_d(variant) indicates the fold increase in binding affinity *versus* the original humanized antibody (Fab-12; Presta *et al.*, 1997) under the specified conditions.

```
Light chain:

                1        10        20        30        40        50
Fab-12          DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIYF
Y0192           DIQLTQSPSSLSASVGDRVTITCRANEQLSNYLNWYQQKPGKAPKVLIYF
Y0317.          DIQLTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKPGKAPKVLIYF

                1        10        20        30        40        50
                       60        70        80        90       100
Fab-12          TSSLHSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQ
Y0192           TSSLHSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQ
Y0317           TSSLHSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYSTVPWTFGQ

                       60        70        80        90       100
                      110       120       130       140       150
Fab-12          GTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV
Y0192           GTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV
Y0317           GTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV

                      110       120       130       140       150
                      160       170       180       190       200
Fab-12          DNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG
Y0192           DNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG
Y0317           DNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG

                      160       170       180       190       200
                      210
Fab-12          LSSPVTKSFNRGEC
Y0192           LSSPVTKSFNRGEC
Y0317           LSSPVTKSFNRGEC

                      210
```

Figure 1 (*legend shown opposite*)

ing affinity (K_d) of the mutant is in fact <0.2 nM, then the IC_{50} in this experiment will appear higher than under conditions of lower VEGF concentration. The result therefore supports the conclusion that the affinity-improved variant is at least 30-fold improved in affinity for VEGF, and that it effectively blocks VEGF activity *in vitro*.

Structure of the complex

In order to compare the structure and binding site of the affinity-improved antibody with that of the parental antibody, we determined the complex structure by X-ray crystallography. Crystals of the complex between the receptor binding domain of VEGF (residues 8 to 109) and the affinity-matured Fab Y0317 were grown as described (see Materials and Methods) and diffracted to a maximum resolution of 2.4 Å. The structure was refined starting from the coordinates of the complex between VEGF and the parent of Fab Y0317, Fab-12 (Muller *et al.*, 1998a), and refined to an R-value of 19.9% (R_{free} = 27.4%) for the reflections between 20 Å and 2.4 Å resolution.

Heavy chain:

```
                1       10        20        30        40        50
Fab-12          EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQAPGKGLEWVGW
Y0192           EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGINWVRQAPGKGLEWVGW
Y0317           EVQLVESGGGLVQPGGSLRLSCAASGYDFTHYGMNWVRQAPGKGLEWVGW

                1       10        20        30        40        50
                       60        70        80        90       100
Fab-12          INTYTGEPTYAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYP
Y0192           INTYTGEPTYAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYP
Y0317           INTYTGEPTYAADFKRRFTFSLDTSKSTAYLQMNSLRAEDTAVYYCAKYP

                 a      60        70        80  abc   90     96
                      110       120       130       140       150
Fab-12          HYYGSSHWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGC
Y0192           HYYGSSHWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGC
Y0317           YYYGTSHWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGC

                100abcdef 110       120       130       140
                     160       170       180       190       200
Fab-12          LVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLG
Y0192           LVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLG
Y0317           LVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLG

                     150       160       170       180       190
                     210       220       230
Fab-12          TQTYICNVNHKPSNTKVDKKVEPKSCDKTHT
Y0192           TQTYICNVNHKPSNTKVDKKVEPKSCDKTHL
Y0317           TQTYICNVNHKPSNTKVDKKVEPKSCDKTHL

                     200       210       220
```

Figure 1. Sequence alignment of the original humanized antibody (Fab-12; Presta *et al.*, 1997), the phage-displayed antibody (Y0192; Muller *et al.*, 1998a) and the affinity-improved antibody (Y0317). Sequential numbering of each chain is shown above the sequences; numbering according to Kabat *et al.* (1987) is shown below. CDR regions are underlined. Positions at which Y0317 differs from Fab-12 are indicated with double underlining.

The final model consists of two Fab fragments bound to the symmetrical poles of the VEGF dimer. Only residues 14–107 of each VEGF monomer are well defined in the electron density, and therefore the six N-terminal and the two C-terminal residues of each monomer were omitted from the model. Each Fab light chain comprises residues 1 to 213, with the C-terminal residue disordered, whereas for each heavy chain residues 138 to 143 as well as the six C-terminal residues are absent from the model. As in the parental Fab complex, two out of 1050 residues, namely T51 in the V_L chain of each Fab fragment, are located in the "disallowed regions" (Laskowski *et al.*, 1993) of the Ramachandran plot; 85 % of all residues have their main-chain torsion angles in the "most favored"

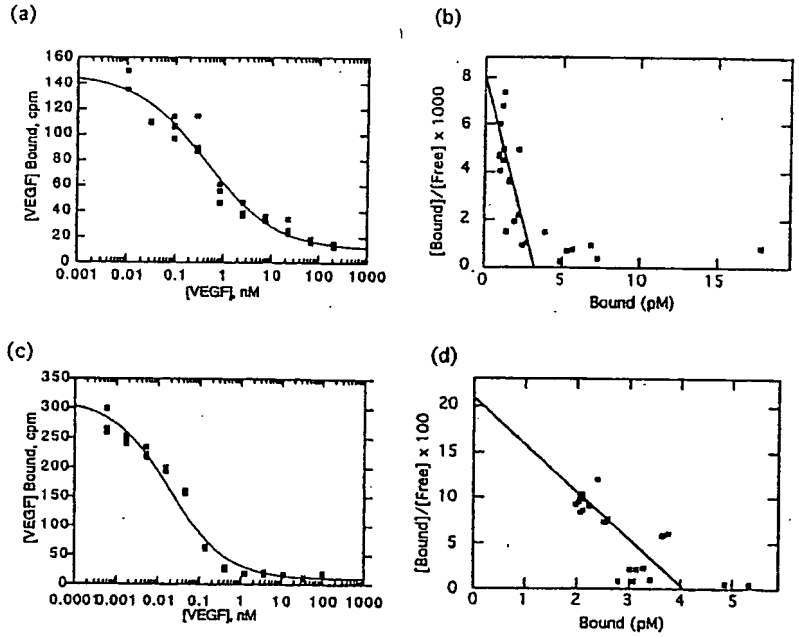


Figure 2. Radiolabeled VEGF binding assay. [^{125}I]VEGF was equilibrated (23 °C) with serial dilutions of unlabeled VEGF and (a) Fab-12 or (c) Y0317. Fabs were captured with an anti-Fab antibody-coated immunosorbant plate. Scatchard analysis (Munson & Rodbard, 1980) with a 1:1 binding model was used to calculate K_d of (b) 433 (±116) pM for Fab-12 and (d) 19.8(±4.3) pM for Y0317.

regions. The average *B*-factor of the model is 51.8 Å^2 and the mobility of the individual domains follows the pattern that was previously observed in the crystal structure of VEGF in complex with the Fab-12, with the constant domain dimer (C_L:C_H1) of one of the Fabs poorly ordered (Muller *et al.*, 1998a).

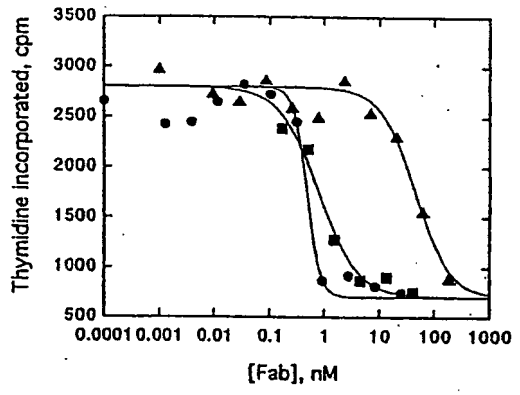


Figure 3. Human umbilical vein endothelial cell (HuVEC) assay of VEGF inhibition. Cells were cultured in the presence of 0.2 nM VEGF and serial dilutions of Fab Y0192 (triangles), Y0238-3 (squares), or Y0313-1 (circles). Cell proliferation was measured by incorporation of [^{3}H]thymidine. Curves show four-parameter fits to the data. Each point represents the mean of three treated wells.

Comparison of the final model with that of the parental Fab-VEGF complex (Muller *et al.*, 1998a) shows that the bound structures are very similar overall (Figure 4(a)) with Y0317 binding to the same site on VEGF as Fab-12 (Figure 4(b)). Side-chains show excellent overlap, and the main-chain structures show very little difference. The most prominent difference in contact residues is at H97Y (Figure 4(c); discussed below), where the tyrosine side-chain packs more favorably with VEGF and a buried water molecule from the parental Fab-VEGF complex is absent in the Y0317-Fab-VEGF complex.

Discussion

Antibody binding selections and affinity improvement

Here we made use of results from alanine-scanning and the previous structure of a humanized antibody-antigen complex to design Fab-phage libraries that randomized the three heavy-chain CDRs as well as one framework region (FR-H3) for improving the binding affinity of an anti-VEGF antibody. Affinity-improved Fab variants were obtained, with the largest effects seen in variants from the CDR-H3 library, although significant improvement was also obtained from mutation of CDR-H1. We therefore combined two mutations from H1 with two from H3, generating a further improved variant, Y0317. By making point mutations, we showed that the 20-fold (Figure 2)

Table 9. Alanine scan of VEGF by ELISA at 37 °C

VEGF(109) variant	IC_{50} (variant)/IC_{50} (VEGF)	
	Fab12-IgG	Y0317-IgG
VEGF(109)	1	1
F17A	1	1
Y21A	1	1
Y45A	4	26
K48A	2	1
Q79A	1	3
I80A	4	5
M81A	>500	930
R82A	>500	4
I83A	>500	9
K84A	3	10
H86A	1	1
Q87A	1	1
G88A	105	87
Q89A	19	6
H90A	1	1
I91A	2	6
G92A	>500	>900
E93A	4	7
M94A	11	25

ELISA assays were carried out using the full-length IgG form of Fab-12 or the IgG form of Y0317 and VEGF(109). Incubation of antibody with VEGF was at 37 °C for five hours. The IC_{50} for inhibition by each Ala mutant was evaluated using a four-parameter equation, and the relative affinities calculated as IC_{50}(mutant VEGF)/IC_{50}(wild-type VEGF). Under these conditions, Fab12-IgG and Y0317-IgG showed IC_{50} values of 9 nM and 1 nM, respectively.

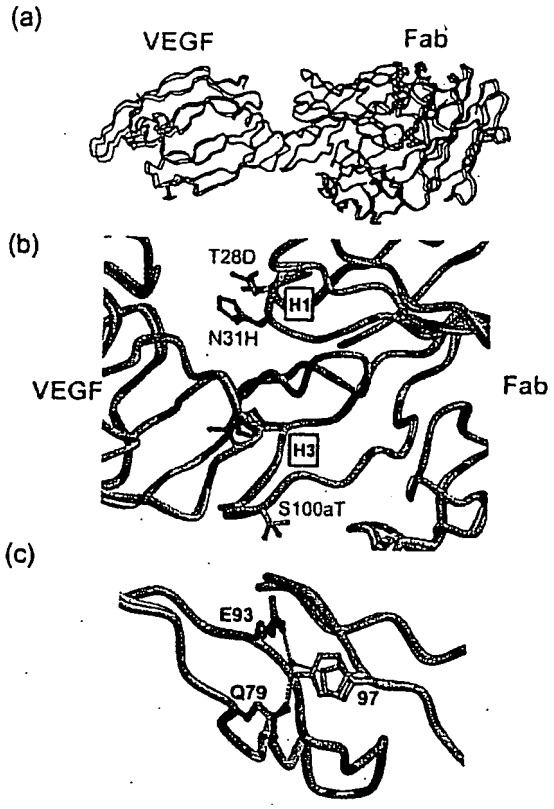


Figure 4. Structure of the affinity-improved Y0317 Fab in complex with VEGF. A superposition of the structure (Muller *et al.*, 1998a) of wild-type humanized antibody Fab-12 (gray) in complex with VEGF (gray) is shown with that of Fab Y0317 (green) in complex with VEGF (yellow). (a) Overall view of the complex, including one Fab molecule bound to one dimer of VEGF (a second Fab molecule is bound at left in the crystal) shows that the binding site for both antibody variants centers on the "80's loop" of VEGF. (b) A view of the four CDR changes between Fab-12 and Y0317 Fab shows that the new D28 and T100a side-chains do not directly contact antigen. However, H31 and Y97 form new contacts. (c) Interactions of H97 and an associated, buried water molecule in the Fab-12 complex, compared with those of Y97 in the Y0317 complex.

to >100-fold (Table 8) affinity improvement in Y0317 can be attributed to two CDR mutations: H97Y and N31H. In fact, H97Y alone improves binding affinty 14-fold.

Despite the relatively slow k_{on} and slow k_{off} of the parental antibody, binding selections described here yielded slower dissociation rates and improved equilibrium dissociation constants. Results of SPR measurements demonstrated that affinity is enhanced mainly through a slower dissociation rate (as opposed to faster association). These results are consistent with the idea of off-rate selection (Hawkins *et al.*, 1992) and with the progressively increased stringency in washing procedures used here (see Materials and Methods and Table 1). Previous binding-optimization efforts have also often yielded larger improvements in k_{off} than in k_{on} (see Lowman & Wells, 1993; Yang *et al.*, 1995; Schier *et al.*, 1996). This may suggest fundamental limitations to the improvements in k_{on} for a given binding site. Even if no conformational changes need occur between free and bound states, the on-rate is limited by the size of the binding interface and the translational and rotational diffusion rates of the binding components (reviewed by Delisi, 1983).

The association rate constants (k_{on}) for both the wild-type Y0192 and the final Y0317 antibodies are relatively slow (about 4×10^4 M^{-1} s^{-1} for both) compared to other antibodies of equal or weaker antigen binding affinity. In fact, the fastest k_{on} identified for any mutant was 6.8×10^4 M^{-1} s^{-1} (Table 6). Typically, k_{on} for antibodies binding to protein antigens, including affinity-matured antibodies, has fallen in the range of 3×10^4 to 1×10^6 M^{-1} s^{-1} (Karlsson *et al.*, 1991; Malmborg *et al.*, 1992; Barbas *et al.*, 1994; Yang *et al.*, 1995; Schier *et al.*, 1996; Wu *et al.*, 1998). In this particular protein-protein interaction, a likely explanation for the slow k_{on} is the high degree of flexibility associated with the binding site both on the Fab and on VEGF. In fact, crystallographic evidence suggests that the "80's loop" region is quite mobile (Muller *et al.*, 1997; Muller *et al.*, 1998b). We are pursuing other strategies to assess whether improvements to k_{on} can be obtained.

The contributions of point mutations in proteins to the free energy of binding or activation are often additive (Wells, 1990). This principle has been used to produce a variety of affinity-improved protein variants based on point or grouped mutations identified by phage display (Lowman & Wells, 1993; Yang *et al.*, 1995) or point-mutant screening (Wu *et al.*, 1998). Considering the initial library selectants Y0238-3 (>ninefold improved in affinity) and Y0243-1 (3.1-fold improved), we would have predicted an improvement of >27-fold for Y0313-1 or Y0317 (Table 7). In fact, a 22-fold improvement is observed (Figure 2) at 25 °C. Addition of the CDR-H1 mutation would be predicted to improve affinity slightly (1.3-fold), but in fact this mutation reduced affinity >twofold (Y0268-1 *versus* Y0313-1; Table 6). Certainly additivity does not always apply, particularly if interacting residues are involved (Wells, 1990). In this case, non-additivity probably results from steric interference between the new Trp in CDR-H2 and the new Tyr in CDR-H3.

To test the energetics of binding by the final Y0317 antibody to VEGF, we made use of a panel of alanine mutants that had been previously constructed for mapping the binding site of the original antibody (Muller *et al.*, 1998a). For these experiments, we made use of the full-length IgG forms of both antibodies. In view of the slow dissociation kinetics for both antibodies, ELISA assays were carried out at 37 °C with incubation for at least five hours to insure that equilibrium was reached. Under these conditions two dramatic differences appear in the Ala-scan of VEGF with respect to Y0317 *versus* Fab-12: both R82A and I83A have small effects on binding in Y0317, but result in large decreases in binding for Fab-12. The reasons for these differences are not clear, but R82 and I83 do have significant surface area (55 Å^2 and 32 Å^2, respectively) buried on binding to VEGF, and make contacts that include residues S100a of CDR-H3 and N52 of CDR-H2 in the wild-type antibody (Muller *et al.*, 1998a).

Structural analysis of the affinity-matured Fab

The structures of a number of antibodies derived from *in vivo* immunization and hybridoma techniques have been determined, in complex with their antigens (reviewed by Nezlin, 1998), and recently, crystallization and preliminary X-ray studies of a chain-shuffled anti-lysozyme scFv antibody in complex with antigen were reported (Küttner *et al.*, 1998). However, to our knowledge, the Y0317 Fab:VEGF structure is the first report of an *in vitro* affinity-matured Fab in complex with antigen. The structural basis of binding affinity improvement is therefore of interest

The Fab fragment of the affinity-matured anti-VEGF antibody Y0317 preserves the structure of the original humanized antibody, Fab-12. Superposition with Fab-12 results in an rmsd of only 0.38 Å for a total of 431 C^{α}-positions, demonstrating the absence of major structural changes between the two molecules. With a total of 1800 Å^2 of solvent-accessible surface buried in each VEGF-Fab interface, the contact area is about 50 Å^2 larger than in the Fab-12 complex. This small increase in buried surface area is mostly due to the exchange of H97 to a tyrosine residue. In the VEGF:Fab-12 complex, H97 buries a solvent-accessible area of 56 Å^2, while the larger tyrosine side-chain of the matured antibody accounts for 86 Å^2 of buried surface. The tyrosine side-chain also affects the hydrogen-bonding pattern and the number of ordered water molecules in the vicinity. In the parental antibody complex, a water molecule near H97 forms two hydrogen bonds to the side-chains of Q79 and E93 of VEGF (Figure 4(c)). In the complex with the affinity-matured Fab, this water molecule is replaced by the hydroxyl group of the newly introduced tyrosine side-chain at position 97. The H97Y mutation therefore not only increases the amount of buried surface area, but also introduces two additional hydrogen bonds between the ligand and Fab-0317 (Figure 4(c)). This is in good agreement with the observation that this single substitution improves VEGF binding affinity by 14-fold (Table 6). We therefore conclude that this single substiution is responsible for the majority of the improvement in binding affinity of Y0317 compared to the parent antibody.

In contrast, despite the availability of the crystal structures of both complexes, it remains uncertain what the structural basis is of the 3.6-fold enhanced binding caused by the N31H mutation. The side-chains of the asparagine and the histidine residues in this position adopt identical conformations in both crystal structures, and the amount of buried surface is not significantly increased in the VEGF:Fab-Y0317 complex. The only difference we can detect is a slight possible improvement in the hydrophobic interactions between the histidine side-chain and the phenyl group of VEGF residue F17, which has rotated slightly compared to the parent complex. It is unclear whether this could contribute to the increased affinity.

Neither of the remaining differences between Fab-12 and Fab-Y0317 has a significant effect on the binding affinity towards VEGF, and the structures show that these residues contribute only marginally to the interface. Some interactions are present between VEGF and the main-chain atoms of the serine and threonine residues in position 100a of the two Fabs, but the side-chains of these residues are not in contact with VEGF. Finally, no contact exist between VEGF and T28 (or D28) of the Fab fragments (the closest point on VEGF to this residue is more than 6 Å distant).

In summary, the analysis and comparison of the two crystal structures are in very good agreement with the results of the binding assays on the various single mutants of the Fab fragments. Although it is not possible to quantify the effects introduced by the amino acid exchanges solely based on the crystal structures, the detailed crystallographic

analysis supports and enables us to interpret the binding data.

Biological implications for antibody inhibition of VEGF

An inhibitory antibody of improved affinity may have improved potency or efficacy in treating diseases associated with VEGF expression. Preceding versions of the anti-VEGF antibody described here, including the murine A4.6.1 (Kim *et al.*, 1993), the humanized version Fab-12 (Presta *et al.*, 1997), as well as Y0192 (Muller *et al.*, 1998a), clearly demonstrated sufficient affinity to effect inhibition of VEGF activity. Here, we show that an affinity-improved variant, Fab Y0317, can inhibit endothelial cell proliferation *in vitro* with least 30-fold greater potency than the parental humanized Fab (Figure 3).

We have limited our optimization strategy to a subset of heavy-chain CDR residues implicated by alanine-scanning and crystallography (Muller *et al.*, 1998a). Furthermore, not all combinations of phage-derived mutations have been tested. One may therefore reasonably ask whether Y0317, with $K_d^{25°} = 20$ pM and $K_d^{37°} = 130$ pM, is the globally optimum variant for binding to this particular epitope (or others) on VEGF. Other affinity optimization efforts have resulted in protein-protein binding affinities in the low picomolar range, from $K_d = 6$ pM to 15 pM (see, e.g. Lowman & Wells, 1993; Schier *et al.*, 1996; Yang *et al.*, 1995). Indeed, we cannot exclude the possibility that higher affinity variants of the A4.6.1 antibody could be produced. However, it seems unlikely that further affinity improvement would greatly enhance biological potency or efficacy because for effective inhibition, the antibody must certainly occupy a significant fraction (perhaps >99 %) of the available (VEGF) binding sites. Serum VEGF concentrations of about 20 pM in normals, and of >300 pM in patients with metastatic carcinoma, have been observed (Kraft *et al.*, 1999). Local or effective concentrations are likely higher. If we conservatively assume the effective concentration of VEGF *in vivo* to be about 400 pM, then 400 pM of even an infinite-affinity Fab would be required to block all sites.

Other factors may limit the improvement in potency of a full-length IgG resulting from an improvement in intrinsic binding affinity of the Fab for antigen. The full-length IgG form of the antibody may benefit from an avidity effect *in vivo*, especially since VEGF is known to associate with proteoglycans on the cell surface (Gitay-Goren *et al.*, 1992). Even in cell-based assays, the IgG form of Fab-12 is a more effective inhibitor than the Fab form (data not shown). Finally, the half-life for dissociation of the affinity-improved antibody is already significant, even on the time-scale of the half-life of clearance for IgG's (days to weeks). The effect of an improved association rate constant for antibody in this system is unknown.

The fact that point (Ala) mutations in the antibody binding site on VEGF sometimes have lesser effects on the binding of Y0317 than on the binding of Fab-12 may suggest that the optimized binding site is more tolerant than the parental one of variations in the antigen. Indeed, Y0317 showed greatly enhanced affinity for murine VEGF over that of Fab-12 (data not shown), though still >100-fold weaker than its affinity for human VEGF. This could provide an advantage against naturally arising VEGF variants.

Materials and Methods

Construction of phage libraries and mutagenesis

A variant of the Fab-12 antibody (a humanized form of murine antibody A4.6.1) was previously identified from phage-displayed Fab libraries for improved expression on phage particles (Muller *et al.*, 1998a). We made use of the plasmid pY0192, a phagemid construct with ampicillin (or carbenicillin) resistance, as the parental ("wild-type") construct for libraries described here. To prevent contamination by wild-type sequence (Lowman *et al.*, 1991; Lowman, 1998), templates with the TAA stop codon at each residue targeted for randomization were prepared from CJ236 *E. coli* cells (Kunkel *et al.*, 1991). Libraries are designated according to the mutagenic oligonucleotides used for their construction: YC265, TCC TGT GCA GCT TCT GGC NNS NNS TTC NNS NNS NNS GGT ATG AAC TGG GTC CG, randomizing residues 27-28, 30-32 in CDR-H1; YC266, GAA TGG GTT GGA TGG ATT AAC NNS NNS NNS GGT NNS CCG ACC TAT GCT GCG G, randomizing residues 52a-54, 56 in CDR-H2; YC103, GAA TGG GTT GGA TGG ATT NNS NNS NNS NNS GGT GAA CCG ACC TAT G, randomizing residues 52-54 in CDR-H2; YC81, C TGT GCA AAG TAC CCG NNS TAT NNS NNS NNS NNS CAC TGG TAT TTC GAC, randomizing residues 97, 99-100b in CDR-H3; and YC101, CGT TTC ACT TTT TCT NNS GAC NNS TCC AAA NNS ACA GCA TAC CTG CAG , randomizing residues 71, 73, and 76 in the "FR-H3" region. An additional library in CDR-H2 was designed to insert three new residues: YC90, GA TGG ATT AAC ACC TAT NNS NNS NNS ACC GGT GAA CCG ACC.

The products of random mutagenesis reactions were electroporated into XL1-Blue *E. coli* cells (Stratagene) and amplified by growing 15-16 hours with M13KO7 helper phage. The complexity of each library, ranging from 2×10^7 to 1.5×10^8 , was estimated based on plating of the initial transformation onto LB plates containing carbenicillin.

Site-directed mutagenesis for point mutations was carried out as above, using appropriate codons to produce the respective mutations, and sequences were confirmed by single-strand DNA sequencing using Sequenase™ (USB).

Phage binding selections

For each round of selection, approximately 10^9-10^{10} phage were screened for binding to plates (Nunc Maxisorp 96-well) coated with 2 μg/ml VEGF(109) in 50 mM carbonate buffer (pH 9.6) and blocked with 5% (w/v) instant milk in 50 mM carbonate buffer, (pH 9.6). Also included were phage prepared from a non-displaying

control phagemid (pCAT), which confers chloramphenicol resistance, as a means of measuring background and enrichment (Lowman & Wells, 1993). Bound phage were eluted with 0.1 M HCl and immediately neutralized with one-third volume of 1 M Tris (pH 8.0). The eluted phage were propagated by infecting XL1 cells for the next selection cycle as described (Lowman, 1998).

In the first cycle, the VEGF plate was incubated with Fab-phage, then was briefly washed to remove bound phage. In the second cycle, binding and washing were followed by a one hour dissociative incubation at room temperature with binding buffer, after which the plate was again washed prior to acid elution. This process was repeated in rounds 3, 4 and 5, except that 1 μM VEGF was included in the dissociative incubation, and the incubation time was increased to 2, 18, and 37 hours, respectively. During these selections, Y0192 phage showed enrichments ranging from 1.5-fold (at the lowest stringency) to 22,000-fold (using a two hour dissociation incubation). However, further increases in stringency (rounds 4-5) resulted in decreasing enrichments for the control phage, with higher enrichments observed for certain libraries, especially the two CDR-H2 libraries and the CDR-H3 library (Table 1).

In cycle 6, a 17 hour dissociative incubation at room temperature was followed by an additional 30 hour incubation at 37 °C (also including VEGF in the buffer). Under these conditions, Y0192-phage showed only slight binding enrichment (20-fold), whereas the CDR-H3 library phage were enriched by 3500-fold. Cycle 7 was carried out with a 63 hour dissociative incubation, after which only small enrichment factors were observed. However, some libraries were continued through eight cycles (with 120 hours of dissociative incubation in the presence of VEGF), after which Fab-phage were still recoverable by acid elution (data not shown).

Purification of Fab

For small-scale preparations, Y0317 Fab and mutants were prepared from *E. coli* shake-flasks as described (Muller *et al.*, 1998a).

For large-scale preparation, whole cell broth was obtained from a ten liter *E. coli* fermentation. The cells were lysed with a Manton-Gaulin homogenizer (two passes at 6000 psi; lysate temperature maintained at 15-25 °C with a heat exchanger). A 5% (v/v) solution of polyethylene imine (PEI), pH 6.0, was added to the lysate to give a final concentration of 0.25% (v/v). The lysate was mixed for 30 minutes at room temperature. The suspension was centrifuged, and the supernatant (containing the Fab) was processed further. The pH of the supernatant was adjusted to 6.0 with 6 M HCl, followed by dilution to a conductivity of 5 mS/cm with purified water. The conditioned supernatant was loaded onto a BakerBond ABx ion-exchange column. Following a wash with the column equilibration buffer, the Fab was eluted with an increasing sodium chloride gradient in the equilibration buffer. Fractions containing the Fab were identified by SDS-PAGE. The BakerBond ABx column fractions were pooled, pH adjusted to 5.5 with 1 M Mes and diluted to a conductivity of 5 mS/cm with purified water. The conditioned BakerBond ABx pool was loaded onto a SP Sepharose HP cation exchange column (Pharmacia). Once again, the Fab was eluted with a sodium chloride-containing gradient. Fractions containing the Fab were identified by SDS-PAGE. The level of purity of Fab (as determined by SDS-PAGE) after this two column purification was >95%.

BIAcore™ binding analysis

The VEGF-binding affinities of Fab fragments were calculated from association and dissociation rate constants measured using a BIAcore™ -2000 surface plasmon resonance system (BIAcore, Inc., Piscataway, NJ). A biosensor chip was activated for covalent coupling of VEGF using *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) according to the supplier's (BIAcore, Inc., Piscataway, NJ) instructions. VEGF(109) or VEGF(165) was buffer-exchanged into 20 mM sodium acetate, pH 4.8 and diluted to approximately 50 μg/ml. Aliquots of VEGF were injected at a flow rate of 2 μl/minute to achieve approximately 700-1400 response units (RU) of coupled protein. A solution of 1 M ethanolamine was injected as a blocking agent.

For kinetics measurements, twofold serial dilutions of Fab were injected in PBS/Tween buffer (0.05% Tween-20 in phosphate-buffered saline) at 25 °C or 37 °C at a flow rate of 10 μl/minute. Equilibrium dissociation constants, K_d values from SPR measurements were calculated as k_{off}/k_{on} (Tables 6 and 8).

Radiolabeled VEGF binding assay

Solution binding affinity of Fabs for VEGF was measured by equilibrating Fab with a minimal concentration of (^{125}I)-labeled VEGF(109) in the presence of a titration series of unlabeled VEGF, then capturing bound VEGF with an anti-Fab antibody-coated plate.

To establish conditions for the assay, microtiter plates (Dynex) were coated overnight with 5 μg/ml of a capturing anti-Fab antibody (Cappel Labs) in 50 mM sodium carbonate (pH 9.6), and subsequently blocked with 2% (w/v) bovine serum albumin in PBS for two to five hours at room temperature (approximately 23 °C). In a non-adsorbant plate (Nunc #269620), 100 pM or 26 pM [^{125}I]VEGF(109) was mixed with serial dilutions of Fab-12 or Fab Y0317, respectively. Fab-12 was incubated overnight; however, the Fab Y0317 incubation was continued for 65 hours to insure that equilibrium was reached. Thereafter, the mixtures were transferred to the capture plate for incubation at room temperature for one hour. The solution was then removed and the plate washed eight times with 0.1% Tween-20 in PBS. When the plates had dried, 150 μl/well of scintillant (MicroScint-20; Packard) was added, and the plates were counted on a Topcount gamma counter (Packard) for ten minutes. Concentrations of each Fab were chosen to give ≤20% of maximal binding.

For competitive binding assays, Dynex plates were coated and blocked as above, and serial threefold dilutions of unlabeled VEGF(109) were made in PBS/Tween buffer in a Nunc plate. [^{125}I]VEGF(109) was added, followed by addition of a fixed concentration of Fab-12 or Fab Y0317. The final concentrations of Fab-12, and Fab Y0317 were 100 pM and 10 pM, respectively. After incubation (as above), bound VEGF was captured and quantified as described above. The binding data was analyzed using a computer program to perform Scatchard analysis (Munson & Rodbard, 1980) for determination of the dissociation binding constants, K_d, for Fab-12 and Fab Y0317.

ELISA assay of VEGF Ala mutants

The binding affinities of VEGF Ala mutants for full-length Fab-12-IgG (known as rhuMAb VEGF) and Y0317-IgG, a full-length IgG form of the improved antibody expressed in CHO cells (V. Chisholm, unpublished results) were measured as previously described (Muller *et al.*, 1997; Muller *et al.*, 1998a) for the murine antibody A4.6.1, except that the temperature was increased to 37 °C, and the incubation time increased to five hours, to insure that equilibrium was reached with the high-affinity antibody.

Cell-based assay of VEGF inhibition

Several versions of the anti-VEGF antibody were tested for their ability to antagonize VEGF(165) induction of the growth of HuVECs (human umbilical vein endothelial cells). The 96-well plates were seeded with 1000 HuVECs per well and fasted in assay medium (F12:DMEM 50:50 supplemented with 1.5% (v/v) dia-filtered fetal bovine serum) for 24 hours.

The concentration of VEGF used for inducing the cells was determined by first titrating to identify the amount of VEGF that can stimulate 80% of maximal DNA synthesis. Fresh assay medium containing fixed amounts of VEGF (0.2 nM final concentration), and increasing concentrations of anti-VEGF Fab or mab were then added. After 40 hours of incubation, DNA synthesis was measured by incorporation of tritiated thymidine. Cells were pulsed with 0.5 μCi per well of [^{3}H]thymidine for 24 hours and harvested for counting, using a TopCount gamma counter.

Crystallization and refinement

The complex between the Fab fragment of affinity-matured, humanized antibody Y0317 Fab and the receptor binding fragment of VEGF (VEGF(109)) was purified and crystallized as described for the analogous complex with the parental humanized Fab-12 fragment (Muller *et al.*, 1998a). The resulting crystals had symmetry consistent with space group $P2_1$ with cell parameters $a = 89.1$ Å, $b = 66.4$ Å, $c = 138.7$ Å, and $\beta = 94.7°$, and were isomorphous with the crystals obtained with the parent complex. A data set was collected from a single frozen crystal at beam line 5.0.2 at the Advanced Light Source, Berkeley, and processed using programs MOSFLM and SCALA (CCP4, 1994). The final data set ($R_{merge} = 7.3\%$) is described in Table 10. Starting with the model of Brookhaven Protein Data Bank entry 1bj1 (Muller *et al.*, 1998a), the structure was refined using the programs X-PLOR (Brünger *et al.*, 1987) and REFMAC (CCP4, 1994). The free R-value was monitored using the identical set of reflections sequestered before refinement of parent complex. The differences in the primary structure between Fab-12 and Fab-Y0317 were modeled using the program O (Jones *et al.*, 1991). After correction for anisotropy and application of a bulk solvent correction, the R-value reached its final value of 19.9% for all reflections greater than 0.2σ (see Table 10; $R_{free} = 27.4\%$).

Table 10. Crystallographic data and refinement statistics

A. *Data collection*	Overall	Last shell
Resolution range (Å)	30-2.4	2.53-2.40
No. of observations	208,257	22,278
Unique reflections	61,742	8900
Completeness (%)	97.4	96.7
Mean $I/\sigma(I)$	13.6	2.7
R_{sym}	0.073	0.38
B. *Refinement*		
Resolution range (Å)	20-2.4	
No. of reflections	61,689	
No. of atoms	8577	
rmsd bond lengths (Å)	0.013	
rmsd angles (deg.)	1.9	
rmsd improper angles (deg.)	0.92	
rmsd B-factors for all bonded atoms, Å^2	3.5	
Number of main-chain torsion angles in disallowed regions of Ramachandran plot[a]	2	

[a] See Laskowski *et al.* (1993).

Protein Data Bank accession number

The coordinates for the VEGF:Y0317 Fab complex have been deposited in the Protein Data Bank, accession number 1cz8.

Acknowledgments

We thank Lyn Deguzman and Tom Zioncheck for providing ^{125}[I]VEGF; Alan Padua and Bill Henzel for quantitative amino acid analysis; James Bourell for mass spectrometry; Vanessa Chisholm and Lynne Krummen for construction of cell lines; and Manuel Baca, Napoleone Ferrara, Yves Muller, Leonard Presta, and James Wells for many helpful discussions.

References

Baca, M., Presta, L. G., O'Connor, S. J. & Wells, J. A. (1997). Antibody humanization using monovalent phage display. *J. Biol. Chem.* **272**, 10678-10684.

Barbas, C. F., III, Hu, D., Dunlop, N., Sawyer, L., Cababa, D., Hendry, R. M., Nara, P. L. & Burton, D. R. (1994). *In vitro* evolution of a neutralizing human antibody to human immunodeficiency virus type 1 to enhance affinity and broaden strain cross-reactivity. *Proc. Natl Acad. Sci. USA*, **91**, 3809-3813.

Bass, S., Greene, R. & Wells, J. A. (1990). Hormone phage: an enrichment method for variant proteins with altered binding properties. *Proteins: Struct. Funct. Genet.* **8**, 309-314.

Brünger, A. T., Kuriyan, J. & Karplus, M. (1987). Crystallographic R factor refinement by molecular dynamics. *Science*, **235**, 458-460.

CCP4 (1994). Programs for protein crystallography. *Acta Crystallog. sect. D*, **50**, 760-763.

Chothia, C. & Lesk, A. M. (1987). Canonical structures for the hypervariable reigions of immunoglobulins. *J. Mol. Biol.* **196**, 901-917.

Delisi, C. (1983). Role of diffusion regulation in receptor-ligand interactions. *Methods Enzymol.* **93**, 95-109.

Fairbrother, W. J., Christinger, H. W., Cochran, A. G., Fuh, G., Keenan, C. J., Quan, C., Shriver, S. K., Tom, J. Y., Wells, J. A. & Cunningham, B. C. (1998). Novel peptides selected to bind vascular endothelial growth factor target the receptor-binding site. *Biochemistry*, **37**, 17754-17764.

Ferrara, N. (1995). The role of vascular endothelial growth factor in pathological angiogenesis. *Breast Cancer Res. Treat.* 36, 127-137.

Ferrara, N. (1999). Vascular endothelial growth factor: molecular and biological aspects. *Curr. Top. Microbiol. Immunol.* 237, 1-30.

Ferrara, N. & Henzel, W. J. (1989). Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem. Biophys. Res. Commun.* 161, 851-858.

Folkman, J. (1995). Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nature Med.* 1, 27-31.

Folkman, J. & Klagsbrun, M. (1987). Angiogenic factors. *Science*, 235, 442-443.

Foote, J. & Winter, G. (1992). Antibody framework residues affecting the conformation of the hypervariable loops. *J. Mol. Biol.* 224, 487-499.

Gitay-Goren, H., Soker, S., Vlodavsky, I. & Neufeld, G. (1992). The binding of vascular endothelial growth factor to its receptors is dependent on cell-surface-associated heparin-like molecules. *J. Biol. Chem.* 267, 6093-6098.

Griffiths, A. D. & Duncan, A. R. (1998). Strategies for selection of antibodies by phage display. *Curr. Opin. Biotechnol.* 9, 102-108.

Hawkins, R. E., Russell, S. J. & Winter, G. (1992). Selection of phage antibodies by binding affinity mimicking affinity maturation. *J. Mol. Biol.* 226, 889-896.

Hawkins, R. E., Russell, S. J., Baier, M. & Winter, G. (1993). The contribution of contact and non-contact residues of antibody in the affinity of binding to antigen. *J. Mol. Biol.* 234, 958-964.

Iruela-Arispe, M. L. & Dvorak, H. F. (1997). Angiogenesis: a dynamic balance of stimulators and inhibitors. *Thromb. Haem.* 78, 672-677.

Jones, T. A., Zhou, J.-Y., Cowan, S. W. & Kjelgaard, M. (1991). Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallog. sect. A*, 47, 110-119.

Kabat, E. A., Wu, T. T., Redi-Miller, M., Perry, H. M. & Gottesman, K. S. (1987). *Sequences of Proteins of Immunological Interest*, 4th edit., National Institutes of Health, Bethesda, MD.

Karlsson, R., Michaelsson, A. & Mattsson, L. (1991). Kinetic analysis of monoclonal antibody-antigen interactions with a new biosensor based analytical system. *J. Immunol. Methods*, 145, 229-240.

Kay, B. K. & Hoess, R. H. (1996). Principles and applications of phage display. In *Phage Display of Peptides and Proteins* (Kay, B. K., Winter, J. & McCafferty, J., eds), pp. 21-34, Academic Press, San Diego.

Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H. S. & Ferrara, N. (1993). Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth *in vivo*. *Nature*, 362, 841-844.

Kraft, A., Weindel, K., Ochs, A., Marth, C., Zmija, J., Schumacher, P., Unger, C., Marmé, D. & Gastl, G. (1999). Vascular endothelial growth factor in the sera and effusions of patients with malignant and nonmalignant disease. *Cancer*, 85, 178-187.

Kunkel, T. A., Bebenek, K. & McClary, J. (1991). Efficient site-directed mutagenesis using uracil-containing DNA. *Methods Enzymol.* 204, 125-139.

Küttner, G., Keitel, T., Gießmann, E., Wessner, H., Scholz, C. & Höhne, W. (1998). A phage library-derived single-chain Fv fragment in complex with turkey egg-white lysozyme: characterization, crystallization and preliminary X-ray analysis. *Mol. Immunol.* 35, 189-194.

Laskowski, R. A., MacArthur, M. W., Moss, D. S. & Thornton, J. M. (1993). Procheck: a program to check the stereochemical quality of protein structures. *J. Appl. Crystallog.* 26, 283-291.

Lowman, H. B. (1998). Phage display of peptide libraries on protein scaffolds. In *Methods in Molecular Biology, Combinatorial Peptide Library Protocols* (Cabilly, S., ed.), vol. 87, pp. 249-264, Humana Press, Totowa, NJ.

Lowman, H. B. & Wells, J. A. (1993). Affinity maturation of human growth hormone by monovalent phage display. *J. Mol. Biol.* 234, 564-578.

Lowman, H. B., Bass, S. H., Simpson, N. & Wells, J. A. (1991). Selecting high-affinity binding proteins by monovalent phage display. *Biochemistry*, 30, 10832-10838.

Malmborg, A.-C., Michaelsson, A., Ohlin, M., Jansson, B. & Borrebaeck, C. A. K. (1992). Real time analysis of antibody-antigen reaction kinetics. *Scan. J. Immunol.* 35, 643-650.

Marks, J. D., Griffiths, A. D., Malmqvist, M., Clackson, T. P., Bye, J. M. & Winter, G. (1992). By-passing immunization: building high affinity human antibodies by chain shuffling. *Biotechnology*, 10, 779-783.

Mordenti, J., Thomsen, K., Licko, V., Chen, H., Meng, Y. G. & Ferrara, N. (1999). Efficacy and concentration-response of murine anti-VEGF monoclonal antibody in tumor-bearing mice and extrapolation to humans. *Toxicol. Pathol.* 27, 14-21.

Muller, Y. A., Li, B., Christinger, H. W., Wells, J. A., Cunningham, B. C. & de Vos, A. M. (1997). Vascular endothelial growth factor: crystal structure and functional mapping of the kinase domain receptor binding site. *Proc. Natl Acad. Sci. USA*, 94, 7192-7197.

Muller, Y. A., Chen, Y., Christinger, H. W., Li, B., Cunningham, B. C., Lowman, H. B. & de Vos, A. M. (1998a). VEGF and the Fab fragment of a humanized neutralizing antibody: crystal structure of the complex at 2.4 Å resolution and mutational analysis of the interface. *Structure*, 6, 1153-1167.

Muller, Y. A., Christinger, H. W., Keyt, B. A. & de Vos, A. M. (1998b). The crystal structure of vascular endothelial growth factor (VEGF) refined to 1.93 Å resolution: multiple copy flexibility and receptor binding. *Structure*, 5, 1325-1338.

Munson, P. & Rodbard, D. (1980). Ligand: a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* 107, 220-239.

Nezlin, R. (1998). *The Immunoglobulins: Structure and Function*, pp. 151-204, Academic Press, San Diego.

Presta, L. G., Chen, H., O'Connor, S. J., Chisholm, V., Meng, Y. G., Krummen, L., Winkler, M. & Ferrara, N. (1997). Humanization of a vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. *Cancer Res.* 47, 4593-4599.

Rader, C. & Barbas, C. F., III (1997). Phage display of combinatorial antibody libraries. *Curr. Opin. Biotechnol.* 8, 503-508.

Schier, R., McCall, A., Adams, G. P., Marshall, K. W., Merritt, H., Yim, M., Crawford, R. S., Weiner, L. M., Marks, C. & Marks, J. D. (1996). Isolation of picomolar affinity anti-c-erbB-2 single-chain Fv by molecular evolution of the complementarity

determining regions in the center of the antibody binding site. *J. Mol. Biol.* **263**, 551-567.

Thompson, J., Pope, T., Tung, J.-S., Chan, C., Hollis, G., Mark, G. & Johnson, K. S. (1996). Affinity maturation of a high-affinity human monoclonal antibody against the third hypervariable loop of human immunodeficiency virus: use of phage display to improve affinity and broaden strain reactivity. *J. Mol. Biol.* **16**, 77-88.

Tramontano, A., Chothia, C. & Lesk, A. M. (1990). Framework residue 71 is a major determinant of the position and conformation of the second hypervariable region in the VH domains of immunoglobulins. *J. Mol. Biol.* **215**, 175-182.

Wells, J. A. (1990). Additivity of mutational effects in proteins. *Biochemistry*, **29**, 8509-8517.

Wu, H., Beuerlein, G., Nie, Y., Smith, H., Lee, B. A., Hensler, M., Huse, W. D. & Watkins, J. D. (1998). Stepwise *in vitro* affinity maturation of vitaxin, an $\alpha_v\beta_3$-specific humanized mAb. *Proc. Natl Acad. Sci. USA*, **95**, 6037-6042.

Xiang, J., Sha, Y., Jia, Z., Prasad, L. & Delbaere, L. T. (1995). Framework residues 71 and 93 of the chimeric B72.3 antibody are major determinants of the conformation of heavy-chain hypervariable loops. *J. Mol. Biol.* **253**, 385-390.

Yang, W.-P., Green, K., Pinz-Sweeney, S., Briones, A. T., Burton, D. R. & Barbas, C. F., III (1995). CDR walking mutagenesis for the affinity maturation of a potent human anti-HIV-1 antibody into the picomolar range. *J. Mol. Biol.* **254**, 392-403.

Edited by I. A. Wilson

(Received 19 July 1999; received in revised form 7 September 1999; accepted 13 September 1999)

```
          1         10         20         30         40
A4.6.1    EIQLVQSGPELKQPGETVRISCKASGYTFTNYGMNWVKQA
           *    *   ** *    *** *  *              *
F(ab)-12  EVQLVESGGGLVQPGGSLRLSCAASGYTFTNYGMNWVRQA
                                    *  ** * *
humIII    EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQA

                  50  a      60         70         80
A4.6.1    PGKGLKWMGWINTYTGEPTYAADFKRRFTFSLETSASTAYL
            * *                              *  *
F(ab)-12  PGKGLEWVGWINTYTGEPTYAADFKRRFTFSLDTSKSTAYL
                  ** **** ***  *** *   * * *  * *
humIII    PGKGLEWVSVISGDGGSTTYADSVKGRFTISRDNSKNTLYL

            abc     90                          110
A4.6.1    QISNLKNDDTATYFCAKYPHYYGSSHWYFDVWGAGTTVTVSS   (SEQ.ID NO:9)
           *** ***    * *                        *  *
F(ab)-12  QMNSLRAEDTAVYYCAKYPHYYGSSHWYFDVWGQGTLVTVSS   (SEQ.ID NO:7)
                         *                    *
humIII    QMNSLRAEDTAVYYCARG----------FDYWGQGTLVTVSS   (SEQ.ID NO:11)
```

FIG._1A

```
          1         10         20         30         40
A4.6.1    DIQMTQTTSSLSASLGDRVIISCSASQDISNYLNWYQQKP
            **       *    * *
F(ab)-12  DIQMTQSPSSLSASVGDRVTITCSASQDISNYLNWYQQKP
                                 *   *      *
humKI     DIQMTQSPSSLSASVGDRVTITCRASQSISNYLAWYQQKP

                  50         60         70         80
A4.6.1    DGTVKVLIYFTSSLHSGVPSRFSGSGSGTDYSLTISNLEP
          ****                              **   * *
F(ab)-12  GKAPKVLIYFTSSLHSGVPSRFSGSGSGTDFTLTISSLQP
            *   **  *
humKI     GKAPKLLIYAASSLESGVPSRFSGSGSGTDFTLTISSLQP

                  90        100
A4.6.1    EDIATYYCQQYSTVPWTFGGGTKLEIKR    (SEQ.ID NO:10)
            *                  *   *
F(ab)-12  EDFATYYCQQYSTVPWTFGQGTKVEIKR    (SEQ.ID NO:8)
                     ***
humKI     EDFATYYCQQYNSLPWTFGQGTKVEIKR    (SEQ.ID NO:12)
```

FIG._1B

```
                10          20          30          40
F(ab)-12    DIQMTQSPSS LSASVGDRVT ITCSASQDIS NYLNWYQQKP
Y0243-1     DIQLTQSPSS LSASVGDRVT ITCRANEQLS NYLNWYQQKP
Y0238-3     DIQLTQSPSS LSASVGDRVT ITCRANEQLS NYLNWYQQKP
Y0313-1     DIQLTQSPSS LSASVGDRVT ITCRANEQLS NYLNWYQQKP
Y0317       DIQLTQSPSS LSASVGDRVT ITCSASQDIS NYLNWYQQKP
                                   CDR-L1
                50          60          70          80
F(ab)-12    GKAPKVLIYF TSSLHSGVPS RFSGSGSGTD FTLTISSLQP
Y0243-1     GKAPKVLIYF TSSLHSGVPS RFSGSGSGTD FTLTISSLQP
Y0238-3     GKAPKVLIYF TSSLHSGVPS RFSGSGSGTD FTLTISSLQP
Y0313-1     GKAPKVLIYF TSSLHSGVPS RFSGSGSGTD FTLTISSLQP
Y0317       GKAPKVLIYF TSSLHSGVPS RFSGSGSGTD FTLTISSLQP
                       CDR-L2
                90          100         110
F(ab)-12    EDFATYYCQQ YSTVPWTFGQ GTKVEIKRTV    (SEQ.ID NO:8)
Y0243-1     EDFATYYCQQ YSTVPWTFGQ GTKVEIKRTV    (SEQ.ID NO:109)
Y0238-3     EDFATYYCQQ YSTVPWTFGQ GTKVEIKRTV    (SEQ.ID NO:111)
Y0313-1     EDFATYYCQQ YSTVPWTFGQ GTKVEIKRTV    (SEQ.ID NO:113)
Y0317       EDFATYYCQQ YSTVPWTFGQ GTKVEIKRTV    (SEQ.ID NO:115)
                       CDR-L3
```

FIG._10A

```
                10          20          30          40
F(ab)-12    EVQLVESGGG LVQPGGSLRL SCAASGYTFT NYGMNWVRQA
Y0243-1     EVQLVESGGG LVQPGGSLRL SCAASGYDFT HYGMNWVRQA
Y0238-3     EVQLVESGGG LVQPGGSLRL SCAASGYTFT NYGINWVRQA
Y0313-1     EVQLVESGGG LVQPGGSLRL SCAASGYDFT HYGMNWVRQA
Y0317       EVQLVESGGG LVQPGGSLRL SCAASGYDFT HYGMNWVRQA
                                   CDR-H1
                50          60          70          80
F(ab)-12    PGKGLEWVGW INTYTGEPTY AADFKRRFTF SLDTSKSTAY
Y0243-1     PGKGLEWVGW INTYTGEPTY AADFKRRFTF SLDTSKSTAY
Y0238-3     PGKGLEWVGW INTYTGEPTY AADFKRRFTF SLDTSKSTAY
Y0313-1     PGKGLEWVGW INTYTGEPTY AADFKRRFTF SLDTSKSTAY
Y0317       PGKGLEWVGW INTYTGEPTY AADFKRRFTF SLDTSKSTAY
                       CDR-H2                CDR-7
                90          100         110
F(ab)-12    LQMNSLRAED TAVYYCAKYP HYYGSSHWYF DVWGQGTL   (SEQ.ID NO:7)
Y0243-1     LQMNSLRAED TAVYYCAKYP HYYGSSHWYF DVWGQGTL   (SEQ.ID NO:110)
Y0238-3     LQMNSLRAED TAVYYCAKYP YYYGTSHWYF DVWGQGTL   (SEQ.ID NO:112)
Y0313-1     LQMNSLRAED TAVYYCAKYP YYYGTSHWYF DVWGQGTL   (SEQ.ID NO:114)
Y0317       LQMNSLRAED TAVYYCAKYP YYYGTSHWYF DVWGQGTL   (SEQ.ID NO:116)
                                   CDR-H3
```

FIG._10B

DEPARTMENT OF HEALTH & HUMAN SERVICES

rhuFab VEGF

Food and Drug Administration
1401 Rockville Pike
Rockville MD 20852-1448

Our Reference: BB-IND 8633

OCT 13 1999

21579

Genentech, Incorporated
Attention: Robert L. Garnick, Ph.D.
Vice President, Regulatory Affairs
1 DNA Way
South San Francisco, CA 94080-4990

Dear Dr. Garnick:

The Center for Biologics Evaluation and Research has received your **Investigational New Drug Application (IND)**. The following product name and BB-IND number have been assigned to this application. They serve only to identify it and do not imply that this Center either endorses or does not endorse your application.

BB-IND #: 8633

SPONSOR: Genentech, Incorporated

PRODUCT NAME: Humanized Monoclonal Antibody Fragment (rhuFab V2) (E. coli, Genentech) to Vascular Endothelial Growth Factor (VEGF), Intravitreal

DATE OF SUBMISSION: October 6, 1999

DATE OF RECEIPT: October 7, 1999

This BB-IND number should be used to identify all future correspondence and submissions, as well as telephone inquiries concerning this IND. Please provide an **original and two copies of every submission to this file**. Please include three originals of all illustrations which do not reproduce well.

It is understood that studies in humans will not be initiated until 30 days after the date of receipt shown above. If this office notifies you, verbally or in writing, of serious deficiencies that require correction before human studies can begin, it is understood that you will continue to withhold such studies until you are notified that the material you have submitted to correct the deficiencies is satisfactory. If such a clinical hold is placed on this file, you will be notified in writing of the reasons for placing the IND on hold.

10-18-99 P02:54 IN
10-18-99 P

You are responsible for compliance with applicable portions of the Public Health Service Act, the Federal Food, Drug, and Cosmetic Act, and the Code of Federal Regulations (CFR). A copy of 21 CFR Part 312, pertaining to INDs, is enclosed. Copies of other pertinent regulations are available from this Center upon request. The following points regarding obligations of an IND sponsor are included for your information only, and are not intended to be comprehensive.

Progress reports are required at intervals not exceeding one year and are due within 60 days of the anniversary of the date that the IND went into effect [21 CFR 312.33]. Any unexpected, fatal or immediately life-threatening reaction associated with use of this product must be reported to this Division by telephone or facsimile transmission no later than seven calendar days after initial receipt of the information, and all serious, unexpected adverse experiences must be reported, in writing, to this Division and to all study centers within fifteen calendar days after initial receipt of this information [21 CFR 312.32].

Charging for an investigational product in a clinical trial under an IND is not permitted without the prior written approval of the FDA.

Prior to use of each new lot of the investigational biologic in clinical trials, please submit the lot number, the results of all tests performed on the lot, and the specifications when established (i.e., the range of acceptable results).

If not included in your submission, please provide copies of the consent forms for each clinical study. A copy of the requirements for and elements of informed consent are enclosed. Also, please provide documentation of the institutional review board approval(s) for each clinical study.

All laboratory or animal studies intended to support the safety of this product should be conducted in compliance with the regulations for "Good Laboratory Practice for Nonclinical Laboratory Studies" (21 CFR Part 58, copies available upon request). If such studies have not been conducted in compliance with these regulations, please provide a statement describing in detail all differences between the practices used and those required in the regulations.

Item 7a of form FDA 1571 requests that either an "environmental assessment," or a "claim for categorical exclusion" from the requirements for environmental assessment, be included in the IND. If you did not include a response to this item with your application, please submit one. See the enclosed information sheet for additional information on how these requirements may be addressed.

Sponsors of INDs for products used to treat life-threatening or severely debilitating diseases are encouraged to consider the interim rule outlined in 21 CFR 312.80 through 312.88.

Telephone inquiries concerning this IND should be made directly to me at (301) 827-5101. Correspondence regarding this file should be addressed as follows:

Center for Biologics Evaluation and Research
Attn: Office of Therapeutics Research and Review
HFM-99, Room 200N
1401 Rockville Pike
Rockville, MD 20852-1448

If we have any comments after we have reviewed this submission, we will contact you.

Sincerely yours,

Kay Schneider

Kay Schneider, M.S.
Consumer Safety Officer
Division of Application Review and Policy
Office of Therapeutics
Research and Review
Center for Biologics
Evaluation and Research

Enclosures (3): 21 CFR Part 312
21 CFR 50.20, 50.25
Information sheet on 21 CFR 25.24

I

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Rockville, MD 20852

JAN 27 2006

Genentech, Inc.
Attention: Robert L. Garnick, Ph.D.
Senior Vice President, Regulatory Affairs, Quality, and Compliance
1 DNA Way
South San Francisco, CA 94080-4990

Dear Dr. Garnick:

We have received your biologics license application (BLA) submitted under section 351 of the Public Health Service Act for the following biological product:

Our Submission Tracking Number (STN): BL #125156/0

Name of Biological Product: Lucentis™ (ranibizumab)

Indication: Treatment for patients with neovascular age-related macular degeneration

Date of Application: December 29, 2005

Date of Receipt: December 30, 2005

User Fee Goal Date: June 30, 2006

All applications for new active ingredients, new dosage forms, new indications, new routes of administration, and new dosing regimens are required to contain an assessment of the safety and effectiveness of the product in pediatric patients unless this requirement is waived or deferred. We note that you have not fulfilled the requirement. We are waiving the requirement for pediatric studies for this application.

If you have not already done so, promptly submit the *content of labeling* (21 CFR 601.14(b)) in electronic format as described at the following website: http://www.fda.gov/oc/datacouncil/spl.html.

We will notify you within 60 days of the receipt date if the application is sufficiently complete to permit a substantive review.

We request that you submit all future correspondence, supporting data, or labeling relating to this application in triplicate, citing the above STN number. Please refer to http://www.fda.gov/cder/biologics/default.htm for important information regarding therapeutic biological products, including the addresses for submissions. Effective August 29, 2005, the new address for all submissions to this application is:

Food and Drug Administration
Center for Drug Evaluation and Research
Therapeutic Biological Products Document Room
5901-B Ammendale Road
Beltsville, MD 20705-1266

If you have any questions, please contact the Regulatory Project Manager, Lori Gorski, at (301) 796-0722.

Sincerely,

Maureen P. Dillon-Parker
Chief, Project Management Staff
Division of Anti-Infective and
Ophthalmology Products
Office of Antimicrobials
Center for Drug Evaluation and Research

J

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Rockville, MD 20852

BLA 125156

MAR 14 2006

Genentech, Inc.
Attention: Robert L. Garnick, Ph.D.
Senior Vice President, Regulatory Affairs, Quality & Compliance
1 DNA Way
South San Francisco, California 94080-4990

Dear Dr. Garnick:

This letter is in regard to your biologics license application (BLA) submitted under section 351 of the Public Health Service Act.

We have completed an initial review of your application dated December 29, 2005, for Lucentis (ranibizumab injection) to determine its acceptability for filing. Under 21 CFR 601.2(a), your application was filed on February 28, 2006. The user fee goal date is June 30, 2006. This acknowledgment of filing does not mean that we have issued a license nor does it represent any evaluation of the adequacy of the data submitted.

At this time, we have not identified any potential review issues. Our filing review is only a preliminary review, and deficiencies may be identified during substantive review of your application. Following a review of the application, we shall advise you in writing of any action we have taken and request additional information if needed.

Please refer to http://www.fda.gov/cder/biologics/default.htm for important information regarding therapeutic biological products, including the addresses for submissions.

Please use the following address for any amendments to your application:

Food and Drug Administration
Center for Drug Evaluation and Research
Therapeutic Biological Products Document Room
5901-B Ammendale Road
Beltsville, MD 20705-1266

If you have any questions, call Lori M. Gorski, Project Manager, at (301) 796-0722.

Sincerely,

Maureen Dillon Parker
Chief, Project Management Staff
Division of Anti-Infective and Ophthalmology Products
Office of Antimicrobial Products
Center for Drug Evaluation and Research

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent of: Paul J. Carter *et al.*-- § 156	Docket No: 22338-80060
Patent No.: 6,407,213	Assignee: Genentech, Inc.
Issued: June 18, 2002	Unit: OPLA
Application No: 08/146,206	

For: METHOD FOR MAKING HUMANIZED ANTIBODIES – Application for § 156 Patent Term Extension

Mail Stop: **Patent Ext.**
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

POWER OF ATTORNEY BY ASSIGNEE

The assignee of the entire right, title, and interest in U.S. Patent No. 6,407,213 (granted on application serial no. 08/146,206), Genentech Inc., hereby appoints the practitioners associated with

CUSTOMER NUMBER 33694

as its attorneys and agents to prosecute the captioned patent application, and to transact all business in the U.S. Patent and Trademark Office connected therewith.

Pursuant to 37 C.F.R. § 3.73(b), the undersigned states that Genentech Inc. is the assignee of the entire right, title, and interest in the captioned patent/application by virtue of an assignment by the inventors to Genentech Inc. recorded at Reel 7035/ Frame 0272.

The undersigned, whose title is supplied below, is authorized to act on behalf of the assignee.

Respectfully submitted,

Genentech, Inc.

08/23/06

Jeffrey S. Kubinec Date
Associate General Counsel – Patent Law

4-19-07

Patent Docket P0709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Paul J. Carter et al. Serial No.: 08/146,206 Filed: November 17, 1993 For: METHOD FOR MAKING HUMANIZED ANTIBODIES	Group Art Unit: 1642 Examiner: Minh-Tam Davis CONFIRMATION NO: **CUSTOMER NO: 09157** **EXPRESS MAIL NUMBERS:** **EV 384 511 097 US** **EV 384 511 106 US** April 17, 2007 Anna Kan Anna Kan

RESPONSE TO NOTICE UNDER 37 CFR 1.251 -PATENT

Mail Stop RECONSTUCTION
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This is responsive to the Notice under 37 CFR 1.251 -Patent , mailed October 17, 2006. The copy of the papers listed in the Notice under 37 CFR 1.251 are a complete and accurate copy of the applicant's record of such papers, except for the following:

1. The PALM INTRANET record states that a Response After Non-Final Action was filed on 07/28/1997. The Response was received by the PTO on 06/27/1997. Please see the enclosed copy.
2. The PALM INTRANET record states that a Notice of Appeal was filed on 08/10/1998. The Notice of Appeal was received by the PTO on 06/26/1998. Please see the enclosed copy.
3. The PALM INTRANET record states that an Extension of Time was filed on 08/10/1998. The Extension of Time was received by the PTO on 06/26/1998. Please see the enclosed copy.
4. The PALM INTRANET record states that an Examiner Interview Summary Record was created on 11/01/2001. Applicants' papers show there was one on 12/11/2001, but not one on 11/01/2001. Please see the enclosed copy.

5. A Request for a Corrected Filing Receipt was mailed on 06/24/1994. Please see the enclosed copy. The PALM INTRANET does not list this.

6. A Request for a Corrected Filing Receipt was mailed on 04/10/1995. Please see the enclosed copy. The PALM INTRANET does not list this.

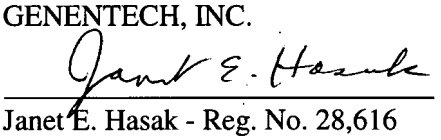
7. A Supplemental Information Disclosure Statement was filed on 10/07/1997. Please see the enclosed copy. The PALM INTRANET does not list this.

8.An Examiner Interview Summary Record was created on 07/16/1999. Please see the enclosed copy. The PALM INTRANET does not list this.

Respectfully submitted,

GENENTECH, INC.

Date: April 17, 2007

By: 

Janet E. Hasak - Reg. No. 28,616
for Wendy M. Lee - Reg. No. 40,378
Telephone: (650) 225-1994

Revised (10/18/95)

In re Patent No.: 6,407,213 B1

Patentee: Carter et al

Patent Date: June 18 2002

Application No.: 08/146,206

Filing Date: November 17 1993

Direct to:

Mail Stop RECONSTRUCTION
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

NOTICE UNDER 37 CFR 1.251 - Patent

Statement (check the appropriate box):

☐ The copy submitted with this reply is a complete and accurate copy of applicant's record of all of the correspondence between the Office and the applicant for the above-identified application (except for U.S. patent documents), and applicant is not aware of any correspondence between the Office and applicant for the above-identified application that is not among applicant's records.

☒ The copy of the paper(s) listed in the notice under 37 CFR 1.251 is/are a complete and accurate copy of applicant's record of such paper(s). Except for the items listed in the Response to Notice under 37 CFR 1.251–Patent

☐ The papers produced by applicant are applicant's complete record of all of the correspondence between the Office and the applicant for the above-identified application (except for U.S. patent documents), and applicant is not aware of any correspondence between the Office and the applicant for the above-identified application that is not among applicant's records.

☐ Applicant does not possess any record of the correspondence between the Office and the applicant for the above-identified application.

April 17, 2007
Date

WMLee
Signature

Wendy Lee
Typed or printed name

A copy of this notice should be returned with the reply.

Burden Hour Statement: This collection of information is required by 37 CFR 1.251. The information is used by the public to reply to a request for copies of correspondence between the applicant and the USPTO in order to reconstruct an application file. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This form is estimated to take 60 minutes to complete. This time will vary depending upon the needs of the individual case. Any comments on the amount of time you are required to complete this form should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.**

Patent Docket P0709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Paul J. Carter et al. Serial No.: 08/146,206 Filed: November 17, 1993 For: METHOD FOR MAKING HUMANIZED ANTIBODIES	Group Art Unit: 1642 Examiner: Minh-Tam Davis Confirmation No: 3992 **Customer No: 09157** **EXPRESS MAIL NUMBERS:** **EV 384 511 097 US** **EV 384 511 106 US** April 17, 2007 Anna Kan Anna Kan

TRANSMITTAL LETTER

Mail Stop RECONSTRUCTION
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This is responsive to the Notice under 37 CFR 1.251 -Patent, mailed October 17, 2006. Transmitted herewith are the following documents:

1. Response to Notice Under 37 CFR 1.251 - Patent
2. Copy of the Notice Under 37 CFR 1.251 - Patent
3. Copy of the PALM INTRANET Listing
4. Copies of Correspondence between PTO and Applicant
5. Copies of References Cited in Information Disclosure Statements

In the event any additional fees are due in connection with the filing of these documents, the Commissioner is authorized to charge such fees to our Deposit Account No. 07-0630.

Respectfully submitted,
GENENTECH, INC.

Date: April 17, 2007

By: Janet E. Hasak

Janet E. Hasak - Reg. No. 28,616
for Wendy M. Lee - Reg. No. 40,378
Telephone: (650) 225-1994

4-19-07

COPY

In re Application of: Paul J. Carter et al.
Serial No.: 08/146,206
Filed On: November 17, 1993
Mailed On: April 17, 2007

Docket No.: P0709P1
By: Janet E. Hasak - Reg. 28,616
for Wendy M. Lee- Reg. 40,378

The following has been received in the U.S. Patent Office on the date stamped:

1. Response to Notice Under 37 CFR 1.251 - Patent
2. Copy of the Notice Under 37 CFR 1.251 - Patent and copy of the PALM INTRANET Listing
3. Copies of Correspondence between PTO and Applicant
4. Copies of References Cited in Information Disclosure Statements

Express Mail No. EV 384 511 097 US
EV 384 511 106 US

EV384511106US 4-19-07

EV384511097US

Patent Docket P0709P1

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Paul J. Carter et al. Serial No.: 08/146,206 Filed: November 17, 1993 For: METHOD FOR MAKING HUMANIZED ANTIBODIES	Group Art Unit: 1642 Examiner: Minh-Tam Davis Confirmation No: 3992 **Customer No: 09157** **EXPRESS MAIL NUMBERS:** **EV 384 511 097 US** **EV 384 511 106 US** April 17, 2007 Anna Kan Anna Kan

TRANSMITTAL LETTER

Mail Stop RECONSTRUCTION
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

This is responsive to the Notice under 37 CFR 1.251 -Patent, mailed October 17, 2006. Transmitted herewith are the following documents:

1. Response to Notice Under 37 CFR 1.251 - Patent
2. Copy of the Notice Under 37 CFR 1.251 - Patent
3. Copy of the PALM INTRANET Listing
4. Copies of Correspondence between PTO and Applicant
5. Copies of References Cited in Information Disclosure Statements

In the event any additional fees are due in connection with the filing of these documents, the Commissioner is authorized to charge such fees to our Deposit Account No. 07-0630.

Respectfully submitted,
GENENTECH, INC.

Date: April 17, 2007

By: Janet E Hasak

Janet E. Hasak - Reg. No. 28,616
for Wendy M. Lee - Reg. No. 40,378
Telephone: (650) 225-1994

Day : Tuesday
Date: 10/17/2006

PALM INTRANET

Time: 12:35:09

Patent Number Information

Application Number: **08/146206**
Assignments
Filing or 371(c) Date: **11/17/1993**
Effective Date: **11/17/1993**
Application Received: **11/17/1993**
Patent Number: **6407213**
Issue Date: **06/18/2002**
Date of Abandonment: **00/00/0000**
Attorney Docket Number: **709P1**
Status: **150 / PATENTED CASE**
Confirmation Number: **3992**

Examiner Number: **73622 / DAVIS, MINH TAM**
Group Art Unit: **1642**
Class/Subclass: **530/387.300**
Lost Case: **YES**
Interference Number:
Unmatched Petition: **NO**
L&R Code: Secrecy Code:**1**
Third Level Review: **NO** Secrecy Order: **NO**
Status Date: **05/31/2002**
Oral Hearing: **NO**

Title of Invention: **METHOD FOR MAKING HUMANIZED ANTIBODIES**

Bar Code	PALM Location	Location Date	Charge to Loc	Charge to Name	Employee Name	Location
08146206	16M1	02/23/2006	16X1	DAVIS, MINH TAM	1600,OUTGOING MAIL	REM/00/A 89

Appln Info | Contents | Petition Info | Atty/Agent Info | Continuity/Reexam | Foreign Data

Search Another: Application# [ ] Search **or Patent#** [ ] Search
PCT / [ ] / [ ] Search **or PG PUBS #** [ ] Search
Attorney Docket # [ ] Search
Bar Code # [ ] Search

To go back use Back button on your browser toolbar.

Back to PALM | ASSIGNMENT | OASIS | Home page

Day : Tuesday
Date: 10/17/2006

Time: 12:39:56

PALM INTRANET

Content Information for 08/146206

Search Another: Application# [ ] Search **or Patent#** [ ] Search

PCT / [ ] / [ ] Search **or PG PUBS #** [ ] Search

Attorney Docket # [ ] Search

Bar Code # [ ] Search

Appln Info | **Contents** | Petition Info | Atty/Agent Info | Continuity/Reexam | Foreign Data

Date	Status	Code	Description
10/17/2006		M2512	MAIL RECONSTRUCTION NOTICE - PATENTED APPLIC
10/17/2006		2512	RECONSTRUCTION NOTICE UNDER 37 CFR 1.251 - PATI
08/29/2006		LFLOST	FILE MARKED LOST
10/30/2002		N423	POST ISSUE COMMUNICATION - CERTIFICATE OF COR
06/20/2002		CRFA	SEQUENCE MOVED TO PUBLIC DATABASE
06/18/2002		PGM/	RECORDATION OF PATENT GRANT MAILED
05/31/2002	150	WPIR	ISSUE NOTIFICATION MAILED
06/18/2002		PTAC	PATENT ISSUE DATE USED IN PTA CALCULATION
05/09/2002		R1021	RECEIPT INTO PUBS
05/04/2002		PILS	APPLICATION IS CONSIDERED READY FOR ISSUE
03/18/2002	95	N084	ISSUE FEE PAYMENT VERIFIED
03/18/2002		DRWF	WORKFLOW - DRAWINGS FINISHED
03/18/2002		DRWM	WORKFLOW - DRAWINGS MATCHED WITH FILE AT CO
05/02/2002		R1021	RECEIPT INTO PUBS
03/15/2002		CSRF	WORKFLOW - CUSTOMER SERVICE REQUEST - FINISH
03/26/2002		R1021	RECEIPT INTO PUBS
03/18/2002		DRWI	WORKFLOW - DRAWINGS RECEIVED AT CONTRACTOI
03/18/2002		DRWR	WORKFLOW - DRAWINGS SENT TO CONTRACTOR
03/18/2002		R85B	WORKFLOW -RECEIVED 85B - UNMATCHED
03/18/2002	94	IFEE	ISSUE FEE PAYMENT RECEIVED
03/15/2002		CSRI	WORKFLOW - CUSTOMER SERVICE REQUEST - BEGIN
01/28/2002		SENT	WORKFLOW - FILE SENT TO CONTRACTOR
01/28/2002	93	R1021	RECEIPT INTO PUBS
01/08/2002		D1220	DISPATCH TO PUBLICATIONS
12/18/2001	92	MN/=.	MAIL NOTICE OF ALLOWANCE

12/18/2001		MN/DR	MAIL FORMAL DRAWINGS REQUIRED
12/14/2001		N/DR	FORMAL DRAWINGS REQUIRED
12/14/2001	90	N/=.	NOTICE OF ALLOWANCE DATA VERIFICATION COMPI
12/14/2001	89	CNTA	NOTICE OF ALLOWABILITY
11/01/2001		EXIN	EXAMINER INTERVIEW SUMMARY RECORD (PTOL - 4
08/29/2001		EXIN	EXAMINER INTERVIEW SUMMARY RECORD (PTOL - 4
10/02/2001		AF/D	AFFIDAVIT(S) (RULE 131 OR 132) OR EXHIBIT(S) RECEI
10/11/2001		FWDX	DATE FORWARDED TO EXAMINER
10/02/2001		SA..	SUPPLEMENTAL RESPONSE
09/04/2001		M844	INFORMATION DISCLOSURE STATEMENT (IDS) FILED
08/16/2001		FWDX	DATE FORWARDED TO EXAMINER
07/30/2001		SA..	SUPPLEMENTAL RESPONSE
07/13/2001		FWDX	DATE FORWARDED TO EXAMINER
07/13/2001		SA..	SUPPLEMENTAL RESPONSE
04/26/2001		EXIN	EXAMINER INTERVIEW SUMMARY RECORD (PTOL - 4
05/02/2001		FWDX	DATE FORWARDED TO EXAMINER
04/26/2001	71	A...	RESPONSE AFTER NON-FINAL ACTION
04/26/2001		XT/G	REQUEST FOR EXTENSION OF TIME - GRANTED
10/25/2000	41	MCTNF	MAIL NON-FINAL REJECTION
10/23/2000	40	CTNF	NON-FINAL REJECTION
09/06/2000		DOCK	CASE DOCKETED TO EXAMINER IN GAU
11/17/1993	19	IEXX	INITIAL EXAM TEAM NN
02/03/2000		CRFE	CRF IS GOOD TECHNICALLY / ENTERED INTO DATAB/
01/11/2000		FWDX	DATE FORWARDED TO EXAMINER
12/28/1999		SA..	SUPPLEMENTAL RESPONSE
01/11/2000		CRFL	CRF DISK HAS BEEN RECEIVED BY PREEXAM / GROUI
11/24/1999	41	MCTMS	MAIL MISCELLANEOUS COMMUNICATION TO APPLIC
11/22/1999	40	CTMS	MISCELLANEOUS ACTION WITH SSP
08/23/1999		EXIN	EXAMINER INTERVIEW SUMMARY RECORD (PTOL - 4
09/07/1999		FWDX	DATE FORWARDED TO EXAMINER
08/30/1999		SA..	SUPPLEMENTAL RESPONSE
07/19/1999		FWDX	DATE FORWARDED TO EXAMINER
07/16/1999		SA..	SUPPLEMENTAL RESPONSE
05/07/1999		FWDX	DATE FORWARDED TO EXAMINER
04/09/1999	71	ELC.	RESPONSE TO ELECTION / RESTRICTION FILED
03/29/1999	41	MCTRS	MAIL RESTRICTION REQUIREMENT
03/26/1999	40	CTRS	REQUIREMENT FOR RESTRICTION / ELECTION

03/12/1999		M844	INFORMATION DISCLOSURE STATEMENT (IDS) FILED
02/01/1999		M844	INFORMATION DISCLOSURE STATEMENT (IDS) FILED
02/01/1999		RQPR	REQUEST FOR FOREIGN PRIORITY (PRIORITY PAPERS
01/07/1999		EXIN	EXAMINER INTERVIEW SUMMARY RECORD (PTOL - 4
01/19/1999		FWDX	DATE FORWARDED TO EXAMINER
01/15/1999		SA..	SUPPLEMENTAL RESPONSE
11/09/1998		FWDX	DATE FORWARDED TO EXAMINER
11/06/1998		SA..	SUPPLEMENTAL RESPONSE
10/16/1998		EXIN	EXAMINER INTERVIEW SUMMARY RECORD (PTOL - 4
08/26/1998		M844	INFORMATION DISCLOSURE STATEMENT (IDS) FILED
08/26/1998		AF/D	AFFIDAVIT(S) (RULE 131 OR 132) OR EXHIBIT(S) RECEI
09/03/1998		FWDX	DATE FORWARDED TO EXAMINER
08/26/1998	71	R129	REQUEST UNDER RULE 129 TO REOPEN PROSECUTION
08/26/1998		MABN3	MAIL EXPRESS ABANDONMENT (DURING EXAMINATI
08/26/1998	168	ABN3	EXPRESS ABANDONMENT (DURING EXAMINATION)
08/10/1998	120	N/AP	NOTICE OF APPEAL FILED
08/10/1998		XT/G	REQUEST FOR EXTENSION OF TIME - GRANTED
08/13/1998		EXIN	EXAMINER INTERVIEW SUMMARY RECORD (PTOL - 4
04/13/1998		C.AD	CORRESPONDENCE ADDRESS CHANGE
12/23/1997	61	MCTFR	MAIL FINAL REJECTION (PTOL - 326)
12/22/1997	60	CTFR	FINAL REJECTION
10/10/1997		FWDX	DATE FORWARDED TO EXAMINER
10/07/1997		SA..	SUPPLEMENTAL RESPONSE
10/10/1997		FWDX	DATE FORWARDED TO EXAMINER
09/01/1997		SA..	SUPPLEMENTAL RESPONSE
10/10/1997		CRFL	CRF DISK HAS BEEN RECEIVED BY PREEXAM / GROUI
09/01/1997		M844	INFORMATION DISCLOSURE STATEMENT (IDS) FILED
10/10/1997		CRFL	CRF DISK HAS BEEN RECEIVED BY PREEXAM / GROUI
10/09/1997		CRFE	CRF IS GOOD TECHNICALLY / ENTERED INTO DATAB/
08/01/1997		FWDX	DATE FORWARDED TO EXAMINER
07/28/1997	71	A...	RESPONSE AFTER NON-FINAL ACTION
06/27/1997		XT/G	REQUEST FOR EXTENSION OF TIME - GRANTED
07/23/1997		EXIN	EXAMINER INTERVIEW SUMMARY RECORD (PTOL - 4
12/23/1996	41	MCTNF	MAIL NON-FINAL REJECTION
12/23/1996	40	CTNF	NON-FINAL REJECTION
12/03/1996		M844	INFORMATION DISCLOSURE STATEMENT (IDS) FILED
12/10/1996		FWDX	DATE FORWARDED TO EXAMINER

12/03/1996	71	R129	REQUEST UNDER RULE 129 TO REOPEN PROSECUTION
12/03/1996		MABN3	MAIL EXPRESS ABANDONMENT (DURING EXAMINATI
12/03/1996	168	ABN3	EXPRESS ABANDONMENT (DURING EXAMINATION)
08/30/1996		XT/G	REQUEST FOR EXTENSION OF TIME - GRANTED
08/30/1996		M844	INFORMATION DISCLOSURE STATEMENT (IDS) FILED
04/01/1996	120	N/AP	NOTICE OF APPEAL FILED
04/01/1996		XT/G	REQUEST FOR EXTENSION OF TIME - GRANTED
04/08/1996		EXIN	EXAMINER INTERVIEW SUMMARY RECORD (PTOL - 4
12/26/1995		M844	INFORMATION DISCLOSURE STATEMENT (IDS) FILED
10/27/1995	61	MCTFR	MAIL FINAL REJECTION (PTOL - 326)
10/26/1995	60	CTFR	FINAL REJECTION
08/03/1995		M844	INFORMATION DISCLOSURE STATEMENT (IDS) FILED
08/18/1995		FWDX	DATE FORWARDED TO EXAMINER
06/12/1995	71	A...	RESPONSE AFTER NON-FINAL ACTION
06/12/1995		XT/G	REQUEST FOR EXTENSION OF TIME - GRANTED
04/17/1995		M844	INFORMATION DISCLOSURE STATEMENT (IDS) FILED
12/09/1994	41	MCTNF	MAIL NON-FINAL REJECTION
12/06/1994	40	CTNF	NON-FINAL REJECTION
10/04/1994		FWDX	DATE FORWARDED TO EXAMINER
09/26/1994	71	ELC.	RESPONSE TO ELECTION / RESTRICTION FILED
08/26/1994	41	MCTRS	MAIL RESTRICTION REQUIREMENT
08/25/1994	40	CTRS	REQUIREMENT FOR RESTRICTION / ELECTION
06/15/1994		CRFE	CRF IS GOOD TECHNICALLY / ENTERED INTO DATABA
06/14/1994		CRFL	CRF DISK HAS BEEN RECEIVED BY PREEXAM / GROUP
06/06/1994		A.PE	PRELIMINARY AMENDMENT
11/17/1993		A.PE	PRELIMINARY AMENDMENT
05/24/1994	30	DOCK	CASE DOCKETED TO EXAMINER IN GAU
05/14/1994		FILM	APPLICATION CAPTURED ON MICROFILM
05/03/1994		COMP	APPLICATION IS NOW COMPLETE
05/09/1994		INCD	NOTICE MAILED--APPLICATION INCOMPLETE--FILING
04/15/1994		CRFD	CRF IS FLAWED TECHNICALLY / NOT ENTERED INTO I
04/07/1994		RTAD	RELEASED TO OIPE
04/04/1994		M903	NOTICE OF DO/EO ACCEPTANCE MAILED
03/31/1994		CRFL	CRF DISK HAS BEEN RECEIVED BY PREEXAM / GROUP
03/14/1994		DKTD	371 APPLICATION PREEXAMINATION DOCKETING
02/19/1994		IBPM	IB PAPER MATCH
12/02/1993		DKTD	371 APPLICATION PREEXAMINATION DOCKETING

12/02/1993		DYWD	APPLICANT DELAY WAIVED
12/02/1993		R331	DEMAND RECEIVED
11/17/1993		R371	RECEIPT OF 371 REQUEST

Appln Info | Contents | Petition Info | Atty/Agent Info | Continuity/Reexam | Foreign Data

To go back use Back button on your browser toolbar.

Back to PALM | ASSIGNMENT | OASIS | Home page

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450
www.uspto.gov

JUN 19 2007

Office of Regulatory Policy
HFD-7
5600 Fishers Lane (Rockwall II Rm 1101)
Rockville, MD 20857

Attention: Beverly Friedman

The attached application for patent term extension of U.S. Patent No. 6,407,213, was filed on August 25, 2006, under 35 U.S.C. § 156. It is noted that patent term extension applications for the same regulatory review period for the human biological product, LUCENTIS® (ranibizumab), have been filed in U.S. Patent No. 7,060,269 (as indicated in a letter to FDA mailed on April 3, 2007) and U.S. Patent No. 6,884,879.

The assistance of your Office is requested in confirming that the product identified in the application, LUCENTIS® (ranibizumab), has been subject to a regulatory review period within the meaning of 35 U.S.C. § 156(g) before its first commercial marketing or use and that the application for patent term extension was filed within the sixty-day period after the product was approved. Since a determination has not been made whether the patent in question claims a product which has been subject to the Federal Food, Drug and Cosmetic Act, or a method of manufacturing or use of such a product, this communication is NOT to be considered as notice which may be made in the future pursuant to 35 U.S.C. § 156(d)(2)(A).

Our review of the application to date indicates that the subject patent would be eligible for extension of the patent term under 35 U.S.C. § 156.

Applicant is advised that despite the statement in compliance with 37 C.F.R. § 1.740(a)(14) regarding payment of the applicable fee by check for submission of a patent term extension application, no check was present and no record exists of the Office cashing the check. Therefore, in accordance with the express authorization provided in the same paragraph, the fee of $1,120 as perscribed in 37 C.F.R. § 1.20(j) is being charged to deposit account no. 18-1260.

Inquiries regarding this communication should be directed to the undersigned at (571) 272-7755 (telephone) or (571) 273-7755 (facsimile).

Mary C. Till
Legal Advisor
Office of Patent Legal Administration
Office of the Deputy Commissioner
for Patent Examination Policy

cc: Jeffrey P. Kushan
Sidley Austin LLP
1501 K Street, N.W.
Washington, DC 20005

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Rockville MD 20857

NOV 2 1 2007

Re: Lucentis
Patent Nos. 6,407,213
6,884,879
Docket Nos. 2007E-0424
2007E-0425

The Honorable Jon Dudas
Under Secretary of Commerce for Intellectual Property
Director of the United States Patent and Trademark Office
Mail Stop Hatch-Waxman PTE
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Director Dudas:

This is in regard to the application for patent term extension for U.S. Patent Nos. 6,407,213 and 6,884,879 filed by Genentech, Inc. under 35 U.S.C. § 156. The human biological product claimed by these patents is Lucentis (ranibizumab), which was assigned biologic license application (BLA) No. 125156/0.

A review of the Food and Drug Administration's official records indicates that this product was subject to a regulatory review period before its commercial marketing or use, as required under 35 U.S.C. § 156(a)(4). Our records also indicate that it represents the first permitted commercial marketing or use of the product, as defined under 35 U.S.C. § 156(f)(1), and interpreted by the courts in *Glaxo Operations UK Ltd. v. Quigg*, 706 F. Supp. 1224 (E.D. Va. 1989), *aff'd*, 894 F. 2d 392 (Fed. Cir. 1990).

The BLA was approved on June 30, 2006, which makes the submission of the patent term extension applications on August 25, 2006, timely within the meaning of 35 U.S.C. § 156(d)(1).

Should you conclude that the subject patents are eligible for patent term extension, please advise us accordingly. As required by 35 U.S.C. § 156(d)(2)(A) we will then determine the applicable regulatory review period, publish the determination in the *Federal Register*, and notify you of our determination.

Please let me know if we can be of further assistance.

Sincerely yours,

Jane A. Axelrad

Jane A. Axelrad
Associate Director for Policy
Center for Drug Evaluation and Research

cc: Jeffrey P. Kushan
SIDLEY AUSTIN LLP
1501 K Street, N.W.
Washington, DC 20005

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450
www.uspto.gov

JAN - 8 2008

Office of Regulatory Policy
HFD - 7
5600 Fishers Lane (Rockwall II Rm. 1101)
Rockville, MD 20857

Attention: Beverly Friedman

Dear Ms. Axelrad:

Transmitted herewith is a copy of the application for patent term extension of U.S. Patent No. 6,407,213. The application was filed on August 25, 2006, under 35 U.S.C. § 156. It is noted that patent term extension applications for the same regulatory review period for the human biological product, LUCENTIS® (ranibizumab), have been filed in U.S. Patent Nos. 6,884,879 and 7,060,269.

The patent claims a product that was subject to regulatory review under the Federal Food, Drug and Cosmetic Act. Subject to final review, the subject patent is considered to be eligible for patent term extension. Thus, a determination by your office of the applicable regulatory review period is necessary. Accordingly, notice and a copy of the application are provided pursuant to 35 U.S.C. § 156(d)(2)(A).

Inquiries regarding this communication should be directed to the undersigned at (571)272-7755 (telephone) or (571) 273-7755 (facsimile).

Mary C. Till
Legal Advisor
Office of Patent Legal Administration
Office of the Deputy Commissioner
for Patent Examination Policy

cc: Jeffrey P. Kushan
Sidley Austin, LLP
1501 K Street, N.W.
Washington, DC 20005

RE: LUCENTIS® (ranibizumab)
FDA Docket No. 2007E-0424

DEPARTMENT OF HEALTH & HUMAN SERVICES

Food and Drug Administration
Rockville MD 20857

APR 28 2008

Re: LUCENTIS - 6,407,213
Docket No.: 2007E-0424
LUCENTIS - 6,884,879
Docket No.: 2007E-0425
LUCENTIS - 7,060,269
Docket No.: 2007E-0146

The Honorable Jon Dudas
Undersecretary of Commerce for Intellectual Property
Director of the United States Patent and Trademark Office
Mail Stop Hatch-Waxman PTE
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Director Dudas:

This is in regard to the applications for patent term extension for U.S. Patent Nos. 6,407,213; 6,884,879; and 7,060,269, filed by Genentech, Inc., under 35 U.S.C. section 156 et seq. We have reviewed the dates contained in the application and have determined the regulatory review period for LUCENTIS (ranibizumab), the human biological product claimed by the patents.

The total length of the regulatory review period for LUCENTIS is 2,430 days. Of this time, 2,247 days occurred during the testing phase and 183 days occurred during the approval phase. These periods of time were derived from the following dates:

1. The date an exemption under subsection 505(i) of the Federal Food, Drug, and Cosmetic Act involving this biologic product became effective: November 6, 1999.

   The applicant claims October 7, 1999, as the date the investigational new drug application (IND) became effective. However, FDA records indicate that the IND effective date was November 6, 1999, which was thirty days after FDA receipt of the IND.

2. The date the application was initially submitted with respect to the human biological product under section 351 of the Public Health Service Act: December 30, 2005.

   The applicant claims December 29, 2005, as the date the biologics license application (BLA) for LUCENTIS (BLA 125156/0) was initially submitted. However, FDA records indicate that BLA 125156/0 was submitted on December 30, 2005.

3. The date the application was approved: June 30, 2006.

   FDA has verified the applicant's claim that BLA 125156/0 was approved on June 30, 2006.

This determination of the regulatory review period by FDA does not take into account the effective date of the patents, nor does it exclude one-half of the testing phase as required by 35 U.S.C. section 156(c)(2).

Please let me know if we can be of further assistance.

Sincerely yours,

Jane A. Axelrad

Jane A. Axelrad
Associate Director for Policy
Center for Drug Evaluation and Research

cc: Jeffrey P. Kushan
SIDLEY AUSTIN LLP
1501 K Street, N.W.
Washington, DC 20005

a person with Medicare could be identified because the sample is small enough to identify participants. CMS would make exceptions if the information is needed for one of the routine uses or if it's required by law.

POLICIES AND PRACTICES FOR STORING, RETRIEVING, ACCESSING, RETAINING, AND DISPOSING OF RECORDS IN THE SYSTEM:

STORAGE:

Records are stored on both tape cartridges (magnetic storage media) and in a DB2 relational database management environment (DASD data storage media).

RETRIEVABILITY:

Information is most frequently retrieved by HICN, provider number (facility, physician, IDs), service dates, and beneficiary state code.

SAFEGUARDS AND PROTECTIONS:

CMS has protections in place for authorized users to make sure they are properly using the data and there is no unauthorized use. Personnel having access to the system have been trained in the Privacy Act and information security requirements. Employees who maintain records in this system cannot use or disclose data until the recipient agrees to implement appropriate management, operational and technical safeguards that will protect the confidentiality, integrity, and availability of the information and information systems.

This system would follow all applicable Federal laws and regulations, and Federal, HHS, and CMS security and data privacy policies and standards. These laws and regulations include but are not limited to: the Privacy Act of 1974; the Federal Information Security Management Act of 2002 (when applicable); the Computer Fraud and Abuse Act of 1986; the Health Insurance Portability and Accountability Act of 1996; the E-Government Act of 2002, the Clinger-Cohen Act of 1996; the Medicare Modernization Act of 2003, and the corresponding implementing regulations. OMB Circular A–130, Management of Federal Resources, Appendix III, Security of Federal Automated Information Resources also applies. Federal, HHS, and CMS policies and standards include but are not limited to all pertinent National Institute of Standards and Technology publications, the HHS Information Systems Program Handbook, and the CMS Information Security Handbook.

RETENTION AND DISPOSAL:

Records are maintained with identifiers for all transactions after they are entered into the system for a period of 20 years. Records are housed in both active and archival files. All claims-related records are encompassed by the document preservation order and will be retained until notification is received from the Department of Justice.

SYSTEM MANAGER AND ADDRESS:

Director, Centers for Beneficiary Choices, CMS, Mail stop C5–19–07, 7500 Security Boulevard, Baltimore, Maryland 21244–1850.

NOTIFICATION PROCEDURE:

For purpose of notification, the subject individual should write to the system manager who will require the system name, and the retrieval selection criteria (e.g., HICN, facility/pharmacy number, service dates, etc.).

RECORD ACCESS PROCEDURE:

For purpose of access, use the same procedures outlined in Notification Procedures above. Requestors should also reasonably specify the record contents being sought. (These procedures are in accordance with Department regulation 45 CFR 5b.5 (a)(2).)

CONTESTING RECORD PROCEDURES:

The subject individual should contact the system manager named above, and reasonably identify the record and specify the information to be contested. State the corrective action sought and the reasons for the correction with supporting justification. (These procedures are in accordance with Department regulation 45 CFR 5b.7.)

RECORD SOURCE CATEGORIES:

Summary prescription drug claim information contained in this system is obtained from the Part D Sponsor daily and monthly drug event transaction reports, Medicare Beneficiary Database (09–70–0530), and other payer information to be provided by the TROOP Facilitator.

SYSTEMS EXEMPTED FROM CERTAIN PROVISIONS OF THE ACT:

None.

[FR Doc. E8–11949 Filed 5–28–08; 8:45 am]
BILLING CODE 4120–03–P

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Food and Drug Administration

[Docket Nos. FDA–2007–E–0461 (formerly Docket No. 2007E–0424), FDA–2007–E–0165 (formerly Docket No. 2007E–0425), FDA–2007–E–0459 (formerly Docket No. 2007E–0146)]

Determination of Regulatory Review Period for Purposes of Patent Extension; LUCENTIS

AGENCY: Food and Drug Administration, HHS.

ACTION: Notice.

SUMMARY: The Food and Drug Administration (FDA) has determined the regulatory review period for LUCENTIS and is publishing this notice of that determination as required by law. FDA has made the determination because of the submission of applications to the Director of Patents and Trademarks, Department of Commerce, for the extension of patents which claim that human biological product.

ADDRESSES: Submit written or electronic comments and petitions to the Division of Dockets Management (HFA–305), Food and Drug Administration, 5630 Fishers Lane, rm. 1061, Rockville, MD 20852. Submit electronic comments to *http://www.regulations.gov.*

FOR FURTHER INFORMATION CONTACT: Beverly Friedman, Center for Drug Evaluation and Research, Food and Drug Administration, 10903 New Hampshire Ave., Bldg. 51, rm. 6222, Silver Spring, MD, 20993–0002, 301–796–3602.

SUPPLEMENTARY INFORMATION: The Drug Price Competition and Patent Term Restoration Act of 1984 (Public Law 98–417) and the Generic Animal Drug and Patent Term Restoration Act (Public Law 100–670) generally provide that a patent may be extended for a period of up to 5 years so long as the patented item (human drug product, animal drug product, medical device, food additive, or color additive) was subject to regulatory review by FDA before the item was marketed. Under these acts, a product's regulatory review period forms the basis for determining the amount of extension an applicant may receive.

A regulatory review period consists of two periods of time: A testing phase and an approval phase. For human biological products, the testing phase begins when the exemption to permit the clinical investigations of the biological product becomes effective

and runs until the approval phase begins. The approval phase starts with the initial submission of an application to market the human biological product and continues until FDA grants permission to market the biological product. Although only a portion of a regulatory review period may count toward the actual amount of extension that the Director of Patents and Trademarks may award (for example, half the testing phase must be subtracted as well as any time that may have occurred before the patent was issued), FDA's determination of the length of a regulatory review period for a human biological product will include all of the testing phase and approval phase as specified in 35 U.S.C. 156(g)(1)(B).

FDA recently approved for marketing the human biologic product LUCENTIS (ranibizumab). LUCENTIS is indicated for the treatment of patients with neovascular (wet) age-related macular degeneration. Subsequent to this approval, the Patent and Trademark Office received patent term restoration applications for LUCENTIS (U.S. Patent Nos. 6,407,213; 6,884,879; and 7,060,269) from Genentech, Inc., and the Patent and Trademark Office requested FDA's assistance in determining this patent's eligibility for patent term restoration. In letters dated July 24, 2007, and November 21, 2007, FDA advised the Patent and Trademark Office that this human biological product had undergone a regulatory review period and that the approval of LUCENTIS represented the first permitted commercial marketing or use of the product. Shortly thereafter, the Patent and Trademark Office requested that FDA determine the product's regulatory review period.

FDA has determined that the applicable regulatory review period for LUCENTIS is 2,430 days. Of this time, 2,247 days occurred during the testing phase of the regulatory review period, while 183 days occurred during the approval phase. These periods of time were derived from the following dates:

1. *The date an exemption under section 505(i) of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. 355(i)) became effective*: November 6, 1999. The applicant claims October 7, 1999, as the date the investigational new drug application (IND) became effective. However, FDA records indicate that the IND effective date was November 6, 1999, which was 30 days after FDA receipt of the IND.

2. *The date the application was initially submitted with respect to the human biological product under section 351 of the Public Health Service Act (42 U.S.C. 262)*: December 30, 2005. The applicant claims December 29, 2005, as the date the biologics license application (BLA) for LUCENTIS (BLA 125156/0) was initially submitted. However, FDA records indicate that BLA 125156/0 was submitted on December 30, 2005.

3. *The date the application was approved*: June 30, 2006. FDA has verified the applicant's claim that BLA 125156/0 was approved on June 30, 2006.

This determination of the regulatory review period establishes the maximum potential length of a patent extension. However, the U.S. Patent and Trademark Office applies several statutory limitations in its calculations of the actual period for patent extension. In its applications for patent extension for U.S. Patent Nos. 6,407,213; 6,884,879; and 7,060,269, this applicant seeks 378 days; 307 days or 17 days, respectively, of patent term extension.

Anyone with knowledge that any of the dates as published are incorrect may submit to the Division of Dockets Management (see **ADDRESSES**) written or electronic comments and ask for a redetermination by July 28, 2008. Furthermore, any interested person may petition FDA for a determination regarding whether the applicant for extension acted with due diligence during the regulatory review period by November 25, 2008. To meet its burden, the petition must contain sufficient facts to merit an FDA investigation. (See H. Rept. 857, part 1, 98th Cong., 2d sess., pp. 41–42, 1984.) Petitions should be in the format specified in 21 CFR 10.30.

Comments and petitions should be submitted to the Division of Dockets Management. Three copies of any mailed information are to be submitted, except that individuals may submit one copy. Comments are to be identified with the docket number found in brackets in the heading of this document. Comments and petitions may be seen in the Division of Dockets Management between 9 a.m. and 4 p.m., Monday through Friday.

Please note that on January 15, 2008, the FDA Division of Dockets Management Web site transitioned to the Federal Dockets Management System (FDMS). FDMS is a Government-wide, electronic docket management system. Electronic comments or submissions will be accepted by FDA only through FDMS at *http://www.regulations.gov.*

Dated: May 8, 2008.

Jane A. Axelrad,

Associate Director for Policy, Center for Drug Evaluation and Research.

[FR Doc. E8–12007 Filed 5–28–08; 8:45 am]

BILLING CODE 4160–01–S

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Food and Drug Administration

[Docket No. FDA–2007–M–0467] (formerly Docket No. 2007M–0408), [Docket No. FDA–2007–M–0481] (formerly Docket No. 2007M–0467), [Docket No. FDA–2007–M–0480] (formerly Docket No. 2007M–0409), [Docket No. FDA–2007–M–0472] (formerly Docket No. 2007M–0413), [Docket No. FDA–2007–M–0468] (formerly Docket No. 2007M–0446), [Docket No. FDA–2007–M–0494] (formerly Docket No. 2007M–0380), [Docket No. FDA–2007–M–0493] (formerly Docket No. 2007M–0411), [Docket No. FDA–2007–M–0492] (formerly Docket No. 2007M–0410), [Docket No. FDA–2007–M–0490] (formerly Docket No. 2007M–0415), [Docket No. FDA–2007–M–0491] (formerly Docket No. 2007M–0447]

Medical Devices; Availability of Safety and Effectiveness Summaries for Premarket Approval Applications

AGENCY: Food and Drug Administration, HHS.

ACTION: Notice.

SUMMARY: The Food and Drug Administration (FDA) is publishing a list of premarket approval applications (PMAs) that have been approved. This list is intended to inform the public of the availability of safety and effectiveness summaries of approved PMAs through the Internet and the agency's Division of Dockets Management.

ADDRESSES: Submit written requests for copies of summaries of safety and effectiveness data to the Division of Dockets Management (HFA–305), Food and Drug Administration, 5630 Fishers Lane, rm. 1061, Rockville, MD 20852. Please cite the appropriate docket number as listed in Table 1 of this document when submitting a written request. See the **SUPPLEMENTARY INFORMATION** section for electronic access to the summaries of safety and effectiveness.

FOR FURTHER INFORMATION CONTACT: Samie Allen, Center for Devices and Radiological Health (HFZ–402), Food and Drug Administration, 9200 Corporate Blvd., Rockville, MD 20850, 240–276–4013.

SUPPLEMENTARY INFORMATION:

DEPARTMENT OF HEALTH & HUMAN SERVICES

Food and Drug Administration
Rockville MD 20857

JAN 8 2009

Re: Lucentis
Docket Nos.: FDA-2007-E-0461
FDA-2007-E-0165
FDA-2007-E-0459

The Honorable Jon Dudas
Under Secretary of Commerce for Intellectual Property
Director of the United States Patent and Trademark Office
Mail Stop Hatch-Waxman PTE
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Director Dudas:

This is in regard to the patent term extension applications for U.S. Patent Nos. 6,407,213; 6,884,879; and 7,060,269 filed by Genentech, Inc., under 35 U.S.C. § 156. The patent claims Lucentis (ranibizumab), biologic license application (BLA) 125156/0.

In the May 29, 2008, issue of the Federal Register (73 Fed. Reg. 30949), the Food and Drug Administration published its determination of this product's regulatory review period, as required under 35 U.S.C. § 156(d)(2)(A). The notice provided that on or before November 25, 2008, 180 days after the publication of the determination, any interested person could file a petition with FDA under 35 U.S.C. § 156(d)(2)(B)(i) for a determination of whether the patent term extension applicant acted with due diligence during the regulatory review period.

The 180-day period for filing a due diligence petition pursuant to this notice has expired and FDA has received no such petition. Therefore, FDA considers the regulatory review period determination to be final.

Please let me know if we can provide further assistance.

Sincerely yours,

Jane A. Axelrad

Jane A. Axelrad
Associate Director for Policy
Center for Drug Evaluation and Research

cc: Jeffrey P. Kushan
SIDLEY AUSTIN LLP
1501 K Street, N.W.
Washington, DC 20005

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450
www.uspto.gov

MAR 26 2009

Jeffrey P. Kushan
Sidley Austin, LLP
1501 K Street, N.W.
Washington, DC 20005

In Re: Patent Term Extension
Application for
U.S. Patent No. 6,407,213

NOTICE OF FINAL DETERMINATION AND REQUIREMENT FOR ELECTION

A determination has been made that U.S. Patent No. 6,407,213, claims of which cover the human biologic drug product LUCENTIS® (ranibizumab), is eligible for patent term extension under 35 U.S.C. § 156. The period of extension has been determined to be 378 days.

A single request for reconsideration of this final determination as to the length of extension of the term of the patent may be made if filed within one month of the date of this notice. Extensions of time under 37 CFR § 1.136(a) are not applicable to this time period.

Applicant also has applied for patent term extension of U.S. Patent No. 6,884,879 and U.S. Patent No. 7,060,269 based on the regulatory review period for the human biologic drug product LUCENTIS® (ranibizumab).

When patent term extension applications are filed for extension of the terms of different patents based upon the same regulatory review period for a product, the certificate of extension is issued to the patent having the earliest date of issuance, unless applicant elects a different patent. In the absence of an election by applicant within ONE MONTH of the date of this notice, and in accordance with 37 CFR 1.785(b), the applications for patent term extension of U.S. Patent No. 6,884,879 and U.S. Patent No. 7,060,269 will be denied. Accordingly, the application for patent term extension of the patent having the earlier date of issuance will be granted, i.e., a certificate of extension will be issued to U.S. Patent No 6,407,213 for a period of 378 days.

In the absence of a request for reconsideration, and if U.S. Patent No. 6,407,213 is elected, the Director will issue to the applicant a certificate of extension, under seal, for a period of 378 days in U.S. Patent No. 6,407,213.

The period of extension, if calculated using the Food and Drug Administration determination of the length of the regulatory review period published in the Federal Register of May 29, 2008 (73 Fed. Reg. 30949), would be 828 days. Under 35 U.S.C. § 156(c):

Period of Extension	=	½ (Testing Phase) + Approval Phase
	=	½ (2,247 days - 956 days) + 183 days
	=	828 days (2.3 years)

Since the regulatory review period began November 6, 1999, before the patent issued (June 18, 2002), only that portion of the regulatory review period occurring after the date the patent issued has been considered in the above determination of the length of the extension period 35 U.S.C. § 156(c). (From November 6, 1999, to and including, June 18, 2002, is 956 days; this period is subtracted for the number of days occurring in the testing phase according to the FDA's determination of the length of the regulatory review period.) No determination of a lack of due diligence under 35 U.S.C. § 156(c)(1) was made.

However, the 14 year exception of 35 U.S.C. § 156(c)(3) operates to limit the term of the extension in the present situation because it provides that the period remaining in the term of the patent measured from the date of approval of the approved product plus any patent term extension cannot exceed fourteen years. The period of extension calculated above, 828 days, would extend the patent from June 18, 2019, to September 23, 2021, which is beyond the 14-year limit (the approval date is June 30, 2006, thus, the 14 year limit is June 30, 2020). The period of extension is thus limited to 378 days, by operation of 35 U.S.C. § 156(c)(3). Accordingly, the period of extension is the number of days to extend the term of the patent from its original expiration date, June 18, 2019, to and including, June 30, 2020, or 378 days.

The limitations of 35 U.S.C. 156(g)(6) do not operate to further reduce the period of extension determined above.

Upon issuance of the certificate of extension, the following information will be published in the Official Gazette:

U.S. Patent No.:	6,407,213
Granted:	June 18, 2002
Original Expiration Date[1]:	June 18, 2019
Applicant:	Paul J. Carter et al.
Owner of Record:	Genentech, Inc.
Title:	Method for Making Humanized Antibodies
Product Trade Name:	LUCENTIS® (ranibizumab)
Term Extended:	378 days
Expiration Date of Extension:	June 30, 2020

[1]Subject to the provisions of 35 U.S.C. § 41(b).

Any correspondence with respect to this matter should be addressed as follows:

By mail: Mail Stop Hatch-Waxman PTE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450.

By FAX: (571) 273-7755

Telephone inquiries related to this determination should be directed to the undersigned at (571) 272-7755.

Mary C. Till
Legal Advisor
Office of Patent Legal Administration
Office of the Deputy Commissioner
for Patent Examination Policy

cc: Office of Regulatory Policy
Food and Drug Administration
10903 New Hampshire Ave., Bldg. 51, Rm. 6222
Silver Spring, MD 20993-0002

Attention: Beverly Friedman

RE: LUCENTIS® (ranibizumab)
Docket No.: FDA-2007-E-0461

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

U.S. Patent No.	**6,407,213 – § 156**	Unit:	OPLA
Serial No.:	**08/ 146,206**		
Confirmation No.:	3992		
Filed:	25 August 2006		
First Inventor:	P.J. CARTER		
Patent Owner:	Genentech, Inc.		
For:	Method for making humanized antibodies Application for patent term extension under 35 U.S.C. § 156		

Mail Stop **Hatch-Waxman PTE**
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

ELECTION UNDER 37 C.F.R. § 1.785(b)

Sir:

This letter responds to the Notice of Final Determination and Requirement for Election mailed in the captioned application for patent term extension on 26 March 2009. The Notice observes that applicant filed applications to extend the terms of U.S. Patent Nos. 6,407,213, 6,884,879, and 7,060,269 based on the regulatory review period for LUCENTIS®. The Notice further states a requirement that applicant elect one of the patents to receive a term extension certificate within a period of one month of the date of the Notice. This election is filed within the stated period and is therefore timely.

Pursuant to § 1.785(b), **applicant elects U.S. Patent No. 6,407,213** to receive a certificate of extension under § 1.780 and 35 U.S.C. § 156(e)(1). Applicant requests that the Director proceed to issue a certificate of extension of U.S. Patent No. 6,407,213 based on the regulatory review period for LUCENTIS® for a period of 378 days, as indicated in the Notice of Final Determination and Requirement for Election issued in this application for patent term extension.

We believe that no fee is due in respect of this election. However, the Director is requested to debit any fee required for entry or consideration of this paper from our Deposit Account No. 18-1260.

Respectfully submitted,

/David L. Fitzgerald/

David L. Fitzgerald, Reg. No. 47,347
Attorney for Genentech, Inc.

24 April 2009

SIDLEY AUSTIN LLP
1501 K Street, NW
Washington, DC 20005

tel. (202) 736-8818
fax (202) 736-8711

Electronic Acknowledgement Receipt

EFS ID:	5212426
Application Number:	08146206
International Application Number:	
Confirmation Number:	3992
Title of Invention:	METHOD FOR MAKING HUMANIZED ANTIBODIES
First Named Inventor/Applicant Name:	PAUL J. CARTER
Customer Number:	33694
Filer:	David Laurence Fitzgerald
Filer Authorized By:	
Attorney Docket Number:	709P1
Receipt Date:	24-APR-2009
Filing Date:	17-NOV-1993
Time Stamp:	10:46:21
Application Type:	U.S. National Stage under 35 USC 371

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Miscellaneous Incoming Letter	Lucentis_213_PTE_election.pdf	78045 b6baae5477e1a50f05bedfe33594cb57572ff209	no	2

Warnings:	
Information:	

Total Files Size (in bytes):	78045

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
United States Patent and Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450
www.uspto.gov

NOV 18 2009

Jeffrey P. Kushan
Sidley Austin, LLP
1501 K Street, N.W.
Washington, DC 20005

In Re: Patent Term Extension
Application for
U.S. Patent No. 6,407,213

Dear Mr. Kushan :

A certificate under 35 U.S.C. § 156 is enclosed extending the term of U.S. Patent No. 6,407,213 for a period of 378 days. While a courtesy copy of this letter is being forwarded to the Food and Drug Administration (FDA), you should directly correspond with the FDA regarding any required changes to the patent expiration dates.

Inquiries regarding this communication should be directed to the undersigned by telephone at (571) 272-7755, or by e-mail at mary.till@uspto.gov.

Mary C. Till
Legal Advisor
Office of Patent Legal Administration
Office of the Deputy Commissioner
for Patent Examination Policy

cc: Office of Regulatory Policy
Food and Drug Administration
10903 New Hampshire Ave., Bldg. 51, Rm. 6222
Silver Spring, MD 20993-0002

Attention: Beverly Friedman

RE: LUCENTIS® (ranibizumab)
Docket No.: FDA-2007-E-0461

UNITED STATES PATENT AND TRADEMARK OFFICE

(12) CERTIFICATE EXTENDING PATENT TERM
UNDER 35 U.S.C. § 156

(68)	PATENT NO.	:	6,407,213
(45)	ISSUED	:	June 18, 2002
(75)	INVENTOR	:	Paul J. Carter et al.
(73)	PATENT OWNER	:	Genentech, Inc.
(95)	PRODUCT	:	LUCENTIS® (ranibizumab)

This is to certify that an application under 35 U.S.C. § 156 has been filed in the United States Patent and Trademark Office, requesting extension of the term of U.S. Patent No. 6,407,213 based upon the regulatory review of the product LUCENTIS® (ranibizumab) by the Food and Drug Administration. Since it appears that the requirements of the law have been met, this certificate extends the term of the patent for the period of

(94) 378 days

from June 18, 2019, the original expiration date of the patent, subject to the payment of maintenance fees as provided by law, with all rights pertaining thereto as provided by 35 U.S.C. § 156(b).

I have caused the seal of the United States Patent and Trademark Office to be affixed this 18th day of November 2009.

David J. Kappos
Under Secretary of Commerce for Intellectual Property and
Director of the United States Patent and Trademark Office

Mail Stop Interference
P.O. Box 1450
Alexandria Va 22313-1450
Tel: 571-272-4683
Fax: 571-273-0042

Paper 1

Filed 2 February 2010

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

PAUL J. CARTER AND LEONARD G. PRESTA
Junior Party
(Patent 6,407,213),

v.

JOHN ROBERT ADAIR, DILJEET SINGH ATHWAL,
AND JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

Patent Interference No. 105,744
(Technology Center 1600)

DECLARATION - Bd.R. 203(b)[1]

Part A. Declaration of interference

An interference is declared (35 U.S.C. § 135(a)) between the above-identified parties. Details of the application(s), patent (if any), reissue application (if any), count(s) and claims designated as corresponding or as not corresponding to the count(s) appear in Parts E and F of this DECLARATION.

[1] "Bd.R. x" may be used as shorthand for "37 C.F.R. § 41.x". 69 Fed. Reg. 49960, 49961 (12 Aug. 2004).

Part B. Judge managing the interference

Administrative Patent Judge Sally Gardner Lane has been designated to manage the interference. Bd. R. 104(a).

Part C. Standing order

A Trial Section STANDING ORDER [SO] (Paper 2) accompanies this DECLARATION. The STANDING ORDER applies to this interference.

Part D. Initial conference call

A telephone conference call to discuss the interference is set for 2:00 p.m. on 16 March 2010 (the Board will initiate the call).

No later than four business days prior to the conference call, each party shall file and serve (SO ¶¶ 10.1 & 105) a list of the motions (Bd. R. 120; Bd. R. 204; SO ¶¶ 104.2.1, 120 & 204) the party intends to file.

A sample schedule for taking action during the motion phase appears as Form 2 in the STANDING ORDER. Counsel are encouraged to discuss the schedule prior to the conference call and to agree on dates for taking action. A typical motion period lasts approximately eight (8) months. Counsel should be prepared to justify any request for a shorter or longer period.

Part E. Identification and order of the parties

Junior Party

Named inventors: Paul J. Carter
San Francisco, CA

Leonard G. Presta
San Francisco, CA

Involved Patent:	6,407,213, issued 18 June 2002, from application 08/146,206, which was filed 17 November 1993, and was based on international application PCT/US92/05126, filed 15 June 1992.
Title:	METHOD FOR MAKING HUMANIZED ANTIBODIES
Assignee:	Genentech, Inc.

Senior Party

Named Inventors:	John Robert Adair High Wycombe, United Kingdom Diljeet Singh Athwal London, United Kingdom John Spencer Emtage Marlow, United Kingdom
Involved Application:	11/284,261, filed 21 November 2005
Title:	HUMANISED ANTIBODIES
Assignee:	Celltech R & D Limited

The senior party is assigned exhibit numbers 1001-1999. The junior party is assigned exhibit numbers 2001-2999. Bd. R. 154(c)(1); SO ¶ 154.2.1. The senior party is responsible for initiating settlement discussions. SO ¶ 126.1.

Part F. Count and claims of the parties

Count 1

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, 71H, 73H, and 78H, utilizing the numbering system set forth in Kabat.

The claims of the parties are:

Carter: 1-82

Adair: 24

The claims of the parties which correspond to Count 1 are:

Carter: 30, 31, 60, 62, 63, 66, 67, 70, 73, 77-81

Adair: 24

The claims of the parties which do not correspond to Count 1, and therefore are not involved in the interference, are:

Carter: 1-29, 32-59, 61, 64, 65, 68, 69, 71, 72, 74-76, 82

Adair: None

The parties are accorded the following benefit for Count 1:

Carter:	PCT/US92/05126, filed 15 June 1992; and 07/715,272, filed 14 June 1991, now abandoned.
Adair:	08/846,658, filed 01 May 1997; 08/303,569, filed 07 September 1994, issued as 5,859,205 on 12 January 1999; 07/743,329, filed on 17 September 1991; PCT/GB90/02017, filed 21 December 1990; and GB 8928874.0, filed 21 December 1989.

Part G. Heading to be used on papers

The following heading must be used on all papers filed in this interference, see SO & 106.1.1:

PAUL J. CARTER AND LEONARD G. PRESTA
Junior Party
(Patent 6,407,213),

v.

JOHN ROBERT ADAIR, DILJEET SINGH ATHWAL,
AND JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

Patent Interference No. 105,744
(Technology Center 1600)

Part H. Order form for requesting file copies

When requesting copies of files, use of SO Form 4 will greatly expedite processing of the request. Please attach a copy of Parts E and F of this DECLARATION with a hand-drawn circle around the patents and applications for which a copy of a file wrapper is requested.

/Sally Gardner Lane/
Administrative Patent Judge

Enc:
Copy of STANDING ORDER
Form PTO-850
Copy U.S. Patent 6,407,213
Copy of claims of 11/284,261

cc (via overnight delivery):

Attorney for Carter:

Sidley Austin, LLP
Attn: DC Patent Docketing
1501 K Street, N.W.
Washington, DC 20005

Attorney for Adair:

Cozen O'Connor, P.C.
1900 Market Street
Philadelphia, PA 19103-3508

Mail Stop Interference
P.O. Box 1450
Alexandria Va 22313-1450
Tel: 571-272-4683
Fax: 571-273-0042

Paper 1

Filed 2 February 2010

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

PAUL J. **CARTER** AND LEONARD G. PRESTA
Junior Party
(Patent 6,407,213),

v.

JOHN ROBERT **ADAIR**, DILJEET SINGH ATHWAL,
AND JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

Patent Interference No. 105,744
(Technology Center 1600)

DECLARATION - Bd.R. 203(b)[1]

Part A. Declaration of interference

An interference is declared (35 U.S.C. § 135(a)) between the above-identified parties. Details of the application(s), patent (if any), reissue application (if any), count(s) and claims designated as corresponding or as not corresponding to the count(s) appear in Parts E and F of this DECLARATION.

[1] "Bd.R. x" may be used as shorthand for "37 C.F.R. § 41.x". 69 Fed. Reg. 49960, 49961 (12 Aug. 2004).

Part B. Judge managing the interference

Administrative Patent Judge Sally Gardner Lane has been designated to manage the interference. Bd. R. 104(a).

Part C. Standing order

A Trial Section STANDING ORDER [SO] (Paper 2) accompanies this DECLARATION. The STANDING ORDER applies to this interference.

Part D. Initial conference call

A telephone conference call to discuss the interference is set for **2:00 p.m. on 16 March 2010** (the Board will initiate the call).

No later than **four business days** prior to the conference call, each party shall file and serve (SO ¶¶ 10.1 & 105) a list of the motions (Bd. R. 120; Bd. R. 204; SO ¶¶ 104.2.1, 120 & 204) the party intends to file.

A sample schedule for taking action during the motion phase appears as Form 2 in the STANDING ORDER. Counsel are encouraged to discuss the schedule prior to the conference call and to agree on dates for taking action. A typical motion period lasts approximately eight (8) months. Counsel should be prepared to justify any request for a shorter or longer period.

Part E. Identification and order of the parties

Junior Party

Named inventors: Paul J. Carter
San Francisco, CA

Leonard G. Presta
San Francisco, CA

Involved Patent:	6,407,213, issued 18 June 2002, from application 08/146,206, which was filed 17 November 1993, and was based on international application PCT/US92/05126, filed 15 June 1992.
Title:	METHOD FOR MAKING HUMANIZED ANTIBODIES
Assignee:	Genentech, Inc.

Senior Party

Named Inventors:	John Robert Adair High Wycombe, United Kingdom Diljeet Singh Athwal London, United Kingdom John Spencer Emtage Marlow, United Kingdom
Involved Application:	11/284,261, filed 21 November 2005
Title:	HUMANISED ANTIBODIES
Assignee:	Celltech R & D Limited

The senior party is assigned exhibit numbers 1001-1999. The junior party is assigned exhibit numbers 2001-2999. Bd. R. 154(c)(1); SO ¶ 154.2.1. The senior party is responsible for initiating settlement discussions. SO ¶ 126.1.

Part F. Count and claims of the parties

Count 1

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, 71H, 73H, and 78H, utilizing the numbering system set forth in Kabat.

The claims of the parties are:

Carter: 1-82

Adair: 24

The claims of the parties which correspond to Count 1 are:

Carter: 30, 31, 60, 62, 63, 66, 67, 70, 73, 77-81

Adair: 24

The claims of the parties which do not correspond to Count 1, and therefore are not involved in the interference, are:

Carter: 1-29, 32-59, 61, 64, 65, 68, 69, 71, 72, 74-76, 82

Adair: None

The parties are accorded the following benefit for Count 1:

Carter: PCT/US92/05126, filed 15 June 1992; and
07/715,272, filed 14 June 1991, now abandoned.

Adair: 08/846,658, filed 01 May 1997;
08/303,569, filed 07 September 1994, issued as 5,859,205 on 12 January 1999;
07/743,329, filed on 17 September 1991;
PCT/GB90/02017, filed 21 December 1990; and
GB 8928874.0, filed 21 December 1989.

Part G. Heading to be used on papers

The following heading must be used on all papers filed in this interference, see SO & 106.1.1:

PAUL J. **CARTER** AND LEONARD G. PRESTA
Junior Party
(Patent 6,407,213),

v.

JOHN ROBERT **ADAIR**, DILJEET SINGH ATHWAL,
AND JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

Patent Interference No. 105,744
(Technology Center 1600)

Part H. Order form for requesting file copies

When requesting copies of files, use of SO Form 4 will greatly expedite processing of the request. Please attach a copy of Parts E and F of this DECLARATION with a hand-drawn circle around the patents and applications for which a copy of a file wrapper is requested.

/Sally Gardner Lane/
Administrative Patent Judge

Enc:
Copy of STANDING ORDER
Form PTO-850
Copy U.S. Patent 6,407,213
Copy of claims of 11/284,261

cc (via overnight delivery):

Attorney for Carter:

Sidley Austin, LLP
Attn: DC Patent Docketing
1501 K Street, N.W.
Washington, DC 20005

Attorney for Adair:

Cozen O'Connor, P.C.
1900 Market Street
Philadelphia, PA 19103-3508

Mail Stop Interference
P.O. Box 1450
Alexandria, Va 22313-1450
Tel: 571-272-4683
Fax: 571-273-0042

Paper 81

Filed 2 September 2010

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

PAUL J. **CARTER** AND LEONARD G. PRESTIA
Junior Party
(Patent 6,407,213),

v.

JOHN ROBERT **ADAIR**, DILGEET SINGH ATHWAL,
and JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

Patent Interference No. 105,744
(Technology Center 1600)

Before SALLY GARDNER LANE, RICHARD TORCZON, and SALLY C. MEDLEY, *Administrative Patent Judges.*

LANE, *Administrative Patent Judge.*

Judgment– Merits – Bd. R. 127

The Carter motion for judgment on the basis that the single involved Adair claim is barred under 35 U.S.C. § 135(b) was granted. (Paper 80). Because Adair no longer has an interfering claim that is not barred under 35 U.S.C. §135(b) it is appropriate to

enter judgment against Adair. *Berman v. Housey*, 291 F.3d 1345, 1351 (Fed. Cir. 2002).

It is

ORDERED that judgment on priority as to Count 1 (Paper 1 at 4), the sole count of the interference, is entered against senior party Adair;

FURTHER ORDERED that claim 24 of Adair application 11/284,261, which claim corresponds to Count 1 (Paper 1 at 4), is FINALLY REFUSED, 35 U.S.C. §135(a):

FURTHER ORDERED that if there is a settlement agreement, the parties are directed to 35 U.S.C. 135(c) and Bd. R. 205; and

FURTHER ORDERED that a copy of this judgment shall be entered into the administrative record of the Carter involved patent and application and the Adair involved application.

cc (via electronic filing):

Attorney for CARTER:

Oliver R. Ashe, Jr., Esq.
ASHE, P.C.
11440 Isaac Newton Square, North
Suite 210
Reston, VA 20190
Tel: 703-467-9001
Email: oashe@ashepc.com

Attorney for ADAIR:
Doreen Yatko Trujillo, Esq.
Michael B. Fein, Esq.
COZEN O'CONNOR P.C.
1900 Market Street
Philadelphia, PA 19103
Tel: 215-665-5593
Email: dtrujillo@cozen.com

Trials@uspto.gov
571-272-7822

Paper No. 23
Entered: March 3, 2017

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

MYLAN PHARMACEUTICALS INC.,
Petitioner,

v.

GENENTECH, INC.,
Patent Owner.

Case IPR2016-01693 (Patent 6,407,213 B1)
Case IPR2016-01694 (Patent 6,407,213 B1)[1]

Before SHERIDAN K. SNEDDEN, ZHENYU YANG, and ROBERT A. POLLOCK, *Administrative Patent Judges*.

YANG, *Administrative Patent Judge*.

DECISION
Termination of the Proceeding Due to Settlement before Institution
37 C.F.R. §§ 42.72, 42.74

---

[1] This order addresses issues that are common to each referenced case. We, therefore, issue a single order that has been entered in each case. For convenience, paper numbers refer to those filed in IPR2016-01693.

On March 7, 2017, pursuant to 35 U.S.C. § 317(a), and with the Board's authorization, the parties filed a Joint Motion to Terminate in each of the above-captioned proceedings. Paper 21. In addition, pursuant to 35 U.S.C. § 317(b) and 37 C.F.R. § 42.74(c), the parties filed a true and correct copy of a Settlement Agreement (Ex. 1132), along with a Joint Request That Settlement Agreement Be Treated as Business Confidential Information, to be kept separate from the publicly available patent files (Paper 22).

Under 35 U.S.C. § 317(a), "[a]n inter partes review instituted under this chapter shall be terminated with respect to any petitioner upon the joint request of the petitioner and patent owner, unless the Office has decided the merits of the proceeding before the request for termination is filed." These cases are in their preliminary stage. No decision on whether to institute a trial has been made in either case.

Under 37 C.F.R. § 42.72, "[t]he Board may terminate a trial without rendering a final written decision, where appropriate, including . . . pursuant to a joint request under 35 U.S.C. 317(a)." After reviewing the Joint Motions to Terminate and the Settlement Agreement, we determine that it is appropriate to terminate the proceedings without rendering a final written decision. Therefore, the Joint Motions to Terminate are GRANTED.

Accordingly, it is

ORDERED that the Joint Motions to Terminate Proceeding are GRANTED and the proceedings are hereby TERMINATED; and

FURTHER ORDERED that the Joint Requests That Settlement Agreement Be Treated as Business Confidential Information are

GRANTED, and the Settlement Agreement will be kept separate from the patent files.

PETITIONER:

Jeffrey Guise
jguise@wsgr.com

Deanne Mazzochi
dmazzochi@rmmslegal.com

Lorelei Westin
lwestin@wsgr.com

Clark Lin
clin@wsgr.com

PATENT OWNER:

David Cavanaugh
David.cavanaugh@wilmerhale.com

Owen Allen
Owen.allen@wilmerhale.com

Adam Brausa
abrausa@durietangri.com

Trials@uspto.gov
571-272-7822

Paper No. 24
Entered: March 3, 2017

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

MYLAN PHARMACEUTICALS INC.,
Petitioner,

v.

GENENTECH, INC.,
Patent Owner.

Case IPR2016-01693 (Patent 6,407,213 B1)
Case IPR2016-01694 (Patent 6,407,213 B1)[1]

Before SHERIDAN K. SNEDDEN, ZHENYU YANG, and ROBERT A. POLLOCK, *Administrative Patent Judges*.

YANG, *Administrative Patent Judge*.

DECISION
Termination of the Proceeding Due to Settlement before Institution
37 C.F.R. §§ 42.72, 42.74

---

[1] This order addresses issues that are common to each referenced case. We, therefore, issue a single order that has been entered in each case. For convenience, paper numbers refer to those filed in IPR2016-01693.

On March 7, 2017, pursuant to 35 U.S.C. § 317(a), and with the Board's authorization, the parties filed a Joint Motion to Terminate in each of the above-captioned proceedings. Paper 21. In addition, pursuant to 35 U.S.C. § 317(b) and 37 C.F.R. § 42.74(c), the parties filed a true and correct copy of a Settlement Agreement (Ex. 1132), along with a Joint Request That Settlement Agreement Be Treated as Business Confidential Information, to be kept separate from the publicly available patent files (Paper 22).

Under 35 U.S.C. § 317(a), "[a]n inter partes review instituted under this chapter shall be terminated with respect to any petitioner upon the joint request of the petitioner and patent owner, unless the Office has decided the merits of the proceeding before the request for termination is filed." These cases are in their preliminary stage. No decision on whether to institute a trial has been made in either case.

Under 37 C.F.R. § 42.72, "[t]he Board may terminate a trial without rendering a final written decision, where appropriate, including . . . pursuant to a joint request under 35 U.S.C. 317(a)." After reviewing the Joint Motions to Terminate and the Settlement Agreement, we determine that it is appropriate to terminate the proceedings without rendering a final written decision. Therefore, the Joint Motions to Terminate are GRANTED.

Accordingly, it is

ORDERED that the Joint Motions to Terminate Proceeding are GRANTED and the proceedings are hereby TERMINATED; and

FURTHER ORDERED that the Joint Requests That Settlement Agreement Be Treated as Business Confidential Information are

GRANTED, and the Settlement Agreement will be kept separate from the patent files.

PETITIONER:

Jeffrey Guise
jguise@wsgr.com

Deanne Mazzochi
dmazzochi@rmmslegal.com

Lorelei Westin
lwestin@wsgr.com

Clark Lin
clin@wsgr.com

PATENT OWNER:

David Cavanaugh
David.cavanaugh@wilmerhale.com

Owen Allen
Owen.allen@wilmerhale.com

Adam Brausa
abrausa@durietangri.com