PTO-103X
(Rev. 7-93)

FILING RECEIPT

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EH

APPLICATION NUMBER	FILING DATE	GRP ART UNIT	FIL FEE REC'D	ATTORNEY DOCKET NO.	DRWGS	TOT CL	IND CL
08/146,206	11/17/93	1804	$1,592.00	709P1	9	18	9

JANET E. HASAK
GENENTECH, INC.
460 POINT SAN BRUNO BOULEVARD
SOUTH SAN FRANCISCO, CA 94080-4990

RECEIVED
MAY 1 3 1994
GENENTECH, INC. LEGAL DEPT.

Receipt is acknowledged of this patent application. It will be considered in its order and you will be notified as to the results of the examination. Be sure to provide the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION when inquiring about this application. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. If an error is noted on this Filing Receipt, please write to the Application Processing Division's Customer Correction Branch within 10 days of receipt. Please provide a copy of the Filing Receipt with the changes noted thereon.

Applicant(s) PAUL J. CARTER, SAN FRANCISCO, CA; LEONARD G. PRESTA, SAN FRANCISCO, CA.

CONTINUING DATA AS CLAIMED BY APPLICANT-
THIS APPLN IS A 371 OF /US92/05126 06/15/92

FOREIGN/PCT APPLICATIONS-PCT PCT/US92/05126 06/15/92

TITLE
IMMUNOGLOBULIN VARIANTS

PRELIMINARY CLASS: 435

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/13, C12P 21/08
C07K 13/00, C12N 5/10
G06F 15/00

A1

(11) International Publication Number: WO 92/22653

(43) International Publication Date: 23 December 1992 (23.12.92)

(21) International Application Number: PCT/US92/05126

(22) International Filing Date: 15 June 1992 (15.06.92)

(30) Priority data:
715,272 14 June 1991 (14.06.91) US

(60) Parent Application or Grant
(63) Related by Continuation
US 715,272 (CIP)
Filed on 14 June 1991 (14.06.91)

(71) Applicant *(for all designated States except US)*: GENENTECH, INC. [US/US]; 460 Point San Bruno Boulevard, South San Francisco, CA 94080 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : CARTER, Paul, J. [GB/US]; 2074 18th Avenue, San Francisco, CA 94116 (US). PRESTA, Leonard, G. [US/US]; 1900 Gough Street, #206, San Francisco, CA 94109 (US).

(74) Agents: ADLER, Carolyn, R. et al.; Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080 (US).

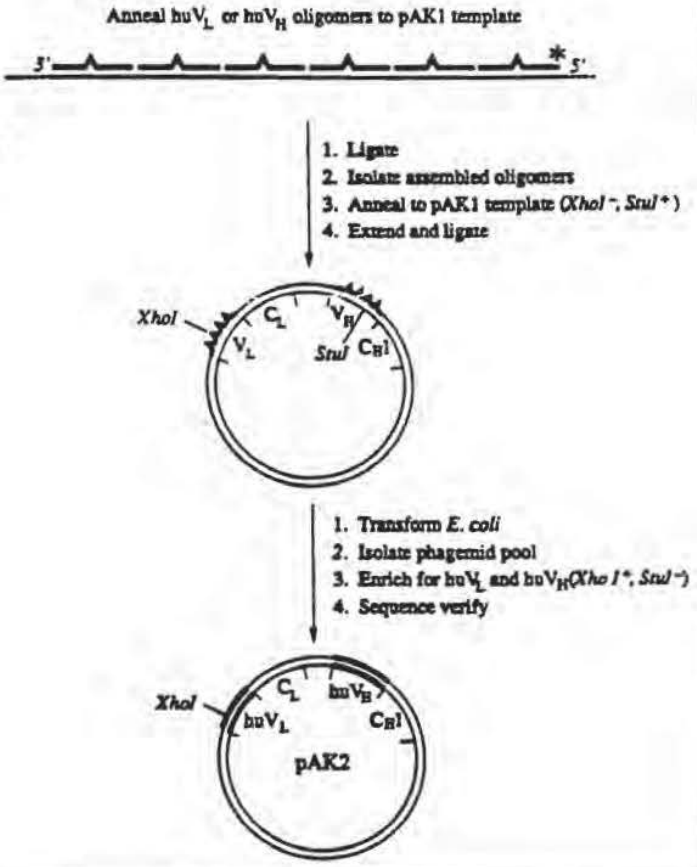
(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHOD FOR MAKING HUMANIZED ANTIBODIES

(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.



08/146,206

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

SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKETT NO.

EXAMINER	
ART UNIT	PAPER NUMBER
	53

DATE MAILED: 8/15/94

EXAMINER INTERVIEW SUMMARY RECORD

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

(1) Julie Burke (PTO) (3) Wendy Lee

(2) Lila Feisee (PTO) (4)

Date of interview 23 Aug 94

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

Exhibit shown or demonstration conducted: ☐ Yes ☐ No. If yes, brief description: None

Agreement ☐ was reached with respect to some or all of the claims in question. ☒ was not reached.

Claims discussed: All pending

Identification of prior art discussed: Reichmann

Description of the general nature of what was agreed to if an agreement was reached, or any other comments: Applicants intend to file TD for claim 111. Applicants intend to add an upper limit to affinity in claim 113 + 128.

(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 below 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 (e.g., items 1-7 on the reverse side of this form). If a response to the last Office action has already been filed, then applicant is given one month from this interview date to provide 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 substance of the interview unless box 1 above is also checked.

Julie C Burke
Examiner's Signature

PTOL-413 (REV. 2-93)

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office

Address : COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

SERIAL NUMBER	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO
08/146,206	11/17/93	CARTER	P 709P1

EXAMINER	
ADAMS, D	

ART UNIT	PAPER NUMBER
1806	10

18M2/0826

JANET E. HASAK
GENENTECH, INC.
460 POINT SAN BRUNO BOULEVARD
SOUTH SAN FRANCISCO, CA 94080-4990

DATE MAILED: 08/26/94

This is a communication from the examiner in charge of your application.
COMMISSIONER OF PATENTS AND TRADEMARKS

☐ This application has been examined ☐ Responsive to communication filed on _____ ☐ This action is made final.

A shortened statutory period for response to this action is set to expire Ø month(s), 30 days from the date of this letter.
Failure to respond within the period for response will cause the application to become abandoned. 35 U.S.C. 133

Part I THE FOLLOWING ATTACHMENT(S) ARE PART OF THIS ACTION:

1. ☐ Notice of References Cited by Examiner, PTO-892.
2. ☐ Notice re Patent Drawing, PTO-948.
3. ☐ Notice of Art Cited by Applicant, PTO-1449.
4. ☐ Notice of Informal Patent Application, Form PTO-152.
5. ☐ Information on How to Effect Drawing Changes, PTO-1474.
6. ☐ _____

Part II SUMMARY OF ACTION

1. ☒ Claims 1-18 are pending in the application.

   Of the above, claims _____ are withdrawn from consideration.

2. ☐ Claims _____ have been cancelled.
3. ☐ Claims _____ are allowed.
4. ☐ Claims _____ are rejected.
5. ☐ Claims _____ are objected to.
6. ☒ Claims 1-18 are subject to restriction or election requirement.
7. ☐ This application has been filed with informal drawings under 37 C.F.R. 1.85 which are acceptable for examination purposes.
8. ☐ Formal drawings are required in response to this Office action.
9. ☐ The corrected or substitute drawings have been received on _____. Under 37 C.F.R. 1.84 these drawings are ☐ acceptable. ☐ not acceptable (see explanation or Notice re Patent Drawing, PTO-948).
10. ☐ The proposed additional or substitute sheet(s) of drawings, filed on _____ has (have) been ☐ approved by the examiner. ☐ disapproved by the examiner (see explanation).
11. ☐ The proposed drawing correction, filed on _____, has been ☐ approved. ☐ disapproved (see explanation).
12. ☐ Acknowledgment is made of the claim for priority under U.S.C. 119. The certified copy has ☐ been received ☐ not been received ☐ been filed in parent application, serial no. _____; filed on _____
13. ☐ Since this application appears to be 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. 11; 453 O.G. 213.
14. ☐ Other

EXAMINER'S ACTION

PTOL-326 (Rev. 9-88)

15. Restriction to one of the following inventions is required under 35 U.S.C. § 121:

I. Claims 1-12, 15 and 18, drawn to a method of making a humanized antibody, classified in Class 435, subclasses 69.6, 69.7, 70.21, 91, 172.2, 240.1, 240.27, 252.3, 320.1 and Class 536, subclass 23.53

II. Claim 13, drawn to a polypeptide, classified in Class 530, subclass 325.

III. Claim 14, drawn to a polypeptide, classified in Class 530, subclass 325.

IV. Claim 16, drawn to a computer, classified in Class 364, subclass 413.

V. Claim 17, drawn to a computer representation, classified in Class 36, subclass 223.3, 223.4, 224.1, 224.91, 225.9 and 226.1

VI. Claim 18, drawn to a method of storing a computer representation, classified in Class 369, subclass 13+

16. The inventions are distinct, each from the other because of the following reasons:

17. The inventions of Groups I-III are not related. The method of making a humanized antibody of Group I is distinct from the polypeptides of either Groups II or III. The polypeptides are not humanized antibodies. Thus the method of Group I is not expected to produce the polypeptides of Groups II or III. The Groups therefore have different issues regarding patentability and enablement and represent patentably distinct subject matter.

18. The inventions of Group I and Group VI are distinct methods. They differ with respect to ingredients and method steps. They have different issues regarding patentability and enablement and represent patentably distinct subject matter.

19. The products of Groups II-V are distinct and unrelated. The peptides of Groups II and III differ chemically and physically from a computer and computer representation. Additionally, the peptides have different sequences and thus differed structures and pharmacokinetic properties. The Groups therefore have different issues regarding patentability and enablement and represent patentably distinct subject matter.

20. The method of Group I is distinct from the products of Groups [IV and V]. The method of Group I can in no manner produce a computer or computer representation as claimed in Groups IV and V. The Groups therefore have different issues regarding patentability and enablement and represent patentably distinct subject matter.

21. The products of Groups II and III can not be produced by the method of Group VI. They therefore have different issues regarding patentability and enablement and represent patentably distinct subject matter.

22. The computer of Group IV is distinct from both a method of storing a computer representation of Group VI and a computer representation Group V. The program required for (1) storing or (2) providing a representation (i.e. word processing text) are distinct components from the architecture of a computer system. Thus the Groups are separate and patentably distinct from each other. They have different issues regarding patentability and enablement.

23. The computer representation of Group V is distinct from a method of storing a computer representation. The logic required for these two applications are distinct and unrelated. The Groups have different issues regarding patentability and enablement and represent patentably distinct subject matter.

24. Because these inventions are distinct for the reasons given above and have acquired a separate status in the art shown by their different classification, in addition to their recognized divergent subject matter, they represent an undue burden on the examiner and restriction for examination purposes as indicated is proper.

25. Applicant is advised that the response to this requirement to be complete must include an election of the invention to be examined even though the requirement be traversed.

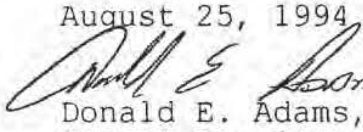
26. Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be amended in compliance with 37 C.F.R. § 1.48(b) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. Any amendment of inventorship must be accompanied by a diligently-filed petition under 37 C.F.R. § 1.48(b) and by the fee required under 37 C.F.R. § 1.17(h).

27. A telephone call was made to Ms. Hasak on August 24, 1994 to request an oral election to the above restriction requirement, but did not result in an election being made.

28. Papers related to this application may be submitted to Group 180 by facsimile transmission. Papers should be faxed to Group 180 via the PTO Fax Center located in Crystal Mall 1. The faxing of such papers must conform with the notice published in the Official Gazette, 1096 OG 30 (November 15, 1989). The CM1 Fax Center telephone number is (703) 308-4227.

29. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Donald E. Adams whose telephone number is (703) 308-0570. The examiner can normally be reached Monday through Thursday from 7:30 am to 6:00 pm. A message may be left on the examiners voice mail service. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Mr. David Lacey can be reached on (703) 308-3535. The fax phone number for Group 180 is (703) 305-3014 or (703) 308-4227. Any inquiry of a general nature or relating to the status of this application should be directed to the Group 180 receptionist whose telephone number is (703) 308-0196.

August 25, 1994



Donald E. Adams, Ph.D.
Patent Examiner
Group 1800

PATENT DOCKET 709P1

#11

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
September 22, 1994
(Date of Deposit)
Elisa R. Hamby
Name of Depositing Party
Elisa R. Hamby
Signature of Depositing Party
9/22/94
Date of Signature

TRANSMITTAL LETTER

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

Sir:

Transmitted herewith is a Response to Restriction Requirement 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 Fee(s)
Total	20	Minus	23	= 0	x 22 =	$ 0
Indep.	7	Minus	10	= 0	x 74 =	$ 0
___ First Presentation of Multiple Dependent Claim					+ 230 =	$ 0
					TOTAL	**$ 0**

__X__ No additional fee is required.

____ The Commissioner is hereby authorized to charge Deposit Account No. 07-0630 in the amount of $. **A duplicate copy of this transmittal is enclosed.**

____ Petition for Extension of Time is enclosed.

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. **A duplicate copy of this sheet is enclosed.**

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: September 22, 1994

By: _____
Wendy M. Lee

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

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

LIMITED RECOGNITION UNDER 37 CFR § 10.9(b)

Wendy M. Lee is hereby given limited recognition under 37 CFR § 10.9(b) as an employee of Genentech, Inc. to prepare and prosecute patent applications and to represent patent applicants wherein Genentech, Inc. is the assignee of record of the entire interest. This limited recognition shall expire on the date appearing below, or when whichever of the following events first occurs prior to December 9, 1994: (i) Wendy M. Lee ceases to lawfully reside in the United States, (ii) Wendy M. Lee's employment with Genentech, Inc. ceases or is terminated, or (iii) Wendy M. Lee ceases to remain or reside in the United States on an H-1 visa.

This document constitutes proof of such recognition. The original of this document is on file in the Office of Enrollment and Discipline of the U.S. Patent and Trademark Office.

Expires: December 9, 1994

Cameron Weiffenbach, Director
Office of Enrollment and Discipline

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

September 22, 1994
(Date of Deposit)

Elisa R. Hamby
Name of Depositing Party

Elisa R. Hamby
Signature of Depositing Party

9/22/94
Date of Signature

RESPONSE TO RESTRICTION REQUIREMENT

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

Sir:

This is responsive to the restriction requirement mailed 8/26/94. The period for response has been set for 30 days making this response due on or before 9/25/94. This response is timely filed. Please amend the application as follows:

IN THE CLAIMS:

Please cancel claims 16-18 without prejudice.

REMARKS

The Examiner required restriction to one of the following inventions under 35 USC §121:

I. Claims 1-12 and 15, drawn to a method of making a humanized antibody.

II. Claim 13 drawn to a polypeptide.

III. Claim 14 drawn to a polypeptide.

IV. Claim 16 drawn to a computer.

V. Claim 17 drawn to a computer representation.

VI. Claim 18 drawn to a method of storing a computer representation.

The Examiner urges that the inventions of Groups I-III are not related insofar as the polypeptides of either Groups II or III are "not humanized antibodies" and are therefore distinct from the method of making a humanized antibody recited in claim 1. The Examiner has also taken the position that the method of Group I is not expected to produce the polypeptides of Groups II or III.

Applicants hereby elect Group I, with traverse. The restriction requirement is submitted to be improper as regards the separate treatment of Groups I, II, and III. The claims in the remaining Groups IV, V, and VI have been canceled from this application, without prejudice to file a continuing application directed thereto.

It is submitted that the inventions of Groups I, II, and III as hereinabove defined are not distinct. These inventions are all respectively related as method of making a humanized antibody (Group I) and the humanized antibody made using the method of claim 1. Applicants submit that the assumption made that the polypeptides of claims 13 and 14 are not humanized antibodies is clearly in error. In particular, claims 13 and 14 encompass the light chain and heavy chain variable domain, respectively, of humanized MAb4D5 made using the method of claim 1 (see page 7, lines 13-21 and Example 1 which describes humanization of muMAb4D5). Surely, the Examiner will agree that the claim encompassing the light chain variable domain of the humanized MAb4D5 (claim 13) and the claim to the heavy chain variable domain of this humanized antibody (claim 14) should be examined together, since both a heavy chain and a light chain are required to form the antibody variable domain. Hence, the separate treatment of Groups II and III is clearly erroneous. Furthermore, since the humanized antibody variable domains of claims 13 and 14 are made using the humanization technique of claim 1, these claims should be examined together.

With respect to the search required to determine the patentability of the inventions defined by the claims of Groups I, II, and III, applicants represent that it is impossible to conduct an exhaustive search for a method of making a humanized antibody without searching for humanized antibodies made using the method. Similarly, the search for the claimed humanized antibody is bound to reveal information concerning the technique for humanizing it. In the same token, a search of the amino acid sequence encoding the humanized heavy chain variable domain of the antibody would lead to the discovery of information concerning the humanized light chain variable domain. Accordingly, performing the entire search covering the method and products made by the method is less burdensome on the Examiner than the separate search, which necessarily involves duplication of searching efforts.

In view of the foregoing arguments, the Examiner is requested to reconsider and withdraw the restriction requirement.

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: Sept 22, 1994 By: _____
Wendy M. Lee

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

```
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                 100                 105

Ser Gly Leu Tyr Asp Tyr Trp Gly Gln Gly Thr Leu Val Thr Val
                 110                 115                 120

Ser Ser
    122
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(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:

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 1               5                  10                  15

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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                 100                 105

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
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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
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                290                 295                 300

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                305                 310                 315

Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val
                320                 325                 330

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

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                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
```

F1

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His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
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Ser Pro Gly Lys
            454
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(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:

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 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                 100                 105

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

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

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                  100                 105

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
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```
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                 100                 105

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

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                 100                 105

Asp Trp Tyr Phe Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val
               110                 115                 120

Ser Ser
   122
```

F 1

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SERIAL NUMBER	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.
08/146,206	11/17/93	CARTER	P ~~.709P1~~

EXAMINER
ADAMS, D

18M2/1209

ART UNIT	PAPER NUMBER
1806	12

JANET E. HASAK
GENENTECH, INC.
460 POINT SAN BRUNO BOULEVARD
SOUTH SAN FRANCISCO, CA 94080-4990

DATE MAILED: 12/09/94

This is a communication from the examiner in charge of your application.
COMMISSIONER OF PATENTS AND TRADEMARKS

Election, Amdt C

☒ This application has been examined ☒ Responsive to communication filed on 9/26/94 ☐ This action is made final.

A shortened statutory period for response to this action is set to expire 3 month(s), 0 days from the date of this letter.
Failure to respond within the period for response will cause the application to become abandoned. 35 U.S.C. 133

Part I THE FOLLOWING ATTACHMENT(S) ARE PART OF THIS ACTION:

1. ☒ Notice of References Cited by Examiner, PTO-892.
2. ☒ Notice re Patent Drawing, PTO-948.
3. ☒ Notice of Art Cited by Applicant, PTO-1449.
4. ☐ Notice of Informal Patent Application, Form PTO-152.
5. ☒ Information on How to Effect Drawing Changes, PTO-1474.
6. ☐ _____

Part II SUMMARY OF ACTION

1. ☒ Claims 1-15 are pending in the application.
   Of the above, claims 13 & 14 are withdrawn from consideration.
2. ☒ Claims 16-18 have been cancelled.
3. ☐ Claims _____ are allowed.
4. ☒ Claims 1-12 & 15 are rejected.
5. ☐ Claims _____ are objected to.
6. ☐ Claims _____ are subject to restriction or election requirement.
7. ☒ This application has been filed with informal drawings under 37 C.F.R. 1.85 which are acceptable for examination purposes.
8. ☐ Formal drawings are required in response to this Office action.
9. ☐ The corrected or substitute drawings have been received on _____. Under 37 C.F.R. 1.84 these drawings are ☐ acceptable, ☐ not acceptable (see explanation or Notice re Patent Drawing, PTO-948).
10. ☐ The proposed additional or substitute sheet(s) of drawings, filed on _____ has (have) been ☐ approved by the examiner. ☐ disapproved by the examiner (see explanation).
11. ☐ The proposed drawing correction, filed on _____, has been ☐ approved. ☐ disapproved (see explanation).
12. ☐ Acknowledgment is made of the claim for priority under U.S.C. 119. The certified copy has ☐ been received ☐ not been received ☐ been filed in parent application, serial no. _____; filed on _____
13. ☐ Since this application appears to be 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. 11; 453 O.G. 213.
14. ☐ Other

EXAMINER'S ACTION

PTOL-326 (Rev. 9-89)

15. Applicant's election with traverse of Group I, claims 1-12 and 15 in Paper No. 11 is acknowledged. The traversal is on the ground(s) that:
(1) the inventions are all respectively related as method of making a humanized antibody. Contrary to applicant's belief the polypeptides of claim 13 (admittedly drawn to the light chain of humanized MAb4D5) and claim 14 (admittedly drawn to the heavy chain of humanized Mab4D5) are not methods.

(2) the assumption made that the polypeptides of claims 13 and 14 are not humanized antibodies is clearly in error. Applicant is invited to reconsider this position since the antibodies of Group I are composed of a heavy and light chains. A polypeptide of just the light chain (claims 13) or just the heavy chain (claim 14) is not an antibody as prepared by Group I. The Groups therefore have different issues regarding patentability and enablement and represent patentably distinct subject matter.

(3) applicants represent that it is impossible to conduct an exhaustive search for a method of making a humanized antibody without searching for humanized antibodies made using the method. To demonstrate the problem with this argument applicant is invited to consider the classification of the Groups found in the restriction requirement. Note that the Groups are classified into distinct classifications. Thus, an exhaustive search would clearly not require searching for polypeptides. Additionally, the light chain (claim 13) and heavy chain (claim 14) are distinct from the antibodies of Group I. Again, a search of Group I would not require the search of a polypeptide.

As a whole applicant's arguments were not found persuasive.
The requirement is still deemed proper and is therefore made FINAL.

16. Claims 16-18 have been cancelled.

17. Claims 13 and 14 have been withdrawn as directed to a non-elected invention.

18. Claims 1-12 and 15 are currently under consideration.

19. The oath or declaration is defective. A new oath or declaration in compliance with 37 C.F.R. § 1.67(a) identifying this application by its Serial Number and filing date is required. See M.P.E.P. §§ 602.01 and 602.02.

20. The oath or declaration is defective because:
It does not state that the person making the oath or declaration in a continuation-in-part application filed under the conditions specified in 35 U.S.C. § 120 which discloses and

claims subject matter in addition to that disclosed in the prior copending application, acknowledges the duty to disclose material information as defined in 37 C.F.R. § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

21. This application has been filed with informal drawings which are acceptable for examination purposes only. Formal drawings will be required when the application is allowed.

22. Applicant is required to submit a proposed drawing correction in response to this Office action. However, correction of the noted defect can be deferred until the application is allowed by the examiner.

23. The following is a quotation of the first paragraph of 35 U.S.C. § 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

24. The specification is objected to under 35 U.S.C. § 112, first paragraph, as failing to provide an adequate written description of the invention and for failing to adequately teach how to make and/or use the invention, i.e. for failing to provide an enabling disclosure.

A) Applicants have not disclosed to one of any skill in the art how to use the claimed antibody or antibody produced by the claimed methods. The scope of the claims reads on any antibody. It is unclear from the specification if the methods or antibodies claimed will all have a diagnostic or therapeutic utility. Applicant has exemplified only one such antibody specifically MAb4D5, as having diagnostic utility for the detection of $p185^{HER2}$. It is unclear if any other antibody will have a diagnostic or therapeutic utility. Determining which other antibodies are useful would be an unpredictable event and would require undue experimentation for a person of any skill in the art to get from what the specification has disclosed to the claimed invention.

25. Claims 1-12 and 15 are rejected under 35 U.S.C. § 112, first paragraph, for the reasons set forth in the objection to the specification.

26. Claims 1, 2, 4-12 and 15 are rejected under 35 U.S.C. § 103 as being unpatentable over Winter [EP 0239400], Riechmann et al. [Nature 332:323-327 (1988)] and Queen et al. [Proc. Natl. Acad. Sci. 86:10029-10033 (1989)]. Briefly the claims are drawn to a method for producing humanized antibodies and humanized antibodies. Winter, teaches the production of altered, chimeric, antibodies by replacing the complementarily determining regions (CDRs), see abstract. Winter, teaches the requirements for CDR fusions, see page 6 to page 8, line 29. Particularly, page 8, lines 11-18, where Winter, teaches that "merely by replacing one or more CDRs with complementary CDRs may not always result in a functional altered antibody..... it will be well within the competence of the man skilled in the art, either by carrying out routine experimentation or by trail and error testing to obtain a functional altered antibody. Note at page 8, last full paragraph that Winter states that framework region replacement and sequence changing may be necessary to obtain a functional humanized antibody. On page 9, lines 13-16, Winter suggests that the antibodies would be of importance for use in human therapy. Winter, teaches a method of producing the antibody, see page 10, paragraph 3 to page 15, paragraph 2. Consistent with Winter, Riechmann et al. teach a method of reshaping human antibodies for therapy by CDR grafting, see whole document and Queen et al. teach the humanization of antibodies by CDR grafting, see entire document. Riechmann et al. teach altering the sequence of the antibody to restore packing or to increase binding affinity, see page 326, first column, first full paragraph. Queen et al. teach the use of computer modeling to assist in the production of humanized antibodies, specifically to predict which amino acids to change thereby effecting molecular interactions, note that of the amino acids predicted to change include those identified by applicant in claims 7 and 10. A person of ordinary skill in the art would have realized that dependent upon the framework region selected and the sequence of the CDR regions amino acid changes would need to be made and they would depend upon the precise amino acid interactions of the polypeptide. The combination of Winter, Riechmann et al. and Queen et al. teach a comprehensive method for producing humanized antibodies which include the steps outlined in applicant's claims. Therefore, it would have been prima facia obvious to a person of ordinary skill in the art at the time the invention was made to take the combined teachings of Winter, Riechmann et al. and Queen et al. to produce a method of making a humanized antibody and to have a humanized antibody for either diagnostic or therapeutic use.

27. Claims 1, 2, 4-12 and 15 are rejected under 35 U.S.C. § 103 as being unpatentable over Winter [EP 0239400], Riechmann et al. [Nature 332:323-327 (1988)] and Queen et al. [Proc. Natl. Acad. Sci. 86:10029-10033 (1989)] in view of In re Durden 226 U.S.P.Q. 359 (Fed. Cir. 1985). Briefly the claims are drawn to a method

for producing humanized antibodies and humanized antibodies. As discussed above the combination of Winter, Riechmann et al. and Queen et al. teach humanized antibodies and methods for their production. Applicant's claimed invention does not appear to differ from what has previously known in the art.

28. Claim 3 is rejected under 35 U.S.C. § 103 as being unpatentable over Winter [EP 0239400], Riechmann et al. [Nature 332:323-327 (1988)] and Queen et al. [Proc. Natl. Acad. Sci. 86:10029-10033 (1989)] as applied to claims 1, 2, 4-12 and 15 and further in view of Roitt [Immunology, published 1985, by Gower Medical Publishing Ltd. (London, England) page 5.5]. Briefly the claim is drawn to a method for producing humanized antibodies having the additional steps of searching the import variable domain sequence for glycosylation sites, determining if any such glycosylation site is reasonable expected to affect the antigen binding or affinity of the antibody and if so substituting the glycosylation site into the consensus sequence. As discussed above the combination of Winter, Riechmann et al. and Queen et al. teach humanized antibodies and methods of producing humanized antibodies. The combination of Winter, Riechmann et al. and Queen et al. do not teach the importance of carbohydrate residues. However, Roitt teaches that antibodies contain carbohydrate residues in the variable region. A person of ordinary skill in the art would realize that carbohydrate residues can produce steric modifications in the folding characteristics of polypeptides. Therefore it would have been prima facia obvious to a person of ordinary skill in the art at the time the invention was made to include a step in the method taught by the combination of Winter, Riechmann et al. and Queen et al. which determines if the presence of carbohydrate residues occur in the variable region that can affect antigen binding and then include in the antibody sequence the appropriate glycosylation signal, by adding the appropriate consensus sequence. A person of ordinary skill in the art would have been motivated to add the additional step of identifying glycosylation that may affect antigen binding to ensure that the antibody produced will have the appropriate binding affinity. A person of ordinary skill in the art would have been motivated to produce such an method to produce antibodies having diagnostic or therapeutic utility.

29. Applicant is invited to include continuing data at the first page of the specifiation which identifies all related applications and noting their current status.

30. No claim allowed.

31. Papers related to this application may be submitted to Group 180 by facsimile transmission. Papers should be faxed to Group

180 via the PTO Fax Center located in Crystal Mall 1. The faxing of such papers must conform with the notice published in the Official Gazette, 1096 OG 30 (November 15, 1989). The CM1 Fax Center telephone number is (703) 308-4227.

32. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Donald E. Adams whose telephone number is (703) 308-0570. The examiner can normally be reached Monday through Thursday from 7:30 am to 6:00 pm. A message may be left on the examiners voice mail service. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Mr. David Lacey can be reached on (703) 308-3535. The fax phone number for Group 180 is (703) 305-3014 or (703) 308-4227. Any inquiry of a general nature or relating to the status of this application should be directed to the Group 180 receptionist whose telephone number is (703) 308-0196.

December 5, 1994

Donald E. Adams, Ph.D.
Patent Examiner
Group 1800

PTO 892 DEA/FCE 1994 U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE NOTICE OF REFERENCES CITED	SERIAL NUMBER 08/146,206	Art Unit 1806	Attachment to Paper Number 12
	APPLICANT(S) : Carter et al.		

U.S. PATENT DOCUMENTS

*		DOCUMENT NUMBER	DATE	NAME(S)	CLASS	SUBCLASS	FILING DATE

FOREIGN PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	COUNTRY	NAME	CLASS	SUBCLASS	PERTINENT DRW	PERTINENT SPEC
	A	0 239 400	09/30/87	EP	Winter	C12N	15/00	-	-
*	B	90/07861	26/07/90	WO	Queen	C12P	21/00	-	-

*		OTHER REFERENCES (INCLUDING AUTHOR, TITLE, DATE, PERTINENT PAGES, ETC.)
	C	Riechmann et al. [Nature 332:323-327 (1988)]
	D	Queen et al. [Proc. Natl. Acad. Sci. 86:10029-10033 (1989)]
	E	Roitt [Immunology, published 1985, by Gower Medical Publishing Ltd. (London, England) page 5.5]
*	F	Tramontano et al. [J. Mol. Biol. 215:175-182 (1990)]

EXAMINER	DATE	* A COPY OF THIS REFERENCE IS NOT BEING FURNISHED WITH THIS OFFICE ACTION. (SEE MPEP SECTION 707.05(a).
[signature]	12/5/94	PAGE 1 OF 1

[signature] 12/05/01

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 239 400**
A2

(12) EUROPEAN PATENT APPLICATION

(21) Application number: 87302620.7

(22) Date of filing: 26.03.87

(51) Int. Cl.⁴: C 12 N 15/00, C 07 K 15/06, C 12 P 21/02

(30) Priority: 27.03.86 GB 8607679

(43) Date of publication of application: 30.09.87 Bulletin 87/40

(84) Designated Contracting States: AT BE CH DE ES FR GB GR IT LI LU NL SE

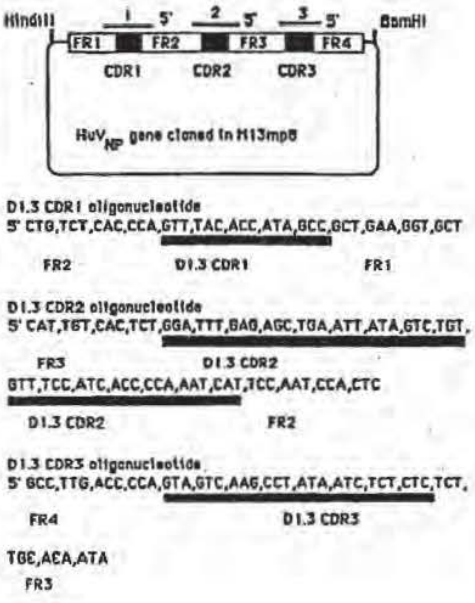
(71) Applicant: Winter, Gregory Paul, 64 Cavendish Avenue, Cambridge (GB)

(72) Inventor: Winter, Gregory Paul, 64 Cavendish Avenue, Cambridge (GB)

(74) Representative: Votier, Sidney David et al, CARPMAELS & RANSFORD 43, Bloomsbury Square, London WC1A 2RA (GB)

(54) Recombinant antibodies and methods for their production.

(57) An altered antibody is produced by replacing the complementarity determining regions (CDRs) of a variable region of an immunoglobulin (Ig) with the CDRs from an Ig of different specificity, using recombinant DNA techniques. The gene coding sequences for producing the altered antibody may be produced by site-directed mutagenesis using long oligonucleotides.



EP 0 239 400 A2

TITLE MODIFIED
see front page

RECOMBINANT DNA PRODUCT AND METHODS

The present invention relates to altered antibodies in which at least parts of the complementarity determining regions (CDRs) in the light or heavy chain variable domains of the antibody have been replaced by analogous parts of CDRs from an antibody of different specificity. The present invention also relates to methods for the production of such altered antibodies.

Natural antibodies, or immunoglobulins, comprise two heavy chains linked together by disulphide bonds and two light chains, one light chain being linked to each of the heavy chains by disulphide bonds. The general structure of an antibody of class IgG (i.e. an immunoglobulin (Ig) of class gamma (G)) is shown schematically in Figure 1 of the accompanying drawings.

Each heavy chain has at one end a variable domain followed by a number of constant domains. Each light chain has a variable domain at one end and a constant domain at its other end, the variable domain being aligned with the variable domain of the heavy chain and the constant domain being aligned with the first constant domain of the heavy chain. The constant domains in the light and heavy chains are not involved directly in binding the antibody to the antigen.

The variable domains of each pair of light and heavy chains form the antigen binding site. The domains on the light and heavy chains have the same general structure and each domain comprises four framework

regions, whose sequences are relatively conserved, connected by three hypervariable or complementarity determining regions (CDRs) (see Kabat, E.A., Wu, T.T., Bilofsky, H., Reid-Miller, M. and Perry, H., in "Sequences of Proteins of Immunological Interest", US Dept. Health and Human Services 1983). The four framework regions largely adopt a β-sheet conformation and the CDRs form loops connecting, and in some cases forming part of, the β-sheet structure. The CDRs are held in close proximity by the framework regions and, with the CDRs from the other domain, contribute to the formation of the antigen binding site.

For a more detailed account of the structure of variable domains, reference may be made to: Poljak, R.J., Amzel, L.M., Avey, H.P., Chen, B.L., Phizackerly, R.P. and Saul, F., PNAS USA, 70, 3305-3310, 1973; Segal, D.M., Padlan, E.A., Cohen, G.H., Rudikoff, S., Potter, M. and Davies, D.R., PNAS USA, 71, 4298-4302, 1974; and Marquart, M., Deisenhofer, J., Huber, R. and Palm, W., J. Mol. Biol., 141, 369-391, 1980.

In recent years advances in molecular biology based on recombinant DNA techniques have provided processes for the production of a wide range of heterologous polypeptides by transformation of host cells with heterologous DNA sequences which code for the production of the desired products.

EP-A-0 088 994 (Schering Corporation) proposes the construction of recombinant DNA vectors comprising a ds DNA sequence which codes for a variable domain of a light or a heavy chain of an Ig specific for a

predetermined ligand. The ds DNA sequence is provided with initiation and termination codons at its 5'- and 3'- termini respectively, but lacks any nucleotides coding for amino acids superfluous to the variable domain. The ds DNA sequence is used to transform bacterial cells. The application does not contemplate variations in the sequence of the variable domain.

EP-A-1 102 634 (Takeda Chemical Industries Limited) describes the cloning and expression in bacterial host organisms of genes coding for the whole or a part of human IgE heavy chain polypeptide, but does not contemplate variations in the sequence of the polypeptide.

EP-A-0 125 023 (Genentech Inc.) proposes the use of recombinant DNA techniques in bacterial cells to produce Ig's which are analogous to those normally found in vertebrate systems and to take advantage of the gene modification techniques proposed therein to construct chimeric Igs or other modified forms of Ig.

The term 'chimeric antibody' is used to describe a protein comprising at least the antigen binding portion of an immunoglobulin molecule (Ig) attached by peptide linkage to at least part of another protein.

It is believed that the proposals set out in the above Genentech application did not lead to the expression of any significant quantities of Ig polypeptide chains, nor to the production of Ig activity, nor to the secretion and assembly of the chains into the desired chimeric Igs.

The production of monoclonal antibodies was first disclosed by Kohler and Milstein (Kohler, G. and Milstein, C., Nature, 256, 495-497, 1975). Such monoclonal antibodies have found widespread use not only as diagnostic reagents (see, for example, 'Immunology for the 80s, Eds. Voller, A., Bartlett, A., and Bidwell, D., MTP Press, Lancaster, 1981) but also in therapy (see, for example, Ritz, J. and Schlossman, S.F., Blood, 59, 1-11, 1982).

The recent emergence of techniques allowing the stable introduction of Ig gene DNA into myeloma cells (see, for example, Oi, V.T., Morrison, S.L., Herzenberg, L.A. and Berg, P., PNAS USA, 80, 825-829, 1983; Neuberger, M.S., EMBO J., 2, 1373-1378, 1983; and Ochi, T., Hawley, R.G., Hawley, T., Schulman, M.J., Traunecker, A., Kohler, G. and Hozumi, N., PNAS USA, 80, 6351-6355, 1983), has opened up the possibility of using in vitro mutagenesis and DNA transfection to construct recombinant Igs possessing novel properties.

However, it is known that the function of an Ig molecule is dependent on its three dimensional structure, which in turn is dependent on its primary amino acid sequence. Thus, changing the amino acid sequence of an Ig may adversely affect its activity. Moreover, a change in the DNA sequence coding for the Ig may affect the ability of the cell containing the DNA sequence to express, secrete or assemble the Ig.

It is therefore not at all clear that it will be possible to produce functional altered antibodies by recombinant DNA techniques.

However, colleagues of the present Inventor have devised a process whereby chimeric antibodies in which both parts of the protein are functional can be secreted. The process, which is disclosed in International Patent Application No. PCT/GB85/00392 (Neuberger et al. and Celltech Limited), comprises:

a) preparing a replicable expression vector including a suitable promoter operably linked to a DNA sequence comprising a first part which encodes at least the variable domain of the heavy or light chain of an Ig molecule and a second part which encodes at least part of a second protein;

b) if necessary, preparing a replicable expression vector including a suitable promoter operably linked to a DNA sequence which encodes at least the variable domain of a complementary light or heavy chain respectively of an Ig molecule;

c) transforming an immortalised mammalian cell line with the or both prepared vectors; and

d) culturing said transformed cell line to produce a chimeric antibody.

The second part of the DNA sequence may encode:

i) at least part, for instance the constant domain of a heavy chain, of an Ig molecule of different species, class or subclass;

ii) at least the active portion or all of an enzyme;

iii) a protein having a known binding specificity;

iv) a protein expressed by a known gene but whose sequence, function or antigenicity is not known; or

v) a protein toxin, such as ricin.

The above Neuberger application only shows the production of chimeric antibodies in which complete variable domains are coded for by the first part of the DNA sequence. It does not show any chimeric antibodies in which the sequence of the variable domain has been altered.

The present invention, in a first aspect, provides an altered antibody in which at least parts of the CDRs in the light or heavy chain variable domains have been replaced by analogous parts of CDRs from an antibody of different specificity

The determination as to what constitutes a CDR and what constitutes a framework region was made on the basis of the amino-acid sequences of a number of Igs. However, from the three dimensional structure of a number of Igs it is apparent that the antigen binding site of an Ig variable domain comprises three looped regions supported on sheet-like structures. The loop regions do not correspond

exactly to the CDRs, although in general there is considerable overlap.

Moreover, not all of the amino-acid residues in the loop regions are solvent accessible and in one case, amino-acid residues in the framework regions are involved in antigen binding.(Amit, A.G., Mariuzza, R.A., Phillips, S.E.V. and Poljak, R.J., Science, 233, 747-753, 1986).

It is also known that the variable regions of the two parts of an antigen binding site are held in the correct orientation by inter-chain non-covalent interactions. These may involve amino-acid residues within the CDRs.

Thus, in order to transfer the antigen binding capacity of one variable domain to another, it may not be necessary to replace all of the CDRs with the complete CDRs from the donor variable region. It may be necessary only to transfer those residues which are accessible from the antigen binding site, and this may involve transferring framework region residues as well as CDR residues.

It may also be necessary to ensure that residues essential for inter-chain interactions are preserved in the acceptor variable domain.

Within a domain, the packing together and orientation of the two disulphide bonded β-sheets (and therefore the ends of the CDR loops) are relatively conserved. However, small shifts in packing and orientation of these β-sheets do occur

(Lesk, A.M. and Chothia, C., J. Mol. Biol., 160, 325-342, 1982). However, the packing together and orientation of heavy and light chain variable domains is relatively conserved (Chothia, C., Novotny, J., Bruccoleri, R. and Karplus, M., J. Mol. Biol., 186, 651-653, 1985). These points will need to be borne in mind when constructing a new antigen biding site so as to ensure that packing and orientation are not altered to the deteriment of antigen binding capacity.

It is thus clear that merely by replacing one or more CDRs with complementary CDRs may not always result in a functional altered antibody. However, given the explanations set out above, it will be well within the competence of the man skilled in the art, either by carrying out routine experimentation or by trial and error testing to obtain a functional altered antibody.

Preferably, the variable domains in both the heavy and light chains have been altered by at least partial CDR replacement and, if necessary, by partial framework region replacement and sequence changing. Although the CDRs may be derived from an antibody of the same class or even subclass as the antibody from which the framework regions are derived, it is envisaged that the CDRs will be derived from an antibody of different class and preferably from an antibody from a different species.

Thus, it is envisaged, for instance, that the CDRs from a mouse antibody could be grafted onto the

framework regions of a human antibody. This arrangement will be of particular use in the therapeutic use of monoclonal antibodies.

At present, when a mouse monoclonal antibody or even a chimeric antibody comprising a complete mouse variable domain is injected into a human, the human body's immune system recognises the mouse variable domain as foreign and produces an immune response thereto. Thus, on subsequent injections of the mouse antibody or chimeric antibody into the human, its effectiveness is considerably reduced by the action of the body's immune system against the foreign antibody. In the altered antibody of the present invention, only the CDRs of the antibody will be foreign to the body, and this should minimise side effects if used for human therapy. Although, for example, human and mouse framework regions have characteristic sequences, there seem to be no characteristic features which distinguish human from mouse CDRs. Thus, an antibody comprised of mouse CDRs in a human framework may well be no more foreign to the body than a genuine human antibody.

Even with the altered antibodies of the present invention, there is likely to be an anti-idiotypic response by the recipient of the altered antibody. This response is directed to the antibody binding region of the altered antibody, It is believed that at least some anti-idiotype antibodies are directed at sites bridging the CDRs and the framework regions. It would therefore be possible to provide a panel of antibodies having the same partial or complete CDR replacements but on a series of different framework regions. Thus, once a first altered antibody became therapeutically ineffective, due to an anti-idiotype

response, a second altered antibody from the series could be used, and so on, to overcome the effect of the anti-idiotype response. Thus, the useful life of the antigen-binding capacity of the altered antibodies could be extended.

Preferably, the altered antibody has the structure of a natural antibody or a fragment thereof. Thus, the altered antibody may comprise a complete antibody, an $(Fab')_2$ fragment, an Fab fragment, a light chain dimer or a heavy chain dimer. Alternatively, the altered antibody may be a chimeric antibody of the type described in the Neuberger application referred to above. The production of such an altered chimeric antibody can be carried out using the methods described below used in conjunction with the methods described in the Neuberger application.

The present invention, in a second aspect, comprises a method for producing such an altered antibody comprising:

a) preparing a first replicable expression vector including a suitable promoter operably linked to a DNA sequence which encodes at least a variable domain of an Ig heavy or light chain, the variable domain comprising framework regions from a first antibody and CDRs comprising at least parts of the CDRs from a second antibody of different specificity;

b) if necessary, preparing a second replicable expression vector including a suitable promoter operably linked to a DNA sequence which encodes at least the variable domain of a complementary Ig light or heavy chain respectively;

c) transforming a cell line with the first or both prepared vectors; and

d) culturing said transformed cell line to produce said altered antibody.

The present invention also includes vectors used to transform the cell line, vectors used in producing the transforming vectors, cell lines transformed with the transforming vectors, cell lines tranformed with preparative vectors, and methods for their production.

Preferably, the cell line which is transformed to produce the altered antibody is an immortalised mammalian cell line, which is advantageously of lymphoid origin, such as a myeloma, hybridoma, trioma or quadroma cell line. The cell line may also comprise a normal lymphoid cell, such as a B-cell, which has been immortalised by transformation with a virus, such as the Epstein-Barr virus. Most preferably, the immortalised cell line is a myeloma cell line or a derivative thereof.

Although the cell line used to produce the altered antibody is preferably a mammalian cell line, any other suitable cell line, such as a bacterial cell line or a yeast cell line, may alternatively be used. In particular, it is envisaged that E. Coli derived bacterial strains could be used.

It is known that some immortalised lymphoid cell lines, such as myeloma cell lines, in their normal state secrete isolated Ig light or heavy chains. If

such a cell line is transformed with the vector prepared in step a) of the process of the invention, it will not be necessary to carry out step b) of the process, provided that the normally secreted chain is complementary to the variable domain of the Ig chain encoded by the vector prepared in step a).

However, where the immortalised cell line does not secrete or does not secrete a complementary chain, it will be necessary to carry out step b). This step may be carried out by further manipulating the vector produced in step a) so that this vector encodes not only the variable domain of an altered antibody light or heavy chain, but also the complementary variable domain.

Alternatively, step b) is carried out by preparing a second vector which is used to transform the immortalised cell line. This alternative leads to easier construct preparation, but may be less preferred than the first alternative in that it may not lead to as efficient production of antibody.

The techniques by which such vectors can be produced and used to transform the immortalised cell lines are well known in the art, and do not form any part of the invention.

In the case where the immortalised cell line secretes a complementary light or heavy chain, the transformed cell line may be produced for example by transforming a suitable bacterial cell with the vector and then fusing the bacterial cell with the immortalised cell line by spheroplast fusion. Alternatively, the DNA may be directly introduced into the immortalised cell line by electroporation.

The DNA sequence encoding the altered variable domain may be prepared by oligonucleotide synthesis. This requires that at least the framework region sequence of the acceptor antibody and at least the CDRs sequences of the donor antibody are known or can be readily determined. Although determining these sequences, the synthesis of the DNA from oligonucleotides and the preparation of suitable vectors is to some extent laborious, it involves the use of known techniques which can readily be carried out by a person skilled in the art in light of the teaching given here.

If it was desired to repeat this strategy to insert a different antigen binding site, it would only require the synthesis of oligonucleotides encoding the CDRs, as the framework oligonucleotides can be re-used.

A convenient variant of this technique would involve making a symthetic gene lacking the CDRs in which the four framework regions are fused together with suitable restriction sites at the junctions. Double stranded synthetic CDR cassettes with sticky ends could then be ligated at the junctions of the framework regions. A protocol for achieving this variant is shown diagrammatically in Figure 6 of the accompanying drawings.

Alternatively, the DNA sequence encoding the altered variable domain may be prepared by primer directed oligonucleotide site-directed mutagenesis. This

technique in essence involves hybridising an oligonucleotide coding for a desired mutation with a single strand of DNA containing the region to be mutated and using the single strand as a template for extension of the oligonulcleotide to produce a strand containing the mutation. This technique, in various forms, is described by : Zoller, M.J. and Smith, M., Nuc. Acids Res., 10, 6487-6500, 1982; Norris, K., Norris F., Christiansen, L. and Fiil, N., Nuc. Acids Res., 11, 5103-5112, 1983; Zoller, M.J. and Smith, M., DNA, 3, 479-488 (1984); Kramer, W., Schughart, K. and Fritz, W.-J., Nuc. Acids Res., 10, 6475-6485, 1982.

For various reasons, this technique in its simplest form does not always produce a high frequency of mutation. An improved technique for introducing both single and multiple mutations in an M13 based vector, has been described by Carter et al. (Carter, P., Bedouelle H. and Winter, G., Nuc. Acids Res., 13, 4431-4443, 1985)

Using a long oligonucleotide, it has proved possible to introduce many changes simultaneously (as in Carter et al., loc. cit.) and thus single oligonucleotides, each encoding a CDR, can be used to introduce the three CDRs from a second antibody into the framework regions of a first antibody. Not only is this technique less laborious than total gene synthesis, but it represents a particularly convenient way of expressing a variable domain of required specificity, as it can be simpler than tailoring an entire V_H domain for insertion into an expression plasmid.

The oligonucleotides used for site-directed mutagenesis may be prepared by oligonucleotide synthesis or may be isolated from DNA coding for the variable domain of the second antibody by use of suitable restriction enzymes. Such long oligonucleotides will generally be at least 30 bases long and may be up to or over 80 bases in length.

The techniques set out above may also be used, where necessary, to produce the vector of part (b) of the process.

The method of the present invention is envisaged as being of particular use in "humanising" non-human monoclonal antibodies. Thus, for instance, a mouse monoclonal antibody against a particular human cancer cell may be produced by techniques well known in the art. The CDRs from the mouse monoclonal antibody may then be partially or totally grafted into the framework regions of a human monoclonal antibody, which is then produced in quantity by a suitable cell line. The product is thus a specifically targetted, essentially human antibody which will recognise the cancer cells, but will not itself be recognised to any significant degree, by a human's immune system, until the anti-idiotype response eventually becomes apparent. Thus, the method and product of the present invention will be of particular use in the clinical environment.

The present invention is now described, by way of example only, with reference to the accompanying drawings, in which:

Figure 1 is a schematic diagram showing the structure of an IgG molecule;

Figure 2 shows the amino acid sequence of the V_H domain of NEWM in comparison with the V_H domain of the BI-8 antibody;

Figure 3 shows the amino acid and nucleotide sequence of the HuV_{NP} gene;

Figure 4 shows a comparison of the results for HuV_{NP}-IgE and MoV_{NP}-IgE in binding inhibition assays;

Figure 5 shows the structure of three oligonucleotides used for site directed mutagenesis;

Figure 6 shows a protocol for the construction of CDR replacements by insertion of CDR cassettes into a vector containing four framework regions fused together;

Figure 7 shows the sequence of the variable domain of antibody D1.3 and the gene coding therefor; and

Figure 8 shows a protocol for the cloning of the D1.3 variable domain gene.

EXAMPLE 1

This example shows the production of an altered antibody in which the variable domain of the heavy chains comprises the framework regions of a human heavy chain and the CDRs from a mouse heavy chain.

The framework regions were derived from the human myeloma heavy chain NEWM, the crystallographic structure of which is known (see Poljak et al., loc. cit. and Reth, M., Hammerling, G.J. and Rajewsky, K., EMBO J., 1, 629-634, 1982.)

The CDRs were derived from the mouse monoclonal antibody B1-8 (see Reth et al., loc. cit.), which binds the hapten NP-cap (4-hydroxy-3-nitrophenyl acetyl-caproic acid: K_{NP-CAP}=1.2 μM).

A gene encoding a variable domain HuV_{NP}, comprising the B1-8 CDRs and the NEWM framework regions, was constructed by gene synthesis as follows.

The amino acid sequence of the V_H domain of NEWM is shown in Figure 2, wherein it is compared to the amino acid sequence of the V_H domain of the B1-8 antibody. The sequence is divided into framework regions and CDRs according to Kabat et al. (loc. cit.). Conserved residues are marked with a line.

The amino acid and nucleotide sequence of the HuV_{NP} gene, in which the CDRs from the B1-8 antibody alternate with the framework regions of the NEWM antibody, is shown in Figure 3. The HuV_{NP} gene was derived by replacing sections of the MoV_{NP} gene in the vector pSV-V_{NP} (see Neuberger, M.S., Williams, G.T., Mitchell, E.B., Jouhal, S., Flanagan, J.G. and Rabbitts, T.H., Nature, 314, 268-270, 1985) by a synthetic fragment encoding the HuV_{NP} domain. Thus the 5' and 3' non-coding sequences, the leader sequence, the L-V intron, five N-terminal and four

C-terminal amino acids are from the MoV_{NP} gene and the rest of the coding sequence is from the synthetic HuV_{NP} fragment.

The oligonucleotides from which the HuV_{NP} fragment was assembled are aligned below the corresponding portion of the HuV_{NP} gene. For convenience in cloning, the ends of oligonucleotides 25 and 26b form a Hind II site followed by a Hind III site, and the sequences of the 25/26b oligonucleotides therefore differ from the HuV_{NP} gene.

The HuV_{NP} synthetic fragment was built as a PstI-Hind III fragment. The nucleotide sequence was derived from the protein sequence using the computer programme ANALYSEQ (Staden, R., Nuc. Acids. Res., 12, 521-538, 1984) with optimal codon usage taken from the sequences of mouse constant domain genes. The oligonucleotides (1 to 26b, 28 in total) vary in size from 14 to 59 residues and were made on a Biosearch SAM or an Applied Biosystems machine, and purified on 8M-urea polyacrylamide gels (see Sanger, F. and Coulson, A., FEBS Lett., 87, 107-110, 1978).

The oligonucleotides were assembled in eight single stranded blocks (A-D) containing oligonucleotides

[1,3,5,7] (Block A), [2,4,6,8] (block A'), [9,11,13a,13b] (Block B), [10a, 10b,12/14] (block B'), [15, 17] (block C), [16,18] (block C'), [19, 21, 23, 25] (block D) and [20, 22/24, 26a, 26b) (block D').

In a typical assembly, for example of block A, 50 pmole of oligonucleotides 1,3,5 and 7 were phosphorylated at the 5' end with T4 polynucleotide kinase and mixed together with 5 pmole of the terminal oligonucleotide [1] which had been phosphorylated with 5 µCi [γ-^{32}P] ATP (Amersham 3000 Ci/mmole). These oligonucleotides were annealed by heating to 80°C and cooling over 30 minutes to room temperature, with unkinased oligonucleotides 2, 4 and 6 as splints, in 150 µl of 50 mM Tris.Cl, pH 7.5, 10 mM $MgCl_2$. For the ligation, ATP (1 mM) and DTT (10mM) were added with 50 U T4 DNA ligase (Anglian Biotechnology Ltd.) and incubated for 30 minutes at room temperature. EDTA was added to 10 mM, the sample was extracted with phenol, precipitated from ethanol, dissolved in 20 µl water and boiled for 1 minute with an equal volume of formamide dyes. The sample was loaded onto and run on a 0.3 mm 8M-urea 10% polyacrylamide gel. A band of the expected size was detected by autoradiography and eluted by soaking.

Two full length single strands were assembled from blocks A to D and A' to D' using splint oligonucleotides. Thus blocks A to D were annealed and ligated in 30 µl as set out in the previous paragraph using 100 pmole of oligonucleotides 10a, 16 and 20 as splints. Blocks A' to D' were ligated using oligonucleotides 7, 13b and 17 as splints.

After phenol/ether extraction, block A-D was annealed with block A'-D', small amounts were cloned in the vector M13mp18 (Yanish-Perron, C., Vieira, J. and Messing, J., Gene, 33, 103-119, 1985) cut with PstI and Hind III, and the gene sequenced by the

dideoxy technique (Sanger, F., Nicklen, S. and Coulson, A.R., PNAS USA, 74, 5463-5467, 1979).

The MoV_{NP} gene was transferred as a Hind III - BamHI fragment from the vector pSV-V_{NP} (Neuberger et al., loc. cit.) to the vector M13mp8 (Messing, J. and Vieira, J., Gene, 19, 269-276, 1982). To facilitate the replacement of MoV_{NP} coding sequences by the synthetic HuV_{NP} fragment, three Hind II sites were removed from the 5' non-coding sequence by site directed mutagenesis, and a new Hind II site was subsequently introduced near the end of the fourth framework region (FR4 in Figure 2). By cutting the vector with PstI and Hind II, most of the V_{NP} fragment can be inserted as a PstI-Hind II fragment. The sequence at the Hind II site was corrected to NEWM FR4 by site directed mutagenesis.

The Hind III - Bam HI fragment, now carrying the HuV_{NP} gene, was excised from M13 and cloned back into pSV-V_{NP} to replace the MoV_{NP} gene and produce a vector pSV-HuV_{NP}. Finally, the genes for the heavy chain constant domains of human Ig E (Flanagan, J.G. and Rabbitts, T.H., EMBO J., 1, 655-660, 1982) were introduced as a Bam HI fragment to give the vector pSV-HuV_{NP}. HE. This was transfected into the myeloma line J558 L by spheroplast fusion.

The sequence of the HuV_{NP} gene in pSV-HuV_{NP}. HE was checked by recloning the Hind III-Bam HI fragment back into M13mp8 (Messing et al., loc. cit.). J558L myeloma cells secrete lambda 1 light chains which have been shown to associate with heavy chains containing the MoV_{NP} variable domain to create a

binding site for NP-cap or the related hapten NIP-Cap (3-iodo-4-hydroxy-5-nitrophenylacetyl-caproic acid) (Reth, M., Hammerling, G.J. and Rajewsky, K., Eur. J. Immunol., 8, 393-400, 1978).

As the plasmid pSV-HuV_{NP}.HE contains the gpt marker, stably transfected myeloma cells could be selected in a medium containing mycophenolic acid. Transfectants secreted an antibody (HuV_{NP}-IgE) with heavy chains comprising a HuV_{NP} variable domain (i.e. a "humanised" mouse variable region) and human γ constant domains, and lambda 1 light chains from the J558L myeloma cells.

The culture supernatants of several gpt^+ clones were assayed by radioimmunoassay and found to contain NIP-cap binding antibody. The antibody secreted by one such clone was purified from culture supernatant by affinity chromatography on NIP-cap Sepharose (Sepharose is a registered trade mark). A polyacrylamide - SDS gel indicated that the protein was indistinguishable from the chimeric antibody MoV_{NP}-IgE (Neuberger et al., loc. cit.).

The HuV_{NP}-IgE antibody competes effectively with the MoV_{NP}-IgE for binding to both anti-human-IgE and to NIP-cap coupled to bovine serum albumin.

Various concentrations of HuV_{NP}-IgE and MoV_{NP}-IgE were used to compete the binding of radiolabelled MoV_{NP}-IgE to polyvinyl microtitre plates coated with (a) Sheep anti-human-IgE antiserum (Seward Laboratories); (b) NIP-cap-bovine serum albumin; (c) Ac38 anti-idiotypic antibody; (d) Ac 146 anti-idiotypic antibody; and (e) rabbit anti-MoV_{NP}

antiserum. Binding was also carried out in the presence of MoV_{NP}-IgM antibody (Neuberger, M.S., Williams, G.T. and Fox, R.O., Nature, 312, 604-608, 1984) or of JW5/1/2 which is an IgM antibody differing from the MoV_{NP}-IgM antibody at 13 residues mainly located in the V_H CDR2 region.

The results of the binding assays are shown in Figure 4, wherein black circles represent HuV_{NP}, white circles MoV_{NP}, black squares MoV_{NP}-IgM and white squares JW5/1/2. Binding is given relative to the binding in the absence of the inhibitor.

The affinities of HuV_{NP}-IgE for NP-cap and NIP-cap were then measured directly using the fluorescence quench technique and compared to those for MoVNP-IgE, using excitation at 295 nm and observing emission at 340 nm (Eisen, H.N., Methods Med. Res., 10, 115-121,1964).

Antibody solutions were diluted to 100 nM in phosphate buffered saline, filtered (0.45 µm pore cellulose acetate) and titrated with NP-cap in the range 0.2 to 20 µM. As a control, mouse DI-3 antibody (Mariuzza, R.A., Jankovic, D.L., Bulot, G., Amit, A.G., Saludjian, P., Le Guern, A., Mazie, J.C. and Poljak, R.J., J. Mol. Biol., 170, 1055-1058, 1983), which does not bind hapten, was titrated in parallel.

Decrease in the ratio of the fluorescence of HuV_{NP}-IgE or HuV_{NP}-IgE to the fluorescence of the D1-3 antibody was taken to be proportional to NP-cap occupancy of the antigen binding sites. The maximum

quench was about 40% for both antibodies, and hapten dissociation constants were determined from least-squares fits of triplicate data sets to a hyperbola.

For NIP-cap, hapten concentration varied from 10 to 300 nM, and about 50% quenching of fluorescence was observed at saturation. Since the antibody concentrations were comparable to the value of the dissociation constants, data were fitted by least squares to an equation describing tight binding inhibition (Segal, I.H., in "Enzyme Kinetics", 73-74, Wiley, New York, 1975).

The binding constants obtained from these data for these antibodies are shown in Table 1 below.

Table 1

	$K_{NP\text{-}cap}$	$K_{NIP\text{-}cap}$
MoV_{NP}-IgE	1.2 µM	0.02 µM
HuV_{NP}-IgE	1.9 µM	0.07 µM

These results show that the affinities of these antibodies are similar and that the change in affinity is less than would be expected for the loss of a hydrogen bond or a van der Waals contact point at the active site of an enzyme.

Thus, it has been shown that it is possible to produce an antibody specific for an artificial small hapten, comprising a variable domain having human framework regions and mouse CDRs, without any significant loss of antigen binding capacity.

As shown in Figure 4(d), the HuV_{NP}-IgE antibody has lost the MoV_{NP} idiotypic determinant recognised by the antibody Ac146. Furthermore, HuV_{NP}-IgE also binds the Ac38 antibody less well (Figure 4(c)), and it is therefore not surprising that HuV_{NP}-IgE has lost many of the determinants recognised by the polyclonal rabbit anti-idiotypic antiserum (Figure 4(e)).

It can thus be seen that, although the HuV_{NP}-IgE antibody has acquired substantially all the antigen binding capacity of the mouse CDRs, it has not acquired any substantial proportion of the mouse antibody's antigenicity.

The results of Figures 4(d) and 4(e) carry a further practical implication. The mouse (or human) CDRs could be transferred from one set of human frameworks (antibody 1) to another (antibody 2). In therapy, anti-idiotypic antibodies generated in response to antibody 1 might well bind poorly to antibody 2. Thus, as the anti-idiotypic response starts to neutralise antibody 1 treatment could be continued with antibody 2, and the CDRs of a desired specificity used more than once.

For instance, the oligonucleotides encoding the CDRs may be used again, but with a set of oligonucleotides encoding a different set of framework regions.

The above work has shown that antigen binding characteristics can be transferred from one framework to another without loss of activity, so

long as the original antibody is specific for a small hapten.

It is known that small haptens generally fit into an antigen binding cleft. However, this may not be true for natural antigens, for instance antigens comprising an epitopic site on a protein or polysaccharide. For such antigens, the antibody may lack a cleft (it may only have a shallow concavity), and surface amino acid residues may play a significant role in antigen binding. It is therefore not readily apparent that the work on artificial antigens shows conclusively that CDR replacement could be used to transfer natural antigen binding properties.

Therefore work was carried out to see if CDR replacement could be used for this purpose. This work also involved using primer-directed, oligonucleotide site-directed mutagenesis using three synthetic oligonucleotides coding for each of the mouse CDRs and the flanking parts of framework regions to produce a variable domain gene similar to the HuV_{NP} gene.

EXAMPLE 2

The three dimensional structure of a complex of lysozyme and the antilysozyme antibody D1.3 (Amit et al., loc. cit.) was solved by X-ray crystallography. There is a large surface of interaction between the antibody and antigen. The antibody has two heavy chains of the mouse IgG1 class (H) and two Kappa light chains (K), and is denoted below as H_2K_2.

The DNA sequence of the heavy chain variable region was determined by making cDNA from the mRNA of the D1.3 hybridoma cells, and cloning into plasmid and M13 vectors. The sequence is shown in Figure 7, in which the boxed residues comprise the three CDRs and the asterisks mark residues which contact lysozyme.

Three synthetic oligonucleotides were then designed to introduce the D1.3 V_HCDRs in place of the V_HCDRs of the HuV_{NP} gene. The Hu_{NP} gene has been cloned into M13mp8 as a BamHI-Hind III fragment, as described above. Each oligonucleotide has 12 nucleotides at the 5' end and 12 nucleotides at the 3' end which are complementary to the appropriate HuV_{NP} framework regions. The central portion of each oligonucleotide encodes either CDR1, CDR2, or CDR3 of the D1.3 antibody, as shown in Figure 5, to which reference is now made. It can be seen from this Figure that these oligonucleotides are 39, 72 and 48 nucleotides long respectively.

10 pmole of D1.3 CDR1 primer was phosphorylated at the 5' end and annealed to 1µg of the M13-HuV_{NP} template and extended with the Klenow fragment of DNA polymerase in the presence of T4 DNA ligase. After an oligonucleotide extension at 15°C, the sample was used to transfect E. Coli strain BHM71/18 mutL and plaques gridded and grown up as infected colonies.

After transfer to nitrocellulose filters, the colonies were probed at room temperature with 10 pmole of D1.3 CDR1 primer labelled at the 5' end

with 30 μCi ^{32}-p-ATP. After a 3" wash at 60°C, autoradiography revealed about 20% of the colonies had hybridised well to the probe. All these techniques are fully described in "Oligonucleotide site-directed mutagenesis in M13" an experimental manual by P. Carter, H. Bedouelle, M.M.Y. Waye and G. Winter 1985 and published by Anglian Biotechnology Limited, Hawkins Road, Colchester, Essex CO2 8JX. Several clones were sequenced, and the replacement of HuV_{NP} CDR1 by D.13 CDR1 was confirmed. This M13 template was used in a second round of mutagenesis with D1.3 CDR2 primer; finally template with both CDRs 1&2 replaced was used in a third round of mutagenesis with D.13 CDR3 primer. In this case, three rounds of mutagenesis were used.

The variable domain containing the D1.3 CDRs was then attached to sequences encoding the heavy chain constant regions of human IgG2 so as to produce a vector encoding a heavy chain Hu*. The vector was transfected into J558L cells as above. The antibody $Hu*_2L_2$ is secreted.

For comparative purposes, the variable region gene for the D1.3 antibody was inserted into a suitable vector and attached to a gene encoding the constant regions of mouse IgG1 to produce a gene encoding a heavy chain H* with the same sequence as H. The protocol for achieving this is shown in Figure 8.

As shown in Figure 8, the gene encoding the D1.3 heavy chain V and C_H1 domains and part of the hinge region are cloned into the M13mp9 vector.

The vector (vector A) is then cut with NcoI, blunted with Klenow polymerase and cut with PstI. The PStI-NcoI fragment is purified and cloned into PstI-HindII cut MV_{NP} vector to replace most of the MV_{NP} coding sequences. The MV_{NP} vector comprises the mouse variable domain gene with its promoter, 5' leader, and 5' and 3' introns cloned into M13mp9. This product is shown as vector B in Figure 8.

Using site directed mutagenesis on the single stranded template of vector B with two primers, the sequence encoding the N-terminal portion of the C_H1 domain and the PstI site near the N-terminus of the V domain are removed. Thus the V domain of D1.3 now replaces that of V_{NP} to produce vector C of Figure 8.

Vector C is then cut with HindIII and BamHI and the fragment formed thereby is inserted into HindIII/BamHI cut M13mp9. The product is cut with Hind III and SacI and the fragment is inserted into PSV-V_{NP} cut with Hind III/SacI so as to replace the V_{NP} variable domain with the D1.3 variable domain. Mouse IgG1 constant domains are cloned into the vector as a SacI fragment to produce vector D of Figure 8.

Vector D of Figure 8 is transfected into J558L cells and the heavy chain H* is secreted in association with the lambda light chain L as an antibody $H^*_2L_2$.

Separated K or L light chains can be produced by treating an appropriate antibody (for instance D1.3 antibody to produce K light chains) with 2-mercaptoethanol in guanidine hydrochloride,

blocking the free interchain sulphydryls with iodoacetamide and separating the dissociated heavy and light chains by HPLC in guanidine hydrochloride.

Different heavy and light chains can be reassociated to produce functional antibodies by mixing the separated heavy and light chains, and dialysing into a non-denaturing buffer to promote re-association and refolding. Properly reassociated and folded antibody molecules can be purified on protein A-sepharose columns. Using appropriate combinations of the above procedures, the following antibodies were prepared.

H_2K_2	(D1 3 antibody)
$H^*_2L_2$	(D1.3 heavy chain, lambda light chain)
$H^*_2K_2$	(recombinant equivalent of D1.3)
$Hu^*_2L_2$	("humanised" D1.3 heavy chain, lambda light chain)
$Hu^*_2K_2$	("humanised" D1.3)

The antibodies containing the lambda light chains were not tested for antigen binding capacity. The other antibodies were, and the results are shown in Table 2.

Table 2

Antibody	Dissociation constant for lysozyme (nM)
D1.3 (H_2K_2)	14.4
D1.3 (H_2K_2) (reassociated)	15.9, 11.4

recombinant D1.3 ($H^*_2K_2$) (reassociated)	9.2
"humanised" D1.3 (Hu_2K_2) (reassociated)	3.5, 3.7

The affinity of the antibodies for lysozyme was determined by fluorescent quenching, with excitation at 290nm and emission observed at 340nm Antibody solutions were diluted to 15-30µg/mg in phosphate buffered saline, filtered (0.45 um-cellulose acetate) and titrated with hen eggwhite lysozyme. There is a quenching of fluorescence on adding the lysozyme to the antibody (>100% quench) and data were fitted by least squares to an equation describing tight binding inhibition (I.H. Segal in Enzyme Kinetics, p73-74, Wiley, New York 1975). Although at first sight the data suggest that the binding of the "humanised" antibody to lysozyme is tighter than in the original D1.3 antibody, this remains to be confirmed. It is clear however that the humanised antibody binds lysozyme with a comparable affinity to D1.3

Further work (with another antibody-CAMPATH1) has shown that CDRs 1,2 and 3 can be exchanged simultaneously, by priming as above with all three primers. 10% hybridisation positives were detected by screening with the CDR1 primer; 30% of these comprised the triple mutant in which all the CDRs were replaced.

It has therefore been shown that CDR replacement can be used not only for artificial antigens (haptens) but also for natural antigens, thereby showing that the present invention will be of therapeutic use.

It will of course be understood that the present invention has been described above purely by way of example, and modifications of detail can be made within the scope of the invention as defined in the appended claims.

CLAIMS

1. An altered antibody in which at least parts of the complementarity determining regions (CDRs) in the light or heavy chain variable domains have been replaced by analogous parts of CDRs from an antibody of different specificity.

2. The altered antibody of claim 1, in which the entire CDRs have been replaced.

3. The altered antibody of claim 1 or claim 2, in which the variable domains in both the heavy and light chains have been altered by CDR replacement.

4. The altered antibody of any one of claims 1 to 3 in which the CDRs from a mouse antibody are grafted onto the framework regions of a human antibody.

5. The altered antibody of any one of claims 1 to 4, which has the structure of a natural antibody or a fragment thereof.

6. A method for producing an altered antibody comprising:

a) preparing a first replicable expression vector including a suitable promoter operably linked to a DNA sequence which encodes at least a variable domain of an Ig heavy or light chain, the variable domain comprising framework regions from a first antibody and CDRs comprising at least parts of the CDRs from a second antibody of different specificity;

b) if necessary, preparing a second replicable expression vector including a suitable promoter operably linked to a DNA sequence which encodes at least the variable domain of a complementary Ig light or heavy chain respectively;

c) transforming a cell line with the first or both prepared vectors; and

d) culturing said transformed cell line to produce said altered antibody.

7. The method of claim 6, in which the cell line which is transformed to produce the altered antibody is an immortalised mammalian cell line.

8. The method of claim 7, in which the immortalised cell line is a myeloma cell line or a derivative thereof.

9. The method of any one of claims 6 to 8, in which the DNA sequence encoding the altered variable domain is prepared by oligonucleotide synthesis.

10. The method of any one of claims 6 to 8, in which the DNA sequence encoding the altered variable domain is prepared by primer directed oligonucleotide site-directed mutagenesis using a long oligonucleotide.

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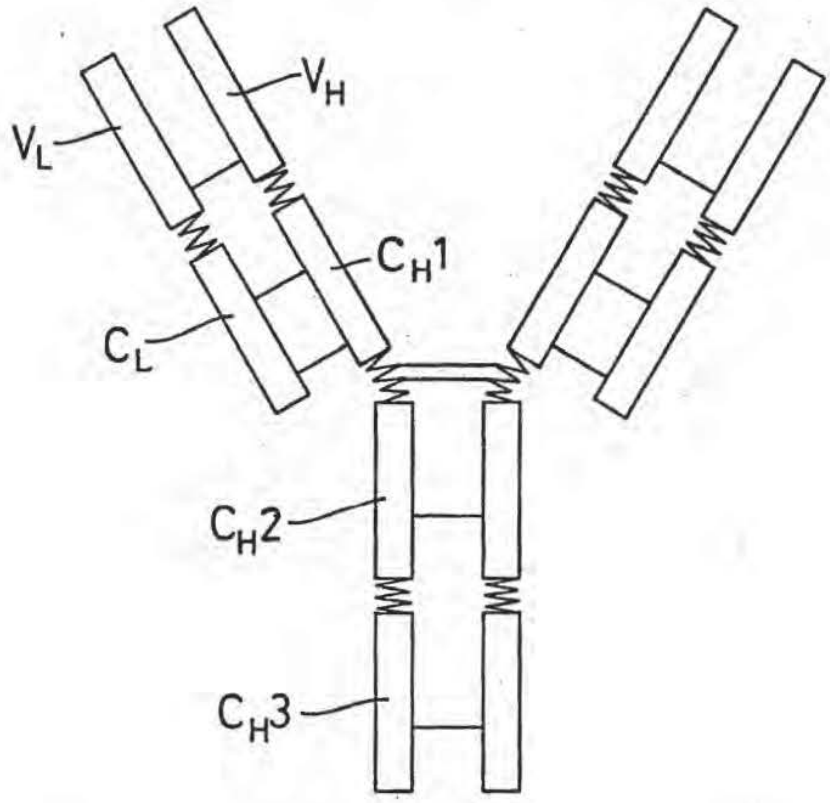
Fig. 1



▭	=	domains
∿∿	=	inter-domain sections
——	=	disulphide bonds
V	=	variable
C	=	constant
L	=	light chain
H	=	heavy chain

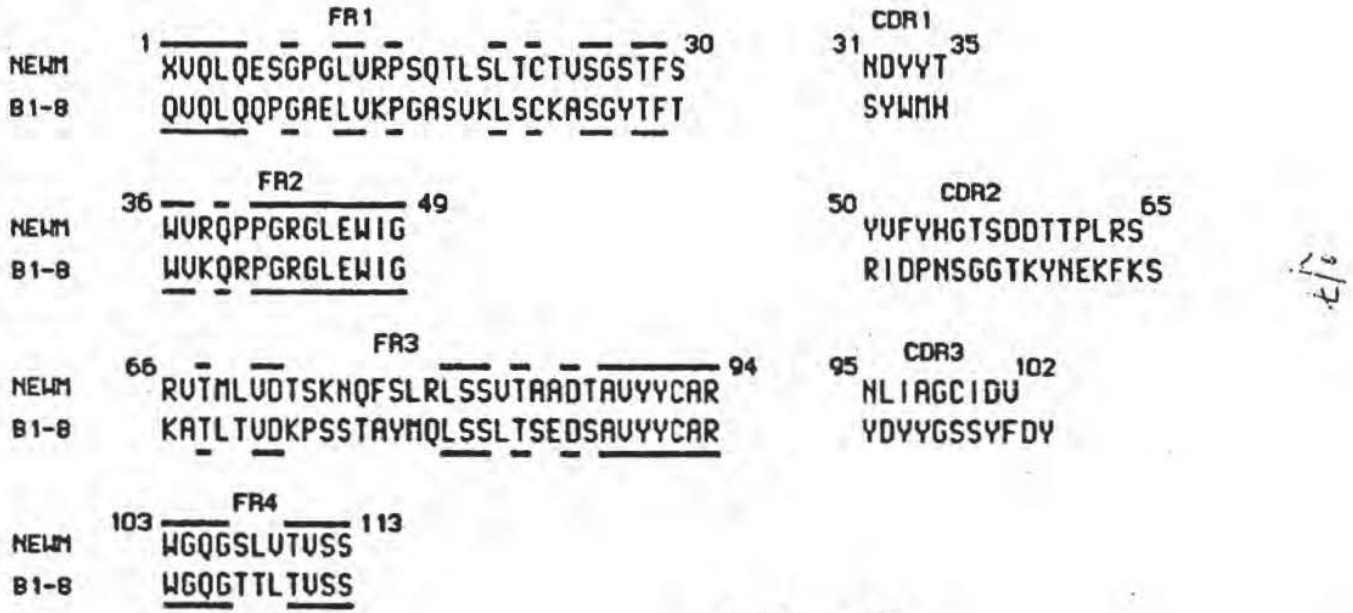


Fig. 2

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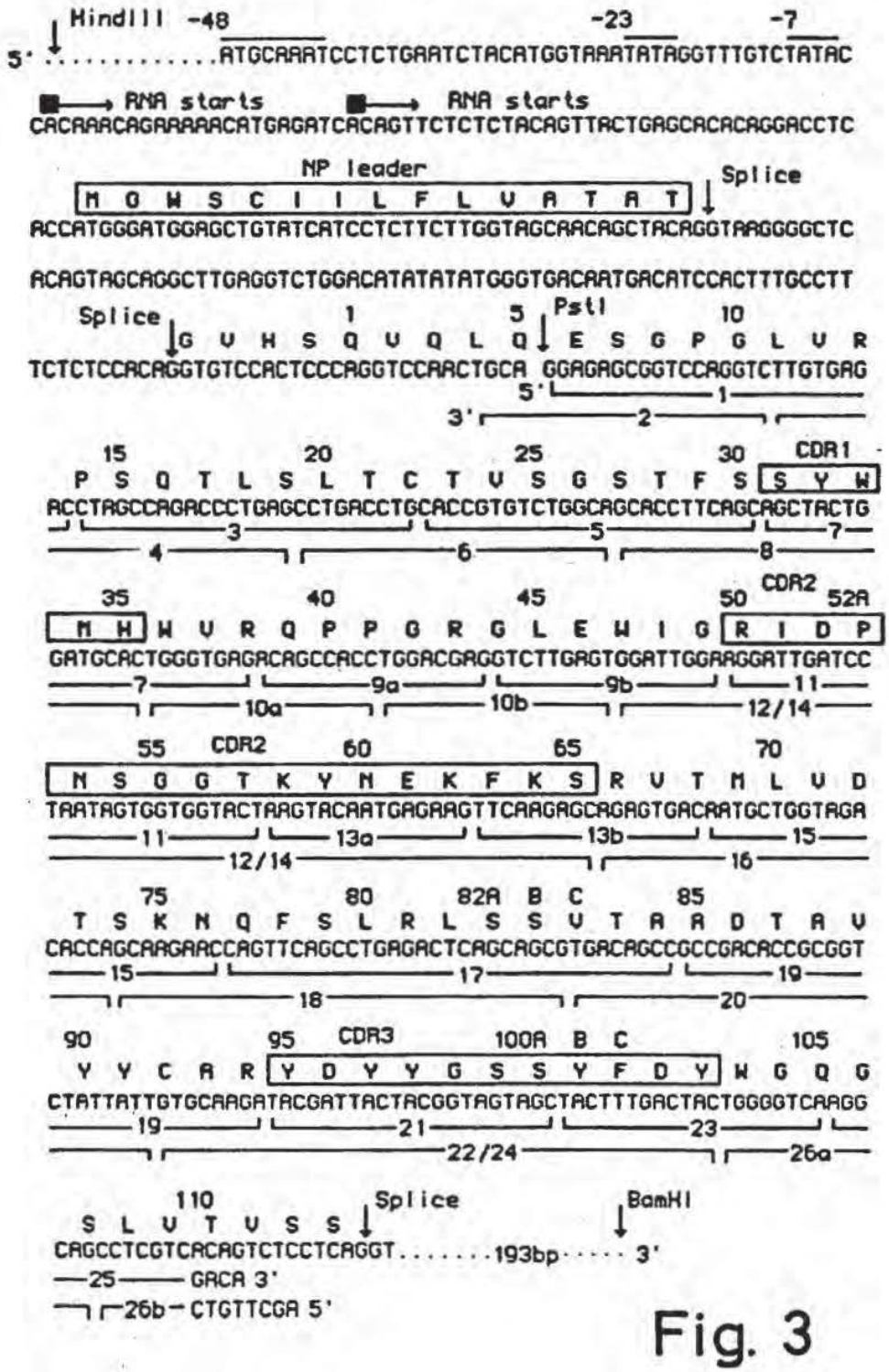
HindIII -48 -23 -7
5' .....ATGCAAATCCTCTGAATCTACATGGTAAATATAGGTTTGTCTATAC
RNA starts RNA starts
CACAAACAGAAAAACATGAGATCACAGTTCTCTCTACAGTTACTGAGCACACAGGACCTC
NP leader
M G W S C I I L F L V A T A T
Splice
ACCATGGGATGGAGCTGTATCATCCTCTTCTTGGTAGCAACAGCTACAGGTAAGGGGCTC
ACAGTAGCAGGCTTGAGGTCTGGACATATATATGGGTGACAATGACATCCACTTTGCCTT
Splice PstI
G V H S Q V Q L Q E S G P G L V R
TCTCTCCACAGGTGTCCACTCCCAGGTCCAACTGCA GGAGAGCGGTCCAGGTCTTGTGAG
CDR1
P S Q T L S L T C T V S G S T F S S Y W
ACCTAGCCAGACCCTGAGCCTGACCTGCACCGTGTCTGGCAGCACCTTCAGCAGCTACTG
CDR2
M H W V R Q P P G R G L E W I G R I D P
GATGCACTGGGTGAGACAGCCACCTGGACGAGGTCTTGAGTGGATTGGAAGGATTGATCC
N S G G T K Y N E K F K S R V T M L V D
TAATAGTGGTGGTACTAAGTACAATGAGAAGTTCAAGAGCAGAGTGACAATGCTGGTAGA
T S K N Q F S L R L S S V T A A D T A V
CACCAGCAAGAACCAGTTCAGCCTGAGACTCAGCAGCGTGACAGCCGCCGACACCGCGGT
CDR3
Y Y C A R Y D Y Y G S S Y F D Y W G Q G
CTATTATTGTGCAAGATACGATTACTACGGTAGTAGCTACTTTGACTACTGGGGTCAAGG
S L V T V S S Splice BamHI
CAGCCTCGTCACAGTCTCCTCAGGT.....193bp..... 3'
25 GACA 3'
26b CTGTTCGA 5'

Fig. 3

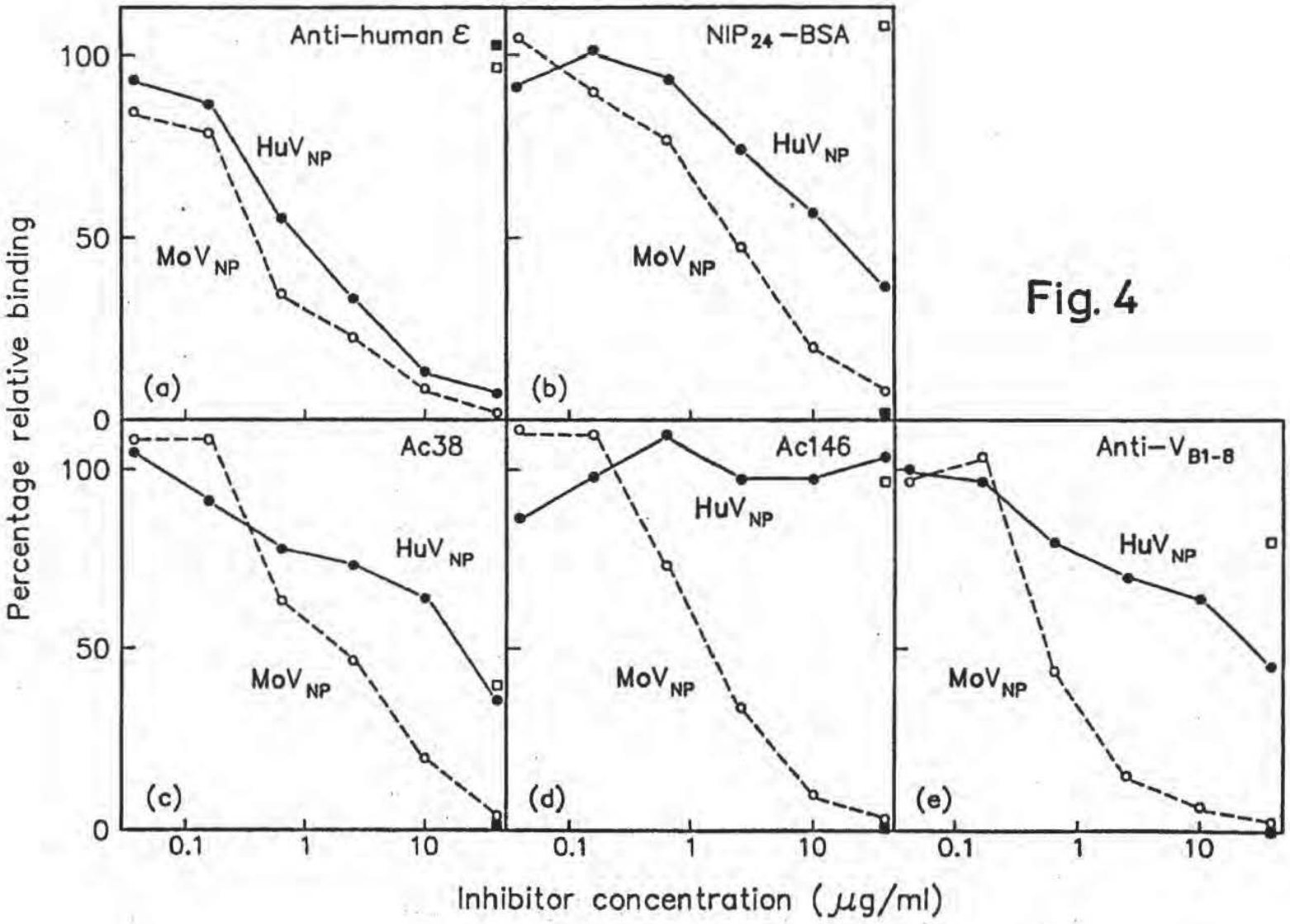

Fig. 4

t/h

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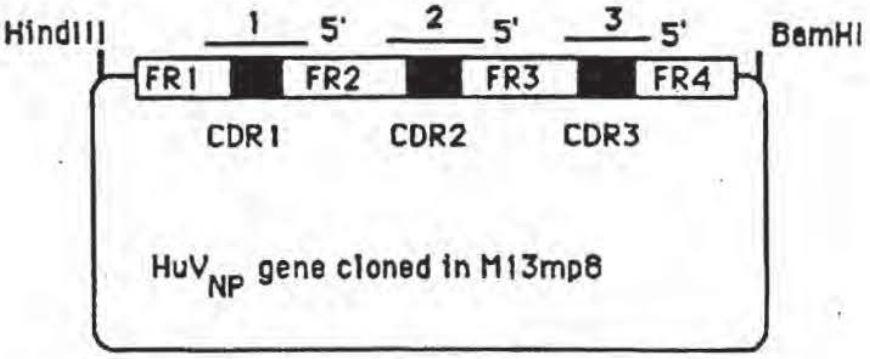
Fig. 5



D1.3 CDR1 oligonucleotide
5' CTG,TCT,CAC,CCA,GTT,TAC,ACC,ATA,GCC,GCT,GAA,GGT,GCT

FR2 D1.3 CDR1 FR1

D1.3 CDR2 oligonucleotide
5' CAT,TGT,CAC,TCT,GGA,TTT,GAG,AGC,TGA,ATT,ATA,GTC,TGT,

FR3 D1.3 CDR2

GTT,TCC,ATC,ACC,CCA,AAT,CAT,TCC,AAT,CCA,CTC

D1.3 CDR2 FR2

D1.3 CDR3 oligonucleotide
5' GCC,TTG,ACC,CCA,GTA,GTC,AAG,CCT,ATA,ATC,TCT,CTC,TCT,

FR4 D1.3 CDR3

TGC,ACA,ATA

FR3

Fig. 6.

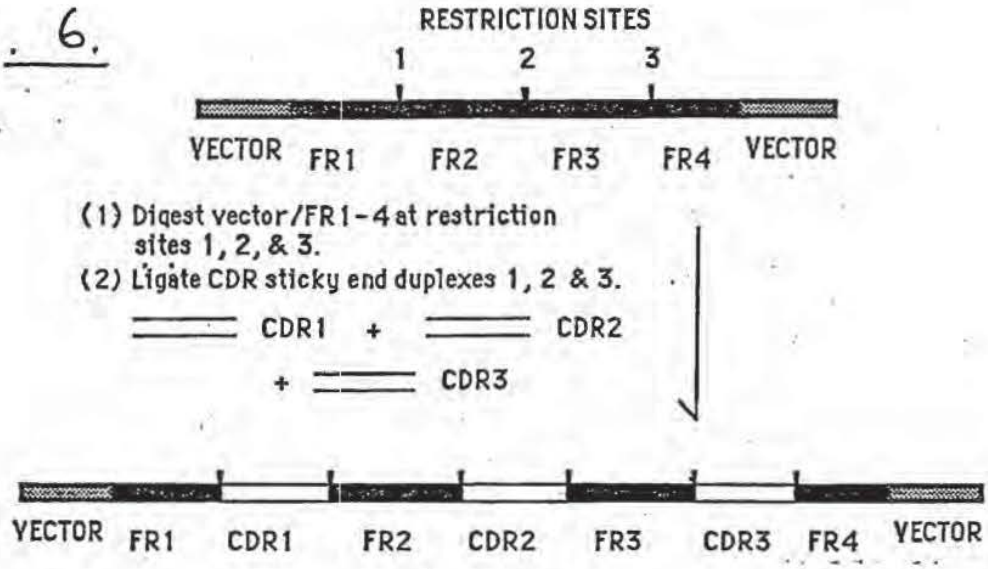


Fig. 7.

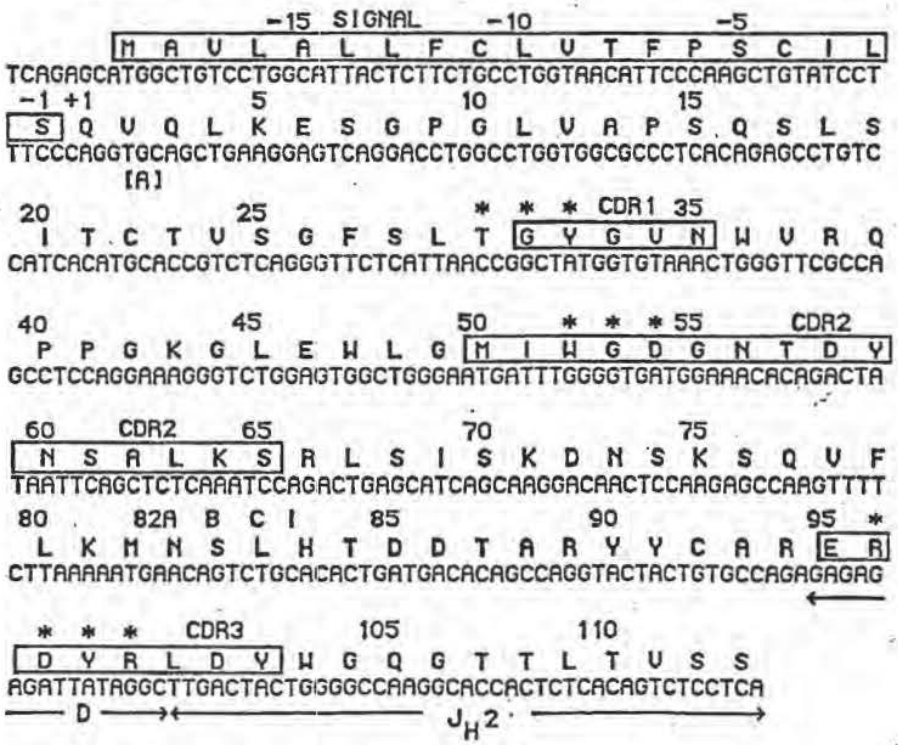


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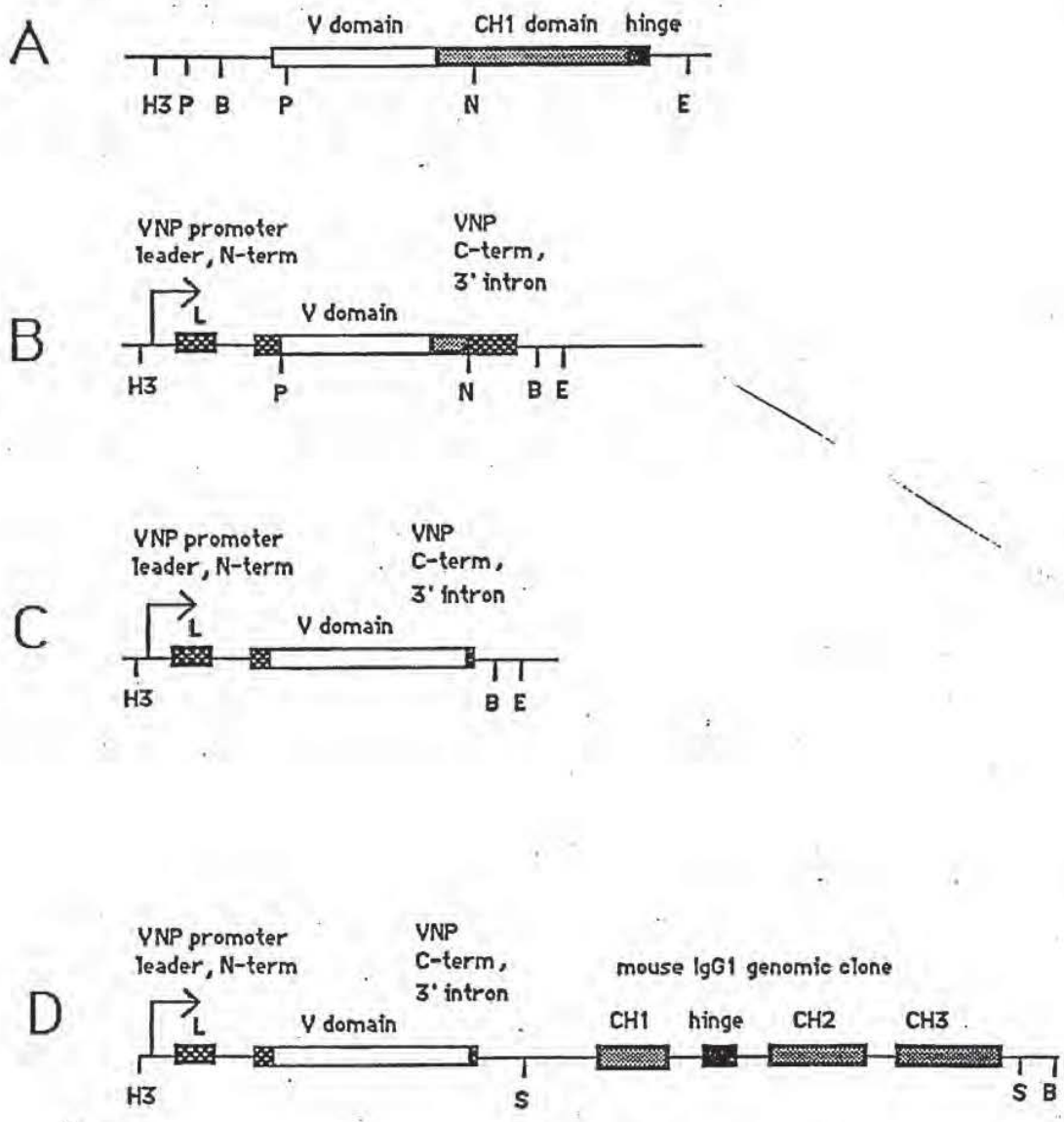
Fig. 8.



H3 = HindIII, P = PstI, B = BamHI, N = NcoI, E = EcoRI, H2 = HindII

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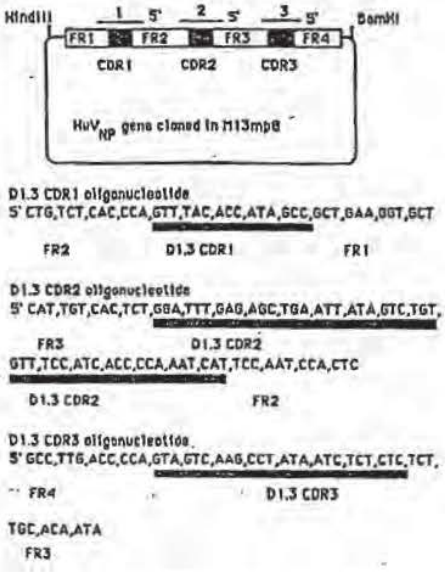
(71) Applicant: **Winter, Gregory Paul, 64 Cavendish Avenue, Cambridge (GB)**

(72) Inventor: **Winter, Gregory Paul, 64 Cavendish Avenue, Cambridge (GB)**

(74) Representative: **Matthews, Heather Clare et al, Keith W Nash & Co Pearl Assurance House 90-92 Regent Street, Cambridge CB2 1DP (GB)**

(54) **Recombinant antibodies and methods for their production.**

(57) An altered antibody is produced by replacing the complementarity determining regions (CDRs) of a variable region of an immunoglobulin (Ig) with the CDRs from an Ig of different specificity, using recombinant DNA techniques. The gene coding sequences for producing the altered antibody may be produced by site-directed mutagenesis using long oligonucleotides.



EP 0 239 400 A3

European Patent Office

EUROPEAN SEARCH REPORT

0239400
Application Number

EP 87 30 2620

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 82, April 1985, pages 2125-2127; P.P. CHEN et al.: "Possible involvement of human D minigenes in the first complementarity-determining region of K light chains" * Whole article * ---	1-10	C 12 N 15/00 C 07 K 15/06 C 12 P 21/02
X,P	NATURE, vol. 321, 29th May 1986, pages 522-525; P.T. JONES et al.: "Replacing the complementarity-determining regions in a human antibody with those from a mouse" * Whole article * ---	1-10	
T	SCIENCE, vol. 239, 25th March 1988, pages 1534-1536; M. VERHOEYEN et al.: "Reshaping human antibodies: grafting an antilysozyme activity" * Whole article * -----	1-10	

TECHNICAL FIELDS SEARCHED (Int. Cl.4)

C 12 N
C 12 P

The present search report has been drawn up for all claims

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THE HAGUE	26-01-1989	CUPIDO M.

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(71) Applicant: PROTEIN DESIGN LABS, INC. [US/US]; 3181 Porter Drive, Palo Alto, CA 94304 (US).

(72) Inventors: QUEEN, Cary, L. ; 1300 Oak Creek Drive, Palo Alto, CA 94304 (US). SELICK, Harold, Edwin ; 1673 Sunnyslope Avenue, Belmont, CA 94002 (US).

(74) Agent: SMITH, William, M.; Townsend and Townsend, One Market Plaza, 2000 Steuart Tower, San Francisco, CA 94105 (US).

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(54) Title: CHIMERIC IMMUNOGLOBULINS SPECIFIC FOR p55 TAC PROTEIN OF THE IL-2 RECEPTOR

(57) Abstract

Novel methods for designing humanized immunoglobulins having one or more complementary determining regions (CDR's) from a donor immunoglobulin and a framework region from a human immunoglobulin comprising first comparing the framework or variable region amino acid sequence of the donor immunoglobulin to corresponding sequences in a collection of human immunoglobulin chains, and selecting as the human immunoglobulin one of the more homologous sequences from the collection. Each humanized immunoglobulin chain may comprise about 3 or more amino acids from the donor immunoglobulin in addition to the CDR's, usually at least one of which is immediately adjacent to a CDR in the donor immunoglobulin. The heavy and light chains may each be designed by using any one or all three additional position criteria. When combined into an intact antibody, the humanized immunoglobulins of the present invention will be substantially non-immunogenic in humans and retain substantially the same affinity as the donor immunoglobulin to the antigen, such as a protein or other compound containing an epitope.

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CHIMERIC IMMUNOGLOBULINS SPECIFIC FOR p55 TAC PROTEIN OF THE IL-2 RECEPTOR

Field of the Invention

The present invention relates generally to the combination of recombinant DNA and monoclonal antibody technologies for developing novel therapeutic agents and, more particularly, to the production of non-immunogenic antibodies and their uses.

Background of the Invention

In mammals, the immune response is mediated by two types of cells that interact specifically with foreign material, i.e., antigens. One of these cell types, B-cells, are responsible for the production of antibodies. The second cell class, T-cells, include a wide variety of cellular subsets controlling the in vivo function of both B-cells and a wide variety of other hematopoietic cells, including T-cells.

One way in which T-cells exert this control is through the production of a lymphokine known as interleukin-2 (IL-2), originally named T-cell growth factor. IL-2's prime function appears to be the stimulation and maintenance of T-cells. Indeed, some immunologists believe that IL-2 may be at the center of the entire immune response (see, Farrar, J., et al., Immunol. Rev. 63:129-166 (1982), which is incorporated herein by reference).

To exert its biological effects, IL-2 interacts with a specific high-affinity membrane receptor (Greene, W., et al., Progress in Hematology XIV, E. Brown, Ed., Grune and Statton, New York (1986), at pgs. 283 ff). The human IL-2 receptor is a complex multichain glycoprotein, with one chain, known as the Tac peptide, being about 55kD in size (see, Leonard, W., et al., J. Biol. Chem. 260:1872 (1985), which is incorporated herein by reference). A gene encoding this protein has been isolated, and predicts a 272 amino acid peptide, including a 21 amino acid signal peptide (see, Leonard, W., et al., Nature 311: 626 (1984)). The 219 NH_2-

terminal amino acids of the p55 Tac protein apparently comprise an extracellular domain (see, Leonard, W., et al., Science, 230:633-639 (1985), which is incorporated herein by reference).

Much of the elucidation of the human IL-2 receptor's structure and function is due to the development of specifically reactive monoclonal antibodies. In particular, one mouse monoclonal antibody, known as anti-Tac (Uchiyama, et al., J. Immunol. 126:1393 (1981)) has shown that IL-2 receptors can be detected on T-cells, but also on cells of the monocyte-macrophage family, Kupffer cells of the liver, Langerhans' cells of the skin and, of course, activated T-cells. Importantly, resting T-cells, B-cells or circulating machrophages typically do not display the IL-2 receptor (Herrmann, et al., J. Exp. Med. 162:1111 (1985)).

The anti-Tac monoclonal antibody has also been used to define lymphocyte functions that require IL-2 interaction, and has been shown to inhibit various T-cell functions, including the generation of cytotoxic and suppressor T lymphocytes in cell culture. Also, based on studies with anti-Tac and other antibodies, a variety of disorders are now associated with improper IL-2 receptor expression by T-cells, in particular adult T-cell leukemia.

More recently, the IL-2 receptor has been shown to be an ideal target for novel therapeutic approaches to T-cell mediated diseases. It has been proposed that IL-2 receptor specific antibodies, such as the anti-Tac monoclonal antibody, can be used either alone or as an immunoconjugate (e.g., with Ricin A, isotopes and the like) to effectively remove cells bearing the IL-2 receptor. These agents can, for example, theoretically eliminate IL-2 receptor-expressing leukemic cells, certain B-cells, or activated T-cells involved in a disease state, yet allow the retention of mature normal T-cells and their precursors to ensure the capability of mounting a normal T-cell immune response as needed. In general, most other T-cell specific agents can destroy essentially all peripheral T-cells, which limits the agents' therapeutic efficacy. Overall, the use of

appropriate monoclonal antibodies specific for the IL-2 receptor may have therapeutic utility in autoimmune diseases, organ transplantation and any unwanted response by activated T-cells. Indeed, clinical trials have been initiated using, e.g., anti-Tac antibodies (see, generally, Waldman, T., et al., Cancer Res. 45:625 (1985) and Waldman, T., Science 232:727-732 (1986), both of which are incorporated herein by reference).

Unfortunately, the use of the anti-Tac and other non-human monoclonal antibodies have certain drawbacks, particularly in repeated therapeutic regimens as explained below. Mouse monoclonal antibodies, for example, do not fix human complement well, and lack other important immunoglobulin functional characteristics when used in humans.

Perhaps more importantly, anti-Tac and other non-human monoclonal antibodies contain substantial stretches of amino acid sequences that will be immunogenic when injected into a human patient. Numerous studies have shown that, after injection of a foreign antibody, the immune response elicited by a patient against an antibody can be quite strong, essentially eliminating the antibody's therapeutic utility after an initial treatment. Moreover, as increasing numbers of different mouse or other antigenic (to humans) monoclonal antibodies can be expected to be developed to treat various diseases, after the first and second treatments with any different non-human antibodies, subsequent treatments even for unrelated therapies can be ineffective or even dangerous in themselves.

While the production of so-called "chimeric antibodies" (e.g., mouse variable regions joined to human constant regions) has proven somewhat successful, a significant immunogenicity problem remains. In general, the production of human immunoglobulins reactive with the human IL-2 receptor, as with many human antigens, has been extremely difficult using typical human monoclonal antibody production techniques. Similarly, utilizing recombinant DNA technology to produce so-called "humanized" antibodies (see,

e.g., EPO Publication No. 0239400), provides uncertain results, in part due to unpredictable binding affinities.

Thus, there is a need for improved forms of human-like immunoglobulins, such as those specific for the human IL-2 receptor, that are substantially non-immunogenic in humans, yet easily and economically produced in a manner suitable for therapeutic formulation and other uses. The present invention fulfills these and other needs.

Summary of the Invention

The present invention provides novel compositions useful, for example, in the treatment of T-cell mediated human disorders, the compositions containing human-like immunoglobulins specifically capable of blocking the binding of human IL-2 to its receptor and/or capable of binding to the p55 Tac protein on human IL-2 receptors. The immunoglobulins can have two pairs of light chain/heavy chain complexes, typically at least one pair having chains comprising mouse complementarity determining regions functionally joined to human framework region segments. For example, mouse complementarity determining regions, with or without additional naturally-associated mouse amino acid residues, can be used to produce human-like antibodies capable of binding to the human IL-2 receptor at affinity levels stronger than about 10^8 M^{-1}.

The immunoglobulins, including binding fragments and other derivatives thereof, of the present invention may be produced readily by a variety of recombinant DNA techniques, with ultimate expression in transfected cells, preferably immortalized eukaryotic cells, such as myeloma or hybridoma cells. Polynucleotides comprising a first sequence coding for human-like immunoglobulin framework regions and a second sequence set coding for the desired immunoglobulin complementarity determining regions can be produced synthetically or by combining appropriate cDNA and genomic DNA segments.

The human-like immunoglobulins may be utilized alone in substantially pure form, or complexed with a

cytotoxic agent, such as a radionuclide, a ribosomal inhibiting protein or a cytotoxic agent active at cell surfaces. All of these compounds will be particularly useful in treating T-cell mediated disorders. The human-like immunoglobulins or their complexes can be prepared in a pharmaceutically accepted dosage form, which will vary depending on the mode of administration.

The present invention also provides novel methods for designing human-like immunoglobulin chains having one or more complementarity determining regions (CDR's) from a donor immunoglobulin and a framework region from a human immunoglobulin, the preferred methods comprising first comparing the framework or variable region amino acid sequence of the donor immunoglobulin to corresponding sequences in a collection of human immunoglobulin chains, and selecting as the human immunoglobulin one of the more homologous sequences from the collection. The human immunoglobulin, or acceptor immunoglobulin, sequence is typically selected from a collection of at least 10 to 20 immunoglobulin chain sequences, and usually will have the highest homology to the donor immunoglobulin sequence of any sequence in the collection. The human immunoglobulin framework sequence will typically have about 65 to 70% homology or more to the donor immunoglobulin framework sequences. The donor immunoglobulin may be either a heavy chain or light chain (or both), and the human collection will contain the same kind of chain. A humanized light and heavy chain can be used to form a complete humanized immunoglobulin or antibody, having two light/heavy chain pairs, with or without partial or full-length human constant regions and other proteins.

In another embodiment of the present invention, either in conjunction with the above comparison step or separately, additional amino acids in an acceptor immunoglobulin chain may be replaced with amino acids form the CDR-donor immunoglobulin chain. More specifically, further optional substitutions of a human framework amino acid of the acceptor immunoglobulin with a corresponding

amino acid from a donor immunoglobulin will be made at positions in the immunoglobulins where:

(a) the amino acid in the human framework region of an acceptor immunoglobulin is rare for that position and the corresponding amino acid in the donor immunoglobulin is common for that position in human immunoglobulin sequences; or

(b) the amino acid is immediately adjacent to one of the CDR's; or

(c) the amino acid is predicted to be within about 3Å of the CDR's in a three-dimensional immunoglobulin model and capable of interacting with the antigen or with the CDR's of the humanized immunoglobulin.

The humanized immunoglobulin chain will typically comprise at least about 3 amino acids from the donor immunoglobulin in addition to the CDR's, usually at least one of which is immediately adjacent to a CDR in the donor immunoglobulin. The heavy and light chains may each be designed by using any one or all three of the position criteria.

When combined into an intact antibody, the humanized light and heavy chains of the present invention will be substantially non-immunogenic in humans and retain substantially the same affinity as the donor immunoglobulin to the antigen (such as a protein or other compound containing an epitope). These affinity levels can vary from about 10^8 M^{-1} or higher, and may be within about 4 fold of the donor immunoglobulin's original affinity to the antigen.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Comparison of sequences of anti-Tac heavy chain (upper lines) and Eu heavy chain (lower lines). The 1-letter code for amino acids is used. The first amino acid on each line is numbered at the left. Identical amino acids in the two sequences are connected by lines. The 3 CDRs are underlined. Other amino acid positions for which the anti-Tac amino acid rather than the Eu amino acid was used in the humanized anti-Tac heavy chain are denoted by an *.

Figure 2. Comparison of sequences of anti-Tac light chain (upper lines) and Eu light chain (lower lines). The single-letter code for amino acids is used. The first amino acid on each line is numbered at the left. Identical amino acids in the two sequences are connected by lines. The 3 CDRs are underlined. Other amino acid positions for which the anti-Tac amino acid rather than the Eu amino acid was used in the humanized anti-Tac heavy chain are denoted by an *.

Figure 3. Nucleotide sequence of the gene for the humanized anti-Tac heavy chain variable region gene. The translated amino acid sequence for the part of the gene encoding protein is shown underneath the nucleotide sequence. The nucleotides TCTAGA at the beginning and end of the gene are Xba I sites. The mature heavy chain sequence begins with amino acid #20 Q.

Figure 4. Nucleotide sequence of the gene for the humanized anti-Tac light chain variable region gene. The translated amino acid sequence for the part of the gene encoding protein is shown underneath the nucleotide sequence. The nucleotides TCTAGA at the beginning and end of the gene are Xba I sites. The mature light chain sequence begins with amino acid #21 D.

Figure 5. A. Sequences of the four oligonucleotides used to synthesize the humanized anti-Tac heavy chain gene, printed 5' to 3'. B. Relative positions of the oligonucleotides. The arrows point in the 3' direction for each oligonucleotide.

Figure 6. (A) Sequences of the four oligonucleotides used to synthesize the humanized anti-Tac light chain gene, printed 5' to 3'. (B) Relative positions of the oligonucleotides. The arrows point in the 3' direction for each oligonucleotide. The position of a Hind III site in the overlap of JFD2 and JFD3 is shown.

Figure 7. Schematic diagram of the plasmid pHuGTAC1 used to express the humanized anti-Tac heavy chain. Relevant restriction sites are shown, and coding regions of the heavy chain are displayed as boxes. The direction of transcription from the immunoglobulin (Ig) promoter is shown by an arrow. E_H = heavy chain enhancer, Hyg = hygromycin resistance gene.

Figure 8. Schematic diagram of the plasmid pHuLTAC used to express the humanized anti-Tac light chain. Relevant restriction sites are shown, and coding regions of the light chain are displayed as boxes. The direction of transcription from the Ig promoter is shown by an arrow.

Figure 9. Fluorocytometry of HUT-102 and Jurkat cells stained with anti-Tac antibody or humanized anti-Tac antibody followed respectively by fluorescein-conjugated goat anti-mouse Ig antibody or goat anti-human Ig antibody, as labeled. In each panel, the dotted curve shows the results when the first antibody was omitted, and the solid curve the results when first and second (conjugated) antibodies were included as described.

Figure 10. (A) Fluorocytometry of HUT-102 cells stained with 0-40 ng of anti-Tac as indicated, then with biotinylated anti-Tac, and then with phycoerythrin-conjugated avidin. (B) Fluorocytometry of HUT-102 cells stained with the indicated antibody, then with biotinylated anti-Tac, and then with phycoerythrin-conjugated avidin.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with one embodiment of the present invention, human-like immunoglobulins specifically reactive with desired epitopes, such as those on the IL-2 receptor on human T-cells, are provided. These immunoglobulins, which have binding affinities of at least about 10^8 M^{-1}, and preferably 10^9 M^{-1} to 10^{10} M^{-1} or stronger, are capable of, e.g., blocking the binding of IL-2 to human IL-2 receptors. The human-like immunoglobulins will have a human-like framework and can have complementarity determining regions (CDR's) from an immunoglobulin, typically a mouse immunoglobulin, specifically reactive with an epitope on p55 Tac protein. The immunoglobulins of the present invention, which can be produced economically in large quantities, find use, for example, in the treatment of T-cell mediated disorders in human patients by a variety of techniques.

The basic antibody structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25kD) and one "heavy" chain (about 50-70kD). The NH_2-terminus of each chain begins a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The COOH terminus of each chain defines a constant region primarily responsible for effector function.

Light chains are classified as either kappa or lambda. Heavy chains are classified (and subclassified) as gamma, mu, alpha, delta, or epsilon, and define the antibody's isotype as IgG, IgM, IgA, IgD and IgE, respectively. Within light and heavy chains, the variable and constant regions are joined by a "J" region of about 12 or more amino acids, with the heavy chain also including a "D" region of about 12 more amino acids. (See, generally, Fundamental Immunology, Paul, W., Ed., Chapter 7, pgs. 131-166, Raven Press, N.Y. (1984), which is incorporated herein by reference.)

The variable regions of each light/heavy chain pair form the antibody binding site. The chains all exhibit the

same general structure of relatively conserved framework regions joined by three hypervariable regions, also called CDR's (see, "Sequences of Proteins of Immunological Interest," Kabat, E., et al., U.S. Department of Health and Human Services, (1983); and Cholthia and Lesk, J. Mol. Biol., 196:901-917 (1987), which are incorporated herein by reference). The CDR's from the two chains of each pair are aligned by the framework regions, enabling binding to a specific epitope.

As used herein, the term "immunoglobulin" refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. The immunoglobulins may exist in a variety of forms besides antibodies; including, for example, Fv, Fab, and $F(ab)_2$, as well as in single chains (e.g., Huston, et al., Proc. Nat. Acad. Sci. U.S.A., 85:5879-5883 (1988) and Bird, et al., Science, 242:423-426 (1988), which are incorporated herein by reference). (See, generally, Hood, et al., "Immunology", Benjamin, N.Y., 2nd ed. (1984), and Hunkapiller and Hood, Nature, 323:15-16 (1986), which are incorporated herein by reference).

Chimeric antibodies are antibodies whose light and heavy chain genes have been constructed, typically by genetic engineering, from immunoglobulin gene segments belonging to different species. For example, the variable (V) segments of the genes from a mouse monoclonal antibody may be joined to human constant (C) segments, such as γ_1 and γ_3. A typical therapeutic chimeric antibody is thus a hybrid protein consisting of the V or antigen-binding domain from a mouse antibody and the C or effector domain from a human antibody (e.g., A.T.C.C. Accession No. CRL 9688 secretes an anti-Tac chimeric antibody), although other mammalian species may be used.

As used herein, the term "framework region" refers to those portions of immunoglobulin light and heavy chain

variable regions that are relatively conserved (i.e., other than the CDR's) among different immunoglobulins in a single species, as defined by Kabat, et al., op. cit. As used herein, a "human-like framework region" is a framework region that in each existing chain comprises at least about 70 or more amino acid residues, typically 75 to 85 or more residues, identical to those in a human immunoglobulin.

As used herein, the term "human-like immunoglobulin" refers to an immunoglobulin comprising a human-like framework and in which any constant region present is substantially homologous to a human immunoglobulin constant region, i.e., at least about 85-90%, preferably about 95% identical. Hence, all parts of a human-like immunoglobulin, except possibly the CDR's, are substantially homologous to corresponding parts of one or more native human immunoglobulin sequences. For example, a human-like immunoglobulin would not encompass a chimeric mouse variable region/human constant region antibody.

In accordance with another general aspect of the present invention, also included are criteria by which a limited number of amino acids in the framework of a human-like or humanized immunoglobulin chain are chosen to be the same as the amino acids at those positions in the donor Ig rather than in the acceptor Ig, in order to increase the affinity of an antibody comprising the humanized immunoglobulin chain.

This aspect of the present invention is based in part on the model that two contributing causes of the loss of affinity in prior means of producing humanized antibodies (using as examples mouse antibodies as the source of CDR's) are:

(1) When the mouse CDR's are combined with the human framework, the amino acids in the framework close to the CDR's become human instead of mouse. Without intending to be bound by theory, we believe that these changed amino acids may slightly distort the CDR's, because they create different electrostatic or hydrophobic forces than in the donor mouse antibody, and the distorted CDR's may not make as

effective contacts with the antigen as the CDR's did in the donor antibody;

(2) Also, amino acids in the original mouse antibody that are close to, but not part of, the CDR's (i.e., still part of the framework), may make contacts with the antigen that contribute to affinity. These amino acids are lost when the antibody is humanized, because all framework amino acids are made human.

To avoid these problems, and to produce humanized antibodies that have a very strong affinity for a desired antigen, the present invention uses the following four criteria for designing humanized immunoglobulins. These criteria may be used singly, or when necessary in combination, to achieve the desired affinity or other characteristics.

Criterion I: As acceptor, use a framework from a particular human immunoglobulin that is unusually homologous to the donor immunoglobulin to be humanized, or use a consensus framework from many human antibodies. 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. Hence, and again without intending to be bound by theory, it is believed that there is a smaller chance of changing an amino acid near the CDR's that distorts their conformation. Moreover, the precise overall shape of a humanized antibody comprising the humanized immunoglobulin chain may more closely resemble the

shape of the donor antibody, also reducing the chance of distorting the CDR's.

Typically, one of the 3-5 most homologous heavy chain variable region sequences in a representative collection of at least about 10 to 20 distinct human heavy chains will be chosen as acceptor to provide the heavy chain framework, and similarly for the light chain. Preferably, one of the 1-3 most homologous variable regions will be used. The selected acceptor immunoglobulin chain will most preferably have at least about 65% homology in the framework region to the donor immunoglobulin.

Regardless of how the acceptor immunoglobulin is chosen, higher affinity may be achieved by selecting a small number of amino acids in the framework of the humanized immunoglobulin chain to be the same as the amino acids at those positions in the donor rather than in the acceptor. The following criteria define what amino acids may be so selected. Preferably, at most or all amino acid positions satisfying one of these criteria, the donor amino acid will in fact be selected.

Criterion II: If an amino acid in the framework of the human acceptor immunoglobulin is unusual (i.e., "rare", which as used herein indicates an amino acid occurring at that position in no more than about 10% of human heavy (respectively light) chain V region sequences in a representative data bank), and if the donor amino acid at that position is typical for human sequences (i.e., "common", which as used herein indicates an amino acid occurring in at least about 25% of sequences in a representative data bank), then the donor amino acid rather than the acceptor may be selected. This criterion helps ensure that an atypical amino acid in the human framework does not disrupt the antibody structure. Moreover, by replacing an unusual amino acid with an amino acid from the donor antibody that happens to be typical for human antibodies, the humanized antibody may be made less immunogenic.

Criterion III: In the positions immediately adjacent to the 3 CDR's in the humanized immunoglobulin chain, the donor amino acid rather than acceptor amino acid may be selected. These amino acids are particularly likely to interact with the amino acids in the CDR's and, if chosen from the acceptor, distort the donor CDR's and reduce affinity. Moreover, the adjacent amino acids may interact directly with the antigen (Amit et al., Science, 233, 747-753 (1986), which is incorporated herein by reference) and selecting these amino acids from the donor may be desirable to keep all the antigen contacts that provide affinity in the original antibody.

Criterion IV: A 3-dimensional model, typically of the original donor antibody, shows that certain amino acids outside of the CDR's are close to the CDR's and have a good probability of interacting with amino acids in the CDR's by hydrogen bonding, Van der Waals forces, hydrophobic interactions, etc. At those amino acid positions, the donor amino acid rather than the acceptor immunoglobulin amino acid may be selected. Amino acids according to this criterion will generally have a side chain atom within about 3 angstrom units of some site in the CDR's and must contain atoms that could interact with the CDR atoms according to established chemical forces, such as those listed above. Computer programs to create models of proteins such as antibodies are generally available and well known to those skilled in the art (see, Loew et al., Int. J. Quant. Chem., Quant. Biol. Symp., 15:55-66 (1988); Bruccoleri et al., Nature, 335, 564-568 (1988); Chothia et al., Science, 233:755-758 (1986), all of which are incorporated herein by reference). These do not form part of the invention. Indeed, because all antibodies have similar structures, the known antibody structures, which are available from the Brookhaven Protein Data Bank, can be used if necessary as rough models of other antibodies. Commercially available computer programs can be used to display these models on a computer monitor, to calculate the distance between atoms, and to estimate the

likelihood of different amino acids interacting (see, Ferrin et al., J. Mol. Graphics, 6:13-27 (1988)).

Humanized or human-like antibodies generally have at least three potential advantages over mouse or in some cases chimeric antibodies for use in human therapy:

1) Because the effector portion is human, it may interact better with the other parts of the human immune system (e.g., destroy the target cells more efficiently by complement- dependent cytotoxicity (CDC) or antibody-dependent cellular cytotoxicity (ADCC)).

2) The human immune system should not recognize the framework or constant region of the humanized antibody as foreign, and therefore the antibody response against such an injected antibody should be less than against a totally foreign mouse antibody or a partially foreign chimeric antibody.

3) Injected mouse antibodies have been reported to have a half-life in the human circulation much shorter than the half-life of normal antibodies (D. Shaw et al., J. Immunol., 138:4534-4538 (1987)). Injected humanized antibodies will presumably have a half-life more similar to naturally occurring human antibodies, allowing smaller and less frequent doses to be given.

The present invention is specifically directed to improved humanized immmunoglobulins (e.g., capable of binding the human IL-2 receptor) with respect to those described in EPA publication no. 0239400. That application, the disclosure of which is excluded from coverage herein, describes, for certain immunoglobulins, substituting CDR's regions in the light or heavy chain variable domains of an acceptor antibody with analogous parts of CDR's (typically solvent accessible) from an antibody of different specificity. Also, that application discusses, for certain immunoglobulins, the possibility of only transferring residues that are (solvent) accessible from the antigen binding site, which residues apparently may include certain framework regions (specifically, residues known to be involved in antigen binding as described in Amit et al.,

Science 233: 747-753 (1986) or perhaps residues essential for inter-chain interactions - but for the selection of which insufficient guidance is provided in that application). Thus, for example, a preferred embodiment of the present invention entails substituting entire CDR's and framework amino acids immediately adjacent one (or preferably each) of the CDR's. In general, any framework residue that also makes contact with the CDR's to, e.g., maintain their conformation (and usually their antigen binding specificity) are specifically included within preferred embodiments of the present invention as described in detail, supra.

In one aspect, the present invention is directed to recombinant DNA segments encoding the heavy and/or light chain CDR's (typically with other amino acid residues as described above) from an immunoglobulin capable of binding to a desired epitope, such as on the human IL-2 receptor (e.g., the anti-Tac monoclonal antibody). The DNA segments encoding these regions will typically be joined to DNA segments encoding appropriate human-like framework regions. For example, the preferred DNA sequences, which on expression code for the polypeptide chains comprising the anti-Tac heavy and light chain hypervariable regions (with human-like framework regions), are shown in Figures 3 and 4, respectively. Due to codon degeneracy and non-critical amino-acid substitutions, other DNA sequences can be readily substituted for those sequences, as detailed below.

The DNA segments will typically further include an expression control DNA sequence operably linked to the human-like antibody coding sequences, including naturally-associated or heterologous promoter regions. Preferably, the expression control sequences will be eukaryotic promoter systems in vectors capable of transforming or transfecting eukaryotic host cells, but control sequences for prokaryotic hosts may also be used. Once the vector has been incorporated into the appropriate host, the host is maintained under conditions suitable for high level expression of the nucleotide sequences, and, as desired, the collection and purification of the light chains, heavy

chains, light/heavy chain dimers or intact antibodies, binding fragments or other immunoglobulin forms may follow.

Human constant region DNA sequences can be isolated in accordance with well known procedures from a variety of human cells, but preferably immortalized B-cells (see, Kabat op. cit. and WP87/02671). For example, the human kappa immunoglobulin constant and J region genes and sequences are described in Heiter et al., Cell 22:197-207 (1980) and the nucleotide sequence of a human immunoglobulin $C_{\gamma 1}$ gene is described in Ellison et al., Nucl. Acid. Res. 10:4071 (1982), both of which are incorporated herein by reference. The CDR's for producing the immunoglobulins of the present invention will be similarly derived from monoclonal antibodies capable of binding to the desired antigen (e.g., the human IL-2 receptor) and produced in any convenient mammalian source, including, mice, rats, rabbits, or other veterbrate capable of producing antibodies by well known methods. Suitable source cells for the DNA sequences and host cells for immunoglobulin expression and secretion can be obtained from a number of sources, such as the American Type Culture Collection ("Catalogue of Cell Lines and Hybridomas," Fifth edition (1985) Rockville, Maryland, U.S.A., which is incorporated herein by reference).

In addition to the human-like immunoglobulins specifically described herein, other "substantially homologous" modified immunoglobulins can be readily designed and manufactured utilizing various recombinant DNA techniques well known to those skilled in the art. For example, for the IL-2 receptor immunoglobulins the framework regions can vary from the sequences in Figures 3 and 4 at the primary structure level by several amino acid substitutions, terminal and intermediate additions and deletions, and the like. Moreover, a variety of different human framework regions may be used singly or in combination as a basis for the human-like immunoglobulins of the present invention. In general, modifications of the genes may be readily accomplished by a variety of well-known techniques, such as site-directed mutagenesis (see, Gillman and Smith, Gene 8:81-97 (1979) and

Roberts, S. et al, Nature 328:731-734 (1987), both of which are incorporated herein by reference).

Alternatively, polypeptide fragments comprising only a portion of the primary antibody structure may be produced, which fragments possess one or more immunoglobulin activities (e.g., complement fixation activity). Also because like many genes, the immunoglobulin-related genes contain separate functional regions, each having one or more distinct biological activities, the genes may be fused to functional regions from other genes (e.g., enzymes, see, commonly assigned U.S.S.N. 132,387, filed Dec. 15, 1987, which is incorporated herein by reference) to produce fusion proteins (e.g., immunotoxins) having novel properties.

The nucleic acid sequences of the present invention capable of ultimately expressing the desired human-like antibodies can be formed from a variety of different polynucleotides (genomic or cDNA, RNA, synthetic oligonucleotides, etc.) and components (e.g., V, J, D, and C regions), as well as by a variety of different techniques. Joining appropriate genomic sequences is presently the most common method of production, but cDNA sequences may also be utilized (see, European Patent Publication No. 0239400 and Reichmann, L., et al., Nature 332:323-327 (1988), both of which are incorporated herein by reference).

As stated previously, the DNA sequences will be expressed in hosts after the sequences have been operably linked to (i.e., positioned to ensure the functioning of) an expression control sequence. These expression vectors are typically replicable in the host organisms either as episomes or as an integral part of the host chromosomal DNA. Commonly, expression vectors will contain selection markers, e.g., tetracycline or neomycin, to permit detection of those cells transformed with the desired DNA sequences (see, e.g., U.S. Patent 4,704,362, which is incorporated herein by reference).

E. coli is one prokaryotic host useful particularly for cloning the DNA sequences of the present invention. Other microbial hosts suitable for use include bacilli, such

as Bacillus subtilus, and other enterobacteriaceae, such as Salmonella, Serratia, and various Pseudomonas species. In these prokaryotic hosts, one can also make expression vectors, which will typically contain expression control sequences compatible with the host cell (e.g., an origin of replication). In addition, any number of a variety of well-known promoters will be present, such as the lactose promoter system, a tryptophan (trp) promoter system, a beta-lactamase promoter system, or a promoter system from phage lambda. The promoters will typically control expression, optionally with an operator sequence, and have ribosome binding site sequences and the like, for initiating and completing transcription and translation.

Other microbes, such as yeast, may also be used for expression. Saccharomyces is a preferred host, with suitable vectors having expression control sequences, such as promoters, including 3-phosphoglycerate kinase or other glycolytic enzymes, and an origin of replication, termination sequences and the like as desired.

In addition to microorganisms, mammalian tissue cell culture may also be used to express and produce the polypeptides of the present invention (see, Winnacker, "From Genes to Clones," VCH Publishers, N.Y., N.Y. (1987), which is incorporated herein by reference). Eukaryotic cells are actually preferred, because a number of suitable host cell lines capable of secreting intact immunoglobulins have been developed in the art, and include the CHO cell lines, various COS cell lines, HeLa cells, myeloma cell lines, etc, but preferably transformed B-cells or hybridomas. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, an enhancer (Queen, C., et al., Immunol. Rev. 89:49-68 (1986), which is incorporated herein by reference), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferred expression control sequences are promoters derived from SV40 with enhancer (see, Mulligan

and Berg, Science 209:1422-1427 (1980), an immunglobulin gene, Adenovirus, Bovine Papilloma Virus, and the like.

The vectors containing the DNA segments of interest (e.g., the heavy and light chain encoding sequences and expression control sequences) can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment or electroporation may be used for other cellular hosts. (See, generally, Maniatis, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, (1982), which is incorporated herein by reference.)

Once expressed, the whole antibodies, their dimers, individual light and heavy chains, or other immunoglobulin forms of the present invention can be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like (see, generally, Scopes, R., Protein Purification, Springer-Verlag, N.Y. (1982)). Substantially pure immunoglobulins of at least about 90 to 95% homogeneity are preferred, and 98 to 99% or more homogeneity most preferred, for pharmaceutical uses. Once purified, partially or to homogeneity as desired, the polypeptides may then be used therapeutically (including extracorporeally) or in developing and performing assay procedures, immunofluorescent stainings, and the like. (See, generally, Immunological Methods, Vols. I and II, Lefkovits and Pernis, eds., Academic Press, New York, N.Y. (1979 and 1981)).

The IL-2 receptor specific antibodies exemplified in the present invention will typically find use individually in treating a T-cell mediated disease state. Generally, where the cell linked to a disease has been identified as IL-2 receptor bearing, then the human-like antibodies capable of blocking the binding of IL-2 to the human IL-2 receptor are suitable (see, U.S.S.N. 085,707, entitled "Treating Human Malignancies and Disorders," which is incorporated herein by

reference). For example, typical disease states suitable for treatment include graft versus host disease and transplant rejection in patients undergoing an organ transplant, such as heart, lungs, kidneys, liver, etc. Other diseases include autoimmune diseases, such as Type I diabetes, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, and myasthenia gravis.

The human-like antibodies of the present invention may also be used in combination with other antibodies, particularly human monoclonal antibodies reactive with other markers on cells responsible for the disease. For example, suitable T-cell markers can include those grouped into the so-called "Clusters of Differentiation," as named by the First International Leukocyte Differentiation Workshop, Leukocyte Typing, Bernard, et al., Eds., Springer-Verlag, N.Y. (1984), which is incorporated herein by reference.

The antibodies can also be used as separately administered compositions given in conjunction with chemotherapeutic or immunosuppressive agents. Typically, the agents will include cyclosporin A or a purine analog (e.g., methotrexate, 6-mercaptopurine, or the like), but numerous additional agents (e.g., cyclophosphamide, prednisone, etc.) well-known to those skilled in the art may also be utilized.

A preferred pharmaceutical composition of the present invention comprises the use of the subject antibodies in immunotoxins. Immunotoxins are characterized by two components and are particularly useful for killing selected cells in vitro or in vivo. One component is a cytotoxic agent which is usually fatal to a cell when attached or absorbed. The second component, known as the "delivery vehicle," provides a means for delivering the toxic agent to a particular cell type, such as cells comprising a carcinoma. The two components are commonly chemically bonded together by any of a variety of well-known chemical procedures. For example, when the cytotoxic agent is a protein and the second component is an intact immunoglobulin, the linkage may be by way of heterobifunctional cross-linkers, e.g., SPDP, carbodiimide, glutaraldehyde, or the like. Production of

various immunotoxins is well-known with the art, and can be found, for example in "Monoclonal Antibody-Toxin Conjugates: Aiming the Magic Bullet," Thorpe et al, Monoclonal Antibodies in Clinical Medicine, Academic Press, pp. 168-190 (1982), which is incorporated herein by reference.

A variety of cytotoxic agents are suitable for use in immunotoxins. Cytotoxic agents can include radionuclides, such as Iodine-131, Yttrium-90, Rhenium-188, and Bismuth-212; a number of chemotherapeutic drugs, such as vindesine, methotrexate, adriamycin, and cisplatinm; and cytotoxic proteins such as ribosomal inhibiting proteins like pokeweed antiviral protein, Pseudomonas exotoxin A, ricin, diphtheria toxin, ricin A chain, etc., or an agent active at the cell surface, such as the phospholipase enzymes (e.g., phospholipase C). (See, generally, commonly assigned U.S.S.N. 07/290,968 filed December 28, 1988), "Chimeric Toxins," Olsnes and Phil, Pharmac. Ther., 25:355-381 (1982), and "Monoclonal Antibodies for Cancer Detection and Therapy," eds. Baldwin and Byers, pp. 159-179, 224-266, Academic Press (1985), all of which are incorporated herein by reference.)

The delivery component of the immunotoxin will include the human-like immunoglobulins of the present invention. Intact immunoglobulins or their binding fragments, such as Fab, are preferably used. Typically, the antibodies in the immunotoxins will be of the human IgM or IgG isotype, but other mammalian constant regions may be utilized as desired.

The human-like antibodies and pharmaceutical compositions thereof of this invention are particularly useful for parenteral administration, i.e., subcutaneously, intramuscularly or intravenously. The compositions for parenteral administration will commonly comprise a solution of the antibody or a cocktail thereof dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, e.g., water, buffered water, 0.4% saline, 0.3% glycine and the like. These solutions are sterile and generally free of particulate matter. These compositions may be sterilized by conventional, well known

sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate, etc. The concentration of antibody in these formulations can vary widely, i.e., from less than about 0.5%, usually at or at least about 1% to as much as 15 or 20% by weight and will be selected primarily based on fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

Thus, a typical pharmaceutical composition for intramuscular injection could be made up to contain 1 ml sterile buffered water, and 50 mg of antibody. A typical composition for intravenous infusion could be made up to contain 250 ml of sterile Ringer's solution, and 150 mg of antibody. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in more detail in, for example, Remington's Pharmaceutical Science, 15th ed., Mack Publishing Company, Easton, Pennsylvania (1980), which is incorporated herein by reference.

The antibodies of this invention can be lyophilized for storage and reconstituted in a suitable carrier prior to use. This technique has been shown to be effective with conventional immune globulins and art-known lyophilization and reconstitution techniques can be employed. It will be appreciated by those skilled in the art that lyophilization and reconstitution can lead to varying degrees of antibody activity loss (e.g., with conventional immune globulins, IgM antibodies tend to have greater activity loss than IgG antibodies) and that use levels may have to be adjusted to compensate.

The compositions containing the present human-like antibodies or a cocktail thereof can be administered for prophylactic and/or therapeutic treatments. In therapeutic application, compositions are administered to a patient

already suffering from a disease, in an amount sufficient to cure or at least partially arrest the disease and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose." Amounts effective for this use will depend upon the severity of the infection and the general state of the patient's own immune system, but generally range from about 1 to about 200 mg of antibody per dose, with dosages of from 5 to 25 mg per patient being more commonly used. It must be kept in mind that the materials of this invention may generally be employed in serious disease states, that is life-threatening or potentially life-threatening situations. In such cases, in view of the minimization of extraneous substances and the lower probability of "foreign substance" rejections which are achieved by the present human-like antibodies of this invention, it is possible and may be felt desirable by the treating physician to administer substantial excesses of these antibodies.

In prophylactic applications, compositions containing the present antibodies or a cocktail thereof are administered to a patient not already in a disease state to enhance the patient's resistance. Such an amount is defined to be a "prophylactically effective dose." In this use, the precise amounts again depend upon the patient's state of health and general level of immunity, but generally range from 0.1 to 25 mg per dose, especially 0.5 to 2.5 mg per patient. A preferred prophylactic use is for the prevention of kidney transplant rejection.

Single or multiple administrations of the compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical formulations should provide a quantity of the antibody(ies) of this invention sufficient to effectively treat the patient.

Human-like antibodies of the present invention can further find a wide variety of utilities <u>in vitro</u>. By way of example, the exemplary antibodies can be utilized for T-cell typing, for isolating specific IL-2 receptor bearing cells or

fragments of the receptor, for vaccine preparation, or the like.

For diagnostic purposes, the antibodies may either be labeled or unlabeled. Unlabeled antibodies can be used in combination with other labeled antibodies (second antibodies) that are reactive with the human-like antibody, such as antibodies specific for human immunoglobulin constant regions. Alternatively, the antibodies can be directly labeled. A wide variety of labels may be employed, such as radionuclides, fluors, enzymes, enzyme substrates, enzyme cofactors, enzyme inhibitors, ligands (particularly haptens), etc. Numerous types of immunoassays are available and are well known to those skilled in the art.

Kits can also be supplied for use with the subject antibodies in the protection against or detection of a cellular activity or for the presence of a selected antigen. Thus, the subject antibody composition of the present invention may be provided, usually in a lyophilized form in a container, either alone or in conjunction with additional antibodies specific for the desired cell type. The antibodies, which may be conjugated to a label or toxin, or unconjugated, are included in the kits with buffers, such as Tris, phosphate, carbonate, etc., stabilizers, biocides, inert proteins, e.g., serum albumin, or the like, and a set of instructions for use. Generally, these materials will be present in less than about 5% wt. based on the amount of active antibody, and usually present in total amount of at least about 0.001% wt. based again on the antibody concentration. Frequently, it will be desirable to include an inert extender or excipient to dilute the active ingredients, where the excipient may be present in from about 1 to 99% wt. of the total composition. Where a second antibody capable of binding to the chimeric antibody is employed in an assay, this will usually be present in a separate vial. The second antibody is typically conjugated to a label and formulated in an analogous manner with the antibody formulations described above.

The following examples are offered by way of illustration, not by limitation.

EXPERIMENTAL

Design of genes for human-like light and heavy chains

The sequence of the human antibody Eu (Sequences of Proteins of Immunological Interest, Kabat, E., et al., U.S. Dept. of Health and Human Services, 1983) was used to provide the framework of the humanized antibody, because the amino acid sequence of the heavy chain of anti-Tac is more homologous to the heavy chain of this antibody than to any other heavy chain sequence in the National Biomedical Foundation Protein Identification Resource.

To select the sequence of the humanized heavy chain, the anti-Tac heavy chain sequence (see, commonly assigned U.S.S.N.'s 186,862 and 223,037, which are incorporated herein by reference) was aligned with the sequence of the Eu heavy chain (Figure 1). At each position, the Eu amino acid was selected for the humanized sequence, unless that position fell in any one of the following categories, in which case the anti-Tac amino acid was selected.

(1) The position fell within a complementarity determining region (CDR), as defined by Kabat, et al., op. cit. (amino acids 31-35, 50-66, 99-106);

(2) The Eu amino acid was unusual for human heavy chains at that position, whereas the anti-Tac amino acid was typical for human heavy chains at that position (amino acids 27, 93, 95, 98, 107-109, 111);

(3) The position was immediately adjacent to a CDR in the amino acid sequence of the anti-Tac heavy chain (amino acids 30 and 67).

(4) 3-dimensional modeling of the anti-Tac antibody suggested that the amino acid was physically close to the antigen binding region (amino acids 48 and 68).

Some amino acids fell in more than one of these categories but are only listed in one.

To select the sequence of the humanized light chain, the anti-Tac light chain sequence was aligned with the sequence of the Eu light chain (Figure 2). The Eu amino acid was selected at each position, unless the position again fell into one of the categories (1) - (4), (with light chain replacing heavy chain in the category definitions):

(1) CDRs (amino acids 24-34, 50-56, 89-97).

(2) Anti-Tac amino acid more typical than Eu (amino acids 48 and 63).

(3) Adjacent to CDRs (no amino acids; Eu and anti-Tac were already the same at all these positions).

(4) Possible 3-dimensional proximity to binding region (amino acid 60).

The actual nucleotide sequence of the heavy (Figure 3) and light chain (Figure 4) genes were selected as follows:

(1) the nucleotide sequences code for the amino acid sequences chosen as described above.

(2) 5' of these coding sequences, the nucleotide sequences code for a leader (signal) sequence, namely the leader of the light chain of the antibody MOPC 63 and the leader of the heavy chain of the antibody PCH 108A (Kabat et al., op. cit.). These leader sequences were chosen as typical of antibodies.

(3) 3' of the coding sequences, the nucleotide sequences are the sequences that follow the mouse light chain J5 segment and the mouse heavy chain J2 segment, which are part of the anti-Tac sequences. These sequences are included because they contain splice donor signals.

(4) At each end of the sequence is an Xba I site to allow cutting at the Xba I sites and cloning into the Xba I site of a vector.

Construction of humanized light and heavy chain genes

To synthesize the heavy chain, four oligonucleotides HES12, HES13, HES14, HES15 (Figure 5A) were synthesized using an Applied Biosystems 380B DNA synthesizer. Two of the oligonucleotides are part of each strand of the heavy chain, and each oligonucleotide overlaps the next one by about 20 nucleotides to allow annealing (Figure 5B). Together, the oligonucleotides cover the entire humanized heavy chain (Figure 3) with a few extra nucleotides at each end to allow cutting at the Xba I sites. The oligonucleotides were purified from polyacrylamide gels.

Each oligonucleotide was phosphorylated using ATP and T4 polynucleotide kinase by standard procedures (see, Maniatis, op. cit.). To anneal the phosphorylated oligonucleotides, they were suspended together in 40 ul of TA (33 mM Tris acetate, pH 7.9, 66 mM potassium acetate, 10 mM magnesium acetate) at a concentration of about 3.75 uM each, heated to 95 deg for 4 min. and cooled slowly to 4 deg. To synthesize the complete gene from the oligonucleotides by synthesizing the opposite strand of each oligonucleotide (Figure 5B), the following components were added in a final volume of 100ul:

10 ul	annealed oligonucleotides
0.16 mM each	deoxyribonucleotide
0.5 mM	ATP
0.5 mM	DTT
100 ug/ml	BSA
3.5 ug/ml	T4 g43 protein (DNA polymerase)
25 ug/ml	T4 g44/62 protein (polymerase accessory protein)
25 ug/ml	45 protein (polymerase accessory protein)

The mixture was incubated at 37 deg for 30 min. Then 10 u of T4 DNA ligase was added and incubation at 37 deg resumed for 30 min. The polymerase and ligase were inactivated by incubation of the reaction at 70 deg for

15 min. To digest the gene with Xba I, to the reaction was added 50 ul of 2x TA containing BSA at 200 ug/ml and DTT at 1 mM, 43 ul of water, and 50 u of Xba I in 5 ul. The reaction was incubated for 3 hr at 37 deg, and run on a gel. The 431 bp Xba I fragment was purified from a gel and cloned into the Xba I site of the plasmid pUC19 by standard methods. Four plasmid isolates were purified and sequenced using the dideoxy method. One of these had the correct sequence (Figure 3).

To synthesize the light chain, four oligonucleotides JFD1, JFD2, JFD3, JFD4 (Figure 6A) were synthesized. Two of the oligonucleotides are part of each strand of the light chain, and each oligonucleotide overlaps the next one by about 20 nucleotides to allow annealing (Figure 6B). Together, the oligonucleotides cover the entire humanized light chain (Figure 4) with a few extra nucleotides at each end to allow cutting at the Xba I sites. The oligonucleotides were purified from polyacrylamide gels.

The light chain gene was synthesized from these olignucleotides in two parts. 0.5 ug each of JFD1 and JFD2 were combined in 20 ul sequenase buffer (40 mM Tris-HCl, pH 7.5, 20 mM magnesium chloride, 50 mM sodium chloride), heated at 70 deg for 3 min and allowed to cool slowly to 23 deg in order for the oligonucleotides to anneal. JFD3 and JFD4 were treated in the same way. Each reaction was made 10 mM in DTT and 0.5 mM in each deoxyribonucleotide and 6.5 u of sequenase (US Biochemicals) was added, in a final volume of 24 ul, and incubated for 1 hr at 37 deg to synthesize the opposite strands of the oligonucleotides. Xba I and Hind III were added to each reaction to digest the DNA (there is a Hind III site in the region where JFD2 and JFD3 overlap and therefore in each of the synthesized DNAs; Figure 6B). The reactions were run on polyacrylamide gels, and the Xba I - Hind III fragments were purified and cloned into pUC18 by standard methods. Several plasmid isolates for each fragment were sequenced by the dideoxy method, and correct ones chosen.

Construction of plasmids to express humanized light and heavy chains

The heavy chain Xba I fragment was isolated from the pUC19 plasmid in which it had been inserted and then inserted into the Xba I site of the vector pVγ1 (see, commonly assigned U.S.S.N. 223,037) in the correct orientation by standard methods, to produce the plasmid pHuGTAC1 (Figure 7). This plasmid will express high levels of a complete heavy chain when transfected into an appropriate host cell.

The two light chain Xba I - Hind III fragments were isolated from the pUC18 plasmids in which they had been inserted. The vector plasmid pVκ1 (see, commonly assigned U.S.S.N. 223,037) was cut with Xba I, dephosphorylated and ligated with the two fragments by standard methods. The desired reaction product has the circular form: vector - Xba I - fragment 1 - Hind III - fragment 2 - Xba I - vector. Several plasmid isolates were analyzed by restriction mapping and sequencing, and one with this form chosen. This plasmid, pHuLTAC (Figure 8), therefore contains the complete humanized light chain (Figure 4) and will express high levels of the light chain when transfected into an appropriate host cell.

Synthesis and affinity of humanized antibody

The plasmids pHuGTAC1 and pHuLTAC were transfected into mouse Sp2/0 cells, and cells that integrated the plasmids were selected on the basis of resistance to mycophenolic acid and/or hygromycin B conferred by the gpt and hyg genes on the plasmids (Figures 7,8) by standard methods. To verify that these cells secreted antibody that binds to the IL-2 receptor, supernatant from the cells was incubated with HUT-102 cells that are known to express the IL-2 receptor. After washing, the cells were incubated with fluorescein-conjugated goat anti-human antibody, washed, and analyzed for fluorescence on a FACSCAN cytofluorometer. The results (Figure 9A), clearly show that the humanized antibody binds to these cells, but not to Jurkat T-cells that do not express the IL-2 receptor (Figure 9D). As controls, the

original mouse anti-Tac antibody was also used to stain these cells (Figure 9B,C), giving similar results.

For further experiments, cells producing the humanized antibody were injected into mice, and the resultant ascites collected. Humanized antibody was purified to substantial homogeneity from the ascites by passage through an affinity column of goat anti-human immunoglobulin antibody, prepared on an Affigel-10 support (Bio-Rad Laboratories, Inc., Richmond, CA) according to standard techniques. To determine the affinity of the humanized antibody relative to the original anti-Tac antibody, a competitive binding experiment was performed. About 5×10^5 HUT-102 cells were incubated with known quantities (10 - 40 ng) of the anti-Tac antibody and the humanized anti-Tac antibody for 10 min at 4 deg. Then 100 ng of biotinylated anti-Tac was added to the cells and incubated for 30 min at 4 deg. This quantity of anti-Tac had previously been determined to be sufficient to saturate the binding sites on the cells, but not to be in large excess. Then the cells were washed twice with 2 ml of phosphate buffered saline (PBS) containing 0.1% sodium azide. The cells were then incubated for 30 min at 4 deg with 250 ng of phycoerythrin-conjugated avidin, which bound to the biotinylated anti-Tac already bound to the cells. The cells were washed again as above, fixed in PBS containing 1% paraformaldehyde, and analyzed for fluorescence on a FACSCAN cytofluorometer.

Use of increasing amounts (10 - 40 ng) of the anti-Tac antibody as competitor in the first step decreased the amount of biotinylated anti-Tac that could bind to the cells in the second step; and therefore the amount of phycoerythrin-conjugated avidin that bound in the last step, thus decreasing fluorescence (Figure 10A). Equivalent amounts (20 ng) of anti-Tac, and humanized anti-Tac used as competitor decreased the fluorescence to approximately the same degree (Figure 10B). This shows that these antibodies have approximately the same affinity (within 3 to 4 fold), because if one had much greater affinity, it would have more

effectively competed with the biotinylated anti-Tac, thus decreasing fluorescence more.

Biological properties of the humanized antibody

For optimal use in treatment of human disease, the humanized antibody should be able to destroy T-cells in the body that express the IL-2 receptor. One mechanism by which antibodies may destroy target cells is antibody-dependent cell-mediated cytotoxicity, abbreviated ADCC (Fundamental Immunology, Paul, W., Ed., Raven Press, New York (1984), at pg. 681), in which the antibody forms a bridge between the target cell and an effector cell such as a macrophage that can lyse the target. To determine whether the humanized antibody and the original mouse anti-Tac antibody can mediate ADCC, a chromium release assay was performed by standard methods. Specifically, human leukemia HUT-102 cells, which express the IL-2 receptor, were incubated with ^{51}Cr to allow them to absorb this radionuclide. The HUT-102 cells were then incubated with an excess of either anti-Tac or humanized anti-Tac antibody. The HUT-102 cells were next incubated for 4 hrs with either a 30:1 or 100:1 ratio of effector cells, which were normal purified human peripheral blood mononuclear cells that had been activated by incubation for about 20 hrs with human recombinant IL-2. Release of ^{51}Cr, which indicated lysis of the target HUT-102 cells, was measured and the background subtracted (Table 1). The results show that at either ratio of effector cells, anti-Tac did not lyse a significant number of the target cells (less than 5%), while the humanized antibody did (more than 20%). Hence, the humanized antibody is likely to be more efficacious than the original mouse antibody in treating T-cell leukemia or other T-cell mediated diseases.

TABLE 1

Percent ^{51}Cr release after ADCC

	Effector: Target ratio	
	30:1	100:1
Antibody		
Anti-Tac	4%	< 1%
Humanized anti-Tac	24%	23%

From the foregoing, it will be appreciated that the human-like immunoglobulins of the present invention offer numerous advantages of other antibodies. For example, in comparison to anti-Tac mouse monoclonal antibodies, the present human-like IL-2 receptor immunoglobulins can be more economically produced and contain substantially less foreign amino acid sequences. This reduced likelihood of antigenicity after injection into a human patient represents a significant therapeutic improvement for immunoglobulins designed in accordance with the above criteria.

Although the present invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the appended claims.

WE CLAIM:

1. A composition comprising a substantially pure human-like immunoglobulin specifically reactive with p55 Tac protein.

2. A composition according to Claim 1, wherein the immunoglobulin comprises two pairs of light/heavy chain dimers, wherein each chain comprises a variable region and a constant region.

3. A composition comprising a substantially pure human-like immunoglobulin capable of inhibiting binding of human interleukin-2 (IL-2) to a human IL-2 receptor.

4. A composition according to Claim 1, wherein the immunoglobulin exhibits a binding affinity to a human IL-2 receptor of about 10^8 M^{-1} or stronger.

5. A composition according to Claim 1, wherein the immunoglobulin comprises complementarity determining regions from one immunoglobulin and framework regions from at least one different immunoglobulin.

6. A recombinant immunoglobulin composition comprising a human-like framework and one or more foreign complementarity determining regions not naturally associated with the framework, wherein said immunoglobulin is capable of binding to a human interleukin-2 receptor.

7. A composition according to Claim 6, wherein the immunoglobulin is an IgG_1 immunoglobulin isotype.

8. A composition according to Claim 6, wherein the mature light and heavy variable region protein sequences are substantially homologous to the mature protein sequences in Figures 3 and 4.

9. A human-like immunoglobulin having two pairs of light chain/heavy chain dimers and capable of specifically reacting with an epitope on a human interleukin-2 receptor with an affinity of at least about 10^8 M^{-1}, said light and heavy chains comprising complementarity determining regions (CDR's) and human-like framework regions, wherein the CDR's are from different immunoglobulin molecules than the framework regions.

10. An immunoglobulin according to Claim 9, which is capable of blocking the binding of interleukin-2 (IL-2) to human IL-2 receptors.

11. A humanized immunoglobulin capable of binding to human interleukin-2 receptors, said immunoglobulin comprising one or more complementarity determining regions (CDR's) from anti-Tac antibody in a human-like framework, wherein the human-like framework region comprises at least one amino acid chosen from the anti-Tac antibody.

12. A humanized immunoglobulin according to Claim 11, having a mature heavy chain variable sequence as shown in Figure 3, and a mature light chain sequence as shown in Figure 4.

13. A humanized immunoglobulin according to Claim 11, wherein an additional amino acid from the anti-Tac antibody is immediately adjacent a CDR.

14. A method of treating T-cell mediated disorders in a human patient, said method comprising administering to said patient a therapeutically effective dose of an immunoglobulin according to Claim 1.

15. An immunoglobulin according to Claim 1 which was produced in a myeloma or hybridoma cell.

16. A polynucleotide molecule comprising a first sequence coding for human-like immunoglobulin framework regions and a second sequence coding for one or more mouse immunoglobulin complementarity determining regions, wherein upon expression said polynucleotide encodes an immunoglobulin specifically reactive with p55 Tac protein and capable of blocking the binding of interleukin-2 (IL-2) to the IL-2 receptor on human T-cells.

17. A cell line transfected with a polynucleotide of Claim 16.

18. A method of designing a humanized immunoglobulin (Ig) chain having one or more complementarity determining regions (CDR's) from a donor Ig and a framework region from a human Ig, said method comprising: comparing the framework or variable region amino acid sequence of the donor Ig light or heavy chain with corresponding sequences in a collection of human Ig chains; and selecting to provide the human Ig light or heavy chain framework one of the about three most homologous sequences from the collection.

19. A method of designing a humanized immunoglobulin chain having a framework region from a human acceptor immunoglobulin and complementarity determining regions (CDR's) from a donor immunoglobulin capable of binding to an antigen, said method comprising the steps of substituting at least one human framework amino acid of the acceptor immunoglobulin with a corresponding amino acid from the donor immunoglobulin at a position in the immunoglobulins where:

(a) the amino acid in the human framework region of the acceptor immunoglobulin is rare for said position and the corresponding amino acid in the donor immunoglobulin is common for said position in human immunoglobulin sequences; or

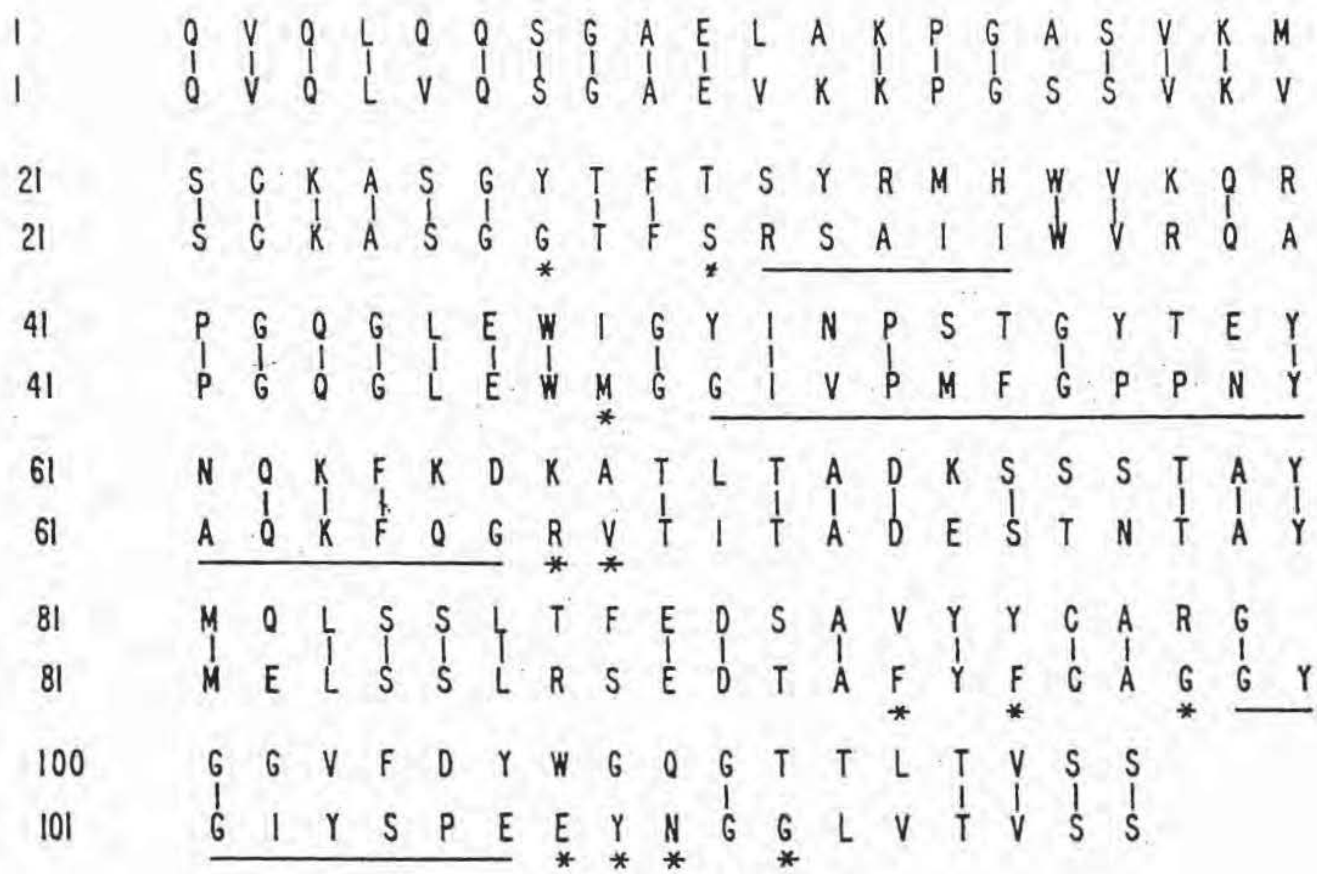
(b) the amino acid is immediately adjacent to one of the CDR's; or

(c) the amino acid is predicted to have a side chain atom within about 3Å of the CDR's in a three-dimensional immunoglobulin model and to be capable of interacting with the antigen or with the CDR's of the humanized immunoglobulin.

20. A method according to Claim 19, wherein the humanized immunoglobulin chain comprises in addition to the CDR's at least three amino acids from the donor immunoglobulin chosen by criteria (a), (b) or (c).

21. A method according to Claim 20, wherein at least one of the amino acids substituted from the donor is immediately adjacent a CDR.

22. A humanized immunoglobulin designed according to Claims 18, 19, or 20.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21 S C K A S G Y T F T S Y R M H W V K Q R
21 S C K A S G G T F S R S A I I W V R Q A
41 P G Q G L E W I G Y I N P S T G Y T E Y
41 P G Q G L E W M G G I V P M F G P P N Y
61 N Q K F K D K A T L T A D K S S S T A Y
61 A Q K F Q G R V T I T A D E S T N T A Y
81 M Q L S S L T F E D S A V Y Y C A R G
81 M E L S S L R S E D T A F Y F C A G G Y
100 G G V F D Y W G Q G T T L T V S S
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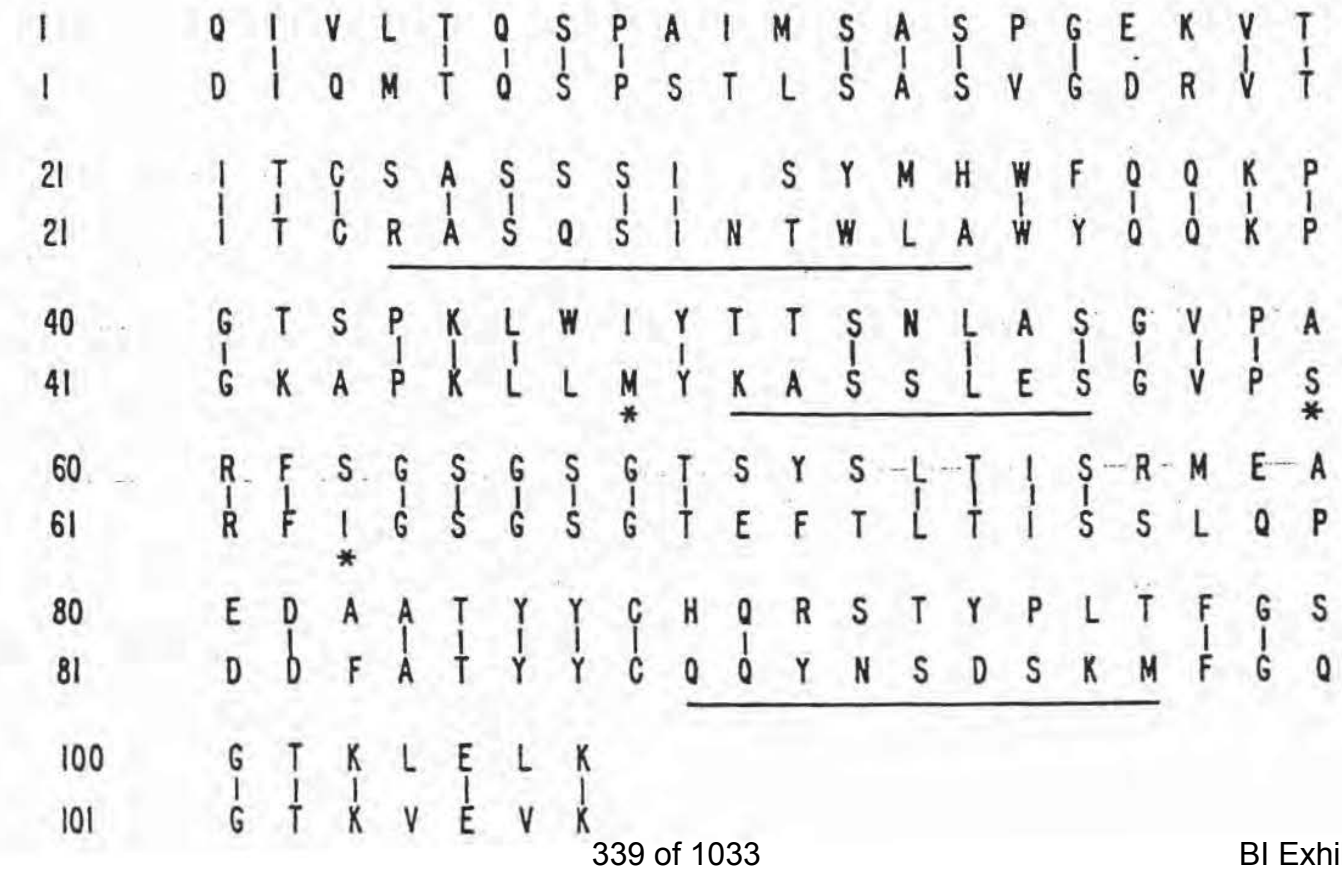
FIG._1.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1 D I Q M T Q S P S T L S A S V G D R V T
21 I T C S A S S S I S Y M H W F Q Q K P
21 I T C R A S Q S I N T W L A W Y Q Q K P
40 G T S P K L W I Y T T S N L A S G V P A
41 G K A P K L L M Y K A S S L E S G V P S
60 R F S G S G S G T S Y S L T I S R M E A
61 R F I G S G S G T E F T L T I S S L Q P
80 E D A A T Y Y C H Q R S T Y P L T F G S
81 D D F A T Y Y C Q Q Y N S D S K M F G Q
100 G T K L E L K
101 G T K V E V K

```
        10          20          30          40          50          60
TCTAGATGGGATGGAGCTGGATCTTTCTCTTCCTCCTGTCAGGTACCGCGGGCGTGCACT
      M  G  W  S  W  I  F  L  F  L  L  S  G  T  A  G  V  H

        70          80          90          100         110         120
CTCAGGTCCAGCTTGTCCAGTCTGGGGCTGAAGTCAAGAAACCTGGCTCGAGCGTGAAGG
S  Q  V  Q  L  V  Q  S  G  A  E  V  K  K  P  G  S  S  V  K

        130         140         150         160         170         180
TCTCCTGCAAGGCTTCTGGCTACACCTTTACTAGCTACAGGATGCACTGGGTAAGGCAGG
V  S  C  K  A  S  G  Y  T  F  T  S  Y  R  M  H  W  V  R  Q

        190         200         210         220         230         240
CCCCTGGACAGGGTCTGGAATGGATTGGATATATTAATCCGTCGACTGGGTATACTGAAT
A  P  G  Q  G  L  E  W  I  G  Y  I  N  P  S  T  G  Y  T  E

        250         260         270         280         290         300
ACAATCAGAAGTTCAAGGACAAGGCAACAATTACTGCAGACGAATCCACCAATACAGCCT
Y  N  Q  K  F  K  D  K  A  T  I  T  A  D  E  S  T  N  T  A

        310         320         330         340         350         360
ACATGGAACTGAGCAGCCTGAGATCTGAGGACACCGCAGTCTATTACTGTGCAAGAGGGG
Y  M  E  L  S  S  L  R  S  E  D  T  A  V  Y  Y  C  A  R  G

        370         380         390         400         410         420
GGGGGGTCTTTGACTACTGGGGCCAAGGAACCCTGGTCACAGTCTCCTCAGGTGAGTCCT
G  G  V  F  D  Y  W  G  Q  G  T  L  V  T  V  S  S

        430
TAAAACCTCTAGA
```

FIG._3.

```
        10          20          30          40          50          60
TCTAGATGGAGACCGATACCCTCCTGCTATGGGTCCTCCTGCTATGGGTCCCAGGATCAA
      M  E  T  D  T  L  L  L  W  V  L  L  L  W  V  P  G  S

        70          80          90          100         110         120
CCGGAGATATTCAGATGACCCAGTCTCCATCTACCCTCTCTGCTAGCGTCGGGGATAGGG
T  G  D  I  Q  M  T  Q  S  P  S  T  L  S  A  S  V  G  D  R

        130         140         150         160         170         180
TCACCATAACCTGCTCTGCCAGCTCAAGTATAAGTTACATGCACTGGTACCAGCAGAAGC
V  T  I  T  C  S  A  S  S  S  I  S  Y  M  H  W  Y  Q  Q  K

        190         200         210         220         230         240
CAGGCAAAGCTCCCAAGCTTCTAATTTATACCACATCCAACCTGGCTTCTGGAGTCCCTG
P  G  K  A  P  K  L  L  I  Y  T  T  S  N  L  A  S  G  V  P

        250         260         270         280         290         300
CTCGCTTCAGTGGCAGTGGATCTGGGACCGAGTTCACCCTCACAATCAGCTCTCTGCAGC
A  R  F  S  G  S  G  S  G  T  E  F  T  L  T  I  S  S  L  Q

        310         320         330         340         350         360
CAGATGATTTCGCCACTTATTACTGCCATCAAAGGAGTACTTACCCACTCACGTTCGGTC
P  D  D  F  A  T  Y  Y  C  H  Q  R  S  T  Y  P  L  T  F  G

        370         380         390         400
AGGGGACCAAGGTGGAGGTCAAACGTAAGTACACTTTTCTAGA
Q  G  T  K  V  E  V  K
```

FIG._4.

A

HES12 AGCTTCTAGATGGGATGGAGCTGGATCTTTCTCTTCCTCCTGTCAGGTACCGCGGGCGTG
CACTCTCAGGTCCAGCTTGTCCAGTCTGGGGCTGAAGTCAAGAAACCTGGCTCGAGCGTG
AAGGTC

HES13 CCCAGTCGACGGATTAATATATCCAATCCATTCCAGACCCTGTCCAGGGGCCTGCCTTAC
CCAGTGCATCCTGTAGCTAGTAAAGGTGTAGCCAGAAGCCTTGCAGGAGACCTTCACGCT
CGAGCCAGG

HES14 TATATTAATCCGTCGACTGGGTATACTGAATACAATCAGAAGTTCAAGGACAAGGCAACA
ATTACTGCAGACGAATCCACCAATACAGCCTACATGGAACTGAGCAGCCTGAGATCTGAG
GACA

HES15 ATATCGTCTAGAGGTTTTAAGGACTCACCTGAGGAGACTGTGACCAGGGTTCCTTGGCCC
CAGTAGTCAAAGACCCCCCCCCCTCTTGCACAGTAATAGACTGCGGTGTCCTCAGATCTC
AGGCTGCT

B

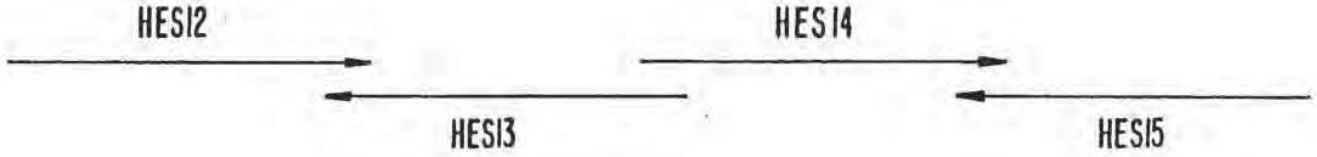

FIG._5.

A

JFD1 CAAATCTAGATGGAGACCGATACCCTCCTGCTATGGGTCCTCCTGCTATGGGTCCCAGGA
TCAACCGGAGATATTCAGATGACCCAGTCTCCATCTACCCTCTCTGCTAGCGTCGGGGAT

JFD2 ATAAATTAGAAGCTTGGGAGCTTTGCCTGGCTTCTGCTGGTACCAGTGCATGTAACTTAT
ACTTGAGCTGGCAGAGCAGGTTATGGTGACCCTATCCCCGACGCTAGCAGAGAG

JFD3 GCTCCCAAGCTTCTAATTTATACCACATCCAACCTGGCTTCTGGAGTCCCTGCTCGCTTC
AGTGGCAGTGGATCTGGGACCGAGTTCACCCTCACAATCAGCTCTCTGCAGCCAGATGAT
TTC

JFD4 TATATCTAGAAAAGTGTACTTACGTTTGACCTCCACCTTGGTCCCCTGACCGAACGTGAG
TGGGTAAGTACTCCTTTGATGGCAGTAATAAGTGGCGAAATCATCTGGCTGCAGAGAGCT
GA

B

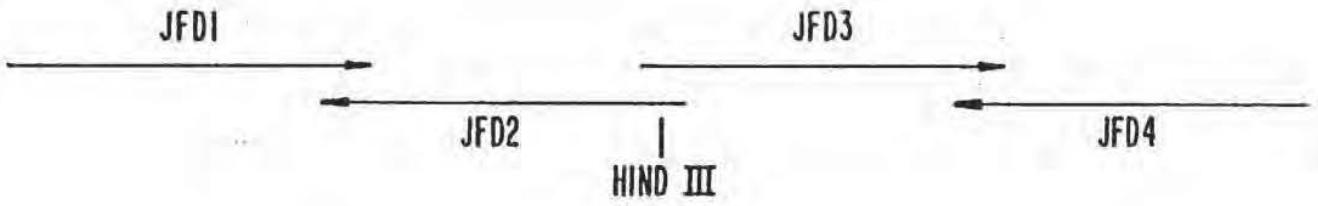

FIG._6.

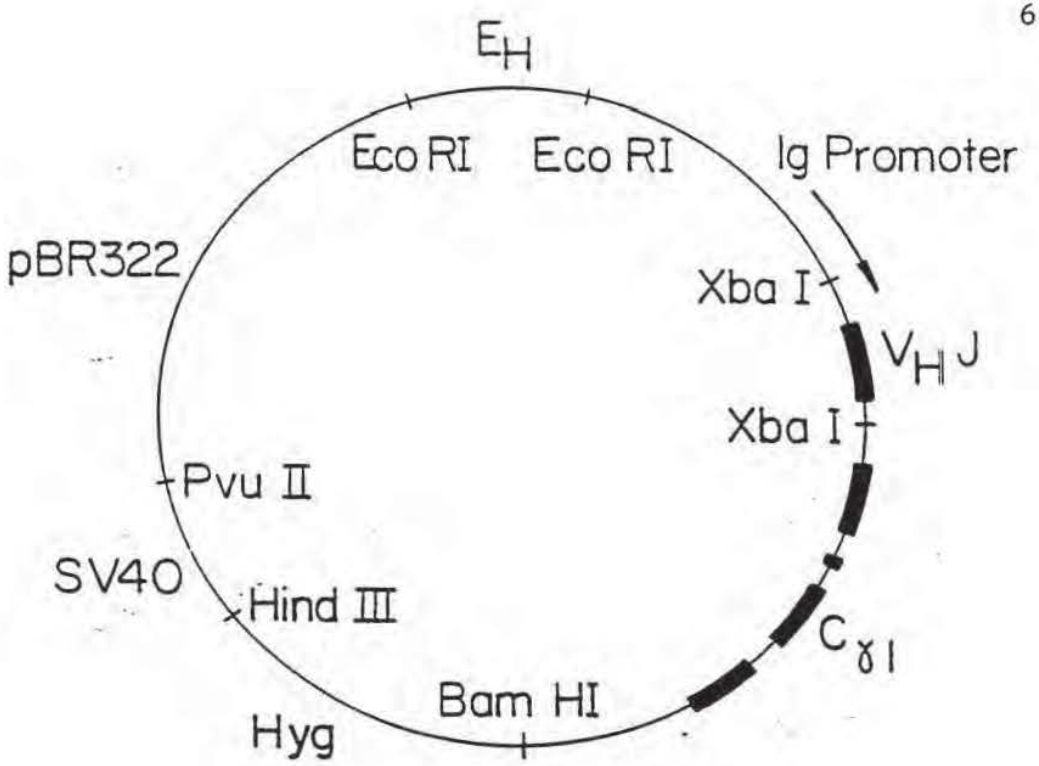

FIG._7.

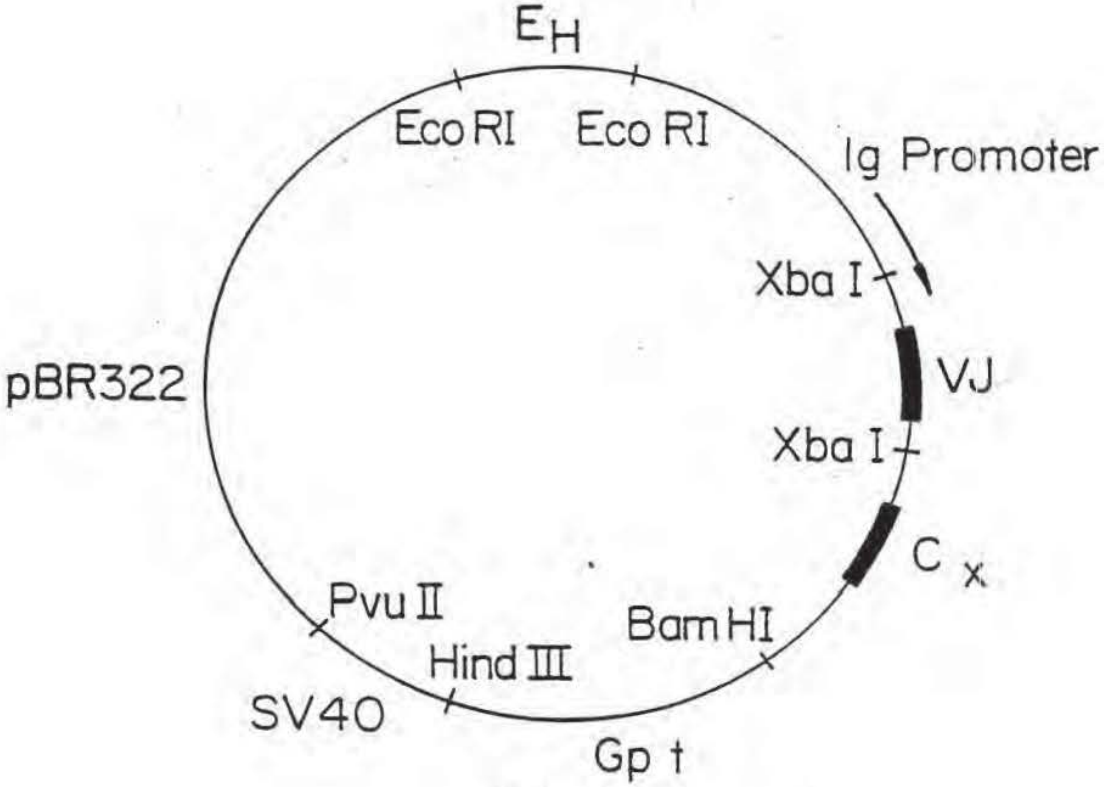

FIG._8.

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ..... because they relate to subject matter [1] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [1], specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

See Attachment Sheet, (page 4).

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☒ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.