Mail Stop Interference
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Filed 2 February 2010

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

PAUL J. **CARTER** AND LEONARD G. PRESTA
Junior Party
(Patent 6,407,213),

v.

JOHN ROBERT **ADAIR**, DILJEET SINGH ATHWAL,
AND JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

Patent Interference No. 105,744
(Technology Center 1600)

DECLARATION - Bd.R. 203(b)[1]

Part A. Declaration of interference

An interference is declared (35 U.S.C. § 135(a)) between the above-identified parties. Details of the application(s), patent (if any), reissue application (if any), count(s) and claims designated as corresponding or as not corresponding to the count(s) appear in Parts E and F of this DECLARATION.

[1] "Bd.R. x" may be used as shorthand for "37 C.F.R. § 41.x". 69 Fed. Reg. 49960, 49961 (12 Aug. 2004).

Part B. Judge managing the interference

Administrative Patent Judge Sally Gardner Lane has been designated to manage the interference. Bd. R. 104(a).

Part C. Standing order

A Trial Section STANDING ORDER [SO] (Paper 2) accompanies this DECLARATION. The STANDING ORDER applies to this interference.

Part D. Initial conference call

A telephone conference call to discuss the interference is set for **2:00 p.m. on 16 March 2010** (the Board will initiate the call).

No later than **four business days** prior to the conference call, each party shall file and serve (SO ¶¶ 10.1 & 105) a list of the motions (Bd. R. 120; Bd. R. 204; SO ¶¶ 104.2.1, 120 & 204) the party intends to file.

A sample schedule for taking action during the motion phase appears as Form 2 in the STANDING ORDER. Counsel are encouraged to discuss the schedule prior to the conference call and to agree on dates for taking action. A typical motion period lasts approximately eight (8) months. Counsel should be prepared to justify any request for a shorter or longer period.

Part E. Identification and order of the parties

Junior Party

Named inventors:	Paul J. Carter San Francisco, CA
	Leonard G. Presta San Francisco, CA

Involved Patent:	6,407,213, issued 18 June 2002, from application 08/146,206, which was filed 17 November 1993, and was based on international application PCT/US92/05126, filed 15 June 1992.
Title:	METHOD FOR MAKING HUMANIZED ANTIBODIES
Assignee:	Genentech, Inc.

Senior Party

Named Inventors:	John Robert Adair High Wycombe, United Kingdom Diljeet Singh Athwal London, United Kingdom John Spencer Emtage Marlow, United Kingdom
Involved Application:	11/284,261, filed 21 November 2005
Title:	HUMANISED ANTIBODIES
Assignee:	Celltech R & D Limited

The senior party is assigned exhibit numbers 1001-1999. The junior party is assigned exhibit numbers 2001-2999. Bd. R. 154(c)(1); SO ¶ 154.2.1. The senior party is responsible for initiating settlement discussions. SO ¶ 126.1.

Part F. Count and claims of the parties

Count 1

A humanized antibody heavy chain variable domain comprising non-human Complementarity Determining Region (CDR) amino acid residues which bind antigen incorporated into a human antibody variable domain, and further comprising a Framework Region (FR) amino acid substitution at a site selected from the group consisting of: 24H, 71H, 73H, and 78H, utilizing the numbering system set forth in Kabat.

The claims of the parties are:

Carter: 1-82

Adair: 24

The claims of the parties which correspond to Count 1 are:

Carter: 30, 31, 60, 62, 63, 66, 67, 70, 73, 77-81

Adair: 24

The claims of the parties which do not correspond to Count 1, and therefore are not involved in the interference, are:

Carter: 1-29, 32-59, 61, 64, 65, 68, 69, 71, 72, 74-76, 82

Adair: None

The parties are accorded the following benefit for Count 1:

Carter:	PCT/US92/05126, filed 15 June 1992; and 07/715,272, filed 14 June 1991, now abandoned.
Adair:	08/846,658, filed 01 May 1997; 08/303,569, filed 07 September 1994, issued as 5,859,205 on 12 January 1999; 07/743,329, filed on 17 September 1991; PCT/GB90/02017, filed 21 December 1990; and GB 8928874.0, filed 21 December 1989.

Part G. Heading to be used on papers

The following heading must be used on all papers filed in this interference, see SO & 106.1.1:

PAUL J. **CARTER** AND LEONARD G. PRESTA
Junior Party
(Patent 6,407,213),

v.

JOHN ROBERT **ADAIR**, DILJEET SINGH ATHWAL,
AND JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

Patent Interference No. 105,744
(Technology Center 1600)

Part H. Order form for requesting file copies

When requesting copies of files, use of SO Form 4 will greatly expedite processing of the request. Please attach a copy of Parts E and F of this DECLARATION with a hand-drawn circle around the patents and applications for which a copy of a file wrapper is requested.

/Sally Gardner Lane/
Administrative Patent Judge

Enc:
Copy of STANDING ORDER
Form PTO-850
Copy U.S. Patent 6,407,213
Copy of claims of 11/284,261

cc (via overnight delivery):

Attorney for Carter:

Sidley Austin, LLP
Attn: DC Patent Docketing
1501 K Street, N.W.
Washington, DC 20005

Attorney for Adair:

Cozen O'Connor, P.C.
1900 Market Street
Philadelphia, PA 19103-3508

Paper ____

UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

ENTERED: 3 January 2006

STANDING ORDER

This Standing Order is entered in, and governs, contested cases assigned to the Trial Division of the Board of Patent Appeals and Interferences. Parties are expected to be familiar with it. The rules reproduced in the Standing Order are current as of the issue date of the Standing Order, but are subject to change. In the event of a change to a rule, the changed rule will control, the Standing Order notwithstanding.

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¶ 1 Applicability of 37 CFR part 1

Part 1 of 37 CFR was drafted principally with patent prosecution in mind, while 37 CFR Part 41 is directed to proceedings before the Board. If Part 41 or the Standing Order expressly requires different procedure than Part 1, then Part 41 or the Standing Order, respectively, will govern. A party that perceives a conflict between Part 1 and either Part 41 or the Standing Order must promptly raise the issue with the Board.

¶ 2 Board organization

¶ 2.1 Trial Division

The Chief Administrative Patent Judge has designated administrative patent judges to constitute the Trial Division, who decide most contested cases, as well as appeals involving patent reexaminations and reissue applications or applications relating to interferences. The Trial Division consists of two sections—

- the Trial Procedures Section and
- the Trial Merits Section.

¶ 2.2 Trial Procedures Section

Within the Trial Division, the Chief Administrative Patent Judge has designated administrative patent judges to constitute the Trial Procedures Section. The principal function of the Trial Procedure Section is to ready interference cases for a merits decision on substantive motions, including interference priority motions. Accordingly,

administrative patent judges will handle all interlocutory matters in contested cases, including but not limited to—

- # declaration of interferences,
- # setting times for taking action, and
- # administering discovery.

¶ 2.3 Trial Merits Section

Within the Trial Division, the Chief Administrative Patent Judge has designated administrative patent judges to constitute the Trial Merits Section. The principal function of the Trial Merits Section is to prepare opinions on the merits of substantive motions, including interference priority motions.

¶ 2.4 Board staff

Board staff other than administrative patent judges may administer some tasks during contested cases. For instance, Trial Division paralegal assistants enter orders authorizing access to files under Bd.R. 109 and authorizing the filing of facsimile transmissions in excess of the five pages permitted under SO ¶ **10.6**. Staff actions are controlling unless countermanded by an administrative patent judge or a Board panel.

¶ 3 Petitions

¶ 3.1 Procedural relief sought through miscellaneous motion

A request for a procedural remedy must be made in the form of a miscellaneous motion. Bd.R. 121(a)(3); SO ¶ **123**. A petition is not appropriate. No fee is required.

¶ 3.2 Petition affecting the merits in a contested case

Any relief affecting the merits of a contested case must be sought in the form of a motion. Bd.R. 121. If the relief would otherwise require the granting of a petition, the petition, including any required fee, must be filed with the motion. Bd.R. 121(c)(2). A party filing a petition in violation of this subparagraph may be subject to sanctions. Bd.R. 128.

¶ 3.3 Petitions under 35 U.S.C. 135(c)

Petitions based on 35 U.S.C. 135(c) are not part of a contested case. Bd.R. 3(b) and Bd.R. 205.

¶ 4 Good cause requirement for untimeliness

¶ 4.1 High standard

The standard for showing good cause under Bd.R. 4(a) is high. Times are set to facilitate the rendering of timely decisions. There are few, if any, circumstances where good cause can be based on the press of other business arising after a time is set by a Board order, particularly where a time period is set or maintained after a conference with counsel.

¶ 4.2 Settlement

An attempt to settle will rarely constitute good cause. Settlement is encouraged, and the administrative patent judge designated to handle a contested case is available to assist in settlement efforts where appropriate, but parties should expect either to settle the contested case or, in the absence of settlement, to meet each deadline.

¶ 5 Pro hac vice

Contested cases can be technically, legally, and procedurally complex. Consequently, a motion to appear pro hac vice will rarely be granted unless the counsel is an experienced litigating attorney and has an established familiarity with the subject matter at issue in the contested case.

The Board may authorize a person other than a registered practitioner to appear as counsel in a specific proceeding.

¶ 6 Publication of opinions

¶ 6.1 Most opinions are publicly available

Most opinions in contested cases will be available for publication during the proceeding. Virtually all become available at the end of the proceeding. The recent experience in contested cases is that redactions are almost never requested.

¶ 6.2 Notice of confidential information

Some opinions are selected for publication to promote public understanding of Trial Division practice or to create uniform practices. If a party believes that its application contains information not otherwise publicly available that should be redacted from any opinion, the party must **within two (2) months** of the initiation of the contested case file as a separate paper a notice specifically identifying such information.

If additional information not otherwise publicly available is introduced into a contested case that a party believes should be redacted from any opinion, the party must promptly file a notice specifically identifying the information.

If, after filing such notice, specifically identified information becomes publicly available (for example, through publication of a collateral application), the party must promptly notify the Board of this change in the status of the information.

¶ 7 Record management

¶ 7.1 Letters between counsel not to be filed

No letter between counsel may be filed unless it is filed as an exhibit cited—

- # in a motion, opposition, or reply, or
- # during cross-examination.

¶ 7.2 No duplicate papers

A party may not file (not even as an appendix or exhibit) a copy of a paper previously filed in the same contested case.

¶ 7.3 Reference to earlier filed paper

A party referring to an earlier filed paper should identify the paper by title and paper number or, if the paper number is not known, by the filing date.

¶ 8 Mandatory notices

¶ 8.1 Real party-in-interest

Within **fourteen (14) days** of the date of the Declaration, each party must file as a separate paper a notice of any and all right, title, or interest in any application or patent involved in the contested case.

¶ 8.2 Related proceedings

Within **fourteen (14) days** of the initiation of a contested case, each party must file and serve as a separate paper a notice identifying the application or patent number of every United States application or patent claiming, or which may claim, the benefit of priority of the filing date of the party's involved patent or application. If there are no such applications or patents the notice must state this fact. If, during the course of the proceeding, a party files an application claiming, or which may claim, the benefit of the filing date of an involved application or patent, a notice of the filing, including the application number, must be promptly served and filed. The Board may order a party to serve on an opponent a copy of any application or patent identified under this paragraph.

¶ 8.3 Notice of judicial review

A party seeking judicial review must notify the Board of the complaint or notice of appeal within 20 days. Bd.R. 8(b). After a contested case ends, administrative tasks remain for the United States Patent and Trademark Office [Office] generally and for the Board particularly. Files need to be distributed, applications need to be allowed or abandoned, and notices of patent claim cancellation need to be published. If the Board

does not receive timely, effective notice of judicial review, it proceeds on the assumption that no review has been sought. The Office may deem an application abandoned or may issue a patent to the opponent. At best, this leaves the litigant with a problem to correct. Failure to provide adequate notice may result in sanctions under Bd.R. 128.

¶ 9 Ownership determined from Office assignment record

In the absence of an assignment of record, the inventor is the presumptive owner of an involved patent or application. It is the responsibility of each party to ensure that its assignments are properly recorded at the Office. Failure to record an assignment can lead to adverse judgment in a contested case.

Cases involving a United States government interest may be referred to the Civil Division of the United States Department of Justice. 28 C.F.R. § 0.45(f).

¶ 10 Communications with the Board

¶ 10.1 Default mode - electronic mail

Electronic mail is the default mode of filing papers in a contested case. See SO ¶ **105**. Papers authorized to be filed electronically must be electronically mailed as an attachment to: **BoxInterferences@USPTO.GOV**. No papers unrelated to the contested case may be filed at this address. Papers relating to the contested case must not be mailed to any other **USPTO.GOV** address.

The subject line may contain only the case number and the assigned administrative patent judge's initials in parentheses, e.g., in Patent Interference 105,000 before Administrative Patent Judge John Doe the subject line would be "105,000 (JD)".

¶ 10.2 Any United States Postal Service, including EXPRESS MAIL

If mailed correspondence is authorized in a contested case, it must be addressed:

Mail Stop INTERFERENCE
Board of Patent Appeals and Interferences
United States Patent and Trademark Office
PO Box 1450
Alexandria Virginia 22313-1450

Caveat: Any delay resulting from an improper address (including any address provided in SO ¶¶ **10.3** & **10.4**) will be attributed to the party.

¶ 10.3 Hand filing

If hand-filed correspondence is authorized in a contested case, the filer must bear in mind that the delivery point lies inside the security zone on the first floor of:

Madison East Building
600 Dulany Street
Alexandria, Virginia

The courier must pass through a magnetometer, and the correspondence must pass through an X-ray sensor. For authorized hand-filed correspondence other than boxes, the correspondence may be deposited in a drop-off box inside the security zone at the first floor lobby of the Madison East Building.

¶ 10.3.1 Boxes

Boxed correspondence cannot be deposited in the drop-off box. Instead, it must be delivered to Madison East, Room 9B55-A. Access to Room 9B55-A is available on business days from 8:30 a.m to 4:45 p.m. (Eastern Time, SO ¶ **100.2**) only.

The courier should approach the security guard station on the first floor of Madison East (near the elevators) and ask the security guard to call the Board at 571-272-9797 to obtain authorization to enter the building for a delivery to Room 9B55-A.

¶ 10.3.2 Correspondence filed after 4:45 p.m.

Authorized hand-filed correspondence filed after 4:45 p.m. (Eastern Time, SO ¶ **100.2**) will receive the next business day's filing date. If the Board receives the paper by 10 a.m. that next business day, it will be deemed to have been timely filed provided the paper was properly served the previous business day.

¶ 10.3.3 Stamped receipt

If a stamped return receipt is required, the courier must personally deliver the correspondence and postcard receipt to Room 9B55-A between 8:30 a.m to 4:45 p.m. (Eastern Time, SO ¶ **100.2**) only, or leave the postcard with the correspondence in the drop-off box. If the postcard is left in the drop-off box, it must include correct postage and the address where the postcard is to be mailed.

¶ 10.4 Overnight delivery services

Papers authorized to be filed using a commercial overnight delivery service (see SO ¶ **10.2** for United States Postal Service) must be addressed:

Board of Patent Appeals and Interferences
Madison Building East, 9th Floor
600 Dulany Street
Alexandria, Virginia 22313

Properly addressed papers filed are deemed filed on the date they are delivered to the overnight delivery service.

¶ 10.5 Telephone calls

Telephone calls to the Board regarding a contested case must be directed to 571-272-9797. A telephone call requesting a conference call must be directed to Trial Division support staff. When arranging a conference call, be prepared to discuss with a Trial Division paralegal why the call is needed and what materials should be before the Board participant during the call.

¶ 10.6 Facsimile

The facsimile number for contested cases is 571-273-0042. Do not send papers exceeding five (5) pages in length without prior permission from Trial Division support staff. Do not file a confirmation copy. Such copies tend to cause duplicate entries (in violation of Bd.R. 7(b)) among other problems.

¶ 11 Ex parte communications

The prohibition on ex parte communications is strictly enforced.

¶ 11.1 Papers

A properly served paper should not result in an ex parte communication.

¶ 11.2 Other forms of communications

Improper communications typically arise in telephone calls, electronic mail messages, or public meetings. Telephone calls should be conference calls with each opposing party included. Electronic mail messages, when authorized, should include the opposing party in the "To" line or the "Cc" line. Discussion of specific pending contested cases should not occur at public meetings.

¶ 11.3 Permissible contacts

The following types of contacts are not ex parte communications—

- # contact of a ministerial nature directed to support staff,
- # contact of a general nature directed to Board management, or
- # contact under a rule requiring reference to a different contested case, provided the contact does not involve the merits of the other contested case.

A call to a Trial Division paralegal to arrange a telephone conference is a typical example of a ministerial contact with support staff. SO ¶ **10.5**. A general comment, made apart from a particular contested case, on a Board rule with examples drawn from actual experiences is an example of the second kind of permissible contact. Identification of a related case pursuant to Bd.R. 8(b) and SO ¶ **8.2** is an example of the third kind of contact.

¶ 11.4 Refusal to participate

The Board may permit a hearing or conference call to take place even if a party refuses to participate. In such instances, the Board may require additional safeguards,

such as the recording of the communication and the entry of the recording into the record.

¶ 12 Citation of authority

¶ 12.1 Copies of authority cited

A party must file and serve a copy of any authority on which it relies. A party must not assume that the opponent or the Board has access to the authority.

An authority reported in (1) United States Reports or West Publishing Company's Supreme Court Reporter, (2) the second or third series of West's Federal Reports, or (3) the Bureau of National Affairs' United States Patents Quarterly is exempt from the requirement to file and serve a copy.

¶ 12.2 Parallel citation

Examples of proper parallel citation as required under Bd.R. 12(a)(2)—

Aelony v. Arni, 547 F.2d 566, 192 USPQ 486 (CCPA 1977).

In re Deckler, 977 F.2d 1449, 24 USPQ2d 1448 (Fed. Cir. 1992).

¶ 12.3 Binding authority

The following types of decisions are binding precedent—

Decisions of the United States Supreme Court.

Decisions of the Federal Circuit, the former Court of Customs and Patent Appeals and the former Court of Claims announced before the close of business on 30 September 1982.

Decisions of the Director of the United States Patent and Trademark Office (formerly the Commissioner of Patents and Trademarks).

- # Decisions of the Board of Patent Appeals and Interferences that have been determined to be binding precedent in accordance with established Board procedures.

- # Trial Division decisions that have been designated as precedential.

Decisions of other federal courts and non-precedential decisions of the Board may be cited, but are not binding precedent.

¶ 12.4 Primary authority

Only primary authority should be cited. Secondary authorities summarizing primary authority are never binding and are inherently less persuasive. Primary authority includes: (1) the United States Code, (2) the Code of Federal Regulations, (3) notices published in the Federal Register or the Official Gazette, and (4) binding precedent.

The Manual of Patent Examining Procedure, a guide for patent examiners prepared by the Office of the Commissioner for Patents, is not itself primary authority except as a statement of patent examining practices.

¶ 20 Fees

No fees are charged for papers filed in the ordinary course of a contested case. Occasionally, a paper filed in a contested case must also comply with another rule. Bd.R. 121(c)(2). Compliance with the other rule may require payment of a fee. If so, then payment must be made at the time the paper is filed.

¶ 100 Days and times

¶ 100.1 Business day

The Board is officially open for business on business days. Occasionally the Board may act or may expressly require a party to act on a day that is not a business day.

¶ 100.2 Time standard

All times stated in the Standing Order and other Board orders (except as expressly provided) are for Eastern Time, the local time for the Board of Patent Appeals and Interferences–either Eastern Standard Time or Eastern Daylight Savings Time, as appropriate.

¶ 101 Notice of proceeding

¶ 101.1 Declaration of interference

The notice initiating an interference is called a Declaration. See 35 U.S.C. 135(a) ("an interference may be declared").

¶ 101.2 Maintaining proper address

While the Board is authorized to provide notice by means other than mailing to the correspondence address of record, it is ultimately the responsibility of the application or patent owner to maintain a proper correspondence address. *Ray v. Lehman*, 55 F.3d 606, 610, 34 USPQ2d 1786, 1788 (Fed. Cir. 1995).

¶ 102 Completion of examination

In most cases the public interest is best served by completion of examination before any contested case is initiated. Exceptions may occur on a case-by-case basis, but the Board may then require parties to address such issues. For example, an applicant might be required to cancel and refile claims that do not correspond to an interference count because such claims are not entitled to a patent term adjustment.

¶ 103 Jurisdiction over related files

¶ 103.1 Access to related files

The Board temporarily may restrict public access to related files, such as paper files for accorded benefit applications, to ensure that the parties can obtain timely copies of such files. See SO ¶ **109**.

¶ 103.2 Action in related files

The Board does not ordinarily take action in a file not involved in the proceeding, but occasionally a decision on a motion will require action in a related file.

¶ 104 Modification of the Standing Order

¶ 104.1 Modification of Standing Order

An administrative patent judge may modify the terms of the Standing Order.

¶ 104.2 Times for motions phases

The Board generally initiates a conference call to set times for the motion periods. For interferences, the date and time for the conference call typically appear in Part D of the Declaration.

¶ 104.2.1 Motions list

A list of proposed motions (see Bd.R. 204(c) and SO ¶ **204**) must typically be filed no later than **four business days** prior to the conference call to set dates. This requirement improves the administration of justice by—

- # helping the Board and counsel arrive at an appropriate schedule for taking action,
- # permitting the Board to determine whether the listed motions are both necessary and sufficient to resolve the issues raised, and
- # revealing the possibility that there may be a dispositive motion, see, e.g., Bd.R. 201 (threshold issue).

With prior Board approval a party may be permitted to file an unlisted motion, but the set times are not likely to be changed to accommodate the unlisted motion.

Sample orders setting times for the preliminary motion (Form **2**) and priority motion (Form **3**) phases of an interference are reproduced in the **Appendix of Forms**. The parties should discuss the appropriate order prior to the conference call and try to agree on dates for taking action. Note that in each order, the parties may usually stipulate changes to the first six times, but not to the last three times.

¶ 104.2.2 Additional discovery

If additional discovery will be needed to support a proposed motion, the movant should include a miscellaneous motion for such discovery in the list of proposed motions. Bd.R. 121(a)(3); SO ¶ **123**.

¶ 105 Electronic filing

¶ 105.1 Filing in paper

Filing in paper is not permitted unless expressly authorized. A party that is unable to comply with the electronic filing requirement must promptly arrange a telephone conference using the procedure for an opposed miscellaneous motion, Bd.R. 123(b)(1)(ii); SO ¶ **123**.

¶ 105.2 Electronic filing procedures

¶ 105.2.1 Time to file

A paper must be filed no later than 5:00 p.m. (Eastern Time, SO ¶ **100.2**) on the day the paper is due.

¶ 105.2.2 Separate files

Each paper (not each page) must be a separate ADOBE® portable document format [pdf] file.

¶ 105.2.3 Papers as an attachment no greater than 1 megabyte

A paper that is not larger than 1 megabyte in size must be electronically mailed as an attachment to: **BoxInterferences@USPTO.GOV**.

No information other than the title of the attachment may appear in the message body, for example, "Jones Motion 1".

The file name of the attachment must concisely identify the document, for example, "Jones Motion 1.pdf".

¶ 105.2.4 Sender

A paper may only be filed from an address at the domain name of the party's counsel of record (or, if no counsel is appointed, the electronic mail address of record for the party).

¶ 105.2.5 Paper larger than 1 megabyte; collections of exhibits

A paper larger than 1 megabyte, or a collection of exhibits, may not be electronically mailed as provided above. Instead, it must be delivered as provided in SO ¶¶ **10.2**-**10.4** on a compact disc or a 3¼ inch diskette that is compatible with MICROSOFT® WINDOWS XP®. If a hearing has been requested, four copies of the disc or diskette must be filed.

¶ 105.3 Service

A paper must be served via electronic mail.

¶ 105.3.1 Simultaneous filing and service

If, when the paper is electronically filed with the Board, the opposing party is included as an addressee, then no additional certificate of service is required. The electronic mail message will function as the certificate of service.

¶ 105.3.2 Delayed service

If the paper is not served via the electronic mail message in which the paper is filed, then the paper must be served in a separate electronic mail message no later than 6:00 p.m. (Eastern Time SO ¶ **100.2**) the day the paper is due. The paper as filed must include a certificate of service stating the time of service in addition to the date and manner of service. Bd.R. 106(f)(3).

¶ 105.4 Format

¶ 105.4.1 Portable document format required

All papers, excluding exhibits, must be filed in ADOBE® portable document format [pdf]. Papers must be filed in **text-searchable** pdf whenever reasonably possible. Each party is responsible for the accuracy of its pdf files.

¶ 105.4.2 Paper size

Each paper created for the contested case should be formatted for printing on 8½ inch × 11 inch paper.

¶ 105.4.3 Waiver of requirements

The following paper formatting requirements in the Board rules are waived for electronically filed papers—

- Bd.R. 106(b)(2) - two-hole punch; and
- Bd.R. 106(c) - working copy.

¶ 105.5 Signature

¶ 105.5.1 Papers other than exhibits

A paper other than an exhibit must be signed using an S-signature (see 37 C.F.R. § 1.4(d)(2)). Board papers will only be signed using an S-signature.

¶ 105.6 Exhibits

An exhibit with a handwritten signature in the exhibit must be scanned to preserve the appearance of the signature even if other portions of the exhibit are converted to a text-searchable format. An affidavit must have an original signature.

¶ 106 Filing and service generally; in paper

A paper or exhibit that is not timely filed and properly served will not ordinarily be considered. One consequence may be that the relief sought is not granted for failure to move or for failure to prove. Cf. SO ¶ **121.3**.

¶ 106.1 General format of papers

¶ 106.1.1 Caption

The heading shown in Part G of the Declaration must be used in all papers other than non-affidavit exhibits. Affidavits other than deposition transcripts must use the heading, but other exhibits do not need to use the heading. Form **1** in the **Appendix of Forms** shows a sample caption for an interference.

¶ 106.1.2 Style

The style of each paper must appear on a single line and must not use the words "et al". Styles for papers other than motions, oppositions, and replies must be simple and descriptive. Examples for papers other than motions, oppositions and replies include—

JONES DESIGNATION OF LEAD ATTORNEY

SMITH DESIGNATION OF REAL PARTY IN INTEREST

JONES REQUEST FOR FILE COPIES

SMITH PRIORITY STATEMENT

JONES SERVICE OF REFERENCES

SMITH CLEAN COPY OF CLAIMS
(with drawing numerals)

SMITH CLEAN COPY OF CLAIMS
(means-plus-function annotation)

¶ 106.1.3 Paper size

The Board's facilities for maintaining paper records are designed to work best with 8½" x 11" paper. Occasionally an exhibit must be reproduced on a larger paper size to preserve detail. In such instances, a larger paper format is permitted, but the paper should be folded to permit entry as a 8½" x 11" paper.

¶ 106.1.4 Line Numbering

For papers other than exhibits, every page (not including the cover sheet, any table of contents, any table of authorities, required appendices, and the certificate of service) must have line numbering to facilitate precise citation between papers. See, e.g., SO ¶ **122.3**.

¶ 106.1.5 Footnotes

The use of footnotes is discouraged. Footnotes must be double-spaced.

¶ 106.2 Combined papers and incorporation not permitted

An opposition must respond to only a single motion and a reply must respond to only a single opposition. Incorporation by reference and combined papers are prohibited to reduce the chance risk of overlooking an argument and to improve the efficiency of decision making. Incorporation of arguments by reference amounts to a self-help increase in the length of the brief and a pointless imposition on the Board's time. Each motion, opposition, and reply must make all arguments accessible to readers, rather than ask them to play archeologist with the record. *DeSilva v. DiLeonardi*, 181 F.3d 865, 866-67 (7th Cir 1999).

The ban on incorporation by reference does not mean that relief in a paper cannot be made expressly contingent on some result arising out of another paper. Indeed, noting such contingencies is strongly encouraged.

¶ 106.3 Transmittal sheets

Do not file a transmittal sheet listing papers being filed *except* an exhibit list may be filed when more than one exhibit is being filed.

¶ 106.4 Service

¶ 106.4.1 Certificate of service

Each paper (other than an exhibit) must have a separate certificate of service, incorporated as the last page of the paper, to permit the Board to verify that each paper has been served.

¶ 106.4.2 Alternatives to EXPRESS MAIL

For service in paper or of electronic media, any mode of service that accomplishes same-day or overnight delivery of the paper (e.g., by hand, facsimile, or a commercial overnight delivery service) may be substituted for EXPRESS MAIL service.

¶ 106.4.3 Papers served but not filed

The following papers must be served on an opponent, but should not be filed with the Board at the time of service—

- An objection to the admissibility of evidence,
- A notice requesting cross-examination, and
- Automatic discovery pursuant to Bd.R. 150(b)(1).

Such papers may be filed later as exhibits if a dispute arises with respect to the paper served. For instance, an objection to the admissibility of evidence may be filed as an exhibit for a motion to exclude. Bd.R. 155(c).

¶ 106.5 Filing in paper

Electronic filing is the default filing mode. SO ¶¶ **10.1** & **105**. The following provisions apply only in the unusual situation that paper filing is authorized—

- The first page of each paper must be pink. Bd.R. 106(b)(1)(ii).
- The paper must have the two holes required in Bd.R. 106(b)(2).
- A working copy must also be filed and must be marked "APJ COPY" at the top first page. Bd.R. 106(c).

¶ 108 Lead and backup counsel

The notice identifying counsel under Bd.R. 108(b) must identify both a lead counsel and a backup lead counsel, and must provide for each the contact information specified in Bd.R. 108(b)(1)-(b)(5).

If lead counsel or backup counsel are not counsel of record (37 CFR §§ 1.32 and 1.34) in the involved application or patent, then a power of attorney must be filed with the Board for entry in the involved patent or application file within the **fourteen (14) day** period of Bd.R. 108(b).

¶ 109 Request for file copies

¶ 109.1 Filing the request

¶ 109.1.1 Time for filing request

A party seeking copies of an involved or benefit file mentioned in the Declaration must, within **fourteen (14) days** of the date of the Declaration, file with the Board (not another part of the Office) a separate paper styled [Name of party] REQUEST FOR FILE COPIES to which is attached a completed FILE COPY REQUEST. See Form **4** in the **Appendix of Forms**. Failure to request copies of files at the beginning of the contested case will rarely constitute a basis for granting an extension of time (Bd.R. 4(a)). Thus, a party should not expect an extension of time based on non-receipt of a requested file if the party did not timely place an order for copies or timely advise the Board of non-receipt of a file.

¶ 109.1.2 Deposit Account

A party may charge the cost of the file request against its deposit account. If so, the party should keep the following points in mind.

¶ 109.1.2.1 Authorization to charge Deposit Account

The individual requesting the file must be authorized to charge a fee to the identified deposit account. "Authorized" means authorized by the records of the Office. "Authorized" to charge a deposit account is *not* the same thing as being an attorney of record in the case—

- # the authorized individual does not have to be a registered practitioner, and
- # a registered practitioner is not automatically authorized to charge a deposit account.

If the individual is not authorized, the Office of Public Records [OPR] will not fill an order even if the Deposit Account has sufficient funds.

¶ 109.1.2.2 Adequate Deposit Account balance

If the balance in a Deposit Account is not sufficient to cover the costs of the requested copies, the order will not be filled. 37 C.F.R. § 1.25.

¶ 109.2 Filling record requests

There are at least three kinds of records—

- # Image File Wrappers [IFW], which are downloaded to a disk and sent to the individual who requested a file copy.
- # Paper files, which are copied and sent in paper form to the individual who requested a file copy.
- # "Artifacts", which includes such things as colored documents, plant patent drawings, or video tapes.

Paper files are copied in one part of OPR and IFW files are downloaded to disks in another part of OPR. An order is considered "filled" when—

An IFW is downloaded, or

A paper file is copied.

Consequently, for the same "file", an IFW portion, an artifact portion, and a paper file portion of the same order may be filled at different times. A party receiving a record from the Office should not be surprised if, for instance, the IFW portion arrives before the paper file portion or the artifact portion.

Patent Cooperation Treaty [PCT] applications

If a benefit or potential benefit application is a PCT application that was filed in the Office, there may be a paper file, but no IFW at this time. If there is a paper file and it is transmitted to OPR, they will copy the PCT paper file and fill an order. The Office does not have copies (IFW or paper) of PCT applications filed elsewhere in the world.

Foreign benefit applications

Foreign benefit applications generally appear as a certified copy in an involved or benefit file. The Office generally does not maintain a separate file for the foreign benefit application. Consequently, before notifying the Board that it has not received a foreign benefit application, a party should first confirm that the application has not been delivered as part of an involved or benefit file.

Copies of a United States patent mentioned in an IFW file

The IFW files generally do not include copies of U.S. Patents cited by the examiner or the applicant. A party receiving a copy of an IFW file should not be surprised that

copies of any U.S. patent cited in the IFW record are not included. Copies of any U.S. patent may be obtained through the PTO's website:

http://www.uspto.gov/patft/index.html

¶ 109.3 Notification on non-receipt

Within **twenty-one (21) days** of the date of the Declaration, the Board forwards all timely requests and necessary files to the Office of Public Records [OPR]. The Trial Division enters an order notifying the parties that their respective orders have been transmitted to OPR. OPR makes the copies, which are shipped via overnight commercial courier within fourteen days of receipt of the order. The shipment may be separated into more than one package. See SO ¶ **109.2**. The transmittal order instructs parties to advise the Board promptly if complete copies are not received consistent with this schedule.

¶ 109.4 Record of contested case

The record for the contested case does not include any involved application or patent or any accorded benefit files, all of which are maintained as separate files. Ordering the record for the contested case will not result in receipt of copies of the involved files. There should rarely be any need for a party to order the record for the contested case during the pendency of the contested case before the Board.

¶ 109.5 Access to paper files at Board

During the pendency of a contested case, involved files (other than files that are maintained as Image File Wrapper records) may be inspected only at the Board. Moreover, paper files are unavailable while OPR is making copies.

¶ 110 Copies of claims

A movant seeking to add a claim must comply with the requirements of Bd.R. 110(c) for the proposed claim.

A movant seeking to have a claim designated as corresponding to a count must provide a clean copy, and where applicable an annotated copy, of the claim as an appendix to the motion unless such copies have already been filed for the claim.

Clean copies of claims and of biotechnology sequences are required because claims and sequences are often spread throughout an application file. In patents, there are often certificates of correction. The filing also provides an early opportunity to identify any divergence between what the party believes its claims to be and what the record actually shows. Annotated copies of the involved claims allow all parties and the Board to understand the precise meaning of limitations in the claims. An example follows:

> An apparatus comprising
> a first valve { **Fig. 2, item 25** },
> means for printing { **page 5, line 8 through page 6, line 1; Fig. 3, items 45 and 46** }, and
> a second valve { **Fig. 3, item 98** } * * *.

¶ 120 Types of notices of basis for requesting relief

The principal types of notices under Bd.R. 120 in interferences are priority statements and motions lists. More detail is provided in Bd.R. 204 and SO ¶ **204**. The Trial Procedures Section will be more flexible in accepting changes in the motions list

than in the priority statement; however, the practice under Bd.R. 120 is intended to be more rigorous than it was prior to the adoption of the current rules on 13 September 2004. An accurate motions list is necessary to provide the Board and the opposing parties adequate notice to plan for the proceeding. Facially inadequate motions lists can no longer be cured by filing whatever motion a party may please whether it was listed or not.

¶ 121 Motions

Relief on the merits of a case must be sought through a substantive or responsive motion. Miscellaneous motions are for seeking procedural relief. All motions require prior Board authorization except unopposed miscellaneous motions, SO ¶ **123**.

¶ 121.1 Title and numbering of motions

Each motion of each party must be numbered consecutively, starting with one, regardless of the type of motion. The title of each motion should state the name of the party, the type of motion, and the motion number. The title of a motion may include a second parenthetical line specifying the nature of the motion. For example—

JONES MISCELLANEOUS MOTION 1
(for additional discovery)

JONES SUBSTANTIVE MOTION 2
(for judgment based on prior art)

JONES RESPONSIVE MOTION 3
(to correct inventorship)

JONES SUBSTANTIVE MOTION 4
(for judgment based on lack of enablement)

¶ 121.2 Page limits in motions

The following page limits (not including a table of contents, a table of authorities, required appendices, and the certificate of service) apply—

priority . . . . . 30 pages

miscellaneous . . . . . 10 pages

other motions . . . . . 20 pages

¶ 121.3 Burden of proof

In addition to complying with any procedural requirements of the rules and the Standing Order, a movant bears a burden to establish its right to any substantive relief requested in the motion. A motion that fails to comply with applicable procedural requirements may be dismissed without reaching the merits, in which case the issue sought to be raised by the motion is deemed not to have been properly presented for decision by the Board. A motion that, while complying with applicable procedural requirements, nevertheless fails to make out a substantive case may be denied on the merits. A motion may be dismissed or denied without considering any opposition, or may be granted without considering a reply. In an interference, the movant should note the presumptions stated in Bd.R. 207.

While the ultimate burden remains with the movant, the burden of production may shift to the opponent after the movant has made out a facially sufficient case. Similarly, the opponent may bare the initial burden with respect to an issue first propounded in the opposition. For example, a movant seeking judgment of anticipation over a reference bears the ultimate burden of establishing anticipation, but the opponent

arguing that the reference is not enabling will have the initial burden of proof on the issue of enablement.

¶ 121.4 Format

Each motion must set out in the following order the precise relief requested and an argument setting out the reasons why relief should be granted. Two examples of a precise statement of relief—

- # Jones moves to be accorded the benefit for count 1 of the filing date of application X, filed 22 January 1993.

- # Jones moves for judgment against Smith on the ground that all of Smith's involved claims are unpatentable under 35 U.S.C. 103 over the combined disclosures of U.S. Patent No. Y and French Patent Z.

¶ 121.5 Appendices

¶ 121.5.1 Appendix 1: List of exhibits

Each motion must include a list of the exhibits cited in the motion. The list must appear as "Appendix 1" to the motion. The list must be ordered by exhibit number. Each exhibit must be listed on a separate line. The listing of each exhibit number should state the exhibit number followed by a concise description of the exhibit. For example:

1048 - Second declaration of Prof. John Doe.

In deciding a motion, the Board ordinarily will not consider an exhibit not listed as provided in this paragraph.

¶ 121.5.2 Appendix 2: Statement of material facts

The statement of material facts for each motion, opposition, or reply must be set forth as "Appendix 2" to the motion, opposition, or reply, respectively. Bd.R. 121(d)(2).

Each fact must be set out as a single, short, numbered declaratory sentence that is capable of being admitted or denied. Citation to the evidence must be specific, for example, by—

- column and line of a patent,
- page, column, and paragraph of a journal article,
- page and line of a deposition transcript,
- page and paragraph of an affidavit (other than a deposition transcript), and
- title and chapter number of a DVD.

A motion may be denied if the facts alleged in Appendix 2 are insufficient to state a claim for which relief may be granted. Facts set out only in the argument portion of a motion may be overlooked and may result in a motion being denied. Citations to an entire document or numerous pages of a cross-examination deposition transcript do not comply with the requirement for a specific citation to the record. The Board will not take on the role of advocate for a party, trying to make out a case the party has not adequately stated.

¶ 121.6 Claim chart alternative

As an alternative to a claim chart, a party may reproduce the complete claim in an appendix. Following each limitation in the claim, and within braces { }, insert in bold a specific citation to the information to be compared to the limitation (such as where a prior art reference describes the limitation). Braces { } must be used instead of brackets [ ] because brackets are used to indicate amended portions of claims in reissue applications.

¶ 122 Oppositions and replies

¶ 122.1 Numbering oppositions and replies

Each opposition must use the same number as the motion it opposes (skipping the number of any motion not opposed), but does not need a descriptive second line. For example (with reference to the examples in SO ¶ **121.1**):

SMITH OPPOSITION 1.

Each reply must use the same number as the opposition to which it replies (again, skipping the number of any opposition for which no reply is filed). For example (with reference to the examples in SO ¶ **121.1**):

JONES REPLY 4.

¶ 122.2 Page limits

As with motions, the page limits for oppositions and replies do not include a table of contents, a table of authorities, required appendices, or a certificate of service.

¶ 122.2.1 Opposition

The page limits for oppositions are the same as those for corresponding motions—

priority . . . . . 30 pages

miscellaneous . . . . . 10 pages

other motions . . . . . 20 pages

¶ 122.2.2 Reply

For replies, the pages limits are—

priority . . . . . 12 pages

miscellaneous . . . . . 5 pages

other motions . . . . . 10 pages

¶ 122.3 Format

¶ 122.3.1 Opposition

An argument stating the reason why relief is opposed must be made in the following manner:

> On page x, lines y-z of the motion, it is argued (or stated factually) that _. The response is _.

¶ 122.3.2 Reply

The argument responsive to statements in the opposition must be made in the following manner:

> On page x, lines y- z of the opposition, it is argued (or stated factually) that _. The response is _.

¶ 122.4 Appendices

¶ 122.4.1 Appendix 1: List of exhibits

Each opposition and reply must include as "Appendix 1" an exhibit list formatted like the list required for motions, SO ¶ **121.5.1**.

¶ 122.4.2 Appendix 2: Statements of material fact

Each opposition and reply must include as "Appendix 2" a statement of material facts formatted like the statement required for motions, SO ¶ **121.5.2**.

As provided in the following paragraphs, the statement of material fact is a compilation of all the facts stated for the motion. That is—

- # The statement of facts in an opposition will include the statement of facts from the motion, and
- # The statement of facts in a reply will include the statement of facts from the motion and the opposition.

Since papers should ordinarily be submitted in text-readable, electronic format, the burden associated with creating the compilation should be relatively small. The statements appear in appendices and thus do not count toward the page limits. The last statement of material facts submitted should be a complete compilation of all facts (and admissions, denials, etc.) relating to the motion.

¶ 122.4.2.1 Opposition

Appendix 2 for the opposition must include a statement of material facts in which,

- # Each material fact alleged in the motion is repeated with concise statement admitting, denying, or stating that the opponent is unable to admit or deny the fact.
- # Any additional material fact upon which the opposition relies, with a citation to the evidence. Any additional material fact must be

consecutively numbered beginning with the next number after the last numbered material fact.

¶ 122.4.2.2 Reply

Appendix 2 for the reply must include a statement of material facts in which,

All the material facts stated in the motion are repeated with the opponent's concise statement admitting, denying, or otherwise addressing the motion fact.

Each material fact alleged in the opposition is repeated with concise statement admitting, denying, or stating that the movant is unable to admit or deny the fact.

Any additional material fact upon which the movant relies, with a citation to the evidence. Any additional material fact must be consecutively numbered beginning with the next number after the last numbered material fact.

¶ 122.5 New issues in replies

A reply that raises a new issue or belatedly presents evidence will not be considered and may be returned. The Board will not attempt to sort proper from improper portions of the reply. Examples of indications that a new issue has been raised in a reply include—

new evidence that is necessary to make out a *prima facie* case for the relief requested in the motion,

new evidence that could have been included with the motion, and

a reply that is longer than the corresponding motion or opposition.

¶ 123 Miscellaneous motions

¶ 123.1 Mandatory conference

Before filing a miscellaneous motion, a party must confer with all opponents and, if agreement cannot be reached, arrange a conference call to the Board official administering the contested case.

¶ 123.2 Timeliness

The movant must explain why the motion is timely.

¶ 123.3 Recording conference calls

The parties, at their expense, may retain the services of a court reporter to record any conference call. A written record is often desirable inasmuch as an oral decision may be made with respect to issues raised during the conference call.

¶ 124 Oral argument

¶ 124.1 Request for oral argument

The time for requesting an oral argument on substantive and responsive motions is normally specifically set in an order. Note that the time set in the order controls over the five-day period set in Bd.R. 124(a). See Bd.R. 104(c).

A precise statement of the issues for which oral argument is requested is helpful in determining whether to authorize a hearing at all and in determining the times to allot to the parties. A request may be granted or denied in whole or in part.

¶ 124.2 Attendance; special needs

A party that does not expect to attend must promptly notify the Board. Such notice must be served on opposing party.

A party should advise the Board as soon as possible before an oral hearing of any special need. Examples of such needs include additional space for a wheel chair or for a stenographer, an easel for posters, or an overhead projector. Parties should not make assumptions about the equipment the Board may have on hand. Such requests should be directed in the first instance to a Trial Division paralegal at 571-272-9797. Ultimately, any special equipment needed for oral argument is the responsibility of the party needing the equipment.

¶ 124.3 Security; access

The parties should consult the Board web page for information regarding parking and security processing:

http://www.uspto.gov/web/offices/dcom/bpai/docs/contacts/visitation_info.htm

Attendees must report to the security station in the lobby of the Madison Building East and present the order setting the hearing along with photo identification to security personnel.

¶ 124.4 Demonstrative exhibits

Four copies (one copy for the record and one working copy for each judge) of each demonstrative exhibit must be filed before, or presented at, oral argument. Demonstrative exhibits must be served at least five business days in advance. Bd.R. 124(d).

Elaborate demonstrative exhibits are more likely to impede than help an oral argument. The most effective demonstrative exhibits tend to be a handout or binder containing the demonstrative exhibits. The pages of each demonstrative exhibit should be numbered to facilitate identification of the exhibits during the hearing, particularly if the hearing is recorded.

¶ 124.5 Transcription of oral argument

Transcription of oral argument is strongly encouraged. The party requesting transcription must arrange for the transcription and pay the costs. Parties are encouraged to share the costs.

When an argument is to be transcribed, the party should notify a Trial Division paralegal assistant (571-272-9797) as soon as possible, but at least one business day prior to oral argument, so that arrangements may be made in the hearing room for the reporter.

The transcriber must use a stenography machine, but may also use a tape recording device as a backup. Microphones at individuals' locations are not authorized.

¶ 125 Rehearing of decisions

¶ 125.1 Time for filing

A request for rehearing must be filed within fourteen (14) days of the decision, Bd.R. 125(c)(1), *unless* a judgment accompanies the decision in which case the request must be filed within thirty (30) days of the judgment, Bd.R. 127(d).

¶ 125.2 Format for request for rehearing

A request for rehearing is, in form, a miscellaneous motion, but no prior conference call is required. The argument responsive to the decision must be made with particularity in the following manner:

> On page _, lines _-_, the opinion states _. The opinion is believed to have overlooked [or misapprehended] _. This point was set forth in _ Motion [or Opposition or Reply] _ at page _, lines _-_.

The request must include as an appendix an evidence list setting forth a list (in numerical order by exhibit number) of each exhibit that the party believes was overlooked or misapprehended.

¶ 125.3 Number of requests

A party may file no more than one request for rehearing per motion decision.

¶ 125.4 New evidence on rehearing

Evidence not already of record at the time of the decision will not be admitted absent a showing of excusable neglect for the belated submission. Bd.R. 4(a).

¶ 126 Settlement discussions required

¶ 126.1 Last-named party initiates

The party named last in the caption set in the declaration is responsible for—

- # initiating any settlement discussions,
- # initially drafting any document, and
- # initiating any conference call required by this paragraph.

The parties may agree to permit another party to undertake the obligations placed upon the last-named party.

The Office encourages settlement of contested cases and has designed this process to facilitate settlement. The last named party in the contested case is required to initiate the mandatory settlement discussion to avoid the perception that initiation of settlement talks indicates weakness.

¶ 126.2 Initial conference

Within **three (3) months** of the date of the Declaration, the parties must conduct a settlement conference and must initiate a conference call with the Board official assigned to the case. During the call, the parties should be prepared to report—

- # the outcome of the settlement discussion;
- # whether the parties are actively engaged in settlement negotiations and, if so, what steps have already been taken toward settlement;
- # whether any settlement negotiations are directed toward obviating the need for filing motions;
- # any issues that are not subject to settlement negotiations; and
- # the status of any settlement negotiations, including how much time might be needed to conclude those negotiations.

The Board official assigned to the contested case is available to facilitate settlement discussions.

¶ 126.3 Subsequent conferences

Unless a different time is set in an order, within **two (2) months** after a panel decision on substantive motions, the parties must conduct another settlement

conference and initiate another conference call with the Board on the conference as provided in the preceding paragraph, SO ¶ **126.2**.

¶ 126.4 Filing notice of conferences

Prior to initiating any conference call required by this paragraph, the parties must file (in cases without electronic filing, preferably by facsimile, SO ¶ **10.6**) a joint statement indicating that a good faith effort has been made to settle the contested case.

¶ 127 Estoppel; rehearing of judgment

¶ 127.1 Estoppel

A substantively or procedurally inadequate motion does not avoid estoppel under Bd.R. 127(a)(1).

¶ 127.2 Rehearing

The rehearing practice under SO ¶¶ **125.2**-**125.4** applies to rehearing of judgments as well. The judgment implementing a decision does not provide a second opportunity to request a rehearing of the underlying decision, SO ¶ **125.3**.

Concurrent decision and judgment

Often a judgment implementing a decision is entered with or shortly after entry of the decision itself such that the fourteen (14) day period for requesting rehearing of a decision (Bd.R. 125(c)(1)) runs concurrently with the thirty (30) day period for requesting rehearing of the judgment (Bd.R. 127(d)). In this situation, a single request for rehearing should be filed within the thirty (30) day period set in Bd.R. 127(d). See SO ¶ **125.1**.

¶ 128 Sanctions

As with other procedural remedies, a party seeks a sanction by filing a miscellaneous motion. SO ¶ **123**.

Examples of conduct likely to lead to sanctions—

- # filing a petition affecting the merits of a pending contested case with a part of the agency other than the Board (SO ¶ **3.2**), and
- # failing to file adequate notice of judicial review with the Board (SO ¶ **8.3**),
- # exceeding the Board-authorized scope for compelled testimony or production (SO ¶ **156.3**),
- # failing to comply with the **Cross Examination Guidelines** (SO ¶ **157.5**),
- # failure to notify the Board promptly of a common interest (SO ¶ **206**), and
- # filing a facially insufficient motion alleging inequitable conduct against an opponent (SO ¶ **208.2**).

¶ 150 Discovery

¶ 150.1 Automatic discovery

Automatic discovery (1) places the parties on a level playing field and (2) reduces any difficulty authenticating documents when a party would like to rely on a document cited in an opponent's specification. A party should have access to documents cited in its opponent's specification, but it may be difficult for the party to locate those documents. The parties should be prepared to promptly file copies of the materials served under Bd.R. 150(b).

¶ 150.2 Requesting additional discovery

Discovery before the Board is significantly different than discovery under the Federal Rules of Civil Procedure. A request for additional discovery must be in the form of a miscellaneous motion. Bd.R. 121(a)(3); SO ¶ **123**. The standard for granting such requests is high and requires specific bases for expecting that the discovery will be productive. Bd.R. 150(a) & (c)(1). Additional discovery is rarely authorized because in contested cases, the party usually has equal or better access to relevant information compared to any other source. Additional discovery is most commonly authorized in the context of cross examination. Bd.R. 150(c)(2). Other situations in which additional discovery might be required include proving an on-sale or public-use bar and proving inequitable conduct intent, but these situations require a solid basis for believing the discovery will be productive.

¶ 151 Challenging admissibility

As with other procedural remedies, a party may seek to prevent the entry of evidence (motion in limine) or to exclude entered evidence by filing a miscellaneous motion. See Bd.R. 121(a)(3); SO ¶¶ **123** & **155**.

¶ 152 Rules of evidence

¶ 152.1 Official notice not automatic

The Board may exercise its discretion to take official notice of the records of the United States Patent and Trademark Office. Bd.R. 152; Fed. R. Evid. 201(c). No party should proceed on the assumption that the Board will take notice of Office records sua sponte. As with other procedural remedies, a party may seek official notice by filing a miscellaneous motion. Bd.R. 121(a)(3); SO ¶ **123**.

¶ 152.2 Hearsay

¶ 152.2.1 Specification

A specification of an involved application or patent is admissible as evidence only to prove what the specification or patent describes. If there is data in the specification upon which a party intends to rely to prove the truth of the data, an affidavit by an individual having first-hand knowledge of how the data was generated (i.e., the individual who performed an experiment reported as an example in the specification) must be filed. This individual may be cross examined.

¶ 152.2.2 Laboratory notebooks

Lab notebooks generally do not fall within the business records exception or the catchall exception. *Chen v. Bouchard*, 347 F.3d 1299, 1308 & n.2, 68 USPQ2d 1705, 1711 & n.2 (Fed. Cir. 2003).

¶ 153 Certification of Office records

Records of the United States Patent and Trademark Office are deemed to be self-authenticating within the meaning of Fed. R. Evid. 902(4), provided the record is available to each party in the proceeding. Parties generally have an opportunity to obtain copies of the involved and benefit files for the contested case.

¶ 154 Form of evidence

¶ 154.1 Records of the Office

¶ 154.1.1 Must be submitted as an exhibit

Records of the United States Patent and Trademark Office, including affidavits filed during examination, are not automatically part of the record before the Board. The first party seeking to rely on the record must submit a copy of the record as an exhibit. As with other exhibits, the opponent will have an opportunity to object to the exhibit. In the case of an affidavit filed during examination, the opponent may cross examine the affiant.

¶ 154.1.2 Reliance on a portion of a file

If a motion (or opposition or reply) relies on any document in the file of a patent or application (including a specification), the entire document must be made an exhibit in the contested case. Each document, however, should be a separate exhibit. Do not submit an entire application file as a single exhibit. A document that is not discussed in the motion (or opposition or reply) must not be filed.

¶ 154.2 Exhibit labels

¶ 154.2.1 Unique and consecutive

Each exhibit from a party must be uniquely and consecutively numbered within the range the Board assigns to the party for the proceeding.

Unless otherwise provided in an order, the party named last in the caption set in the declaration is assigned the range 1001-1999, while the first-named party is assigned 2001-2999.

¶ 154.2.2 Material covered on first page

If an exhibit label covers important material on the first page of an exhibit, a copy of the first page of the exhibit must be reproduced and presented as page 1-a of the exhibit.

¶ 154.3 Filing of exhibits

¶ 154.3.1 Electronically

See SO ¶ **105.2**.

¶ 154.3.2 In paper

A set of original exhibits must be filed in a box, an accordion folder, or a comparable folder containing the exhibits in numerical order, separated by a divider that conspicuously identifies each exhibit by number.

If any party requests oral argument, three (3) separate additional sets of exhibits must also be filed; otherwise, one (1) additional set of exhibits must be filed.

¶ 154.3.3 Repeated submission of the same exhibit

Multiple copies of the same exhibit with different exhibit numbers from the same party are prohibited. Bd.R. 7(b).

¶ 154.4 Exhibit list

A current list must be served whenever evidence is served.

The exhibit list must be filed with the exhibits.

¶ 154.5 Evidence on DVD digital disc

Use of a DVD digital disc [DVD] may be appropriate to present some evidence, such as video of a deposition or of an experiment. The Board may also require live testimony before the Board.

¶ 154.5.1 Transcript of deposition

A DVD of a deposition may be filed in addition to, but not instead of, a text transcript of the deposition. Ordinarily the proponent of the direct testimony is responsible for filing the transcript regardless of which party is filing the DVD. Bd.R. 157(f).

¶ 154.5.2 Format

¶ 154.5.2.1 MPEG-2 encoded DVD R media

The Board will not consider a DVD that does not operate on Board equipment. The Board has equipment to operate a DVD properly encoded in MPEG-2 format on DVD R media. The DVD must be indexed (e.g., title and chapter numbers) so that reference to and viewing of a particular portion of the video may be made. Any references to a DVD exhibit must specifically refer to a particular portion in the DVD (e.g., title number and chapter number), analogous to citing a specific particular page and line number in a text

transcript. Four copies of the DVD must be filed. One copy of the DVD must be served on all opponents.

¶ 154.5.2.2 Deposition DVD

A deposition DVD must show only the head and upper torso of the witness. Except for breaks, the DVD must contain the entire deposition of the witness. For example, the DVD should show whether the witness took a long time to answer a particular question or had to review documents unless review of documents takes place off the record during a break.

¶ 154.5.3 Costs

A party recording an experiment or other event, including a deposition, is responsible for all costs of preparing the DVD. The proponent of the direct testimony remains responsible for the other reasonable costs of the deposition, including any court reporter and required transcripts. Bd.R. 157(f). If a party records a deposition, but subsequently decides not to file the DVD prepared, another party may cause the DVD to be prepared and must assume responsibility for the costs of having the DVD prepared. The parties may agree to divide the costs of preparing a DVD in any proportion.

¶ 154.5.4 Notice of intent to video-record cross examination

A party that intends to prepare a DVD of cross-examination must file and serve a notice of intent to prepare a DVD on each opponent before the date of the cross examination. The timing of the notice depends on which party intends to video-record the cross examination—

If the proponent of the direct testimony, notice must be served at least five (5) business days before the cross examination; else

If the opponent, notice must be served at least three (3) business days before the cross examination.

¶ 155 Challenging admissibility

¶ 155.1 Objections

¶ 155.1.1 Objecting to served evidence

An objection to the admissibility of evidence must not be filed except as part of a motion to exclude.

¶ 155.1.2 Waiver of untimely objections

If an objection could have been made before the filing of supplemental evidence and an objection was not made, the objection is waived.

¶ 155.1.3 Responding to an objection

No immediate response to an objection is required other than the filing of supplemental evidence in response to the objection if the proponent of the evidence chooses to do so. Any attack on the correctness of an objection must come in response to a motion to exclude based on the objection. Any other response to an objection is neither necessary nor desirable.

¶ 155.1.4 Serving supplemental evidence

Supplemental evidence responding to an objection to the admissibility of evidence must not be filed until it is used as an exhibit.

¶ 155.2 Motion to exclude evidence; motion in limine

¶ 155.2.1 Form of motion

As with other motions seeking a procedural remedy, motions to exclude and in limine are miscellaneous motions. Bd.R. 121(a)(3); SO ¶ **123**.

¶ 155.2.2 Content

A motion to exclude evidence must—

- # identify where in the record the objection was originally made,
- # identify where in the record the evidence to be excluded was relied upon by an opponent, and
- # address objections to exhibits (in whole or in part) in exhibit numerical order.

When a timely objection has been made (see SO ¶ **155.1**), no conference call is necessary to file a motion to exclude.

¶ 155.3 Time to file objections and motions to exclude

Times for filing objections and for filing and serving motions to exclude are set in the order setting times for motions. Generally, the order will set a later date for filing of objections and motions to exclude than the default time for a miscellaneous motion set in the rule because supplemental evidence may cure any defect in the evidence.

¶ 156 Compelled testimony and production

¶ 156.1 Form of motion

A miscellaneous motion under Bd.R. 156(a) must comply with the requirements for a miscellaneous motion, Bd.R. 121(a)(3), SO ¶ **123**, and must be filed sufficiently in

advance that any authorized testimony can be served with the motion, opposition or reply it is intended to support.

¶ 156.2 Alternative procedures for compelled testimony

If a motion to compel testimony is granted, testimony may be—

ex parte, subject to subsequent cross examination, or

inter partes.

In moving for, or opposing, such testimony, the parties should discuss which procedure is appropriate.

¶ 156.3 Admissibility of compelled testimony and production

Compelled testimony and production will only be admitted to the extent the Board has authorized. Significant deviation from the Board-authorized scope may result in sanctions.

¶ 157 Direct testimony; cross examination

¶ 157.1 Direct testimony as affidavit

Direct testimony, other than compelled testimony, must be in the form of an affidavit. Bd.R.157(a). "Affidavit" is defined to include a declaration or ex parte deposition. Bd.R. 2.

¶ 157.2 Required paragraph for affidavits

Affiants have been known to announce belatedly that appearing for cross examination at a reasonable time and place in the United States might not be possible. Consequently, the following paragraph must be included on the signature page of all

affidavits to prevent surprise and hardship to the party relying on the testimony of the witness:

> In signing this [affidavit], I understand that the [affidavit] will be filed as evidence in a contested case before the Board of Patent Appeals and Interferences of the United States Patent and Trademark Office. I acknowledge that I may be subject to cross examination in the case and that cross examination will take place within the United States. If cross examination is required of me, I will appear for cross examination within the United States during the time allotted for cross examination.

¶ 157.3 Cross examination

The party relying on an affiant must make the affiant available for cross examination; otherwise, the affidavit may be excluded. The parties must confer to reach agreement on reasonable times, dates, and locations for cross examination of witnesses.

¶ 157.3.1 Start date

Unless the parties otherwise agree, cross examination of an affiant may begin no earlier than twenty-one (21) days after service of the affidavit.

¶ 157.3.2 End date

Unless the parties otherwise agree,

- # Cross examination of an affiant relied upon in a motion other than a miscellaneous motion must occur at least ten (10) days before the opposition to the motion is due.

- # Cross examination of an affiant relied upon in an opposition to a motion other than a miscellaneous motion must take place at least ten (10) days before a reply is due.

¶ 157.3.3 Notice of cross examination

Since cross examination builds on testimony that has already been served, the notice requirement is simplified.

The party seeking the cross examination (rather than the proponent of the testimony) prepares the notice requesting cross examination. The notice must be served at least two business days before cross examination, but must not be filed (except as an exhibit to a miscellaneous motion, SO ¶ **123**, in the event of a dispute). The notice must identify the time and place of the deposition, but not the other items required under Bd.R. 157(c)(4).

¶ 157.3.4 Live testimony

Cross-examination might be ordered to take place in the presence of an administrative patent judge. Examples of where such testimony has been ordered include contested cases where inventorship, derivation, or inequitable conduct has been an issue; where testimony has been given through an interpreter; or where close supervision is warranted.

¶ 157.4 Order of cross examination

While a party requesting cross examination may choose the order of the witnesses, Bd.R.157(c)(2), the order must be reasonable.

¶ 157.5 Cross examination guidelines

The **Cross Examination Guidelines** appended to the Standing Order apply to all cross examination in this contested case.

¶ 157.6 Transcript

¶ 157.6.1 Proponent responsible

The proponent of the direct testimony is responsible for securing the services of a court reporter and providing a copy of any transcript to every opponent.

¶ 157.6.2 Filing transcript

An uncertified copy of each deposition transcript must be filed as an exhibit. A certified transcript of testimony need not be filed unless required by the Board.

¶ 157.7 Observations on cross examinations

Cross examination may occur after a party has filed its last substantive paper on an issue (e.g., after the reply). Such cross examination may result in testimony that should be called to the Board's attention but does not merit a motion to exclude. The Board may authorize the filing of observations to identify such testimony and responses to observations. In practice, few parties file observations.

The party taking the cross examination files the observations. The opposing party may file a response to an observation. The opposing party may not file observations without express prior authorization.

An observation must be a concise statement of the relevance of precisely identified testimony to a precisely identified argument or portion of an exhibit (including another part of the same testimony). Any response should be equally concise. An observation (or response) is not an opportunity to raise new issues, to re-argue issues, or to pursue objections. Each observation should be in the following form:

> In exhibit _, on page _, lines_, the witness testified _. This testimony is relevant to the _ on page _ of _. The testimony is relevant because _.

The entire observation should not exceed one short paragraph. The Board might refuse entry of excessively long or argumentative observations (or responses).

¶ 158 Expert testimony; tests and data

¶ 158.1 Expert testimony

¶ 158.1.1 Basis for testimony must be provided

Each affidavit expressing an opinion of an expert must disclose the underlying facts or data upon which the opinion is based. Bd.R. 152; Fed. R. Evid. 705. Opinions expressed without disclosing the underlying facts or data may be given little, or no, weight.

¶ 158.1.2 Expert testimony on patent law

Affidavits of patent law experts on issues of law generally will not be admitted in evidence.

¶ 158.2 Tests and data

¶ 158.2.1 Explanation

Explanation of tests or data must come in the form of an affidavit, preferably accompanied by citation to relevant pages of standard texts (which should be filed as exhibits). In addition to providing the explanation required in Bd.R. 158(b), the proponent of the test evidence should provide any other information it believes would assist the Board in understanding the significance of the test or the data.

¶ 200 Interference procedure

¶ 200.1 Patent claim scope

Every claim before the Board, including a patent claim, is given the broadest reasonable scope consistent with the disclosure with which the claim appears.

¶ 200.2 Pendency

Requests for a stay or an extension are viewed with strong disfavor. Delay in one part of an interference may result in shortening of another part of an interference. An applicant may be required to disclaim patent term for a period related to the delay.

¶ 203 Suggesting another interference; adding an application or a patent

¶ 203.1 Suggesting another interference

Occasionally the declaration of another interference between the same parties or a subset of the parties may be appropriate, for instance, because it would be more faster, less expensive, or more fair. A suggestion to declare another interference must be in the form of a miscellaneous motion. Bd.R. 121(a)(3); SO ¶ **123**. The motion must comply with the requirements of Bd.R. 202(a). The motion must certify that each opponent has been served with a copy of the application file for the applications or patents that would be involved in the new interference.

¶ 203.2 Adding an application or a patent

A suggestion to add an application or patent to an interference must be in the form of a miscellaneous motion. Bd.R. 121(a)(3); SO ¶ **123**. The motion must—

Identify the application or patent to be added;

Certify that a complete copy of the application file for the application or patent has been served on all opponents;

Indicate which claims of the patent or application should be designated as corresponding to the count (and if there is more than one count, which count); and

Explain whether there are alternative remedies; if so, why alternative remedies are not adequate; and what attempts, if any, have been made to have the examiner recommend declaration of another interference involving the application or patent sought to be added to the interference.

¶ 204 Motions list

The motions list is a tool for planning the course of the proceeding, eliminating unnecessary costs and delay, and avoiding abusive practices. All substantive and anticipated responsive motions must be listed on the motions list. No substantive motions or responsive motions may be filed without prior Board authorization. If the need for an unlisted motion arises, the movant should initiate a conference call to obtain such authorization.

¶ 205 Settlement agreements

¶ 205.1 Notice under 35 U.S.C. 135(c)

Notice is hereby given of the requirement of 35 U.S.C. 135(c) for filing in the Office a copy of any agreement "in connection with or in contemplation of the termination of the interference."

¶ 205.2 Petitions under 35 U.S.C. 135(c)

The Chief Administrative Patent Judge decides petitions for acceptance of untimely filed agreements and petitions for access to agreements. A petition under § 135(c) must be formatted as a miscellaneous motion, Bd.R. 121(a)(3), SO ¶ **123**, and served on all parties, Bd.R. 106(e), but no prior Board authorization is required.

¶ 206 Common interests in the invention

The Board generally relies on assignment records of the United States Patent and Trademark Office (SO ¶ **9**) and notices of real parties-in-interest to determine whether common interests in involved cases may require judgment under Bd.R. 206. Failure to notify the Board promptly of a common interest may lead to sanctions, including adverse judgment. Bd.R. 128.

¶ 207 Applicability of prior art

Prior art asserted against an opponent's involved claims is presumed to render the movant's involved claims unpatentable as well. Even if the movant does not adequately contest the presumption, however, the Board may exercise its discretion not to hold the movant's claims unpatentable if in deciding the motion the Board determines the presumption is not appropriate.

¶ 208 Specific substantive motions

The following subparagraphs provide procedural guidance on the necessary content of substantive and responsive motions. Motions must, however, also be substantively sufficient. Thus, even perfect compliance with this guidance does not assure that a movant will satisfy its burden of proof.

¶ 208.1 Obviousness

When obviousness (35 U.S.C. 103) is the basis for a motion for judgment, if a reference does not teach or suggest a limitation, that fact must be explicitly identified as a difference in the statement of material facts. The argument portion of the motion must account for the difference.

An explanation must be made in the body of the motion (not an appendix) why the subject matter of the claim, as a whole, would have been obvious to a person having ordinary skill in the art notwithstanding any difference.

Proving subject matter is not obvious

Proving that subject matter is not obvious may require a negative proof: that no prior art in combination with the subject matter of the count render the claimed subject matter obvious. A party may be able to satisfy its burden of production with testimony from a knowledgeable witness certifying that there is no known prior art that would have overcome the differences between the subject matter of the count and the subject matter of the claim.

¶ 208.2 Adding or substituting a count

A motion to add or substitute a count must—

Propose a new count.

Show why the new count is patentably distinct from every other count the movant believes should remain.

For each of movant's claims, show why the claim does or does not correspond to the proposed new count. Bd.R. 207(b)(1). If none of movant's claims would correspond, then contingently move to add no more than one claim that would correspond (SO ¶ **208.5.1**).

For each opponent's claim that movant believes should correspond to the count, show why the claim corresponds to the proposed new count. If no claim of an opponent would correspond, then contingently move to add no more than one claim to the opponent's involved application or patent.

Accorded benefit

The motion must show why the movant should be accorded any benefit for the proposed count.

The opponent will be presumed to be entitled to its earliest accorded benefit for a proposed count. The movant may overcome this presumption by showing in the motion why the opponent should not be accorded benefit of an earlier application with respect to the proposed count.

¶ 208.3 Claim correspondence

A change in claim correspondence requires comparison of the claim to a count. See Bd.R. 121(e).

¶ 208.3.1 Designating a claim as corresponding

A motion to have a claim designated as corresponding to a count must show why the subject matter of the count, if treated as prior art, would have anticipated or rendered obvious the subject matter of the claim. Bd.R. 207(b)(2).

If no clean or annotated copy of the claim has been filed, then a clean copy, and where applicable an annotated copy, of the claim must be filed as an appendix to the motion. SO ¶ **110**.

A movant seeking to have its own claim designated as corresponding to a count may stipulate that the claim corresponds to the count. The Board is not bound to accept the stipulation. For instance, a stipulation of correspondence that constitutes the sole basis for maintaining an interference-in-fact might be viewed with some skepticism.

¶ 208.3.2 Designating a claim as not corresponding

A motion to have a claim designated as not corresponding to a count must show why the subject matter of the count, if treated as prior art, would not have anticipated or rendered obvious the subject matter of the claim. Bd.R. 207(b)(2).

A movant may not seek to have all of a party's claims designated as not corresponding to the count. Instead, such relief should be sought by way of a motion for judgment of no interference-in-fact.

¶ 208.4 Benefit motions

¶ 208.4.1 For additional accorded benefit

A motion to be accorded the benefit of an application for a particular count must—

- # Certify that a copy of the application has been served on each opponent. See Bd.R. 154(b) (requiring certified translation of documents not in English).

- # If the application was originally filed in the United States Patent and Trademark Office, certify that a complete copy of the application file has been served on each opponent.

- # Show that the application includes at least one constructive reduction to practice of the count. Bd.R. 201 ("Constructive reduction to practice").

¶ 208.4.2 Attacking accorded benefit

A motion to attack the benefit of an application accorded for a count must explain why the application does not provide a constructive reduction to practice for the count. Bd.R. 201 ("Constructive reduction to practice").

¶ 208.5 Responsive motions

¶ 208.5.1 Adding a claim

The claim may only be added to an involved application or patent. Note the requirement under Bd.R. 110(c) to provide clean and annotated copies of the claim. Adding a patent claim generally requires the filing of a reissue application. If the claim is to be added to the movant's application or patent, an amendment (or if necessary a reissue application, see SO ¶ **208.5.4**) must be filed with the motion. A motion to add a claim must—

- # Show the written description for the claim in the disclosure of the involved application or patent to which it would be added.
- # Certify that the movant is not aware of any reason why the claim is not patentable.

A certification that is inconsistent with the prosecution history of an involved or benefit file will be accorded no weight unless the inconsistency is explained. Similarly, if a claim is added to overcome a patentability problem raised in a motion, the motion to add the claim must explain why the proposed claim would overcome the problem.

A motion to add a claim must also comply with the requirements of 37 C.F.R. § 1.75 to the extent they are consistent with the Standing Order. No more than one claim may

be added in response to a motion unless the Board expressly authorizes the addition of more claims.

A claim will not be added if it does not correspond to a count. Correspondence may be stipulated for the movant's claim, but must be shown for an opponent's claim. A showing of claim correspondence requires comparison of the claim to a count. See Bd.R. 121(e).

Opposing a motion to add a claim to an opponent's involved application or patent

If the movant is seeking to add a claim to an opponent's involved application or patent, the opponent may oppose on the basis that the claim is not patentable to the opponent. If the motion is granted, the opponent must add the claim. Failure to add the claim will be treated as a concession that the subject matter of the claim is not patentable to the opponent. Cf. *In re Ogiue*, 517 F.2d 1382, 1390, 186 USPQ 227, 235 (CCPA 1975) (refusal to add claim to permit interference treated as concession).

¶ 208.5.2 Amending a claim

To ensure clarity in the record, amendment of claims is not permitted. Instead, a party may cancel the existing claim and add a new claim as provided above. SO ¶ 208.5.1.

¶ 208.5.3 Substituting an application of the movant

Occasionally it may be necessary to substitute an application for an involved application or patent. For example, the application may be necessary to support an added claim or to provide a better basis for seeking an earlier accorded benefit. A motion to substitute an application must—

Identify the application.

Certify that a complete copy of the application file has been served on each opponent.

¶ 208.5.4 Adding a reissue application

A motion to add the movant's own reissue application must stipulate that every added claim (compared to the original patent) corresponds to a count in the interference. If the reissue application has not been filed in the Office, it must be filed directly with the Board. The Board will see that a filing receipt is promptly issued. Filing directly with the Board avoids processing delays that might prevent consideration of the motion to add the reissue application. The motion must—

Certify that a complete copy of the reissue application file has been served on each opponent.

Make the showings required for adding a claim (SO ¶ **208.5.1**).

Ordinarily, the filing of a reissue application in itself will not precipitate a change in the accorded benefit. If the movant believes that addition of the reissue application would entitle it to different accorded benefit, it should file a contingent motion to change the accorded benefit (SO ¶ **208.4**).

¶ 208.6 Priority

A party seeking judgment on the basis of priority must file a substantive motion seeking such relief. Bd.R. 121(a)(1)(iii).

When diligence is an issue in priority, the priority motion must include as an appendix a diligence chart. The diligence chart must (1) list all days from the beginning of diligence through the end of diligence, (2) state what happened on each day, and

(3) cite the page and line of the motion on which the listed day is discussed. In a case where the invention is "B", an example would be:

Monday, April 5, 2004	Ordered chemical C	p. 17, lines 4-7
Tuesday, April 6, 2004	Waited for chemical C	p. 17, lines 4-7
Wednesday, April 7, 2004	Chemical C arrived	p. 17, lines 4-7
Thursday, April 8, 2004	Used chemical C to make "A"	p. 17, lines 7-8
Friday, April 9, 2004	Sick--not at work	p. 17, lines 18-24
Saturday, April 10, 2004	Weekend	p. 18, lines 2-8
Sunday, April 11, 2004	Weekend	p. 18, lines 2-8
Monday, April 12, 2004	Scheduled vacation	p. 18, lines 2-8
Tuesday April 13, 2004	Scheduled vacation	p. 18, lines 2-8
Wednesday, April 14, 2004	Used "A" to make "B"	p. 17, lines 9-15

Every date gap in the diligence showing must be explained. The fact that there is a gap does not per se establish lack of reasonable diligence. The fact that there is no gap does not per se establish reasonable diligence.

¶ 208.7 Inequitable conduct

A motion alleging inequitable conduct must make out a facially sufficient case of inequitable conduct or fraud. Additional discovery (Bd.R. 150(c)) or a request to take testimony (Bd.R. 156), asserted to be necessary to make out a facially sufficient case, will rarely be authorized. An allegation of inequitable conduct or fraud that fails to make out a facially sufficient case may result in sanctions or a referral to the Office of Enrollment and Discipline.

/Michael R. Fleming/
Chief Administrative Patent Judge

/Marc L. Caroff/
Administrative Patent Judge

/William F. Pate, III/
Administrative Patent Judge

Board of Patent Appeals and Interferences

Trial Division

/John C. Martin/
Administrative Patent Judge

/Fred E. McKelvey/
Senior Administrative Patent Judge

/Richard E. Schafer/
Administrative Patent Judge

/Teddy S. Gron/
Administrative Patent Judge

/Jameson Lee/
Administrative Patent Judge

/Adriene Lepiane Hanlon/
Administrative Patent Judge

/Richard Torczon/
Administrative Patent Judge

/Hubert C. Lorin/
Administrative Patent Judge

/Carol A. Spiegel/
Administrative Patent Judge

/Romulo H. Delmendo/
Administrative Patent Judge

/Sally Gardner Lane/
Administrative Patent Judge

/Sally C. Medley/
Administrative Patent Judge

/Michael P. Tierney/
Administrative Patent Judge

/James T. Moore/
Administrative Patent Judge

/Linda R. Gaudette/
Administrative Patent Judge

/Mark Nagumo/
Administrative Patent Judge

APPENDIX OF FORMS

Form 1. Standard caption for an interference

Filed on behalf of:
[Name of filing party] Paper No. [leave blank]
By: [Name of lead counsel]
[Name of backup counsel]
[Street address]
[City, State, and ZIP Code]
[Telephone number]
[Facsimile number]
[Electronic mail address]

UNITED STATES PATENT AND TRADEMARK OFFICE

_____

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

_____

[Name of junior party]
([Involved application or patent number])
Junior Party,

v.

[Name of Senior party]
([Involved application or patent number])
Senior Party.

_____

Patent Interference [interference number] ([APJ initials])

_____

[TITLE OF PAPER]

Form 2. Typical schedule for motions other than interference priority

Times shown from date of order.

The parties may typically change these six times by stipulation

TIME PERIOD 1 . . . . . Week 6
File substantive motions
File (but serve one week later) priority statements

TIME PERIOD 2 . . . . . Week 9
File responsive motions to motions
filed in TIME PERIOD 1

TIME PERIOD 3 . . . . . Week 15
File oppositions to all motions

TIME PERIOD 4 . . . . . Week 21
File replies

TIME PERIOD 5 . . . . . Week 27
File request for oral argument
File motions to exclude
File observations

TIME PERIOD 6 . . . . . Week 30
File oppositions to motions to exclude
File response to observations

The parties cannot change these three times by stipulation

TIME PERIOD 7 . . . . . Week 32
File replies to oppositions to motions to exclude

TIME PERIOD 8 . . . . . Week 33
File exhibits
File sets of motions

TIME PERIOD 9 . . . . . Week 35
Present oral argument

Form 3. Typical schedule for priority motions in an interference

Times shown from date of order.

The parties may typically change these six times by stipulation

TIME PERIOD 11 . . . . . Week 6
Junior party only file priority brief and serve
(but do not file) priority evidence

TIME PERIOD 12 . . . . . Week 12
Senior party only file priority brief and serve
(but do not file) priority evidence

TIME PERIOD 13 . . . . . Week 18
File opposition to priority briefs
Serve (but do not file) opposition evidence

TIME PERIOD 14 . . . . . Week 24
File reply
Serve (but do not file) reply evidence

TIME PERIOD 15 . . . . . Week 30
Request hearing
File list of issues to be considered
File observations
File motion to exclude

TIME PERIOD 16 . . . . . Week 33
File response to observations
File opposition to motion to exclude

The parties cannot change these three times by stipulation

TIME PERIOD 17 . . . . . Week 35
File reply to opposition to motion to exclude

TIME PERIOD 18 (Last Time) . . . . . Week 36
File and serve the exhibits
File sets of priority motions

TIME PERIOD 19 . . . . . Week 38
Present oral argument

Form 4. File copy request

FILE COPY REQUEST
Contested Case No. [Contested Case number]

Attach a copy of sections E and F of the DECLARATION to this REQUEST. On the copy, circle each patent and application that you are requesting.

Include the following information to facilitate processing of this REQUEST:

1. Charge fees to USPTO Deposit Account No. _____

2. Complete address, including street, city, state, zip code and telephone number (do not list a Post Office box because file copies are sent by commercial overnight courier).

_____

_____

_____

_____

3. Telephone, including area code: _____

APPENDIX: CROSS EXAMINATION GUIDELINES

Introduction

Cross examination can be a useful tool for determining the facts in a case. In contested cases, direct testimony is usually presented by affidavit, Bd.R. 157(a), while cross examination occurs by oral deposition. Bd.R. 157(b).

Cross examination should be a question-and-answer conversation between the examining lawyer and the witness. The defending lawyer must not act as an intermediary, interpreting questions, deciding which questions the witness should answer and helping the witness formulate answers. The witness comes to the cross examination to be questioned. It is the witness, and not the lawyer, who is testifying.

The cross-examination guidelines below are essentially the deposition guidelines set out in *Hall v. Clifton Precision*, 150 F.R.D. 525 (E.D. Pa. 1993) (Gawthrop, J.) The only significant difference, which results from Bd.R. 157(e)(4), is that certain objections must be noted on the record.

Failure to adhere strictly to these guidelines may be a basis for a sanction under Bd.R. 128, which could include a requirement that the witness, on very short notice, may be directed to appear before the Board or elsewhere, as may be appropriate, coupled with any appropriate award of compensatory damages under Bd.R. 128(b)(6). In addition, cross examination undertaken contrary to these guidelines may result in exclusion of an affidavit from evidence or in the assignment of little, if any weight, to the direct testimony of a witness who was cross examined.

Guideline [1]

At the beginning of a cross examination, the party conducting the cross examination must instruct the witness on the record to ask deposing counsel, rather than the witness's own counsel, for clarifications, definitions or explanations of any words, questions or documents presented during the cross examination. The witness must follow these instructions.

Guideline [2]

A party may not direct or request that a witness not answer a question unless:

(a) a party has objected to the question on the ground that the answer would:
    (1) reveal privileged material or
    (2) violate a limitation the Board has imposed and
(b) counsel immediately places a conference call to the Board official assigned to the contested case asking for a ruling on the objection.

Under these circumstances, (i) the cross examination shall be suspended, (ii) the conference call immediately shall be placed to the Board official assigned to the contested case, and (iii) all counsel must be prepared to explain their respective positions during the call. The court reporter for the cross examination shall be available to record the conference call and to read back questions to which an objection has been made.

If the Board cannot be reached, then the party directing a witness not to answer shall within **two (2) business days** file with the Board (and not to the Office Mail Room or any other part of the Office) a miscellaneous motion seeking relief. Bd.R. 121(a)(3), SO ¶ **123**. Any opposition must be filed within **two (2) business days** of service of the motion. While a reply can be filed, the motion will likely be decided before the reply is filed.

Guideline [3]

Counsel must not make objections or statements that even remotely suggest an answer to a witness. Any objection to evidence during cross examination must be stated concisely and in a non-argumentative and non-suggestive manner and must include the legal basis for the objection. Examining counsel must not address the correctness of an objection, but may instead continue with questions to the witness, the objection having been noted on the record as required under Bd.R. 157(e)(4).[1]

Guideline [4]

Counsel and their witness-clients shall not engage in private, off-the-record conferences during cross examinations or during breaks or recesses, except for the purpose of deciding whether to assert a privilege.[2]

---

[1] With respect to this guideline, the following observation by Judge Gawthrop, 150 F.R.D. at 530 n.10, is highly relevant:

> I also note that a favorite objection or interjection of lawyers is, "I don't understand the question; therefore the witness doesn't understand the question." This is not a proper objection. If the witness needs clarification, the witness may ask the deposing lawyer for clarification. A lawyer's purported lack of understanding is not a proper reason to interrupt a deposition. In addition, counsel are not permitted to state on the record their interpretations of questions, since those interpretations are irrelevant and often suggestive of a particularly desired answer.

By way of example, the following comments by defending counsel generally are viewed as suggesting an answer to a witness:

(a) Objection, vague.
(b) Objection to the form of the question.
(c) Take your time in answering the question.
(d) Look at the document before you answer.
(e) Counsel, do you want to show the witness the document?

[2] The term "witness-clients" in the context of this guideline includes all witnesses who are employed by, or otherwise under the control of, the real party-in-interest, including retained expert witnesses, as well as the individual or individuals named in the caption of the contested case. With respect to this guideline, the following observation by Judge Gawthrop, 150 F.R.D. at 528, is highly relevant:

> The fact that there is no judge in the room to prevent private conferences does not mean that such conferences should or may occur. The underlying reason for preventing private conferences is still present: they tend, at the very least, to give the appearance of obstructing the truth.

Guideline [5]

Any conferences that occur pursuant to, or in violation of, guideline [4] are a proper subject for inquiry by deposing counsel to ascertain whether there has been any witness-coaching and, if so, the nature of that coaching.

Guideline [6]

Any conferences that occur pursuant to, or in violation of, guideline [4] shall be noted on the record by the counsel who participated in the conference. The purpose and outcome of the conference shall also be noted on the record.

Guideline [7]

Counsel taking cross-examination shall provide to defending counsel a copy of all documents shown to the witness during the cross examination. The copies shall be provided either before the cross examination begins or contemporaneously with the showing of each document to the witness. The witness and defending counsel do not have a right to discuss documents privately before the witness answers questions about the documents.

APPENDIX: INDEX OF TIMES

Times running from initiation/declaration

Notice of lead and backup counsel (Bd.R. 108(b)) . . . . . 14 days

Clean copy of claims (Bd.R. 110(a)) . . . . . 14 days

Notice of real party-in-interest (SO ¶ **8.1**) . . . . . 14 days

Notice of related proceedings (SO ¶ **8.2**) . . . . . 14 days

Request for file copies (SO ¶ **109.1**) . . . . . 14 days

Annotated copy of claims (Bd.R. 110(b)) . . . . . 28 days

Notice of confidential information (SO ¶ **6.2**) . . . . . 2 months

Initial settlement conference (SO ¶ **126.2**) . . . . . 3 months

Default times before a triggering event

Filing of motions list (SO ¶ **104.2.1**) before conference . . . . . 4 business days

Service of demonstrative exhibit before oral argument (Bd.R. 124(d)) . 5 business days

Notice of transcription before oral argument (SO ¶ **124.5**) . . . . . 1 business day

End of cross examination before opposition or reply (SO ¶ **157.3.2**) . . . . . 10 days

List of documents and things for cross examination before conference call (Bd.R. 157(c)(3)) . . . . . 3 business days

Notice of deposition before deposition (Bd.R. 157(c)(4)) . . . . . 2 business days

Conference call regarding interpreter before deposition (Bd.R. 157(d)) 5 business days

Default times after a triggering event

Notice of change in real party-in-interest (Bd.R. 8(a)(1)) . . . . . 20 days

Notice of change in related proceedings (Bd.R. 8(a)(2)) . . . . . 20 days

Notice of missing or incomplete copies (Bd.R. 109(c)) . . . . . 21 days

Notice of change in counsel (SO ¶ **108**) . . . . . 14 days

Service of requested automatic discovery materials (Bd.R. 150(b)(1)) . . . . . 21 days

Objection to admissibility of evidence (Bd.R. 155(b)(1)) . . . . . 5 business days

Service of supplemental evidence (Bd.R. 155(b)(2)) . . . . . 10 business days

Start of cross examination of affiant after service of affidavit (SO ¶ **157.3.1**) . . . 21 days

Opposition to motion (other than miscellaneous motion) (Bd.R. 123(a)(1)) . . . . 30 days

Reply to opposition (other than miscellaneous motion) (Bd.R. 123(a)(2)) . . . . . 30 days

Responsive motion (Bd.R. 123(a)(3)) . . . . . 30 days

Opposition to miscellaneous motion (Bd.R. 123(b)(2)(i)) . . . . . 5 business days

Reply to opposition to miscellaneous motion (Bd.R. 123(b)(2)(ii)) . . . . 3 business days

Request oral argument (Bd.R. 124(a)) . . . . . 5 business days

Request for rehearing of decision (Bd.R. 125(c)(1)) . . . . . 14 days

Identification of arbitrator after arbitration agreement (Bd.R. 126(a)(3)(iii)) . . . . 30 days

Copy of executed arbitration agreement (Bd.R. 126(b)(4)) . . . . . 20 days

Arbitration award after date of award (Bd.R. 126(d)(4)) . . . . . 20 days

Settlement conference after substantive motions decision (SO ¶ **126.3**) . . . . . 2 months

Request for rehearing of judgment (Bd.R. 127(d)) . . . . . 30 days

Notice of judicial review (Bd.R. 8(b)) . . . . . 20 days

Filed on behalf of: **Adair** Paper No:_____
By: Doreen Yatko Trujillo
Michael B. Fein
Cozen O'Connor P.C.
1900 Market St.
Philadelphia, PA 19103
Telephone: (215) 665-5593
Facsimile: (215) 701-2005
dtrujillo@cozen.com

UNITED STATES PATENT AND TRADEMARK OFFICE

_____

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

_____

PAUL J. **CARTER** AND LEONARD G. PRESTA
Junior Party
(Patent 6,407,213),

v.

JOHN ROBERT **ADAIR,** DILJEET SINGH ATHWAL,
AND JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

Patent Interference No. 105,744
(Technology Center 1600)

ADAIR DESIGNATION OF LEAD COUNSEL

Pursuant to Bd. R. 108(b) and SO ¶ 108, the party Adair designates Doreen Yatko Trujillo, Reg. No. 35,719, as its lead counsel and Michael B. Fein, Reg. No. 25,333, as its backup lead counsel.

Please direct all future correspondence and telephone calls in connection with this interference to the following as lead counsel and backup lead counsel:

Lead Counsel:

Doreen Yatko Trujillo
Cozen O'Connor P.C.
1900 Market St.
Philadelphia, PA 19103
Telephone: (215) 665-5593
Facsimile: (215) 701-2005
dtrujillo@cozen.com

If the above-designated lead counsel should not be available, backup lead counsel, Michael B. Fein, can be contacted at the same address as given above.

Backup Lead Counsel:

Michael B. Fein
Telephone: (215) 665-4622
Facsimile: (215) 701-2246
mfein@cozen.com

Respectfully submitted,

/Doreen Yatko Trujillo/
DOREEN YATKO TRUJILLO
Registration No. 35,719
Lead Counsel for Adair

Date: February 16, 2010

Cozen O'Connor P.C.
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Philadelphia, PA 19103
Telephone: (215) 665-5593
Facsimile: (215) 701-2005
dtrujillo@cozen.com

Certificate of Service

This will certify that a true copy of this paper was served this date on the party Carter by Federal Express overnight mail directed to:

SIDLEY AUSTIN, LLP
Attn: DC Patent Docketing
1501 K Street, N.W.
Washington, DC 20005

Date: February 16, 2010

/Doreen Yatko Trujillo/
Doreen Yatko Trujillo

Filed on behalf of: **Adair** Paper No:_____
By: Doreen Yatko Trujillo
Michael B. Fein
Cozen O'Connor P.C.
1900 Market St.
Philadelphia, PA 19103
Telephone: (215) 665-5593
Facsimile: (215) 701-2005
dtrujillo@cozen.com

UNITED STATES PATENT AND TRADEMARK OFFICE

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BEFORE THE BOARD OF PATENT APPEALS
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PAUL J. **CARTER** AND LEONARD G. PRESTA
Junior Party
(Patent 6,407,213),

v.

JOHN ROBERT **ADAIR,** DILJEET SINGH ATHWAL,
AND JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

Patent Interference No. 105,744
(Technology Center 1600)

ADAIR CLEAN COPY OF CLAIMS

Pursuant to Bd. R. 110(a) and SO ¶ 110, a clean copy of all the claims currently pending in U.S. Application Serial No. 11/284,261 is enclosed herewith.

Respectfully submitted,

/Doreen Yatko Trujillo/
DOREEN YATKO TRUJILLO
Registration No. 35,719
Lead Counsel for Adair

Date: February 16, 2010

Cozen O'Connor P.C.
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Philadelphia, PA 19103
Telephone: (215) 665-5593
Facsimile: (215) 01-2005
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SIDLEY AUSTIN, LLP
Attn: DC Patent Docketing
1501 K Street, N.W.
Washington, DC 20005

Date: February 16, 2010

/Doreen Yatko Trujillo/
Doreen Yatko Trujillo

Claim 24: A humanised antibody comprising a heavy chain variable domain comprising non-human complementarity determining region amino acid residues which bind an antigen and a human framework region wherein said framework region comprises a non-human amino acid substitution at a residue selected from the group consisting of 23, 24, 49, 71, 73, and 78, and combinations thereof, as numbered according to Kabat.

Filed on behalf of: **Adair**
By: Doreen Yatko Trujillo
Michael B. Fein
Cozen O'Connor P.C.
1900 Market St.
Philadelphia, PA 19103
Telephone: (215) 665-5593
Facsimile: (215) 701-2005
dtrujillo@cozen.com

Paper No:_____

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AND JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

Patent Interference No. 105,744
(Technology Center 1600)

ADAIR NOTICE OF RELATED PROCEEDINGS

Pursuant to Standing Order ¶ 8.2, the party Adair hereby states that no application claims the benefit of priority of the filing date of the party's involved application.

Pursuant to Bd. R. 41.8, Adair advises that it is involved in two other interferences involving applications that claim priority to Application Serial No. 08/846,658, to which the present application also claims priority. They are Interference Nos. 105,688 and 105,705, involving U.S. Patent Nos. 5,585,089 and 6,180,379, respectively.

Respectfully submitted,

/Doreen Yatko Trujillo/
DOREEN YATKO TRUJILLO
Registration No. 35,719
Lead Counsel for Adair

Date: February 16, 2010

Cozen O'Connor P.C.
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1501 K Street, N.W.
Washington, DC 20005

Date: February 16, 2010

/Doreen Yatko Trujillo/
Doreen Yatko Trujillo

Filed on behalf of: **Adair**
By: Doreen Yatko Trujillo
Michael B. Fein
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1900 Market St.
Philadelphia, PA 19103
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Paper No:_____

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS
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PAUL J. **CARTER** AND LEONARD G. PRESTA
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(Patent 6,407,213),

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JOHN ROBERT **ADAIR,** DILJEET SINGH ATHWAL,
AND JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

Patent Interference No. 105,744
(Technology Center 1600)

ADAIR REQUEST FOR FILE COPIES

A completed File Copy Request, and Sections E and F of the Declaration, are attached.

Respectfully submitted,

/Doreen Yatko Trujillo/
DOREEN YATKO TRUJILLO
Registration No. 35,719
Lead Counsel for Adair

Date: February 16, 2010

Cozen O'Connor P.C.
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Attn: DC Patent Docketing
1501 K Street, N.W.
Washington, DC 20005

Date: February 16, 2010

/Doreen Yatko Trujillo/
Doreen Yatko Trujillo

Involved Patent:	6,407,213, issued 18 June 2002, from application 08/146,206, which was filed 17 November 1993, and was based on international application PCT/US92/05126, filed 15 June 1992.
Title:	METHOD FOR MAKING HUMANIZED ANTIBODIES
Assignee:	Genentech, Inc.

Senior Party

Named Inventors:	John Robert Adair High Wycombe, United Kingdom Diljeet Singh Athwal London, United Kingdom John Spencer Emtage Marlow, United Kingdom
Involved Application:	11/284,261, filed 21 November 2005
Title:	HUMANISED ANTIBODIES
Assignee:	Celltech R & D Limited

The senior party is assigned exhibit numbers 1001-1999. The junior party is assigned exhibit numbers 2001-2999. Bd. R. 154(c)(1); SO ¶ 154.2.1. The senior party is responsible for initiating settlement discussions. SO ¶ 126.1.

Part B. Judge managing the interference

Administrative Patent Judge Sally Gardner Lane has been designated to manage the interference. Bd. R. 104(a).

Part C. Standing order

A Trial Section STANDING ORDER [SO] (Paper 2) accompanies this DECLARATION. The STANDING ORDER applies to this interference.

Part D. Initial conference call

A telephone conference call to discuss the interference is set for **2:00 p.m. on 16 March 2010** (the Board will initiate the call).

No later than **four business days** prior to the conference call, each party shall file and serve (SO ¶¶ 10.1 & 105) a list of the motions (Bd. R. 120; Bd. R. 204; SO ¶¶ 104.2.1, 120 & 204) the party intends to file.

A sample schedule for taking action during the motion phase appears as Form 2 in the STANDING ORDER. Counsel are encouraged to discuss the schedule prior to the conference call and to agree on dates for taking action. A typical motion period lasts approximately eight (8) months. Counsel should be prepared to justify any request for a shorter or longer period.

Part E. Identification and order of the parties

Junior Party

Named inventors: Paul J. Carter
San Francisco, CA

Leonard G. Presta
San Francisco, CA

Part F. Count and claims of the parties

Count 1

A humanized antibody heavy chain variable domain comprising non-human Complementarity Determining Region (CDR) amino acid residues which bind antigen incorporated into a human antibody variable domain, and further comprising a Framework Region (FR) amino acid substitution at a site selected from the group consisting of: 24H, 71H, 73H, and 78H, utilizing the numbering system set forth in Kabat.

The claims of the parties are:

Carter: 1-82

Adair: 24

The claims of the parties which correspond to Count 1 are:

Carter: 30, 31, 60, 62, 63, 66, 67, 70, 73, 77-81

Adair: 24

The claims of the parties which do not correspond to Count 1, and therefore are not involved in the interference, are:

Carter: 1-29, 32-59, 61, 64, 65, 68, 69, 71, 72, 74-76, 82

Adair: None

The parties are accorded the following benefit for Count 1:

Carter: PCT/US92/05126, filed 15 June 1992; and
07/715,272, filed 14 June 1991, now abandoned.

Adair: 08/846,658, filed 01 May 1997;
08/303,569, filed 07 September 1994, issued as 5,859,205 on 12 January 1999;
07/743,329, filed on 17 September 1991;
PCT/GB90/02017, filed 21 December 1990; and
GB 8928874.0, filed 21 December 1989.

Part G. Heading to be used on papers

The following heading must be used on all papers filed in this interference, see SO & 106.1.1:

PAUL J. **CARTER** AND LEONARD G. PRESTA
Junior Party
(Patent 6,407,213),

v.

JOHN ROBERT **ADAIR**, DILJEET SINGH ATHWAL,
AND JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

Patent Interference No. 105,744
(Technology Center 1600)

Form 4. File copy request

FILE COPY REQUEST
Contested Case No. [Contested Case number]

Attach a copy of sections E and F of the DECLARATION to this REQUEST. On the copy, circle each patent and application that you are requesting.

Include the following information to facilitate processing of this REQUEST:

1. Charge fees to USPTO Deposit Account No. 503111

2. Complete address, including street, city, state, zip code and telephone number (do not list a Post Office box because file copies are sent by commercial overnight courier).

Doreen Yatko Trujillo
Cozen O'Connor P.C.
1900 Market St.
Philadelphia, PA 19103

3. Telephone, including area code: 215-665-5593

Paper No. _____

Filed on behalf of: Party Carter

Filed: February 19, 2010

By: Oliver R. Ashe, Jr., Esq.
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Jeffrey P. Kushan, Esq.
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1501 K Street, N.W.
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Fax: (202) 736-8711
E-mail: jkushan@sidley.com

UNITED STATES PATENT AND TRADEMARK OFFICE

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BEFORE THE BOARD OF PATENT APPEALS
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AND JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

_____

Patent Interference 105,744 (SGL)
Technology Center 1600

_____

CARTER DESIGNATION OF LEAD AND BACKUP LEAD COUNSEL

CARTER DESIGNATION OF LEAD AND BACKUP LEAD COUNSEL

In accordance with 37 C.F.R. § 41.108(b) and ¶ 108 of the Standing Order (Paper No. 2), Carter hereby identifies the following lead and backup lead counsel in the above-identified interference:

Lead Counsel:	Oliver R. Ashe, Jr. Registration No. 40,491
Backup Lead Counsel:	Jeffrey P. Kushan Registration No. 43,401

The respective mailing address, telephone number, facsimile number, and internet e-mail address for Mr. Ashe and Mr. Kushan are as follows:

By:

Oliver R. Ashe, Jr., Esq.
ASHE, P.C.
11440 Isaac Newton Sq. North
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Reston, VA 20190
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Washington, DC 20005
Tel.: (202) 736-8914
Fax: (202) 736-8711
E-mail: jkushan@sidley.com

Carter is concurrently filing a paper titled "Carter Submission of Power of Attorney," which includes an appointment of attorney for Mr. Ashe.

Respectfully submitted,

February 19, 2010

/Oliver R. Ashe, Jr./
Oliver R. Ashe, Jr.
Registration No. 40,491
Counsel for Party Carter

ASHE, P.C.
11440 Isaac Newton Sq. North
Suite 210
Reston, VA 20190
Tel.: (703) 467-9001
Fax: (703) 467-9002
E-mail: oashe@ashepc.com

CERTIFICATE OF FILING

The undersigned certifies that a copy of the paper entitled **"CARTER DESIGNATION OF LEAD AND BACKUP LEAD COUNSEL"** was filed this 19th day of February, 2010, by e-mail, to:

The Board of Patent Appeals and Interferences
Madison Building East, 9th Floor
600 Dulany Street
Alexandria, VA 22314
Tel.: 571-272-9797
Fax: 571-273-0042
E-mail: BoxInterferences@USPTO.GOV

February 19, 2010 /Oliver R. Ashe, Jr./
Oliver R. Ashe, Jr.

CERTIFICATE OF SERVICE

The undersigned hereby certifies that a copy of the paper entitled **"CARTER DESIGNATION OF LEAD AND BACKUP LEAD COUNSEL"** was served this 19th day of February, 2010, by e-mail, on the Attorney of Record for Adair:

Doreen Yatko Trujillo, Esq.
Cozen O'Connor P.C.
1900 Market Street, 7th Floor
Philadelphia, PA 19103
Tel.: 215-665-6593
Fax: 215-701-2005
E-mail: dtrujillo@cozen.com

February 19, 2010 /Oliver R. Ashe, Jr./
Oliver R. Ashe, Jr.

Paper No. _____

Filed on behalf of: Party Carter

Filed: February 19, 2010

By: Oliver R. Ashe, Jr., Esq.
ASHE, P.C.
11440 Isaac Newton Sq. North
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Reston, VA 20190
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E-mail: jkushan@sidley.com

UNITED STATES PATENT AND TRADEMARK OFFICE

_____

BEFORE THE BOARD OF PATENT APPEALS
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(Patent 6,407,213),

v.

JOHN ROBERT **ADAIR**, DILJEET SINGH ATHWAL,
AND JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

_____

Patent Interference 105,744 (SGL)
Technology Center 1600

_____

CARTER SUBMISSION OF POWER OF ATTORNEY

CARTER SUBMISSION OF POWER OF ATTORNEY

In accordance with 37 C.F.R. § 41.108(a) and ¶ 108 of the Standing Order (Paper No. 2), attached hereto is a Power of Attorney and Statement Under 37 C.F.R. § 3.73(b), recognizing for representation the attorney associated with Customer Number 000066181 on U.S. Patent Application No. 08/146,206, filed November 17, 1993, now U.S. Patent No. 6,407,213, issued June 18, 2002.

Carter is concurrently filing a paper titled "Carter Designation of Lead and Backup Lead Counsel" in which Oliver R. Ashe, Jr. and Jeffrey P. Kushan are identified as Lead Counsel (Mr. Ashe) and Back-up Lead Counsel (Mr. Kushan) for Carter in the above-identified interference.

Respectfully submitted,

February 19, 2010

/Oliver R. Ashe, Jr./
Oliver R. Ashe, Jr.
Registration No. 40,491
Counsel for Party Carter

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11440 Isaac Newton Sq. North
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Reston, VA 20190
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Fax: (703) 467-9002
E-mail: oashe@ashepc.com

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants :	Paul J. Carter et al.	Conf. No.:	3992
Appln No. :	08/146,206	Examiner:	Davis, Minh Tam B
Filed:	November 17, 1993	Art Unit:	1642
U.S. Patent No.:	6,407,213	Issued:	June 18, 2002
For :	*METHOD FOR MAKING HUMANIZED ANTIBODIES*		

Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

POWER OF ATTORNEY
AND
STATEMENT UNDER 37 C.F.R. § 3.73(B)

The assignee of the entire right, title, and interest in U.S. Patent No. 6,407,213, Genentech, Inc., hereby appoints the attorney associated with

Customer Number 000066181

as attorney for the above-identified patent to represent the assignee in any and all interference proceedings and to transact all business in the Patent and Trademark Office connected therewith.

Pursuant to 37 C.F.R. § 3.73(b), the undersigned states that Genentech, Inc. is the assignee of the entire right, title and interest in the above-identified patent application by virtue of the assignment from the inventors to Genentech, Inc., as recorded in U.S. Patent No. 6,407,213 on June 28, 1994, at Reel 007035, Frame 0272.

The undersigned, whose title is supplied below, is authorized to act on behalf of the assignee.

Respectfully submitted,
Genentech, Inc.

Feb. 19, 2010
Date

Atulya R. Agarwal
Name:
Title: **Atulya R. Agarwal, Ph.D., J.D.**
Associate General Counsel, Director
Genentech, Inc.
Authorized Corporate Signatory

CERTIFICATE OF FILING

The undersigned certifies that a copy of the paper entitled **"CARTER SUBMISSION OF POWER OF ATTORNEY"** attaching a copy of the Power of Attorney and Statement Under 37 C.F.R. § 3.73(b) was filed this 19th day of February, 2010, by e-mail, to:

The Board of Patent Appeals and Interferences
Madison Building East, 9th Floor
600 Dulany Street
Alexandria, VA 22314
Tel.: 571-272-9797
Fax: 571-273-0042
E-mail: BoxInterferences@USPTO.GOV

February 19, 2010 /Oliver R. Ashe, Jr./
Oliver R. Ashe, Jr.

CERTIFICATE OF SERVICE

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Doreen Yatko Trujillo, Esq.
Cozen O'Connor P.C.
1900 Market Street, 7th Floor
Philadelphia, PA 19103
Tel.: 215-665-6593
Fax: 215-701-2005
E-mail: dtrujillo@cozen.com

February 19, 2010 /Oliver R. Ashe, Jr./
Oliver R. Ashe, Jr.

Paper No. _____

Filed on behalf of: Party Carter

Filed: February 19, 2010

By: Oliver R. Ashe, Jr., Esq.
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Reston, VA 20190
Tel.: (703) 467-9001
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Jeffrey P. Kushan, Esq.
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1501 K Street, N.W.
Washington, DC 20005
Tel.: (202) 736-8914
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E-mail: jkushan@sidley.com

UNITED STATES PATENT AND TRADEMARK OFFICE

_____

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

_____

PAUL J. **CARTER** AND LEONARD G. PRESTA
Junior Party
(Patent 6,407,213),

v.

JOHN ROBERT **ADAIR**, DILJEET SINGH ATHWAL,
AND JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

_____

Patent Interference 105,744 (SGL)
Technology Center 1600

_____

CARTER NOTICE OF REAL PARTY IN INTEREST

CARTER NOTICE OF REAL PARTY IN INTEREST

In accordance with 37 C.F.R. § 41.8(a)(1) and ¶ 8.1 of the Standing Order (Paper No. 2), Carter hereby notifies the Board that any and all right, title, or interest in the involved U.S. Patent No. 6,407,213 is assigned to Genentech, Inc.

Respectfully submitted,

February 19, 2010

/Oliver R. Ashe, Jr./
Oliver R. Ashe, Jr.
Registration No. 40,491
Counsel for Party Carter

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11440 Isaac Newton Sq. North
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Fax: (703) 467-9002
E-mail: oashe@ashepc.com

CERTIFICATE OF FILING

The undersigned certifies that a copy of the paper entitled **"CARTER NOTICE OF REAL PARTY IN INTEREST"** was filed this 19th day of February, 2010, by e-mail, to:

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February 19, 2010 /Oliver R. Ashe, Jr./
Oliver R. Ashe, Jr.

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February 19, 2010 /Oliver R. Ashe, Jr./
Oliver R. Ashe, Jr.

Paper No. _____
Filed on behalf of: Party Carter
Filed: February 19, 2010

By: Oliver R. Ashe, Jr., Esq.
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UNITED STATES PATENT AND TRADEMARK OFFICE

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BEFORE THE BOARD OF PATENT APPEALS
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Junior Party
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v.

JOHN ROBERT **ADAIR**, DILJEET SINGH ATHWAL,
AND JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

_____

Patent Interference 105,744 (SGL)
Technology Center 1600

_____

CARTER NOTICE OF RELATED PROCEEDINGS

CARTER NOTICE OF RELATED PROCEEDINGS

In accordance with ¶ 8.2 of the Standing Order (Paper No. 2), Carter hereby notifies the Board that the following applications claim the benefit of priority of the November 17, 1993, filing date of Carter's involved U.S. Patent No. 6,407,213.

1. U.S. Patent Application No. 08/439,004, filed May 11, 1995, now abandoned;
2. U.S. Patent Application No. 09/115,800, filed July 15, 1998, now abandoned;
3. U.S. Patent Application No. 09/705,392, filed November 2, 2000, now U.S. Patent No. 6,719,971, issued April 13, 2004;
4. U.S. Patent Application No. 09/705,398, filed November 2, 2000, now U.S. Patent No. 6,800,738, issued October 5, 2004;
5. U.S. Patent Application No. 09/705,686, filed November 2, 2000, now U.S. Patent No. 6,639,055, issued October 28, 2003;
6. U.S. Patent Application No. 10/835,641, filed April 30, 2004, now abandoned;
7. U.S. Patent Application No. 11/444,791, filed June 1, 2006, now abandoned; and
8. U.S. Patent Application No. 11/969,430, now pending.

Respectfully submitted,

February 19, 2010

/Oliver R. Ashe, Jr./
Oliver R. Ashe, Jr.
Registration No. 40,491
Counsel for Party Carter

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February 19, 2010 /Oliver R. Ashe, Jr./
Oliver R. Ashe, Jr.

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February 19, 2010 /Oliver R. Ashe, Jr./
Oliver R. Ashe, Jr.

Paper No. _____
Filed on behalf of: Party Carter
Filed: February 19, 2010

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E-mail: jkushan@sidley.com

UNITED STATES PATENT AND TRADEMARK OFFICE

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BEFORE THE BOARD OF PATENT APPEALS
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PAUL J. **CARTER** AND LEONARD G. PRESTA
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v.

JOHN ROBERT **ADAIR**, DILJEET SINGH ATHWAL,
AND JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

_____

Patent Interference 105,744 (SGL)
Technology Center 1600

_____

CARTER CLEAN COPY OF CLAIMS

CARTER CLEAN COPY OF CLAIMS

In accordance with 37 C.F.R. § 41.110(a), Carter herewith provides a clean copy of Carter's involved claim, *i.e.*, claims 30, 31, 60, 62, 63, 66, 67, 70, 73, and 77-81 of U.S. Patent No. 6,407,213.

Respectfully submitted,

February 19, 2010

/Oliver R. Ashe, Jr./
Oliver R. Ashe, Jr.
Registration No. 40,491
Counsel for Party Carter

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E-mail: oashe@ashepc.com

Clean Copy of Carter's Involved Claims in U.S. Patent No. 6,407,213

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

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

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

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

63. A humanized antibody which lacks immunogenicity compared to a non-human parent antibody upon repeated administration to a human patient in order to treat a

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

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

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

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

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

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

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

79. A humanized variant of a non-human parent antibody which binds an antigen, wherein the humanized variant comprises Complementarity Determining Region

(CDR) amino acid residues of the non-human parent antibody incorporated into a human antibody variable domain, and further comprises Framework Region (FR) substitutions at heavy chain positions 71H, 73H, 78H and 93H, utilizing the numbering system set forth in Kabat.

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

(a) noncovalently binds antigen directly;

(b) interacts with a CDR; or

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

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

CERTIFICATE OF FILING

The undersigned certifies that a copy of the paper entitled **"CARTER CLEAN COPY OF CLAIMS"** attaching a clean set of Carter's involved claims was filed this 19th day of February, 2010, by e-mail, to:

The Board of Patent Appeals and Interferences
Madison Building East, 9th Floor
600 Dulany Street
Alexandria, VA 22314
Tel.: 571-272-9797
Fax: 571-273-0042
E-mail: BoxInterferences@USPTO.GOV

February 19, 2010 /Oliver R. Ashe, Jr./
Oliver R. Ashe, Jr.

CERTIFICATE OF SERVICE

The undersigned hereby certifies that a copy of the paper entitled **"CARTER CLEAN COPY OF CLAIMS"** attaching a clean set of Carter's involved claims was served this 19th day of February, 2010, by e-mail, on the Attorney of Record for Adair:

Doreen Yatko Trujillo, Esq.
Cozen O'Connor P.C.
1900 Market Street, 7th Floor
Philadelphia, PA 19103
Tel.: 215-665-6593
Fax: 215-701-2005
E-mail: dtrujillo@cozen.com

February 19, 2010 /Oliver R. Ashe, Jr./
Oliver R. Ashe, Jr.

Paper No. _____

Filed on behalf of: Party Carter

Filed: February 19, 2010

By: Oliver R. Ashe, Jr., Esq.
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Fax: (703) 467-9002
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Jeffrey P. Kushan, Esq.
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Washington, DC 20005
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Fax: (202) 736-8711
E-mail: jkushan@sidley.com

UNITED STATES PATENT AND TRADEMARK OFFICE

_____

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

_____

PAUL J. **CARTER** AND LEONARD G. PRESTA
Junior Party
(Patent 6,407,213),

v.

JOHN ROBERT **ADAIR**, DILJEET SINGH ATHWAL,
AND JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

_____

Patent Interference 105,744 (SGL)
Technology Center 1600

_____

CARTER REQUEST FOR FILE COPIES

CARTER REQUEST FOR FILE COPIES

In accordance with 37 C.F.R. § 41.109(a) and ¶ 109.1.1 of the Standing Order (Paper No. 2), Carter hereby requests copies of the following files:

Adair Files:

(i) U.S. Patent Application No. 11/284,261, filed November 21, 2005;

(ii) U.S. Patent Application No. 08/846,658, filed May 1, 1997;

(iii) U.S. Patent Application No. 08/303,569, filed September 7, 1994, now U.S. Patent No. 5,859,205, issued January 12, 1999;

(iv) U.S. Patent Application No. 07/743,329, filed September 17, 1991, now abandoned;

(v) PCT Application No. PCT/GB90/02017, filed December 21, 1990; and

(vi) UK Application No. GB 8928874.0, filed December 21, 1989.

Carter Files:

(i) U.S. Patent Application No. 08/146,206, filed November 17, 1993, now U.S. Patent No. 6,407,213, issued June 18, 2002;

(ii) PCT Application No. PCT/US92/05126, filed June 15, 1992; and

(iii) U.S. Patent Application No. 07/715,272, filed June 14, 1991, now abandoned.

Please ship the files to:

Oliver R. Ashe, Jr., Esq.
ASHE, P.C.
11440 Isaac Newton Sq. North
Suite 210
Reston, VA 20190
Tel.: (703) 467-9001
Fax: (703) 467-9002

In accordance with Part H of the Notice Declaring Interference (Paper No. 1) and ¶ 109 of the Standing Order (Paper No. 2), a copy of Parts E and F of the Notice Declaring Interference with a hand-drawn circle around the patents and applications for which a copy of a file wrapper is requested and a copy of completed File Copy Request (SO Form 4) are attached hereto.

Respectfully submitted,

February 19, 2010

/Oliver R. Ashe, Jr./
Oliver R. Ashe, Jr.
Registration No. 40,491
Counsel for Party Carter

ASHE, P.C.
11440 Isaac Newton Sq. North
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Fax: (703) 467-9002
E-mail: oashe@ashepc.com

Part B. Judge managing the interference

Administrative Patent Judge Sally Gardner Lane has been designated to manage the interference. Bd. R. 104(a).

Part C. Standing order

A Trial Section STANDING ORDER [SO] (Paper 2) accompanies this DECLARATION. The STANDING ORDER applies to this interference.

Part D. Initial conference call

A telephone conference call to discuss the interference is set for 2:00 p.m. on 16 March 2010 (the Board will initiate the call).

No later than four business days prior to the conference call, each party shall file and serve (SO ¶¶ 10.1 & 105) a list of the motions (Bd. R. 120; Bd. R. 204; SO ¶¶ 104.2.1, 120 & 204) the party intends to file.

A sample schedule for taking action during the motion phase appears as Form 2 in the STANDING ORDER. Counsel are encouraged to discuss the schedule prior to the conference call and to agree on dates for taking action. A typical motion period lasts approximately eight (8) months. Counsel should be prepared to justify any request for a shorter or longer period.

Part E. Identification and order of the parties

Junior Party

Named inventors: Paul J. Carter
San Francisco, CA

Leonard G. Presta
San Francisco, CA

Involved Patent:	6,407,213, issued 18 June 2002, from application 08/146,206, which was filed 17 November 1993, and was based on international application PCT/US92/05126, filed 15 June 1992.
Title:	METHOD FOR MAKING HUMANIZED ANTIBODIES
Assignee:	Genentech, Inc.

Senior Party

Named Inventors:	John Robert Adair High Wycombe, United Kingdom Diljeet Singh Athwal London, United Kingdom John Spencer Emtage Marlow, United Kingdom
Involved Application:	11/284,261, filed 21 November 2005
Title:	HUMANISED ANTIBODIES
Assignee:	Celltech R & D Limited

The senior party is assigned exhibit numbers 1001-1999. The junior party is assigned exhibit numbers 2001-2999. Bd. R. 154(c)(1); SO ¶ 154.2.1. The senior party is responsible for initiating settlement discussions. SO ¶ 126.1.

Part F. Count and claims of the parties

Count 1

A humanized antibody heavy chain variable domain comprising non-human Complementarity Determining Region (CDR) amino acid residues which bind antigen incorporated into a human antibody variable domain, and further comprising a Framework Region (FR) amino acid substitution at a site selected from the group consisting of: 24H, 71H, 73H, and 78H, utilizing the numbering system set forth in Kabat.

The claims of the parties are:

Carter: 1-82

Adair: 24

The claims of the parties which correspond to Count 1 are:

Carter: 30, 31, 60, 62, 63, 66, 67, 70, 73, 77-81

Adair: 24

The claims of the parties which do not correspond to Count 1, and therefore are not involved in the interference, are:

Carter: 1-29, 32-59, 61, 64, 65, 68, 69, 71, 72, 74-76, 82

Adair: None

The parties are accorded the following benefit for Count 1:

Carter:	PCT/US92/05126, filed 15 June 1992; and 07/715,272, filed 14 June 1991, now abandoned.
Adair:	08/846,658, filed 01 May 1997; 08/303,569, filed 07 September 1994, issued as 5,859,205 on 12 January 1999; 07/743,329, filed on 17 September 1991; PCT/GB90/02017, filed 21 December 1990; and GB 8928874.0, filed 21 December 1989.

Part G. Heading to be used on papers

The following heading must be used on all papers filed in this interference, see SO & 106.1.1:

PAUL J. CARTER AND LEONARD G. PRESTA
Junior Party
(Patent 6,407,213),

v.

JOHN ROBERT ADAIR, DILJEET SINGH ATHWAL,
AND JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

Patent Interference No. 105,744
(Technology Center 1600)

Form 4. File Copy request

FILE COPY REQUEST
Contested Case No.: Interference No. 105,744

Attach a copy of sections E and F of the DECLARATION to this REQUEST. On the copy, circle each patent and application that you are requesting.

Include the following information to facilitate processing of this REQUEST:

1. Charge fees to USPTO Deposit Account No. **50-4028**

2. Complete address, including street, city, state, zip code and telephone number (do not list a Post Office box because file copies are sent by commercial overnight courier).

Oliver R. Ashe, Jr.

ASHE, P.C.

11440 Isaac Newton Sq. North, Suite 210

Reston, VA 20190

3. Telephone, including area code: **(703) 467-9001 ext. 201**

CERTIFICATE OF FILING

The undersigned certifies that a copy of the paper entitled **"CARTER REQUEST FOR FILE COPIES"** attaching Parts E and F of the Notice Declaring Interference and completed SO Form 4 was filed this 19th day of February, 2010, by e-mail, to:

The Board of Patent Appeals and Interferences
Madison Building East, 9th Floor
600 Dulany Street
Alexandria, VA 22314
Tel.: 571-272-9797
Fax: 571-273-0042
E-mail: BoxInterferences@USPTO.GOV

February 19, 2010 /Oliver R. Ashe, Jr./
Oliver R. Ashe, Jr.

CERTIFICATE OF SERVICE

The undersigned hereby certifies that a copy of the paper entitled **"CARTER REQUEST FOR FILE COPIES"** attaching Parts E and F of the Notice Declaring Interference and completed SO Form 4 was served this 19th day of February, 2010, by e-mail, on the Attorney of Record for Adair:

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1900 Market Street, 7th Floor
Philadelphia, PA 19103
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Fax: 215-701-2005
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February 19, 2010 /Oliver R. Ashe, Jr./
Oliver R. Ashe, Jr.

Filed by: Trial Section
Mail Stop Interferences
Board of Patent Appeals & Interferences
U.S. Patent & Trademark Office
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Paper 14
Entered: 23 February 2010

UNITED STATES PATENT AND TRADEMARK OFFICE
BOARD OF PATENT APPEALS AND INTERFERENCES

Patent Interference 105,744 (SGL)
Technology Center 1600

PAUL J. CARTER and LEONARD G. PRESTA
Junior Party,
Patent 6,407,213,

v.

JOHN ROBERT ADAIR, DILJEET SINGH ATHWAL
and JOHN SPENCER EMTAGE
Senior Party,
Application 11/284,261.

Order – Bd.R. 109(b) – Authorizing copies of Office records

(Transmitting file copy requests to the Office of Public Records)

The requests of the parties for file copies, along with those requested files at the Board, have been forwarded to the Office of Public Records of the United States Patent and Trademark Office. It is the responsibility of each party to inform the Board if a complete copy is not received **within 21**

days of this order. Bd.R. 109(c). If a party does not promptly notify the Board of missing or incomplete files, the party may not request an extension of time, Bd.R. 4(a), due to non-receipt of files.

Note that files come in many different formats, see SO ¶ 109.2, and thus different files may arrive at different times.

Upon consideration of the receipt of requests for files (see Part H of the DECLARATION), it is---

ORDERED that the notice to the Board that a file copy has not been received or is incomplete must be transmitted via electronic mail addressed to BoxInterferences@uspto.gov, with "Attention Paralegal" in the subject line of the electronic mail message.

/Yolunda R. Townes/
Paralegal Specialist
Trial Division

cc: Office of Public Records, USPTO
Revised: 23 January 2006

cc (via electronic filing):

Attorney for Carter:

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Tel: 703-467-9001
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Attorney for Adair:

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Paper 15
Filed: 24 February 2010

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

PAUL J. **CARTER** AND LEONARD G. PRESTIA
Junior Party
(Patent 6,407,213),

v.

JOHN ROBERT **ADAIR**, DILGEET SINGH ATHWAL, and JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

Patent Interference No. 105,744
(Technology Center 1600)

ORDER – Miscellaneous – 104(a)

A conference call was held on 24 February 2010 at approximately 10:30 am.

Paritcipating in the call were:

(1) Oliver Ashe and Jeffrey P. Kushan for Junior Party Carter,

(2) Doreen Trujillo and Michael B. Fein for Senior Party Adair, and

(3) Sally Gardner Lane, Administrative Patent Judge.

There was a delay in receipt of the declaration of the interference and other relevant papers by Carter. Moreover, the parties expressed that other delays have been caused by the major snowstorm that occurred in the Washington, D.C. area the week of February 8th.

The parties moved for a delay of the initial conference call. The motion is GRANTED.

It is

ORDERED that the conference call is rescheduled for **15 April 2010 at 10:00 am,** and

FURTHER ORDERED that the call will be initiated by the Board.

/Sally Gardner Lane/
Administrative Patent Judge

cc (via overnight delivery):

Attorney for Carter:

Oliver R. Ashe, Jr., Esq.
ASHE, P.C.
11440 Isaac Newton Square North
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Jeffrey P. Kushan, Esq.
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Attorney for Adair:

Doreen Yatko Trujillo, Esq.
Michael B. Fein, Esq
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Filed 3 March 2010

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

PAUL J. **CARTER** AND LEONARD G. PRESTIA
Junior Party
(Patent 6,407,213),

v.

JOHN ROBERT **ADAIR**, DILGEET SINGH ATHWAL, and JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

Patent Interference No. 105,744
(Technology Center 1600)

ORDER – Miscellaneous – 104(a)

A conference call was held on 3 March 2010 at approximately 2:30 pm.

Participating in the call were:

(1) Oliver Ashe for Carter,

(2) Doreen Trujillo for Adair, and

(3) Sally Gardner Lane, Administrative Patent Judge.

The parties asked for the call to determine if they are required to file an annotated copy of the claims, and if so, for additional time to file the annotated copy.

Parties are required to file "[f]or each involved claim having a limitation that is illustrated in a drawing or biotechnology material sequence, file an annotated copy of the claim indicating in bold face between braces ({}) where each limitation is shown in the drawing or sequence." Bd. R. 110(b)(1).

The parties indicated that, and a review of the claims indicates, that the claims do not recite any particular sequence. (Clean claim copies, Adair at Paper 5 and Carter at Paper 12). Under these particular circumstances, the parties need not file annotated copies of the claims.

It is

ORDERED that the parties need not file annotated copies of the claims. Bd. R. 110(b)(1).

/Sally Gardner Lane/
Administrative Patent Judge

cc (electronic filing):

Attorney for Carter:

Oliver R. Ashe, Jr., Esq.
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Jeffrey P. Kushan, Esq.
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Tel: 202-736-8914
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Attorney for Adair:

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Michael B. Fein, Esq.
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Email: dtrujillo@cozen.com
Email: mfein@cozen.com

Paper No. _____
Filed on behalf of: Party Carter
Filed: March 16, 2010

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UNITED STATES PATENT AND TRADEMARK OFFICE

_____

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

_____

PAUL J. **CARTER** AND LEONARD G. PRESTA
Junior Party
(Patent 6,407,213),

v.

JOHN ROBERT **ADAIR**, DILJEET SINGH ATHWAL,
AND JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

_____

Patent Interference 105,744 (SGL)
Technology Center 1600

_____

CARTER NOTICE OF INCOMPLETE FILE COPIES

CARTER NOTICE OF INCOMPLETE FILE COPIES

In accordance with the instructions in the Order dated February 23, 2010 (Paper No. 14, Order - Bd.R. 109(b) - Authorizing copies of Office records), Carter hereby notifies the Board that Carter has received a copy of all requested file histories.

The following papers are either missing from the files, incomplete (*e.g.*, page(s) missing), or illegible:

File	Paper requested	Reason for request
GB 8928874.0 to Adair et al.	GB Application No. 8928874.0, filed December 21, 1989.	Illegible (faint text)
USSN 08/303,569 to Adair et al.	Office Action mailed May 16, 1995 (Paper No. 37).	Page 1 (front page) is missing
	Protest Under 37 CFR § 1.291, filed April 1, 1997 (Paper No. 45).	Missing
	Communication mailed June 23, 1997 (Paper No. 46).	Missing

In view of the substantive nature of the above-identified documents, Carter respectfully requests the Board to promptly forward copies of these documents to the undersigned. In the event the Board does not have immediate access to these documents, immediate production should be required from Adair. See Bd.R. 150. The documents should be directed to:

Oliver R. Ashe, Jr.
ASHE, P.C.
11440 Isaac Newton Square North
Suite 210
Reston, VA 20190

Please charge any associated costs to Deposit Account No. 50-4028.

Respectfully submitted,

March 16, 2010

/Oliver R. Ashe, Jr./
Oliver R. Ashe, Jr.
Registration No. 40,491
Counsel for Party Carter

CERTIFICATE OF FILING

The undersigned certifies that a copy of the paper entitled **"CARTER NOTICE OF INCOMPLETE FILE COPIES"** was filed this 16th day of March, 2010, by e-mail, to:

The Board of Patent Appeals and Interferences
Madison Building East, 9th Floor
600 Dulany Street
Alexandria, VA 22314
Tel.: 571-272-9797
Fax: 571-273-0042
E-mail: BoxInterferences@USPTO.GOV

March 16, 2010 /Oliver R. Ashe, Jr./
Oliver R. Ashe, Jr.

CERTIFICATE OF SERVICE

The undersigned hereby certifies that a copy of the paper entitled **"CARTER NOTICE OF INCOMPLETE FILE COPIES"** was served this 16th day of March, 2010, by e-mail, on the Attorney of Record for Adair:

Doreen Yatko Trujillo, Esq.
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1900 Market Street, 7th Floor
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Tel.: 215-665-6593
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E-mail: dtrujillo@cozen.com

March 16, 2010 /Oliver R. Ashe, Jr./
Oliver R. Ashe, Jr.

Filed on behalf of: **Adair**
By: Doreen Yatko Trujillo
Michael B. Fein
Cozen O'Connor P.C.
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Paper No:_____
Date Filed: March 16, 2010

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

PAUL J. **CARTER** AND LEONARD G. PRESTA
Junior Party
(Patent 6,407,213),

v.

JOHN ROBERT **ADAIR,** DILJEET SINGH ATHWAL,
AND JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

Patent Interference No. 105,744
(Technology Center 1600)

ADAIR NOTICE OF INCOMPLETE FILE COPIES

In accordance with the instructions in the Order dated February 23, 2010 (Paper No. 14, Order - Bd.R. 109(b) - Authorizing copies of Office records), Adair hereby notifies the Board that Adair has received a copy of all requested file histories. However, as indicated in the attached documents, Adair has not received most of the references cited by or to the Patent Office in the file history of U.S. Application Serial No. 07/715,272. References Adair received are crossed-out. Also, Adair was only able to open the first 2230 of 6819 pages of the file wrapper for Application Serial No. 08/146,206. For pages beyond 2230, there is a message of an I/O error. Both file histories were supplied on diskette.

Respectfully submitted,

/Doreen Yatko Trujillo/
DOREEN YATKO TRUJILLO
Registration No. 35,719
Lead Counsel for Adair

Date: March 16, 2010

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Certificate of Service

This will certify that a true copy of this paper and attachments was served this date on the party Carter by electronic mail directed to :

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Fax: (703) 467-9002
E-mail: oashe@ashepc.com

Date: March 16, 2010

/Doreen Yatko Trujillo/
Doreen Yatko Trujillo

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186

PATENT DOCKET 708

#19 10. 5-18-95

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of

Paul J. Carter et al.

Serial No. 07/715272

Filed: June 14, 1991

For: Immunoglobulin Variants

Group Art Unit:

Examiner:

460 Point San Bruno Boulevard
South San Francisco, CA 94080
(415) 266-2614

INFORMATION DISCLOSURE STATEMENT

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

Sir:

The following items are supplied to the United States Patent and Trademark Office to advance the prosecution of the subject application.

Chothia *et al.*, *J. Mol. Biol.* **186**:651-663 (1985)

Novotny and Haber, *Proc. Natl. Acad. Sci. USA* **82**:4592-4596 (1985)

Cabilly *et al.*, U.S. patent No. 4,816,567

~~Morrison, S. L. *et al., Proc. Natl. Acad. Sci. USA* **81**:6851-6855 (1984)~~

Boulianne, G. L. *et al.*, *Nature* **312**:643-646 (1984)

Neuberger, M. S. *et al.*, *Nature* **314**:268-270 (1985)

Brüggemann, M. *et al.*, *J. Exp. Med.* **166**:1351-1361 (1987)

Riechmann, L. *et al.*, *Nature* **332**:323-327 (1988)

Love *et al.*, *Methods in Enzymology* **178**:515-527 (1989)

Bindon *et al.*, *J. Exp. Med.* **168**:127-142 (1988)

Jones, P. T. *et al.*, *Nature* **321**:522-525 (1986)

Verhoeyen, M. *et al.*, *Science* **239**:1534-1536 (1988)

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Co *et al.*, *Proc. Natl. Acad. Sci. USA* **88**:2869-2873 (1991)

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Brown *et al.*, *Proc. Natl. Acad. Sci. USA* **88**:2663-2667 (1991)

Junghans *et al.*, *Cancer Research* **50**:1495-1502 (1990)

Davies, D. R. *et al.*, *Ann. Rev. Biochem.* **59**:439-473 (1990)

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Chothia, C. *et al.*, *Nature* **342**:877-883 (1989)

Tramontano, A. *et al.*, *J. Mol. Biol.* **215**:175-182 (1990)

Margolies *et al.*, *Proc. Natl. Acad. Sci. USA* **72**:2180-2184 (1975)

Pluckthun, *Biotechnology* **9**:545-51 (1991)

Spiegelberg *et al.*, *Biochemistry* **9**:4217-4223 (1970)

Wallick *et al.*, *J. Exp. Med.* **168**:1099-1109 (1988)

Sox *et al.*, *Proc. Natl. Acad. Sci. USA* **66**:975-982 (1970)

Margni *et al.*, *Ann. Rev. Immunol.***6**:535-554 (1988)

Fendly, B. M. *et al.*, *Cancer Res.* **50**:1550-1558 (1990)

~~Neuberger *et al.*, *Nature* **312**:604-608 (1984)~~

~~Takeda *et al.*, *Nature* **314**:452-454 (1985)~~

Snow and Amzel, *Protein: Structure, Function, and Genetics* **1**:267-279, Alan R. Liss, Inc. pubs. (1986)

Cheetham, J., *Protein Engineering*, **2(3)**: 170-172 (1988)

WO 91/09967, pub. 07/11/91, Adair *et al.*

One copy of each item cited above is supplied, along with a completed Form PTO-1449. The Examiner is requested to make the citations of record.

This submission is understood to complement the results of the Examiner's own independent search. The submission of this Disclosure Statement should not be construed as a representation that a search was made, or that the cited itms are inclusive of all the relevant and amterial citations that may be available publicly.

The citation of any item is not an admission that the item is prior art. The right is reserved to antedate any item in adherence with standard procedures.

Respectfully submitted,
GENENTECH, INC.

Carolyn R. Adler
Reg. No. 32,324

Dated: April 30, 1992

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

Atty Docket No.	Serial No.
709	07/715,272

LIST OF DISCLOSURES CITED BY APPLICANT

(Use several sheets if necessary)

Applicant	#14
Paul J. Carter et al.	
Filing Date	**Group**
June 14, 1991	1806

U.S. PATENT DOCUMENTS

*Examiner Initials		Document Number	Date	Name	Class	Subclass	Filing Date
	AA						
	AB						
	AC						
	AD						
	AE						
	AF						
	AG						
	AH						
	AI						
	AJ						
	AK						

FOREIGN PATENT DOCUMENTS

		Document Number	Date	Country	Class	Subclass	Translation Yes	Translation No
CR	AL	WO 90/07861	7/26/90	PCT				
	AM							
	AN							
	AO							
	AP							

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

CR	AR	Carter et al., Proc. Natl. Acad. Sci., 89: 4285-4289 (1992)
	AS	
	AT	
	AU	
	AV	
	AW	
	AX	

Examiner [signature] Date Considered 4/29/93

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

TO SEPARATE, ...D TOP AND BOTTOM EDGES, SNAP--APART AN... DISCARD CARBON

FORM PTO-892 (REV. 2-92) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

NOTICE OF REFERENCES CITED

SERIAL NO.	GROUP ART UNIT	ATTACHMENT TO PAPER NUMBER
07/715,272	1806	26

APPLICANT(S): Carter et al.

U.S. PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	NAME	CLASS	SUB-CLASS	FILING DATE IF APPROPRIATE
	A						
	B						
	C						
	D						
	E						
	F						
	G						
	H						
	I						
	J						
	K						

FOREIGN PATENT DOCUMENTS

*		DOCUMENT NO.	DATE	COUNTRY	NAME	CLASS	SUB-CLASS	PERTINENT SHTS. DWG	PERTINENT PP. SPEC.
	L								
	M								
	N								
	O								
	P								
	Q								

OTHER REFERENCES (Including Author, Title, Date, Pertinent Pages, Etc.)

R ~~Queen et al. PNAS 86: 10029-10033 (1989)~~

S ~~Fendly et al. Cancer Research 5: 1550-1558 (1...~~

T Hudziak et al. J. Molecular and Cellular Biology 1989 p. 1165-1172

U

EXAMINER: [signature] DATE: 1/11/94

...th this office action. ...on 707.05 (a).)

* A copy of this reference...

Filed on behalf of: **Adair**
By: Doreen Yatko Trujillo
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Paper No:_____
March 18, 2010

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

PAUL J. **CARTER** AND LEONARD G. PRESTA
Junior Party
(Patent 6,407,213),

v.

JOHN ROBERT **ADAIR,** DILJEET SINGH ATHWAL,
AND JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

Patent Interference No. 105,744
(Technology Center 1600)

ADAIR NOTICE OF PRODUCTION OF DOCUMENTS

Adair is today forwarding, by overnight mail, copies of the following documents that Carter identified as missing from the file history of U.S. Application Serial No. 08/303,569 in "Carter Notice of Incomplete File Copies" filed March 16, 2010:

1. Office Action mailed May 16, 1995(Paper No. 37), Page 1 (front page) and Office Action;

2. Protest Under 37 CFR § 1.291, filed April 1, 1997 ("Protest", Paper No. 45); and

3. Communication mailed June 23, 1997 (Paper No. 46).

As discussed and agreed to by Mr. Ashe, all attachments to the Protest, with the exception of the two U.S. patents, were also forwarded.

Respectfully submitted,

/Doreen Yatko Trujillo/
DOREEN YATKO TRUJILLO
Registration No. 35,719
Lead Counsel for Adair

Date: February 16, 2010

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Certificate of Service

This will certify that a true copy of this paper and the documents referenced herein were served this date on the party Carter by Federal Express overnight mail directed to:

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Date: March 18, 2010

/Doreen Yatko Trujillo/
Doreen Yatko Trujillo

Filed on behalf of: **Adair**
By: Doreen Yatko Trujillo
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Paper No:_____
Filed: April 9, 2010

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

PAUL J. **CARTER** AND LEONARD G. PRESTA
Junior Party
(Patent 6,407,213),

v.

JOHN ROBERT **ADAIR,** DILJEET SINGH ATHWAL,
AND JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

Patent Interference No. 105,744
(Technology Center 1600)

ADAIR MOTIONS LIST

Pursuant to the Declaration of Interference, Bd. R. 120, Bd. R. 204(B), and SO ¶¶ 104.2.1., 120, and 204, Adair submits the following list of proposed motions.

1. Motion under Bd. R. 208(a)(2) and SO ¶ 208.2 to add the following count:

> A humanized antibody light chain variable domain comprising non-human Complementarity Determining Region (CDR) amino acid residues which bind antigen incorporated into a human antibody variable domain, and further comprising a Framework Region (FR) amino acid substitution at residue 58L, utilizing the numbering system set forth in Kabat.

2. Contingent upon the granting of Motion 1, a motion under Bd. R. 208(a)(2) and SO ¶ 208.2 to add the following claim to Adair's application:

> Claim 26 (new). A humanised antibody comprising a light chain variable domain comprising non-human complementarity determining region amino acid residues which bind an antigen and a human framework region wherein said framework region comprises a non-human amino acid substitution at residue 58 as numbered according to Kabat.

3. Contingent upon the granting of Motion 1, a motion under Bd. R. 208(a)(2) and SO ¶ 208.2 to designate the following claims of U.S. Patent No. 6,407,213 ("the '213 patent") as corresponding to the new count: 1-3, 9, 38, and 39.

4. Motion under Bd. Rule 121(a)(1)(iii) and SO ¶ 208.6 for judgment on priority.

5. Motion under Bd. Rule 208(a)(2) and SO ¶ 208.3.1 to designate claims 65 (as corrected by the Certificate of Correction to depend from claim 79), 68, 71, 75, 76, and 82 of the '213 patent as corresponding to Count 1.

6. Motion under Bd. Rule 121(a)(1)(iii) for judgment that the claims of the '213 patent designated as corresponding to the count are unpatentable under 35 U.S.C. § 112, first paragraph, for failure to comply with the written description requirement. There is no written descriptive support in the specification of the '213 patent for the subgenus of residues recited in the involved claims. In particular, there is no written descriptive

support for the subgenus of residues recited in claim 30 regarding an antibody which binds $p185^{HER2}$.

7. Motion under Bd. R. 121(a)(1)(iii) for judgment that all involved claims of the '213 patent are unpatentable under 35 U.S.C. §102(b) as anticipated by, or, under 35 U.S.C. §103(a) as obvious over, at least one or more of the following references, or combinations thereof:

- Slide presentation by Dr. Riechmann at the "Advances in the Application of Monoclonal Antibodies in Clinical Oncology" held at the Wolfson Institute in London, England May 6-8, 1987 (as evidenced by Declaration of Dr. Riechmann submitted by Genentech, Inc. in the Opposition of European Patent No. 451,216B1, and present in the file history of the '213 patent);
- Chothia et al., *J. Mol. Biol.*, 186:651-663 (1985);
- Sheriff et al., *Proc. Natl. Acad. Sci., USA*, 84:8075-8079 (1987);
- WO 88/09344 (published December 1, 1988);
- Foote et al., *Nova acta Leopoldina 61*, 269:103-110 (1989);
- Queen et al., *Proc. Natl. Acad. Sci. USA*, 86:10029-10033 (1989);
- WO 90/07861 (published July 26, 1990);
- Co et al., *Proc. Natl. Acad. Sci.,*88:2869-2873, April, 1991;

Adair's position is that the '213 patent is not entitled to priority earlier than its filing date of June 15, 1992. If the '213 patent is entitled to priority, then WO 90/07861 and Co et al. become references under 35 U.S.C. §102(a).

8. Motion under Bd. R. 121(a)(1)(iii) for judgment that claims of the '213 patent are unpatentable under 35 U.S.C. §102(e) as anticipated by, or, under 35 U.S.C. §103(a)

as obvious over, at least one or more of the following references, or combinations thereof with each other or in combination with one or more of the references listed in paragraph 7:

- U.S. Patent No. 5,132,405 (priority to May 21, 1987);
- U.S. Patent No. 6,548,640 (priority to May 3, 1988);
- U.S. Patent No. 6,632,927 (priority to December 21, 1990); and
- U.S. Patent No. 5,859,205 (priority to December 21, 1990).

9. Motion under Bd. R. 208(a)(2) and SO 208.2 to add an additional count, wherein the additional count is directed to the subject matter of claim 1 of U.S. Patent No. 6,639,055 ("the '055 patent") or claim 24 of U.S. Application Serial No. 11/284,260. The '055 patent claims priority to the '213 patent. Adair is currently pursuing an interference with the '055 patent in their Application Serial No. 11/284,260.

10. Contingent upon the granting of Motion 9, a motion under Bd. R. 208(a)(2) and 203(d) and SO ¶¶ 123 and 203.2 to add the '055 patent.

11. Contingent upon the granting of Motion 9, a motion under Bd. R. 208(a)(2) and 203(d) and SO ¶¶ 123 and 203.2 to add Adair Application Serial No. 11/284,260.

Respectfully submitted,

/Doreen Yatko Trujillo/
DOREEN YATKO TRUJILLO
Registration No. 35,719
Lead Counsel for Adair

Date: April 9, 2010

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Certificate of Service

This will certify that a true copy of this paper was served this date on the party Carter by electronic mail directed to:

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Date: April 9, 2010

/Doreen Yatko Trujillo/
Doreen Yatko Trujillo

Paper No. _____
Filed on behalf of: Party Carter
Filed: April 9, 2010

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UNITED STATES PATENT AND TRADEMARK OFFICE

_____

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

_____

PAUL J. **CARTER** AND LEONARD G. PRESTA
Junior Party
(Patent 6,407,213),

v.

JOHN ROBERT **ADAIR**, DILJEET SINGH ATHWAL,
AND JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

_____

Patent Interference 105,744 (SGL)
Technology Center 1600

_____

CARTER MOTIONS LIST

CARTER MOTIONS LIST

Pursuant to Part D of the Declaration of Interference (Paper No. 1), 37 C.F.R. §§ 41.120 and 204, and ¶¶ 104.2.1, 120, and 204 of the Standing Order (Paper No. 2), Carter herein provides a list of motions that Carter presently intends to file.

1. A motion under 37 C.F.R. § 41.121(a)(1)(iii) seeking judgment that Adair's claim 24 is not patentable to Adair under 35 U.S.C. § 135(b)(1). The claims presented by Adair prior to the critical date were not patentable to Adair and/or were not the same as or for the same or substantially the same subject matter as any of the involved claims of Carter's U.S. Patent No. 6,407,213 ("the '213 patent"). Adair's involved claim 24 was presented over three years after the issuance of the involved Carter '213 patent and is not entitled to the benefit of any pre-critical date claim. For at least these reasons, Adair claim 24 is barred under 35 U.S.C. § 135(b)(1). This motion should be treated as raising a threshold issue. 37 C.F.R. § 41.201.

2. A motion under 37 C.F.R. § 41.121(a)(1)(iii) seeking judgment that Adair's involved claim 24 is not patentable under 35 U.S.C. § 112, first paragraph, for failure to satisfy the written description requirement. Adair's involved specification does not describe a humanized antibody wherein a "framework region comprises a non-human amino acid substitution at a residue selected from the group consisting of 23, 24, 49, 71, 73, and 78, and combinations thereof" as recited in Adair's involved claim 24. This motion should be treated as raising a threshold issue. 37 C.F.R. § 41.201.

3. A motion under 37 C.F.R. § 41.121(a)(1)(iii) seeking judgment that Adair's involved claim 24 is not patentable under 35 U.S.C. § 112, second paragraph, because it is indefinite in its recitation of a humanized antibody wherein a "framework region comprises a

non-human amino acid substitution at a residue selected from the group consisting of 23, 24, 49, 71, 73, and 78, and combinations thereof."

4. A motion under 37 C.F.R. § 41.121(a)(1)(iii) seeking judgment that, applying a two-way test, an interference-in-fact does not exist between Adair's involved claim 24 and any of Carter's involved patent claims. This motion should be treated as raising a threshold issue. 37 C.F.R. § 41.201.

5. A motion under 37 C.F.R. §§ 41.121(a)(1)(i) and 41.208(a)(2) to designate Carter's involved '213 patent claims 30, 31, 60, 62, 63, 67, 80 and 81 as not corresponding to Count 1. Count 1, if treated as prior art, would not have anticipated or rendered obvious the subject matter of these claims.

6. A motion under 37 C.F.R. §§ 41.121(a)(1)(ii) and 41.208(a)(3) to deny Adair priority benefit of Adair U.K. Application No. 8928874.0 and/or PCT/GB90/02017 because the applications do not provide a described and/or enabled anticipation under 35 U.S.C. 102(g)(1) of the subject matter of Count 1.

7. A motion under 37 C.F.R. § 41.121(a)(1)(iii) seeking judgment that Adair's involved claim 24 is not patentable under 35 U.S.C. §§ 102 and/or 103. The primary references to be addressed in the motion are Chothia et al., *Nature*, 342:877-883 (1989) and Tramontano et al., *J. Mol. Biol.,* 215:175-182 (1990) and, possibly, other related evidence under 35 U.S.C. 102(g). This motion may, in part, address whether Adair claim 24 is entitled to the benefit of one or more of its earlier-filed applications under 35 U.S.C. §§ 119/120. In the event Carter determines that additional primary references will be addressed in this motion, Carter will promptly give notice to Adair and the Board and serve copies of such references on Adair.

8. A motion under 37 C.F.R. § 41.208(a)(4) seeking judgment based on derivation and/or priority.

9. Pursuant to 37 C.F.R. § 41.127(a)(1), the Board should be aware that Adair has requested an interference between Adair's U.S. Patent Application 11/284,260 ("the '260 application") and Carter's U.S. Patent 6,639,055 ("the '055 patent"). In an Office Action dated December 28, 2009, concerning the Adair '260 application, the Examiner correctly noted that examination of the Adair application has not been completed as required under 37 C.F.R. § 41.102(a) and that consideration of a potential interference is premature, citing MPEP 2303. Moreover, in the event such an interference is declared, Carter would maintain that Adair lacks standing to provoke or maintain an interference with the Carter '055 patent and that Adair's claim is not patentable to Adair.

Respectfully submitted,

April 9, 2010

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CERTIFICATE OF FILING

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April 9, 2010 /Oliver R. Ashe, Jr./
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CERTIFICATE OF SERVICE

The undersigned hereby certifies that a copy of the paper entitled **"CARTER MOTIONS LIST"** was served this 9th day of April, 2010, by e-mail, on the Attorney of Record for Adair:

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Paper No. _____

Filed on behalf of: Party Carter

Filed: April 26, 2010

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UNITED STATES PATENT AND TRADEMARK OFFICE

_____

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

_____

PAUL J. **CARTER** AND LEONARD G. PRESTA
Junior Party
(Patent 6,407,213),

v.

JOHN ROBERT **ADAIR**, DILJEET SINGH ATHWAL,
AND JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

_____

Patent Interference 105,744 (SGL)
Technology Center 1600

_____

CARTER'S TEST DOCUMENT PURSUANT TO WEB PORTAL INSTRUCTIONS

Mail Stop Interference
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Filed 27 April 2010

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

PAUL J. **CARTER** AND LEONARD G. PRESTIA
Junior Party
(Patent 6,407,213),

v.

JOHN ROBERT **ADAIR**, DILGEET SINGH ATHWAL,
and JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

Patent Interference No. 105,744
(Technology Center 1600)

Order - Motion Times - Bd.R. 104(c)

A. Conference call

A conference call was held on 22 April 2010 at approximately 2:00 p.m.

Participating in the call were:

(1) Oliver Ashe and Jeffrey Kushan for Carter,

(2) Doreen Trujillo for Adair, and

(3) Sally Gardner Lane, Administrative Patent Judge.

B. Relevant discussion during conference call

The principal purpose of the conference call was to set times for taking action on motions (other than miscellaneous motions) in the interference. Each party has filed a list of motions that it wishes to file.

Modified schedule

At the beginning of the call the Ms. Trujillo, counsel for Adair informed the Board that she soon will be undergoing hip replacement surgery and does not expect to return to work until the beginning part of June. Mr. Ashe, counsel for Carter, expressed a willingness to cooperate and adjust the schedule to accommodate Ms. Trujillo's unavailability.

Given the particular circumstances, the schedule has been adjusted so that Carter will file its threshold motions first and, if necessary, remaining motions will be filed at a time to allow Ms. Trujillo adequate time to prepare the authorized motions after returning to work. However, as discussed during the call, if, in light of either of the Carter threshold motions, an Adair opposition or responsive motion is authorized, it would be expected that the opposition or responsive motion would be filed as soon as possible (with a precise time to be set if, and when, necessary).

Adair request for a second interference

Adair has proposed an interference with Carter patent 6,639,055 in its application 11/284,260 ('260). Adair confirmed that *ex parte* prosecution in the '260 application is not complete. Declaring an interference involving the '260 claims would be premature at this time. However, Adair should inform the Board if its claims become allowable and

at that time Adair can seek authorization to file a miscellaneous motion suggesting an interference. (Bd. R. 102 and Standing Order at ¶ 203.1).

Authorized Motions

Only the motions specifically authorized in this Order may be filed. If a party finds that a responsive motion is necessary, then a conference call should be arranged well prior to the time such motion would be due.

Each party is authorized to file a motion for priority.[1] Priority motions are deferred. In addition either party may move to defer its opposition to any motion for unpatentability over prior art based upon the party's priority statement.

Carter

Expedited schedule:

Carter is authorized to file the following two threshold motions on or before **01 June 2010**:

1. A motion seeking judgment that the single Adair claim is barred under 35 U.S.C. § 135(b) (1) (item #1 on the Carter list), and

2. A motion seeking judgment that the single Adair claim is unpatentable under the written description requirement of 35 U.S.C. § 112, ¶1 (item 2 of the Carter list).

No Adair opposition or responsive motion to either of the Carter threshold motions is authorized at this time. If an opposition to either motion is necessary, a conference call will be scheduled to decide upon an appropriate time table. At that time Adair should be prepared to discuss any responsive motion it might wish to file.

1 In addition derivation briefing, if any, will be deferred. If a party wishes to allege derivation it must do so in its priority statement.

Regular schedule:

1. Carter is authorized to file the motions at items 3 (§ 112, ¶ 2), 4 (no interference in fact), 6 (benefit) and 7 (unpatentability over prior art) on its list in accordance with the schedule set out in the appendix.

Adair

Expedited schedule:

Adair has not asked to file any motion as a threshold motion.

Regular schedule:

1. Adair is authorized to file the motion at item 5 (Carter claim designations).

2. Adair is authorized to file a single motion that all the Carter claims are unpatentable over the prior art cited at items 7 and 8 of the Adair list. As discussed during the call, Adair has filed an unacceptably long list of prior art. Adair agreed that, at a reasonable time prior to the time it files its motion, Adair will inform Carter what references it intends to rely upon and, as to the § 103 issue, which will be relied upon as primary and which will be relied upon as secondary references. If the number of references relied upon is unreasonable the motion may be returned to Adair.

Adair requested to file other motions however Adair is not authorized to file these motions for the following reasons.

1. The motions at items 1, 9 and 10 were said to relate to the ‘260 application. As discussed above, because that *ex parte* prosecution has not been completed a miscellaneous motion suggesting a second interference is not authorized. (Bd. R. 102 and Standing Order at ¶ 203.1).

2. The motion at item 2 seeks to present a claim said to be directed to subject matter at claims 1-3, 9, 38 and 39 of the involved Carter patent. Adair indicated that it could claim this subject matter in a pending application. In such case, the claim(s) could be examined by a patent examiner to determine there is allowable subject matter to Adair. Since the claims can be presented elsewhere Adair is not authorized to file the motion at item 2.

4. Adair conceded that if its motion at item 6 were to be granted, the subject matter of the Count would not change. An interference is principally about the priority contest. If the granting of the motion would not change that contest, then its filing usually will not be authorized. Bd. R. 208.

Notwithstanding the Standing Order (e.g., 10 at ¶ 121.2 and ¶122.2), the page limit on motions and oppositions is 25 pages and the page limit for replies is 10 pages. The page limit does not include a table of contents, a table of authorities, a certificate of service, or required appendices **except for the page limit does include the statement of material facts (SO ¶ 121.5.2).**[2] In other words, the statement of material facts counts toward the 25 page limit.

C. Time periods associated with motions

In accordance with discussion during the telephone conference call, the TIME PERIODS described below are set out in an Appendix to this ORDER. Action specified for each TIME PERIOD must be completed by the date specified for the TIME PERIOD.

2 The page limit does not include a party's response to its opponent's statements of material facts.

The parties are authorized to stipulate different times (earlier or later, but not later than TIME PERIOD 7) for TIME PERIODS 1 through 6.[3] A notice of the stipulation must be promptly filed. The notice must be in the form of a photocopy of the Appendix attached to this ORDER with old dates crossed out and new dates inserted by hand. The parties may not stipulate an extension of TIME PERIODS 7-9.

1. TIME PERIOD 1

a. File and serve all authorized motions and

b. Serve but do not file evidence in support of these motions.

If no party files a motion, the SENIOR PARTY must arrange a conference call with the parties and the Board so that appropriate adjustments to the schedule may be made.

2. TIME PERIOD 2

a. File and serve responsive motions (Bd.R. 121(a)(2)) in response to an opponent's motion filed during TIME PERIOD 1 and

b. Serve but do not file evidence in support of these responsive motions.

3. TIME PERIOD 3

a. File and serve oppositions to all motions, including responsive motions and

[3] In stipulating different times, the parties should consider the effect of the stipulation on times (1) to object to evidence (5 business days, Bd.R. 155(b)(1)), (2) to supplement evidence (10 business days, Bd.R. 155(b)(2)), (3) to begin cross examination (no earlier than 21 days after service, SO & 157.3.1) and (4) to conclude cross examination (at least 10 days before the opposition or reply is due, SO & 157.3.2).

b. Serve but do not file evidence in support of these oppositions.

4. TIME PERIOD 4

a. File and serve replies to all oppositions and

b. Serve but do not file evidence in support of these replies.

5. TIME PERIOD 5

a. File and serve any request for oral argument on motions,

b. File and serve motions to exclude evidence (Bd.R. 155(c); SO & 155.2), and

c. File and serve observations on cross examination (SO & 157.7) of reply testimony.

6. TIME PERIOD 6

a. File and serve oppositions to an opponent's motion to exclude evidence and

b. File and serve any response to observations.

7. TIME PERIOD 7

File and serve replies to oppositions to motions to exclude evidence.

D. Deposition transcripts

Transcripts of cross examinations and depositions taken under 35 U.S.C. 24 must be served, but not filed until the exhibits are filed.

E. Serving exhibits relied upon in motions

An exhibit, including an affidavit, cited in connection with a motion, opposition, reply, or affidavit, must be served, but not filed,[4] with the motion, opposition, reply or affidavit in which the exhibit is first mentioned.

F. TIME PERIOD 8: Filing the record for decision on motions

1. File an original set of your exhibits and one working copy of your exhibits;
2. For each of your motions, file one folder (or three folders if an oral argument is set each) containing:
    a. The motion,
    b. Any corresponding opposition,
    c. Any corresponding reply,
    d. Any corresponding observations, and
    e. Any corresponding response to the observations.
3. File CD-ROM a party elects to file.

G. TIME PERIOD 9: Default oral argument date

If a request for oral argument (Bd.R. 124(a); TIME PERIOD 5) is granted, the default date for such argument is TIME PERIOD 9. No oral argument will occur if either no argument is requested or granted.

H. Priority statements

1. At TIME PERIOD 1:

[4] Except when the Board sets an expedited schedule for a particular motion, in which case, all exhibits mentioned in that motion or the corresponding opposition or reply must be filed with the motion, opposition, reply, or affidavit in which the exhibit is first mentioned.

a. File but do not serve a priority statement (Bd.R. 120; Bd.R. 204(a)).

b. File and serve a notice advising each opponent of the filing of the priority statement.

2. A junior party who does not file a priority statement shall not have access to the priority statement of any other party.

3. **Within one (1) week** after TIME PERIOD 1, serve a copy of the priority statement upon each opponent (except for a junior party barred under ¶ H.2 above).

/Sally Gardner Lane/
Administrative Patent Judge

Revised 3 January 2006

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Appendix--ORDER - RULE 123(a)
(Times for substantive motions; priority deferred)

Interference 105,744

CARTER THRESHOLD MOTIONS……**1 June 2010**

ADAIR OPPOSITION, RESPONSIVE MOTIONS……to be set if needed

TIME PERIOD 1 (all other authorized motions).....**9 August 2010**
File motions
File (but serve one week later) priority statements

TIME PERIOD 2.....**20 September 2010**
File responsive motions (none authorized at this time)
filed in TIME PERIOD 1

TIME PERIOD 3.....**2 November 2010**
File oppositions to all motions

TIME PERIOD 4.....**14 December 2010**
File all replies

TIME PERIOD 5.....**4 January 2011**
File request for oral argument
File motions to exclude
File observations

TIME PERIOD 6.....**18 January 2011**
File oppositions to motions to exclude
File response to observations

TIME PERIOD 7.....**1 February 2011**
File replies to oppositions to motions to exclude

TIME PERIOD 8.....**8 February 2011**
File exhibits
File sets of motions
File any CD-ROMs

TIME PERIOD 9.....**to be set**
Default oral argument date (if ordered)

Paper No. _____
Filed on behalf of: Party Carter
Filed: April 29, 2010

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UNITED STATES PATENT AND TRADEMARK OFFICE

_____

BEFORE THE BOARD OF PATENT APPEALS
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_____

PAUL J. **CARTER** AND LEONARD G. PRESTA
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(Patent 6,407,213),

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JOHN ROBERT **ADAIR**, DILJEET SINGH ATHWAL,
AND JOHN SPENCER EMTAGE
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_____

Patent Interference 105,744 (SGL)
Technology Center 1600

_____

CARTER'S REQUEST FOR CLARIFICATION OF PAPER NO. 23

CARTER'S REQUEST FOR CLARIFICATION OF PAPER NO. 23

The Order - Motion Times - Bd. R. 104(c) dated April 27, 2010 (Paper No. 23) does not expressly address the proposed motion identified in item 5 of Carter Motions List (to designate Carter's involved claims 30, 31, 60, 62-63, 67, and 80-81 as not corresponding to Count 1). Carter respectfully requests the Board to clarify whether Carter is authorized to file such a motion.

Respectfully submitted,

April 29, 2010

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April 29, 2010 /Oliver R. Ashe, Jr./
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CERTIFICATE OF SERVICE

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Filed 3 May 2010

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

PAUL J. **CARTER** AND LEONARD G. PRESTIA
Junior Party
(Patent 6,407,213),

v.

JOHN ROBERT **ADAIR**, DILGEET SINGH ATHWAL,
and JOHN SPENCER EMTAGE
Senior Party
(Application No. 11/284,261),

Patent Interference No. 105,744
(Technology Center 1600)

ORDER – Miscellaneous – 104(a)

Carter requests clarification (Paper 24) of the Order setting motions times. (Paper 23). As noted by Carter, the Order does not discuss whether Carter is authorized to file the proposed motion to have certain Carter claims designated as not corresponding to the Count (item 5 on the Carter list).

It is

ORDERED that Carter is authorized to file a motion to designate Carter claims 30, 31, 60, 62-63, 67, and 80-81 as not corresponding to the Count; and

FURTHER ORDERED that the motion shall be filed on the regular (non-expedited) schedule.

/Sally Gardner Lane/
Administrative Patent Judge

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US006407213B1

(12) **United States Patent**
Carter et al.

(10) **Patent No.: US 6,407,213 B1**
(45) **Date of Patent: Jun. 18, 2002**

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

(75) Inventors: **Paul J. Carter**; **Leonard G. Presta**, both of San Francisco, CA (US)

(73) Assignee: **Genentech, Inc.**, South San Francisco, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **08/146,206**

(22) PCT Filed: **Jun. 15, 1992**

(86) PCT No.: **PCT/US92/05126**

§ 371 (c)(1),
(2), (4) Date: **Nov. 17, 1993**

Related U.S. Application Data

(63) Continuation-in-part of application No. 07/715,272, filed on Jun. 14, 1991, now abandoned.

(51) **Int. Cl.**[7] **C07K 16/00**

(52) **U.S. Cl.** **530/387.3**; 435/69.6; 435/69.7; 435/70.21; 435/91; 536/23.53; 424/133.1

(58) **Field of Search** 435/69.6, 69.7, 435/70.21, 91, 172.2, 240.1, 240.27, 252.3, 320.1, 328; 536/23.53; 424/133.1; 530/387.3

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(57) **ABSTRACT**

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

82 Claims, 9 Drawing Sheets

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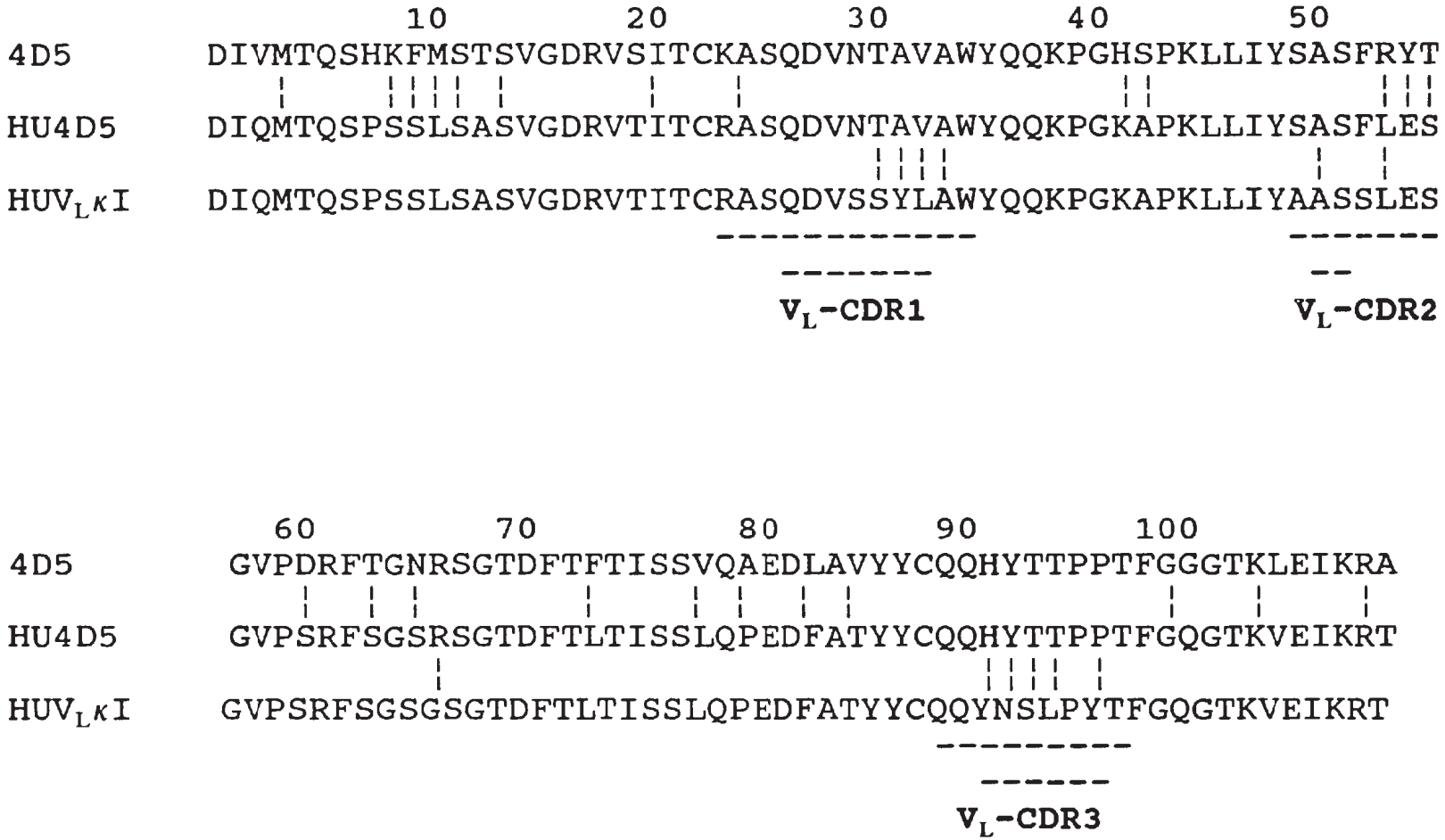
FIG. 1A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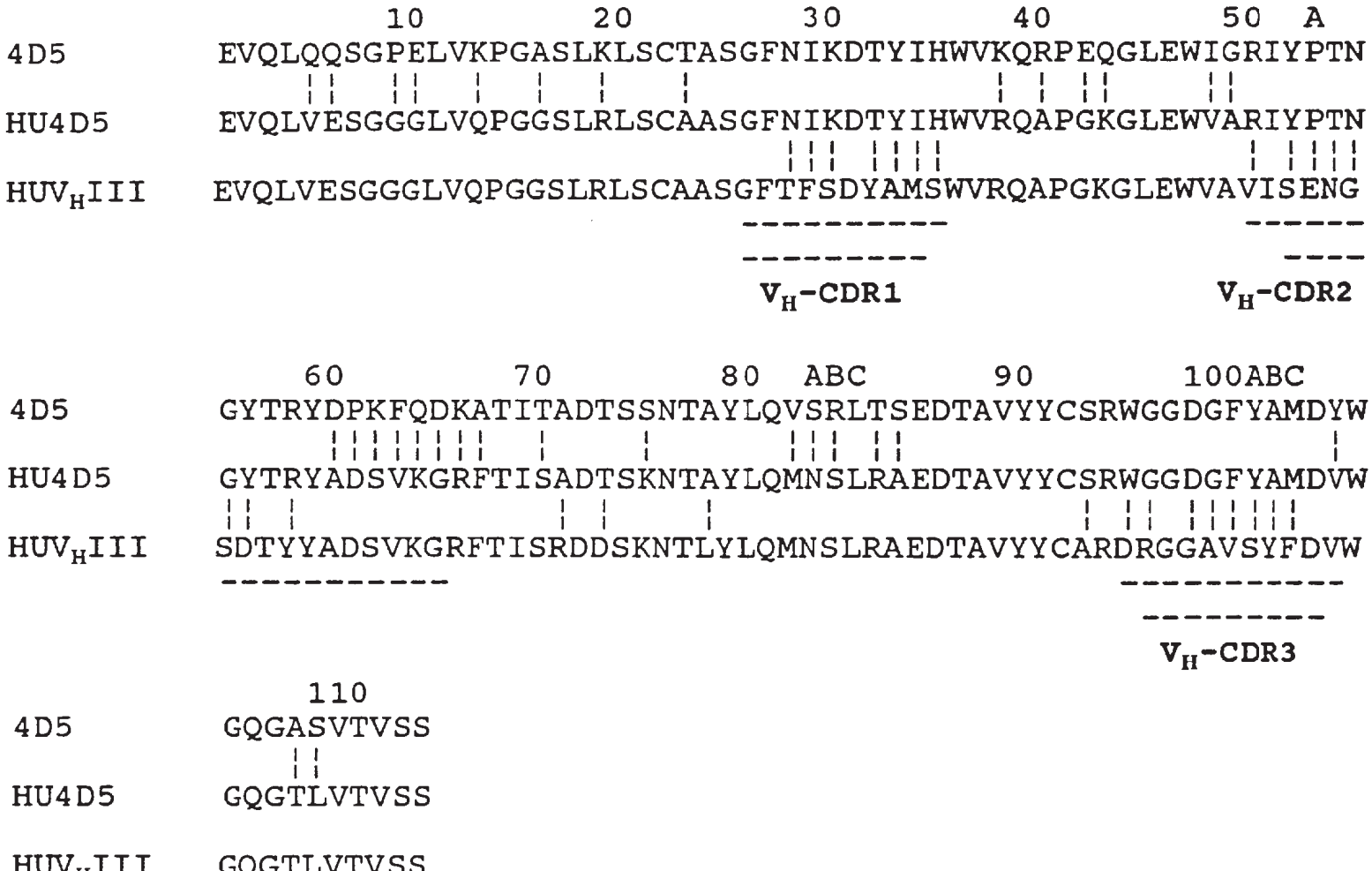
HU4D5 DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLES
HUVLκI DIQMTQSPSSLSASVGDRVTITCRASQDVSSYLAWYQQKPGKAPKLLIYAASSLES
VL-CDR1
VL-CDR2
60 70 80 90 100
4D5 GVPDRFTGNRSGTDFTFTISSVQAEDLAVYYCQQHYTTPPTFGGGTKLEIKRA
HU4D5 GVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRT
HUVLκI GVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYNSLPYTFGQGTKVEIKRT
VL-CDR3

FIG. 1B


10 20 30 40 50 A
4D5 EVQLQQSGPELVKPGASLKLSCTASGFNIKDTYIHWVKQRPEQGLEWIGRIYPTN
HU4D5 EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTN
HUV$_H$III EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYAMSWVRQAPGKGLEWVAVISENG
V$_H$-CDR1
V$_H$-CDR2
60 70 80 ABC 90 100ABC
4D5 GYTRYDPKFQDKATITADTSSNTAYLQVSRLTSEDTAVYYCSRWGGDGFYAMDYW
HU4D5 GYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMDVW
HUV$_H$III SDTYYADSVKGRFTISRDDSKNTLYLQMNSLRAEDTAVYYCARDRGGAVSYFDVW
V$_H$-CDR3
110
4D5 GQGASVTVSS
HU4D5 GQGTLVTVSS
HUV$_H$III GQGTLVTVSS

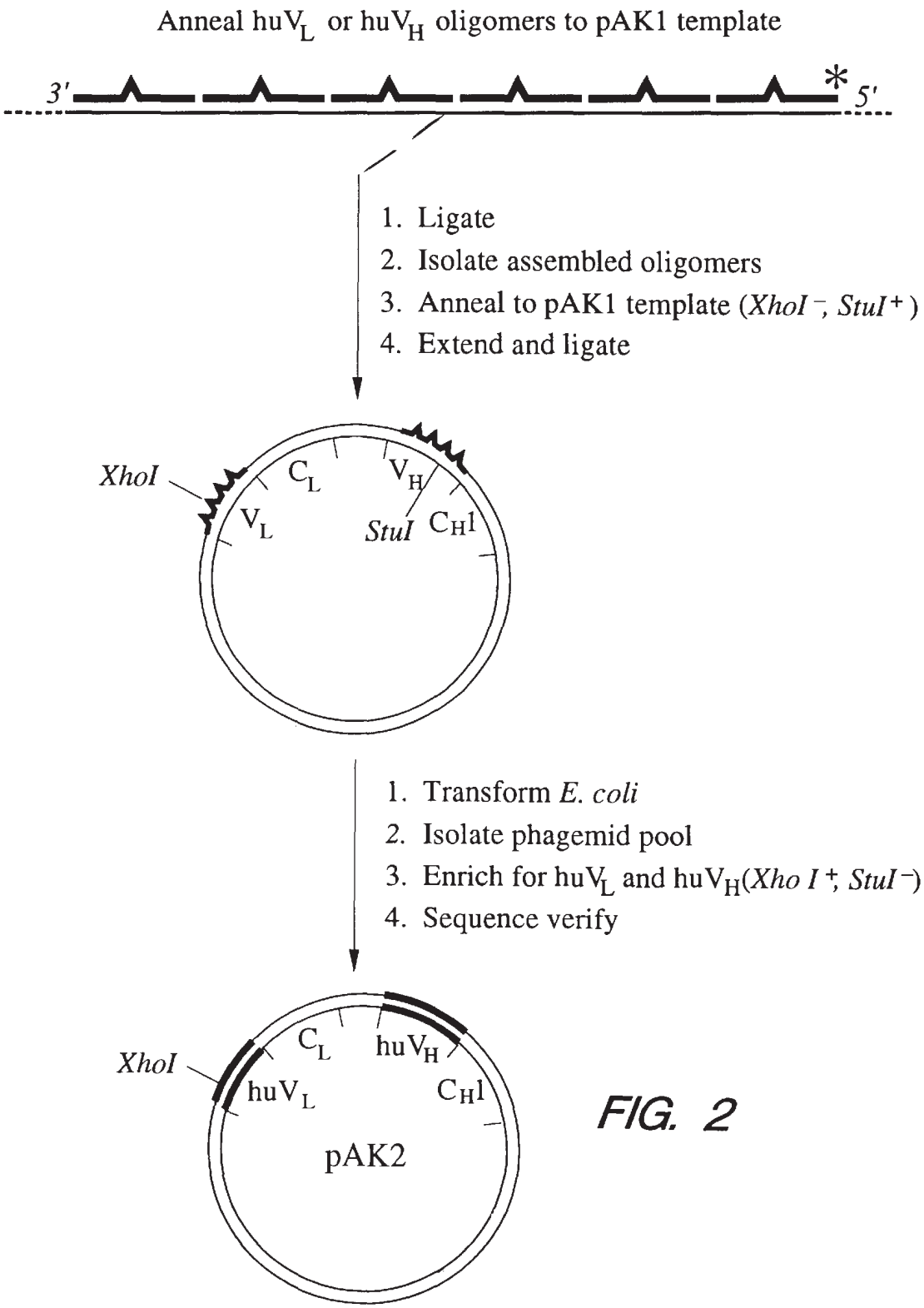

Anneal huV$_L$ or huV$_H$ oligomers to pAK1 template
3'
*
5'
1. Ligate
2. Isolate assembled oligomers
3. Anneal to pAK1 template (*XhoI*$^-$, *StuI*$^+$)
4. Extend and ligate
XhoI
C$_L$
V$_H$
V$_L$
StuI
C$_H$1
1. Transform *E. coli*
2. Isolate phagemid pool
3. Enrich for huV$_L$ and huV$_H$(*Xho I*$^+$,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

FIG. 2

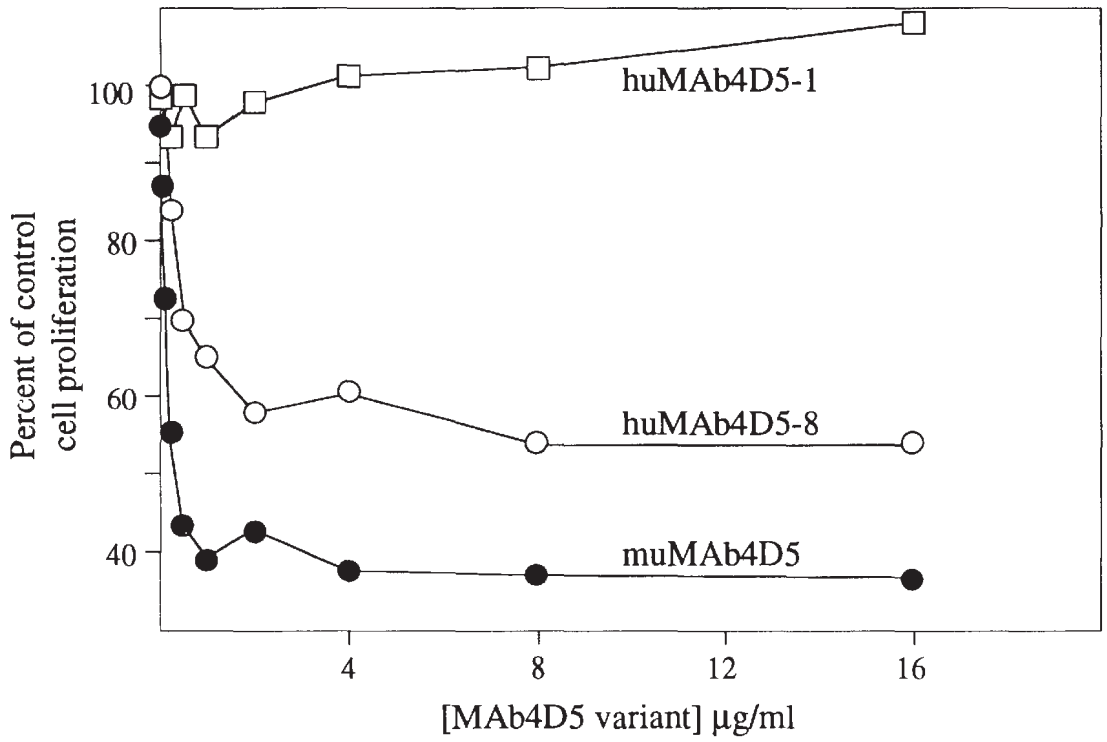

Percent of control cell proliferation
100
80
60
40
huMAb4D5-1
huMAb4D5-8
muMAb4D5
4
8
12
16
[MAb4D5 variant] µg/ml

FIG. 3

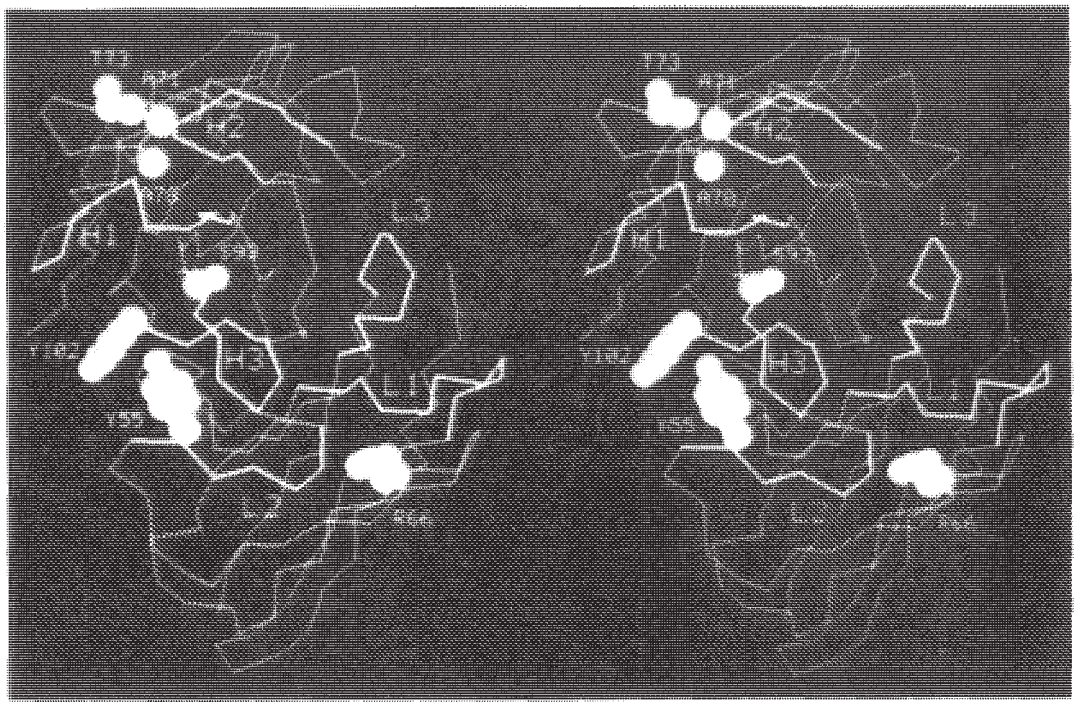


FIG. 4

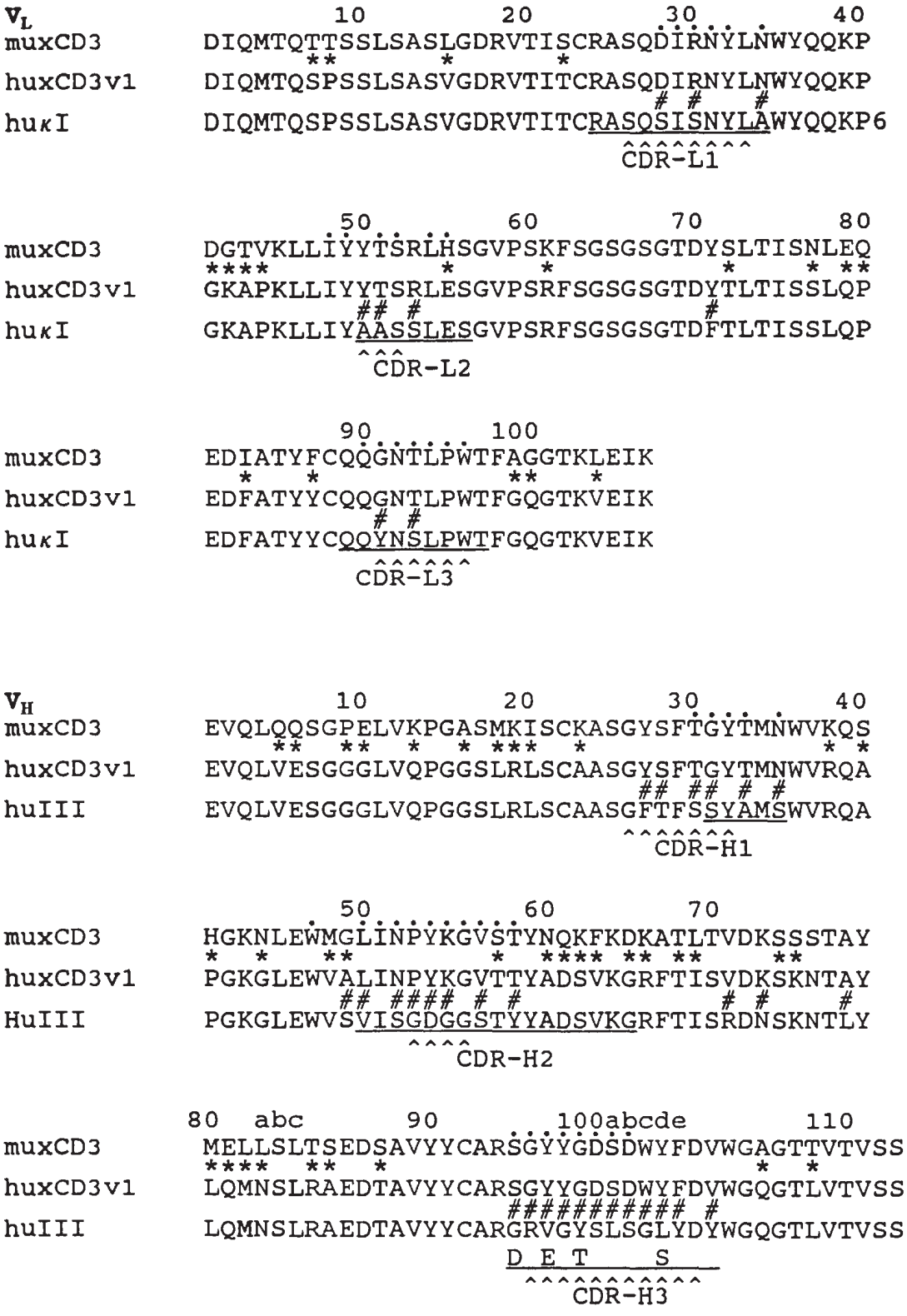

VL
muxCD3 DIQMTQTTSSLSASLGDRVTISCRASQDIRNYLNWYQQKP
huxCD3v1 DIQMTQSPSSLSASVGDRVTITCRASQDIRNYLNWYQQKP
huκI DIQMTQSPSSLSASVGDRVTITCRASQSISNYLAWYQQKP6
CDR-L1
muxCD3 DGTVKLLIYYTSRLHSGVPSKFSGSGSGTDYSLTISNLEQ
huxCD3v1 GKAPKLLIYYTSRLESGVPSRFSGSGSGTDYTLTISSLQP
huκI GKAPKLLIYAASSLESGVPSRFSGSGSGTDFTLTISSLQP
CDR-L2
muxCD3 EDIATYFCQQGNTLPWTFAGGTKLEIK
huxCD3v1 EDFATYYCQQGNTLPWTFGQGTKVEIK
huκI EDFATYYCQQYNSLPWTFGQGTKVEIK
CDR-L3
VH
muxCD3 EVQLQQSGPELVKPGASMKISCKASGYSFTGYTMNWVKQS
huxCD3v1 EVQLVESGGGLVQPGGSLRLSCAASGYSFTGYTMNWVRQA
huIII EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQA
CDR-H1
muxCD3 HGKNLEWMGLINPYKGVSTYNQKFKDKATLTVDKSSSTAY
huxCD3v1 PGKGLEWVALINPYKGVTTYADSVKGRFTISVDKSKNTAY
HuIII PGKGLEWVSVISGDGGSTYYADSVKGRFTISRDNSKNTLY
CDR-H2
muxCD3 MELLSLTSEDSAVYYCARSGYYGDSDWYFDVWGAGTTVTVSS
huxCD3v1 LQMNSLRAEDTAVYYCARSGYYGDSDWYFDVWGQGTLVTVSS
huIII LQMNSLRAEDTAVYYCARGRVGYSLSGLYDYWGQGTLVTVSS
D E T S
CDR-H3

FIG. 5

FIG. 6A-1

```
                                        10        20        30
H52H4-160                       QVQLQQSGPELVKPGASVKISCKTSGYTFTE
                                .*** .**  **.**.*...** ********
pH52-8.0    MGWSCIILFLVATATGVHSEVQLVESGGGLVQPGGSLRLSCATSGYTFTE
                    10        20        30        40        50

                   40        50        60        70        80
H52H4-160   YTMHWMKQSHGKSLEWIGGFNPKNGGSSHNQRFMDKATLAVDKSTSTAYM
            ******.*. **.***..*.******.********. *..**********
pH52-8.0    YTMHWMRQAPGKGLEWVAGINPKNGGTSHNQRFMDRFTISVDKSTSTAYM
                    60        70        80        90        100

                  90       100       110       120       130
H52H4-160   ELRSLTSEDSGIYYCARWRGLNYGFDVRYFDVWGAGTTVTVSSASTKGPS
            .. ** .**...********************** ** ************
pH52-8.0    QMNSLRAEDTAVYYCARWRGLNYGFDVRYFDVWGQGTLVTVSSASTKGPS
                    110       120       130       140       150

                 140       150       160       170       180
H52H4-160   VFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL
            ****** *.*** .**********************************
pH52-8.0    VFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVL
                    160       170       180       190       200

                 190       200       210       220       230
H52H4-160   QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTH
            ************** **..***** ***.********** ** * *
pH52-8.0    QSSGLYSLSSVVTVTSSNFGTQTYTCNVDHKPSNTKVDKTVERKCC---V
                    210       220       230       240

                 240       250       260       270       280
H52H4-160   TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVK
             *******  ..**********************************.
pH52-8.0    ECPPCPAPP-VAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQ
            250       260       270       280       290
```

FIG. 6A-2

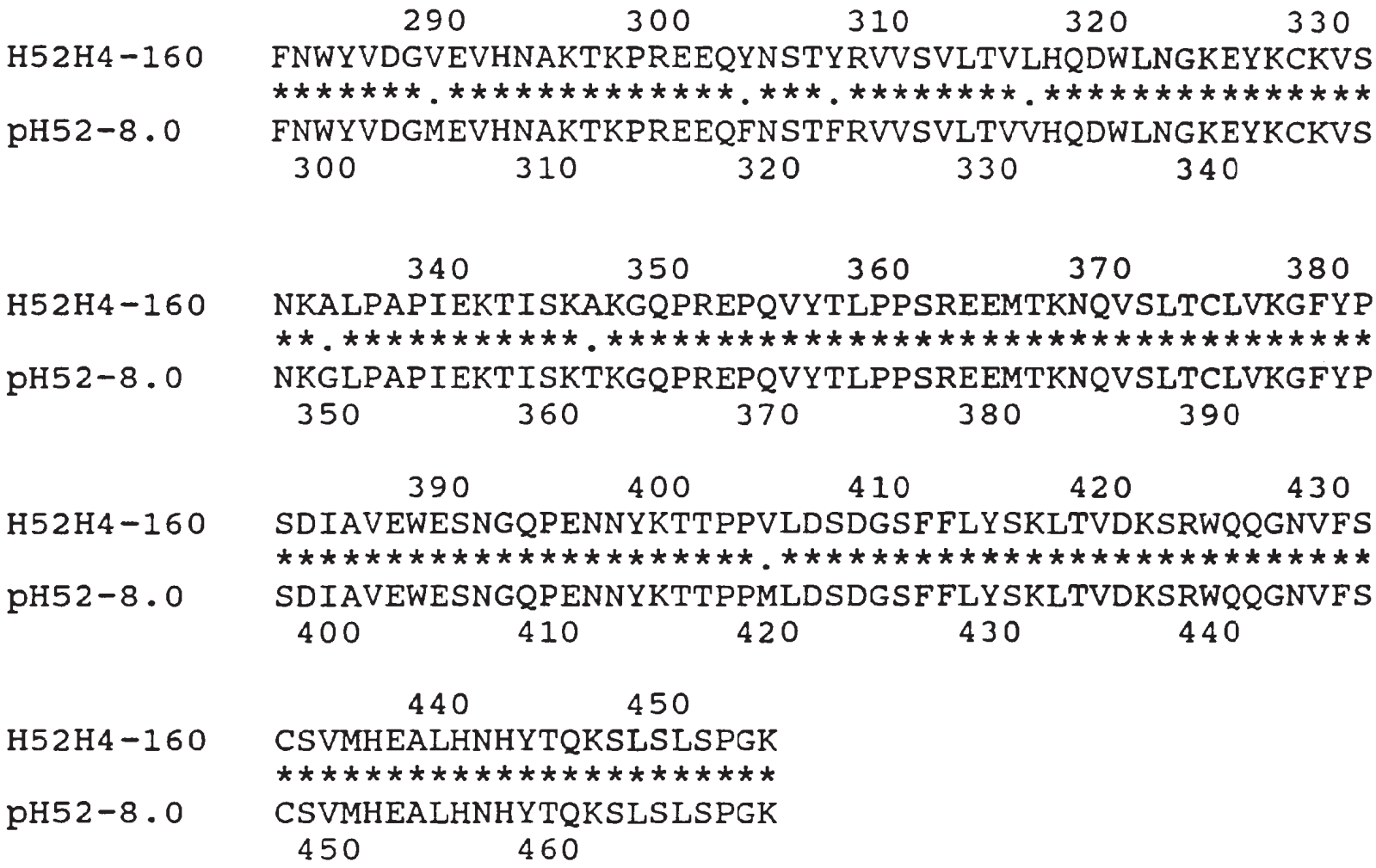

290 300 310 320 330
H52H4-160 FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVS
pH52-8.0 FNWYVDGMEVHNAKTKPREEQFNSTFRVVSVLTVVHQDWLNGKEYKCKVS
300 310 320 330 340
340 350 360 370 380
H52H4-160 NKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYP
pH52-8.0 NKGLPAPIEKTISKTKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYP
350 360 370 380 390
390 400 410 420 430
H52H4-160 SDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFS
pH52-8.0 SDIAVEWESNGQPENNYKTTPPMLDSDGSFFLYSKLTVDKSRWQQGNVFS
400 410 420 430 440
440 450
H52H4-160 CSVMHEALHNHYTQKSLSLSPGK
pH52-8.0 CSVMHEALHNHYTQKSLSLSPGK
450 460

FIG. 6B

```
                                        10        20        30
H52L6-158                          DVQMTQTTSSLSASLGDRVTINCRASQDINN
                                   *.****. ******.****** *********
pH52-9.0    MGWSCIILFLVATATGVHSDIQMTQSPSSLSASVGDRVTITCRASQDINN
                    10        20        30        40        50

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

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

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

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

METHOD FOR MAKING HUMANIZED ANTIBODIES

CROSS REFERENCES

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

FIELD OF THE INVENTION

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

BACKGROUND OF THE INVENTION

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

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

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

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

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

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

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

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

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

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

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

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

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

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

The proto-oncogene HER2 (human epidermal growth factor receptor 2) encodes a protein tyrosine kinase ($p185^{HER2}$) that is related to and somewhat homologous to the human epidermal growth factor receptor (see Coussens, L. et al., *Science* 230:1132–1139 (1985); Yamamoto, T. et al., *Nature* 319:230–234 (1986); King, C. R. et al., *Science* 229:974–976 (1985)). HER2 is also known in the field as c-erbB-2, and sometimes by the name of the rat homolog, neu. Amplification and/or overexpression of HER2 is associated with multiple human malignancies and appears to be integrally involved in progression of 25–30% of human breast and ovarian cancers (Slamon, D. J. et al., *Science* 235:177–182 (1987), Slamon, D. J. et al., *Science* 244:707–712 (1989)). Furthermore, the extent of amplification is inversely correlated with the observed median patient survival time (Slamon, supra, Science 1989).

The murine monoclonal antibody known as muMAb4D5 (Fendly, B. M. et al., *Cancer Res.* 50:1550–1558 (1990)), directed against the extracellular domain (ECD) of $p185^{HER2}$, specifically inhibits the growth of tumor cell lines overexpressing $p185^{HER2}$ in monolayer culture or in soft agar (Hudziak, R. M. et al., *Molec. Cell. Biol* 9:1165–1172 (1989); Lupu, R. et al., *Science* 249:1552–1555 (1990)). MuMAb4D5 also has the potential of enhancing tumor cell sensitivity to tumor necrosis factor, an important effector molecule in macrophage-mediated tumor cell cytotoxicity (Hudziak, supra, 1989; Shepard, H. M. and Lewis, G. D. *J. Clinical Immunology* 8:333–395 (1988)). Thus muMAb4D5 has potential for clinical intervention in and imaging of carcinomas in which $p185^{HER2}$ is overexpressed. The muMAb4D5 and its uses are described in PCT application WO 89/06692 published Jul. 27, 1989. This murine antibody was deposited with the ATCC and designated ATCC CRL 10463. However, this antibody may be immunogenic in humans.

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

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

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

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

SUMMARY OF THE INVENTION

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

a. obtaining the amino acid sequences of at least a portion of an import antibody variable domain and of a consensus variable domain;

b. identifying Complementarity Determining Region (CDR) amino acid sequences in the import and the human variable domain sequences;

c. substituting an import CDR amino acid sequence for the corresponding human CDR amino acid sequence;

d. aligning the amino acid sequences of a Framework Region (FR) of the import antibody and the corresponding FR of the consensus antibody;

e. identifying import antibody FR residues in the aligned FR sequences that are non-homologous to the corresponding consensus antibody residues;

f. determining if the non-homologous import amino acid residue is reasonably expected to have at least one of the following effects:

1. non-covalently binds antigen directly,

2. interacts with a CDR; or

3. participates in the V_L–V_H interface; and

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

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

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

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

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

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

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

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

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

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

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

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

1. SEQ. ID NO. 1, which is the light chain variable domain of a humanized version of muMAb4D5: DIQMTOSPSSLSASVGDRVTITCRASQD-VNTAVAWYQQKPGKAPKLLIYSASFLES-GVPSRFSGSRSGTDFTLTISSLQPEDFA-TYYCQQHYTTPPTFGQGTKVEIKRT

2. SEQ. ID NO. 2, which is the heavy chain variable domain of a humanized version of muMAb4D5): EVQLVESGGGLVOPGGSLRLSCAASGFNIK DTYIHWVRQAPGKGLEWVARIYPTNGYTRY ADSVKGRFTISADTSKNTAYLQMNSLRAED TAVYYCSRWGGDGFYAMDVWGQGTLVTVSS

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

SEQ. ID NO. 3 (light chain): DDIOMTQSPSSLSAS-VGDRVTITCRASQDVSSYLAWYQQKPGKAPKLL IYAASSLESGVPSRFSGSGSGTDFTLTISSLQP EDFATYYCQQYNSLPYTFGQGTKVEIKRT, and

SEQ. ID NO. 4 (heavy chain): EVQLVESGGGLVQPG GSLRLSCAASGFTFSDYAMSWVRQAPGKGL EWVAVISENGGYTRYADSVKGRFTISADTSKNT AYLQMNSLRAEDTAWYCSRWGGDGFYAMD VWGQGTLVTVSS

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1**A shows the comparison of the V_L domain amino acid residues of muMAb4D5, huMAb4D5, and a consensus sequence (FIG. **1**A, SEQ.ID NO. 5, SEQ. ID NO. 1 and SEQ. ID NO. 3, respectively). FIG. **1**B shows the comparison between the V_H domain amino acid residues of the muMAb4D5, huMAb4D5, and a consensus sequence (FIG. **1**B, SEQ. ID NO. 6, SEQ. ID NO. 2 and SEQ. ID NO. 4, respectively). Both FIGS. **1**A and **1**B use the generally accepted numbering scheme from Kabat, E. A., et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md. (1987)). In both FIG. **1**A

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

FIG. 2 shows a scheme for humanization of muMAb4D5 V_L and V_H by gene conversion mutagenesis.

FIG. 3 shows the inhibition of SK-BR-3 proliferation by MAb4D5 variants. Relative cell proliferation was determined as described (Hudziak, R. M. et al., *Molec. Cell. Biol.* 9:1165–1172 (1989)) and data (average of triplicate determinations) are presented as a percentage of results with untreated cultures for muMAb4D5 (●), huMAb4D5-8 (○) and huMAb4D5-1 (□).

FIG. 4 shows a stereo view of α-carbon tracing for a model of huMAb4D5-8 V_L and V_H. The CDR residues (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) are shown in bold and side chains of V_H residues A71, T73, A78, S93, Y102 and V_L residues Y55 plus R66 (see Table 3) are shown.

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

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

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

DETAILED DESCRIPTION OF THE INVENTION

Definitions

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

The murine monoclonal antibody known as muMAb4D5 (Fendly, B. M. et al., *Cancer Res.* 50:1550–1558 (1990)) is directed against the extracellular domain (ECD) of $p185^{HER2}$. The muMAb4D5 and its uses are described in PCT application WO 89/06692 published Jul. 27, 1989. This murine antibody was deposited with the ATCC and designated ATCC CRL 10463. In this description and claims, the terms muMAb4D5, chMAb4D5 and huMAb4D5 represent murine, chimerized and humanized versions of the monoclonal antibody 4D5, respectively.

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

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

In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains (Fab, Fab', $F(ab')_2$, Fabc, Fv) in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. Ordinarily, the antibody will contain both the light chain as well as at least the variable domain of a heavy chain. The antibody also may include the CH1, hinge, CH2, CH3, and CH4 regions of the heavy chain.

The humanized antibody will be selected from any class of immunoglobulins, including IgM, IgG, IgD, IgA and IgE, and any isotype, including IgG1, IgG2, IgG3 and lgG4. Usually the constant domain is a complement fixing constant domain where it is desired that the humanized antibody exhibit cytotoxic activity, and the class is typically IgG_1. Where such cytotoxic activity is not desirable, the constant domain may be of the IgG_2 class. The humanized antibody may comprise sequences from more than one class or isotype, and selecting particular constant domains to optimize desired effector functions is within the ordinary skill in the art.

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

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

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

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

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

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

A residue that interacts with a CDR side chain is one that is reasonably expected to form a noncovalent bond with a CDR side chain, generally either a salt bridge or hydrogen bond. Such residues are identified by three dimensional positioning of their side chains. A salt or ion bridge could be expected to form between two side chains positioned within about 2.5–3.2 Angstroms of one another that bear opposite charges, for example a lysinyl and a glutamyl pairing. A hydrogen bond could be expected to form between the side chains of residue pairs such as seryl or threonyl with aspartyl or glutamyl (or other hydrogen accepting residues). Such pairings are well known in the protein chemistry art and will be apparent to the artisan upon three dimensional modeling of the candidate immunoglobulin.

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

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

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

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

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

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

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

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

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

Class	Heavy Chain	Subclasses	Light Chain	Molecular Formula
IgG	γ	$\gamma1$, $\gamma2$, $\gamma3$, $\gamma4$	κ or λ	$(\gamma_2\kappa_2)$, $(\gamma_2\lambda_2)$
IgA	α	$\alpha1$, $\alpha2$	κ or λ	$(\alpha_2\kappa_2)_n{}^8$, $(\alpha_2\lambda_2)_n{}^8$
IgM	μ	none	κ or λ	$(\mu_2\kappa_2)_5$, $(\mu_2\lambda_2)_5$
IgD	δ	none	κ or λ	$(\delta_2\kappa_2)$, $(\delta_2\lambda_2)$
IgE	ϵ	none	κ or λ	$(\epsilon_2\kappa_2)$, $(\epsilon_2\lambda_2)$

($^8{}_n$ may equal 1, 2, or 3)

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

DIQMTQSPSSLSASVGDRVTITCRASQD-VSSYLAWYQQKPGKAPKLLIYAASSLES-GVPSRFSGSGSGTDFTLTISSLQPEDFA-TYYCQQYNSLPYTFGQGTKVEIKRT (SEQ. ID NO. 3);

the V_H consensus domain has the amino acid sequence:

EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYAMSW VRQAPGKGLEWVAVISENGGYTRYADSVKGRFT ISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGD GFYAMDVWGQGTLVTVSS (SEQ. ID NO. 4).

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

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

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

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

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

This invention is also directed to novel polypeptides, and in certain aspects, isolated novel humanized anti-p185^{HER2} antibodies are provided. These novel anti-p185^{HER2} antibodies are sometimes collectively referred to herein as huMAb4D5, and also sometimes as the light or heavy chain variable domains of huMAb4D5, and are defined herein to be any polypeptide sequence which possesses a biological property of a polypeptide comprising the following polypeptide sequence:

DIQMTQSPSSLSASVGDRVTITCRASODVNTAVAWY QQKPGKAPKLLIYSASFLESGVPSRFSGSRSGT DFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTK VEIKRT (SEQ. ID NO. 1, which is the light chain variable domain of huMAb4D5); or

EVQLVESGGGLVOPGGSLRLSCAASGFNIKDTYIHW VRQAPGKGLEWVARIYPTNGYTRYADSVKGRFT ISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGD GFYAMDVWGQGTLVTVSS (SEQ. ID NO. 2, which is the heavy chain variable domain of huMAb4D5).

"Biological property", as relates for example to anti-p185^{HER2}, for the purposes herein means an in vivo effector or antigen-binding function or activity that is directly or indirectly performed by huMAb4D5 (whether in its native or denatured conformation). Effector functions include p185^{HER2} binding, any hormonal or hormonal antagonist activity, any mitogenic or agonist or antagonist activity, any

cytotoxic activity. An antigenic function means possession of an epitope or antigenic site that is capable of cross-reacting with antibodies raised against the polypeptide sequence of huMAb4D5.

Biologically active huMAb4D5 is defined herein as a polypeptide that shares an effector function of huMAb4D5. A principal known effector function of huMAb4D5 is its ability to bind to $p185^{HER2}$.

Thus, the biologically active and antigenically active huMAb4D5 polypeptides that are the subject of certain embodiments of this invention include the sequence of the entire translated nucleotide sequence of huMAb4D5; mature huMAb4D5; fragments thereof having a consecutive sequence of at least 5, 10, 15, 20, 25, 30 or 40 amino acid residues comprising sequences from muMAb4D5 plus residues from the human FR of huMAb4D5; amino acid sequence variants of huMAb4D5 wherein an amino acid residue has been inserted N- or C-terminal to, or within, huMAb4D5 or its fragment as defined above; amino acid sequence variants of huMAb4D5 or its fragment as defined above wherein an amino acid residue of huMAb4D5 or its fragment as defined above has been substituted by another residue, including predetermined mutations by, e.g., site-directed or PCR mutagenesis; derivatives of huMAb4D5 or its fragments as defined above wherein huMAb4D5 or its fragments have been covalent modified, by substitution, chemical, enzymatic, or other appropriate means, with a moiety other than a naturally occurring amino acid; and glycosylation variants of huMAb4D5 (insertion of a glycosylation site or deletion of any glycosylation site by deletion, insertion or substitution of suitable residues). Such fragments and variants exclude any polypeptide heretofore identified, including muMAb4D5 or any known polypeptide fragment, which are anticipatory order 35 U.S.C. 102 as well as polypeptides obvious thereover under 35 U.S.C. 103.

An "isolated" polypeptide means polypeptide which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, for example, a polypeptide product comprising huMAb4D5 will be purified from a cell culture or other synthetic environment (1) to greater than 95% by weight of protein as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a gas- or liquid-phase sequenator (such as a commercially available Applied Biosystems sequenator Model 470, 477, or 473), or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated huMAb4D5 includes huMAb4D5 in situ within recombinant cells since at least one component of the huMAb4D5 natural environment will not be present. Ordinarily, however, isolated huMAb4D5 will be prepared by at least one purification step.

In accordance with this invention, huMAb4D5 nucleic acid is RNA or DNA containing greater than ten bases that encodes a biologically or antigenically active huMAb4D5, is complementary to nucleic acid sequence encoding such huMAb4D5, or hybridizes to nucleic acid sequence encoding such huMAb4D5 and remains stably bound to it under stringent conditions, and comprises nucleic acid from a muMAb4D5 CDR and a human FR region.

Preferably, the huMAb4D5 nucleic acid encodes a polypeptide sharing at least 75% sequence identity, more preferably at least 80%, still more preferably at least 85%, even more preferably at 90%, and most preferably 95%, with the huMAb4D5 amino acid sequence. Preferably, a nucleic acid molecule that hybridizes to the huMAb4D5 nucleic acid contains at least 20, more preferably 40, and most preferably 90 bases. Such hybridizing or complementary nucleic acid, however, is further defined as being novel under 35 U.S.C. 102 and unobvious under 35 U.S.C. 103 over any prior art nucleic acid.

Stringent conditions are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0/1% $NaDodSO_4$ at 50° C.; (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serumalbumin/0/1% Ficoll/0/1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42° C.; or (3) employ 50% formamide, 5×SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5×Denhardt's solution, sonicated salmon sperm DNA (50 g/ml), 0.1% SDS, and 10% dextran sulfate at 42 C., with washes at 42 C. in 0.2×SSC and 0.1% SDS.

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

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

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

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

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

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

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

Suitable Methods for Practicing the Invention

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

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

Molecular Modeling

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

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

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

Step 1

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

TABLE I

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

TABLE I-continued

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

[a]Four-letter code for Protein Data Bank file.
[b]Residue numbers for the crystal structures are taken from the Protein Data Bank files. Residue numbers for the consensus structure are according to Kabat et al.
[c]Root-mean-square deviation in Å for (N, Cα, C) atoms superimposed on 2FB4.
[d]Root-mean-square deviation in Å for (N, Cα, C) atoms superimposed on 2HFL.

Step 2

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

Step 3

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

Step 4

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

Step 5

In order to correct these deviations, the final step was to subject the "average" structure to 50 cycles of energy minimization (DISCOVER program, Biosym Technologies) using the AMBER (Weiner, S. J. et. al., *J. Amer. Chem. Soc.,* 106: 765–784 (1984)) parameter set with only the Ca coordinates fixed (i.e. all other atoms are allowed to move) (energy minimization is described below). This allowed any deviant bond lengths and angles to assume a standard (chemically acceptable) geometry. See Table II.

TABLE II

Average Bond Lengths and Angles for "Average" (Before) and Energy-Minimized Consensus (After 50 Cycles) Structures

	$V_L\kappa$ before (Å)	$V_L\kappa$ after (Å)	V_H before (Å)	V_H after (Å)	Standard Geometry (Å)
N—Cα	1.459(0.012)	1.451(0.004)	1.451(0.023)	1.452(0.004)	1.449
Cα-C	1.515(0.012)	1.523(0.005)	1.507(0.033)	1.542(0.005)	1.522
O=C	1.208(0.062)	1.229(0.003)	1.160(0.177)	1.231(0.003)	1.229
C—N	1.288(0.049)	1.337(0.002)	1.282(0.065)	1.335(0.004)	1.335
Cα-Cβ	1.508(0.026)	1.530(0.002)	1.499(0.039)	1.530(0.002)	1.526
	(*)	(*)	(*)	(*)	(*)
C—N—Cα	123.5(4.2)	123.8(1.1)	125.3(4.6)	124.0(1.1)	121.9
N—Cα-C	110.0(4.0)	109.5(1.9)	110.3(2.8)	109.5(1.6)	110.1
Cα-C—N	116.6(4.0)	116.6(1.2)	117.6(5.2)	116.6(0.8)	116.6
O=C—N	123.1(4.1)	123.4(0.6)	122.2(4.9)	123.3(0.4)	122.9
N—Cα-Cβ	110.3(2.1)	109.8(0.7)	110.6(2.5)	109.8(0.6)	109.5
Cβ-Cα-C	111.4(2.4)	111.1(0.7)	111.2(2.2)	111.1(0.6)	111.1

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

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

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

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

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

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

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

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

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

Methods for Obtaining a Humanized Antibody Sequence

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

While these steps may be taken in different order, typically a structure for the candidate humanized antibody is created by transferring the at least one CDR from the non-human, import sequence into the consensus human structure, after the entire corresponding human CDR has been removed. The humanized antibody may contain human replacements of the non-human import residues at positions within CDRs as defined by sequence variability (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) or as defined by structural variability (Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901–917 (1987)). For example, huMAb4D5 contains human replacements of the muMAb4D5 residues at three positions within CDRs as defined by sequence variability (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) but not as defined by structural variability (Chothia, C. & Lesk, A. M., *J. Mol Bol.* 196:901–917 (1987)): V_L-CDR1 K24R, V_L-CDR2 R54L and V_L-CDR2 T56S.

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

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

a. obtaining the amino acid sequences of at least a portion of an import antibody variable domain and of a consensus human variable domain;

b. identifying Complementarity Determining Region (CDR) amino acid sequences in the import and the human variable domain sequences;

c. substituting an import CDR amino acid sequence for the corresponding human CDR amino acid sequence;

d. aligning the amino acid sequences of a Framework Region (FR) of the import antibody and the corresponding FR of the consensus antibody;

e. identifying import antibody FR residues in the aligned FR sequences that are non-homologous to the corresponding consensus antibody residues;

f. determining if the non-homologous import amino acid residue is reasonably expected to have at least one of the following effects:

1. non-covalently binds antigen directly,

2. interacts with a CDR; or

3. participates in the V_L–V_H interface; and

g. for any non-homologous import antibody amino acid residue which is reasonably expected to have at least

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

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

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

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

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

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

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

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

Preferably, the non-CDR residue substituted at the consensus FR site is the residue found at the corresponding location of the non-human antibody. If desired, one may utilize the other method steps described above for determining whether a particular amino acid residue can reasonably be expected to have undesirable effects, and remedying those effects.

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

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

Antibodies

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

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

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

Animals are typically immunized against the immunogenic conjugates or derivatives by combining 1 mg or 1 μg of conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with ⅕ to 1/10 the original amount of conjugate in Freund's complete adjuvant (or other suitable adjuvant) by subcutaneous injection at multiple sites. 7 to 14 days later animals are bled and the serum is assayed for antigen titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking agent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are used to enhance the immune response.

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

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

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

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

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

Amino Acid Sequence Variants

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

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

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

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

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

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

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

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

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

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

Substantial modifications in function or immunological identity of the target polypeptide are accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, lb) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on common side chain properties:

(1) hydrophobic: norleucine, met, ala, val, leu, ile;

(2) neutral hydrophilic: cys, ser, thr;

(3) acidic: asp, glu;

(4) basic: asn, gin, his, lys, arg;

(5) residues that influence chain orientation: gly, pro; and

(6) aromatic: trp, tyr, phe.

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

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

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

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

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

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

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

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

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

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

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

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

The alternative method involves two or more rounds of mutagenesis to produce the desired mutant. The first round is as described for the single mutants: wild-type DNA is used for the template, an oligonucleotide encoding the first desired amino acid substitution(s) is annealed to this template, and the heteroduplex DNA molecule is then generated. The second round of mutagenesis utilizes the mutated DNA produced in the first round of mutagenesis as the template. Thus, this template already contains one or more mutations. The oligonucleotide encoding the additional desired amino acid substitution(s) is then annealed to this template, and the resulting strand of DNA now encodes mutations from both the first and second rounds of mutagenesis. This resultant DNA can be used as a template in a third round of mutagenesis, and so on.

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

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

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

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

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

Insertion of DNA into a Cloning Vehicle

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

(a) Signal Sequence Component

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

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

(b) Origin of Replication Component

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

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

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

(c) Selection Gene Component

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

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

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

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

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

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

(d) Promoter Component

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

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

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

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

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

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

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

also contains the SV40 viral origin of replication. Fiers et al., *Nature,* 273:113 (1978); Mulligan and Berg, *Science,* 209: 1422–1427 (1980); Pavlakis et al., *Proc. Natl. Acad. Sci. USA,* 78: 7398–7402 (1981). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. Greenaway et al., *Gene,* 18: 355–360 (1982). A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Pat. No. 4,419,446. A modification of this system is described in U.S. Pat. No. 4,601,978. See also Gray et al., *Nature,* 29: 503–508 (1982) on expressing cDNA encoding immune interferon in monkey cells; , Reyes et al., *Nature,* 297: 598–601 (1982) on expression of human β-interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus, Canaani and Berg, *Proc. Natl. Acad. Sci. USA,* 79: 5166–5170 (1982) on expression of the human interferon β1 gene in cultured mouse and rabbit cells, and Gorman et al., *Proc. Natl. Aced. Sci. USA,* 79: 6777–6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter.

(e) Enhancer Element Component

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

(f) Transcription Termination Component

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

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

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced is by the method of Messing et al., *Nucleic Acids Res.,* 9: 309 (1981) or by the method of Maxam et al., *Methods in Enzymology* 65: 499 (1980).

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

Other methods, vectors, and host cells suitable for adaptation to the synthesis of the target polypeptide in recombinant vertebrate cell culture are described in Gething et al., *Nature,* 293: 620–625 [1981]; Mantei et al., *Nature,* 281: 40–46 [1979]; Levinson et al.,; EP 117,060; and EP 117,058. A particularly useful plasmid for mammalian cell culture expression of the target polypeptide is pRK5 (EP pub. no. 307,247) or pSVI6B.

Selection and Transformation of Host Cells

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

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

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

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

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

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, $CaPO_4$ and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

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

Culturina the Host Cells

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

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

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

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

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

Detecting Gene Amplification/Expression

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

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

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

Purification of the Target Polypeptide

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

When the target polypeptide is expressed in a recombinant cell other than one of human origin, the target polypeptide is completely free of proteins or polypeptides of human origin. However, it is necessary to purify the target polypeptide from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to the target polypeptide. As a first step, the culture medium or lysate is centrifuged to remove particulate cell debris. The membrane and soluble protein fractions are then separated. The target polypeptide may then be purified from the soluble protein fraction and from the membrane fraction of the culture lysate, depending on whether the target polypeptide is membrane bound. The following procedures are exemplary of suitable purification procedures: fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; and protein A Sepharose columns to remove contaminants such as IgG.

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

Covalent Modifications of Target Polypeptides

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

Cysteinyl residues most commonly are reacted with α-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α-bromo-β-(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides,3-nitro-2-pyridyl disulfide, methyl2-pyridyldisulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Diagnostic and Related Uses of the Antibodies

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

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

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

The label used (and this is also useful to label antigen nucleic acid for use as a probe) is any detectable functionality that does not interfere with the binding of analyte and its binding partner. Numerous labels are known for use in immunoassay, examples including moieties that may be detected directly, such as fluorochrome, chemiluminescent, and radioactive labels, as well as moieties, such as enzymes, that must be reacted or derivatized to be detected. Examples of such labels include the radioisotopes ^{32}P, ^{14}C, ^{125}I, ^{3}H, and ^{131}I, fluorophores such as rare earth chelates or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, luceriferases, e.g., firefly luciferase and bacterial luciferase (U.S. Pat. No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, horseradish peroxidase (HRP), alkaline phosphatase, β-galactosidase, glucoamylase, lysozyme, saccharide oxidases, e.g., glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase, heterocyclic oxidases such as uricase and xanthine oxidase, coupled with an enzyme that employs hydrogen peroxide to oxidize a dye precursor such as HRP, lactoperoxidase, or microperoxidase, biotintavidin, spin labels, bacteriophage labels, stable free radicals, and the like.

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

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

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

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

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

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

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

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

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

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

Immunotoxins

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

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

The cytotoxic moiety of the immunotoxin may be a cytotoxic drug or an enzymatically active toxin of bacterial, fungal, plant or animal origin, or an enzymatically active fragment of such a toxin. Enzymatically active toxins and fragments thereof used are diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins, dianthin proteins, *Phytolaca americana* proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin, enomycin and the tricothecenes. In another embodiment, the antibodies are conjugated to small molecule anticancer drugs such as cis-platin or 5FU. Conjugates of the monoclonal antibody and such cytotoxic moieties are made using a variety of bifunctional protein coupling agents. Examples of such reagents are SPDP, IT, bifunctional derivatives of imidoesters such as dimethyl adipimidate HCI, active esters such as disuccinimidyl suberate, aldehydes such as glutaraldehyde, bis-azido compounds such as bis (p-azidobenzoyl) hexanediamine, bis-diazonium derivatives such as bis-(p-diazoniumbenzoyl)-ethylenediamine, diisocyanates such as tolylene 2,6-diisocyanate and bis-active fluorine compounds such as 1,5-difluoro-2,4-dinitrobenzene. The lysing portion of a toxin may be joined to the Fab fragment of the antibodies.

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

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

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

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

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

In a further embodiment toxin-conjugates are made with Fab or $F(ab')_2$ fragments. Because of their relatively small size these fragments can better penetrate tissue to reach infected cells.

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

Antibody Dependent Cellular Cytotoxicity

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

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

Textbook of Immunology, 2nd Edition, Williams & Wilkins, p. 218 (1984)). This includes their ability to activate complement and to mediate antibody-dependent cellular cytotoxicity (ADCC) as effected by leukocytes. Antibodies of different classes and subclasses differ in this respect, as do antibodies from the same subclass but different species; according to the present invention, antibodies of those classes having the desired biological activity are prepared. Preparation of these antibodies involves the selection of antibody constant domains are their incorporation in the humanized antibody by known technique. For example, mouse immunoglobulins of the IgG3 and lgG2a class are capable of activating serum complement upon binding to the target cells which express the cognate antigen, and therefore humanized antibodies which incorporate IgG3 and lgG2a effector functions are desirable for certain therapeutic applications.

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

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

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

Therapeutic and Other Uses of the Antibodies

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

For parenteral administration the antibodies will be formulated in a unit dosage injectable form (solution, suspension, emulsion) in association with a pharmaceutically acceptable parenteral vehicle. Such vehicles are inherently nontoxic, and non-therapeutic. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Nonaqueous vehicles such as fixed oils and ethyl oleate can also be used. Liposomes may be used as carriers. The vehicle may contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, e.g., buffers and preservatives. The antibodies will typically be formulated in such vehicles at concentrations of about 1 mg/ml to 10 mg/ml.

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

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

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

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

Deposit of Materials

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

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

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

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

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

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

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

EXAMPLES

Example 1

Humanization of muMAb4D5

Here we report the chimerization of muMAb4D5 (chMAb4D5) and the rapid and simultaneous humanization of heavy (V_H) and light (V_L) chain variable region genes using a novel "gene conversion mutagenesis" strategy. Eight humanized variants (huMAb4D5) were constructed to probe the importance of several FR residues identified by our molecular modeling or previously proposed to be critical to the conformation of particular CDRs (see Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901–917 (1987); Chothia, C. et al., *Nature* 342:877–883 (1989); Tramontano, A. et al., *J. Mol. Biol.* 215:175–182 (1990)). Efficient transient expression of humanized variants in non-myeloma cells allowed us to rapidly investigate the relationship between binding affinity for $p185^{HER2}$ ECD and anti-proliferative activity against $p185^{HER2}$ overexpressing carcinoma cells.

Materials and Methods

Cloning of Variable Region Genes. The muMAb4D5 V_H and V_L genes were isolated by polymerase chain reaction (PCR) amplification of mRNA from the corresponding hybridoma (Fendly, B. M. et al., *Cancer Res.* 50:1550–1558 (1990)) as described by Orlandi et al. (Orlandi, R. et al., *Proc. Natl. Acad. Sci. USA* 86:3833–3837 (1989)). Amino terminal sequencing of muMAb4D5 V_L and V_H was used to design the sense strand PCR primers, whereas the anti-sense PCR primers were based upon consensus sequences of murine framework residues (Orlandi, R. et al., *Proc. Natl. Acad. Sci. USA* 86:3833–3837 (1989); Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) incorporating restriction sites for directional cloning shown by underlining and listed after the sequences: V_L sense, 5'-TCC GATATCCAGCTGACCCAGTCTCCA-3' (SEQ. ID NO. 7), EcoRV; V_L anti-sense, 5'-GTTTGATCTCCAGCTT GGTACCHSCDCCGAA-3' (SEQ. ID NO. 8), Asp718; V_H sense, 5'-AGGTSMARCTGCAGSAGTCWGG-3' (SEQ. ID NO. 9), PstI and V_H anti-sense, 5'-TGAGGAGAC GGTGACCGTGGTCCCTTGGCCCCAG-3' (SEQ. ID. NO. 10), BstEII; where H=A or C or T, S=C or G, D=A or G or T, M=A or C, R=A or G and W=A or T. The PCR products were cloned into pUC119 (Vieira, J. & Messing, J., *Methods Enzymol.* 153:3–11 (1987)) and five clones for each variable domain sequenced by the dideoxy method (Sanger, F. et al., *Proc. Natl. Acad. Sci. USA* 74:5463–5467 (1977)).

Molecular Modelling. Models for muMAb4D5 V_H and V_L domains were constructed separately from consensus coordinates based upon seven Fab structures from the Brookhaven protein data bank (entries 1FB4, 2RHE, 2MCP, 3FAB, 1FBJ, 2HFL and 1REI). The Fab fragment KOL (Marquart, M. et al., *J. Mol. Biol.* 141:369–391 (1980)) was first chosen as a template for V_L and V_H domains and additional structures were then superimposed upon this structure using their main chain atom coordinates (INSIGHT program, Siosym Technologies). The distance from the template Cα to the analogous Cα in each of the superimposed structures was calculated for each residue position. If all (or nearly all) Cα—Cα distances for a given residue were ≦1 Å, then that position was included in the consensus structure. In most cases the β-sheet framework residues satisfied these criteria whereas the CDR loops did not. For each of these selected residues the average coordinates for individual N, Cα, C, O and Cβ atoms were calculated and then corrected for resultant deviations from non-standard bond geometry by 50 cycles of energy minimization using the DISCOVER program (Biosym Technologies) with the AMBER forcefield (Weiner, S. J. et al., *J. Amer. Chem. Soc.* 106:765–784 (1984)) and Cα coordinates fixed. The side chains of highly conserved residues, such as the disulfide-bridged cysteine residues, were then incorporated into the resultant consensus structure. Next the sequences of muMAb4D5 V_L and V_H were incorporated starting with the CDR residues and using the tabulations of CDR conformations from Chothia et al. (Chothia, C. et al., *Nature* 342:877–883 (1989)) as a guide. Side-chain conformations were chosen on the basis of Fab crystal structures, rotamer libraries (Ponder, J. W. & Richards, F. M., *J. Mol. Biol.* 193:775–791 (1987)) and packing considerations. Since V_H-COR3 could not be assigned a definite backbone conformation from these criteria, two models were created from a search of similar sized loops using the INSIGHT program. A third model was derived using packing and solvent exposure considerations. Each model was then subjected to 5000 cycles of energy minimization.

In humanizing muMAb4D5, consensus human sequences were first derived from the most abundant subclasses in the sequence compilation of Kabat et al. (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)), namely V_L κ subgroup I and V_H group III, and a molecular model generated for these sequences using the methods described above. A structure for huMAb4D5 was created by transferring the CDRs from the muMAb4D5 model into the consensus human structure. All huMAb4D5 variants contain human replacements of muMAb4D5 residues at three positions within CDRs as defined by sequence variability (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) but notas defined by structural variability (Chothia, C. & Lesk, A. M., *J. Mol. Biol.* 196:901–917 (1987)):

V_L-CDR1 K24R, V_L-CDR2 R54L and V_L-CDR2 T56S. Differences between muMAb4D5 and the human consensus framework residues (FIG. 1) were individually modeled to investigate their possible influence on CDR conformation and/or binding to the $p185^{HER2}$ ECD.

Construction of Chimeric Genes. Genes encoding chMAb4D5 light and heavy chains were separately assembled in previously described phagemid vectors containing ihe human cytomegalovirus enhancer and promoter, a 5' intron and SV40 polyadenylation signal (Gorman, C. M. et al., *DNA & Prot. Engin. Tech.* 2:3–10 (1990)). Briefly, gene segments encoding muMAb4D5 V_L (FIG. 1A) and REI human κ_1 light chain C_L (Palm, W. & Hilschmann, N., *Z. Physiol. Chem.* 356:167–191 (1975)) were precisely joined as were genes for muMAb4D5 V_H (FIG. 1B) and human γ1 constant region (Capon, D. J. et al., *Nature* 337:525–531 (1989)) by simple subcloning (Boyle, A., in *Current Protocols in Molecular Biology,* Chapter 3 (F. A. Ausubel et al., eds., Greene Publishing & Wiley-Interscience, New York, 1990)) and site-directed mutagenesis (Carter, P., in *Mutagenesis: A Practical Approach*, Chapter 1 (IRL Press, Oxford, UK 1991)). The γ1 isotype was chosen as it has been found to be the preferred human isotype for supporting ADCC and complement dependent cytotoxicity using matched sets of chimeric (Brüggemann, M. et al., *J. Exp. Med.* 166:1351–1361 (1987)) or humanized antibodies (Riechmann, L. et al., *Nature* 332:323–327 (1988)). The PCR-generated V_L and V_H fragments (FIG. 1) were subsequently mutagenized so that they faithfully represent the sequence of muMAb4D5 determined at the protein level: V_H Q1E, V_L V_{104}L and T109A (variants are denoted by the amino acid residue and number followed by the replacement amino acid). The human γ1 constant regions are identical to those reported by Ellison et al. (Ellison, J. W. et al., *Nucleic Acids Res.* 13:4071–4079 (1982)) except for the mutations E359D and M361L (Eu numbering, as in Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) which we installed to convert the antibody from the naturally rare A allotype to the much more common non-A allotype (Tramontano, A. et al., *J. Mol. Biol.* 215:175–182 (1990)). This was an attempt to reduce the risk of anti-allotype antibodies interfering with therapy.

Construction of Humanized Genes. Genes encoding chMAb4D5 light chain and heavy chain Fd fragment (V_H and C_H1 domains) were subcloned together into pUC119 (Vieira, J. & Messing, J., *Methods Enzymol.* 153:3–11 (1987)) to create pAK1 and simultaneously humanized in a single step (FIG. 2). Briefly, sets of 6 contiguous oligonucleotides were designed to humanize V_H and V_L (FIG. 1). These oligonucleotides are 28 to 83 nucleotides in length, contain zero to 19 mismatches to the murine antibody template and are constrained to have 8 or 9 perfectly matched residues at each end to promote efficient annealing and ligation of adjacent oligonucleotides. The sets of V_H and V_L humanization oligonucleotides (5 pmol each) were phosphorylated with either ATP or γ-^{32}P-ATP (Carter, P. *Methods Enzymol.* 154: 382–403 (1987)) and separately annealed with 3.7 pmol of pAK1 template in 40 μl 10 mM Tris-HCl (pH 8.0) and 10 mM $MgCl_2$ by cooling from 100° C. to room temperature over ~30 min. The annealed oligonucleotides were joined by incubation with T4 DNA ligase (12 units; New England Biolabs) in the presence of 2 μl 5 mM ATP and 2 μl 0.1 M DTT for 10 min at 14° C. After electrophoresis on a 6% acrylamide sequencing gel the assembled oligonucleotides were located by autoradiography and recovered by electroelution. The assembled oligonucleotides (~0.3 pmol each) were simultaneously annealed to 0.15 pmol single-stranded deoxyuridine-containing pAK1 prepared according to Kunkel et al. (Kunkel, T. A. et al., *Methods Enzymol.* 154:367–382 (1987)) in 10 μl 40 mM Tris-HCl (pH 7.5) and 16 mM $MgCl_2$ as above. Heteroduplex DNA was constructed by extending the primers with T7 DNA polymerase and transformed into *E. coli* BMH 71–18 mutL as previously described (Carter, P., in *Mutagenesis: A Practical Approach,* Chapter 1 (IRL Press, Oxford, UK 1991)). The resultant phagemid DNA pool was enriched first for huV_L by restriction purification using XhoI and then for huV_H by restriction selection using StuI as described in Carter, P., in *Mutagenesis: A Practical Approach,* Chapter 1 (IRL Press, Oxford, UK 1991); and in Wells, J. A. et al., *Phil. Trans. R. Soc. Lond., A* 317:415–423 (1986). Resultant clones containing both huV_L and huV_H genes were identified by nucleotide sequencing (Sanger, F. et al., *Proc. Natl. Acad. Sci. USA* 74:5463–5467 (1977)) and designated pAK2. Additional humanized variants were generated by site-directed mutagenesis (Carter, P., in *Mutagenesis: A Practical Approach,* Chapter 1 (IRL Press, Oxford, UK 1991)). The muMAb4D5 V_L and V_H gene segments in the transient expression vectors described above were then precisely replaced with their humanized versions.

Expression and Purification of MAb4D5 Variants. Appropriate MAb4D5 light and heavy chain cDNA expression vectors were co-transfected into an adenovirus transformed human embryonic kidney cell line, 293 (Graham, F. L. et al., *J. Gen. Virol.* 36:59–72 (1977)) using a high efficiency procedure (Gorman, C. M. et al., *DNA & Prot. Engin. Tech.* 2:3–10 (1990); Gorman, C., in *DNA Cloning,* vol II, pp 143–190 (D. M. Glover, ed., IRL Press, Oxford, UK 1985)). Media were harvested daily for up to 5 days and the cells re-fed with serum free media. Antibodies were recovered from the media and affinity purified on protein A sepharose CL-4B (Pharmacia) as described by the manufacturer. The eluted antibody was buffer-exchanged into phosphate-buffered saline by G25 gel filtration, concentrated by ultrafiltration (Centriprep-30 or Centricon-100, Amicon), sterile-filtered (Millex-GV, Millipore) and stored at 4° C. The concentration of antibody was determined by using both total immunoglobulin and antigen binding ELISAs. The standard used was huMAb4D5-5, whose concentration had been determined by amino acid composition analysis.

Cell Proliferation Assay. The effect of MAb4D5 variants upon proliferation of the human mammary adenocarcinoma cell line, SK-BR-3, was investigated as previously described (Fendly, B. M. et al., *Cancer Res.* 50:1550–1558 (1990)) using saturating MAb4D5 concentrations.

Affinity Measurements. The antigen binding affinity of MAb4D5 variants was determined using a secreted form of the $p185^{HER2}$ ECD prepared as described in Fendly, B. M. et al., *J. Biol. Resp. Mod.* 9:449–455 (1990). Briefly, antibody and $p185^{HER2}$ ECD were incubated in solution until equilibrium was found to be reached. The concentration of free antibody was then determined by ELISA using immobilized $p185^{HER2}$ ECD and used to calculate affinity (K_d) according to Friguet et al. (Friguet, B. et al., *J. Immunol. Methods* 77:305–319 (1985)).

Results

Humanization of muMAb4D5. The muMAb4D5 V_L and V_H gene segments were first cloned by PCR and sequenced (FIG. 1). The variable genes were then simultaneously humanized by gene conversion mutagenesis using preassembled oligonucleotides (FIG. 2). A 311-mer oligonucle-

otide containing 39 mismatches to the template directed 24 simultaneous amino acid changes required to humanize muMAb4D5 V_L. Humanization of muMAb4D5 V_H required 32 amino acid changes which were installed with a 361-mer containing 59 mismatches to the muMAb4D5 template. Two out of 8 clones sequenced precisely encode huMAb4D5-5, although one of these clones contained a single nucleotide imperfection. The 6 other clones were essentially humanized but contained a small number of errors: <3 nucleotide changes and <1 single nucleotide deletion per kilobase. Additional humanized variants (Table 3) were constructed by site-directed mutagenesis of huMAb4D5-5.

Expression levels of huMAb4D5 variants were in the range of 7 to 15 μg/ml as judged by ELISA using immobilized $p185^{HER2}$ ECD. Successive harvests of five 10 cm plates allowed 200 μg to 500 mg of each variant to be produced in a week. Antibodies affinity purified on protein A gave a single band on a Coomassie blue stained SDS polyacrylamide gel of mobility consistent with the expected M_r of ~150 kDa. Electrophoresis under reducing conditions gave 2 bands consistent with the expected M_r of free heavy (48 kDa) and light (23 kDa) chains (not shown). Amino terminal sequence analysis (10-cycles) gave the mixed sequence expected (see FIG. **1**) from an equimolar combination of light and heavy chains (not shown).

huMAb4D5 Variants. In general, the FR residues were chosen from consensus human sequences (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) and CDR residues from muMAb4D5. Additional variants were constructed by replacing selected human residues in huMAb4D5-1 with their muMAb4D5 counterparts. These are V_H residues 71, 73, 78, 93 plus 102 and V_L residues 55 plus 66 identified by our molecular modeling. V_H residue 71 has previously been proposed by others (Tramontano, A. et al., *J. Mol. Biol.* 215:175–182 (1990)) to be critical to the conformation of V_H-CDR2. Amino acid sequence differences between huMAb4D5 variant molecules are shown in Table 3, together with their $p185^{HER2}$ ECD binding affinity and maximal anti-proliferative activities against SK-BR-3 cells. Very similar K_d values were obtained for binding of MAb4D5 variants to either SK-BR-3 cells or to $p185^{HER2}$ ECD (Table 3). However, K_d estimates derived from binding of MAb4D5 variants to $p185^{HER2}$ ECD were more reproducible with smaller standard errors and consumed much smaller quantities of antibody than binding measurements with whole cells.

The most potent humanized variant designed by molecular modeling, huMAb4D5-8, contains 5 FR residues from muMAb4D5. This antibody binds the $p185^{HER2}$ ECD 3-fold more tightly than does muMAb4D5 itself (Table 3) and has comparable anti-proliferative activity with SK-BR-3 cells (FIG. **3**). In contrast, huMAb4D5-1 is the most humanized but least potent muMAb4D5 variant, created by simply installing the muMAb4D5 CDRs into the consensus human sequences. huMAb4D5-1 binds the $p185^{HER2}$ ECD 80-fold less tightly than does the murine antibody and has no detectable anti-proliferative activity at the highest antibody concentration investigated (16 μg/ml).

The anti-proliferative activity of huMAb4D5 variants against $p185^{HER2}$ overexpressing SK-BR-3 cells is not simply correlated with their binding affinity for the $p185^{HER2}$ ECD. For example, installation of three murine residues into the V_H domain of huMAb4D5-2 (D73T, L78A and A93S) to create huMAb4D5-3 does not change the antigen binding affinity but does confer significant anti-proliferative activity (Table 3).

The importance of V_H residue 71 (Tramontano, A. et al., *J. Mol. Biol.* 215:175–182 (1990)) is supported by the observed 5-fold increase in affinity for $p185^{HER2}$ ECD on replacement of R71 in huMAb4D5-1 with the corresponding murine residue, alanine (huMAb4D5-2). In contrast, replacing V_H L78 in huMAb4D5-4 with the murine residue, alanine (huMAb4D5-5), does not significantly change the affinity for the $p185^{HER2}$ ECD or change anti-proliferative activity, suggesting that residue 78 is not of critical functional significance to huMAb4D5 and its ability to interact properly with the extracellular domain of $p185^{HER2}$.

V_L residue 66 is usually a glycine in human and murine κ chain sequences (Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md., 1987)) but an arginine occupies this position in the muMAb4D5 κ light chain. The side chain of residue 66 is likely to affect the conformation of V_L-CDR1 and V_L-CDR2 and the hairpin turn at 68–69 (FIG. **4**). Consistent with the importance of this residue, the mutation V_L G66R (huMAb4D5-3→huMAb4D5-5) increases the affinity for the $p185^{HER2}$ ECD by 4-fold with a concomitant increase in anti-proliferative activity.

From molecular modeling it appears that the tyrosyl side chain of muMAb4D5 V_L residue 55 may either stabilize the conformation of V_H-CDR3 or provide an interaction at the V_L–V_H interface. The latter function may be dependent upon the presence of V_H Y102. In the context of huMAb4D5-5 the mutations V_L E55Y (huMAb4D5-6) and V_H V102Y (huMAb4D5-7) individually increase the affinity for $p185^{HER2}$ ECD by 5-fold and 2-fold respectively, whereas together (huMAb4D5-8) they increase the affinity by 11-fold. This is consistent with either proposed role of V_L Y55 and V_H Y102.

Secondary Immune Function of huMAb4D5-8. MuMAb4D5 inhibits the growth of human breast tumor cells which overexpress $p185^{HER2}$ (Hudziak, R. M. et al., *Molec. Cell. Biol.* 9:1165–1172 (1989)). The antibody, however, does not offer the possibility of direct tumor cytotoxic effects. This possibility does arise in huMAb4D5-8 as a result of its high affinity (Kd_d=0.1 μM) and its human IgG_1 subtype. Table 4 compares the ADCC mediated by huMAb4D5-8 with muMAb4D5 on a normal lung epithelial cell line, WI-38, which expresses a low level of $p185^{HER2}$ and on SK-BR-3, which expresses a high level of $p185^{HER2}$. The results demonstrate that: (1) huMAb4D5 has a greatly enhanced ability to carry out ADCC as compared with its murine parent; and (2) that this activity may be selective for cell types which overexpress $p185^{HER2}$.

Discussion

MuMAb4D5 is potentially useful for human therapy since it is cytostatic towards human breast and ovarian tumor lines overexpressing the HER2-encoded $p185^{HER2}$ receptor-like tyrosine kinase. Since both breast and ovarian carcinomas are chronic diseases it is anticipated that the optimal MAb4D5 variant molecule for therapy will have low immunogenicity and will be cytotoxic rather than solely cytostatic in effect. Humanization of muMAb4D5 should accomplish these goals. We have identified 5 different huMAb4D5 variants which bind tightly to $p185^{HER2}$ ECD ($K_d \leqq 1$ nM) and which have significant anti-proliferative activity (Table 3). Furthermore huMAb4D5-8 but not muMAb4D5 mediates ADCC against human tumor cell lines overexpressing $p185^{HER2}$ in the presence of human effector cells (Table 4) as anticipated for a human γ1 isotype (Brcüggemann, M. et al., *J. Exp. Med.* 166:1351–1361 (1987); Riechmann, L. et al., *Nature* 332:323–327 (1988)).

Rapid humanization of huMAb4D5 was facilitated by the gene conversion mutagenesis strategy developed here using long preassembled oligonucleotides. This method requires less than half the amount of synthetic DNA as does total gene synthesis and does not require convenient restriction sites in the target DNA. Our method appears to be simpler and more reliable than a variant protocol recently reported (Rostapshov, V. M. et al., *FEBS Lett.* 249: 379–382 (1989)). Transient expression of huMAb4D5 in human embryonic kidney 293 cells permitted the isolation of a few hundred micrograms of huMAb4D5 variants for rapid characterization by growth inhibition and antigen binding affinity assays. Furthermore, different combinations of light and heavy chain were readily tested by co-transfection of corresponding cDNA expression vectors.

The crucial role of molecular modeling in the humanization of muMAb4D5 is illustrated by the designed variant huMAb4D5-8 which binds the $p185^{HER2}$ ECD 250-fold more tightly than the simple CDR loop swap variant, huMAb4D5-1. It has previously been shown that the antigen binding affinity of a humanized antibody can be increased by mutagenesis based upon molecular modelling (Riechmann, L. et al., *Nature* 332:323–327 (1988); Queen, C. et al., *Proc. Natl. Acad. Sci. USA* 86:10029–10033 (1989)). Here we have extended this earlier work by others with a designed humanized antibody which binds its antigen 3-fold more tightly than the parent rodent antibody. While this result is gratifying, assessment of the success of the molecular modeling must await the outcome of X-ray structure determination. From analysis of huMAb4D5 variants (Table 3) it is apparent that their anti-proliferative activity is not a simple function of their binding affinity for $p185^{HER2}$ ECD. For example the huMAb4D5–8 variant binds $p185^{HER2}$ 3-fold more tightly than muMAb4D5 but the humanized variant is slightly less potent in blocking the proliferation of SK-SR-3 cells. Additional huMAb4D5 variants are currently being constructed in an attempt to identify residues triggering the anti-proliferative activity and in an attempt to enhance this activity.

In addition to retaining tight receptor binding and the ability to inhibit cell growth, the huMAb4D5-8 also confers a secondary immune function (ADCC). This allows for direct cytotoxic activity of the humanized molecule in the presence of human effector cells. The apparent selectivity of the cytotoxic activity for cell types which overexpress $p185^{HER2}$ allows for the evolution of a straightforward clinic approach to those human cancers characterized by overexpression of the HER2 protooncogene.

TABLE 3

$p185^{HER2}$ ECD binding affinity and anti-proliferative activities of MAb4D5 variants

	V_H Residue*					V_L Residue*			
MAb4D5 cell Variant proliferation‡	71 FR3	73 FR3	78 FR3	93 FR3	102 CDR3	55 CDR2	56 FR3	K_d† nM	Relative
huMAb4D5-1	R	D	L	A	V	E	G	25	102
huMAb4D5-2	Ala	D	L	A	V	E	G	4.7	101
huMAb4D5-3	Ala	Thr	Ala	Ser	V	E	G	4.4	66
huMAb4D5-4	Ala	Thr	L	Ser	V	E	Arg	0.82	56
huMAb4D5-5	Ala	Thre	Ala	Ser	V	E	Arg	1.1	48
huMAb4D5-6	Ala	Thr	Ala	Ser	V	Tyr	Arg	0.22	51
huMAb4D5-7	Ala	Thr	Ala	Ser	Tyr	E	Arg	0.62	53
huMAb4D5-8	Ala	Thr	Ala	Ser	Tyr	Tyr	Arg	0.10	54
muMAb4D5	Ala	Thr	Ala	Ser	Tyr	Tyr	Arg	0.30	37

*Human and murine residues are shown in one letter and three letter amino acid code respectively.
†K_d values for the $p185^{HER2}$ ECD were determined using the method of Friguet et al. (43) and the standard error of each estimate is ≦ ± 10%.
‡Proliferation of SK-BR-3 cells incubated for 96 hr with MAb4D5 variants shown as a percentage of the untreated control as described (Hudziak, R. M. et al., Molec. Cell. Biol. 9: 1165–1172 (1989)). Data represent the maximal anti-proliferative effect for each variant (see FIG. 3A) calculated as the mean of triplicate determinations at a MAb4D5 concentration of 8 μg/ml. Data are all taken from the same experiment with an estimated standard error of ≦ ± 15%.

TABLE 4

Selectivity of antibody dependent tumor cell cytotoxicity mediated by huMAb4D5-8

Effector:Target ratio†	WI-38* muMAb4D5	WI-38* huMAb4D5-8	SK-BR-3 muMAb4D5	SK-BR-3 huMAb4D5-8
A.‡				
25:1	<1.0	9.3	7.5	40.6
12.5:1	<1.0	11.1	4.7	36.8
6.25:1	<1.0	8.9	0.9	35.2
3.13:1	<1.0	8.5	4.6	19.6
B.				
25:1	<1.0	3.1	6.1	33.4
12.5:1	<1.0	1.7	5.5	26.2
6.25:1	1.3	2.2	2.0	21.0
3.13:1	<1.0	0.8	2.4	13.4

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

Example 2
Schematic Method for Humanizing an Antibody Sequence

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

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

1. ascertain a consensus human variable domain amino acid sequence and prepare from it a consensus structural model.
2. prepare model of import (the non-human domain to be humanized) variable domain sequences and note structural differences with respect to consensus human model.
3. identify CDR sequences in human and in import, both by using Kabat (supra, 1987) and crystal structure criteria. If there is any difference in CDR identity from the different criteria, use of crystal structure definition of the CDR, but retain the Kabat residues as important framework residues to import.
4. substitute import CDR sequences for human CDR sequences to obtain initial "humanized" sequence.
5. compare import non-CDR variable domain sequence to the humanized sequence and note divergences.
6. Proceed through the following analysis for each amino acid residue where the import diverges from the humanized.
   a. If the humanized residue represents a residue which is generally highly conserved across all species, use the residue in the humanized sequence. If the residue is not conserved across all species, proceed with the analysis described in 6b.
   b. If the residue is not generally conserved across all species, ask if the residue is generally conserved in humans.
      i. If the residue is generally conserved in humans but the import residue differs, examine the structural models of the import and human sequences and determine if the import residue would be likely to affect the binding or biological activity of the CDRs by considering 1) could it bind antigen directly and 2) could it affect the conformation of the CDR. If the conclusion is that an affect on the CDRs is likely, substitute the import residue. If the conclusion is that a CDR affect is unlikely, leave the humanized residue unchanged.
      ii. If the residue is also not generally conserved in humans, examine the structural models of the import and human sequences and determine if the import residue would be likely to affect the binding or biological activity of the CDRs be considering 1) could it bind antigen directly and 2) could it affect the conformation of the CDR. If the conclusion is that an affect on the CDRs is likely, substitute the import residue. If the conclusion is that a CDR affect is unlikely, proceed to the next step.
         a) Examine the structural models of the import and human sequences and determine if the residue is exposed on the surface of the domain or is buried within. If the residue is exposed, use the residue in the humanized sequence. If the residue is buried, proceed to the next step.
            (i) Examine the structural models of the import and human sequences and determine if the residue is likely to affect the V_L–V_H interface. Residues involved with the interface include: 34L, 36L, 38L, 43L, 33L, 36L, 85L, 87L, 89L, 91L, 96L, 98L, 35H, 37H, 39H, 43H, 45H, 47H, 60H, 91H, 93H, 95H, 100H, and 103H. If no effect is likely, use the residue in the humanized sequence. If some affect is likely, substitute the import residue.
7. Search the import sequence, the consensus sequence and the humanized sequence for glycosylation sites outside the CDRs, and determine if this glycosylation site is likely to have any affect on antigen binding and/or biological activity. If no effect is likely, use the human sequence at that site; if some affect is likely, eliminate the glycosylation site or use the import sequence at that site.
8. After completing the above analysis, determine the planned humanized sequence and prepare and test a sample. If the sample does not bind well to the target antigen, examine the particular residues listed below, regardless of the question of residue identity between the import and humanized residues.
   a. Examine particular peripheral (non-CDR) variable domain residues that may, due to their position, possibly interact directly with a macromolecular antigen, including the following residues (where the * indicates residues which have been found to interact with antigen based on crystal structures):
      i. Variable light domain: 36, 46, 49$^-$, 63–70
      ii. Variable heavy domain: 2, 47$^-$, 68, 70, 73–76.
   b. Examine particular variable domain residues which could interact with, or otherwise affect, the conformation of variable domain CDRs, including the following (not including CDR residues themselves, since it is assumed that, because the CDRs interact with one another, any residue in one CDR could potentially affect the conformation of another CDR residue) (L=LIGHT, H=HEAVY, residues appearing in bold are indicated to be structurally important according the Chothia et al., Nature 342:877 (1989), and residues appearing in italic were altered during humanization by Queen et al. (PDL), Proc. Natl. Acad. Sci. USA 86:10029 (1989) and Proc. Natl. Acad. Sci. USA 88:2869 (1991).):
      i. Variable light domain:
         a) CDR-1 (residues 24L–34L): 2L, 4L, 66L-69L, 71L
         b) CDR-2 (residues 50L–56L): 35L, 46L, 47L, 48L, 49L, 58L, 62L, 64L–66L, 71L, 73L
         c) CDR-3 (residues 89L–97L): 2L, 4L, 36L, 98L, 37H, 45H, 47H, 58H, 60H
      ii. Variable heavy domain:
         a) CDR-1 (residues 26H–35H): 2H, 4H, 24H, 36H, 71H, 73H, 76H, 78H, 92H, 94H
         b) CDR-2 (residues 50H–55H): 49H, 69H, 69H, 71H, 73H, 78H
         c) CDR-3 (residues 95H–102H): examine all residues as possible interaction partners with this loop, because this loop varies in size and conformation much more than the other CDRs.
9. If after step 8 the humanized variable domain still is lacking in desired binding, repeat step 8. In addition, re-investigate any buried residues which might affect the V_L–V_H interface (but which would not directly affect CDR conformation). Additionally, evaluate the accessibility of non-CDR residues to solvent.

Example 3

Engineering a Humanized Bisnecific F(ab')$_2$ Fragment

This example demonstrates the construction of a humanized bispecific antibody (BsF(ab')$_2$v1 by separate *E. coli* expression of each Fab' arm followed by directed chemical coupling in vitro. BsF(ab')$_2$v1 (anti-CD3/anti-p185^{HER2}) was demonstrated to retarget the cytotoxic activity of human

$CD3^+$CTL in vitro against the human breast tumor cell line, SK-BR-3, which overexpresses the $p185^{HER2}$ product of the protooncogene HER2. This example demonstrates the minimalistic humanization strategy of installing as few murine residues as possible into a human antibody in order to recruit antigen-binding affinity and biological properties comparable to that of the murine parent antibody. This strategy proved very successful for the anti-$p185^{HER2}$arm of BsF $(ab')_2$v1. In contrast BsF$(ab')_2$ v1 binds to T cells via its anti-CD3 arm much less efficiently than does the chimeric BsF$(ab')_2$ which contains the variable domains of the murine parent anti-CD3 antibody. Here we have constructed additional BsF$(ab')_2$ fragments containing variant anti-CD3 arms with selected murine residues restored in an attempt to improve antibody binding to T cells. One such variant, Ss F$(ab')_2$v9, was created by replacing six residues in the second hypervariable loop of the anti-CD3 heavy chain variable domain of BsF$(ab')_2$v1 with their counterparts from the murine parent anti-CD3 antibody. BsF$(ab')_2$v9 binds to T cells (Jurkat) much more efficiently than does BsF$(ab')_2$v1 and almost as efficiently as the chimeric BsF$(ab')_2$. This improvement in the efficiency of T cell binding of the humanized BsF$(ab')_2$ is an important step in its development as a potential therapeutic agent for the treatment of $p185^{HER2}$-overexpressing cancers.

Bispecific antibodies (BsAbs) with specificities for tumor-associated antigens and surface markers on immune effector cells have proved effective for retargeting effector cells to kill tumor targets both in vitro and in vivo (reviewed by Fanger, M. W. et al., *Immunol. Today* 10: 92–99 (1989); Fanger, M. W. et al., *Immunol. Today* 12: 51–54 (1991); and Nelson, H., *Cancer Cells* 3: 163–172 (1991)). BsF$(ab')_2$ fragments have often been used in preference to intact BsAbs in retargeted cellular cytotoxicity to avoid the risk of killing innocent bystander cells binding to the Fc region of the antibody. An additional advantage of BsF$(ab')_2$ over intact BsAbs is that they are generally much simpler to prepare free of contaminating monospecific molecules (reviewed by Songsivilai, S. and Lachmann, P. J., *Clin. Exp. Immunol.* 79: 315–321 (1990) and Nolan, O. and O'Kennedy, R., *Biochim. Biophys. Acta* 1040: 1–11 (1990)).

BsF$(ab')_2$ fragments are traditionally constructed by directed chemical coupling of Fab' fragments obtained by limited proteolysis plus mild reduction of the parent rodent monoclonal Ab (Brennan, M. et al., *Science* 229, 81–83 (1985) and Glennie, M. J. et al., *J. Immunol.* 139: 2367–2375 (1987)). One such BsF$(ab')_2$ fragment (anti-glioma associated antigen/anti-CD3) was found to have clinical efficacy in glioma patients (Nitta, T. et al., *Lancet* 335: 368–371 (1990) and another BsF$(ab')_2$ (anti-indium chelate/anti-carcinoembryonic antigen) allowed clinical imaging of colorectal carcinoma (Stickney, D. R. et al., *Antibody, Immunoconj. Radiopharm.* 2: 1–13 (1989)). Future SsF$(ab')_2$ destined for clinical applications are likely to be constructed from antibodies which are either human or at least "humanized" (Riechmann, L. et al., *Nature* 332: 323–327 (1988) to reduce their immunogenicity (Hale, G. et al., *Lancet i:* 1394–1399 (1988)).

Recently a facile route to a fully humanized BsF$(ab')_2$ fragment designed for tumor immunotherapy has been demonstrated (Shalaby, M. R. et al., *J. Exp. Med.* 175: 217–225 (1992)). This approach involves separate *E. coli* expression of each Fab' arm followed by traditional directed chemical coupling in vitro to form the BsF$(ab')_2$. One arm of the BsF$(ab')_2$ was a humanized version (Carter, P. et al., *Proc. Natl. Aced. Sci. USA* (1992a) and Carter, P., et al., *Bio/Technology* 10: 163–167 (1992b)) of the murine monoclonal Ab 4D5 which is directed against the $p185^{HER2}$ product of the protooncogene HER2 (c-erbB-2) (Fendly, B. M. et al. *Cancer Res.* 50: 1550–1558 (1989)). The humanization of the antibody 4D5 is shown in Example 1 of this application. The second arm was a minimalistically humanized anti-CD3 antibody (Shalaby et al. supra) which was created by installing the CDR loops from the variable domains of the murine parent monoclonal Ab UCHT1 (Beverley, P. C. L. and Callard, R. E., *Eur. J. Immunol.* 11: 329–334 (1981)) into the humanized anti-$p185^{HER2}$ antibody. The BsF$(ab')_2$ fragment containing the most potent humanized anti-CD3 variant (v1) was demonstrated by flow cytometry to bind specifically to a tumor target overexpressing $p185^{HER2}$ and to human peripheral blood mononuclear cells carrying CD3. In addition, BsF$(ab')_2$v1 enhanced the cytotoxic effects of activated human CTL 4-fold against SK-SR-3 tumor cells overexpressing $p185^{HER2}$. The example descries efforts to improve the antigen binding affinity of the humanized anti-CD3 arm by the judicious recruitment of a small number of additional murine residues into the minimalistically humanized anti-CD3 variable domains.

Materials and Methods

Construction of Mutations in the Anti-CD3 Variable Region Genes

The construction of genes encoding humanized anti-CD3 variant 1 (v1) variable light (V_1) and heavy (V_H) chain domains in phagemid pUC119 has been described (Shalaby et al. supra). Additional anti-CD3 variants were generated using an efficient site-directed mutagenesis method (Carter, P., *Mutagenesis: a practical approach,* (M. J. McPherson, Ed.), Chapter 1, IRL Press, Oxford, UK (1991)) using mismatched oligonucleotides which either install or remove unique restriction sites. Oligonucleotides used are listed below using lowercase to indicate the targeted mutations. Corresponding coding changes are denoted by the starting amino acid in one letter code followed by the residue numbered according to Kabat, E. A. et al., *Sequences of Proteins of Immunological Interest,* 5th edition, National Institutes of Health, Bethesda, Md., USA (1991), then the replacement amino acid and finally the identity of the anti-CD3 variant:

HX11, 5' GTAGATAAATCCtctAACACAGC-CTAtCTGCAAATG 3' (SEQ.ID. NO. 11) V_HK75S, v6;

HX12, 5' GTAGATAAATCCAAAtctACAGC-CTAtCTGCAAATG 3' (SEQ.ID. NO. 12) V_H N76S, v7;

HX13, 5' GTAGATAAATCCtcttctACAGC-CTAtCTGCAAATG 3' (SEQ.ID. NO. 13) V_H K75S:N76S, v8;

X14, 5' CTTATAAAGGTGTTtCcACCTATaaCcA-gAaatTCAAGGatCGTTTCACgATAtc-CGTAGATAAATCC 3' (SEO.ID.NO. 14) V_H T57S:A60N:D61Q:S62K:V63F:G65D, v9;

LX6, 5' CTATACCTCCCGTCTgcatTCTGGAGTCCC 3' (SEQ.ID. NO. 15) V_L E55H, v11.

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

E. coli Expression of Fab' Fragments

The expression plasmid, pAK19, for the co-secretion of light chain and heavy chain Fd' fragment of the most preferred humanized anti-$p185^{HER2}$ variant, HuMAb4D5-8, is described in Carter et al., 1992b, supre. Briefly, the Fab' expression unit is bicistronic with both chains under the

transcriptional control of the ohoA promoter. Genes encoding humanized V_L and V_H domains are precisely fused on their 5' side to a gene segment encoding the heat-stable enterotoxin II signal sequence and on their 3' side to human k_1 C_L and IgG1C_H1 constant domain genes, respectively. The C_H1gene is immediately followed by a sequence encoding the hinge sequence CysAlaAla and followed by a bacteriophage λ t_o transcriptional terminator. Fab' expression plasmids for chimeric and humanized anti-CD3 variants (v1 to v4, Shalaby et al.,supra; v6 to v12, this study) were created from pAK19 by precisely replacing anti-p185^{HER2} V_L and V_H gene segments with those encoding murine and corresponding humanized variants of the anti-CD3 antibody, respectively, by sub-cloning and site-directed mutagenesis. The Fab' expression plasmid for the most potent humanized anti-CD3 variant identified in this study (v9) is designated pAK22. The anti-p185^{HER2} Fab' fragment was secreted from *E. coli* K12 strain 25F2 containing plasmid pAK19 grown for 32 to 40 hr at 37° C. in an aerated 10 liter fermentor. The final cell density was 120–150 OD_{550} and the titer of soluble and functional anti-p185^{HER2} Fab' was 1–2 g/liter as judged by antigen binding ELISA (Carter et al., 1992b, suora). Anti-CD3 Fab' variants were secreted from *E. coli* containing corresponding expression plasmids using very similar fermentation protocols. The highest expression titers of chimeric and humanized anti-CD3 variants were 200 mgaliter and 700 mgaliter, respectively, as judged by total immunoglobulin ELISA.

Construction of BsF(ab')$_2$ Fragments

Fab' fragments were directly recovered from *E. coli* fermentation pastes in the free thiol form (Fab'-SH) by affinity purification on Streptococcal protein G at pH 5 in the presence of EDTA (Carter et al., 1992b supra). Thioether linked BsF(ab')$_2$ fragments (anti-p185^{HER2}/anti-CD3) were constructed by the procedure of Glennie et al. supra with the following modifications. Anti-p185^{HER2} Fab'-SH in 100 mM Tris acetate, 5 mM EDTA (pH 5.0) was reacted with 0.1 vol of 40 mM N,N'-1,2-phenylenedimalemide (o-PDM) in dimethyl formamide for ~1.5 hr at 20° C. Excess o-PDM was removed by protein G purification of the Fab' maleimide derivative (Fab'-mal) followed by buffer exchange into 20 mM sodium acetate, 5 mM EDTA (pH 5.3) (coupling buffer) using centriprep-30 concentrators (Amicon). The total concentration of Fab' variants was estimated from the measured absorbance at 280 nm (HuMAb4D5-8 Fab' $e^{0.1\%}$=1.56, Carter et al., 1992b, supra). The free thiol content of Fab' preparations was estimated by reaction with 5,5' -dithiobis (2-nitrobenzoic acid) as described by Creighton, T. E., *Protein structure: a practical approach,* (T. E. Creighton, Ed.), Chapter 7, IRL Press, Oxford, UK (1990). Equimolar amounts of anti-p185^{HER2} Fab'-mal (assuming quantitative reaction of Fab'-SH with o-PDM) and each anti-CD3 Fab'-SH variant were coupled together at a combined concentration of 1 to 2.5 mg/ml in the coupling buffer for 14 to 48 hr at 4° C. The coupling reaction was adjusted to 4 mM cysteine at pH 7.0 and incubated for 15 min at 20 ° C. to reduce any unwanted disulfide-linked F(ab')$_2$ formed. These reduction conditions are sufficient to reduce inter-heavy chain disulfide bonds with virtually no reduction of the disulfide between light and heavy chains. Any free thiols generated were then blocked with 50 mM iodoacetamide. BsF(ab')$_2$ was isolated from the coupling reaction by S100-HR (Pharmacia) size exclusion chromatography (2.5 cm×100 cm) in the presence of PBS. The BsF(ab')$_2$ samples were passed through a 0.2 mm filter flash frozen in liquid nitrogen and stored at −70° C.

Flow Cytometric Analysis of F(ab')$_2$Binding to Jurkat Cells

The Jurkat human acute T cell leukemia cell line was purchased from the American Type Culture Collection (Manassas Va.) (ATCC TIB 152) and grown as. recommended by the ATCC. Aliquots of 10^6 Jurkat cells were incubated with appropriate concentrations of BsF(ab')$_2$ (anti-p185^{HER2}/anti-CD3 variant) or control mono-specific anti-p185^{HER2} F(ab')$_2$ in PBS plus 0.1% (w/v) bovine serum albumin and 10 mM sodium azide for 45 min at 4° C. The cells were washed and then incubated with fluorescein-conjugated goat anti-human F(ab')$_2$ (Organon Teknika, West Chester, Pa.) for 45 min at 4° C. Cells were washed and analyzed on a FACScan® (Becton Dickinson and Co., Mountain View, Calif.). Cells (8×10^3) were acquired by list mode and gated by forward light scatter versus side light scatter excluding dead cells and debris.

Results

Design of Humanized anti-CD3 Variants

The most potent humanized anti-CD3 variant previously identified, v1, differs from the murine parent antibody, UCHT1 at 19 out of 107 amino acid residues within V_L and at 37 out of 122 positions within V_H (Shalaby et al.,supra) 1992). Here we recruited back additional murine residues into anti-CD3 v1 in an attempt to improve the binding affinity for CD3. The strategy chosen was a compromise between minimizing both the number of additional murine residues recruited and the number of anti-CD3 variants to be analyzed. We focused our attentions on a few CDR residues which were originally kept as human sequences in our minimalistic humanization regime. Thus human residues in V_H CDR2 of anti-CD3 v1 were replaced en bloc with their murine counterparts to give anti-CD3 v9: T57S:A60N:D61Q:S62K:V63F:G65D (SEQ ID NO:20). Similarly, the human residue E55 in V_L CDR2 of anti-CD3 v_1 was replaced with histidine from the murine anti-CD3 antibody to generate anti-CD3 v11. In addition, V_H framework region (FR) residues 75 and 76 in anti-CD3 v1 were also replaced with their murine counterparts to create anti-CD3 v8: K75S:N76S. V_H residues 75 and 76 are located in a loop close to V_H CDR1 and CDR2 and therefore might influence antigen binding. Additional variants created by combining mutations at these three sites are described below.

Preparation of BsF(ab')$_2$ Fragments

Soluble and functional anti-p185^{HER2} and anti-CD3 Fab' fragments were recovered directly from corresponding *E. coli* fermentation pastes with the single hinge cysteine predominantly in the free thiol form (75–100% Fab'-SH) by affinity purification on Streptococcal protein G at pH 5 in the presence of EDTA (Carter et al., 1992b, supra). Thioether-linked BsF(ab')$_2$ fragments were then constructed by directed coupling using o-PDM as described by Glennie et al., supra. One arm was always the most potent humanized anti-p185^{HER2} variant, HuMAb4D5-8 (Carter et al., 1992a, supra) and the other either a chimeric or humanized variant of the anti-CD3 antibody. Anti-p185^{HER2} Fab'-SH was reacted with o-PDM to form the maleimide derivative (Fab'-mal) and then coupled to the Fab'-SH for each anti-CD3 variant. F(ab')$_2$ was then purified away from unreacted Fab' by size exclusion chromatography as shown for a representative preparation (BsF(ab')$_2$ v8) in data not shown. The F(ab')$_2$ fragment represents ~54% of the total amount of antibody fragments (by mass) as judged by integration of the chromatograph peaks.

SDS-PAGE analysis of this BsF(ab')$_2$v8 preparation under non-reducing conditions gave one major band with the expected mobility (M, ~96 kD) as well as several very minor

bands (data not shown). Amino-terminal sequence analysis of the major band after electroblotting on to polyvinylidene difluoride 76 are located in a loop close to V_H CDR1 and CDR2 and therefore might membrane Matsudaira, P., *J. Biol. Chem.* 262: 10035–10038 (1987) gave the expected mixed sequence from a stoichiometric 1:1 mixture of light and heavy chains (V_L/V_H: D/E, I/V, Q/D, M/L, T/V, D/E, S/S) expected for BsF(ab')$_2$. The amino terminal region of both light chains are identical as are both heavy chains and correspond to consensus human FR sequences. We have previously demonstrated that F(ab')$_2$ constructed by directed chemical coupling carry both anti-p185^{HER2} and anti-CD3 antigen specificities (Shalaby et al., supra). The level of contamination of the BsF(ab')$_2$ with monospecific F(ab')$_2$ iS likely to be very low since mock coupling reactions with either anti-p185^{HER2} w Fab'-mal or anti-CD3 Fab'-SH alone did not yield detectable quantities of F(ab')$_2$. Furthermore the coupling reaction was subjected to a mild reduction step followed by alkylation to remove trace amounts of disulfide-linked F(ab')$_2$ that might be present. SDS-PAGE of the purified F(ab')$_2$ under reducing conditions gave two major bands with electrophoretic mobility and amino terminal sequence anticipated for free light chain and thioether-linked heavy chain dimers.

Scanning LASER densitometry of a o-PDM coupled F(ab')$_2$ preparation suggest that the minor species together represent ~10% of the protein. These minor contaminants were characterized by amino terminal sequence analysis and were tentatively identified on the basis of stoichiometry of light and heavy chain sequences and their electrophoretic mobility (data not shown). These data are consistent with the minor contaminants including imperfect F(ab')$_2$ in which the disulfide bond between light and heavy chains is missing in one or both arms, trace amounts of Fab' and heavy chain thioether-linked to light chain.

Binding of BsF(ab')$_2$ to Jurkat Cells

Binding of BsF(ab')$_2$ containing different anti-CD3 variants to Jurkat cells (human acute T cell leukemia) was investigated by flow cytometry (data not shown). BsF(ab')$_2$v9 binds much more efficiently to Jurkat cells than does our starting molecule, BsF(ab')$_2$vl, and almost as efficiently as the chimeric BsF(ab')$_2$. Installation of additional murine residues into anti-CD3 v9 to create v10 (V_HK75S:N76S) and v12 (V_HK75S:N76S plus V_L E55H) did not further improve binding of corresponding BsF(ab')$_2$to Jurkat cells. Nor did recruitment of these murine residues into anti-CD3 v1 improve Jurkat binding: V_HK75S (v6), V_HN76S (v7), V_HK75S:N76S (V8), V_LE55H (v11) (not shown). BsF(ab')$_2$v9 was chosen for future study since it is amongst the most efficient variants in binding to Jurkat cells and contains fewest murine residues in the humanized anti-CD3 arm. A monospecific anti-p185^{HEE2} F(ab')$_2$ did not show significant binding to Jurkat cells consistent with the interaction being mediated through the anti-CD3 arm.

Discussion

A minimalistic strategy was chosen to humanize the anti-p185^{HER2} (Carter et al., 1992a, supra) and anti-CD3 arms (Shalaby et al., supra) of the BsF(ab')$_2$ in this study in an attempt to minimize the potential immunogenicity of the resulting humanized antibody in the clinic. Thus we tried to install the minimum number of murine CDR and FR residues into the context of consensus human variable domain sequences as required to recruit antigen-binding affinity and biological properties comparable to the murine parent antibody. Molecular modeling was used firstly to predict the murine FR residues which might be important to antigen binding and secondly to predict the murine CDR residues that might not be required. A small number of humanized variants were then constructed to test these predictions.

Our humanization strategy was very successful for the anti-p185^{HER2} antibody where one out of eight humanized variants (HuMAb4D5-8, IgG1) was identified that bound the p185^{HER2} antigen ~3-fold more tightly than the parent murine antibody (Carter et al., 1992a, supra). HuMAb4D5-8 contains a total of five murine FR residues and nine murine CDR residues, including V_H CDR2 residues 60–65, were discarded in favor of human counterparts. In contrast, BsF(ab')$_2$v1 containing the most potent humanized anti-CD3 variant out of four originally constructed (Shalaby et al., supra) binds J6 cells with an affinity (K_d) of 140 nM which is ~70-fold weaker than that of the corresponding chimeric BsF(ab')$_2$.

Here we have restored T cell binding of the humanized anti-CD3 close to that of the chimeric variant by replacing six human residues in V_H CDR2 with their murine counterparts: T57S:A60N:D61Q:S62K:V63F:G65D (anti-CD3 v9, FIG. **5**). It appears more likely that these murine residues enhance antigen binding indirectly by influencing the conformation of residues in the N-terminal part of V_H CDR2 rather than by directly contacting antigen. Firstly, only N-terminal residues in V_H CDR2 (50–58) have been found to contact antigen in one or more of eight crystallographic structures of antibody/antigen complexes (Kabat et al., supra; and Mian, I. S. et al., *J. Mol. Biol* 217: 133–151 (1991), FIG. **5**). Secondly, molecular modeling suggests that residues in the C-terminal part of V_H CDR2 are at least partially buried (FIG. **5**). BsF(ab')$_2$v9 binds to SK-BR-3 breast tumor cells with equal efficiency to BsF(ab')$_2$v1 and chimeric BsF(ab')$_2$ as anticipated since the anti-p185^{HER2} arm is identical in all of these molecules (Shalaby et al., supra, not shown).

Our novel approach to the construction of BsF(ab')$_2$ fragments exploits an *E. coli* expression system which secretes humanized Fab' fragments at gram per liter titers and permits their direct recovery as Fab'-SH (Carter et al., 1992b, supra). Traditional directed chemical coupling of Fab'-SH fragments is then used to form BsF(ab')$_2$ in vitro (Brennan et al.,supra; and Glennie et al., supra). This route to Fab'-SH obviates problems which are inherent in their generation from intact antibodies: differences in susceptibility to proteolysis and nonspecific cleavage resulting in heterogeneity, low yield as well as partial reduction that is not completely selective for the hinge disulfide bonds. The strategy of using *E. coli*-derived Fab'-SH containing a single hinge cysteine abolishes some sources of heterogeneity in BsF(ab')$_2$ preparation such as intra-hinge disulfide formation and contamination with intact parent antibody whilst greatly diminishes others, eg. formation of F(ab')$_3$ fragments.

BsF(ab')2 fragments constructed here were thioether-linked as originally described by Glennie et al., supra with future in vivo testing of these molecules in mind. Thioether bonds, unlike disulfide bonds, are not susceptible to cleavage by trace amounts of thiol, which led to the proposal that thioether-linked F(ab')$_2$ may be more stable than disulfide-linked F(ab')$_2$ in vivo (Glennie et al., supra). This hypothesis is supported by our preliminary pharmacokinetic experiments in normal mice which suggest that thioether-linked BsF(ab')$_2$ v1 has a 3-fold longer plasma residence time than BsF(ab')$_2$ v1 linked by a single disulfide bond. Disulfide and thioether-linked chimeric BsF(ab')$_2$ were found to be indistinguishable in their efficiency of cell binding and in their retargeting of CTL cytotoxicity, which suggests that o-PDM directed coupling does not compromise binding of the

$BsF(ab')_2$ to either antigen (not shown). Nevertheless the nature of the linkage appears not to be critical since a disulfide-linked $BsF(ab')_2$ (murine anti-$p185^{HER2}$/murine anti-CD3) was recently shown by others (Nishimura et al., Int. *J. Cancer* 50: 800–804 (1992) to have potent anti-tumor activity in nude mice. Our previous study (Shalaby et al., supra) together with this one and that of Nishimura, T. et al., supra improve the potential for using $BsF(ab')_2$ in targeted immunotherapy of $p185^{HER2}$-overexpressing cancers in humans.

Example 4

Humanization of an anti-CD18 Antibody

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

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 26

(2) INFORMATION FOR SEQ ID NO:1:

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

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

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

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

Thr Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                 35                  40                  45

Leu Leu Ile Tyr Ser Ala Ser Phe Leu Glu Ser Gly Val Pro Ser
                 50                  55                  60

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

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

His Tyr Thr Thr Pro Pro Thr Phe Gly Gln Gly Thr Lys Val Glu
                 95                 1 00                 1 05

Ile Lys Arg Thr
            109
```

(2) INFORMATION FOR SEQ ID NO:2:

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

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

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Asn Ile Lys
                 20                  25                  30

Asp Thr Tyr Ile His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                 35                  40                  45

Glu Trp Val Ala Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr
                 50                  55                  60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Ala Asp Thr Ser
                 65                  70                  75
```

-continued

```
Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                 80                  85                  90

Thr Ala Val Tyr Tyr Cys Ser Arg Trp Gly Gly Asp Gly Phe Tyr
                 95                 1 00                 1 05

Ala Met Asp Val Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
                110                 115                  120
```

(2) INFORMATION FOR SEQ ID NO:3:

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

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

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

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

Ser Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                 35                  40                  45

Leu Leu Ile Tyr Ala Ala Ser Ser Leu Glu Ser Gly Val Pro Ser
                 50                  55                  60

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

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

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

Ile Lys Arg Thr
            109
```

(2) INFORMATION FOR SEQ ID NO:4:

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

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

```
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
                 20                  25                  30

Asp Tyr Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                 35                  40                  45

Glu Trp Val Ala Val Ile Ser Glu Asn Gly Ser Asp Thr Tyr Tyr
                 50                  55                  60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser
                 65                  70                  75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                 80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Asp Arg Gly Gly Ala Val Ser
                 95                 1 00                 1 05

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

-continued

(2) INFORMATION FOR SEQ ID NO:5:

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

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

Asp Ile Val Met Thr Gln Ser His Lys Phe Met Ser Thr Ser Val
1 5 10 15

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

Thr Ala Val Ala Trp Tyr Gln Gln Lys Pro Gly His Ser Pro Lys
35 40 45

Leu Leu Ile Tyr Ser Ala Ser Phe Arg Tyr Thr Gly Val Pro Asp
50 55 60

Arg Phe Thr Gly Asn Arg Ser Gly Thr Asp Phe Thr Phe Thr Ile
65 70 75

Ser Ser Val Gln Ala Glu Asp Leu Ala Val Tyr Tyr Cys Gln Gln
80 85 90

His Tyr Thr Thr Pro Pro Thr Phe Gly Gly Gly Thr Lys Leu Glu
95 1 00 1 05

Ile Lys Arg Ala
109

(2) INFORMATION FOR SEQ ID NO:6:

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

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

Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly
1 5 10 15

Ala Ser Leu Lys Leu Ser Cys Thr Ala Ser Gly Phe Asn Ile Lys
20 25 30

Asp Thr Tyr Ile His Trp Val Lys Gln Arg Pro Glu Gln Gly Leu
35 40 45

Glu Trp Ile Gly Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr
50 55 60

Asp Pro Lys Phe Gln Asp Lys Ala Thr Ile Thr Ala Asp Thr Ser
65 70 75

Ser Asn Thr Ala Tyr Leu Gln Val Ser Arg Leu Thr Ser Glu Asp
80 85 90

Thr Ala Val Tyr Tyr Cys Ser Arg Trp Gly Gly Asp Gly Phe Tyr
95 1 00 1 05

Ala Met Asp Tyr Trp Gly Gln Gly Ala Ser Val Thr Val Ser Ser
110 115 120

(2) INFORMATION FOR SEQ ID NO:7:

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

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

TCCGATATCC AGCTGACCCA GTCTCCA 27

-continued

(2) INFORMATION FOR SEQ ID NO:8:

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

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

GTTTGATCTC CAGCTTGGTA CCHSCDCCGA A 31

(2) INFORMATION FOR SEQ ID NO:9:

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

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

AGGTSMARCT GCAGSAGTCW GG 22

(2) INFORMATION FOR SEQ ID NO:10:

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

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

TGAGGAGACG GTGACCGTGG TCCCTTGGCC CCAG 34

(2) INFORMATION FOR SEQ ID NO:11:

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

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

GTAGATAAAT CCTCTAACAC AGCCTATCTG CAAATG 36

(2) INFORMATION FOR SEQ ID NO:12:

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

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

GTAGATAAAT CCAAATCTAC AGCCTATCTG CAAATG 36

(2) INFORMATION FOR SEQ ID NO:13:

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

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

GTAGATAAAT CCTCTTCTAC AGCCTATCTG CAAATG 36

-continued

(2) INFORMATION FOR SEQ ID NO:14:

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

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

CTTATAAAGG TGTTTCCACC TATAACCAGA AATTCAAGGA TCGTTTCACG 50

ATATCCGTAG ATAAATCC 68

(2) INFORMATION FOR SEQ ID NO:15:

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

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

CTATACCTCC CGTCTGCATT CTGGAGTCCC 30

(2) INFORMATION FOR SEQ ID NO:16:

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

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

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

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

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

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

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

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

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

Ile Lys
107

(2) INFORMATION FOR SEQ ID NO:17:

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

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

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

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

Asn Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys

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                35                  40                  45

 Leu Leu Ile Tyr Tyr Thr Ser Arg Leu Glu Ser Gly Val Pro Ser
                50                  55                  60

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

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

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

 Ile Lys
     107


(2) INFORMATION FOR SEQ ID NO:18:

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

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

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

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

 Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys
                35                  40                  45

 Leu Leu Ile Tyr Ala Ala Ser Ser Leu Glu Ser Gly Val Pro Ser
                50                  55                  60

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

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

 Tyr Asn Ser Leu Pro Trp Thr Phe Gly Gln Gly Thr Lys Val Glu
                95                 1 00                 1 05

 Ile Lys
     107


(2) INFORMATION FOR SEQ ID NO:19:

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

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

 Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly
   1               5                  10                  15

 Ala Ser Met Lys Ile Ser Cys Lys Ala Ser Gly Tyr Ser Phe Thr
                20                  25                  30

 Gly Tyr Thr Met Asn Trp Val Lys Gln Ser His Gly Lys Asn Leu
                35                  40                  45

 Glu Trp Met Gly Leu Ile Asn Pro Tyr Lys Gly Val Ser Thr Tyr
                50                  55                  60

 Asn Gln Lys Phe Lys Asp Lys Ala Thr Leu Thr Val Asp Lys Ser
                65                  70                  75

 Ser Ser Thr Ala Tyr Met Glu Leu Leu Ser Leu Thr Ser Glu Asp
                80                  85                  90
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-continued

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Ser Ala Val Tyr Tyr Cys Ala Arg Ser Gly Tyr Tyr Gly Asp Ser
                 95                 1 00                 1 05

Asp Trp Tyr Phe Asp Val Trp Gly Ala Gly Thr Thr Val Thr Val
                110                  115                  120

Ser Ser
    122


(2) INFORMATION FOR SEQ ID NO:20:

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

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

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Ser Phe Thr
                 20                  25                  30

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

Glu Trp Val Ala Leu Ile Asn Pro Tyr Lys Gly Val Ser Thr Tyr
                 50                  55                  60

Asn Gln Lys Phe Lys Asp Arg Phe Thr Ile Ser Val Asp Lys Ser
                 65                  70                  75

Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                 80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Ser Gly Tyr Tyr Gly Asp Ser
                 95                 1 00                 1 05

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

Ser Ser
    122


(2) INFORMATION FOR SEQ ID NO:21:

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

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

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
                 20                  25                  30

Ser Tyr Ala Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
                 35                  40                  45

Glu Trp Val Ser Val Ile Ser Gly Asp Gly Gly Ser Thr Tyr Tyr
                 50                  55                  60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser
                 65                  70                  75

Lys Asn Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                 80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Gly Arg Val Gly Tyr Ser Leu
                 95                 1 00                 1 05

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

-continued

Ser Ser
122

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 454 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Gln Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly
1 5 10 15

Ala Ser Val Lys Ile Ser Cys Lys Thr Ser Gly Tyr Thr Phe Thr
20 25 30

Glu Tyr Thr Met His Trp Met Lys Gln Ser His Gly Lys Ser Leu
35 40 45

Glu Trp Ile Gly Gly Phe Asn Pro Lys Asn Gly Gly Ser Ser His
50 55 60

Asn Gln Arg Phe Met Asp Lys Ala Thr Leu Ala Val Asp Lys Ser
65 70 75

Thr Ser Thr Ala Tyr Met Glu Leu Arg Ser Leu Thr Ser Glu Asp
80 85 90

Ser Gly Ile Tyr Tyr Cys Ala Arg Trp Arg Gly Leu Asn Tyr Gly
95 1 00 1 05

Phe Asp Val Arg Tyr Phe Asp Val Trp Gly Ala Gly Thr Thr Val
110 115 120

Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu
125 130 135

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

Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp
155 160 165

Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val
170 175 180

Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val
185 190 195

Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
200 205 210

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys
215 220 225

Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu
230 235 240

Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys
245 250 255

Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val
260 265 270

Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr
275 280 285

Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
290 295 300

Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val
305 310 315

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

-continued

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

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

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

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

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

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

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

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

Ser Pro Gly Lys
            454
```

(2) INFORMATION FOR SEQ ID NO:23:

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

-continued

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Ser Pro Gly Lys
            469


(2) INFORMATION FOR SEQ ID NO:24:

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

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

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

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

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

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

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

-continued

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

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

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

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

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

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

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

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

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

Arg Gly Glu Cys
            214
```

(2) INFORMATION FOR SEQ ID NO:25:

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

-continued

```
Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val
                200                 205                 210

Tyr Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr
                215                 220                 225

Lys Ser Phe Asn Arg Gly Glu Cys
                230          233


(2) INFORMATION FOR SEQ ID NO:26:

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

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

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly
  1               5                  10                  15

Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Ser Phe Thr
                20                  25                  30

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

Glu Trp Val Ala Leu Ile Asn Pro Tyr Lys Gly Val Thr Thr Tyr
                50                  55                  60

Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser
                65                  70                  75

Lys Asn Thr Ala Tyr Leu Gln Met Asn Ser Leu Arg Ala Glu Asp
                80                  85                  90

Thr Ala Val Tyr Tyr Cys Ala Arg Ser Gly Tyr Tyr Gly Asp Ser
                95                 1 00                1 05

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

Ser Ser
    122
```

We claim:

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

(a) noncovalently binds antigen directly;

(b) interacts with a CDR; or

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

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

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

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

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

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

Column 88,
Line 63, please delete "63" and insert therefor -- 79 --.

Signed and Sealed this

Third Day of December, 2002

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

PTO/SB/05 (05-05)
Approved for use through 07/31/2006. OMB 0651-0032
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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 C.F.R. 1.53(b))

Attorney Docket No.	CARP0001-112
First Inventor	John R. Adair et al.
Title	HUMANISED ANTIBODIES
Express Mail Label No.	EV146 601 565US

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1. ☒ **Fee Transmittal Form (e.g., PTO/SB/17)**
   (Submit an original and a duplicate for fee processing)
2. ☐ **Applicant claims small entity status.**
   See 37 CFR 1.27.
3. ☒ **Specification** [*Total Pages* 70]
   Both the claims and abstract must start on a new page
   (For information on the preferred arrangement, see MPEP 608.01(a))
4. ☒ **Drawing(s)** (*35 U.S.C.113*) [*Total Sheets* 18]
5. **Oath or Declaration** [*Total Sheets* 03]
   a. ☐ Newly executed (original or copy)
   b. ☒ **Copy from a prior application (37 CFR 1.63 (d))**
   (for a continuation/divisional with Box 18 completed)
   i. ☐ DELETION OF INVENTOR(S)
   Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).
6. ☒ **Application Data Sheet.** See 37 CFR 1.76
7. ☐ **CD-ROM or CD-R** in duplicate, large table or Computer Program *(Appendix)*
   ☐ Landscape Table on CD
8. **Nucleotide and/or Amino Acid Sequence Submission**
   (if applicable, items a.-c. are required)
   a. Computer Readable Form (CRF)
   i. ☐ Computer Readable Form (CRF)
   ii. ☒ **Transfer Request (37 CFR 1.821(e))**
   b. Specification Sequence Listing on:
   i. ☐ CD-ROM or CD-R (2 copies); or
   ii. ☒ **Paper Copy**
   c. ☒ **Statements verifying identity of above copies**

ADDRESS TO: Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

ACCOMPANYING APPLICATIONS PARTS

9. ☐ **Assignment Papers** (cover sheet & document(s))
   Name of Assignee _____
10. ☐ **37 C.F.R. 3.73(b) Statement** *(when there is an assignee)* ☒ **Copy of Power of Attorney**
11. ☐ **English Translation Document** *(if applicable)*
12. ☐ **Information Disclosure Statement** (PTO/SB/08 or PTO-1449)
    ☐ Copies of foreign patent documents, publications & other information
13. ☒ **Preliminary Amendment and Request for Interference under 37 C.F.R. § 42.202**
14. ☒ **Return Receipt Postcard** (MPEP 503)
    (Should be specifically itemized)
15. ☒ **Certified Copy of Priority Document Was Received in Parent Application Serial No. 07/743,329, Filed September 17, 1991** *(if foreign priority is claimed)*
16. ☐ **Nonpublication Request** under 35 U.S.C. 122(b)(2)(B)(i). Applicant must attach form PTO/SB/35 or equivalent.
17. ☒ Other: Copy of Change of Correspondence Address – Application dated December 23, 2002, from Application Serial No. 08/846,658, Filed May 1, 1997.

18. If a CONTINUING APPLICATION, *check appropriate box, and supply the requisite information below and in the first sentence of the specification following the title, or in an Application Data Sheet under 37 CFR 1.76:*

☒ **Continuation** ☐ Divisional ☐ Continuation-in-part (CIP) of prior Application No. Serial No. 08/846,658, Filed May 1, 1997, which is a Continuation of Application Serial No. 08/303,569, Filed September 7, 1994, now U.S. Patent No. 5,859,205, Issued January 12, 1999, which is a File Wrapper Continuation of Application Serial No. 07/743,329, Filed September 17, 1991, Abandoned, which is a 35 U.S.C. 371 National Stage Application of International Application No. PCT/GB90/02017, International Filing Date of December 21, 1990, which claims priority to Great Britain Application No. 8928874.0, Filed December 21, 1989, all applications incorporated by reference herein in their entireties.

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

19. CORRESPONDENCE ADDRESS

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

Name					
Address					
City		*State*		*Zip Code*	
Country		*Telephone*		*Email address*	

Signature	Doreen Yatko Trujillo	Date	November 21, 2005
Name (Print/Type)	Doreen Yatko Trujillo	*Registration No. (Attorney/Agent)*	35,719

PTO/SB/17 (12-04v2)
Approved for use through 07/31/2006. OMB 0651-0032
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

Effective on 12/08/2004.
Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).

FEE TRANSMITTAL for FY 2005

☐ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT	($) 1,000.00

Complete if Known	
Application Number	Not Yet Assigned
Filing Date	November 21, 2005
First Named Inventor	John R. Adair et al.
Examiner Name	Not Yet Assigned
Art Unit	Not Yet Assigned
Attorney Docket No.	CARP0001-112

METHOD OF PAYMENT (check all that apply)

☐ Check ☐ Credit Card ☐ Money Order ☐ None ☐ Other (please identify) : _____

☒ Deposit Account Deposit Account Number: 50-1275 Deposit Account Name: Cozen O'Connor, P.C.

For the above-identified deposit account, the Director is hereby authorized to: (check all that apply)

☒ Charge fee(s) indicated below ☐ Charge fee(s) indicated below, **except for the filing fee**

☒ Charge any additional fee(s) or underpayments of fee(s) Under 37 CFR 1.16 and 1.17 ☒ Credit any overpayments

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES

	FILING FEES		SEARCH FEES		EXAMINATION FEES		
		Small Entity		Small Entity		Small Entity	
Application Type	Fee ($)	Fee($)	Fee($)	Fee($)	Fee($)	Fee($)	Fees Paid ($)
Utility	300	150	500	250	200	100	$1,000.00
Design	200	100	100	50	130	65	_____
Plant	200	100	300	150	160	80	_____
Reissue	300	150	500	250	600	300	_____
Provisional	200	100	0	0	0	0	_____

2. EXCESS CLAIM FEES

Fee Description	Fee ($)	Small Entity Fee ($)
Each claim over 20 (including Reissues)	50	25
Each independent claim over 3 (including Reissues)	200	100
Multiple dependent claims	360	180

Total Claims	Extra Claims	Fee($)	Fee Paid ($)
02	- 20 or HP= 00	x _____	= _____

HP = highest number of total claims paid for, if greater than 20.

Indep. Claims	Extra Claims	Fee($)	Fee Paid ($)
01	- 03 or HP= 00	x _____	= _____

HP = highest number of independent claims paid for, if greater than 3.

Multiple Dependent Claims	
Fee ($)	Fee Paid ($)
_____	_____

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets	Extra Sheets	Number of each additional 50 or fraction thereof	Fee ($)	Fee Paid ($)
_____	- _____ = _____ /50 =	_____ (round **up** to a whole number) x	_____ =	_____

4. OTHER FEE(S)

	Fees Paid ($)
Non-English Specification, $130 fee (no small entity discount)	_____
Other (e.g., late filing surcharge) : _____	_____

SUBMITTED BY

Signature	Doreen Yatko Trujillo	Registration No. (Attorney/Agent) 35,719	Telephone (215) 665-5593
Name (Print/Type)	Doreen Yatko Trujillo		Date November 21, 200

PTO/SB/17 (12-04v2)
Approved for use through 07/31/2006. OMB 0651-0032
U.S. Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

Effective on 12/08/2004.
Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).

FEE TRANSMITTAL for FY 2005

☐ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT	($) 1,000.00

Complete if Known	
Application Number	Not Yet Assigned
Filing Date	November 21, 2005
First Named Inventor	John R. Adair et al.
Examiner Name	Not Yet Assigned
Art Unit	Not Yet Assigned
Attorney Docket No.	CARP0001-112

METHOD OF PAYMENT (check all that apply)

☐ Check ☐ Credit Card ☐ Money Order ☐ None ☐ Other (please identify) : _____

☒ Deposit Account Deposit Account Number: 50-1275 Deposit Account Name: Cozen O'Connor, P.C.

For the above-identified deposit account, the Director is hereby authorized to: (check all that apply)

☒ Charge fee(s) indicated below ☐ Charge fee(s) indicated below, **except for the filing fee**

☒ Charge any additional fee(s) or underpayments of fee(s) Under 37 CFR 1.16 and 1.17 ☒ Credit any overpayments

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FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES

	FILING FEES		SEARCH FEES		EXAMINATION FEES		
		Small Entity		Small Entity		Small Entity	
Application Type	Fee ($)	Fee($)	Fee($)	Fee($)	Fee($)	Fee($)	Fees Paid ($)
Utility	300	150	500	250	200	100	$1,000.00
Design	200	100	100	50	130	65	_____
Plant	200	100	300	150	160	80	_____
Reissue	300	150	500	250	600	300	_____
Provisional	200	100	0	0	0	0	_____

2. EXCESS CLAIM FEES

Fee Description	Fee ($)	Small Entity Fee ($)
Each claim over 20 (including Reissues)	50	25
Each independent claim over 3 (including Reissues)	200	100
Multiple dependent claims	360	180

Total Claims	Extra Claims	Fee($)	Fee Paid ($)
02 - 20 or HP=	00	x _____	= _____

HP = highest number of total claims paid for, if greater than 20.

Indep. Claims	Extra Claims	Fee($)	Fee Paid ($)
01 - 03 or HP=	00	x _____	= _____

HP = highest number of independent claims paid for, if greater than 3.

Multiple Dependent Claims	
Fee ($)	Fee Paid ($)
_____	_____

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets	Extra Sheets	Number of each additional 50 or fraction thereof	Fee ($)	Fee Paid ($)
_____ - _____ =	_____ /50 =	_____ (round **up** to a whole number) x	_____ =	_____

4. OTHER FEE(S)

	Fees Paid ($)
Non-English Specification, $130 fee (no small entity discount)	_____
Other (e.g., late filing surcharge) : _____	_____

SUBMITTED BY

Signature	Doreen Yatko Trujillo	Registration No. (Attorney/Agent) 35,719	Telephone (215) 665-5593
Name (Print/Type)	Doreen Yatko Trujillo		Date November 21, 200

HUMANISED ANTIBODIES

Field of the Invention

The present invention relates to humanised antibody molecules, to processes for their production using recombinant DNA technology, and to their therapeutic uses.

The term "humanised antibody molecule" is used to describe a molecule having an antigen binding site derived from an immunoglobulin from a non-human species, and remaining immunoglobulin-derived parts of the molecule being derived from a human immunoglobulin. The antigen binding site typically comprises complementarity determining regions (CDRs) which determine the binding specificity of the antibody molecule and which are carried on appropriate framework regions in the variable domains. There are 3 CDRs (CDR1, CDR2 and CDR3) in each of the heavy and light chain variable domains.

In the description, reference is made to a number of publications by number. The publications are listed in numerical order at the end of the description.

Background of the Invention

Natural immunoglobulins have been known for many years, as have the various fragments thereof, such as the Fab, $(Fab')_2$ and Fc fragments, which can be derived by enzymatic cleavage. Natural immunoglobulins comprise a generally Y-shaped molecule having an antigen-binding site towards the end of each upper arm. The remainder of the structure, and particularly the stem of the Y, mediates the effector functions associated with immunoglobulins.

Natural immunoglobulins have been used in assay, diagnosis and, to a more limited extent, therapy. However, such uses, especially in therapy, were hindered until recently by the polyclonal nature of natural immunoglobulins. A significant step towards the realisation of the potential

of immunoglobulins as therapeutic agents was the discovery of procedures for the production of monoclonal antibodies (MAbs) of defined specificity (1).

However, most MAbs are produced by hybridomas which are fusions of rodent spleen cells with rodent myeloma cells. They are therefore essentially rodent proteins. There are very few reports of the production of human MAbs.

Since most available MAbs are of rodent origin, they are naturally antigenic in humans and thus can give rise to an undesirable immune response termed the HAMA (Human Anti-Mouse Antibody) response. Therefore, the use of rodent MAbs as therapeutic agents in humans is inherently limited by the fact that the human subject will mount an immunological response to the MAb and will either remove it entirely or at least reduce its effectiveness. In practice, MAbs of rodent origin may not be used in patients for more than one or a few treatments as a HAMA response soon develops rendering the MAb ineffective as well as giving rise to undesirable reactions. For instance, OKT3 a mouse IgG2a/k MAb which recognises an antigen in the T-cell receptor-CD3 complex has been approved for use in many countries throughout the world as an immunosuppressant in the treatment of acute allograft rejection [Chatenoud et al (2) and Jeffers et al (3)]. However, in view of the rodent nature of this and other such MAbs, a significant HAMA response which may include a major anti-idiotype component, may build up on use. Clearly, it would be highly desirable to diminish or abolish this undesirable HAMA response and thus enlarge the areas of use of these very useful antibodies.

Proposals have therefore been made to render non-human MAbs less antigenic in humans. Such techniques can be generically termed "humanisation" techniques. These

techniques typically involve the use of recombinant DNA technology to manipulate DNA sequences encoding the polypeptide chains of the antibody molecule.

Early methods for humanising MAbs involved production of chimeric antibodies in which an antigen binding site comprising the complete variable domains of one antibody is linked to constant domains derived from another antibody. Methods for carrying out such chimerisation procedures are described in EP0120694 (Celltech Limited), EP0125023 (Genentech Inc. and City of Hope), EP-A-0 171496 (Res. Dev. Corp. Japan), EP-A-0 173 494 (Stanford University), and WO 86/01533 (Celltech Limited). This latter Celltech application (WO 86/01533) discloses a process for preparing an antibody molecule having the variable domains from a mouse MAb and the constant domains from a human immunoglobulin. Such humanised chimeric antibodies, however, still contain a significant proportion of non-human amino acid sequence, i.e. the complete non-human variable domains, and thus may still elicit some HAMA response, particularly if administered over a prolonged period [Begent et al (ref. 4)].

In an alternative approach, described in EP-A-0239400 (Winter), the complementarity determining regions (CDRs) of a mouse MAb have been grafted onto the framework regions of the variable domains of a human immunoglobulin by site directed mutagenesis using long oligonucleotides. The present invention relates to humanised antibody molecules prepared according to this alternative approach, i.e. CDR-grafted humanised antibody molecules. Such CDR-grafted humanised antibodies are much less likely to give rise to a HAMA response than humanised chimeric antibodies in view of the much lower proportion of non-human amino acid sequence which they contain.

The earliest work on humanising MAbs by CDR-grafting was carried out on MAbs recognising synthetic antigens, such as the NP or NIP antigens. However, examples in which a mouse MAb recognising lysozyme and a rat MAb recognising an antigen on human T-cells were humanised by CDR-grafting have been described by Verhoeyen et al (5) and Riechmann et al (6) respectively. The preparation of CDR-grafted antibody to the antigen on human T cells is also described in WO 89/07452 (Medical Research Council).

In Riechmann et al/Medical Research Council it was found that transfer of the CDR regions alone [as defined by Kabat refs. (7) and (8)] was not sufficient to provide satisfactory antigen binding activity in the CDR-grafted product. Riechmann et al found that it was necessary to convert a serine residue at position 27 of the human sequence to the corresponding rat phenylalanine residue to obtain a CDR-grafted product having improved antigen binding activity. This residue at position 27 of the heavy chain is within the structural loop adjacent to CDR1. A further construct which additionally contained a human serine to rat tyrosine change at position 30 of the heavy chain did not have a significantly altered binding activity over the humanised antibody with the serine to phenylalanine change at position 27 alone. These results indicate that changes to residues of the human sequence outside the CDR regions, in particular in the structural loop adjacent to CDR1, may be necessary to obtain effective antigen binding activity for CDR-grafted antibodies which recognise more complex antigens. Even so the binding affinity of the best CDR-grafted antibodies obtained was still significantly less than the original MAb.

Very recently Queen et al (9) have described the preparation of a humanised antibody that binds to the

interleukin 2 receptor, by combining the CDRs of a murine MAb (anti-Tac) with human immunoglobulin framework and constant regions. The human framework regions were chosen to maximise homology with the anti-Tac MAb sequence. In addition computer modelling was used to identify framework amino acid residues which were likely to interact with the CDRs or antigen, and mouse amino acids were used at these positions in the humanised antibody.

In WO 90/07861 Queen et al propose four criteria for designing humanised immunoglobulins. The first criterion is to use as the human acceptor the framework from a particular human immunoglobulin that is unusually homologous to the non-human donor immunoglobulin to be humanised, or to use a consensus framework from many human antibodies. The second criterion is to use the donor amino acid rather than the acceptor if the human acceptor residue is unusual and the donor residue is typical for human sequences at a specific residue of the framework. The third criterion is to use the donor framework amino acid residue rather than the acceptor at positions immediately adjacent to the CDRs. The fourth criterion is to use the donor amino acid residue at framework positions at which the amino acid is predicted to have a side chain atom within about 3 Å of the CDRs in a three-dimensional immunoglobulin model and to be capable of interacting with the antigen or with the CDRs of the humanised immunoglobulin. It is proposed that criteria two, three or four may be applied in addition or alternatively to criterion one, and may be applied singly or in any combination.

WO 90/07861 describes in detail the preparation of a single CDR-grafted humanised antibody, a humanised antibody having specificity for the p55 Tac protein of the

IL-2 receptor. The combination of all four criteria, as above, were employed in designing this humanised antibody, the variable region frameworks of the human antibody Eu (7) being used as acceptor. In the resultant humanised antibody the donor CDRs were as defined by Kabat et al (7 and 8) and in addition the mouse donor residues were used in place of the human acceptor residues, at positions 27, 30, 48, 66, 67, 89, 91, 94, 103, 104, 105 and 107 in the heavy chain and at positions 48, 60 and 63 in the light chain, of the variable region frameworks. The humanised anti-Tac antibody obtained is reported to have an affinity for p55 of 3×10^9 M^{-1}, about one-third of that of the murine MAb.

We have further investigated the preparation of CDR-grafted humanised antibody molecules and have identified a hierarchy of positions within the framework of the variable regions (i.e. outside both the Kabat CDRs and structural loops of the variable regions) at which the amino acid identities of the residues are important for obtaining CDR-grafted products with satisfactory binding affinity. This has enabled us to establish a protocol for obtaining satisfactory CDR-grafted products which may be applied very widely irrespective of the level of homology between the donor immunoglobulin and acceptor framework. The set of residues which we have identified as being of critical importance does not coincide with the residues identified by Queen et al (9).

Summary of the Invention

Accordingly, in a first aspect the invention provides a CDR-grafted antibody heavy chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 6, 23 and/or 24, 48 and/or 49, 71 and/or 73, 75 and/or 76 and/or 78 and 88 and/or 91.

In preferred embodiments, the heavy chain framework comprises donor residues at positions 23, 24, 49, 71, 73 and 78 or at positions 23, 24 and 49. The residues at positions 71, 73 and 78 of the heavy chain framework are preferably either all acceptor or all donor residues.

In particularly preferred embodiments the heavy chain framework additionally comprises donor residues at one, some or all of positions 6, 37, 48 and 94. Also it is particularly preferred that residues at positions of the heavy chain framework which are commonly conserved across species, i.e. positions 2, 4, 25, 36, 39, 47, 93, 103, 104, 106 and 107, if not conserved between donor and acceptor, additionally comprise donor residues. Most preferably the heavy chain framework additionally comprises donor residues at positions 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.

In addition the heavy chain framework optionally comprises donor residues at one, some or all of positions:
1 and 3,
72 and 76,
69 (if 48 is different between donor and acceptor),
38 and 46 (if 48 is the donor residue),
80 and 20 (if 69 is the donor residue),
67,
82 and 18 (if 67 is the donor residue),
91,
88, and
any one or more of 9, 11, 41, 87, 108, 110 and 112.

In the first and other aspects of the present invention reference is made to CDR-grafted antibody products comprising acceptor framework and donor antigen binding regions. It will be appreciated that the invention is widely applicable to the CDR-grafting of antibodies in

general. Thus, the donor and acceptor antibodies may be derived from animals of the same species and even same antibody class or sub-class. More usually, however, the donor and acceptor antibodies are derived from animals of different species. Typically the donor antibody is a non-human antibody, such as a rodent MAb, and the acceptor antibody is a human antibody.

In the first and other aspects of the present invention, the donor antigen binding region typically comprises at least one CDR from the donor antibody. Usually the donor antigen binding region comprises at least two and preferably all three CDRs of each of the heavy chain and/or light chain variable regions. The CDRs may comprise the Kabat CDRs, the structural loop CDRs or a composite of the Kabat and structural loop CDRs and any combination of any of these. Preferably, the antigen binding regions of the CDR-grafted heavy chain variable domain comprise CDRs corresponding to the Kabat CDRs at CDR2 (residues 50-65) and CDR3 (residues 95-100) and a composite of the Kabat and structural loop CDRs at CDR1 (residues 26-35).

The residue designations given above and elsewhere in the present application are numbered according to the Kabat numbering [refs. (7) and (8)]. Thus the residue designations do not always correspond directly with the linear numbering of the amino acid residues. The actual linear amino acid sequence may contain fewer or additional amino acids than in the strict Kabat numbering corresponding to a shortening of, or insertion into, a structural component, whether framework or CDR, of the basic variable domain structure. For example, the heavy chain variable region of the anti-Tac antibody described by Queen et al (9) contains a single amino acid insert (residue 52a) after residue 52 of CDR2 and a three amino

acid insert (residues 82a, 82b and 82c) after framework residue 82, in the Kabat numbering. The correct Kabat numbering of residues may be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence.

The invention also provides in a second aspect a CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 1 and/or 3 and 46 and/or 47. Preferably the CDR grafted light chain of the second aspect comprises donor residues at positions 46 and/or 47.

The invention also provides in a third aspect a CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 46, 48, 58 and 71.

In a preferred embodiment of the third aspect, the framework comprises donor residues at all of positions 46, 48, 58 and 71.

In particularly preferred embodiments of the second and third aspects, the framework additionally comprises donor residues at positions 36, 44, 47, 85 and 87. Similarly positions of the light chain framework which are commonly conserved across species, i.e. positions 2, 4, 6, 35, 49, 62, 64-69, 98, 99, 101 and 102, if not conserved between donor and acceptor, additionally comprise donor residues. Most preferably the light chain framework additionally comprises donor residues at positions 2, 4, 6, 35, 36, 38, 44, 47, 49, 62, 64-69, 85, 87, 98, 99, 101 and 102.

In addition the framework of the second or third aspects optionally comprises donor residues at one, some or all of positions:
1 and 3,
63,
60 (if 60 and 54 are able to form at potential saltbridge),
70 (if 70 and 24 are able to form a potential saltbridge),
73 and 21 (if 47 is different between donor and acceptor),
37 and 45 (if 47 is different between donor and acceptor),
and
any one or more of 10, 12, 40, 80, 103 and 105.

Preferably, the antigen binding regions of the CDR-grafted light chain variable domain comprise CDRs corresponding to the Kabat CDRs at CDR1 (residue 24-34), CDR2 (residues 50-56) and CDR3 (residues 89-97).

The invention further provides in a fourth aspect a CDR-grafted antibody molecule comprising at least one CDR-grafted heavy chain and at least one CDR-grafted light chain according to the first and second or first and third aspects of the invention.

The humanised antibody molecules and chains of the present invention may comprise: a complete antibody molecule, having full length heavy and light chains; a fragment thereof, such as a Fab, $(Fab')_2$ or FV fragment; a light chain or heavy chain monomer or dimer; or a single chain antibody, e.g. a single chain FV in which heavy and light chain variable regions are joined by a peptide linker; or any other CDR-grafted molecule with the same specificity as the original donor antibody. Similarly the CDR-grafted heavy and light chain variable region may be combined with other antibody domains as appropriate.

Also the heavy or light chains or humanised antibody molecules of the present invention may have attached to them an effector or reporter molecule. For instance, it may have a macrocycle, for chelating a heavy metal atom, or a toxin, such as ricin, attached to it by a covalent bridging structure. Alternatively, the procedures of recombinant DNA technology may be used to produce an immunoglobulin molecule in which the Fc fragment or CH3 domain of a complete immunoglobulin molecule has been replaced by, or has attached thereto by peptide linkage, a functional non-immunoglobulin protein, such as an enzyme or toxin molecule.

Any appropriate acceptor variable region framework sequences may be used having regard to class/type of the donor antibody from which the antigen binding regions are derived. Preferably, the type of acceptor framework used is of the same/similar class/type as the donor antibody. Conveniently, the framework may be chosen to maximise/optimise homology with the donor antibody sequence particularly at positions close or adjacent to the CDRs. However, a high level of homology between donor and acceptor sequences is not important for application of the present invention. The present invention identifies a hierarchy of framework residue positions at which donor residues may be important or desirable for obtaining a CDR-grafted antibody product having satisfactory binding properties. The CDR-grafted products usually have binding affinities of at least 10^5 M^{-1}, preferably at least about 10^8 M^{-1}, or especially in the range 10^8-10^{12} M^{-1}. In principle, the present invention is applicable to any combination of donor and acceptor antibodies irrespective of the level of homology between their sequences. A protocol for applying the invention to any particular donor-acceptor antibody pair is given hereinafter. Examples of human frameworks which may be

used are KOL, NEWM, REI, EU, LAY and POM (refs. 4 and 5) and the like; for instance KOL and NEWM for the heavy chain and REI for the light chain and EU, LAY and POM for both the heavy chain and the light chain.

Also the constant region domains of the products of the invention may be selected having regard to the proposed function of the antibody in particular the effector functions which may be required. For example, the constant region domains may be human IgA, IgE, IgG or IgM domains. In particular, IgG human constant region domains may be used, especially of the IgG1 and IgG3 isotypes, when the humanised antibody molecule is intended for therapeutic uses, and antibody effector functions are required. Alternatively, IgG2 and IgG4 isotypes may be used when the humanised antibody molecule is intended for therapeutic purposes and antibody effector functions are not required, e.g. for simple blocking of lymphokine activity.

However, the remainder of the antibody molecules need not comprise only protein sequences from immunoglobulins. For instance, a gene may be constructed in which a DNA sequence encoding part of a human immunoglobulin chain is fused to a DNA sequence encoding the amino acid sequence of a functional polypeptide such as an effector or reporter molecule.

Preferably the CDR-grafted antibody heavy and light chain and antibody molecule products are produced by recombinant DNA technology.

Thus in further aspects the invention also includes DNA sequences coding for the CDR-grafted heavy and light chains, cloning and expression vectors containing the DNA sequences, host cells transformed with the DNA sequences

and processes for producing the CDR-grafted chains and antibody molecules comprising expressing the DNA sequences in the transformed host cells.

The general methods by which the vectors may be constructed, transfection methods and culture methods are well known per se and form no part of the invention. Such methods are shown, for instance, in references 10 and 11.

The DNA sequences which encode the donor amino acid sequence may be obtained by methods well known in the art. For example the donor coding sequences may be obtained by genomic cloning, or cDNA cloning from suitable hybridoma cell lines. Positive clones may be screened using appropriate probes for the heavy and light chain genes in question. Also PCR cloning may be used.

DNA coding for acceptor, e.g. human acceptor, sequences may be obtained in any appropriate way. For example DNA sequences coding for preferred human acceptor frameworks such as KOL, REI, EU and NEWM, are widely available to workers in the art.

The standard techniques of molecular biology may be used to prepare DNA sequences coding for the CDR-grafted products. Desired DNA sequences may be synthesised completely or in part using oligonucleotide synthesis techniques. Site-directed mutagenesis and polymerase chain reaction (PCR) techniques may be used as appropriate. For example oligonucleotide directed synthesis as described by Jones et al (ref. 20) may be used. Also oligonucleotide directed mutagenesis of a pre-exising variable region as, for example, described by Verhoeyen et al (ref. 5) or Riechmann et al (ref. 6) may be used. Also enzymatic filling in of gapped

oligonucleotides using T_4 DNA polymerase as, for example, described by Queen et al (ref. 9) may be used.

Any suitable host cell/vector system may be used for expression of the DNA sequences coding for the CDR-grafted heavy and light chains. Bacterial e.g. E. coli, and other microbial systems may be used, in particular for expression of antibody fragments such as FAb and $(Fab')_2$ fragments, and especially FV fragments and single chain antibody fragments e.g. single chain FVs. Eucaryotic e.g. mammalian host cell expression systems may be used for production of larger CDR-grafted antibody products, including complete antibody molecules. Suitable mammalian host cells include CHO cells and myeloma or hybridoma cell lines.

Thus, in a further aspect the present invention provides a process for producing a CDR-grafted antibody product comprising:

(a) producing in an expression vector an operon having a DNA sequence which encodes an antibody heavy chain according to the first aspect of the invention;

and/or

(b) producing in an expression vector an operon having a DNA sequence which encodes a complementary antibody light chain according to the second or third aspect of the invention;

(c) transfecting a host cell with the or each vector; and

(d) culturing the transfected cell line to produce the CDR-grafted antibody product.

The CDR-grafted product may comprise only heavy or light chain derived polypeptide, in which case only a heavy chain or light chain polypeptide coding sequence is used to transfect the host cells.
For production of products comprising both heavy and light chains, the cell line may be transfected with two vectors, the first vector may contain an operon encoding a light chain-derived polypeptide and the second vector containing an operon encoding a heavy chain-derived polypeptide. Preferably, the vectors are identical, except in so far as the coding sequences and selectable markers are concerned, so as to ensure as far as possible that each polypeptide chain is equally expressed. Alternatively, a single vector may be used, the vector including the sequences encoding both light chain- and heavy chain-derived polypeptides.

The DNA in the coding sequences for the light and heavy chains may comprise cDNA or genomic DNA or both. However, it is preferred that the DNA sequence encoding the heavy or light chain comprises at least partially, genomic DNA, preferably a fusion of cDNA and genomic DNA.

The present invention is applicable to antibodies of any appropriate specificity. Advantageously, however, the invention may be applied to the humanisation of non-human antibodies which are used for <u>in vivo</u> therapy or diagnosis. Thus the antibodies may be site-specific antibodies such as tumour-specific or cell surface-specific antibodies, suitable for use in <u>in vivo</u> therapy or diagnosis, e.g. tumour imaging. Examples of cell surface-specific antibodies are anti-T cell antibodies, such as anti-CD3, and CD4 and adhesion molecules, such as CR3, ICAM and ELAM. The antibodies may have specificity for interleukins (including lymphokines, growth factors and stimulating factors), hormones and other biologically active compounds, and receptors for any of these. For

example, the antibodies may have specificity for any of the following: Interferons α, β, γ or δ, IL1, IL2, IL3, or IL4, etc., TNF, GCSF, GMCSF, EPO, hGH, or insulin, etc.

The the present invention also includes therapeutic and diagnostic compositions comprising the CDR-grafted products of the invention and uses of such compositions in therapy and diagnosis.

Accordingly in a further aspect the invention provides a therapeutic or diagnostic composition comprising a CDR-grafted antibody heavy or light chain or molecule according to previous aspects of the invention in combination with a pharmaceutically acceptable carrier, diluent or excipient.

Accordingly also the invention provides a method of therapy or diagnosis comprising administering an effective amount of a CDR-grafted antibody heavy or light chain or molecule according to previous aspects of the invention to a human or animal subject.

A preferred protocol for obtaining CDR-grafted antibody heavy and light chains in accordance with the present invention is set out below together with the rationale by which we have derived this protocol. This protocol and rationale are given without prejudice to the generality of the invention as hereinbefore described and defined.

Protocol

It is first of all necessary to sequence the DNA coding for the heavy and light chain variable regions of the donor antibody, to determine their amino acid sequences. It is also necessary to choose appropriate acceptor heavy and light chain variable regions, of known amino acid sequence. The CDR-grafted chain is then designed

starting from the basis of the acceptor sequence. It will be appreciated that in some cases the donor and acceptor amino acid residues may be identical at a particular position and thus no change of acceptor framework residue is required.

1. As a first step donor residues are substituted for acceptor residues in the CDRs. For this purpose the CDRs are preferably defined as follows:

Heavy chain	-	CDR1:	residues 26-35
	-	CDR2:	residues 50-65
	-	CDR3:	residues 95-102
Light chain	-	CDR1:	residues 24-34
	-	CDR2:	residues 50-56
	-	CDR3:	residues 89-97

The positions at which donor residues are to be substituted for acceptor in the framework are then chosen as follows, first of all with respect to the heavy chain and subsequently with respect to the light chain.

2. <u>Heavy Chain</u>

2.1 Choose donor residues at all of positions 23, 24, 49, 71, 73 and 78 of the heavy chain or all of positions 23, 24 and 49 (71, 73 and 78 are always either all donor or all acceptor).

2.2 Check that the following have the same amino acid in donor and acceptor sequences, and if not preferably choose the donor: 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.

2.3 To further optimise affinity consider choosing donor residues at one, some or any of:

i. 1, 3
ii. 72, 76
iii. If 48 is different between donor and acceptor sequences, consider 69
iv. If at 48 the donor residue is chosen, consider 38 and 46
v. If at 69 the donor residue is chosen, consider 80 and then 20
vi. 67
vii. If at 67 the donor residue is chosen, consider 82 and then 18
viii. 91
ix. 88
x. 9, 11, 41, 87, 108, 110, 112

3. Light Chain

3.1 Choose donor at 46, 48, 58 and 71

3.2 Check that the following have the same amino acid in donor and acceptor sequences, if not preferably choose donor:

2, 4, 6, 35, 38, 44, 47, 49, 62, 64-69 inclusive, 85, 87, 98, 99, 101 and 102

3.3 To further optimise affinity consider choosing donor residues at one, some or any of:

i. 1, 3
ii. 63

iii. 60, if 60 and 54 are able to form potential saltbridge
iv. 70, if 70 and 24 are able to form potential saltbridge
v. 73, and 21 if 47 is different between donor and acceptor
vi. 37, and 45 if 47 is different between donor and acceptor
vii. 10, 12, 40, 80, 103, 105

Rationale

In order to transfer the binding site of an antibody into a different acceptor framework, a number of factors need to be considered.

1. The extent of the CDRs

The CDRs (Complementary Determining Regions) were defined by Wu and Kabat (refs. 4 and 5) on the basis of an analysis of the variability of different regions of antibody variable regions. Three regions per domain were recognised. In the light chain the sequences are 24-34, 50-56, 89-97 (numbering according to Kabat (ref. 4), Eu Index) inclusive and in the heavy chain the sequences are 31-35, 50-65 and 95-102 inclusive.

When antibody structures became available it became apparent that these CDR regions corresponded in the main to loop regions which extended from the β barrel framework of the light and heavy variable domains. For H1 there was a discrepancy in that the loop was from 26 to 32 inclusive and for H2 the loop was 52 to 56 and for L2 from 50 to 53. However, with the exception of H1 the CDR regions encompassed the loop regions and extended into the β strand

frameworks. In H1 residue 26 tends to be a serine and 27 a phenylalanine or tyrosine, residue 29 is a phenylalanine in most cases. Residues 28 and 30 which are surface residues exposed to solvent might be involved in antigen-binding. A prudent definition of the H1 CDR therefore would include residues 26-35 to include both the loop region and the hypervariable residues 33-35.

It is of interest to note the example of Riechmann et al (ref. 3), who used the residue 31-35 choice for CDR-H1. In order to produce efficient antigen binding, residue 27 also needed to be recruited from the donor (rat) antibody.

2. Non-CDR residues which contribute to antigen binding

By examination of available X-ray structures we have identified a number of residues which may have an effect on net antigen binding and which can be demonstrated by experiment. These residues can be sub-divided into a number of groups.

2.1 Surface residues near CDR [all numbering as in Kabat et al (ref. 7)].

2.1.1. Heavy Chain - Key residues are 23, 71 and 73. Other residues which may contribute to a lesser extent are 1, 3 and 76. Finally 25 is usually conserved but the murine residue should be used if there is a difference.

2.1.2 Light Chain - Many residues close to the CDRs, e.g. 63, 65, 67 and 69 are conserved. If conserved none of the surface residues in the light chain are likely to have a major effect. However, if the murine residue at these positions

is unusual, then it would be of benefit to analyse the likely contribution more closely. Other residues which may also contribute to binding are 1 and 3, and also 60 and 70 if the residues at these positions and at 54 and 24 respectively are potentially able to form a salt bridge i.e. 60 + 54; 70 + 24.

2.2 Packing residues near the CDRs.

2.2.1. Heavy Chain - Key residues are 24, 49 and 78. Other key residues would be 36 if not a tryptophan, 94 if not an arginine, 104 and 106 if not glycines and 107 if not a threonine. Residues which may make a further contribution to stable packing of the heavy chain and hence improved affinity are 2, 4, 6, 38, 46, 67 and 69. 67 packs against the CDR residue 63 and this pair could be either both mouse or both human. Finally, residues which contribute to packing in this region but from a longer range are 18, 20, 80, 82 and 86. 82 packs against 67 and in turn 18 packs against 82. 80 packs against 69 and in turn 20 packs against 80. 86 forms an H bond network with 38 and 46. Many of the mouse-human differences appear minor e.g. Leu-Ile, but could have an minor impact on correct packing which could translate into altered positioning of the CDRs.

2.2.2. Light Chain - Key residues are 48, 58 and 71. Other key residues would be 6 if not glutamine, 35 if not tryptophan, 62 if not phenylalanine or tryosine, 64, 66, 68, 99 and 101 if not glycines and 102 if not a threonine. Residues which make a further contribution are 2, 4, 37, 45 and 47. Finally residues 73 and 21 and 19 may make long distance packing contributions of a minor nature.

2.3. Residues at the variable domain interface between heavy and light chains - In both the light and heavy chains most of the non-CDR interface residues are conserved. If a conserved residue is replaced by a residue of different character, e.g. size or charge, it should be considered for retention as the murine residue.

2.3.1. Heavy Chain - Residues which need to be considered are 37 if the residue is not a valine but is of larger side chain volume or has a charge or polarity. Other residues are 39 if not a glutamine, 45 if not a leucine, 47 if not a tryptophan, 91 if not a phenylalanine or tyrosine, 93 if not an alanine and 103 if not a tryptophan. Residue 89 is also at the interface but is not in a position where the side chain could be of great impact.

2.3.2. Light Chain - Residues which need to be considered are 36, if not a tyrosine, 38 if not a glutamine, 44 if not a proline, 46, 49 if not a tyrosine, residue 85, residue 87 if not a tyrosine and 98 if not a phenylalanine.

2.4. Variable-Constant region interface - The elbow angle between variable and constant regions may be affected by alterations in packing of key residues in the variable region against the constant region which may affect the position of V_L and V_H with respect to one another. Therefore it is worth noting the residues likely to be in contact with the constant region. In the heavy chain the surface residues potentially in contact with the variable region are conserved between mouse and human antibodies therefore the variable region contact residues may influence the V-C interaction. In the light chain the amino acids found at a number of the constant

region contact points vary, and the V & C regions are not in such close proximity as the heavy chain. Therefore the influences of the light chain V-C interface may be minor.

2.4.1. Heavy Chain - Contact residues are 7, 11, 41, 87, 108, 110, 112.

2.4.2. Light Chain - In the light chain potentially contacting residues are 10, 12, 40, 80, 83, 103 and 105.

The above analysis coupled with our considerable practical experimental experience in the CDR-grafting of a number of different antibodies have lead us to the protocol given above.

The present invention is now described, by way of example only, with reference to the accompanying Figures 1 - 13.

Brief Description of the Figures

Figure 1 shows DNA and amino acid sequences of the OKT3 light chain;

Figure 2 shows DNA and amino acid sequences of the OKT3 heavy chain;

Figure 3 shows the alignment of the OKT3 light variable region amino acid sequence with that of the light variable region of the human antibody REI;

Figure 4 shows the alignment of the OKT3 heavy variable region amino acid sequence with that of the heavy variable region of the human antibody KOL;

Figure 5 shows the heavy variable region amino acid sequences of OKT3, KOL and various corresponding CDR grafts;

Figure 6 shows the light variable region amino acid sequences of OKT3, REI and various corresponding CDR grafts;

Figure 7 shows a graph of binding assay results for various grafted OKT3 antibodies'

Figure 8 shows a graph of blocking assay results for various grafted OKT3 antibodies;

Figure 9 shows a similar graph of blocking assay results;

Figure 10 shows similar graphs for both binding assay and blocking assay results;

Figure 11 shows further similar graphs for both binding assay and blocking assay results;

Figure 12 shows a graph of competition assay results for a minimally grafted OKT3 antibody compared with the OKT3 murine reference standard, and

Figure 13 shows a similar graph of competition assay results comparing a fully grafted OKT3 antibody with the murine reference standard.

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

EXAMPLE 1

CDR-GRAFTING OF OKT3

MATERIAL AND METHODS

1. INCOMING CELLS

Hybridoma cells producing antibody OKT3 were provided by Ortho (seedlot 4882.1) and were grown up in antibiotic free Dulbecco's Modified Eagles Medium (DMEM) supplemented with glutamine and 5% foetal calf serum, and divided to provide both an overgrown supernatant for evaluation and cells for extraction of RNA. The overgrown supernatant was shown to contain 250 ug/mL murine IgG2a/kappa antibody. The supernatant was negative for murine lambda light chain and IgG1, IgG2b, IgG3, IgA and IgM heavy chain. 20mL of supernatant was assayed to confirm that the antibody present was OKT3.

2. MOLECULAR BIOLOGY PROCEDURES

Basic molecular biology procedures were as described in Maniatis et al (ref. 9) with, in some cases, minor modifications. DNA sequencing was performed as described in Sanger et al (ref. 11) and the Amersham International Plc sequencing handbook. Site directed mutagenesis was as described in Kramer et al (ref. 12) and the Anglian Biotechnology Ltd. handbook. COS cell expression and metabolic labelling studies were as described in Whittle et al (ref. 13)

3. RESEARCH ASSAYS

3.1. ASSEMBLY ASSAYS

Assembly assays were performed on supernatants from transfected COS cells to determine the amount of intact IgG present.

3.1.1. COS CELLS TRANSFECTED WITH MOUSE OKT3 GENES

The assembly assay for intact mouse IgG in COS cell supernatants was an ELISA with the following format:

96 well microtitre plates were coated with F(ab')2 goat anti-mouse IgG Fc. The plates were washed in water and samples added for 1 hour at room temperature. The plates were washed and F(ab')2 goat anti-mouse IgG F(ab')2 (HRPO conjugated) was then added. Substrate was added to reveal the reaction. UPC10, a mouse IgG2a myeloma, was used as a standard.

3.1.2. COS AND CHO CELLS TRANSFECTED WITH CHIMERIC OR CDR-GRAFTED OKT3 GENES

The assembly assay for chimeric or CDR-grafted antibody in COS cell supernatants was an ELISA with the following format:

96 well microtitre plates were coated with F(ab')2 goat anti-human IgG Fc. The plates were washed and samples added and incubated for 1 hour at room temperature. The plates were washed and monoclonal mouse anti-human kappa chain was added for 1 hour at room temperature.

The plates were washed and F(ab')2 goat anti-mouse IgG Fc (HRPO conjugated) was added. Enzyme substrate was added to reveal the reaction. Chimeric B72.3 (IgG4) (ref. 13) was used as a standard. The use of a monoclonal anti-kappa chain in this assay allows grafted antibodies to be read from the chimeric standard.

3.2. ASSAY FOR ANTIGEN BINDING ACTIVITY

Material from COS cell supernatants was assayed for OKT3 antigen binding activity onto CD3 positive cells in a direct assay. The procedure was as follows:

HUT 78 cells (human T cell line, CD3 positive) were maintained in culture. Monolayers of HUT 78 cells were prepared onto 96 well ELISA plates using poly-L-lysine and glutaraldehyde. Samples were added to the monolayers for 1 hour at room temperature.

The plates were washed gently using PBS. F(ab')2 goat anti-human IgG Fc (HRPO conjugated) or F(ab')2 goat anti-mouse IgG Fc (HRPO conjugated) was added as appropriate for humanised or mouse samples. Substrate was added to reveal the reaction.

The negative control for the cell-based assay was chimeric B72.3. The positive control was mouse Orthomune OKT3 or chimeric OKT3, when available. This cell-based assay was difficult to perform, and an alternative assay was developed for CDR-grafted OKT3 which was more sensitive and easier to carry out.

In this system CDR-grafted OKT3 produced by COS cells was tested for its ability to bind to the CD3-positive HPB-ALL (human peripheral blood acute lymphocytic leukemia) cell line. It was also tested for its ability to block the binding of murine OKT3 to these cells. Binding was measured by the following procedure: HPB-ALL cells were harvested from tissue culture. Cells were incubated at 4°C for 1 hour with various dilutions of test antibody, positive control antibody, or negative control antibody. The cells were washed once and incubated at 4°C for 1 hour with an FITC-labelled goat anti-human IgG (Fc-

specific, mouse absorbed). The cells were washed twice and analysed by cytofluorography. Chimeric OKT3 was used as a positive control for direct binding. Cells incubated with mock- transfected COS cell supernatant, followed by the FITC-labelled goat anti-human IgG, provided the negative control. To test the ability of CDR-grafted OKT3 to block murine OKT3 binding, the HPB-ALL cells were incubated at 4°C for 1 hour with various dilutions of test antibody or control antibody. A fixed saturating amount of FITC OKT3 was added. The samples were incubated for 1 hour at 4°C, washed twice and analysed by cytofluorography. FITC-labelled OKT3 was used as a positive control to determine maximum binding. Unlabelled murine OKT3 served as a reference standard for blocking. Negative controls were unstained cells with or without mock-transfected cell supernatant. The ability of the CDR-grafted OKT3 light chain to bind CD3-positive cells and block the binding of murine OKT3 was initially tested in combination with the chimeric OKT3 heavy chain. The chimeric OKT3 heavy chain is composed of the murine OKT3 variable region and the human IgG4 constant region. The chimeric heavy chain gene is expressed in the same expression vector used for the CDR-grafted genes. The CDR-grafted light chain expression vector and the chimeric heavy chain expression vector were co-transfected into COS cells. The fully chimeric OKT3 antibody (chimeric light chain and chimeric heavy chain) was found to be fully capable of binding to CD3 positive cells and blocking the binding of murine OKT3 to these cells.

3.3 DETERMINATION OF RELATIVE BINDING AFFINITY

The relative binding affinities of CDR-grafted

anti-CD3 monoclonal antibodies were determined by competition binding (ref. 6) using the HPB-ALL human T cell line as a source of CD3 antigen, and fluorescein-conjugated murine OKT3 (Fl-OKT3) of known binding affinity as a tracer antibody. The binding affinity of Fl-OKT3 tracer antibody was determined by a direct binding assay in which increasing amounts of Fl-OKT3 were incubated with HPB-ALL ($5x10^5$) in PBS with 5% foetal calf serum for 60 min. at 4°C. Cells were washed, and the fluorescence intensity was determined on a FACScan flow cytometer calibrated with quantitative microbead standards (Flow Cytometry Standards, Research Triangle Park, NC). Fluorescence intensity per antibody molecule (F/P ratio) was determined by using microbeads which have a predetermined number of mouse IgG antibody binding sites (Simply Cellular beads, Flow Cytometry Standards). F/P equals the fluorescence intensity of beads saturated with Fl-OKT3 divided by the number of binding sites per bead. The amount of bound and free Fl-OKT3 was calculated from the mean fluorescence intensity per cell, and the ratio of bound/free was plotted against the number of moles of antibody bound. A linear fit was used to determine the affinity of binding (absolute value of the slope).

For competitive binding, increasing amounts of competitor antibody were added to a sub-saturating dose of Fl-OKT3 and incubated with $5x10^5$ HPB-ALL in 200 Ml of PBS with 5% foetal calf serum, for 60 min at 4°C. The fluorescence intensities of the cells were measured on a FACScan flow cytometer calibrated with quantitative microbead standards. The concentrations of bound and free Fl-OKT3 were calculated. The affinities of competing anti-

bodies were calculated from the equation [X]-[ORT3] = (1/Kx) - (1/Ka), where Ka is the affinity of murine ORT3, Kx is the affinity of competitor X, [ ] is the concentration of competitor antibody at which bound/free binding is R/2, and R is the maximal bound/free binding.

4. cDNA LIBRARY CONSTRUCTION

4.1. mRNA PREPARATION AND cDNA SYNTHESIS

ORT3 producing cells were grown as described above and 1.2 x 10^9 cells harvested and mRNA extracted using the guanidinium/LiCl extraction procedure. cDNA was prepared by priming from Oligo-dT to generate full length cDNA. The cDNA was methylated and EcoR1 linkers added for cloning.

4.2. LIBRARY CONSTRUCTION

The cDNA library was ligated to pSP65 vector DNA which had been EcoR1 cut and the 5′ phosphate groups removed by calf intestinal phosphatase (EcoR1/CIP). The ligation was used to transform high transformation efficiency Escherichia coli (E.coli) HB101. A cDNA library was prepared. 3600 colonies were screened for the light chain and 10000 colonies were screened for the heavy chain.

5. SCREENING

E.coli colonies positive for either heavy or light chain probes were identified by oligonucleotide screening using the oligonucleotides:
5′ TCCAGATGTTAACTGCTCAC for the light chain, which is complementary to a sequence in the mouse kappa constant region, and 5′ CAGGGGCCAGTGGATGGATAGAC for the heavy chain which is complementary to a sequence in the mouse IgG2a constant CH1 domain region. 12 light chain and 9 heavy chain clones

were identified and taken for second round screening. Positive clones from the second round of screening were grown up and DNA prepared. The sizes of the gene inserts were estimated by gel electrophoresis and inserts of a size capable of containing a full length cDNA were subcloned into M13 for DNA sequencing.

6. DNA SEQUENCING

Clones representing four size classes for both heavy and light chains were obtained in M13. DNA sequence for the 5′ untranslated regions, signal sequences, variable regions and 3′ untranslated regions of full length cDNAs [Figures 1(a) and 2(a)] were obtained and the corresponding amino acid sequences predicted [(Figures 1(b) and 2(b)]. In Figure 1(a) the untranslated DNA regions are shown in uppercase, and in both Figures 1 and 2 the signal sequences are underlined.

7. CONSTRUCTION OF cDNA EXPRESSION VECTORS

Celltech expression vectors are based on the plasmid pEE6hCMV (ref. 14). A polylinker for the insertion of genes to be expressed has been introduced after the major immediate early promoter/enhancer of the human Cytomegalovirus (hCMV). Marker genes for selection of the plasmid in transfected eukaryotic cells can be inserted as BamH1 cassettes in the unique BamH1 site of pEE6 hCMV; for instance, the neo marker to provide pEE6 hCMV neo. It is usual practice to insert the neo and gpt markers prior to insertion of the gene of interest, whereas the GS marker is inserted last because of the presence of internal EcoR1 sites in the cassette.

The selectable markers are expressed from the SV40 late promoter which also provides an origin of replication so that the vectors can be used for expression in the COS cell transient expression system.
The mouse sequences were excised from the M13 based vectors described above as EcoR1 fragments and cloned into either pEE6-hCMV-neo for the heavy chain and into EE6-hCMV-gpt for the light chain to yield vectors pJA136 and pJA135 respectively.

8. EXPRESSION OF cDNAS IN COS CELLS

Plasmids pJA135 and pJA136 were co-transfected into COS cells and supernatant from the transient expression experiment was shown to contain assembled antibody which bound to T-cell enriched lymphocytes. Metabolic labelling experiments using ^{35}S methionine showed expression and assembly of heavy and light chains.

9. CONSTRUCTION OF CHIMERIC GENES

Construction of chimeric genes followed a previously described strategy [Whittle et al (ref. 13)]. A restriction site near the 3′ end of the variable domain sequence is identified and used to attach an oligonucleotide adapter coding for the remainder of the mouse variable region and a suitable restriction site for attachment to the constant region of choice.

9.1. LIGHT CHAIN GENE CONSTRUCTION

The mouse light chain cDNA sequence contains an AvaI site near the 3′ end of the variable region [Fig. 1(a)]. The majority of the sequence of the variable region was isolated as a 396 bp. EcoR1-AvaI fragment. An oligonucleotide adapter was designed to replace the remainder of the 3′

region of the variable region from the AvaI site and to include the 5' residues of the human constant region up to and including a unique NarI site which had been previously engineered into the constant region.
A HindIII site was introduced to act as a marker for insertion of the linker.
The linker was ligated to the V_L fragment and the 413 bp EcoRI-NarI adapted fragment was purified from the ligation mixture.
The constant region was isolated as an NarI-BamHI fragment from an M13 clone NW361 and was ligated with the variable region DNA into an EcoRI/BamHI/ClP pSP65 treated vector in a three way reaction to yield plasmid JA143. Clones were isolated after transformation into E.coli and the linker and junction sequences were confirmed by the presence of the HindIII site and by DNA sequencing.

9.2 LIGHT CHAIN GENE CONSTRUCTION - VERSION 2

The construction of the first chimeric light chain gene produces a fusion of mouse and human amino acid sequences at the variable-constant region junction. In the case of the OKT3 light chain the amino acids at the chimera junction are:

.....Leu-Glu-Ile-Asn-Arg/ -/Thr-Val-Ala -Ala
VARIABLE CONSTANT

This arrangement of sequence introduces a potential site for Asparagine (Asn) linked (N-linked) glycosylation at the V-C junction. Therefore, a second version of the chimeric light chain oligonucleotide adapter was designed in which the threonine (Thr), the first amino acid of the human constant region, was replaced with the equivalent amino acid from the mouse constant region, Alanine (Ala).

An internal HindIII site was not included in this adapter, to differentiate the two chimeric light chain genes.
The variable region fragment was isolated as a 376 bp EcoR1-AvaI fragment. The oligonucleotide linker was ligated to NarI cut pNW36I and then the adapted 396bp constant region was isolated after recutting the modified pNW361 with EcoR1. The variable region fragment and the modified constant region fragment were ligated directly into EcoR1/C1P treated pEE6hCMVneo to yield pJA137. Initially all clones examined had the insert in the incorrect orientation. Therefore, the insert was re-isolated and recloned to turn the insert round and yield plasmid pJA141. Several clones with the insert in the correct orientation were obtained and the adapter sequence of one was confirmed by DNA sequencing

9.3. HEAVY CHAIN GENE CONSTRUCTION

9.3.1. CHOICE OF HEAVY CHAIN GENE ISOTYPE

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

9.3.2. GENE CONSTRUCTION

The heavy chain cDNA sequence showed a BanI site near the 3′ end of the variable region [Fig. 2(a)]. The majority of the sequence of the variable region was isolated as a 426bp. EcoR1/C1P/BanI fragment. An oligonucleotide adapter was designated to replace the remainder of the 3′ region of the variable region from the BanI site up to and including a unique HindIII site which had been previously engineered into the first two amino acids of the constant region.
The linker was ligated to the V_H fragment and the EcoR1-HindIII adapted fragment was purified from the ligation mixture.

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

10. CONSTRUCTION OF CHIMERIC EXPRESSION VECTORS

10.1. neo AND gpt VECTORS

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

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

The chimeric heavy chain gene was isolated from pJA142 as a 2.5Kbp EcoR1/BamH1 fragment and cloned into the EcoR1/Bc11/C1P treated vector fragment of a derivative of pEE6hCMVgpt to yield plasmid pJA144.

10.2. GS SEPARATE VECTORS

GS versions of pJA141 and pJA144 were constructed by replacing the neo and gpt cassettes by a BamH1/Sa11/C1P treatment of the plasmids, isolation of the vector fragment and ligation to a GS-containing fragment from the plasmid pRO49 to yield the light chain vector pJA179 and the heavy chain vector pJA180.

10.3. GS SINGLE VECTOR CONSTRUCTION

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

and with transcription of the genes being head to tail e.g. cL>cH>GS were constructed. These plasmids were made by treating pJA179 or pJA180 with BamH1/ClP and ligating in a Bgl11/Hind111 hCMV promoter cassette along with either the Hind111/BamH1 fragment from pJA141 into pJA180 to give the cH-cL-GS plasmid pJA182 or the Hind111/BamH1 fragment from pJA144 into pJA179 to give the cL-cH-GS plasmid pJA181.

11. EXPRESSION OF CHIMERIC GENES

11.1. EXPRESSION IN COS CELLS

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

11.2 EXPRESSION IN CHINESE HAMSTER OVARY (CHO) CELLS

Stable cell lines have been prepared from plasmids PJA141/pJA144 and from pJA179/pJA180, pJA181 and pJA182 by transfection into CHO cells.

12. CDR-GRAFTING

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

12.1. VARIABLE REGION ANALYSIS

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

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

(a) By examination of antibody X-ray crystal structures the antigen binding surface can be predominantly located on a series of loops, three per domain, which extend from the B-barrel framework.

(b) By analysis of antibody variable domain sequences regions of hypervariability [termed the Complementarity Determining Regions (CDRs) by Wu and Kabat (ref. 5)] can be identified. In the most but not all cases these CDRs correspond to, but extend a short way beyond, the loop regions noted above.

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

12.1.1. LIGHT CHAIN

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

N - near to CDR (From X-ray Structures)

P - Packing B - Buried Non-Packing

S - Surface E - Exposed

I - Interface * - Interface

- Packing/Part Exposed

? - Non-CDR Residues which may require to be left as Mouse sequence.

Residues underlined in Figure 3 are amino acids. RE1 was chosen as the human framework because the light chain is a kappa chain and the kappa variable regions show higher homology with the mouse sequences than a lambda light variable region, e.g. KOL (see below). RE1 was chosen in preference to another kappa light chain because the X-ray structure of the light chain has been determined so that a structural examination of individual residues could be made.

12.1.2. HEAVY CHAIN

Similarly Figure 4 shows an alignment of sequences for the human framework region KOL and the OKT3 heavy variable region. The structural loops and CDRs believed to correspond to the antigen binding region are marked. Also marked are a number of other residues which may also contribute to antigen binding as described in 12.1(c). The residue type key and other indicators used in Figure 4 are the same as those used in Figure 3. KOL was chosen as the heavy chain framework because the X-ray structure has been determined to a better resolution than, for example, NEWM and also the sequence alignment of OKT3 heavy variable region showed a slightly better homology to KOL than to NEWM.

12.2. DESIGN OF VARIABLE GENES

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

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

12.3. GENE CONSTRUCTION

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

13. CONSTRUCTION OF EXPRESSION VECTORS

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

TABLE 1 CDR-GRAFTED GENE CONSTRUCTS

CODE	MOUSE SEQUENCE CONTENT	METHOD OF CONSTRUCTION	KOZAK SEQUENCE -	KOZAK SEQUENCE +
LIGHT CHAIN	ALL HUMAN FRAMEWORK RE1			
121	26-32, 50-56, 91-96 inclusive	SDM and gene assembly	+	n.d.
121A	26-32, 50-56, 91-96 inclusive +1, 3, 46, 47	Partial gene assembly	n.d.	+
121B	26-32, 50-56, 91-96 inclusive + 46, 47	Partial gene assembly	n.d.	+
221	24-24, 50-56, 91-96 inclusive	Partial gene assembly	+	+
221A	24-34, 50-56, 91-96 inclusive +1, 3, 46, 47	Partial gene assembly	+	+
221B	24-34, 50-56, 91-96 inclusive +1, 3	Partial gene assembly	+	+
221C	24-34, 50-56, 91-96 inclusive	Partial gene assembly	+	+
HEAVY CHAIN	ALL HUMAN FRAMEWORK KOL			
121	26-32, 50-56, 95-100B inclusive	Gene assembly	n.d.	+
131	26-32, 50-58, 95-100B inclusive	Gene assembly	n.d.	+
141	26-32, 50-65, 95-100B inclusive	Partial gene assembly	+	n.d.
321	26-35, 50-56, 95-100B inclusive	Partial gene assembly	+	n.d.
331	26-35, 50-58, 95-100B inclusive	Partial gene assembly	+	
		Gene assembly		+
341	26-35, 50-65, 95-100B inclusive	SDM	+	
		Partial gene assembly		+
341A	26-35, 50-65, 95-100B inclusive +6, 23, 24, 48, 49, 71, 73, 76, 78, 88, 91 (+63 = human)	Gene assembly	n.d.	+
341B	26-35, 50-65, 95-100B inclusive + 48, 49, 71, 73, 76, 78, 88, 91 (+63 + human)	Gene assembly	n.d.	+

KEY

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

14. EXPRESSION OF CDR-GRAFTED GENES

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

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

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

When direct binding to antigen on HUT 78 cells was measured, a construct designed to include mouse sequence based on loop length (gL121) did not lead to active antibody in association with mH or cH. A construct designed to include mouse sequence based on Kabat CDRs (gL221) demonstrated some weak binding in association with mH or cH. However, when framework residues 1, 3, 46, 47 were changed from the human to the murine OKT3 equivalents based on the arguments outlined in Section 12.1 antigen binding was demonstrated when both of the new constructs, which were termed 121A and 221A were co-expressed with cH. When the effects of these residues were examined in more detail, it appears that residues 1 and 3 are not major contributing residues as the product of the gL221B gene shows little detectable binding activity in association with cH. The light chain product of gL221C, in which mouse sequences are present at 46 and 47, shows good binding activity in association with cH.

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

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

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

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

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

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

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

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

14.3 PRODUCTION OF FULLY CDR-GRAFTED ANTIBODY

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

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

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

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

An analysis of the above results is given below.

15. DISCUSSION OF CDR-GRAFTING RESULTS

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

15.1. LIGHT CHAIN

15.1.1. EXTENT OF THE CDRs

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

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

15.1.2. FRAMEWORK RESIDUES

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

see Figure 3 and Table 1) was made, cloned in EE6hCMVneo and co-expressed with cH (pJA144). The resultant antibody was well expressed and showed good binding activity. When the related genes gL221B (gL221 + D1Q, Q3V) and gL221C (gL221 + L46R, L47W) were made and similarly tested, while both genes produced antibody when co-expressed with cH, only the gL221C/cH combination showed good antigen binding. When the gL121A (gL121 + D1Q, Q3V, L46R, L47W) gene was made and co-expressed with cH, antibody was produced which also bound to antigen.

15.2. HEAVY CHAIN

15.2.1. EXTENT OF THE CDRs

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

being degraded internally. In some experiments trace amounts of antibody could be detected in ^{35}S labelling studies.
As no net antibody was produced, analysis of these constructs was not pursued further.
When, however, a combination of the loop choice and the Kabat choice for CDR1 was tested (mouse amino acids 26-35 inclusive) and in which residues 31 (Ser to Arg), 33 (Ala to Thr), and 35 (Tyr to His) were changed from the human residues to the mouse residue and compared to the first series, antibody was produced for gH321, kgH331 and kgH341 when co-expressed with cL. Expression was generally low and could not be markedly improved by the insertion of the Kozak consensus sequence 5′ to the ATG of the signal sequence of the gene, as distinct from the case of the gL genes where such insertion led to a 2-5 fold increase in net antibody production. However, only in the case of gH341/mL or kgH341/cL could marginal antigen binding activity be demonstrated. When the kgH341 gene was co-expressed with kgL221A, the net yield of antibody was too low to give a signal above the background level in the antigen binding assay.

15.2.2. FRAMEWORK RESIDUES

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

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

15.3 INTERIM CONCLUSIONS

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

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

16. FURTHER CDR-GRAFTING EXPERIMENTS

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

gH341A (plasmid pJA185) with either mouse OKT3 or human KOL residues at 6, 23, 24, 48, 49, 63, 71, 73, 76, 78, 88 and 91, as indicated. The CDR-grafted light chain genes used in these further experiments were gL221, gL221A, gL221B and gL221C as described above.

TABLE 2

OKT3 HEAVY CHAIN CDR GRAFTS

1. gH341 and derivatives

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

OKT3 LIGHT CHAIN CDR GRAFTS

2. gL221 and derivatives

RES NUM	1	3	46	47	
OKT3vl	Q	V	R	W	
GL221	D	Q	L	L	DA221
gL221A	Q	V	R	W	DA221A
gL221B	Q	V	L	L	DA221B
GL221C	D	Q	R	W	DA221C
REI	D	Q	L	L	

MURINE RESIDUES ARE UNDERLINED

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

The results of the assays for various grafted heavy chains co-expressed with the gL221C light chain are given in Figures 7 and 8 (for the JA184, JA185, JA197 and JA198 constructs - see Table 2), in Figure 9 (for the JA183, JA184, JA185 and JA197 constructs) in Figure 10 (for the chimeric, JA185, JA199, JA204, JA205, JA207, JA208 and JA209 constructs) and in Figure 11 (for the JA183, JA184, JA185, JA198, JA203, JA205 and JA206 constructs).

The basic grafted product without any human to murine changes in the variable frameworks, i.e. gL221 co-expressed with gh341 (JA178), and also the "fully grafted" product, having most human to murine changes in the grafted heavy chain framework, i.e. gL221C co-expressed with gh341A (JA185), were assayed for relative binding affinity in a competition assay against murine OKT3 reference standard, using HPB-ALL cells. The assay used was as described above in section 3.3. The results obtained are given in Figure 12 for the basic grafted product and in Figure 13 for the fully grafted product. These results indicate that the basic grafted product has neglibible binding ability as compared with the OKT3 murine reference standard; whereas the "fully grafted" product has a binding ability very similar to that of the OKT3 murine reference standard.

The binding and blocking assay results indicate the following:

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

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

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

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

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

EXAMPLE 2

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

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

THE LIGHT CHAIN

The human acceptor framework used for the grafted light chains was RE1. The preferred LCDR2 light chain has human to mouse changes at positions 33, 34, 38, 49 and 89 in addition to the structural loop CDRs. Of these changed positions, positions 33, 34 and 89 fall within the preferred extended CDRs of the present invention (positions 33 and 34 in CDR1 and position 89 in CDR3). The human to murine changes at positions 38 and 49 corresponds to positions at which the amino acid residues are preferably donor murine amino acid residues in accordance with the present invention.

A comparison of the amino acid sequences of the donor murine light chain variable domain and the RE1 human acceptor light chain variable further reveals that the murine and human residues are identical at all of positions 46, 48 and 71 and at all of positions 2, 4, 6, 35, 36, 44, 47, 62, 64-69, 85, 87, 98, 99 and 101 and 102. However the amino acid residue at position 58 in LCDR2 is

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

THE HEAVY CHAIN

The human acceptor framework used for the grafted heavy chains was KOL.

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

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

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

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

EXAMPLE 3

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

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

(a) B72.3 Light Chain

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

CDR Number	Residues
1	24-34
2	50-56
3	90-96

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

B72.3 cH/B72.3 cL

and B72.3 cH/B72.3 gL

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

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

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

(b) B72.3 heavy chain

i. Choice of framework

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

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

Comparison of the B72.3 heavy chain sequence with those in Kabat (ref. 4) for human heavy chains showed clearly that B72.3 had poor homology for KOL and NEWM (for which crystal structures are available) but was very homologous to the heavy chain for EU.

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

CDR Number	Residues
1	27-36
2	50-63
3	93-102

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

ii. Results with grafted heavy chain genes

Expression of grafted heavy chain genes containing all human framework regions with either gL or cL genes produced a grafted antibody with little ability to bind to mucin. The grafted antibody had about 1% the activity of the chimeric antibody.

In these experiments, however, it was noted that the activity of the grafted antibody could be increased to ~ 10% of B72.3 by exposure to pHs of 2-3.5.

This observation provided a clue as to how the activity of the grafted antibody could be improved without acid treatment. It was postulated that acid exposure brought about the protonation of an acidic residue (pKa of aspartic acid = 3.86 and of glutamine acid = 4.25) which in turn caused a change in structure of the CDR loops, or allowed better access of antigen.

From comparison of the sequences of B72.3 (ref. 13) and EU (refs. 4 and 5), it was clear that, in going from the mouse to human frameworks, only two positions had been changed in such a way that acidic residues had been introduced. These

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

Which of these positions might be important was determined by examining the crystal structure of the KOL antibody. In KOL heavy chain, position 81 is far removed from either of the CDR loops.

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

iii. Framework changes in B72.3 gH gene

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

iv. Other framework changes

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

v. Other

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

Comparison of the B72.3 murine and EU heavy chain sequences reveals that the mouse and human residues are identical at positions 23, 24, 71 and 78.
Thus the mutated CDR-grafted B72.3 heavy chain corresponds to a preferred embodiment of the present invention.

EXAMPLE 4

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

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

The human EU framework was used as the acceptor framework for both heavy and light chains. The CDR-grafted antibody currently of choice is provided by co-expression of grafted light chain gL221A and grafted heavy chain gH341D which has a binding affinity for ICAM 1 of about 75% of that of the corresponding mouse-human chimeric antibody.

LIGHT CHAIN

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

HEAVY CHAIN

gH341D has murine CDRs at positions 26-35 (CDR1), 50-56 (CDR2) and94-100B (CDR3). In addition murine residues were used in gH341D at positions 24, 48, 69, 71, 73, 80, 88 and 91. Comparison of the murine anti-ICAM 1 and human EU heavy chain amino acid sequences are identical at positions 23, 49 and 78.

EXAMPLE 5

CDR-Grafting of murine anti-TNFα antibodies

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

61E71

A similar analysis as described above (Example 1, Section 12.1.) was done for 61E71 and for the heavy chain 10 residues were identified at 23, 24, 48, 49, 68, 69, 71, 73, 75 and 88 as residues to potentially retain as murine. The human frameworks chosen for CDR-grafting of this antibody, and the hTNF3 and 101.4 antibodies were RE1 for the light chain and KOL for the heavy chain.
Three genes were built, the first of which contained 23, 24, 48, 49, 71 and 73 [gH341(6)] as murine residues. The second gene also had 75 and 88 as murine residues [gH341(8)] while the third gene additionally had 68, 69, 75 and 88 as murine residues [gH341(10)]. Each was co-expressed with gL221, the minimum grafted light chain (CDRs only). The gL221/gH341(6) and gL221/gH341(8) antibodies both bound as well to TNF as murine 61E71. The gL221/gH341(10) antibody did not express and this combination was not taken further.
Subsequently the gL221/gH341(6) antibody was assessed in an L929 cell competition assay in which the antibody competes against the TNF receptor on L929 cells for binding to TNF in solution. In this assay the gL221/gH341(6) antibody was approximately 10% as active as murine 61E71.

hTNF1

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

Heavy Chain

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

Light Chain

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

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

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

hTNF3

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

101.4

101.4 is a further murine monoclonal antibody able to recognise human TNF-a. The heavy chain of this antibody shows good homology to KOL and so the CDR-grafting has been based on RE1 for the light chain and KOL for the heavy chain. Several grafted heavy chain genes have been constructed with conservative choices for the CDR's (gH341) and which have one or a small number of non-CDR residues at positions 73, 78 or 77-79 inclusive, as the mouse amino acids. These have been co-expressed with cL or gL221. In all cases binding to TNF equivalent to the chimeric antibody is seen and when co-expressed with cL the resultant antibodies are able to compete well in the L929 assay. However, with gL221 the resultant antibodies

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

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

A number of other antibodies including antibodies having specificity for interleukins e.g. IL1 and cancer markers such as carcinoembryonic antigen (CEA) e.g. the monoclonal antibody A5B7 (ref. 21), have been successfully CDR-grafted according to the present invention.
It will be appreciated that the foregoing examples are given by way of illustration only and are not intended to limit the scope of the claimed invention. Changes and modifications may be made to the methods described whilst still falling within the spirit and scope of the invention.

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CLAIMS

1. A CDR-grafted antibody heavy chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 6, 23 and/or 24, 48 and/or 49, 71 and/or 73, 75 and/or 76 and/or 78 and 88 and/or 91.

2. A CDR-grafted heavy chain according to Claim 1 comprising donor residues at positions 23, 24, 49, 71, 73 and 78, or at positions 23, 24 and 49.

3. A CDR-grafted heavy chain according to Claim 2 comprising donor residues at positions 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.

4. A CDR-grafted heavy chain according to Claim 2 or 3, comprising donor residues at one, some or all of positions:
1 and 3,
69 (if 48 is different between donor and acceptor),
38 and 46 (if 48 is the donor residue),
67,
82 and 18 (if 67 is the donor residue),
91, and
any one or more of 9, 11, 41, 87, 108, 110 and 112.

5. A CDR-grafted heavy chain according to any of the preceding comprising donor CDRs at positions 26-35, 50-65 and 95-100.

6. A CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 1 and/or 3 and 46 and/or 47.

7. A CDR-grafted light chain according to Claim 6 comprising donor residues at positions 46 and 47.

8. A CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 46, 48, 58 and 71.

9. A CDR-grafted light chain according to Claim 8 comprising donor residues at positions 46, 48, 58 and 71.

10. A CDR-grafted light chain according to Claim 8 or 9, comprising donor residues at positions 2, 4, 6, 35, 36, 38, 44, 47, 49, 62, 64-69, 85, 87, 98, 99, 101 and 102.

11. A CDR-grafted light chain according to Claim 9 or 10, comprising donor residues at one, some or all of positions:
1 and 3,
63,
60 (if 60 and 54 are able to form a potential saltbridge),
70 (if 70 and 24 are able to form a potential saltbridge),
73 and 21 (if 47 is different between donor and acceptor),
37 and 45 (if 47 if different between donor and acceptor), and
any one or more of 10, 12, 40, 83, 103 and 105.

12. A CDR-grafted light chain according to any one of Claims 6-11, comprising donor CDRs at positions 24-34, 50-56 and 89-97.

13. A CDR-grafted antibody molecule comprising at least one CDR-grafted heavy chain according to any one of Claims 1-5 and at least one CDR-grafted light chain according to any one of Claims 6-12.

14. A CDR-grafted antibody molecule according to Claim 13, which is a site-specific antibody molecule.

15. A CDR-grafted antibody molecule according to Claim 13 which has specificity for an interleukin, hormone or other biologically active compound or a receptor therefor.

16. A CDR-grafted antibody heavy or light chain or molecule according to any one of the preceding claims comprising human acceptor residues and non-human donor residues.

17. A DNA sequence which codes for a CDR-grafted heavy chain according to Claim 1 or a CDR-grafted light chain according to Claim 6 or Claim 8.

18. A cloning or expression vector containing a DNA sequence according to Claim 17.

19. A host cell transformed with a DNA sequence according to Claim 17.

20. A process for the production of a CDR-grafted antibody sequence according to Claim 17 in a transformed host cell.

21. A process for producing a CDR-grafted antibody product comprising:

(a) producing in an expression vector an operon having a DNA sequence which encodes an antibody heavy chain according to Claim 1;

and/or

(b) producing in an expression vector an operon having a DNA sequence which encodes a complementary antibody light chain according to Claim 6 or Claim 8;

(c) transfecting a host cell with the or each vector; and

(d) culturing the transfected cell line to produce the CDR-grafted antibody product.

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

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

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(57) Abstract

CDR-grafted antibody heavy and light chains comprise acceptor framework and donor antigen binding regions, the heavy chains comprising donor residues at at least one of positions (6, 23) and/or (24, 48) and/or (49, 71) and/or (73, 75) and/or (76) and/or (78) and (88) and/or (91). The CDR-grafted light chains comprise donor residues at at least one of positions (1) and/or (3) and (46) and/or (47) or at at least one of positons (46, 48, 58) and (71). The CDR-grafted antibodies are preferably humanised antibodies, having non human, e.g. rodent, donor and human acceptor frameworks, and may be used for *in vivo* therapy and diagnosis. A generally applicable protocol is disclosed for obtaining CDR-grafted antibodies.

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```
  1  GAATTCCCAA AGACAAAatg gattttcaag tgcagatttt cagcttcctg
 51  ctaatcagtg cctcagtcat aatatccaga ggacaaattg ttctcaccca
101  gtctccagca atcatgtctg catctccagg ggagaaggtc accatgacct
151  gcagtgccag ctcaagtgta agttacatga actggtacca gcagaagtca
201  ggcacctccc ccaaaagatg gatttatgac acatccaaac tggcttctgg
251  agtccctgct cacttcaggg gcagtgggtc tgggacctct tactctctca
301  caatcagcgg catggaggct gaagatgctg ccacttatta ctgccagcag
351  tggagtagta  accattcac gttcggctcg gggacaaagt tggaaataaa
401  ccgggctgat actgcaccaa ctgtatccat cttcccacca tccagtgagc
451  agttaacatc tggaggtgcc tcagtcgtgt gcttcttgaa caacttctac
501  cccaaagaca tcaatgtcaa gtggaagatt gatggcagtg aacgacaaaa
551  tggcgtcctg aacagttgga ctgatcagga cagcaaagac agcacctaca
601  gcatgagcag caccctcacg ttgaccaagg acgagtatga acgacataac
651  agctatacct gtgaggccac tcacaagaca tcaacttcac ccattgtcaa
701  gagcttcaac aggaatgagt gtTAGAGACA AAGGTCCTGA GACGCCACCA
751  CCAGCTCCCA GCTCCATCCT ATCTTCCCTT CTAAGGTCTT GGAGGCTTCC
801  CCACAAGCGC tTACCACTGT TGCGGTGCTC TAAACCTCCT CCCACCTCCT
851  TCTCCTCCTC CTCCCTTTCC TTGGCTTTTA TCATGCTAAT ATTTGCAGAA
901  AATATTCAAT AAAGTGAGTC TTTGCCTTGA AAAAAAAAAA AAA
(SEQ ID NO:4)
```

FIG. 1a

```
  1  MDFQVQIFSF LLISASVIIS RGQQIVLTQSP AIMSASPGEK VTMTCSASSS
 51  VSYMNWYQQK SGTSPKRWIY DTSKLASGVP AHFRGSGSGT  SYSLTISGME
101  AEDAATYYCQ QWSSNPFTFG SGTKLEINRA DTAPTVSIFP  PSSEQLTSGG
151  ASVVCFLNNF YPKDINVKWK IDGSERQNGV LNSWTDQDSK  DSTYSMSSTL
201  TLTKDEYERH NSYTCEATHK TSTSPIVKSF NRNEC* (SEQ ID NO:5)
```

FIG. 1b

```
   1  GAATTCCCCT CTCCACAGAC ACTGAAAACT CTGACTCAAC ATGGAAAGGC
  51  ACTGGATCTT TCTACTCCTG TTGTCAGTAA CTGCAGGTGT CCACTCCCAG
 101  GTCCAGCTGC AGCAGTCTGG GGCTGAACTG GCAAGACCTG GGGCCTCAGT
 151  GAAGATGTCC TGCAAGGCTT CTGGCTACAC CTTTACTAGG TACACGATGC
 201  ACTGGGTAAA ACAGAGGCCT GGACAGGGTC TGGAATGGAT TGGATACATT
 251  ATTCCTAGCC GTGGTTATAC TAATTACAAT CAGAAGTTCA AGGACAAGGC
 301  CACATTGACT ACAGACAAAT CCTCCAGCAC AGCCTACATG CAACTGAGCA
 351  GCCTGACATC TGAGGACTCT GCAGTCTATT ACTGTGCAAG ATATTATGAT
 401  GATCATTACT GCCTTGACTA CTGGGGCCAA GGCACCACTC TCACAGTCTC
 451  CTCAGCCAAA ACAACAGCCC CATCGGTCTA TCCACTGGCC CCTGTGTGTG
 501  GAGATACAAC TGGCTCCTCG GTGACTCTAG GATGCCTGGT CAAGGGTTAT
 551  TTCCCTGAGC CAGTGACCTT GACCTGGAAC TCTGGATCCC TGTCCAGTGG
 601  TGTGCACACC TTCCCAGCTG TCCTGCAGTC TGACCTCTAC ACCCTCAGCA
 651  GCTCAGTGAC TGTAACCTCG AGCACCTGGC CCAGCCAGTC CATCACCTGC
 701  AATGTGGCCC ACCCGGCAAG CAGCACCAAG GTGGACAAGA AAATTGAGCC
 801  ACCTCTTGGG TGGACCATCC GTCTTCATCT TCCCTCCAAA GATCAAGGAT
 851  GTACTCATGA TCTCCCTGAG CCCCATAGTC ACATGTGTGG TGGTGGATGT
 901  GAGCGAGGAT GACCCAGATG TCCAGATCAG CTGGTTTGTG AACAACGTGG
 951  AAGTACACAC AGCTCAGACA CAAACCCATA GAGAGGATTA CAACAGTACT
1001  CTCCGGGTGG TCAGTGCCCT CCCCATCCAG CACCAGGACT GGATGAGTGG
1051  CAAGGAGTTC AAATGCAAGG TCAACAACAA AGACCTCCCA GCGCCCATCG
1101  AGAGAACCAT CTCAAAACCC AAAGGGTCAG TAAGAGCTCC ACAGGTATAT
1151  GTCTTGCCTC CACCAGAAGA AGAGATGACT AAGAAACAGG TCACTCTGAC
1201  CTGCATGGTC ACAGACTTCA TGCCTGAAGA CATTTACGTG GAGTGGACCA
1251  ACAACGGGAA AACAGAGCTA AACTACAAGA ACACTGAACC AGTCCTGGAC
1301  TCTGATGGTT CTTACTTCAT GTACAGCAAG CTGAGAGTGG AAAAGAAGAA
1351  CTGGGTGGAA AGAAATAGCT ACTCCTGTTC AGTGGTCCAC GAGGGTCTGC
1401  ACAATCACCA CACGACTAAG AGCTTCTCCC GGACTCCGGG TAAATGAGCT
1451  CAGCACCCAC AAAACTCTCA GGTCCAAAGA GAGACCCACA CTCATCTCCA
1501  TGCTTCCCTT GTATAAATAA AGCACCCAGC AATGCCTGGG ACCATGTAAA
1551  AAAAAAAAAA AAAGGAATTC  (SEQ ID NO:6)
```

FIG. 2a

OKT 3 HEAVY CHAIN PROTEIN SEQUENCE DEDUCED FROM DNA SEQUENCE

```
  1  MERHWIFLLL LSVTAGVHSQ VQLQQSGAEL ARPGASVKMS CKASGYTFTR
 51  YTMHWVKQRP GQGLEWIGYI NPSRGYTNYN QKFKDKATLT TDKSSSTAYM
101  QLSSLTSEDS AVYYCARYYD DHYCLDYWGQ GTTLTVSSAK TTAPSVYPLA
151  PVCGDTTGSS VTLGCLVKGY FPEPVTLTWN SGSLSSGVHT FPAVLQSDLY
201  TLSSSVTVTS STWPSQSITC NVAHPASSTK VDKKIEPRGP TIKPCPPCKC
251  PAPNLLGGPS VFIFPPKIKD VLMISLSPIV TCVVVDVSED DPDVQISWFV
301  NNVEVHTAQT QTHREDYNST LRVVSALPIQ HQDWMSGKEF KCKVNNKDLP
351  APIERTISKP KGSVRAPQVY VLPPPEEEMT KKQVTLTCMV TDFMPEDIYV
401  EWTNNGKTEL NYKNTEPVLD SDGSYFMYSK LRVEKKNWVE RNSYSCSVVH
451  EGLHNHHTTK SFSRTPGK*  (SEQ ID NO:7)
```

FIG. 2b

```
                1                       23                      42
                NN      N                 N         N    N
RES TYPE        SBspSPESssBSbSsSssPSPSPsPSsse*s*p*Pi^ISsSe
Okt3vl          QIVLTQSPAIMSASPGEKVTMTCSASS.SVSYMNWYQQKSGT
REI             DIQMTQSPSSLSASVGDRVTITCQASQDIIKYLNWYQQIPGK
                ? ?
                  CDR1    (LOOP)          *******
                  CDR1    (KABAT)        **********


                          56                                    85
                N  NN
RES TYPE        *IsiPpIeesesssSBEsePsPSBSSEsPspsPsseesSPePb
Okt3vl          SPKRWIYDTSKLASGVPAHFRGSGSGTSYSLTISGMEAEDAAT
REI             APKLLIYEASNLQAGVPSRFSGSGSGTDYTFTISSLQPEDIAT (SEQ
ID NO:8)
                ?  ??                              ?  ?
                       *******  CDR2 (LOOP/KABAT)

                              102   108
RES TYPE        PiPIPies**iPIIsPPSPSPSS
Okt3vl          YYCQQWSSNPFTFGSGTKLEINR (SEQ ID NO:29)
REIvl           YYCQQYQSLPYTFGQGTKLQITR (SEQ ID NO:9)
                              ?    ?
                    ******       CDR3 (LOOP)
                  *********      CRD3(KABAT)
```

FIG. 3

```
           NN N                    23 26    32 35  N39  43
RES TYPE   SESPs^SBssS^sSSsSpSpSPsPSEbSBssBePi^PIpiesss
Okt3h      QVQLQQSGAELARPGASVKMSCKASGYTFTRYTMNHWVKQRPGQ
KOL        QVQLVESGGGVVQPGRSLRLSCSSSGFIFSSYAMYWVRQAPGK
                ?                  ??
                                          ******     CDR1 (LOOP)
                                              *****  CDR1 (KABAT)

                      52a   60     65    N N  N   82abc     89
RES TYPE   IIeIppp^sssssssss^ps^pSSsbSpseSsSseSp^pSpsSBssS^ePb
Okt3vh     GLEWIGYINPSRGYTNTNQKFKRKATLTTDKSSSTAYMQLSSLTSEDSAV
KOL        GLEWVAIIWDDGSDQHYADSVKGRFTISRDNSKNTLFLQMDSLPPEDTGV
              ??                       ? ?  ?  ?             ?
                   ***********          CDR2   (LOOP)
                   ********************  CDR2   (KABAT)


            92 N                   107    113
RES TYPE   PiPIEissssiiisssbibi*EIPIP*spSBSS
Okt3vh     YYCARYYDDHY.......CLDYWGQGTTLTVSS   (SEQ ID NO:30)
KOL        YFCARDGGHGFCSSASCFGPDYWGQGTPVTVSS   (SEQ ID NO:10)
                ****************  CRD4 (KABAT/LOOP)
```

FIG. 4

OKT 3 HEAVY CHAIN CDR GRAFTS

1. gh341 and derivatives

	1 26 35 39 43	
Okt3vh	QVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPGQ	
gH341	QVQLVESGGGVVQOGRSLRLSCSSSGYTFTRYTMHWVRQAPGK	JA178
gH341A	QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK	JA185
gH341E	QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK	JA198
gH341*	QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK	JA207
gH341*	QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK	JA209
gH341D	QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK	JA197
gH341*	QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK	JA199
gH341C	QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK	JA184
gH341*	QVQLVQSGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK	JA203
gH341*	QVQLVESGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK	JA205
gH341B	QVQLVESGGGVVQPGRSLRLSCSSSGYTFTRYTMHWVRQAPGK	JA183
gH341*	QVQLVQSGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK	JA204
gH341*	QVQLVESGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK	JA206
gH341*	QVQLVQSGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK	JA208
KOL	QVQLVESGGGVVQPGRSLRLSCSSSGFIFSSYAMYWVRQAPGK	

FIG. 5a

```
          44    50              65                  83
Okt3vh    GLEWIGYINPSRGYTNYNQKFKDKATLTTDKSSSTAYMQLSSLT
gH341     GLEWVAYINPSRGYTNYNQKFKDRFTISRDNSKNTLFLQMDSLR   JA178
gH341A    GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKSTAFLQMDSLR   JA185

gH341E    GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKSTAFLQMDSLR   JA198
gH341*    GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKNTAFLQMDSLR   JA207
gH341*    GLEWIGYINPSRGYTNYNQKVKDRFTISRDNSKNTAFLQMDSLR   JA209
gH341D    GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKNTLFLQMDSLR   JA197
gH341*    GLEWIGYINPSRGYTNYNQKVKDRFTISRDNSKNTLFLQMDSLR   JA199
gH341C    GLEWVAYINPSRGYTNYNQKFKDRFTISRDNSKNTLFLQMDSLR   JA184

gH341*    GLEWIGYINPSRGYTNYNOKVKDRFTISTDKSKSTAFLQMDSLR   JA207
gH341*    GLEWIGYINPSRGYTNYNOKVKDRFTISTDKSKSTAFLQMDSLR   JA205
gH341B    GLEWIGYINPSRGYTNYNOKVKDRFTISTDKSKSTAFLQMDSLR   JA183
gH341*    GLEWIGYINPSRGYTNYNOKVKDRFTISTDKSKSTAFLQMDSLR   JA204
gH341*    GLEWIGYINPSRGYTNYNOKVKDRFTISTDKSKSTAFLQMDSLR   JA206
gH341*    GLEWIGYINPSRGYTNYNOKVKDRFTISTDKSKNTAFLQMDSLR   JA208
KOL       GLEWVAIIWDDGSDQHYADSVKGRFTISRDNSKNTLFLQMDSLR
```

FIG. 5b

	84 95 102 113		SEQ ID NO:
Okt3vh	SEDSAVYYCARYYDDHY.....CLDYWGQGTTLTVSS		30
gH341	PEDTGVYFCARYYDDHY.....CLDYWGQGTTLTVSS	JA178	11
gH341A	PEDTAVYYCARYYDDHY.....CLDYWGQGTTLTVSS	JA185	12
gH341E	PEDTGVYFCARYYDDHY.....CLDYWGQGTTLTVSS	JA198	13
gH341*	PEDTGVYFCARYYDDHY.....CLDYWGQGTTLTVSS	JA207	14
gH341D	PEDTGVYFCARYYDDHY.....CLDYWGQGTTLTVSS	JA197	15
gH341*	PEDTGVYFCARYYDDHY.....CLDYWGQGTTLTVSS	JA209	16
gH341*	PEDTGVYFCARYYDDHY.....CLDYWGQGTTLTVSS	JA199	17
gH341C	PEDTGVYFCARYYDDHY.....CLDYWGQGTTLTVSS	JA184	18
gH341*	PEDTAVYYCARYYDDHY.....CLDYWGQGTTLTVSS	JA203	19
gH341*	PEDTAVYYCARYYDDHY.....CLDYWGQGTTLTVSS	JA205	20
gH341B	PEDTAVYYCARYYDDHY.....CLDYWGQGTTLTVSS	JA183	21
gH341*	PEDTGVYFCARYYDDHY.....CLDYWGQGTTLTVSS	JA204	22
gH341*	PEDTGVYFCARYYDDHY.....CLDYWGQGTTLTVSS	JA206	23
gH341*	PEDTGVYFCARYYDDHY.....CLDYWGQGTTLTVSS	JA208	24
KOL	PEDTGVYFCARDGGHGFCSSASCFGPDYWGQGTPVTVSS		10

FIG. 5c

OKT3 LIGHT CHAIN CDR GRAFTING

1. gL221 and derivatives

```
          1                        24        34      42
Okt3vl    QIVLTQSPAOMSASPGEKVTMTCSASS.SVSYMNWYQQKSGT
gL221     DIQMTQSPSSLSASVGDRVTITCSASS.SVSYMNWYQQTPGK
gL221A    QIVMTQSPSSLSASVGDRVTITCSASS.SVSYMNWYQQTPGK
gL221B    QIVMTQSPSSLSASVGDRVTITCSASS.SVSYMNWYQQTPGK
gL221C    DIQMTQSPSSLSASVGDRVTITCSASS.SVSYMNWYQQTPGK
REI       DIQMTQSPSSLSASVGDRVTITCQASQDIIKYLNWYQQTPGK

          43     50    56                            85
Okt3vl    SPKRWIYDTSKLASGVPAHFRGSGSGTSYSLTISGMEAEDAAT
gL221     APKLLIYDTSKLASGVPSRFSGSGSGTDYTFTISSLQPEDIAT
gL221A    APKRWIYDTSKLASGVPSRFSGSGSGTDYTFTISSLQPEDIAT
gL221B    APKLLIYDTSKLASGVPSRFSGSGSGTDYTFTISSLQPEDIAT
gL221C    APKRWIYDTSKLASGVPSRFSGSGSGTDYTFTISSLQPEDIAT
REI       APKLLIYEASNLQAGVPSRFSGSGSGTDYTFTISSLQPEDIAT (SEQ ID NO:8)

          86   91   96          108
Okt3vl    YYCQQWSSNPFTFGSGTKLEINR     (SEQ ID NO:29)
gL221     YYCQQWSSNPETFGQGTKLQITR     (SEQ ID NO:25)
gL221A    YYCQQWSSNPETFGQGTKLQITR     (SEQ ID NO:26)
gL221B    YYCQQWSSNPETFGQGTKLQITR     (SEQ ID NO:27)
gL221C    YYCQQWSSNPETFGQGTKLQITR     (SEQ ID NO:28)
REI       YYCQQYQSLPYTFGQGTKLQITR     (SEQ ID NO:9)
```

CDR'S ARE UNDERLINED

FRAMEWORK RESIDUES INCLUDED IN THE GENE ARE DOUBLE UNDERLINED

FIG. 6

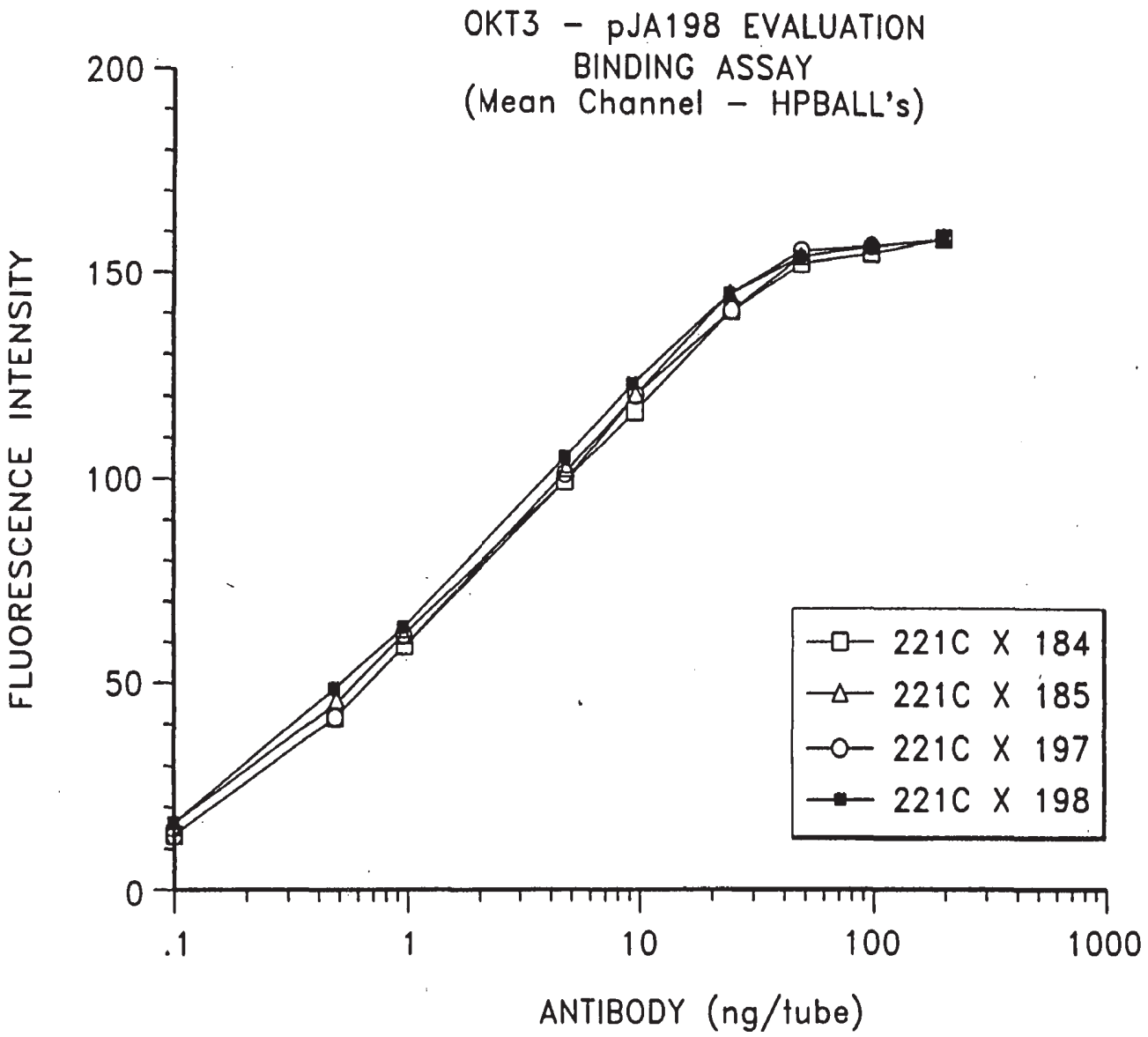


FIG. 7

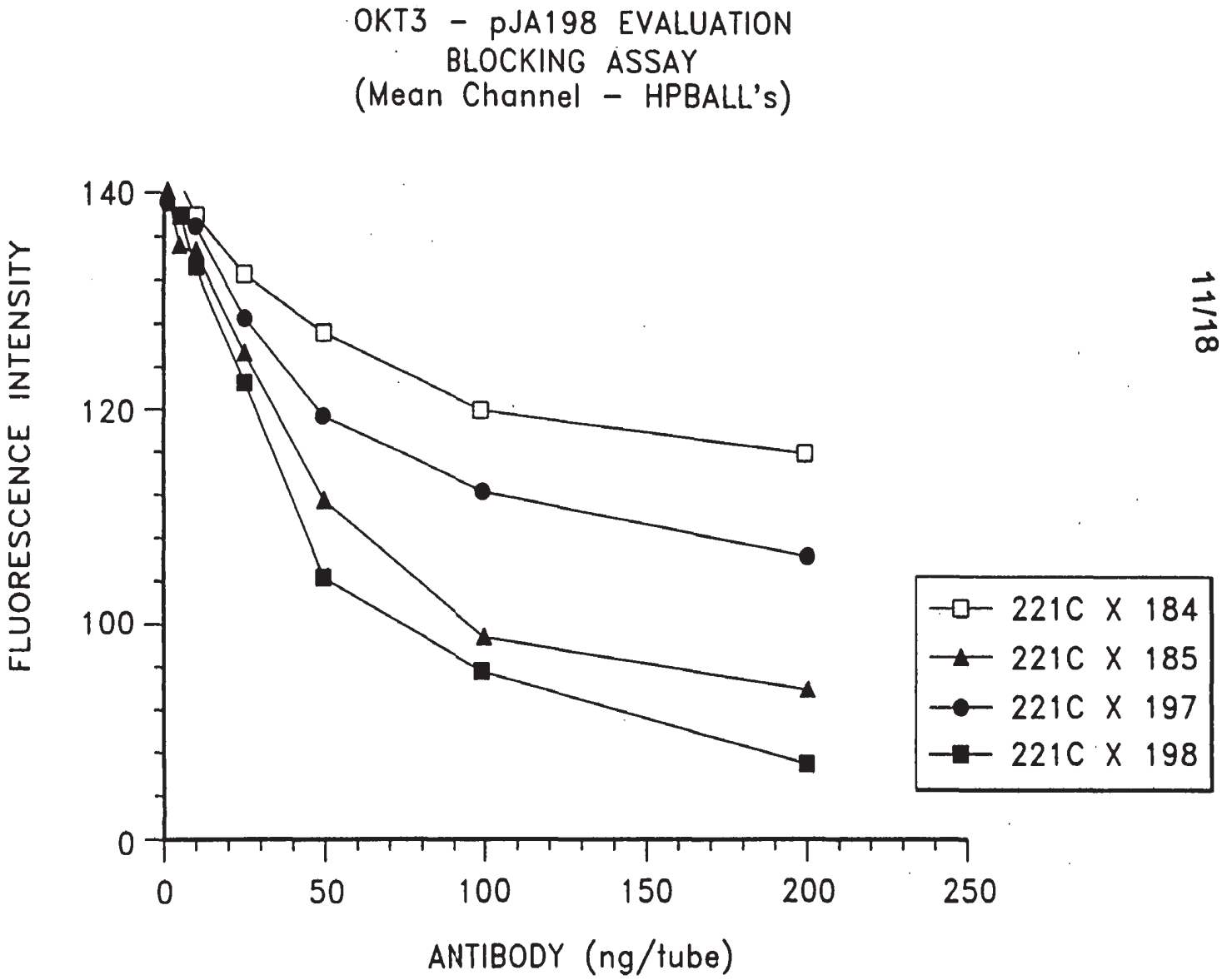


FIG. 8

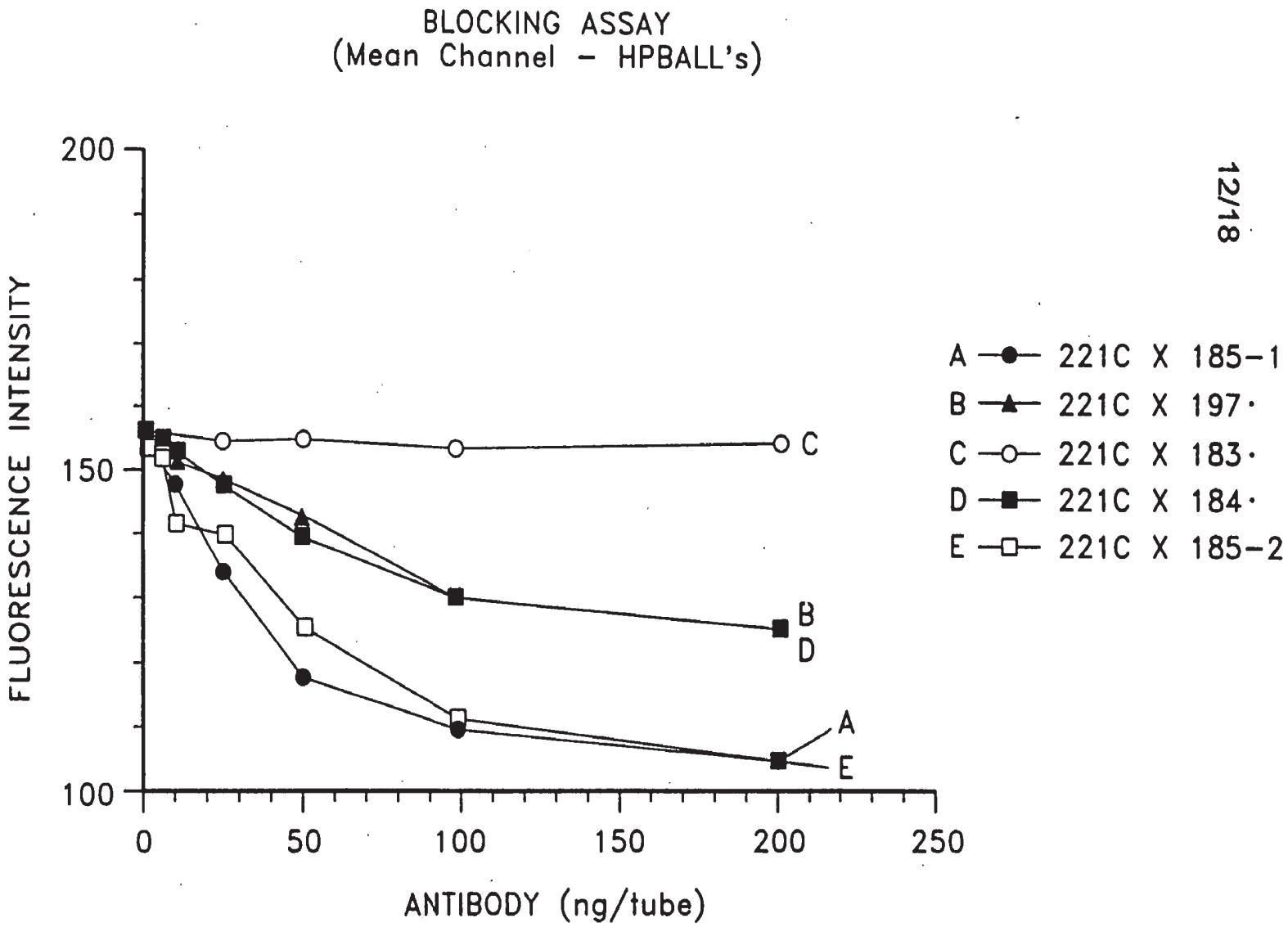
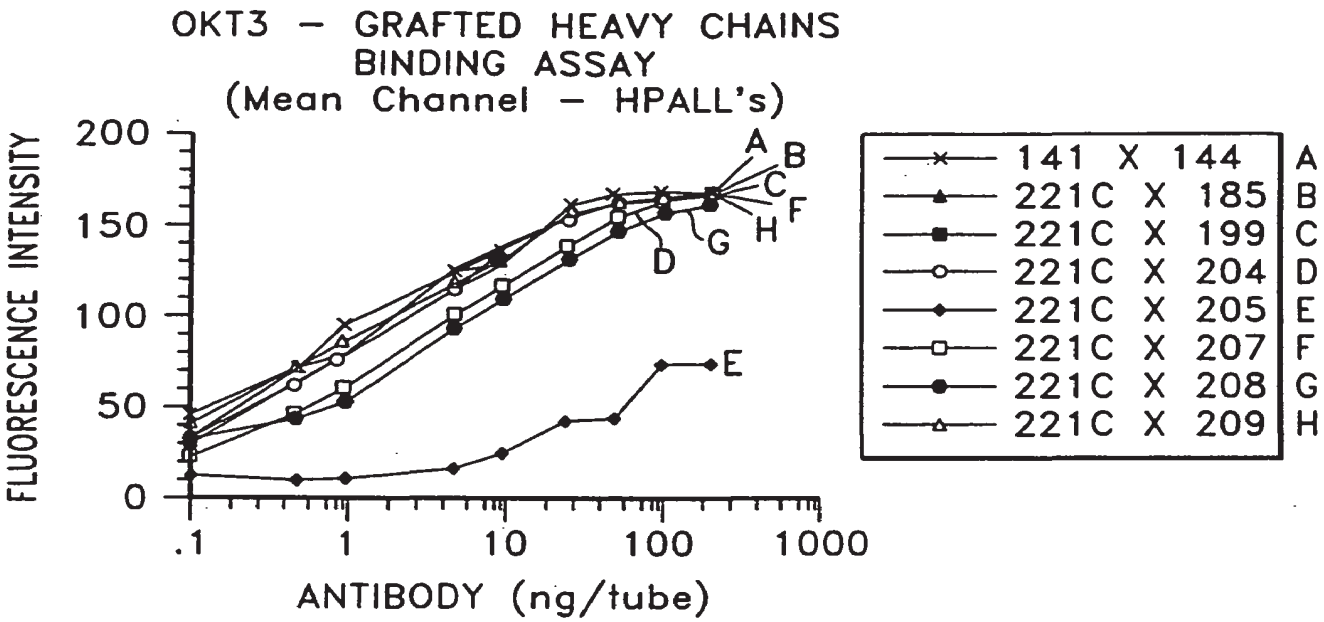


FIG. 9



(205)	_,__,24,48,49,71,73,76,78,88,91,
(208)	6,__,24,48,49,71,73,__,78,__,__,
(204)	6,__,24,48,49,71,73,76,78,__,__,
(199)	6,23,24,48,49,__,__,__,__,__,__,
(207)	6,23,24,48,49,71,73,__,78,__,__,
(185)	6,23,24,48,49,71,73,76,78,88,91,
(209)	6,23,24,48,49,__,__,__,78,__,__,
141 X 144	

FIG. 10a

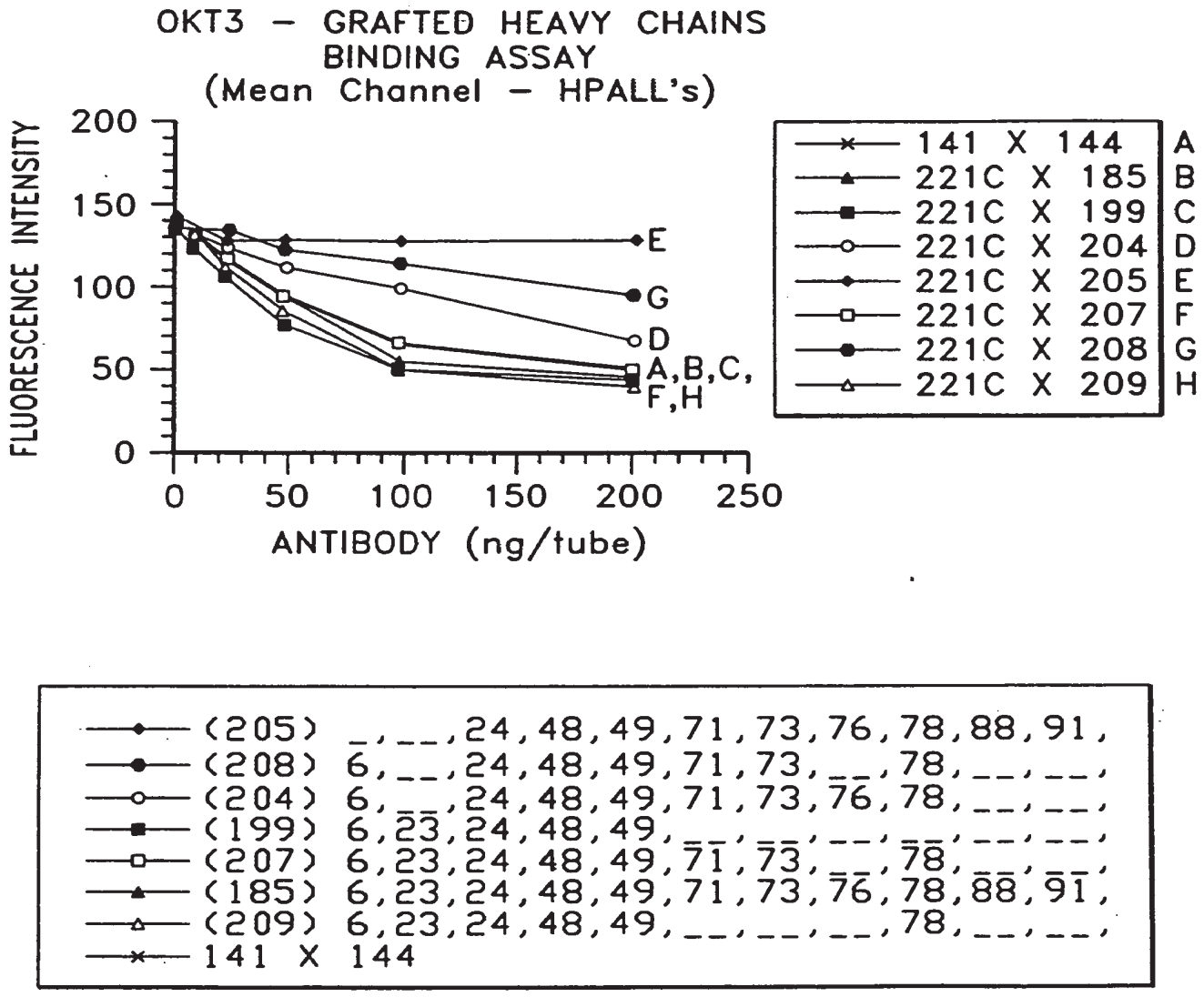


FIG. 10b

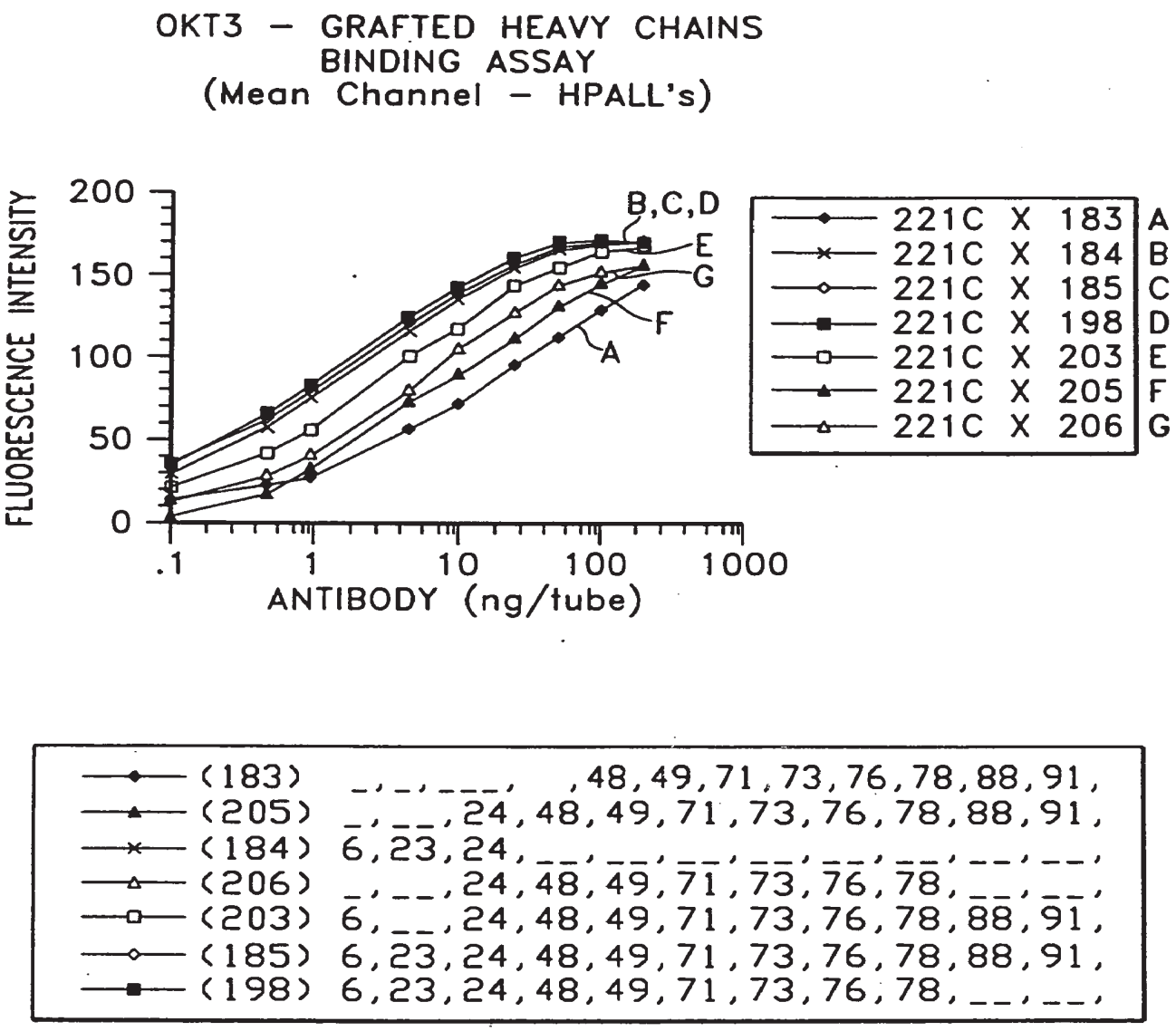


FIG. 11a

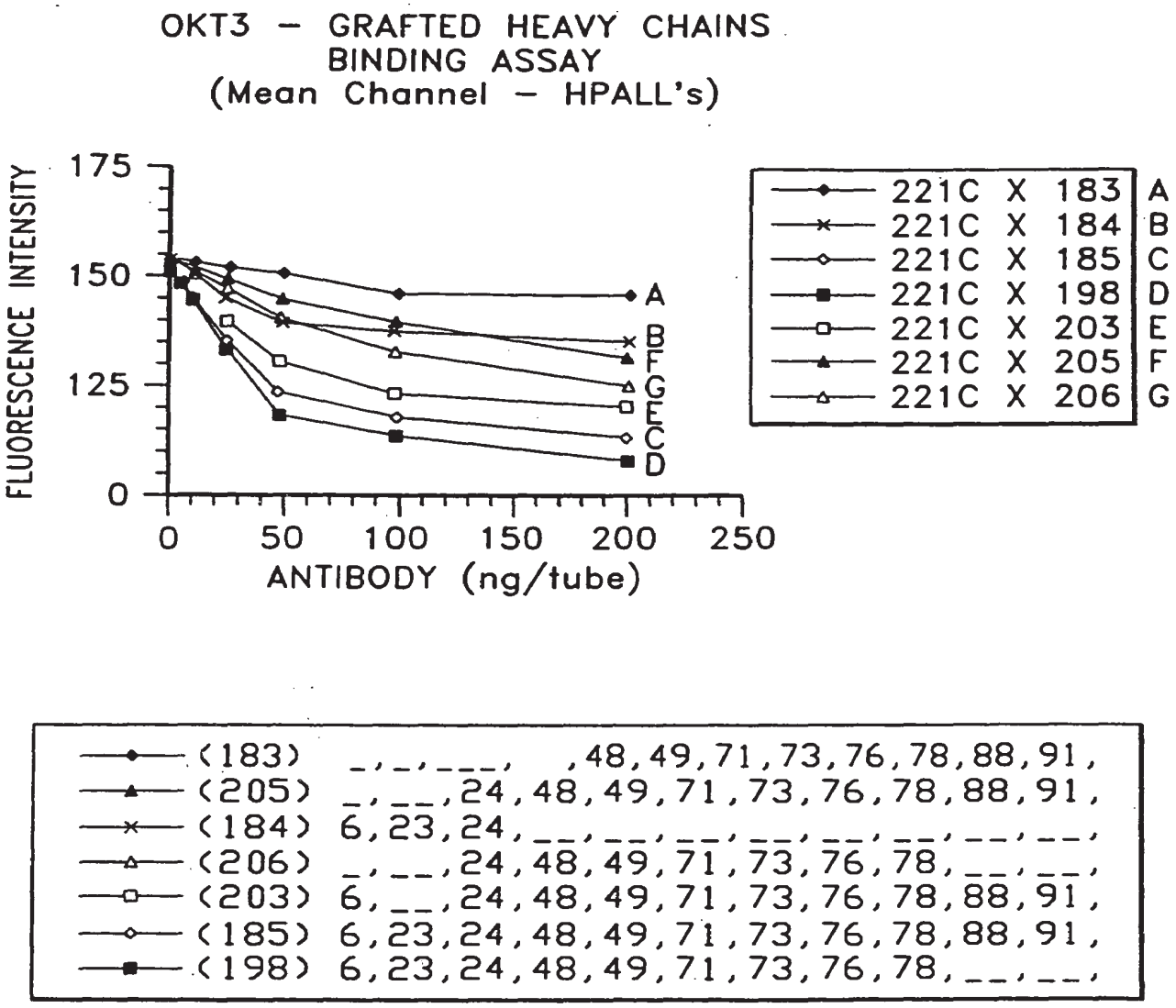


FIG. 11b

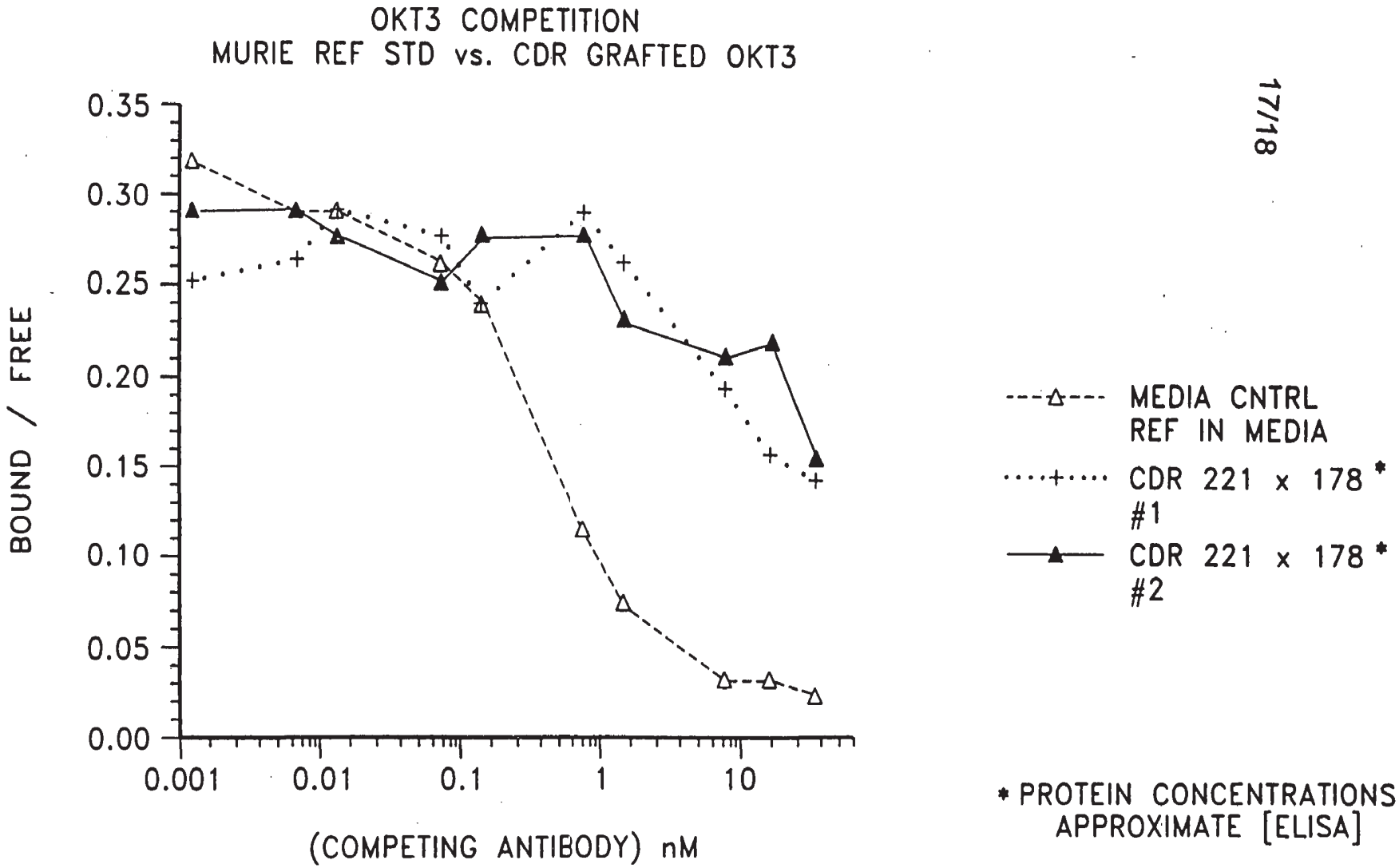


FIG. 12

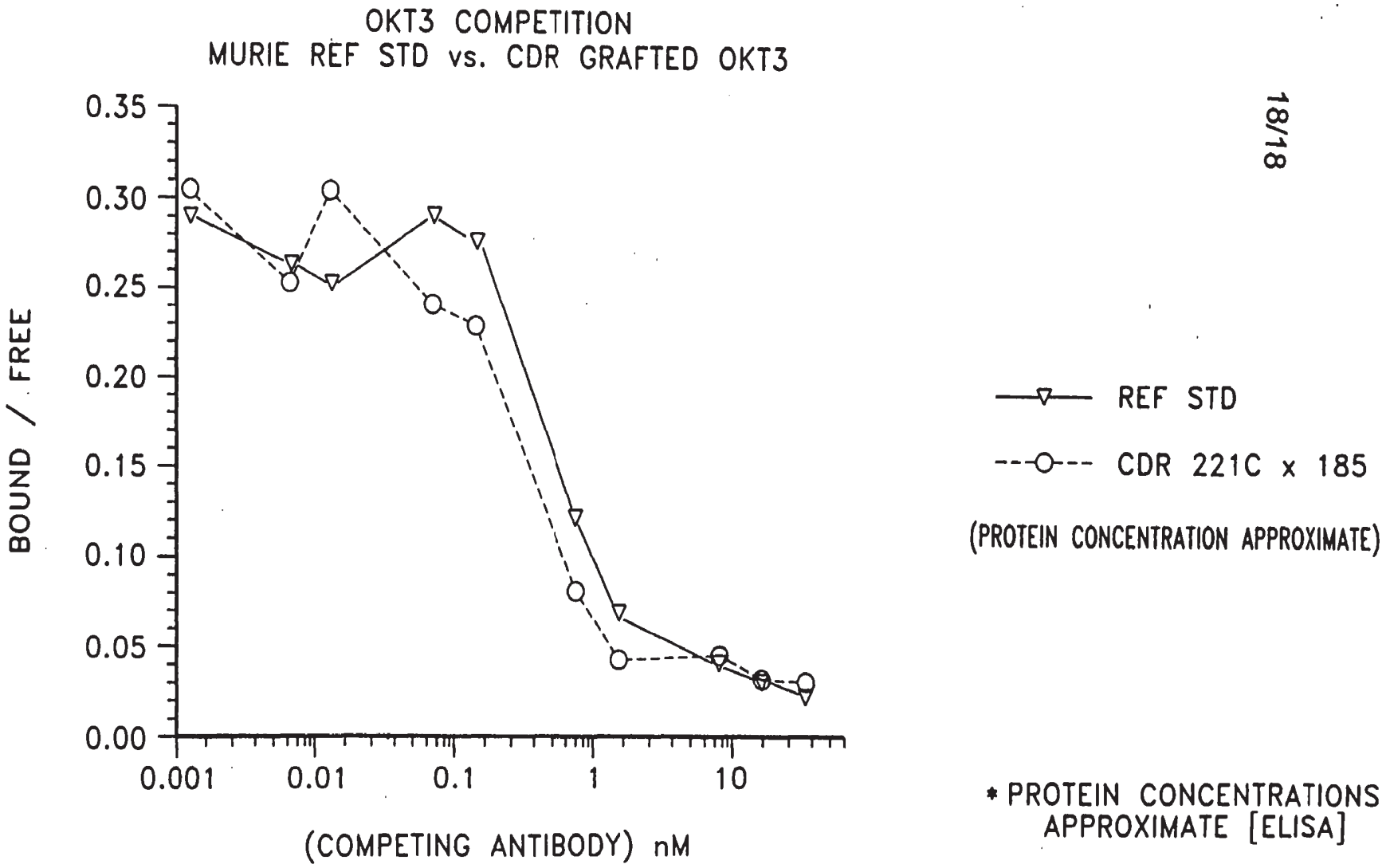


FIG. 13

DOCKET NO.: CARP-0009 PATENT

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

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

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

___ is attached hereto.

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

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

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

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

Country	Number	Date Filed	Priority Claimed
U.K.	8928874.0	21.12.89	yes
			___
			___

PTO-19

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

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

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

Francis A. Paintin

Registration Nos. 19,386

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

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One Liberty Place - 46th Floor
Philadelphia, PA 19103
Telephone No. **215-568-3100.**

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

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

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4	Full Name	Inventor's Signature	Date
	Residence	Citizenship	
	Post Office Address		
5	Full Name	Inventor's Signature	Date
	Residence	Citizenship	
	Post Office Address		

DOCKET NO.: CARP0001-112 **PATENT**
PRELIMINARY AMENDMENT AND REQUEST FOR INTERFERENCE UNDER 37 C.F.R. § 42.202 DATED NOVEMBER 21, 2005

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: John R. Adair, Diljeet S. Athwal and John S. Emtage

Serial No.: Not Yet Assigned **Art Unit: Not Yet Assigned**

Filing Date: November 21, 2005 **Examiner: Not Yet Assigned**

For: HUMANISED ANTIBODIES

Customer No.: 34132

EXPRESS MAIL LABEL NO.: EV146 601 565US
DATE OF DEPOSIT: November 21, 2005

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

PRELIMINARY AMENDMENT
AND
REQUEST FOR INTERFERENCE UNDER 37 CFR § 42.202

Applicant respectfully requests entry of the following amendments prior to the calculation of filing fees:

Amendments To The Specification begin on page 2 of this paper.

Listing of the Claims, reflecting current amendments, begins on page 3 of this paper.

Remarks begin on page 4 of this paper.

The **Conclusion** is found on page 12.

Appendix A is found on page 14.

Appendix B is found on page 17.

Appendix C is found on page 19.

Appendix D is found on page 20.

Appendix E is found on page 21.

Carter Exhibit 2003
Carter v. Adair
Interference No. 105,744

AMENDMENTS TO THE SPECIFICATION

Please replace the paragraph containing the cross-reference to related applications on page 1 of the specification with the following:

This application is a Continuation of U.S. Application Serial No. 08/846,658, Filed May 1, 1997, which is a Continuation of U.S. Application Serial No. 08/303,569, Filed September 7, 1994, now U.S. Patent No. 5,859,205, Issued January 12, 1999, which is a File Wrapper Continuation of U.S. Application Serial No. 07/743,329, Filed September 17, 1991, Abandoned, which is a 35 U.S.C. 371 National Stage Application of International Application No. PCT/GB90/02017, International Filing Date of December 21, 1990, which claims priority to Great Britain Application No. 8928874.0, Filed December 21, 1989, all applications are incorporated by reference herein in their entireties.

LISTING OF CLAIMS

This listing of claims represents the current status of the claims.

Claims 1-23 **(cancelled)**

Claim 24 **(new)** A humanised antibody heavy chain variable domain comprising non-human complementarity determining region amino acid residues which bind an antigen and a human framework region wherein said framework region comprises an amino acid substitution at a residue selected from the group consisting of 23, 24, 49, 71, 73, and 78, and combinations thereof, as numbered according to Kabat.

Claim 25 **(new)** A humanised antibody comprising the variable domain of claim 24.

REMARKS

Claims 24 to 25 are pending. Applicants hereby request an interference in accordance with 37 CFR § 42.202. It is noted that U.S. Patent No. 6,407,213, whose claims present the basis for an interference, is classified in Class 530, and was examined by Examiners Minh-Tam Davis and Anthony C. Caputa.

Compliance with 37 CFR § 42.202

Applicants respectfully submit that all requirements of 37 CFR § 42.202 have been met and respectfully request examination of the present application and declaration of an interference.

(a) Identifying The Patent

Applicants request that an interference be declared between Applicants' above-identified application and U.S. Patent No. 6,407,213 B1 (hereinafter the 213 patent), issued to Carter et al. on June 18, 2002, a copy of which is enclosed herewith.

(b) Compliance with 35 USC § 135(b)

Although the present rules do not require a showing of compliance under 35 USC § 135(b), Applicants submit the following to advance the examination of the present application to allowability. The present application is a Continuation of U.S. Application Serial No. 08/846,658, Filed May 1, 1997, which is a Continuation of U.S. Application Serial No. 08/303,569, Filed September 7, 1994, now U.S. Patent No. 5,859,205, Issued January 12, 1999, which is a File Wrapper Continuation of U.S. Application Serial No. 07/743,329, Filed September 17, 1991, Abandoned, which is a 35 U.S.C. 371 National Stage Application of International Application No. PCT/GB90/02017, International Filing Date of December 21, 1990 (hereinafter "the PCT application," a copy of which is enclosed herewith), which claims priority to Great Britain Application No. 8928874.0, Filed December 21, 1989. Claims 1-23 as filed in the PCT application are attached as Appendix A.

Under 35 USC § 135(b)(1), Applicants must show that they had a claim to the same, or substantially the same, subject mater as a claim of the 213 patent within one year of the issuance of the 213 patent, or June 18, 2003. The 213 patent issued on June 18, 2002. The PCT application was filed on December 21, 1990, over 10 years earlier than the 213 patent issued. The time limit of Section 135(b)(1) has been complied with fully. See *Corbett v. Chisholm*, 196 USPQ 337 (CCPA 1977).

To meet the "same or substantially the same invention" requirement of Section 135(b)(1), Applicants must show that their claim contained all material limitations, i.e. limitations necessary to patentability, of the claim of the 213 patent alleged to be to the same, or substantially the same, invention. *Corbett v. Chisholm*, 196 USPQ 337 (C.C.P.A. 1977), *citing Wetmore v. Miller*, 477 F.2d 960, 177 USPQ 699 (C.C.P.A. 19730).

As is evident from Appendix A, Applicants made a claim for the same, or substantially the same, subject matter as a claim of the 213 patent well before the issuance of the 213 patent. Claim 16 of the PCT application, as depending from claim 8, is to substantially the same subject matter as at least claim 1 of the 213 patent. For the Office's convenience, all three claims are duplicated below.

> **Claim 8 of the PCT application**: A CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 46, 48, **58** and 71.

> **Claim 16 of the PCT application**: A CDR-grafted antibody heavy or **light chain** or molecule according to any one of the preceding claims comprising human acceptor residues and non-human donor residues.

> **Claim 1 of the 213 patent**: A humanized antibody variable domain comprising non-human Complementarity Determining Region (CDR) amino acid residues

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

Both claim 16 of the PCT application, as it depends from claim 8, and claim 1 of the 213 patent are directed to variable domains comprising non-human Complementarity Determining Regions ("CDRs") that bind antigen, i.e., antigen binding regions, human framework regions, and a non-human framework amino acid at residue 58 of the light chain, using Kabat numbering. Both claims also comprise a human framework region; claim 1 of the 213 patent simply recites it differently -- if one incorporates non-human CDR amino acid residues into a human antibody variable domain, one ends up with a human framework region. Further, the 213 patent defines "humanized antibody" as an antibody having a framework region "having substantially the amino acid sequence of a human immunoglobulin," i.e., a human framework region (see column 8, lines 11-17, of the 213 patent). "Antigen binding regions," as recited in claim 8 of the PCT application, refers to CDRs (see page 8, lines 10-13, of the PCT application). As indicated on page 8, lines 22-24 of the PCT application, all numbering is according to Kabat. Claim 16 implicitly contains the recitation that the amino acid be substituted. As indicated on page 17, lines 1-19 of the PCT application, substitution of the non-human framework residues for the human framework residues is required when the donor and framework residue at any of the recited positions differ. While Applicants' claim recites a "light **chain**" and claim 1 of the 213 patent recites a "variable domain," Applicants submit that this is merely a difference in scope, and not a material difference. *See Corbett.*

(c) Presentation Of A Proposed Count

Applicants present in Appendix B, attached hereto, a proposed count that is presented in the "alternative" format as claim 30 or claim 80 of the 213 patent or Applicants' claim 24. All alternatives are to the same patentable invention.

All alternatives recite that the variable domain comprises non-human complementarity determining region amino acid residues which bind an antigen. All alternatives also comprise a human framework region. As discussed above for compliance with Section 135(b), the 213 patent defines "humanized antibody" as an antibody having a framework region "having substantially the amino acid sequence of a human immunoglobulin," i.e., a human framework region (see column 8, lines 11-17, of the 213 patent). And, if one incorporates non-human CDR amino acid residues into a human antibody variable domain, as recited in claims 30 and 80, one ends up with a human framework region. Further, all alternatives require only that one of the listed framework residues be substituted. Claims 30 and 80 of the 213 patent and Applicants' claim 24 recite substituting, *inter alia*, residue 78 of the heavy chain.

Although claim 30 of the 213 patent and Applicants' claim 24 do not recite the alternative recitations (a) through (c) of claim 80 of the 213 patent, e.g., "noncovalently binds antigen directly," such limitations are clearly implicit for all the framework residue substitutions recited in claim 80.

Although claims 30 and 80 of the 213 patent do not recite that the variable domain is from the light chain, it clearly must be the case when a light chain framework residue, i.e., one designated with an "L," is to be substituted.

Claim 30 of the 213 patent recites an "antibody" while the remaining alternatives recite a "variable domain." The antibody recitation is anticipated by and/or obvious over the recitation of variable domain and *vice versa*. "Humanized antibody" is defined in the 213 patent to comprise, *inter alia*, at least one variable domain (see column 8, lines 26-28, of the 213 patent).

Claim 30 of the 213 patent further recites a specific antibody target. The alternatives of the count that do not recite a particular target are clearly anticipated thereby. Considering the target, i.e., the tyrosine kinase receptor for HER2, $p185^{HER2}$, Applicants submit that claim 30 is clearly obvious over the other alternatives of the count for the reasons that follow.

The import of $p185^{HER2}$ to cancer, particularly breast cancer, had been well documented well before the priority date of the 213 patent. See, for example, Slamon, D.J. et al., *Science*, 235:177-182,1987. The development of antibodies against $p185^{HER2}$ for use in therapy had also

been well documented, as was the use of antibodies other than hybridoma-produced monoclonal antibodies. Applicants respectfully submit that, in view of the knowledge of the art at the time, a humanized antibody against $p185^{HER2}$ would have been obvious over the remaining alternatives of the count.

(d) Identification of claims corresponding to the count

Applicants identify all of the 213 patent claims, claims 1-82, and all of Applicants' pending claims, claims 24-25, as corresponding to the proposed count. All of said claims are either anticipated by, or obvious over, the proposed count as required by 37 CFR § 41.207(b)(2).

More specifically, the alternatives of the proposed count recite framework residue substitutions recited in claims 1, 5-28, 34-63, 66, 70-77, and 79 of the 213 patent.

Claims 2, 31, 67, and 81 of the 213 patent recite that the substituted residue is the residue found at the corresponding location of the non-human antibody from which the non-human CDR amino acids are obtained. Such a claim is anticipated by the proposed count – the goal of substituting framework residues is to improve affinity to approach that of the antibody from which the CDRs are obtained. See, for example, Riechmann, et al., *Nature*, 332:323-327, 1988.

Claims 3, 32, 68, and 82 of the 213 patent recite that no human framework residue other than those set forth in the claim from which they depend has been substituted. Such claims are anticipated by, or clearly obvious over, the proposed count in view of the use of the Markush language "selected from the group consisting of." The transitional phrase "consisting of" is closed, meaning that it excludes any element not listed. See MPEP 2111.03.

Claims 29 and 78 recite an antibody comprising the humanized variable domains of the claims from which they depend. Such claims are clearly anticipated by claim 30 of the proposed count.

While claims 4, 33, 62 and 69 of the 213 patent further recite that the variable domain is a "consensus" human variable domain, use of such framework regions in making humanized antibodies was known by the priority date of the 213 patent. See, for example, column 13, lines

6-9, of U.S. Patent No. 5,530,101, issued to Queen et al., filed on December 19, 1990. Claims 62 and 69 are, thus, obvious in view of the proposed count and the art.

The recitation in claim 63 that the humanized antibody lacks immunogenicity when compared to the non-human parent upon repeated administration is the very goal of humanization and is, thus, implicit. The motivation for preparing humanized antibodies was to reduce immunogenicity. *See* column 1, lines 51-58, of the 213 patent, discussing the "Background of the Invention." References from the early 1980's are cited therein disclosing the anti-globulin response to rodent monoclonal antibodies. Claim 63 is, at a minimum, obvious over the proposed count and the art.

The alternatives recited in claim 64 include the alternatives recited in claim 80 of the count. Claim 64 is, thus, anticipated by the proposed count.

The recitation in claim 65 that the variant of claim 63 binds the antigen "**up to** 3-fold more in the binding affinity than the parent antibody binds antigen" broadly includes variants that have binding affinities equal to **and** less than the parent. As recognized in the "Background of the Invention" section of the 213 patent, at column 3, lines 50-55, humanizing antibody while **retaining** high affinity for antigen was difficult to achieve; achieving lower affinity was not. This recitation, thus, is also either anticipated by, or obvious over, the proposed count in view of the art.

All of Applicants claims, claims 24-25, are anticipated by or obvious over the proposed count. Claim 25 is anticipated by claim 30 of the count.

(e) Interference-In-Fact

"An interference-in-fact exists if the subject matter of a claim of one party would, if prior art, have anticipated or rendered obvious the subject matter of a claim of the opposing party and vice versa." 37 CFR § 41.203(a) (2004). Applicants set forth in attached Appendix C a comparison of claim 66 of the 213 patent with Applicants' claim 24, both of which correspond to the present count.

As is clear from Appendix C, each claim anticipates and/or renders obvious the other. Both claims recite a humanized variable domain. Both claims are directed to a heavy chain

variable domain. Both claims also recite that the variable domain comprises non-human complementarity determining region amino acid residues which bind an antigen. Both claims also comprise a human framework region; claim 66 of the 213 patent simply recites it differently -- if one incorporates non-human CDR amino acid residues into a human antibody variable domain, one ends up with a human framework region. Indeed, as discussed above, the 213 patent defines "humanized antibody" as an antibody having a framework region "having substantially the amino acid sequence of a human immunoglobulin," i.e., a human framework region (see column 8, lines 11-17, of the 213 patent). Both claims recite a single amino acid substitution in the framework region to be selected from a Markush group listed thereafter; both recite that residue 24 of the heavy chain is to be substituted. Finally, both claims recite that numbering is according to Kabat.

(f) Support for Applicants Claims

In attached Appendix D, Applicants illustrate the representative support in their disclosure for the limitations of their claims 24 to 25. There is, of course, additional support in Applicants' application omitted herein for the sake of brevity. In Appendix E, Applicants show support for their claim 24, filed December 21, 1989. Methods for preparing exemplary antibodies having framework substitutions are described, *inter alia*, on pages 18-23, Sections 13.1.1 through 15.3, of the GB priority application.

(g) Applicants Will Prevail on Priority

US Serial No. 08/146,206, which issued as the 213 patent, was filed on June 15, 1992 as a PCT continuation-in-part of U.S. Serial No. 07/715,272, filed June 14, 1991.

The present application is a continuation of Application Serial No. 08/846,658, filed May 1, 1997, which is a continuation of Application Serial No. 08/303,569, filed September 7, 1994, now U.S. Patent No. 5,859,205, which is a continuation of Application Serial No. 07/743,329, filed as PCT/GB90/02017, filed December 21, 1990 and which claims priority benefit of GB 8928874.0, filed December 21, 1989. Applicants' earliest constructive reduction to practice date to which they are entitled is at least as early as December 21, 1989, but not later than December

21, 1990. Regardless, both dates are earlier than the earliest filing date of the 213 patent , or June 14, 1991.

CONCLUSION

The Requested Interference Should Be Declared

Early consideration and indication of allowability of all pending claims is respectfully requested. For an interference to be declared, however, only one claim needs to be allowable. MPEP § 2307.02. Should the present examination involve rejections of applicant's claims that would have been equally applicable against the 213 patent claims, applicants respectfully note MPEP § 2307.02, which requires the approval of the Group Director for such a rejection. Applicants are presumptively the prior inventors of the claimed subject matter and only desire an interference to prove that they are the actual prior inventors. Their opportunity to do so should not be unduly delayed. In view of the foregoing, Applicants respectfully request that an interference be declared between the present application and the 213 patent.

To assist the Examiner, Applicants note the following.

(1) the proposed count for the interference should be the Proposed Count set forth in Appendix B.

(2) the claims of the 213 patent which should be designated as corresponding to the count are claims 1-82, all of the issued claims of the patent.

(3) the claims of Applicants that should be designated as corresponding to the count are claims 24-25, all pending claims of the Applicants' above-identified application.

(4) no claims of either party should be designated as not corresponding to the count since none are directed to a separate patentable invention when considering the proposed count.

The examiner is requested to contact the undersigned attorney if an interview, telephonic or personal, would facilitate allowance of the claims or declaration of an interference.

Respectfully submitted,

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Date: November 21, 2005

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Attachments: **Appendices A-E**
Copy of U.S. Patent No. 6,407,213 B1
Copy of PCT Publication WO 91/09967

APPENDIX A

1. A CDR-grafted antibody heavy chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 6, 23 and/or 24, 48 and/or 49, 71 and/or 73, 75 and/or 76 and/or 78 and 88 and/or 91.

2. A CDR-grafted heavy chain according to claim 1 comprising donor residues at positions 23, 24, 49, 71, 73 and 78, or at positions 23, 24 and 49.

3. A CDR-grafted heavy chain according to claim 2 comprising donor residues at positions 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.

4. A CDR-grafted heavy chain according to claim 2 or 3, comprising donor residues at one, some or all of positions: 1 and 3, 69 (if 48 is different between donor and acceptor), 38 and 46 (if 48 is the donor residue), 67, 82 and IS (if 67 is the donor residue), 91, and any one or more of 9, 11, 41, 87, 108, 110 and 112.

5. A CDR-grafted heavy chain according to any of the preceding comprising donor CDRs at positions 26-35, 50-65 and 95-100.

6. A CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 1 and/or 3 and 46 and/or 47.

7. A CDR-grafted light chain according to claim 6 comprising donor residues at positions 46 and 47.

8. A CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor

residues at at least one of positions 46, 48, 58 and 71.

9. A CDR-grafted light chain according to claim 8 comprising donor residues at positions 46, 48, 58 and 71.

10. A CDR-grafted light chain according to claim 8 or 9, comprising donor residues at positions 2, 4, 6, 35, 36, 38, 44, 47, 49, 62, 64-69, 85, 87, 98, 99, 101 and 102.

11. A CDR-grafted light chain according to claim 9 or 10, comprising donor residues at one, some or all of positions: 1 and 3, 63, 60 (if 60 and 54 are able to form a potential saltbridge), 70 (if 70 and 24 are able to form a potential saltbridge), 73 and 21 (if 47 is different between donor and acceptor), 37 and 45 (if 47 if different between donor and acceptor), and any one or more of 10, 12, 40, 83, 103 and 105.

12. A CDR-grafted light chain according to any one of claims 6-11, comprising donor CDRs at positions 24-34, 50-56 and 89-97.

13. A CDR-grafted antibody molecule comprising at least one CDR-grafted heavy chain according to any one of claims 1-5 and at least one CDR-grafted light chain according to any one of claims 6-12.

14. A CDR-grafted antibody molecule according to claim 13, which is a site-specific antibody molecule.

15. A CDR-grafted antibody molecule according to claim 13 which has specificity for an interleukin, hormone or other biologically active compound or a receptor therefor.

16. A CDR-grafted antibody heavy or light chain or molecule according to any one of the preceding claims comprising human acceptor residues and non-human donor residues.

17. A DNA sequence which codes for a CDR-grafted heavy chain according to claim 1 or a

CDR-grafted light chain according to claim 6 or claim 8.

18. A cloning or expression vector containing a DNA sequence according to claim 17.

19. A host cell transformed with a DNA sequence according to claim 17.

20. A process for the production of a CDR-grafted antibody sequence according to claim 17 in a transformed host cell.

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

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

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

APPENDIX B

Proposed Count for interference

Claim 30 of the 213 patent

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

OR

Claim 80 of the 213 patent

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

(a) noncovalently binds antigen directly;

(b) interacts with a CDR; or

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

OR

Applicants' claim 24

A humanised antibody **heavy chain** variable domain comprising non-human complementarity determining region amino acid residues which bind an antigen and a human framework region wherein said framework region comprises an amino acid substitution at a residue selected from the group consisting of 23, 24, 49, 71, 73, and **78**, and combinations thereof, as numbered according to Kabat.

APPENDIX C

Comparison of Applicants' claim 24 and claim 66 of the 213 patent

Applicants' Claim 24	213 patent Claim 66
Claim 24 – A humanised antibody heavy chain variable domain comprising	A humanized antibody heavy chain variable domain comprising
non-human complementarity determining region amino acid residues which bind an antigen and	non-human Complementarity Determining Region (CDR) amino acid residues which bind an antigen
a human framework region wherein said framework region comprises an amino acid substitution at a residue selected from the group consisting of	incorporated into a human antibody variable domain, and further comprising a Framework Region (FR) amino acid substitution at a site selected from the group consisting of:
23, **24**, 49, 71, 73, and 78, and combinations thereof,	**24H**, 73H, 76H, 78H, and 93H
as numbered according to Kabat.	utilizing the numbering system as set forth in Kabat.

APPENDIX D

Support for Applicants' claims in Applicants' Present Specification

Claim	Present Specification
Claim 24 -- A humanised antibody heavy chain variable domain comprising	page 6, lines 29-31
non-human complementarity determining region amino acid residues which bind an antigen and	page 7, line 29, through page 8, line 7 and page 17, lines 6-7
a human framework region wherein said framework region comprises an amino acid substitution at a residue selected from the group consisting of	page 7, line 29, through page 8, line 7 and page 17, lines 1-19
23, 24, 49, 71, 73, and 78, and combinations thereof,	page 7, lines 1-3
as numbered according to Kabat.	page 8, lines 22-24
Claim 25 -- A humanised antibody comprising the variable domain of claim 24.	see above support for claim 24

APPENDIX E

Support For Applicants' Claims in the GB Application, filed December 21, 1989

Claim	1989 GB Application
Claim 24 – A humanised antibody heavy chain variable domain comprising	page 5, lines 8-10
non-human complementarity determining region amino acid residues which bind an antigen and	page 5, lines 8-21
a human framework region wherein said framework region comprises an amino acid substitution at a residue selected from the group consisting of	page 5, line 10 and page 26, lines 31-33
23, 24, 49, 71, 73, and 78, and combinations thereof,	page 6, lines 8-10 and Table 1
as numbered according to Kabat.	page 6, lines 5-7

DOCKET NO.: CARP0001-112 **PATENT**
APPLICATION SERIAL NO. 11/284,261

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re application of: **John R. Adair et al.** Confirmation No. **5305**

Serial No.: **11/284,261** Art Unit: **1643**

Filed: **November 21, 2005** Examiner: **Anne Gussow**

Title: **HUMANISED ANTIBODIES**

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MAIL STOP AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

REQUEST FOR RECONSIDERATION

Dear Sir:

This paper is being filed in response to the Non-Final Office Action dated as mailed March 9, 2009. Applicants hereby petition for a three-month extension of time to respond to the Non-Final Office Action and request the Commissioner to charge Deposit Account 50-3111 the appropriate extension of time fee.

Listing of the Claims, begin on page 2.

Remarks begin on page 3.

The **Conclusion** is found on page 5.

Carter Exhibit 2004
Carter v. Adair
Interference No. 105,744

LISTING OF THE CLAIMS

Claims 1-23 (**cancelled**)

Claim 24 (**currently amended**): A humanised antibody comprising a heavy chain variable domain comprising non-human complementarity determining region amino acid residues which bind an antigen and a human framework region wherein said framework region comprises a non-human amino acid substitution at a residue selected from the group consisting of 23, 24, 49, 71, 73, and 78, and combinations thereof, as numbered according to Kabat.

Claim 25 (**cancelled**)

REMARKS

Claims 24 and 25 were pending. All pending claims were rejected in the Non-Final Rejection. In view of the foregoing amendments and arguments that follow, Applicants respectfully request withdrawal of all rejections upon reconsideration.

Applicants acknowledge with appreciation the Office's withdrawal of the objections to claim 24 under 35 U.S.C. 112, second paragraph, as being indefinite.

Rejection Under 35 U.S.C. § 112, First Paragraph

Claim 24 was amended with the RCE filing and was again rejected as allegedly being indefinite. The Office alleges that the specification is enabling for a humanized antibody comprising a heavy chain variable domain and a light chain variable domain, with all 6 CDRs, and does not provide enablement for a humanized antibody heavy chain variable domain alone. The Office is clearly disregarding the fact that the CDR-grafted chains can be combined with other chains, as disclosed in the specification, including chimeric and mouse chains. Thus, it is not necessary for the claims to recite both chains. Applicants traverse this rejection but have amended claim 24 to recite a humanized antibody comprising a heavy chain variable domain.

Applicants respectfully submit that this rejection has been overcome.

Rejection Under 35 U.S.C. 102(e)

Claims 24 and 25 were rejected under 35 U.S.C. 102(e) as allegedly being anticipated by Queen, et al US Patent 5,585,089 the "'089 patent". Claim 25 has been cancelled. Applicants traverse this rejection as it applies to claim 24.

With all due respect, the Office has apparently misread the claims. Initially, the office states that the claims recite that the framework region comprises a **non-human** amino acid

substitution at a residue selected from 23, 24, 49, 71, 73, and 78, and combinations thereof. The Office then asserts that the "'089 patent" teaches an antibody comprising a humanized heavy chain variable domain comprising human residues at positions except the CDRs, and the following framework positions—27, 93, 95, 98, 107-109, 11, 30, 67, 48, and 68. The Office concludes, thus, that all the remaining framework positions are the human antibody allegedly taught in the "'089 patent". The Office then states that, since the claims recite substitution to **human** residues in the heavy chain in residues 23, 24, 49, 71, 73, and 78, and the "'089 patent" teaches human residues in all of those positions, the claim limitations are met. As the Office previously acknowledged, however, these residues are **non-human** residues in claim 24. The "'089 patent", thus, does not anticipate the Applicants invention.

Applicants respectfully request that this rejection be withdrawn.

CONCLUSION

Applicants respectfully submit that claim 24 is allowable and early allowance of the same. If a telephonic conversation with Applicants' attorney would help expedite the prosecution of the above-identified application, the Examiner is urged to call the undersigned at 215-665-5593.

Respectfully submitted,

/Doreen Yatko Trujillo/

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Date: September 9, 2009

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(54) Title: HUMANISED ANTIBODIES

(57) Abstract

CDR-grafted antibody heavy and light chains comprise acceptor framework and donor antigen binding regions, the heavy chains comprising donor residues at at least one of positions (6, 23) and/or (24, 48) and/or (49, 71) and/or (73, 75) and/or (76) and/or (78) and (88) and/or (91). The CDR-grafted light chains comprise donor residues at at least one of positions (1) and/or (3) and (46) and/or (47) or at at least one of positons (46, 48, 58) and (71). The CDR-grafted antibodies are preferably humanised antibodies, having non human, e.g. rodent, donor and human acceptor frameworks, and may be used for *in vivo* therapy and diagnosis. A generally applicable protocol is disclosed for obtaining CDR-grafted antibodies.

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HUMANISED ANTIBODIES

Field of the Invention

The present invention relates to humanised antibody molecules, to processes for their production using recombinant DNA technology, and to their therapeutic uses.

The term "humanised antibody molecule" is used to describe a molecule having an antigen binding site derived from an immunoglobulin from a non-human species, and remaining immunoglobulin-derived parts of the molecule being derived from a human immunoglobulin. The antigen binding site typically comprises complementarity determining regions (CDRs) which determine the binding specificity of the antibody molecule and which are carried on appropriate framework regions in the variable domains. There are 3 CDRs (CDR1, CDR2 and CDR3) in each of the heavy and light chain variable domains.

In the description, reference is made to a number of publications by number. The publications are listed in numerical order at the end of the description.

Background of the Invention

Natural immunoglobulins have been known for many years, as have the various fragments thereof, such as the Fab, $(Fab')_2$ and Fc fragments, which can be derived by enzymatic cleavage. Natural immunoglobulins comprise a generally Y-shaped molecule having an antigen-binding site towards the end of each upper arm. The remainder of the structure, and particularly the stem of the Y, mediates the effector functions associated with immunoglobulins.

Natural immunoglobulins have been used in assay, diagnosis and, to a more limited extent, therapy. However, such uses, especially in therapy, were hindered until recently by the polyclonal nature of natural immunoglobulins. A significant step towards the realisation of the potential

of immunoglobulins as therapeutic agents was the discovery of procedures for the production of monoclonal antibodies (MAbs) of defined specificity (1).

However, most MAbs are produced by hybridomas which are fusions of rodent spleen cells with rodent myeloma cells. They are therefore essentially rodent proteins. There are very few reports of the production of human MAbs.

Since most available MAbs are of rodent origin, they are naturally antigenic in humans and thus can give rise to an undesirable immune response termed the HAMA (Human Anti-Mouse Antibody) response. Therefore, the use of rodent MAbs as therapeutic agents in humans is inherently limited by the fact that the human subject will mount an immunological response to the MAb and will either remove it entirely or at least reduce its effectiveness. In practice, MAbs of rodent origin may not be used in patients for more than one or a few treatments as a HAMA response soon develops rendering the MAb ineffective as well as giving rise to undesirable reactions. For instance, OKT3 a mouse IgG2a/k MAb which recognises an antigen in the T-cell receptor-CD3 complex has been approved for use in many countries throughout the world as an immunosuppressant in the treatment of acute allograft rejection [Chatenoud et al (2) and Jeffers et al (3)]. However, in view of the rodent nature of this and other such MAbs, a significant HAMA response which may include a major anti-idiotype component, may build up on use. Clearly, it would be highly desirable to diminish or abolish this undesirable HAMA response and thus enlarge the areas of use of these very useful antibodies.

Proposals have therefore been made to render non-human MAbs less antigenic in humans. Such techniques can be generically termed "humanisation" techniques. These

techniques typically involve the use of recombinant DNA technology to manipulate DNA sequences encoding the polypeptide chains of the antibody molecule.

Early methods for humanising MAbs involved production of chimeric antibodies in which an antigen binding site comprising the complete variable domains of one antibody is linked to constant domains derived from another antibody. Methods for carrying out such chimerisation procedures are described in EP0120694 (Celltech Limited), EP0125023 (Genentech Inc. and City of Hope), EP-A-0 171496 (Res. Dev. Corp. Japan), EP-A-0 173 494 (Stanford University), and WO 86/01533 (Celltech Limited). This latter Celltech application (WO 86/01533) discloses a process for preparing an antibody molecule having the variable domains from a mouse MAb and the constant domains from a human immunoglobulin. Such humanised chimeric antibodies, however, still contain a significant proportion of non-human amino acid sequence, i.e. the complete non-human variable domains, and thus may still elicit some HAMA response, particularly if administered over a prolonged period [Begent et al (ref. 4)].

In an alternative approach, described in EP-A-0239400 (Winter), the complementarity determining regions (CDRs) of a mouse MAb have been grafted onto the framework regions of the variable domains of a human immunoglobulin by site directed mutagenesis using long oligonucleotides. The present invention relates to humanised antibody molecules prepared according to this alternative approach, i.e. CDR-grafted humanised antibody molecules. Such CDR-grafted humanised antibodies are much less likely to give rise to a HAMA response than humanised chimeric antibodies in view of the much lower proportion of non-human amino acid sequence which they contain.

The earliest work on humanising MAbs by CDR-grafting was carried out on MAbs recognising synthetic antigens, such as the NP or NIP antigens. However, examples in which a mouse MAb recognising lysozyme and a rat MAb recognising an antigen on human T-cells were humanised by CDR-grafting have been described by Verhoeyen et al (5) and Riechmann et al (6) respectively. The preparation of CDR-grafted antibody to the antigen on human T cells is also described in WO 89/07452 (Medical Research Council).

In Riechmann et al/Medical Research Council it was found that transfer of the CDR regions alone [as defined by Kabat refs. (7) and (8)] was not sufficient to provide satisfactory antigen binding activity in the CDR-grafted product. Riechmann et al found that it was necessary to convert a serine residue at position 27 of the human sequence to the corresponding rat phenylalanine residue to obtain a CDR-grafted product having improved antigen binding activity. This residue at position 27 of the heavy chain is within the structural loop adjacent to CDR1. A further construct which additionally contained a human serine to rat tyrosine change at position 30 of the heavy chain did not have a significantly altered binding activity over the humanised antibody with the serine to phenylalanine change at position 27 alone. These results indicate that changes to residues of the human sequence outside the CDR regions, in particular in the structural loop adjacent to CDR1, may be necessary to obtain effective antigen binding activity for CDR-grafted antibodies which recognise more complex antigens. Even so the binding affinity of the best CDR-grafted antibodies obtained was still significantly less than the original MAb.

Very recently Queen et al (9) have described the preparation of a humanised antibody that binds to the

interleukin 2 receptor, by combining the CDRs of a murine MAb (anti-Tac) with human immunoglobulin framework and constant regions. The human framework regions were chosen to maximise homology with the anti-Tac MAb sequence. In addition computer modelling was used to identify framework amino acid residues which were likely to interact with the CDRs or antigen, and mouse amino acids were used at these positions in the humanised antibody.

In WO 90/07861 Queen et al propose four criteria for designing humanised immunoglobulins. The first criterion is to use as the human acceptor the framework from a particular human immunoglobulin that is unusually homologous to the non-human donor immunoglobulin to be humanised, or to use a consensus framework from many human antibodies. The second criterion is to use the donor amino acid rather than the acceptor if the human acceptor residue is unusual and the donor residue is typical for human sequences at a specific residue of the framework. The third criterion is to use the donor framework amino acid residue rather than the acceptor at positions immediately adjacent to the CDRs. The fourth criterion is to use the donor amino acid residue at framework positions at which the amino acid is predicted to have a side chain atom within about 3 Å of the CDRs in a three-dimensional immunoglobulin model and to be capable of interacting with the antigen or with the CDRs of the humanised immunoglobulin. It is proposed that criteria two, three or four may be applied in addition or alternatively to criterion one, and may be applied singly or in any combination.

WO 90/07861 describes in detail the preparation of a single CDR-grafted humanised antibody, a humanised antibody having specificity for the p55 Tac protein of the

IL-2 receptor. The combination of all four criteria, as above, were employed in designing this humanised antibody, the variable region frameworks of the human antibody Eu (7) being used as acceptor. In the resultant humanised antibody the donor CDRs were as defined by Kabat et al (7 and 8) and in addition the mouse donor residues were used in place of the human acceptor residues, at positions 27, 30, 48, 66, 67, 89, 91, 94, 103, 104, 105 and 107 in the heavy chain and at positions 48, 60 and 63 in the light chain, of the variable region frameworks. The humanised anti-Tac antibody obtained is reported to have an affinity for p55 of 3×10^9 M^{-1}, about one-third of that of the murine MAb.

We have further investigated the preparation of CDR-grafted humanised antibody molecules and have identified a hierarchy of positions within the framework of the variable regions (i.e. outside both the Kabat CDRs and structural loops of the variable regions) at which the amino acid identities of the residues are important for obtaining CDR-grafted products with satisfactory binding affinity. This has enabled us to establish a protocol for obtaining satisfactory CDR-grafted products which may be applied very widely irrespective of the level of homology between the donor immunoglobulin and acceptor framework. The set of residues which we have identified as being of critical importance does not coincide with the residues identified by Queen et al (9).

Summary of the Invention

Accordingly, in a first aspect the invention provides a CDR-grafted antibody heavy chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 6, 23 and/or 24, 48 and/or 49, 71 and/or 73, 75 and/or 76 and/or 78 and 88 and/or 91.

In preferred embodiments, the heavy chain framework comprises donor residues at positions 23, 24, 49, 71, 73 and 78 or at positions 23, 24 and 49. The residues at positions 71, 73 and 78 of the heavy chain framework are preferably either all acceptor or all donor residues.

In particularly preferred embodiments the heavy chain framework additionally comprises donor residues at one, some or all of positions 6, 37, 48 and 94. Also it is particularly preferred that residues at positions of the heavy chain framework which are commonly conserved across species, i.e. positions 2, 4, 25, 36, 39, 47, 93, 103, 104, 106 and 107, if not conserved between donor and acceptor, additionally comprise donor residues. Most preferably the heavy chain framework additionally comprises donor residues at positions 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.

In addition the heavy chain framework optionally comprises donor residues at one, some or all of positions:
1 and 3,
72 and 76,
69 (if 48 is different between donor and acceptor),
38 and 46 (if 48 is the donor residue),
80 and 20 (if 69 is the donor residue),
67,
82 and 18 (if 67 is the donor residue),
91,
88, and
any one or more of 9, 11, 41, 87, 108, 110 and 112.

In the first and other aspects of the present invention reference is made to CDR-grafted antibody products comprising acceptor framework and donor antigen binding regions. It will be appreciated that the invention is widely applicable to the CDR-grafting of antibodies in

general. Thus, the donor and acceptor antibodies may be derived from animals of the same species and even same antibody class or sub-class. More usually, however, the donor and acceptor antibodies are derived from animals of different species. Typically the donor antibody is a non-human antibody, such as a rodent MAb, and the acceptor antibody is a human antibody.

In the first and other aspects of the present invention, the donor antigen binding region typically comprises at least one CDR from the donor antibody. Usually the donor antigen binding region comprises at least two and preferably all three CDRs of each of the heavy chain and/or light chain variable regions. The CDRs may comprise the Kabat CDRs, the structural loop CDRs or a composite of the Kabat and structural loop CDRs and any combination of any of these. Preferably, the antigen binding regions of the CDR-grafted heavy chain variable domain comprise CDRs corresponding to the Kabat CDRs at CDR2 (residues 50-65) and CDR3 (residues 95-100) and a composite of the Kabat and structural loop CDRs at CDR1 (residues 26-35).

The residue designations given above and elsewhere in the present application are numbered according to the Kabat numbering [refs. (7) and (8)]. Thus the residue designations do not always correspond directly with the linear numbering of the amino acid residues. The actual linear amino acid sequence may contain fewer or additional amino acids than in the strict Kabat numbering corresponding to a shortening of, or insertion into, a structural component, whether framework or CDR, of the basic variable domain structure. For example, the heavy chain variable region of the anti-Tac antibody described by Queen et al (9) contains a single amino acid insert (residue 52a) after residue 52 of CDR2 and a three amino

acid insert (residues 82a, 82b and 82c) after framework residue 82, in the Kabat numbering. The correct Kabat numbering of residues may be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence.

The invention also provides in a second aspect a CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 1 and/or 3 and 46 and/or 47. Preferably the CDR grafted light chain of the second aspect comprises donor residues at positions 46 and/or 47.

The invention also provides in a third aspect a CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 46, 48, 58 and 71.

In a preferred embodiment of the third aspect, the framework comprises donor residues at all of positions 46, 48, 58 and 71.

In particularly preferred embodiments of the second and third aspects, the framework additionally comprises donor residues at positions 36, 44, 47, 85 and 87. Similarly positions of the light chain framework which are commonly conserved across species, i.e. positions 2, 4, 6, 35, 49, 62, 64-69, 98, 99, 101 and 102, if not conserved between donor and acceptor, additionally comprise donor residues. Most preferably the light chain framework additionally comprises donor residues at positions 2, 4, 6, 35, 36, 38, 44, 47, 49, 62, 64-69, 85, 87, 98, 99, 101 and 102.

In addition the framework of the second or third aspects optionally comprises donor residues at one, some or all of positions:
1 and 3,
63,
60 (if 60 and 54 are able to form at potential saltbridge),
70 (if 70 and 24 are able to form a potential saltbridge),
73 and 21 (if 47 is different between donor and acceptor),
37 and 45 (if 47 is different between donor and acceptor),
and
any one or more of 10, 12, 40, 80, 103 and 105.

Preferably, the antigen binding regions of the CDR-grafted light chain variable domain comprise CDRs corresponding to the Kabat CDRs at CDR1 (residue 24-34), CDR2 (residues 50-56) and CDR3 (residues 89-97).

The invention further provides in a fourth aspect a CDR-grafted antibody molecule comprising at least one CDR-grafted heavy chain and at least one CDR-grafted light chain according to the first and second or first and third aspects of the invention.

The humanised antibody molecules and chains of the present invention may comprise: a complete antibody molecule, having full length heavy and light chains; a fragment thereof, such as a Fab, $(Fab')_2$ or FV fragment; a light chain or heavy chain monomer or dimer; or a single chain antibody, e.g. a single chain FV in which heavy and light chain variable regions are joined by a peptide linker; or any other CDR-grafted molecule with the same specificity as the original donor antibody. Similarly the CDR-grafted heavy and light chain variable region may be combined with other antibody domains as appropriate.

Also the heavy or light chains or humanised antibody molecules of the present invention may have attached to them an effector or reporter molecule. For instance, it may have a macrocycle, for chelating a heavy metal atom, or a toxin, such as ricin, attached to it by a covalent bridging structure. Alternatively, the procedures of recombinant DNA technology may be used to produce an immunoglobulin molecule in which the Fc fragment or CH3 domain of a complete immunoglobulin molecule has been replaced by, or has attached thereto by peptide linkage, a functional non-immunoglobulin protein, such as an enzyme or toxin molecule.

Any appropriate acceptor variable region framework sequences may be used having regard to class/type of the donor antibody from which the antigen binding regions are derived. Preferably, the type of acceptor framework used is of the same/similar class/type as the donor antibody. Conveniently, the framework may be chosen to maximise/optimise homology with the donor antibody sequence particularly at positions close or adjacent to the CDRs. However, a high level of homology between donor and acceptor sequences is not important for application of the present invention. The present invention identifies a hierarchy of framework residue positions at which donor residues may be important or desirable for obtaining a CDR-grafted antibody product having satisfactory binding properties. The CDR-grafted products usually have binding affinities of at least 10^5 M^{-1}, preferably at least about 10^8 M^{-1}, or especially in the range 10^8-10^{12} M^{-1}. In principle, the present invention is applicable to any combination of donor and acceptor antibodies irrespective of the level of homology between their sequences. A protocol for applying the invention to any particular donor-acceptor antibody pair is given hereinafter. Examples of human frameworks which may be

used are KOL, NEWM, REI, EU, LAY and POM (refs. 4 and 5) and the like; for instance KOL and NEWM for the heavy chain and REI for the light chain and EU, LAY and POM for both the heavy chain and the light chain.

Also the constant region domains of the products of the invention may be selected having regard to the proposed function of the antibody in particular the effector functions which may be required. For example, the constant region domains may be human IgA, IgE, IgG or IgM domains. In particular, IgG human constant region domains may be used, especially of the IgG1 and IgG3 isotypes, when the humanised antibody molecule is intended for therapeutic uses, and antibody effector functions are required. Alternatively, IgG2 and IgG4 isotypes may be used when the humanised antibody molecule is intended for therapeutic purposes and antibody effector functions are not required, e.g. for simple blocking of lymphokine activity.

However, the remainder of the antibody molecules need not comprise only protein sequences from immunoglobulins. For instance, a gene may be constructed in which a DNA sequence encoding part of a human immunoglobulin chain is fused to a DNA sequence encoding the amino acid sequence of a functional polypeptide such as an effector or reporter molecule.

Preferably the CDR-grafted antibody heavy and light chain and antibody molecule products are produced by recombinant DNA technology.

Thus in further aspects the invention also includes DNA sequences coding for the CDR-grafted heavy and light chains, cloning and expression vectors containing the DNA sequences, host cells transformed with the DNA sequences

and processes for producing the CDR-grafted chains and antibody molecules comprising expressing the DNA sequences in the transformed host cells.

The general methods by which the vectors may be constructed, transfection methods and culture methods are well known per se and form no part of the invention. Such methods are shown, for instance, in references 10 and 11.

The DNA sequences which encode the donor amino acid sequence may be obtained by methods well known in the art. For example the donor coding sequences may be obtained by genomic cloning, or cDNA cloning from suitable hybridoma cell lines. Positive clones may be screened using appropriate probes for the heavy and light chain genes in question. Also PCR cloning may be used.

DNA coding for acceptor, e.g. human acceptor, sequences may be obtained in any appropriate way. For example DNA sequences coding for preferred human acceptor frameworks such as KOL, REI, EU and NEWM, are widely available to workers in the art.

The standard techniques of molecular biology may be used to prepare DNA sequences coding for the CDR-grafted products. Desired DNA sequences may be synthesised completely or in part using oligonucleotide synthesis techniques. Site-directed mutagenesis and polymerase chain reaction (PCR) techniques may be used as appropriate. For example oligonucleotide directed synthesis as described by Jones et al (ref. 20) may be used. Also oligonucleotide directed mutagenesis of a pre-exising variable region as, for example, described by Verhoeyen et al (ref. 5) or Riechmann et al (ref. 6) may be used. Also enzymatic filling in of gapped

oligonucleotides using T_4 DNA polymerase as, for example, described by Queen et al (ref. 9) may be used.

Any suitable host cell/vector system may be used for expression of the DNA sequences coding for the CDR-grafted heavy and light chains. Bacterial e.g. E. coli, and other microbial systems may be used, in particular for expression of antibody fragments such as FAb and $(Fab')_2$ fragments, and especially FV fragments and single chain antibody fragments e.g. single chain FVs. Eucaryotic e.g. mammalian host cell expression systems may be used for production of larger CDR-grafted antibody products, including complete antibody molecules. Suitable mammalian host cells include CHO cells and myeloma or hybridoma cell lines.

Thus, in a further aspect the present invention provides a process for producing a CDR-grafted antibody product comprising:

(a) producing in an expression vector an operon having a DNA sequence which encodes an antibody heavy chain according to the first aspect of the invention;

and/or

(b) producing in an expression vector an operon having a DNA sequence which encodes a complementary antibody light chain according to the second or third aspect of the invention;

(c) transfecting a host cell with the or each vector; and

(d) culturing the transfected cell line to produce the CDR-grafted antibody product.

The CDR-grafted product may comprise only heavy or light chain derived polypeptide, in which case only a heavy chain or light chain polypeptide coding sequence is used to transfect the host cells.

For production of products comprising both heavy and light chains, the cell line may be transfected with two vectors, the first vector may contain an operon encoding a light chain-derived polypeptide and the second vector containing an operon encoding a heavy chain-derived polypeptide. Preferably, the vectors are identical, except in so far as the coding sequences and selectable markers are concerned, so as to ensure as far as possible that each polypeptide chain is equally expressed. Alternatively, a single vector may be used, the vector including the sequences encoding both light chain- and heavy chain-derived polypeptides.

The DNA in the coding sequences for the light and heavy chains may comprise cDNA or genomic DNA or both. However, it is preferred that the DNA sequence encoding the heavy or light chain comprises at least partially, genomic DNA, preferably a fusion of cDNA and genomic DNA.

The present invention is applicable to antibodies of any appropriate specificity. Advantageously, however, the invention may be applied to the humanisation of non-human antibodies which are used for <u>in vivo</u> therapy or diagnosis. Thus the antibodies may be site-specific antibodies such as tumour-specific or cell surface-specific antibodies, suitable for use in <u>in vivo</u> therapy or diagnosis, e.g. tumour imaging. Examples of cell surface-specific antibodies are anti-T cell antibodies, such as anti-CD3, and CD4 and adhesion molecules, such as CR3, ICAM and ELAM. The antibodies may have specificity for interleukins (including lymphokines, growth factors and stimulating factors), hormones and other biologically active compounds, and receptors for any of these. For

example, the antibodies may have specificity for any of the following: Interferons α, β, γ or δ, IL1, IL2, IL3, or IL4, etc., TNF, GCSF, GMCSF, EPO, hGH, or insulin, etc.

The the present invention also includes therapeutic and diagnostic compositions comprising the CDR-grafted products of the invention and uses of such compositions in therapy and diagnosis.

Accordingly in a further aspect the invention provides a therapeutic or diagnostic composition comprising a CDR-grafted antibody heavy or light chain or molecule according to previous aspects of the invention in combination with a pharmaceutically acceptable carrier, diluent or excipient.

Accordingly also the invention provides a method of therapy or diagnosis comprising administering an effective amount of a CDR-grafted antibody heavy or light chain or molecule according to previous aspects of the invention to a human or animal subject.

A preferred protocol for obtaining CDR-grafted antibody heavy and light chains in accordance with the present invention is set out below together with the rationale by which we have derived this protocol. This protocol and rationale are given without prejudice to the generality of the invention as hereinbefore described and defined.

Protocol

It is first of all necessary to sequence the DNA coding for the heavy and light chain variable regions of the donor antibody, to determine their amino acid sequences. It is also necessary to choose appropriate acceptor heavy and light chain variable regions, of known amino acid sequence. The CDR-grafted chain is then designed

starting from the basis of the acceptor sequence. It will be appreciated that in some cases the donor and acceptor amino acid residues may be identical at a particular position and thus no change of acceptor framework residue is required.

1. As a first step donor residues are substituted for acceptor residues in the CDRs. For this purpose the CDRs are preferably defined as follows:

Heavy chain	-	CDR1:	residues 26-35
	-	CDR2:	residues 50-65
	-	CDR3:	residues 95-102
Light chain	-	CDR1:	residues 24-34
	-	CDR2:	residues 50-56
	-	CDR3:	residues 89-97

The positions at which donor residues are to be substituted for acceptor in the framework are then chosen as follows, first of all with respect to the heavy chain and subsequently with respect to the light chain.

2. Heavy Chain

2.1 Choose donor residues at all of positions 23, 24, 49, 71, 73 and 78 of the heavy chain or all of positions 23, 24 and 49 (71, 73 and 78 are always either all donor or all acceptor).

2.2 Check that the following have the same amino acid in donor and acceptor sequences, and if not preferably choose the donor: 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.

2.3 To further optimise affinity consider choosing donor residues at one, some or any of:

i. 1, 3
ii. 72, 76
iii. If 48 is different between donor and acceptor sequences, consider 69
iv. If at 48 the donor residue is chosen, consider 38 and 46
v. If at 69 the donor residue is chosen, consider 80 and then 20
vi. 67
vii. If at 67 the donor residue is chosen, consider 82 and then 18
viii. 91
ix. 88
x. 9, 11, 41, 87, 108, 110, 112

3. Light Chain

3.1 Choose donor at 46, 48, 58 and 71

3.2 Check that the following have the same amino acid in donor and acceptor sequences, if not preferably choose donor:

2, 4, 6, 35, 38, 44, 47, 49, 62, 64-69 inclusive, 85, 87, 98, 99, 101 and 102

3.3 To further optimise affinity consider choosing donor residues at one, some or any of:

i. 1, 3
ii. 63

iii. 60, if 60 and 54 are able to form potential saltbridge

iv. 70, if 70 and 24 are able to form potential saltbridge

v. 73, and 21 if 47 is different between donor and acceptor

vi. 37, and 45 if 47 is different between donor and acceptor

vii. 10, 12, 40, 80, 103, 105

Rationale

In order to transfer the binding site of an antibody into a different acceptor framework, a number of factors need to be considered.

1. The extent of the CDRs

The CDRs (Complementary Determining Regions) were defined by Wu and Kabat (refs. 4 and 5) on the basis of an analysis of the variability of different regions of antibody variable regions. Three regions per domain were recognised. In the light chain the sequences are 24-34, 50-56, 89-97 (numbering according to Kabat (ref. 4), Eu Index) inclusive and in the heavy chain the sequences are 31-35, 50-65 and 95-102 inclusive.

When antibody structures became available it became apparent that these CDR regions corresponded in the main to loop regions which extended from the β barrel framework of the light and heavy variable domains. For H1 there was a discrepancy in that the loop was from 26 to 32 inclusive and for H2 the loop was 52 to 56 and for L2 from 50 to 53. However, with the exception of H1 the CDR regions encompassed the loop regions and extended into the β strand

frameworks. In H1 residue 26 tends to be a serine and 27 a phenylalanine or tyrosine, residue 29 is a phenylalanine in most cases. Residues 28 and 30 which are surface residues exposed to solvent might be involved in antigen-binding. A prudent definition of the H1 CDR therefore would include residues 26-35 to include both the loop region and the hypervariable residues 33-35.

It is of interest to note the example of Riechmann et al (ref. 3), who used the residue 31-35 choice for CDR-H1. In order to produce efficient antigen binding, residue 27 also needed to be recruited from the donor (rat) antibody.

2. Non-CDR residues which contribute to antigen binding

By examination of available X-ray structures we have identified a number of residues which may have an effect on net antigen binding and which can be demonstrated by experiment. These residues can be sub-divided into a number of groups.

2.1 Surface residues near CDR [all numbering as in Kabat et al (ref. 7)].

2.1.1. Heavy Chain - Key residues are 23, 71 and 73. Other residues which may contribute to a lesser extent are 1, 3 and 76. Finally 25 is usually conserved but the murine residue should be used if there is a difference.

2.1.2 Light Chain - Many residues close to the CDRs, e.g. 63, 65, 67 and 69 are conserved. If conserved none of the surface residues in the light chain are likely to have a major effect. However, if the murine residue at these positions

is unusual, then it would be of benefit to analyse the likely contribution more closely. Other residues which may also contribute to binding are 1 and 3, and also 60 and 70 if the residues at these positions and at 54 and 24 respectively are potentially able to form a salt bridge i.e. 60 + 54; 70 + 24.

2.2 Packing residues near the CDRs.

2.2.1. Heavy Chain - Key residues are 24, 49 and 78. Other key residues would be 36 if not a tryptophan, 94 if not an arginine, 104 and 106 if not glycines and 107 if not a threonine. Residues which may make a further contribution to stable packing of the heavy chain and hence improved affinity are 2, 4, 6, 38, 46, 67 and 69. 67 packs against the CDR residue 63 and this pair could be either both mouse or both human. Finally, residues which contribute to packing in this region but from a longer range are 18, 20, 80, 82 and 86. 82 packs against 67 and in turn 18 packs against 82. 80 packs against 69 and in turn 20 packs against 80. 86 forms an H bond network with 38 and 46. Many of the mouse-human differences appear minor e.g. Leu-Ile, but could have an minor impact on correct packing which could translate into altered positioning of the CDRs.

2.2.2. Light Chain - Key residues are 48, 58 and 71. Other key residues would be 6 if not glutamine, 35 if not tryptophan, 62 if not phenylalanine or tryosine, 64, 66, 68, 99 and 101 if not glycines and 102 if not a threonine. Residues which make a further contribution are 2, 4, 37, 45 and 47. Finally residues 73 and 21 and 19 may make long distance packing contributions of a minor nature.

2.3. Residues at the variable domain interface between heavy and light chains - In both the light and heavy chains most of the non-CDR interface residues are conserved. If a conserved residue is replaced by a residue of different character, e.g. size or charge, it should be considered for retention as the murine residue.

2.3.1. Heavy Chain - Residues which need to be considered are 37 if the residue is not a valine but is of larger side chain volume or has a charge or polarity. Other residues are 39 if not a glutamine, 45 if not a leucine, 47 if not a tryptophan, 91 if not a phenylalanine or tyrosine, 93 if not an alanine and 103 if not a tryptophan. Residue 89 is also at the interface but is not in a position where the side chain could be of great impact.

2.3.2. Light Chain - Residues which need to be considered are 36, if not a tyrosine, 38 if not a glutamine, 44 if not a proline, 46, 49 if not a tyrosine, residue 85, residue 87 if not a tyrosine and 98 if not a phenylalanine.

2.4. Variable-Constant region interface - The elbow angle between variable and constant regions may be affected by alterations in packing of key residues in the variable region against the constant region which may affect the position of V_L and V_H with respect to one another. Therefore it is worth noting the residues likely to be in contact with the constant region. In the heavy chain the surface residues potentially in contact with the variable region are conserved between mouse and human antibodies therefore the variable region contact residues may influence the V-C interaction. In the light chain the amino acids found at a number of the constant

region contact points vary, and the V & C regions are not in such close proximity as the heavy chain. Therefore the influences of the light chain V-C interface may be minor.

2.4.1. Heavy Chain - Contact residues are 7, 11, 41, 87, 108, 110, 112.

2.4.2. Light Chain - In the light chain potentially contacting residues are 10, 12, 40, 80, 83, 103 and 105.

The above analysis coupled with our considerable practical experimental experience in the CDR-grafting of a number of different antibodies have lead us to the protocol given above.

The present invention is now described, by way of example only, with reference to the accompanying Figures 1 - 13.

Brief Description of the Figures

Figure 1 shows DNA and amino acid sequences of the OKT3 light chain;

Figure 2 shows DNA and amino acid sequences of the OKT3 heavy chain;

Figure 3 shows the alignment of the OKT3 light variable region amino acid sequence with that of the light variable region of the human antibody REI;

Figure 4 shows the alignment of the OKT3 heavy variable region amino acid sequence with that of the heavy variable region of the human antibody KOL;

Figure 5 shows the heavy variable region amino acid sequences of OKT3, KOL and various corresponding CDR grafts;

Figure 6 shows the light variable region amino acid sequences of OKT3, REI and various corresponding CDR grafts;

Figure 7 shows a graph of binding assay results for various grafted OKT3 antibodies'

Figure 8 shows a graph of blocking assay results for various grafted OKT3 antibodies;

Figure 9 shows a similar graph of blocking assay results;

Figure 10 shows similar graphs for both binding assay and blocking assay results;

Figure 11 shows further similar graphs for both binding assay and blocking assay results;

Figure 12 shows a graph of competition assay results for a minimally grafted OKT3 antibody compared with the OKT3 murine reference standard, and

Figure 13 shows a similar graph of competition assay results comparing a fully grafted OKT3 antibody with the murine reference standard.

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

EXAMPLE 1

CDR-GRAFTING OF OKT3

MATERIAL AND METHODS

1. INCOMING CELLS

Hybridoma cells producing antibody OKT3 were provided by Ortho (seedlot 4882.1) and were grown up in antibiotic free Dulbecco's Modified Eagles Medium (DMEM) supplemented with glutamine and 5% foetal calf serum, and divided to provide both an overgrown supernatant for evaluation and cells for extraction of RNA. The overgrown supernatant was shown to contain 250 ug/mL murine IgG2a/kappa antibody. The supernatant was negative for murine lambda light chain and IgG1, IgG2b, IgG3, IgA and IgM heavy chain. 20mL of supernatant was assayed to confirm that the antibody present was OKT3.

2. MOLECULAR BIOLOGY PROCEDURES

Basic molecular biology procedures were as described in Maniatis et al (ref. 9) with, in some cases, minor modifications. DNA sequencing was performed as described in Sanger et al (ref. 11) and the Amersham International Plc sequencing handbook. Site directed mutagenesis was as described in Kramer et al (ref. 12) and the Anglian Biotechnology Ltd. handbook. COS cell expression and metabolic labelling studies were as described in Whittle et al (ref. 13)

3. RESEARCH ASSAYS

3.1. ASSEMBLY ASSAYS

Assembly assays were performed on supernatants from transfected COS cells to determine the amount of intact IgG present.

3.1.1. COS CELLS TRANSFECTED WITH MOUSE OKT3 GENES

The assembly assay for intact mouse IgG in COS cell supernatants was an ELISA with the following format:

96 well microtitre plates were coated with F(ab')2 goat anti-mouse IgG Fc. The plates were washed in water and samples added for 1 hour at room temperature. The plates were washed and F(ab')2 goat anti-mouse IgG F(ab')2 (HRPO conjugated) was then added. Substrate was added to reveal the reaction. UPC10, a mouse IgG2a myeloma, was used as a standard.

3.1.2. COS AND CHO CELLS TRANSFECTED WITH CHIMERIC OR CDR-GRAFTED OKT3 GENES

The assembly assay for chimeric or CDR-grafted antibody in COS cell supernatants was an ELISA with the following format:

96 well microtitre plates were coated with F(ab')2 goat anti-human IgG Fc. The plates were washed and samples added and incubated for 1 hour at room temperature. The plates were washed and monoclonal mouse anti-human kappa chain was added for 1 hour at room temperature.

The plates were washed and F(ab')2 goat anti-mouse IgG Fc (HRPO conjugated) was added. Enzyme substrate was added to reveal the reaction.

Chimeric B72.3 (IgG4) (ref. 13) was used as a standard. The use of a monoclonal anti-kappa chain in this assay allows grafted antibodies to be read from the chimeric standard.

3.2. ASSAY FOR ANTIGEN BINDING ACTIVITY

Material from COS cell supernatants was assayed for OKT3 antigen binding activity onto CD3 positive cells in a direct assay. The procedure was as follows:

HUT 78 cells (human T cell line, CD3 positive) were maintained in culture. Monolayers of HUT 78 cells were prepared onto 96 well ELISA plates using poly-L-lysine and glutaraldehyde. Samples were added to the monolayers for 1 hour at room temperature.

The plates were washed gently using PBS. F(ab')2 goat anti-human IgG Fc (HRPO conjugated) or F(ab')2 goat anti-mouse IgG Fc (HRPO conjugated) was added as appropriate for humanised or mouse samples. Substrate was added to reveal the reaction.

The negative control for the cell-based assay was chimeric B72.3. The positive control was mouse Orthomune OKT3 or chimeric OKT3, when available. This cell-based assay was difficult to perform, and an alternative assay was developed for CDR-grafted OKT3 which was more sensitive and easier to carry out.

In this system CDR-grafted OKT3 produced by COS cells was tested for its ability to bind to the CD3-positive HPB-ALL (human peripheral blood acute lymphocytic leukemia) cell line. It was also tested for its ability to block the binding of murine OKT3 to these cells. Binding was measured by the following procedure: HPB-ALL cells were harvested from tissue culture. Cells were incubated at 4°C for 1 hour with various dilutions of test antibody, positive control antibody, or negative control antibody. The cells were washed once and incubated at 4°C for 1 hour with an FITC-labelled goat anti-human IgG (Fc-

specific, mouse absorbed). The cells were washed twice and analysed by cytofluorography. Chimeric OKT3 was used as a positive control for direct binding. Cells incubated with mock- transfected COS cell supernatant, followed by the FITC-labelled goat anti-human IgG, provided the negative control. To test the ability of CDR-grafted OKT3 to block murine OKT3 binding, the HPB-ALL cells were incubated at 4°C for 1 hour with various dilutions of test antibody or control antibody. A fixed saturating amount of FITC OKT3 was added. The samples were incubated for 1 hour at 4°C, washed twice and analysed by cytofluorography. FITC-labelled OKT3 was used as a positive control to determine maximum binding. Unlabelled murine OKT3 served as a reference standard for blocking. Negative controls were unstained cells with or without mock-transfected cell supernatant. The ability of the CDR-grafted OKT3 light chain to bind CD3-positive cells and block the binding of murine OKT3 was initially tested in combination with the chimeric OKT3 heavy chain. The chimeric OKT3 heavy chain is composed of the murine OKT3 variable region and the human IgG4 constant region. The chimeric heavy chain gene is expressed in the same expression vector used for the CDR-grafted genes. The CDR-grafted light chain expression vector and the chimeric heavy chain expression vector were co-transfected into COS cells. The fully chimeric OKT3 antibody (chimeric light chain and chimeric heavy chain) was found to be fully capable of binding to CD3 positive cells and blocking the binding of murine OKT3 to these cells.

3.3 DETERMINATION OF RELATIVE BINDING AFFINITY

The relative binding affinities of CDR-grafted

anti-CD3 monoclonal antibodies were determined by competition binding (ref. 6) using the HPB-ALL human T cell line as a source of CD3 antigen, and fluorescein-conjugated murine OKT3 (Fl-OKT3) of known binding affinity as a tracer antibody. The binding affinity of Fl-OKT3 tracer antibody was determined by a direct binding assay in which increasing amounts of Fl-OKT3 were incubated with HPB-ALL ($5x10^5$) in PBS with 5% foetal calf serum for 60 min. at 4°C. Cells were washed, and the fluorescence intensity was determined on a FACScan flow cytometer calibrated with quantitative microbead standards (Flow Cytometry Standards, Research Triangle Park, NC). Fluorescence intensity per antibody molecule (F/P ratio) was determined by using microbeads which have a predetermined number of mouse IgG antibody binding sites (Simply Cellular beads, Flow Cytometry Standards). F/P equals the fluorescence intensity of beads saturated with Fl-OKT3 divided by the number of binding sites per bead. The amount of bound and free Fl-OKT3 was calculated from the mean fluorescence intensity per cell, and the ratio of bound/free was plotted against the number of moles of antibody bound. A linear fit was used to determine the affinity of binding (absolute value of the slope).

For competitive binding, increasing amounts of competitor antibody were added to a sub-saturating dose of Fl-OKT3 and incubated with $5x10^5$ HPB-ALL in 200 Ml of PBS with 5% foetal calf serum, for 60 min at 4°C. The fluorescence intensities of the cells were measured on a FACScan flow cytometer calibrated with quantitative microbead standards. The concentrations of bound and free Fl-OKT3 were calculated. The affinities of competing anti-

bodies were calculated from the equation [X]-[OKT3] = (1/Kx) - (1/Ka), where Ka is the affinity of murine OKT3, Kx is the affinity of competitor X, [ ] is the concentration of competitor antibody at which bound/free binding is R/2, and R is the maximal bound/free binding.

4. cDNA LIBRARY CONSTRUCTION

4.1. mRNA PREPARATION AND cDNA SYNTHESIS

OKT3 producing cells were grown as described above and 1.2 x 10^9 cells harvested and mRNA extracted using the guanidinium/LiCl extraction procedure. cDNA was prepared by priming from Oligo-dT to generate full length cDNA. The cDNA was methylated and EcoR1 linkers added for cloning.

4.2. LIBRARY CONSTRUCTION

The cDNA library was ligated to pSP65 vector DNA which had been EcoR1 cut and the 5′ phosphate groups removed by calf intestinal phosphatase (EcoR1/CIP). The ligation was used to transform high transformation efficiency Escherichia coli (E.coli) HB101. A cDNA library was prepared. 3600 colonies were screened for the light chain and 10000 colonies were screened for the heavy chain.

5. SCREENING

E.coli colonies positive for either heavy or light chain probes were identified by oligonucleotide screening using the oligonucleotides:
5′ TCCAGATGTTAACTGCTCAC for the light chain, which is complementary to a sequence in the mouse kappa constant region, and 5′ CAGGGGCCAGTGGATGGATAGAC for the heavy chain which is complementary to a sequence in the mouse IgG2a constant CH1 domain region. 12 light chain and 9 heavy chain clones

were identified and taken for second round screening. Positive clones from the second round of screening were grown up and DNA prepared. The sizes of the gene inserts were estimated by gel electrophoresis and inserts of a size capable of containing a full length cDNA were subcloned into M13 for DNA sequencing.

6. DNA SEQUENCING

Clones representing four size classes for both heavy and light chains were obtained in M13. DNA sequence for the 5′ untranslated regions, signal sequences, variable regions and 3′ untranslated regions of full length cDNAs [Figures 1(a) and 2(a)] were obtained and the corresponding amino acid sequences predicted [(Figures 1(b) and 2(b)]. In Figure 1(a) the untranslated DNA regions are shown in uppercase, and in both Figures 1 and 2 the signal sequences are underlined.

7. CONSTRUCTION OF cDNA EXPRESSION VECTORS

Celltech expression vectors are based on the plasmid pEE6hCMV (ref. 14). A polylinker for the insertion of genes to be expressed has been introduced after the major immediate early promoter/enhancer of the human Cytomegalovirus (hCMV). Marker genes for selection of the plasmid in transfected eukaryotic cells can be inserted as BamH1 cassettes in the unique BamH1 site of pEE6 hCMV; for instance, the neo marker to provide pEE6 hCMV neo. It is usual practice to insert the neo and gpt markers prior to insertion of the gene of interest, whereas the GS marker is inserted last because of the presence of internal EcoR1 sites in the cassette.

The selectable markers are expressed from the SV40 late promoter which also provides an origin of replication so that the vectors can be used for expression in the COS cell transient expression system.

The mouse sequences were excised from the M13 based vectors described above as EcoR1 fragments and cloned into either pEE6-hCMV-neo for the heavy chain and into EE6-hCMV-gpt for the light chain to yield vectors pJA136 and pJA135 respectively.

8. EXPRESSION OF cDNAS IN COS CELLS

Plasmids pJA135 and pJA136 were co-transfected into COS cells and supernatant from the transient expression experiment was shown to contain assembled antibody which bound to T-cell enriched lymphocytes. Metabolic labelling experiments using ^{35}S methionine showed expression and assembly of heavy and light chains.

9. CONSTRUCTION OF CHIMERIC GENES

Construction of chimeric genes followed a previously described strategy [Whittle et al (ref. 13)]. A restriction site near the 3′ end of the variable domain sequence is identified and used to attach an oligonucleotide adapter coding for the remainder of the mouse variable region and a suitable restriction site for attachment to the constant region of choice.

9.1. LIGHT CHAIN GENE CONSTRUCTION

The mouse light chain cDNA sequence contains an AvaI site near the 3′ end of the variable region [Fig. 1(a)]. The majority of the sequence of the variable region was isolated as a 396 bp. EcoR1-AvaI fragment. An oligonucleotide adapter was designed to replace the remainder of the 3′

region of the variable region from the AvaI site and to include the 5′ residues of the human constant region up to and including a unique NarI site which had been previously engineered into the constant region.

A HindIII site was introduced to act as a marker for insertion of the linker.

The linker was ligated to the V_L fragment and the 413 bp EcoRI-NarI adapted fragment was purified from the ligation mixture.

The constant region was isolated as an NarI-BamHI fragment from an M13 clone NW361 and was ligated with the variable region DNA into an EcoRI/BamHI/ClP pSP65 treated vector in a three way reaction to yield plasmid JA143. Clones were isolated after transformation into E.coli and the linker and junction sequences were confirmed by the presence of the HindIII site and by DNA sequencing.

9.2 LIGHT CHAIN GENE CONSTRUCTION - VERSION 2

The construction of the first chimeric light chain gene produces a fusion of mouse and human amino acid sequences at the variable-constant region junction. In the case of the OKT3 light chain the amino acids at the chimera junction are:

.....Leu-Glu-Ile-Asn-Arg/ -/Thr-Val-Ala -Ala

VARIABLE CONSTANT

This arrangement of sequence introduces a potential site for Asparagine (Asn) linked (N-linked) glycosylation at the V-C junction. Therefore, a second version of the chimeric light chain oligonucleotide adapter was designed in which the threonine (Thr), the first amino acid of the human constant region, was replaced with the equivalent amino acid from the mouse constant region, Alanine (Ala).

An internal Hind111 site was not included in this adapter, to differentiate the two chimeric light chain genes.

The variable region fragment was isolated as a 376 bp EcoR1-Aval fragment. The oligonucleotide linker was ligated to Nar1 cut pNW361 and then the adapted 396bp constant region was isolated after recutting the modified pNW361 with EcoR1. The variable region fragment and the modified constant region fragment were ligated directly into EcoR1/C1P treated pEE6hCMVneo to yield pJA137. Initially all clones examined had the insert in the incorrect orientation. Therefore, the insert was re-isolated and recloned to turn the insert round and yield plasmid pJA141. Several clones with the insert in the correct orientation were obtained and the adapter sequence of one was confirmed by DNA sequencing

9.3. HEAVY CHAIN GENE CONSTRUCTION

9.3.1. CHOICE OF HEAVY CHAIN GENE ISOTYPE

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

9.3.2. GENE CONSTRUCTION

The heavy chain cDNA sequence showed a Ban1 site near the 3′ end of the variable region [Fig. 2(a)]. The majority of the sequence of the variable region was isolated as a 426bp. EcoR1/C1P/Ban1 fragment. An oligonucleotide adapter was designated to replace the remainder of the 3′ region of the variable region from the Ban1 site up to and including a unique HindIII site which had been previously engineered into the first two amino acids of the constant region.

The linker was ligated to the V_H fragment and the EcoR1-Hind111 adapted fragment was purified from the ligation mixture.

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

10. CONSTRUCTION OF CHIMERIC EXPRESSION VECTORS

10.1. neo AND gpt VECTORS

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

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

The chimeric heavy chain gene was isolated from pJA142 as a 2.5Kbp EcoR1/BamH1 fragment and cloned into the EcoR1/Bc11/C1P treated vector fragment of a derivative of pEE6hCMVgpt to yield plasmid pJA144.

10.2. GS SEPARATE VECTORS

GS versions of pJA141 and pJA144 were constructed by replacing the neo and gpt cassettes by a BamH1/Sa11/C1P treatment of the plasmids, isolation of the vector fragment and ligation to a GS-containing fragment from the plasmid pRO49 to yield the light chain vector pJA179 and the heavy chain vector pJA180.

10.3. GS SINGLE VECTOR CONSTRUCTION

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

and with transcription of the genes being head to tail e.g. cL>cH>GS were constructed. These plasmids were made by treating pJA179 or pJA180 with BamH1/C1P and ligating in a Bgl11/Hind111 hCMV promoter cassette along with either the Hind111/BamH1 fragment from pJA141 into pJA180 to give the cH-cL-GS plasmid pJA182 or the Hind111/BamH1 fragment from pJA144 into pJA179 to give the cL-cH-GS plasmid pJA181.

11. EXPRESSION OF CHIMERIC GENES

11.1. EXPRESSION IN COS CELLS

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

11.2 EXPRESSION IN CHINESE HAMSTER OVARY (CHO) CELLS

Stable cell lines have been prepared from plasmids PJA141/pJA144 and from pJA179/pJA180, pJA181 and pJA182 by transfection into CHO cells.

12. CDR-GRAFTING

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

12.1. VARIABLE REGION ANALYSIS

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

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

(a) By examination of antibody X-ray crystal structures the antigen binding surface can be predominantly located on a series of loops, three per domain, which extend from the B-barrel framework.

(b) By analysis of antibody variable domain sequences regions of hypervariability [termed the Complementarity Determining Regions (CDRs) by Wu and Kabat (ref. 5)] can be identified. In the most but not all cases these CDRs correspond to, but extend a short way beyond, the loop regions noted above.

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

12.1.1. LIGHT CHAIN

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

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

Residues underlined in Figure 3 are amino acids. RE1 was chosen as the human framework because the light chain is a kappa chain and the kappa variable regions show higher homology with the mouse sequences than a lambda light variable region, e.g. KOL (see below). RE1 was chosen in preference to another kappa light chain because the X-ray structure of the light chain has been determined so that a structural examination of individual residues could be made.

12.1.2. HEAVY CHAIN

Similarly Figure 4 shows an alignment of sequences for the human framework region KOL and the OKT3 heavy variable region. The structural loops and CDRs believed to correspond to the antigen binding region are marked. Also marked are a number of other residues which may also contribute to antigen binding as described in 12.1(c). The residue type key and other indicators used in Figure 4 are the same as those used in Figure 3. KOL was chosen as the heavy chain framework because the X-ray structure has been determined to a better resolution than, for example, NEWM and also the sequence alignment of OKT3 heavy variable region showed a slightly better homology to KOL than to NEWM.

12.2. DESIGN OF VARIABLE GENES

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

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

12.3. GENE CONSTRUCTION

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

13. CONSTRUCTION OF EXPRESSION VECTORS

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

TABLE 1 CDR-GRAFTED GENE CONSTRUCTS

CODE	MOUSE SEQUENCE CONTENT	METHOD OF CONSTRUCTION	KOZAK SEQUENCE -	KOZAK SEQUENCE +
LIGHT CHAIN	ALL HUMAN FRAMEWORK RE1			
121	26-32, 50-56, 91-96 inclusive	SDM and gene assembly	+	n.d.
121A	26-32, 50-56, 91-96 inclusive +1, 3, 46, 47	Partial gene assembly	n.d.	+
121B	26-32, 50-56, 91-96 inclusive + 46, 47	Partial gene assembly	n.d.	+
221	24-24, 50-56, 91-96 inclusive	Partial gene assembly	+	+
221A	24-34, 50-56, 91-96 inclusive +1, 3, 46, 47	Partial gene assembly	+	+
221B	24-34, 50-56, 91-96 inclusive +1, 3	Partial gene assembly	+	+
221C	24-34, 50-56, 91-96 inclusive	Partial gene assembly	+	+
HEAVY CHAIN	ALL HUMAN FRAMEWORK KOL			
121	26-32, 50-56, 95-100B inclusive	Gene assembly	n.d.	+
131	26-32, 50-58, 95-100B inclusive	Gene assembly	n.d.	+
141	26-32, 50-65, 95-100B inclusive	Partial gene assembly	+	n.d.
321	26-35, 50-56, 95-100B inclusive	Partial gene assembly	+	n.d.
331	26-35, 50-58, 95-100B inclusive	Partial gene assembly	+	
		Gene assembly		+
341	26-35, 50-65, 95-100B inclusive	SDM	+	
		Partial gene assembly		+
341A	26-35, 50-65, 95-100B inclusive +6, 23, 24, 48, 49, 71, 73, 76, 78, 88, 91 (+63 = human)	Gene assembly	n.d.	+
341B	26-35, 50-65, 95-100B inclusive + 48, 49, 71, 73, 76, 78, 88, 91 (+63 + human)	Gene assembly	n.d.	+

KEY

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

14. EXPRESSION OF CDR-GRAFTED GENES

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

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

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

When direct binding to antigen on HUT 78 cells was measured, a construct designed to include mouse sequence based on loop length (gL121) did not lead to active antibody in association with mH or cH. A construct designed to include mouse sequence based on Kabat CDRs (gL221) demonstrated some weak binding in association with mH or cH. However, when framework residues 1, 3, 46, 47 were changed from the human to the murine OKT3 equivalents based on the arguments outlined in Section 12.1 antigen binding was demonstrated when both of the new constructs, which were termed 121A and 221A were co-expressed with cH. When the effects of these residues were examined in more detail, it appears that residues 1 and 3 are not major contributing residues as the product of the gL221B gene shows little detectable binding activity in association with cH. The light chain product of gL221C, in which mouse sequences are present at 46 and 47, shows good binding activity in association with cH.

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

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

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

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

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

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

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

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

14.3 PRODUCTION OF FULLY CDR-GRAFTED ANTIBODY

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

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

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

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

An analysis of the above results is given below.

15. DISCUSSION OF CDR-GRAFTING RESULTS

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

15.1. LIGHT CHAIN

15.1.1. EXTENT OF THE CDRs

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

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

15.1.2. FRAMEWORK RESIDUES

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

see Figure 3 and Table 1) was made, cloned in EE6hCMVneo and co-expressed with cH (pJA144). The resultant antibody was well expressed and showed good binding activity. When the related genes gL221B (gL221 + D1Q, Q3V) and gL221C (gL221 + L46R, L47W) were made and similarly tested, while both genes produced antibody when co-expressed with cH, only the gL221C/cH combination showed good antigen binding. When the gL121A (gL121 + D1Q, Q3V, L46R, L47W) gene was made and co-expressed with cH, antibody was produced which also bound to antigen.

15.2. HEAVY CHAIN

15.2.1. EXTENT OF THE CDRs

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

being degraded internally. In some experiments trace amounts of antibody could be detected in ^{35}S labelling studies.
As no net antibody was produced, analysis of these constructs was not pursued further.
When, however, a combination of the loop choice and the Kabat choice for CDR1 was tested (mouse amino acids 26-35 inclusive) and in which residues 31 (Ser to Arg), 33 (Ala to Thr), and 35 (Tyr to His) were changed from the human residues to the mouse residue and compared to the first series, antibody was produced for gH321, kgH331 and kgH341 when co-expressed with cL. Expression was generally low and could not be markedly improved by the insertion of the Kozak consensus sequence 5′ to the ATG of the signal sequence of the gene, as distinct from the case of the gL genes where such insertion led to a 2-5 fold increase in net antibody production. However, only in the case of gH341/mL or kgH341/cL could marginal antigen binding activity be demonstrated. When the kgH341 gene was co-expressed with kgL221A, the net yield of antibody was too low to give a signal above the background level in the antigen binding assay.

15.2.2. FRAMEWORK RESIDUES

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

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

15.3 INTERIM CONCLUSIONS

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

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

16. <u>FURTHER CDR-GRAFTING EXPERIMENTS</u>

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

gH341A (plasmid pJA185) with either mouse OKT3 or human KOL residues at 6, 23, 24, 48, 49, 63, 71, 73, 76, 78, 88 and 91, as indicated. The CDR-grafted light chain genes used in these further experiments were gL221, gL221A, gL221B and gL221C as described above.

TABLE 2

OKT3 HEAVY CHAIN CDR GRAFTS

1. gH341 and derivatives

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

OKT3 LIGHT CHAIN CDR GRAFTS

2. gL221 and derivatives

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

MURINE RESIDUES ARE UNDERLINED

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

The results of the assays for various grafted heavy chains co-expressed with the gL221C light chain are given in Figures 7 and 8 (for the JA184, JA185, JA197 and JA198 constructs - see Table 2), in Figure 9 (for the JA183, JA184, JA185 and JA197 constructs) in Figure 10 (for the chimeric, JA185, JA199, JA204, JA205, JA207, JA208 and JA209 constructs) and in Figure 11 (for the JA183, JA184, JA185, JA198, JA203, JA205 and JA206 constructs).

The basic grafted product without any human to murine changes in the variable frameworks, i.e. gL221 co-expressed with gh341 (JA178), and also the "fully grafted" product, having most human to murine changes in the grafted heavy chain framework, i.e. gL221C co-expressed with gh341A (JA185), were assayed for relative binding affinity in a competition assay against murine OKT3 reference standard, using HPB-ALL cells. The assay used was as described above in section 3.3. The results obtained are given in Figure 12 for the basic grafted product and in Figure 13 for the fully grafted product. These results indicate that the basic grafted product has neglibible binding ability as compared with the OKT3 murine reference standard; whereas the "fully grafted" product has a binding ability very similar to that of the OKT3 murine reference standard.

The binding and blocking assay results indicate the following:

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

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

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

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

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

EXAMPLE 2

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

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

THE LIGHT CHAIN

The human acceptor framework used for the grafted light chains was RE1. The preferred LCDR2 light chain has human to mouse changes at positions 33, 34, 38, 49 and 89 in addition to the structural loop CDRs. Of these changed positions, positions 33, 34 and 89 fall within the preferred extended CDRs of the present invention (positions 33 and 34 in CDR1 and position 89 in CDR3). The human to murine changes at positions 38 and 49 corresponds to positions at which the amino acid residues are preferably donor murine amino acid residues in accordance with the present invention.

A comparison of the amino acid sequences of the donor murine light chain variable domain and the RE1 human acceptor light chain variable further reveals that the murine and human residues are identical at all of positions 46, 48 and 71 and at all of positions 2, 4, 6, 35, 36, 44, 47, 62, 64-69, 85, 87, 98, 99 and 101 and 102. However the amino acid residue at position 58 in LCDR2 is

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

THE HEAVY CHAIN

The human acceptor framework used for the grafted heavy chains was KOL.

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

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

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

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

EXAMPLE 3

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

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

(a) B72.3 Light Chain

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

CDR Number	Residues
1	24-34
2	50-56
3	90-96

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

B72.3 cH/B72.3 cL

and B72.3 cH/B72.3 gL

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

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

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

(b) B72.3 heavy chain

i. Choice of framework

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

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

Comparison of the B72.3 heavy chain sequence with those in Kabat (ref. 4) for human heavy chains showed clearly that B72.3 had poor homology for KOL and NEWM (for which crystal structures are available) but was very homologous to the heavy chain for EU.

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

CDR Number	Residues
1	27-36
2	50-63
3	93-102

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

ii. Results with grafted heavy chain genes

Expression of grafted heavy chain genes containing all human framework regions with either gL or cL genes produced a grafted antibody with little ability to bind to mucin. The grafted antibody had about 1% the activity of the chimeric antibody.

In these experiments, however, it was noted that the activity of the grafted antibody could be increased to ~ 10% of B72.3 by exposure to pHs of 2-3.5.

This observation provided a clue as to how the activity of the grafted antibody could be improved without acid treatment. It was postulated that acid exposure brought about the protonation of an acidic residue (pKa of aspartic acid = 3.86 and of glutamine acid = 4.25) which in turn caused a change in structure of the CDR loops, or allowed better access of antigen.

From comparison of the sequences of B72.3 (ref. 13) and EU (refs. 4 and 5), it was clear that, in going from the mouse to human frameworks, only two positions had been changed in such a way that acidic residues had been introduced. These

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

Which of these positions might be important was determined by examining the crystal structure of the KOL antibody. In KOL heavy chain, position 81 is far removed from either of the CDR loops.

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

iii. Framework changes in B72.3 gH gene

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

iv. Other framework changes

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

v. Other

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

Comparison of the B72.3 murine and EU heavy chain sequences reveals that the mouse and human residues are identical at positions 23, 24, 71 and 78.
Thus the mutated CDR-grafted B72.3 heavy chain corresponds to a preferred embodiment of the present invention.

EXAMPLE 4

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

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

The human EU framework was used as the acceptor framework for both heavy and light chains. The CDR-grafted antibody currently of choice is provided by co-expression of grafted light chain gL221A and grafted heavy chain gH341D which has a binding affinity for ICAM 1 of about 75% of that of the corresponding mouse-human chimeric antibody.

LIGHT CHAIN

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

HEAVY CHAIN

gH341D has murine CDRs at positions 26-35 (CDR1), 50-56 (CDR2) and94-100B (CDR3). In addition murine residues were used in gH341D at positions 24, 48, 69, 71, 73, 80, 88 and 91. Comparison of the murine anti-ICAM 1 and human EU heavy chain amino acid sequences are identical at positions 23, 49 and 78.

EXAMPLE 5

CDR-Grafting of murine anti-TNFα antibodies

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

61E71

A similar analysis as described above (Example 1, Section 12.1.) was done for 61E71 and for the heavy chain 10 residues were identified at 23, 24, 48, 49, 68, 69, 71, 73, 75 and 88 as residues to potentially retain as murine. The human frameworks chosen for CDR-grafting of this antibody, and the hTNF3 and 101.4 antibodies were RE1 for the light chain and KOL for the heavy chain.
Three genes were built, the first of which contained 23, 24, 48, 49, 71 and 73 [gH341(6)] as murine residues. The second gene also had 75 and 88 as murine residues [gH341(8)] while the third gene additionally had 68, 69, 75 and 88 as murine residues [gH341(10)]. Each was co-expressed with gL221, the minimum grafted light chain (CDRs only). The gL221/gH341(6) and gL221/gH341(8) antibodies both bound as well to TNF as murine 61E71. The gL221/gH341(10) antibody did not express and this combination was not taken further.
Subsequently the gL221/gH341(6) antibody was assessed in an L929 cell competition assay in which the antibody competes against the TNF receptor on L929 cells for binding to TNF in solution. In this assay the gL221/gH341(6) antibody was approximately 10% as active as murine 61E71.

hTNF1

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

Heavy Chain

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

Light Chain

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

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

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

hTNF3

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

101.4

101.4 is a further murine monoclonal antibody able to recognise human TNF-a. The heavy chain of this antibody shows good homology to KOL and so the CDR-grafting has been based on RE1 for the light chain and KOL for the heavy chain. Several grafted heavy chain genes have been constructed with conservative choices for the CDR's (gH341) and which have one or a small number of non-CDR residues at positions 73, 78 or 77-79 inclusive, as the mouse amino acids. These have been co-expressed with cL or gL221. In all cases binding to TNF equivalent to the chimeric antibody is seen and when co-expressed with cL the resultant antibodies are able to compete well in the L929 assay. However, with gL221 the resultant antibodies

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

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

A number of other antibodies including antibodies having specificity for interleukins e.g. IL1 and cancer markers such as carcinoembryonic antigen (CEA) e.g. the monoclonal antibody A5B7 (ref. 21), have been successfully CDR-grafted according to the present invention.
It will be appreciated that the foregoing examples are given by way of illustration only and are not intended to limit the scope of the claimed invention. Changes and modifications may be made to the methods described whilst still falling within the spirit and scope of the invention.

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CLAIMS

1. A CDR-grafted antibody heavy chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 6, 23 and/or 24, 48 and/or 49, 71 and/or 73, 75 and/or 76 and/or 78 and 88 and/or 91.

2. A CDR-grafted heavy chain according to Claim 1 comprising donor residues at positions 23, 24, 49, 71, 73 and 78, or at positions 23, 24 and 49.

3. A CDR-grafted heavy chain according to Claim 2 comprising donor residues at positions 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.

4. A CDR-grafted heavy chain according to Claim 2 or 3, comprising donor residues at one, some or all of positions:
1 and 3,
69 (if 48 is different between donor and acceptor),
38 and 46 (if 48 is the donor residue),
67,
82 and 18 (if 67 is the donor residue),
91, and
any one or more of 9, 11, 41, 87, 108, 110 and 112.

5. A CDR-grafted heavy chain according to any of the preceding comprising donor CDRs at positions 26-35, 50-65 and 95-100.

6. A CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 1 and/or 3 and 46 and/or 47.

7. A CDR-grafted light chain according to Claim 6 comprising donor residues at positions 46 and 47.

8. A CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 46, 48, 58 and 71.

9. A CDR-grafted light chain according to Claim 8 comprising donor residues at positions 46, 48, 58 and 71.

10. A CDR-grafted light chain according to Claim 8 or 9, comprising donor residues at positions 2, 4, 6, 35, 36, 38, 44, 47, 49, 62, 64-69, 85, 87, 98, 99, 101 and 102.

11. A CDR-grafted light chain according to Claim 9 or 10, comprising donor residues at one, some or all of positions:
1 and 3,
63,
60 (if 60 and 54 are able to form a potential saltbridge),
70 (if 70 and 24 are able to form a potential saltbridge),
73 and 21 (if 47 is different between donor and acceptor),
37 and 45 (if 47 if different between donor and acceptor), and
any one or more of 10, 12, 40, 83, 103 and 105.

12. A CDR-grafted light chain according to any one of Claims 6-11, comprising donor CDRs at positions 24-34, 50-56 and 89-97.

13. A CDR-grafted antibody molecule comprising at least one CDR-grafted heavy chain according to any one of Claims 1-5 and at least one CDR-grafted light chain according to any one of Claims 6-12.

14. A CDR-grafted antibody molecule according to Claim 13, which is a site-specific antibody molecule.

15. A CDR-grafted antibody molecule according to Claim 13 which has specificity for an interleukin, hormone or other biologically active compound or a receptor therefor.

16. A CDR-grafted antibody heavy or light chain or molecule according to any one of the preceding claims comprising human acceptor residues and non-human donor residues.

17. A DNA sequence which codes for a CDR-grafted heavy chain according to Claim 1 or a CDR-grafted light chain according to Claim 6 or Claim 8.

18. A cloning or expression vector containing a DNA sequence according to Claim 17.

19. A host cell transformed with a DNA sequence according to Claim 17.

20. A process for the production of a CDR-grafted antibody sequence according to Claim 17 in a transformed host cell.

21. A process for producing a CDR-grafted antibody product comprising:

(a) producing in an expression vector an operon having a DNA sequence which encodes an antibody heavy chain according to Claim 1;

and/or

(b) producing in an expression vector an operon having a DNA sequence which encodes a complementary antibody light chain according to Claim 6 or Claim 8;

(c) transfecting a host cell with the or each vector; and

(d) culturing the transfected cell line to produce the CDR-grafted antibody product.

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

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

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   1  GAATTCCCAA AGACAAAatg gattttcaag tgcagatttt cagcttcctg
  51  ctaatcagtg cctcagtcat aatatccaga ggacaaattg ttctcaccca
 101  gtctccagca atcatgtctg catctccagg ggagaaggtc accatgacct
 151  gcagtgccag ctcaagtgta agttacatga actggtacca gcagaagtca
 201  ggcacctccc ccaaaagatg gatttatgac acatccaaac tggcttctgg
 251  agtccctgct cacttcaggg gcagtgggtc tgggacctct tactctctca
 301  caatcagcgg catggaggct gaagatgctg ccacttatta ctgccagcag
 351  tggagtagta acccattcac gttcggctcg gggacaaagt tggaaataaa
 401  ccgggctgat actgcaccaa ctgtatccat cttcccacca tccagtgagc
 451  agttaacatc tggaggtgcc tcagtcgtgt gcttcttgaa caacttctac
 501  cccaaagaca tcaatgtcaa gtggaagatt gatggcagtg aacgacaaaa
 551  tggcgtcctg aacagttgga ctgatcagga cagcaaagac agcacctaca
 601  gcatgagcag caccctcacg ttgaccaagg acgagtatga acgacataac
 651  agctatacct gtgaggccac tcacaagaca tcaacttcac ccattgtcaa
 701  gagcttcaac aggaatgagt gtTAGAGACA AAGGTCCTGA GACGCCACCA
 751  CCAGCTCCCA GCTCCATCCT ATCTTCCCTT CTAAGGTCTT GGAGGCTTCC
 801  CCACAAGCGC tTACCACTGT TGCGGTGCTC tAAACCTCCT CCCACCTCCT
 851  TCTCCTCCTC CTCCCTTTCC TTGGCTTTTA TCATGCTAAT ATTTGCAGAA
 901  AATATTCAAT AAAGTGAGTC TTTGCCTTGA AAAAAAAAAA AAA
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Fig. 1(a)

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   1  MDFQVQIFSF LLISASVIIS RGQIVLTQSP AIMSASPGEK VTMTCSASSS
  51  VSYMNWYQQK SGTSPKRWIY DTSKLASGVP AHFRGSGSGT SYSLTISGME
 101  AEDAATYYCQ QWSSNPFTFG SGTKLEINRA DTAPTVSIFP PSSEQLTSGG
 151  ASVVCFLNNF YPKDINVKWK IDGSERQNGV LNSWTDQDSK DSTYSMSSTL
 201  TLTKDEYERH NSYTCEATHK TSTSPIVKSF NRNEC*
```

Fig. 1(b)

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

Fig. 2(a)

OKT 3 HEAVY CHAIN PROTEIN SEQUENCE DEDUCED FROM DNA SEQUENCE

```
  1  MERHWIFLLL LSVTAGVHSQ VQLQQSGAEL ARPGASVKMS CKASGYTFTR
 51  YTMHWVKQRP GQGLEWIGYI NPSRGYTNYN QKFKDKATLT TDKSSSTAYM
101  QLSSLTSEDS AVYYCARYYD DHYCLDYWGQ GTTLTVSSAK TTAPSVYPLA
151  PVCGDTTGSS VTLGCLVKGY FPEPVTLTWN SGSLSSGVHT FPAVLQSDLY
201  TLSSSVTVTS STWPSQSITC NVAHPASSTK VDKKIEPRGP TIKPCPPCKC
251  PAPNLLGGPS VFIFPPKIKD VLMISLSPIV TCVVVDVSED DPDVQISWFV
301  NNVEVHTAQT QTHREDYNST LRVVSALPIQ HQDWMSGKEF KCKVNNKDLP
351  APIERTISKP KGSVRAPQVY VLPPPEEEMT KKQVTLTCMV TDFMPEDIYV
401  EWTNNGKTEL NYKNTEPVLD SDGSYFMYSK LRVEKKNWVE RNSYSCSVVH
451  EGLHNHHTTK SFSRTPGK*
```

Fig. 2(b)

```
                1                       23                  42
                NN      N                 N        N    N
RES TYPE        SBspSPESssBSbSsSssPSPSPsPSsse*s*p*Pi^ISsSe
Okt3vl          QIVLTQSPAIMSASPGEKVTMTCSASS.SVSYMNWYQQKSGT
REI             DIQMTQSPSSLSASVGDRVTITCQASQDIIKYLNWYQQTPGK
                ? ?
                  CDR1    (LOOP)          *******
                  CDR1    (KABAT)        ***********


                          56                          85
                N  NN
RES TYPE        *IsiPpIeesesssSBEsePsPSBSSEsPspsPsseesSPePb
Okt3vl          SPKRWIYDTSKLASGVPAHFRGSGSGTSYSLTISGMEAEDAAT
REI             APKLLIYEASNLQAGVPSRFSGSGSGTDYTFTISSLQPEDIAT
                ?  ??                        ?  ?
                      *******  CDR2 (LOOP/KABAT)


                           102   108
RES TYPE        PiPIPies**iPIIsPPSPSPSS
Okt3vl          YYCQQWSSNPFTFGSGTKLEINR
REIvl           YYCQQYQSLPYTFGQGTKLQITR
                           ?    ?
                    ******        CDR3 (LOOP)
                  *********       CRD3 (KABAT)
```

Fig. 3

```
          NN N                    23 26    32 35 N39  43
RES TYPE  SESPs^SBssS^sSSsSpSpSPsPSEbSBssBePiPIpiesss
Okt3h     QVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPGQ
KOL       QVQLVESGGGVVQPGRSLRLSCSSSGFIFSSYAMYWVRQAPGK
               ?                ??
                                      ******    CDR1 (LOOP)
                                          ***** CDR1 (KABAT)

                   52a      60  65     N N  N    82abc     89
RES TYPE IIeIppp^sssssssss^ps^pSSsbSpseSsSseSp^pSpsSBssS^ePb
Okt3vh   GLEWIGYINPSRGYTNTNQKFKDKATLTTDKSSSTAYMQLSSLTSEDSAV
KOL      GLEWVAIIWDDGSDQHYADSVKGRFTISRDNSKNTLFLQMDSLRPEDTGV
            ??                   ? ?  ?  ?             ?
               ************        CDR2  (LOOP)
               ******************  CDR2  (KABAT)

          92 N                   107    113
RES TYPE  PiPIEissssiiisssbibi*EIPIP*spSBSS
Okt3vh    YYCARYYDDHY.......CLDYWGQGTTLTVSS
KOL       YFCARDGGHGFCSSASCFGPDYWGQGTPVTVSS
               ***************** CRD3 (KABAT/LOOP)
```

Fig. 4

OKT 3 HEAVY CHAIN CDR GRAFTS

1. gh341 and derivatives

```
          1                              26        35  39  43
Okt3vh    QVQLQQSGAELARPGASVKMSCKASGYTFTRYTMHWVKQRPGQ
gH341     QVQLVESGGGVVQPGRSLRLSCSSSGYTFTRYTMHWVRQAPGK   JA178
gH341A    QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK   JA185

gH341E    QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK   JA198
gH341*    QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK   JA207
gH341*    QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK   JA209
gH341D    QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK   JA197
gH341*    QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK   JA199
gH341C    QVQLVQSGGGVVQPGRSLRLSCKASGYTFTRYTMHWVRQAPGK   JA184

gH341*    QVQLVQSGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK   JA203
gH341*    QVQLVESGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK   JA205
gH341B    QVQLVESGGGVVQPGRSLRLSCSSSGYTFTRYTMHWVRQAPGK   JA183
gH341*    QVQLVQSGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK   JA204
gH341*    QVQLVESGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK   JA206
gH341*    QVQLVQSGGGVVQPGRSLRLSCSASGYTFTRYTMHWVRQAPGK   JA208
KOL       QVQLVESGGGVVQPGRSLRLSCSSSGFIFSSYAMYWVRQAPGK
```

Fig. 5(i)

```
          44     50                  65                        83
Okt3vh    GLEWIGYINPSRGYTNYNQKFKDKATLTTDKSSSTAYMQLSSLT
gH341     GLEWVAYINPSRGYTNYNQKFKDRFTISRDNSKNTLFLQMDSLR JA178
gH341A    GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKSTAFLQMDSLR JA185

gH341E    GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKSTAFLQMDSLR JA198
gH341*    GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKNTAFLQMDSLR JA207
gH341*    GLEWIGYINPSRGYTNYNQKVKDRFTISRDNSKNTAFLQMDSLR JA209
gH341D    GLEWIGYINPSRGYTNYNQKVKDRFTISTDKSKNTLFLQMDSLR JA197
gH341*    GLEWIGYINPSRGYTNYNQKVKDRFTISRDNSKNTLFLQMDSLR JA199
gH341C    GLEWVAYINPSRGYTNYNQKFKDRFTISRDNSKNTLFLQMDSLR JA184

gH341*    GLEWIGYINPSRGYTNYNOKVKDRFTISTDKSKSTAFLQMDSLR JA207
gH341*    GLEWIGYINPSRGYTNYNOKVKDRFTISTDKSKSTAFLQMDSLR JA205
gH341B    GLEWIGYINPSRGYTNYNOKVKDRFTISTDKSKSTAFLQMDSLR JA183
gH341*    GLEWIGYINPSRGYTNYNOKVKDRFTISTDKSKSTAFLQMDSLR JA204
gH341*    GLEWIGYINPSRGYTNYNOKVKDRFTISTDKSKSTAFLQMDSLR JA206
gH341*    GLEWIGYINPSRGYTNYNOKVKDRFTISTDKSKNTAFLQMDSLR JA208
KOL       GLEWVAIIWDDGSDQHYADSVKGRFTISRDNSKNTLFLQMDSLR
```

Fig. 5(ii)

```
          84          95              102         113
Okt3vh    SEDSAVYYCARYYDDHY.......CLDYWGQGTTLTVSS
gH341     PEDTGVYFCARYYDDHY.......CLDYWGQGTTLTVSS   JA178
gH341A    PEDTAVYYCARYYDDHY.......CLDYWGQGTTLTVSS   JA185

gH341E    PEDTGVYFCARYYDDHY.......CLDYWGQGTTLTVSS   JA198
gH341*    PEDTGVYFCARYYDDHY.......CLDYWGQGTTLTVSS   JA207
gH341D    PEDTGVYFCARYYDDHY.......CLDYWGQGTTLTVSS   JA197
gH341*    PEDTGVYFCARYYDDHY.......CLDYWGQGTTLTVSS   JA209
gH341*    PEDTGVYFCARYYDDHY.......CLDYWGQGTTLTVSS   JA199
gH341C    PEDTGVYFCARYYDDHY.......CLDYWGQGTTLTVSS   JA184

gH341*    PEDTAVYYCARYYDDHY.......CLDYWGQGTTLTVSS   JA203
gH341*    PEDTAVYYCARYYDDHY.......CLDYWGQGTTLTVSS   JA205
gH341B    PEDTAVYYCARYYDDHY.......CLDYWGQGTTLTVSS   JA183
gH341*    PEDTGVYFCARYYDDHY.......CLDYWGQGTTLTVSS   JA204
gH341*    PEDTGVYFCARYYDDHY.......CLDYWGQGTTLTVSS   JA206
gH341*    PEDTGVYFCARYYDDHY.......CLDYWGQGTTLTVSS   JA208
KOL       PEDTGVYFCARDGGHGFCSSASCFGPDYWGQGTPVTVSS
```

Fig. 5(iii)

OKT3 LIGHT CHAIN CDR GRAFTING

1. gL221 and derivatives

```
          1                        24        34       42
Okt3vl    QIVLTQSPAIMSASPGEKVTMTCSASS.SVSYMNWYQQKSGT
gL221     DIQMTQSPSSLSASVGDRVTITCSASS.SVSYMNWYQQTPGK
gL221A    QIVMTQSPSSLSASVGDRVTITCSASS.SVSYMNWYQQTPGK
gL221B    QIVMTQSPSSLSASVGDRVTITCSASS.SVSYMNWYQQTPGK
gL221C    DIQMTQSPSSLSASVGDRVTITCSASS.SVSYMNWYQQTPGK
REI       DIQMTQSPSSLSASVGDRVTITCQASQDIIKYLNWYQQTPGK

          43     50     56                          85
Okt3vl    SPKRWIYDTSKLASGVPAHFRGSGSGTSYSLTISGMEAEDAAT
gL221     APKLLIYDTSKLASGVPSRFSGSGSGTDYTFTISSLQPEDIAT
gL221A    APKRWIYDTSKLASGVPSRFSGSGSGTDYTFTISSLQPEDIAT
gL221B    APKRWIYDTSKLASGVPSRFSGSGSGTDYTFTISSLQPEDIAT
gL221C    APKRWIYDTSKLASGVPSRFSGSGSGTDYTFTISSLQPEDIAT
REI       APKLLIYEASNLQAGVPSRFSGSGSGTDYTFTISSLQPEDIAT

          86   91   96          108
Okt3vl    YYCQQWSSNPFTFGSGTKLEINR
gL221     YYCQQWSSNPFTFGQGTKLQITR
gL221A    YYCQQWSSNPFTFGQGTKLQITR
gL221B    YYCQQWSSNPFTFGQGTKLQITR
gL221C    YYCQQWSSNPFTFGQGTKLQITR
REI       YYCQQYQSLPYTFGQGTKLQITR
```

CDR'S ARE UNDERLINED

FRAMEWORK RESIDUES INCLUDED IN THE GENE ARE DOUBLE UNDERLINED

Fig. 6

Fig. 7

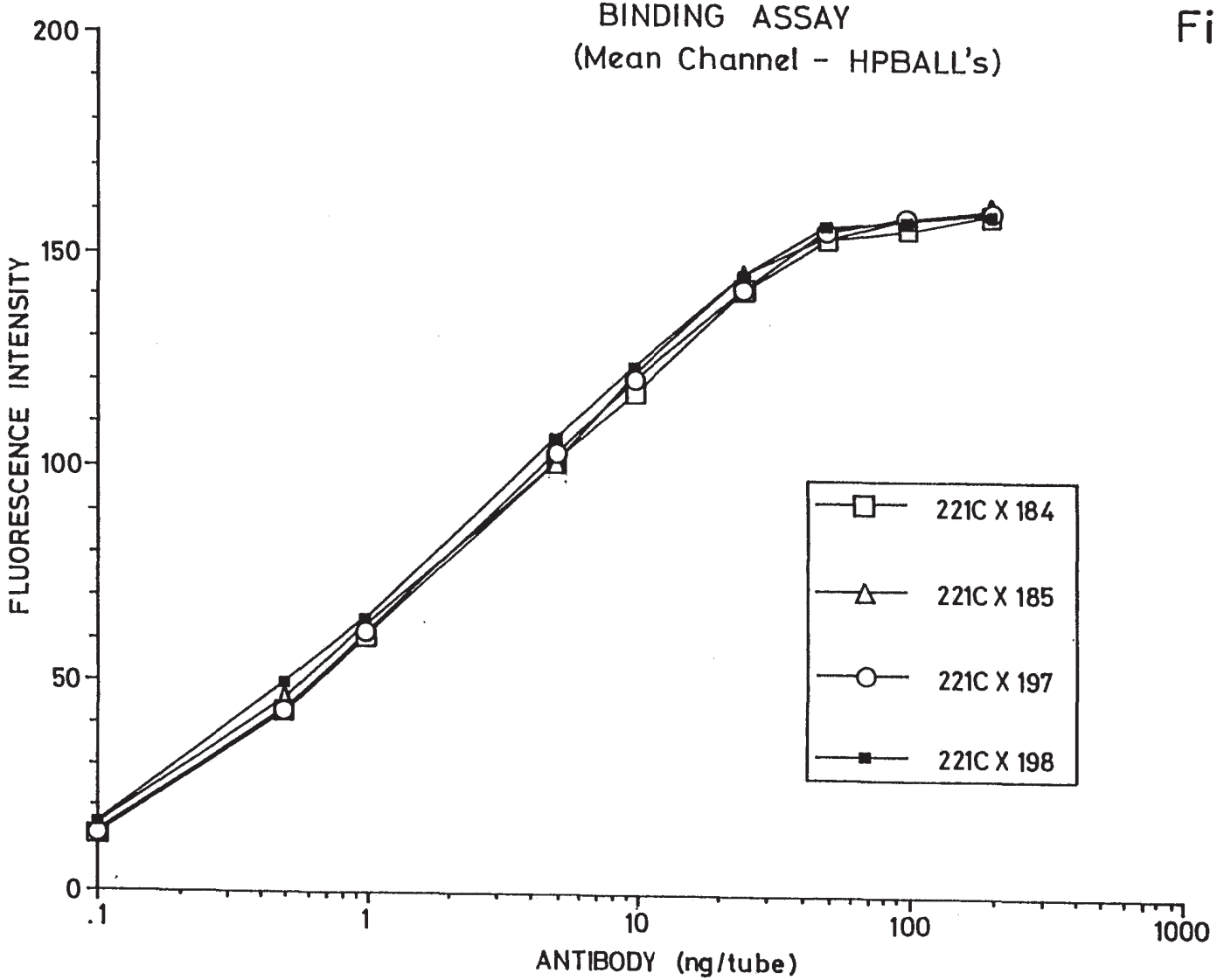
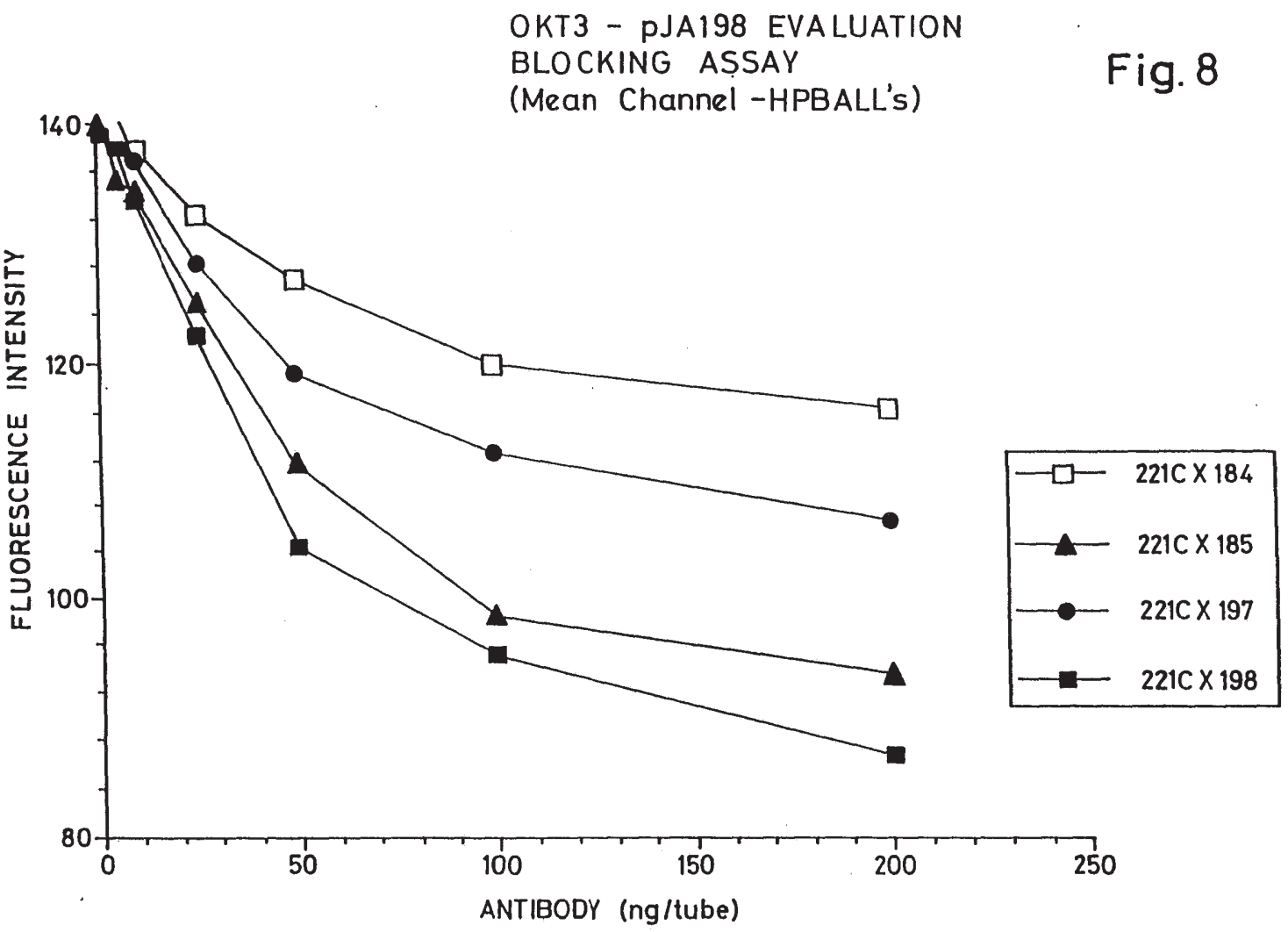


Fig. 8



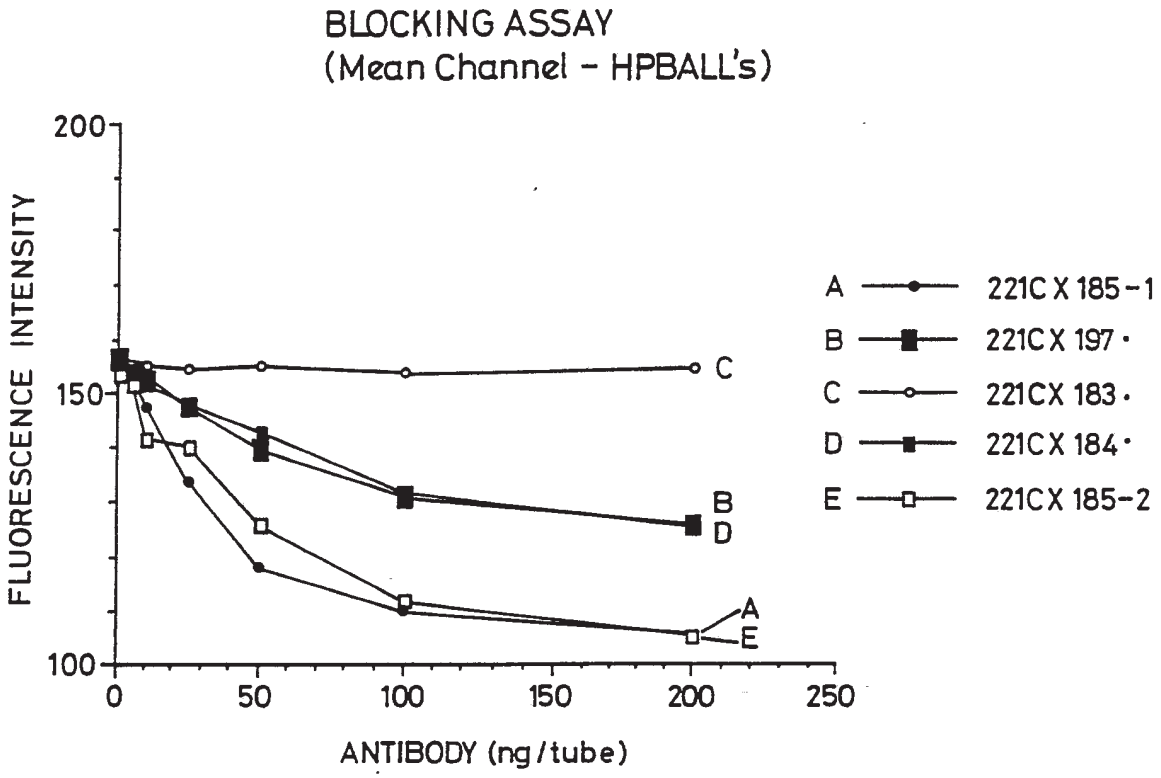
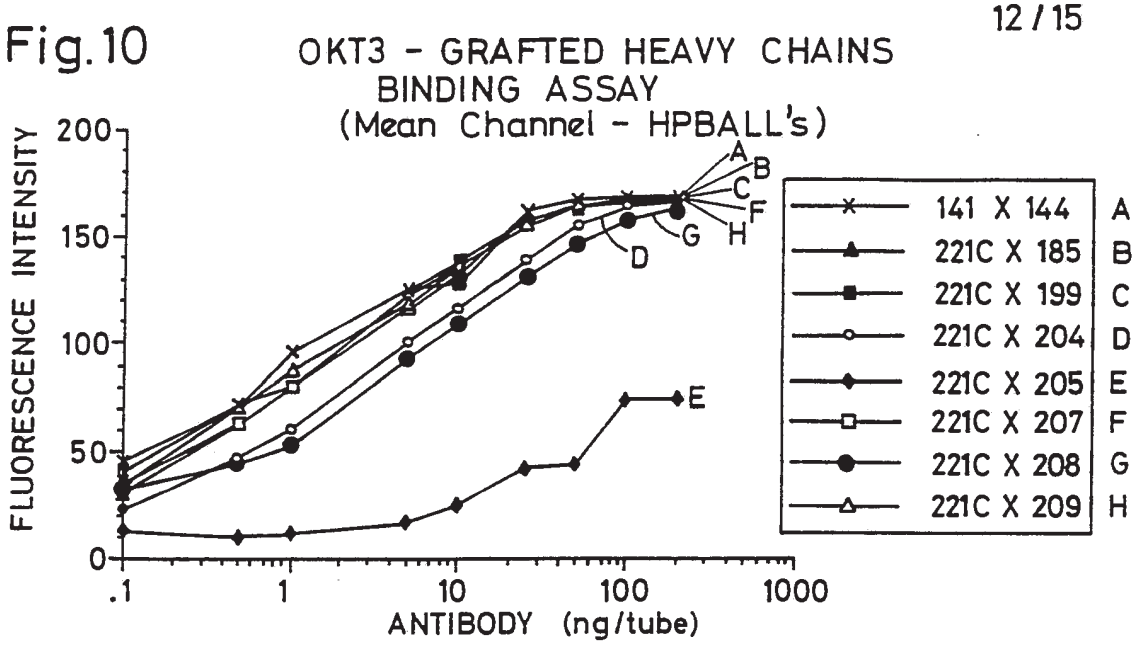
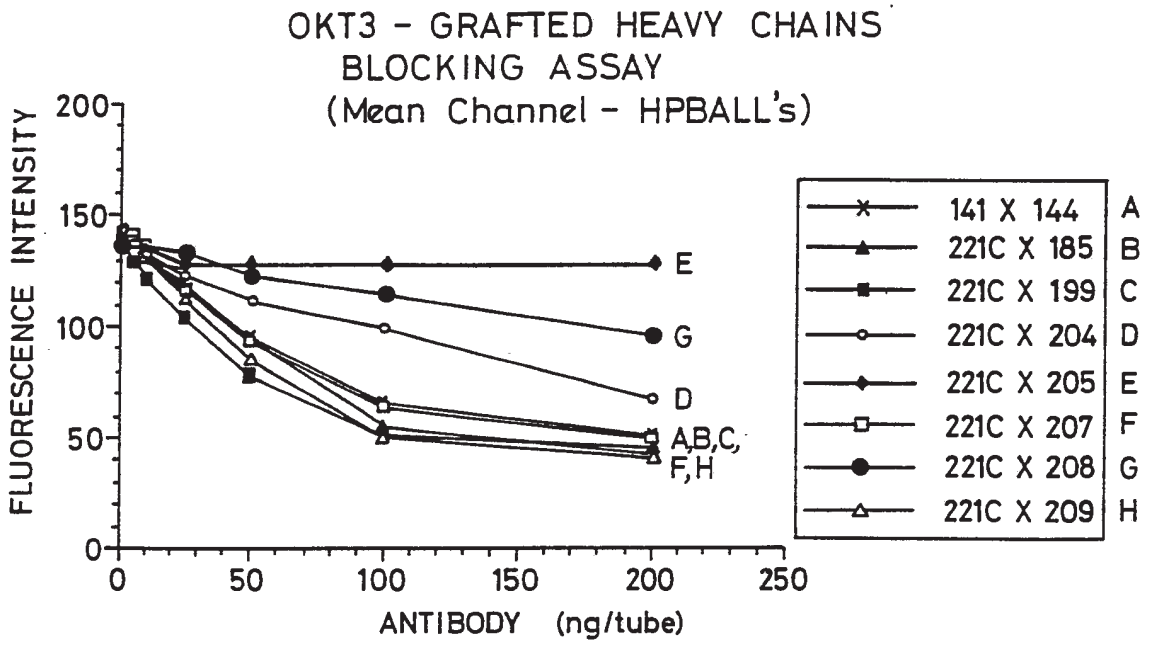


Fig. 9

Fig.10





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(208)	6,__,24,48,49,71,73,__,78,__,__,
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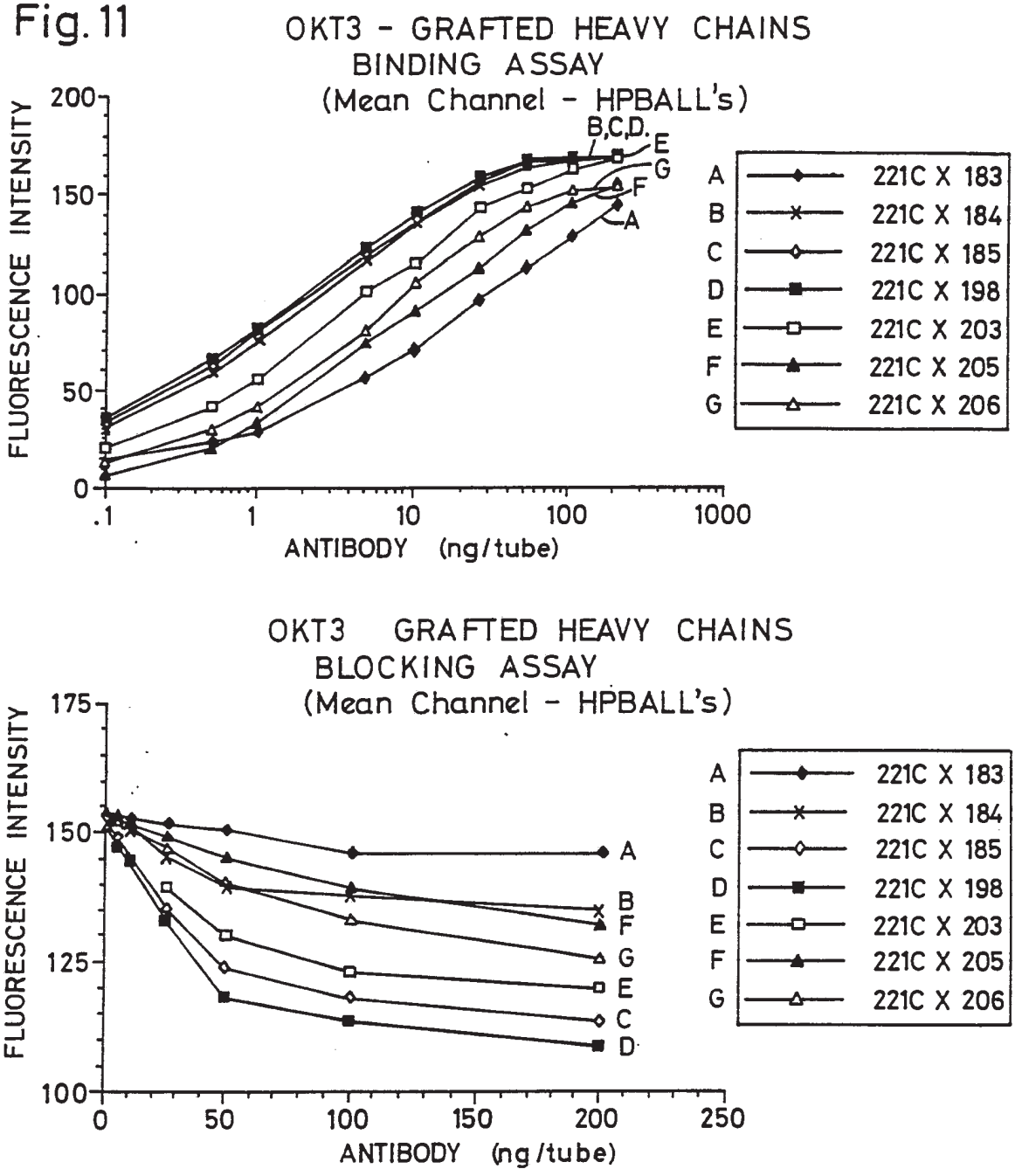
(199)	6,23,24,48,49,__,__,__,__,__,__,
(207)	6,23,24,48,49,71,73,__,78,__,__,
(185)	6,23,24,48,49,71,73,76,78,88,91,
(209)	6,23,24,48,49,__,__,__,78,__,__,
141 X 144	

Fig. 11



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

Fig. 12

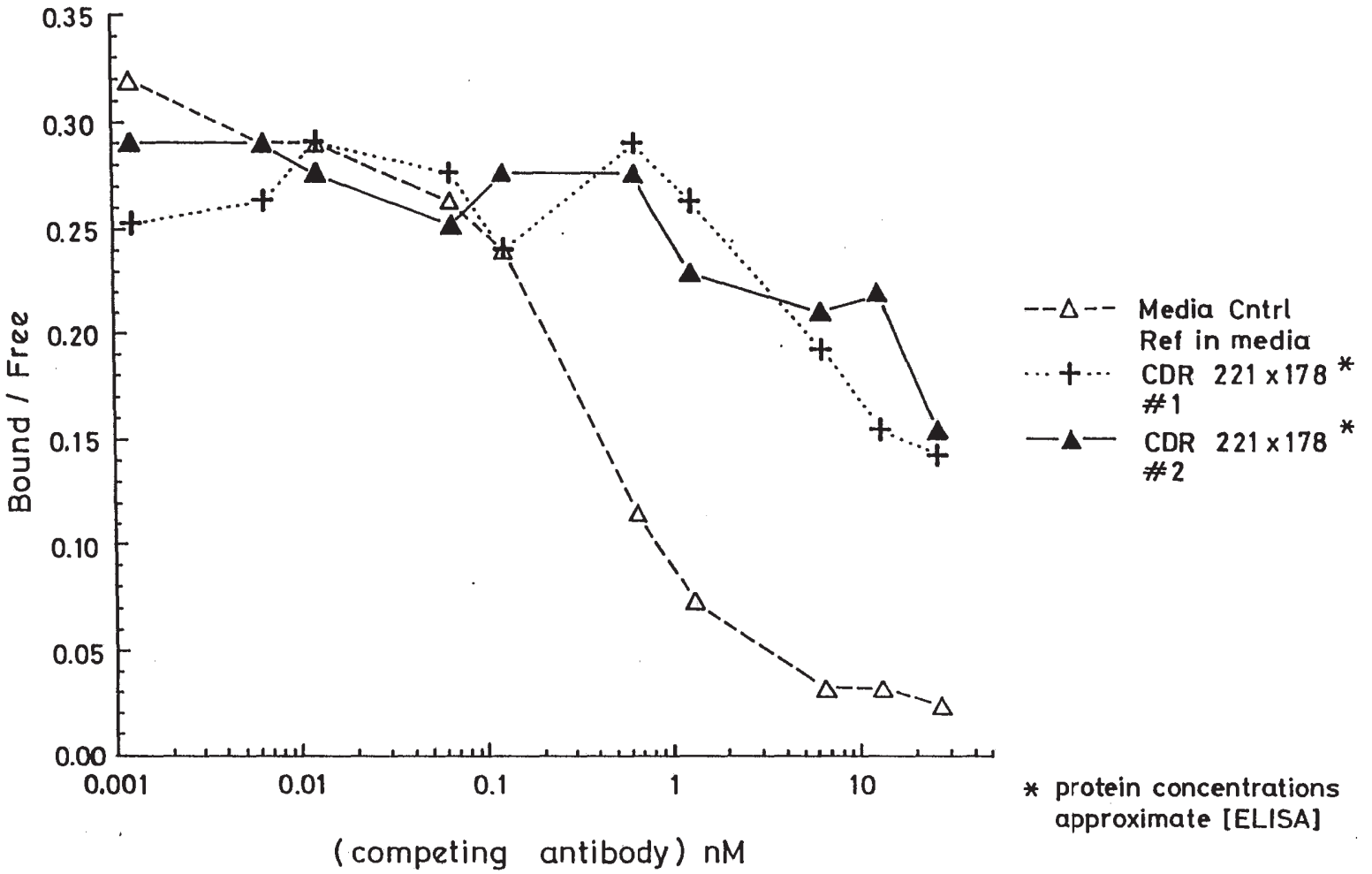
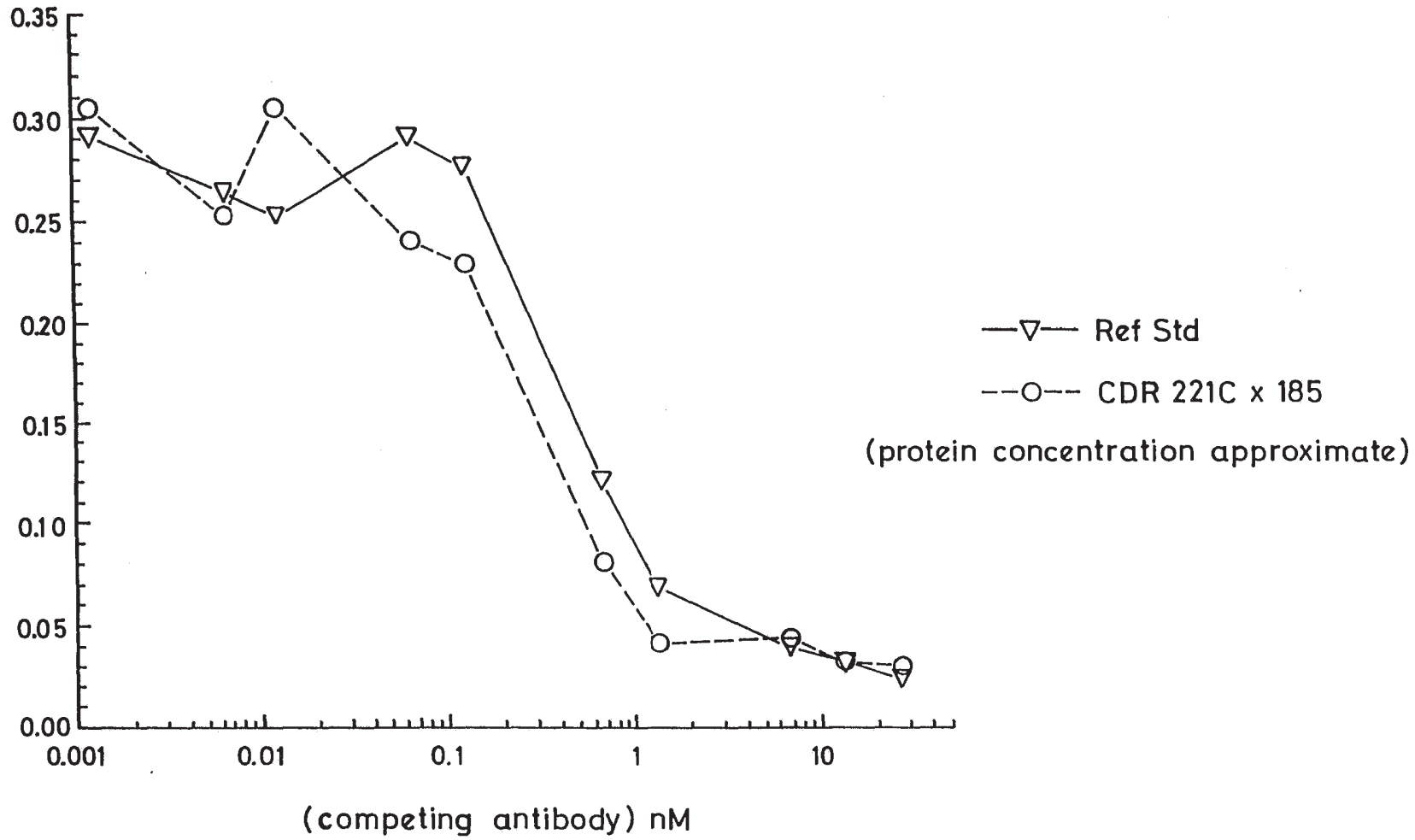


Fig. 13



INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 90/02017

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 P 21/08, C 12 N 15/13, A 61 K 39/395, C 07 K 15/06
C 12 N 5/10, 15/62

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 12 P; C 12 N; A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	EP, A1, 0403156 (GENZYME CORPORATION ET AL.) 19 December 1990, see examples 8-12 and corresponding tables	1,6,8, 13,14-22
Y	Proc. Natl. Acad. Sci. USA, vol. 86, December 1989, C. Queen et al.: "A humanized antibody that binds to the interleukin 2 receptor ", see page 10029- page 10033 see the whole document and in particular page 10031 right col. - page 10032, left col. and page 10033 left col.	1,6,8, 13,14-22
Y	EP, A1, 0328404 (MEDICAL RESEARCH COUNCIL ET AL.) 16 August 1989, see pages 1-3, page 9, lines 49-54 and the claims	1,6,8, 13,14-22

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
11th April 1991	17. 05. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	F.W. HECK

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Nature, vol. 332, March 1988, L. Riechmann et al.: "Reshaping human antibodies for therapy ", see page 323 - page 327 see in particular page 327, right col.	1,6,8, 13,144-22
A	Nature, vol. 321, May 1986, P.T. Jones et al.: "Replacing the complementarity-determining regions in a human antibody with those from a mouse ", see page 522 - page 525 see the whole document	1-22
A	Nature, vol. 328, August 1987, S. Roberts et al.: "Generation of an antibody with enhanced affinity and specificity for its antigen by protein engineering ", see page 731 - page. 734 see the whole document	1,6
A	Science, vol. 239, 1988, M. Verhoeyen et al.: "Reshaping Human Antibodies: Grafting an Antilysozyme Activity ", see page 1534 - page 1536 see the whole document	1,6
A	EP, A2, 0239400 (WINTER, GREGORY PAUL) 30 September 1987, see the whole document	1,6,17-22
A	EP, A1, 0323806 (CIBA-GEIGY AG) 12 July 1989, see pages 2-6	1,6,17-22
A	Nature, vol. 341, October 1989, E.S. Ward et al.: "Binding activities of a repertoire of single immunoglobulin variable domains secreted from Escherichia coli ", see page 544 - page 546	1,6

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/GB 90/02017

SA 43080

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 28/02/91 The European Patent office is in no way liable for theseparticulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A1- 0403156	19/12/90	NONE		
EP-A1- 0328404	16/08/89	AU-D-	3062689	06/09/89
		GB-A-	2216126	04/10/89
		WO-A-	89/07452	24/08/89
EP-A2- 0239400	30/09/87	GB-A-B-	2188638	07/10/87
		JP-A-	62296890	24/12/87
EP-A1- 0323806	12/07/89	AU-D-	2759588	06/07/89
		JP-A-	2154696	14/06/90

For more details about this annex : see Official Journal of the European patent Office, No. 12/82

EPO FORM P0479

15
Ins 1

HUMANISED ANTIBODIES

Field of the Invention

The present invention relates to humanised antibody molecules, to processes for their production using recombinant DNA technology, and to their therapeutic uses.

The term "humanised antibody molecule" is used to describe a molecule having an antigen binding site derived from an immunoglobulin from a non-human species, and remaining immunoglobulin-derived parts of the molecule being derived from a human immunoglobulin. The antigen binding site typically comprises complementarity determining regions (CDRs) which determine the binding specificity of the antibody molecule and which are carried on appropriate framework regions in the variable domains. There are 3 CDRs (CDR1, CDR2 and CDR3) in each of the heavy and light chain variable domains.

In the description, reference is made to a number of publications by number. The publications are listed in numerical order at the end of the description.

Background of the Invention

Natural immunoglobulins have been known for many years, as have the various fragments thereof, such as the Fab, $(Fab')_2$ and Fc fragments, which can be derived by enzymatic cleavage. Natural immunoglobulins comprise a generally Y-shaped molecule having an antigen-binding site towards the end of each upper arm. The remainder of the structure, and particularly the stem of the Y, mediates the effector functions associated with immunoglobulins.

Natural immunoglobulins have been used in assay, diagnosis and, to a more limited extent, therapy. However, such uses, especially in therapy, were hindered until recently by the polyclonal nature of natural immunoglobulins. A significant step towards the realisation of the potential

2

of immunoglobulins as therapeutic agents was the discovery of procedures for the production of monoclonal antibodies (MAbs) of defined specificity (1).

However, most MAbs are produced by hybridomas which are fusions of rodent spleen cells with rodent myeloma cells. They are therefore essentially rodent proteins. There are very few reports of the production of human MAbs.

Since most available MAbs are of rodent origin, they are naturally antigenic in humans and thus can give rise to an undesirable immune response termed the HAMA (Human Anti-Mouse Antibody) response. Therefore, the use of rodent MAbs as therapeutic agents in humans is inherently limited by the fact that the human subject will mount an immunological response to the MAb and will either remove it entirely or at least reduce its effectiveness. In practice, MAbs of rodent origin may not be used in patients for more than one or a few treatments as a HAMA response soon develops rendering the MAb ineffective as well as giving rise to undesirable reactions. For instance, OKT3 a mouse IgG2a/k MAb which recognises an antigen in the T-cell receptor-CD3 complex has been approved for use in many countries throughout the world as an immunosuppressant in the treatment of acute allograft rejection [Chatenoud et al (2) and Jeffers et al (3)]. However, in view of the rodent nature of this and other such MAbs, a significant HAMA response which may include a major anti-idiotype component, may build up on use. Clearly, it would be highly desirable to diminish or abolish this undesirable HAMA response and thus enlarge the areas of use of these very useful antibodies.

Proposals have therefore been made to render non-human MAbs less antigenic in humans. Such techniques can be generically termed "humanisation" techniques. These

techniques typically involve the use of recombinant DNA technology to manipulate DNA sequences encoding the polypeptide chains of the antibody molecule.

Early methods for humanising MAbs involved production of chimeric antibodies in which an antigen binding site comprising the complete variable domains of one antibody is linked to constant domains derived from another antibody. Methods for carrying out such chimerisation procedures are described in EP0120694 (Celltech Limited), EP0125023 (Genentech Inc. and City of Hope), EP-A-0 171496 (Res. Dev. Corp. Japan), EP-A-0 173 494 (Stanford University), and WO 86/01533 (Celltech Limited). This latter Celltech application (WO 86/01533) discloses a process for preparing an antibody molecule having the variable domains from a mouse MAb and the constant domains from a human immunoglobulin. Such humanised chimeric antibodies, however, still contain a significant proportion of non-human amino acid sequence, i.e. the complete non-human variable domains, and thus may still elicit some HAMA response, particularly if administered over a prolonged period [Begent et al (ref. 4)].

In an alternative approach, described in EP-A-0239400 (Winter), the complementarity determining regions (CDRs) of a mouse MAb have been grafted onto the framework regions of the variable domains of a human immunoglobulin by site directed mutagenesis using long oligonucleotides. The present invention relates to humanised antibody molecules prepared according to this alternative approach, i.e. CDR-grafted humanised antibody molecules. Such CDR-grafted humanised antibodies are much less likely to give rise to a HAMA response than humanised chimeric antibodies in view of the much lower proportion of non-human amino acid sequence which they contain.

The earliest work on humanising MAbs by CDR-grafting was carried out on MAbs recognising synthetic antigens, such as the NP or NIP antigens. However, examples in which a mouse MAb recognising lysozyme and a rat MAb recognising an antigen on human T-cells were humanised by CDR-grafting have been described by Verhoeyen et al (5) and Riechmann et al (6) respectively. The preparation of CDR-grafted antibody to the antigen on human T cells is also described in WO 89/07452 (Medical Research Council).

In Riechmann et al/Medical Research Council it was found that transfer of the CDR regions alone [as defined by Kabat refs. (7) and (8)] was not sufficient to provide satisfactory antigen binding activity in the CDR-grafted product. Riechmann et al found that it was necessary to convert a serine residue at position 27 of the human sequence to the corresponding rat phenylalanine residue to obtain a CDR-grafted product having improved antigen binding activity. This residue at position 27 of the heavy chain is within the structural loop adjacent to CDR1. A further construct which additionally contained a human serine to rat tyrosine change at position 30 of the heavy chain did not have a significantly altered binding activity over the humanised antibody with the serine to phenylalanine change at position 27 alone. These results indicate that changes to residues of the human sequence outside the CDR regions, in particular in the structural loop adjacent to CDR1, may be necessary to obtain effective antigen binding activity for CDR-grafted antibodies which recognise more complex antigens. Even so the binding affinity of the best CDR-grafted antibodies obtained was still significantly less than the original MAb.

Very recently Queen et al (9) have described the preparation of a humanised antibody that binds to the

interleukin 2 receptor, by combining the CDRs of a murine MAb (anti-Tac) with human immunoglobulin framework and constant regions. The human framework regions were chosen to maximise homology with the anti-Tac MAb sequence. In addition computer modelling was used to identify framework amino acid residues which were likely to interact with the CDRs or antigen, and mouse amino acids were used at these positions in the humanised antibody.

In WO 90/07861 Queen et al propose four criteria for designing humanised immunoglobulins. The first criterion is to use as the human acceptor the framework from a particular human immunoglobulin that is unusually homologous to the non-human donor immunoglobulin to be humanised, or to use a consensus framework from many human antibodies. The second criterion is to use the donor amino acid rather than the acceptor if the human acceptor residue is unusual and the donor residue is typical for human sequences at a specific residue of the framework. The third criterion is to use the donor framework amino acid residue rather than the acceptor at positions immediately adjacent to the CDRs. The fourth criterion is to use the donor amino acid residue at framework positions at which the amino acid is predicted to have a side chain atom within about 3 Å of the CDRs in a three-dimensional immunoglobulin model and to be capable of interacting with the antigen or with the CDRs of the humanised immunoglobulin. It is proposed that criteria two, three or four may be applied in addition or alternatively to criterion one, and may be applied singly or in any combination.

WO 90/07861 describes in detail the preparation of a single CDR-grafted humanised antibody, a humanised antibody having specificity for the p55 Tac protein of the

IL-2 receptor. The combination of all four criteria, as above, were employed in designing this humanised antibody, the variable region frameworks of the human antibody Eu (7) being used as acceptor. In the resultant humanised antibody the donor CDRs were as defined by Kabat et al (7 and 8) and in addition the mouse donor residues were used in place of the human acceptor residues, at positions 27, 30, 48, 66, 67, 89, 91, 94, 103, 104, 105 and 107 in the heavy chain and at positions 48, 60 and 63 in the light chain, of the variable region frameworks. The humanised anti-Tac antibody obtained is reported to have an affinity for p55 of 3×10^9 M^{-1}, about one-third of that of the murine MAb.

We have further investigated the preparation of CDR-grafted humanised antibody molecules and have identified a hierarchy of positions within the framework of the variable regions (i.e. outside both the Kabat CDRs and structural loops of the variable regions) at which the amino acid identities of the residues are important for obtaining CDR-grafted products with satisfactory binding affinity. This has enabled us to establish a protocol for obtaining satisfactory CDR-grafted products which may be applied very widely irrespective of the level of homology between the donor immunoglobulin and acceptor framework. The set of residues which we have identified as being of critical importance does not coincide with the residues identified by Queen et al (9).

Summary of the Invention

Accordingly, in a first aspect the invention provides a CDR-grafted antibody heavy chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 6, 23 and/or 24, 48 and/or 49, 71 and/or 73, 75 and/or 76 and/or 78 and 88 and/or 91.

In preferred embodiments, the heavy chain framework comprises donor residues at positions 23, 24, 49, 71, 73 and 78 or at positions 23, 24 and 49. The residues at positions 71, 73 and 78 of the heavy chain framework are preferably either all acceptor or all donor residues.

In particularly preferred embodiments the heavy chain framework additionally comprises donor residues at one, some or all of positions 6, 37, 48 and 94. Also it is particularly preferred that residues at positions of the heavy chain framework which are commonly conserved across species, i.e. positions 2, 4, 25, 36, 39, 47, 93, 103, 104, 106 and 107, if not conserved between donor and acceptor, additionally comprise donor residues. Most preferably the heavy chain framework additionally comprises donor residues at positions 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.

In addition the heavy chain framework optionally comprises donor residues at one, some or all of positions:
1 and 3,
72 and 76,
69 (if 48 is different between donor and acceptor),
38 and 46 (if 48 is the donor residue),
80 and 20 (if 69 is the donor residue),
67,
82 and 18 (if 67 is the donor residue),
91,
88, and
any one or more of 9, 11, 41, 87, 108, 110 and 112.

In the first and other aspects of the present invention reference is made to CDR-grafted antibody products comprising acceptor framework and donor antigen binding regions. It will be appreciated that the invention is widely applicable to the CDR-grafting of antibodies in

general. Thus, the donor and acceptor antibodies may be derived from animals of the same species and even same antibody class or sub-class. More usually, however, the donor and acceptor antibodies are derived from animals of different species. Typically the donor antibody is a non-human antibody, such as a rodent MAb, and the acceptor antibody is a human antibody.

In the first and other aspects of the present invention, the donor antigen binding region typically comprises at least one CDR from the donor antibody. Usually the donor antigen binding region comprises at least two and preferably all three CDRs of each of the heavy chain and/or light chain variable regions. The CDRs may comprise the Kabat CDRs, the structural loop CDRs or a composite of the Kabat and structural loop CDRs and any combination of any of these. Preferably, the antigen binding regions of the CDR-grafted heavy chain variable domain comprise CDRs corresponding to the Kabat CDRs at CDR2 (residues 50-65) and CDR3 (residues 95-100) and a composite of the Kabat and structural loop CDRs at CDR1 (residues 26-35).

The residue designations given above and elsewhere in the present application are numbered according to the Kabat numbering [refs. (7) and (8)]. Thus the residue designations do not always correspond directly with the linear numbering of the amino acid residues. The actual linear amino acid sequence may contain fewer or additional amino acids than in the strict Kabat numbering corresponding to a shortening of, or insertion into, a structural component, whether framework or CDR, of the basic variable domain structure. For example, the heavy chain variable region of the anti-Tac antibody described by Queen et al (9) contains a single amino acid insert (residue 52a) after residue 52 of CDR2 and a three amino

acid insert (residues 82a, 82b and 82c) after framework residue 82, in the Kabat numbering. The correct Kabat numbering of residues may be determined for a given antibody by alignment at regions of homology of the sequence of the antibody with a "standard" Kabat numbered sequence.

The invention also provides in a second aspect a CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 1 and/or 3 and 46 and/or 47. Preferably the CDR grafted light chain of the second aspect comprises donor residues at positions 46 and/or 47.

The invention also provides in a third aspect a CDR-grafted antibody light chain having a variable region domain comprising acceptor framework and donor antigen binding regions wherein the framework comprises donor residues at at least one of positions 46, 48, 58 and 71.

In a preferred embodiment of the third aspect, the framework comprises donor residues at all of positions 46, 48, 58 and 71.

In particularly preferred embodiments of the second and third aspects, the framework additionally comprises donor residues at positions 36, 44, 47, 85 and 87. Similarly positions of the light chain framework which are commonly conserved across species, i.e. positions 2, 4, 6, 35, 49, 62, 64-69, 98, 99, 101 and 102, if not conserved between donor and acceptor, additionally comprise donor residues. Most preferably the light chain framework additionally comprises donor residues at positions 2, 4, 6, 35, 36, 38, 44, 47, 49, 62, 64-69, 85, 87, 98, 99, 101 and 102.

In addition the framework of the second or third aspects optionally comprises donor residues at one, some or all of positions:
1 and 3,
63,
60 (if 60 and 54 are able to form at potential saltbridge),
70 (if 70 and 24 are able to form a potential saltbridge),
73 and 21 (if 47 is different between donor and acceptor),
37 and 45 (if 47 is different between donor and acceptor), and
any one or more of 10, 12, 40, 80, 103 and 105.

Preferably, the antigen binding regions of the CDR-grafted light chain variable domain comprise CDRs corresponding to the Kabat CDRs at CDR1 (residue 24-34), CDR2 (residues 50-56) and CDR3 (residues 89-97).

The invention further provides in a fourth aspect a CDR-grafted antibody molecule comprising at least one CDR-grafted heavy chain and at least one CDR-grafted light chain according to the first and second or first and third aspects of the invention.

The humanised antibody molecules and chains of the present invention may comprise: a complete antibody molecule, having full length heavy and light chains; a fragment thereof, such as a Fab, $(Fab')_2$ or FV fragment; a light chain or heavy chain monomer or dimer; or a single chain antibody, e.g. a single chain FV in which heavy and light chain variable regions are joined by a peptide linker; or any other CDR-grafted molecule with the same specificity as the original donor antibody. Similarly the CDR-grafted heavy and light chain variable region may be combined with other antibody domains as appropriate.

Also the heavy or light chains or humanised antibody molecules of the present invention may have attached to them an effector or reporter molecule. For instance, it may have a macrocycle, for chelating a heavy metal atom, or a toxin, such as ricin, attached to it by a covalent bridging structure. Alternatively, the procedures of recombinant DNA technology may be used to produce an immunoglobulin molecule in which the Fc fragment or CH3 domain of a complete immunoglobulin molecule has been replaced by, or has attached thereto by peptide linkage, a functional non-immunoglobulin protein, such as an enzyme or toxin molecule.

Any appropriate acceptor variable region framework sequences may be used having regard to class/type of the donor antibody from which the antigen binding regions are derived. Preferably, the type of acceptor framework used is of the same/similar class/type as the donor antibody. Conveniently, the framework may be chosen to maximise/optimise homology with the donor antibody sequence particularly at positions close or adjacent to the CDRs. However, a high level of homology between donor and acceptor sequences is not important for application of the present invention. The present invention identifies a hierarchy of framework residue positions at which donor residues may be important or desirable for obtaining a CDR-grafted antibody product having satisfactory binding properties. The CDR-grafted products usually have binding affinities of at least 10^5 M^{-1}, preferably at least about 10^8 M^{-1}, or especially in the range 10^8-10^{12} M^{-1}. In principle, the present invention is applicable to any combination of donor and acceptor antibodies irrespective of the level of homology between their sequences. A protocol for applying the invention to any particular donor-acceptor antibody pair is given hereinafter. Examples of human frameworks which may be

used are KOL, NEWM, REI, EU, LAY and POM (refs. 4 and 5) and the like; for instance KOL and NEWM for the heavy chain and REI for the light chain and EU, LAY and POM for both the heavy chain and the light chain.

Also the constant region domains of the products of the invention may be selected having regard to the proposed function of the antibody in particular the effector functions which may be required. For example, the constant region domains may be human IgA, IgE, IgG or IgM domains. In particular, IgG human constant region domains may be used, especially of the IgG1 and IgG3 isotypes, when the humanised antibody molecule is intended for therapeutic uses, and antibody effector functions are required. Alternatively, IgG2 and IgG4 isotypes may be used when the humanised antibody molecule is intended for therapeutic purposes and antibody effector functions are not required, e.g. for simple blocking of lymphokine activity.

However, the remainder of the antibody molecules need not comprise only protein sequences from immunoglobulins. For instance, a gene may be constructed in which a DNA sequence encoding part of a human immunoglobulin chain is fused to a DNA sequence encoding the amino acid sequence of a functional polypeptide such as an effector or reporter molecule.

Preferably the CDR-grafted antibody heavy and light chain and antibody molecule products are produced by recombinant DNA technology.

Thus in further aspects the invention also includes DNA sequences coding for the CDR-grafted heavy and light chains, cloning and expression vectors containing the DNA sequences, host cells transformed with the DNA sequences

and processes for producing the CDR-grafted chains and antibody molecules comprising expressing the DNA sequences in the transformed host cells.

The general methods by which the vectors may be constructed, transfection methods and culture methods are well known per se and form no part of the invention. Such methods are shown, for instance, in references 10 and 11.

The DNA sequences which encode the donor amino acid sequence may be obtained by methods well known in the art. For example the donor coding sequences may be obtained by genomic cloning, or cDNA cloning from suitable hybridoma cell lines. Positive clones may be screened using appropriate probes for the heavy and light chain genes in question. Also PCR cloning may be used.

DNA coding for acceptor, e.g. human acceptor, sequences may be obtained in any appropriate way. For example DNA sequences coding for preferred human acceptor frameworks such as KOL, REI, EU and NEWM, are widely available to workers in the art.

The standard techniques of molecular biology may be used to prepare DNA sequences coding for the CDR-grafted products. Desired DNA sequences may be synthesised completely or in part using oligonucleotide synthesis techniques. Site-directed mutagenesis and polymerase chain reaction (PCR) techniques may be used as appropriate. For example oligonucleotide directed synthesis as described by Jones et al (ref. 20) may be used. Also oligonucleotide directed mutagenesis of a pre-exising variable region as, for example, described by Verhoeyen et al (ref. 5) or Riechmann et al (ref. 6) may be used. Also enzymatic filling in of gapped

oligonucleotides using T_4 DNA polymerase as, for example, described by Queen et al (ref. 9) may be used.

Any suitable host cell/vector system may be used for expression of the DNA sequences coding for the CDR-grafted heavy and light chains. Bacterial e.g. E. coli, and other microbial systems may be used, in particular for expression of antibody fragments such as FAb and $(Fab')_2$ fragments, and especially FV fragments and single chain antibody fragments e.g. single chain FVs. Eucaryotic e.g. mammalian host cell expression systems may be used for production of larger CDR-grafted antibody products, including complete antibody molecules. Suitable mammalian host cells include CHO cells and myeloma or hybridoma cell lines.

Thus, in a further aspect the present invention provides a process for producing a CDR-grafted antibody product comprising:

(a) producing in an expression vector an operon having a DNA sequence which encodes an antibody heavy chain according to the first aspect of the invention;

and/or

(b) producing in an expression vector an operon having a DNA sequence which encodes a complementary antibody light chain according to the second or third aspect of the invention;

(c) transfecting a host cell with the or each vector; and

(d) culturing the transfected cell line to produce the CDR-grafted antibody product.

The CDR-grafted product may comprise only heavy or light chain derived polypeptide, in which case only a heavy chain or light chain polypeptide coding sequence is used to transfect the host cells.
For production of products comprising both heavy and light chains, the cell line may be transfected with two vectors, the first vector may contain an operon encoding a light chain-derived polypeptide and the second vector containing an operon encoding a heavy chain-derived polypeptide. Preferably, the vectors are identical, except in so far as the coding sequences and selectable markers are concerned, so as to ensure as far as possible that each polypeptide chain is equally expressed. Alternatively, a single vector may be used, the vector including the sequences encoding both light chain- and heavy chain-derived polypeptides.

The DNA in the coding sequences for the light and heavy chains may comprise cDNA or genomic DNA or both. However, it is preferred that the DNA sequence encoding the heavy or light chain comprises at least partially, genomic DNA, preferably a fusion of cDNA and genomic DNA.

The present invention is applicable to antibodies of any appropriate specificity. Advantageously, however, the invention may be applied to the humanisation of non-human antibodies which are used for in vivo therapy or diagnosis. Thus the antibodies may be site-specific antibodies such as tumour-specific or cell surface-specific antibodies, suitable for use in in vivo therapy or diagnosis, e.g. tumour imaging. Examples of cell surface-specific antibodies are anti-T cell antibodies, such as anti-CD3, and CD4 and adhesion molecules, such as CR3, ICAM and ELAM. The antibodies may have specificity for interleukins (including lymphokines, growth factors and stimulating factors), hormones and other biologically active compounds, and receptors for any of these. For

example, the antibodies may have specificity for any of the following: Interferons α, β, γ or δ, IL1, IL2, IL3, or IL4, etc., TNF, GCSF, GMCSF, EPO, hGH, or insulin, etc.

The the present invention also includes therapeutic and diagnostic compositions comprising the CDR-grafted products of the invention and uses of such compositions in therapy and diagnosis.

Accordingly in a further aspect the invention provides a therapeutic or diagnostic composition comprising a CDR-grafted antibody heavy or light chain or molecule according to previous aspects of the invention in combination with a pharmaceutically acceptable carrier, diluent or excipient.

Accordingly also the invention provides a method of therapy or diagnosis comprising administering an effective amount of a CDR-grafted antibody heavy or light chain or molecule according to previous aspects of the invention to a human or animal subject.

A preferred protocol for obtaining CDR-grafted antibody heavy and light chains in accordance with the present invention is set out below together with the rationale by which we have derived this protocol. This protocol and rationale are given without prejudice to the generality of the invention as hereinbefore described and defined.

Protocol

It is first of all necessary to sequence the DNA coding for the heavy and light chain variable regions of the donor antibody, to determine their amino acid sequences. It is also necessary to choose appropriate acceptor heavy and light chain variable regions, of known amino acid sequence. The CDR-grafted chain is then designed

starting from the basis of the acceptor sequence. It will be appreciated that in some cases the donor and acceptor amino acid residues may be identical at a particular position and thus no change of acceptor framework residue is required.

1. As a first step donor residues are substituted for acceptor residues in the CDRs. For this purpose the CDRs are preferably defined as follows:

Heavy chain	-	CDR1:	residues 26-35
	-	CDR2:	residues 50-65
	-	CDR3:	residues 95-102
Light chain	-	CDR1:	residues 24-34
	-	CDR2:	residues 50-56
	-	CDR3:	residues 89-97

The positions at which donor residues are to be substituted for acceptor in the framework are then chosen as follows, first of all with respect to the heavy chain and subsequently with respect to the light chain.

2. Heavy Chain

2.1 Choose donor residues at all of positions 23, 24, 49, 71, 73 and 78 of the heavy chain or all of positions 23, 24 and 49 (71, 73 and 78 are always either all donor or all acceptor).

2.2 Check that the following have the same amino acid in donor and acceptor sequences, and if not preferably choose the donor: 2, 4, 6, 25, 36, 37, 39, 47, 48, 93, 94, 103, 104, 106 and 107.

2.3 To further optimise affinity consider choosing donor residues at one, some or any of:

i. 1, 3
ii. 72, 76
iii. If 48 is different between donor and acceptor sequences, consider 69
iv. If at 48 the donor residue is chosen, consider 38 and 46
v. If at 69 the donor residue is chosen, consider 80 and then 20
vi. 67
vii. If at 67 the donor residue is chosen, consider 82 and then 18
viii. 91
ix. 88
x. 9, 11, 41, 87, 108, 110, 112

3. Light Chain

3.1 Choose donor at 46, 48, 58 and 71

3.2 Check that the following have the same amino acid in donor and acceptor sequences, if not preferably choose donor:

2, 4, 6, 35, 38, 44, 47, 49, 62, 64-69 inclusive, 85, 87, 98, 99, 101 and 102

3.3 To further optimise affinity consider choosing donor residues at one, some or any of:

i. 1, 3
ii. 63

iii. 60, if 60 and 54 are able to form potential saltbridge

iv. 70, if 70 and 24 are able to form potential saltbridge

v. 73, and 21 if 47 is different between donor and acceptor

vi. 37, and 45 if 47 is different between donor and acceptor

vii. 10, 12, 40, 80, 103, 105

Rationale

In order to transfer the binding site of an antibody into a different acceptor framework, a number of factors need to be considered.

1. The extent of the CDRs

The CDRs (Complementary Determining Regions) were defined by Wu and Kabat (refs. 4 and 5) on the basis of an analysis of the variability of different regions of antibody variable regions. Three regions per domain were recognised. In the light chain the sequences are 24-34, 50-56, 89-97 (numbering according to Kabat (ref. 4), Eu Index) inclusive and in the heavy chain the sequences are 31-35, 50-65 and 95-102 inclusive.

When antibody structures became available it became apparent that these CDR regions corresponded in the main to loop regions which extended from the β barrel framework of the light and heavy variable domains. For H1 there was a discrepancy in that the loop was from 26 to 32 inclusive and for H2 the loop was 52 to 56 and for L2 from 50 to 53. However, with the exception of H1 the CDR regions encompassed the loop regions and extended into the β strand

frameworks. In H1 residue 26 tends to be a serine and 27 a phenylalanine or tyrosine, residue 29 is a phenylalanine in most cases. Residues 28 and 30 which are surface residues exposed to solvent might be involved in antigen-binding. A prudent definition of the H1 CDR therefore would include residues 26-35 to include both the loop region and the hypervariable residues 33-35.

It is of interest to note the example of Riechmann et al (ref. 3), who used the residue 31-35 choice for CDR-H1. In order to produce efficient antigen binding, residue 27 also needed to be recruited from the donor (rat) antibody.

2. Non-CDR residues which contribute to antigen binding

By examination of available X-ray structures we have identified a number of residues which may have an effect on net antigen binding and which can be demonstrated by experiment. These residues can be sub-divided into a number of groups.

2.1 Surface residues near CDR [all numbering as in Kabat et al (ref. 7)].

2.1.1. Heavy Chain - Key residues are 23, 71 and 73. Other residues which may contribute to a lesser extent are 1, 3 and 76. Finally 25 is usually conserved but the murine residue should be used if there is a difference.

2.1.2 Light Chain - Many residues close to the CDRs, e.g. 63, 65, 67 and 69 are conserved. If conserved none of the surface residues in the light chain are likely to have a major effect. However, if the murine residue at these positions

is unusual, then it would be of benefit to analyse the likely contribution more closely. Other residues which may also contribute to binding are 1 and 3, and also 60 and 70 if the residues at these positions and at 54 and 24 respectively are potentially able to form a salt bridge i.e. 60 + 54; 70 + 24.

2.2 Packing residues near the CDRs.

2.2.1. Heavy Chain - Key residues are 24, 49 and 78. Other key residues would be 36 if not a tryptophan, 94 if not an arginine, 104 and 106 if not glycines and 107 if not a threonine. Residues which may make a further contribution to stable packing of the heavy chain and hence improved affinity are 2, 4, 6, 38, 46, 67 and 69. 67 packs against the CDR residue 63 and this pair could be either both mouse or both human. Finally, residues which contribute to packing in this region but from a longer range are 18, 20, 80, 82 and 86. 82 packs against 67 and in turn 18 packs against 82. 80 packs against 69 and in turn 20 packs against 80. 86 forms an H bond network with 38 and 46. Many of the mouse-human differences appear minor e.g. Leu-Ile, but could have an minor impact on correct packing which could translate into altered positioning of the CDRs.

2.2.2. Light Chain - Key residues are 48, 58 and 71. Other key residues would be 6 if not glutamine, 35 if not tryptophan, 62 if not phenylalanine or tryosine, 64, 66, 68, 99 and 101 if not glycines and 102 if not a threonine. Residues which make a further contribution are 2, 4, 37, 45 and 47. Finally residues 73 and 21 and 19 may make long distance packing contributions of a minor nature.

2.3. Residues at the variable domain interface between heavy and light chains - In both the light and heavy chains most of the non-CDR interface residues are conserved. If a conserved residue is replaced by a residue of different character, e.g. size or charge, it should be considered for retention as the murine residue.

2.3.1. Heavy Chain - Residues which need to be considered are 37 if the residue is not a valine but is of larger side chain volume or has a charge or polarity. Other residues are 39 if not a glutamine, 45 if not a leucine, 47 if not a tryptophan, 91 if not a phenylalanine or tyrosine, 93 if not an alanine and 103 if not a tryptophan. Residue 89 is also at the interface but is not in a position where the side chain could be of great impact.

2.3.2. Light Chain - Residues which need to be considered are 36, if not a tyrosine, 38 if not a glutamine, 44 if not a proline, 46, 49 if not a tyrosine, residue 85, residue 87 if not a tyrosine and 98 if not a phenylalanine.

2.4. Variable-Constant region interface - The elbow angle between variable and constant regions may be affected by alterations in packing of key residues in the variable region against the constant region which may affect the position of V_L and V_H with respect to one another. Therefore it is worth noting the residues likely to be in contact with the constant region. In the heavy chain the surface residues potentially in contact with the variable region are conserved between mouse and human antibodies therefore the variable region contact residues may influence the V-C interaction. In the light chain the amino acids found at a number of the constant

region contact points vary, and the V & C regions are not in such close proximity as the heavy chain. Therefore the influences of the light chain V-C interface may be minor.

2.4.1. Heavy Chain - Contact residues are 7, 11, 41, 87, 108, 110, 112.

2.4.2. Light Chain - In the light chain potentially contacting residues are 10, 12, 40, 80, 83, 103 and 105.

The above analysis coupled with our considerable practical experimental experience in the CDR-grafting of a number of different antibodies have lead us to the protocol given above.

The present invention is now described, by way of example only, with reference to the accompanying Figures 1 - 13.

Brief Description of the Figures

Figure 1 shows DNA and amino acid sequences of the OKT3 light chain;

Figure 2 shows DNA and amino acid sequences of the OKT3 heavy chain;

Figure 3 shows the alignment of the OKT3 light variable region amino acid sequence with that of the light variable region of the human antibody REI;

Figure 4 shows the alignment of the OKT3 heavy variable region amino acid sequence with that of the heavy variable region of the human antibody KOL;

Figure 5 shows the heavy variable region amino acid sequences of OKT3, KOL and various corresponding CDR grafts;

Figure 6 shows the light variable region amino acid sequences of OKT3, REI and various corresponding CDR grafts;

Figure 7 shows a graph of binding assay results for various grafted OKT3 antibodies'

Figure 8 shows a graph of blocking assay results for various grafted OKT3 antibodies;

Figure 9 shows a similar graph of blocking assay results;

Figure 10a and b shows similar graphs for both binding assay and blocking assay results;

Figure 11a and b shows further similar graphs for both binding assay and blocking assay results;

Figure 12 shows a graph of competition assay results for a minimally grafted OKT3 antibody compared with the OKT3 murine reference standard, and

Figure 13 shows a similar graph of competition assay results comparing a fully grafted OKT3 antibody with the murine reference standard.

DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

EXAMPLE 1

CDR-GRAFTING OF OKT3

MATERIAL AND METHODS

1. INCOMING CELLS

Hybridoma cells producing antibody OKT3 were provided by Ortho (seedlot 4882.1) and were grown up in antibiotic free Dulbecco's Modified Eagles Medium (DMEM) supplemented with glutamine and 5% foetal calf serum, and divided to provide both an overgrown supernatant for evaluation and cells for extraction of RNA. The overgrown supernatant was shown to contain 250 ug/mL murine IgG2a/kappa antibody. The supernatant was negative for murine lambda light chain and IgG1, IgG2b, IgG3, IgA and IgM heavy chain. 20mL of supernatant was assayed to confirm that the antibody present was OKT3.

2. MOLECULAR BIOLOGY PROCEDURES

Basic molecular biology procedures were as described in Maniatis et al (ref. 9) with, in some cases, minor modifications. DNA sequencing was performed as described in Sanger et al (ref. 11) and the Amersham International Plc sequencing handbook. Site directed mutagenesis was as described in Kramer et al (ref. 12) and the Anglian Biotechnology Ltd. handbook. COS cell expression and metabolic labelling studies were as described in Whittle et al (ref. 13)

3. RESEARCH ASSAYS

3.1. ASSEMBLY ASSAYS

Assembly assays were performed on supernatants from transfected COS cells to determine the amount of intact IgG present.

3.1.1. COS CELLS TRANSFECTED WITH MOUSE OKT3 GENES

The assembly assay for intact mouse IgG in COS cell supernatants was an ELISA with the following format:

96 well microtitre plates were coated with F(ab')2 goat anti-mouse IgG Fc. The plates were washed in water and samples added for 1 hour at room temperature. The plates were washed and F(ab')2 goat anti-mouse IgG F(ab')2 (HRPO conjugated) was then added. Substrate was added to reveal the reaction. UPC10, a mouse IgG2a myeloma, was used as a standard.

3.1.2. COS AND CHO CELLS TRANSFECTED WITH CHIMERIC OR CDR-GRAFTED OKT3 GENES

The assembly assay for chimeric or CDR-grafted antibody in COS cell supernatants was an ELISA with the following format:

96 well microtitre plates were coated with F(ab')2 goat anti-human IgG Fc. The plates were washed and samples added and incubated for 1 hour at room temperature. The plates were washed and monoclonal mouse anti-human kappa chain was added for 1 hour at room temperature.

The plates were washed and F(ab')2 goat anti-mouse IgG Fc (HRPO conjugated) was added. Enzyme substrate was added to reveal the reaction. Chimeric B72.3 (IgG4) (ref. 13) was used as a standard. The use of a monoclonal anti-kappa chain in this assay allows grafted antibodies to be read from the chimeric standard.

3.2. ASSAY FOR ANTIGEN BINDING ACTIVITY

Material from COS cell supernatants was assayed for OKT3 antigen binding activity onto CD3 positive cells in a direct assay. The procedure was as follows:

HUT 78 cells (human T cell line, CD3 positive) were maintained in culture. Monolayers of HUT 78 cells were prepared onto 96 well ELISA plates using poly-L-lysine and glutaraldehyde. Samples were added to the monolayers for 1 hour at room temperature.

The plates were washed gently using PBS. F(ab')2 goat anti-human IgG Fc (HRPO conjugated) or F(ab')2 goat anti-mouse IgG Fc (HRPO conjugated) was added as appropriate for humanised or mouse samples. Substrate was added to reveal the reaction.

The negative control for the cell-based assay was chimeric B72.3. The positive control was mouse Orthomune OKT3 or chimeric OKT3, when available. This cell-based assay was difficult to perform, and an alternative assay was developed for CDR-grafted OKT3 which was more sensitive and easier to carry out.

In this system CDR-grafted OKT3 produced by COS cells was tested for its ability to bind to the CD3-positive HPB-ALL (human peripheral blood acute lymphocytic leukemia) cell line. It was also tested for its ability to block the binding of murine OKT3 to these cells. Binding was measured by the following procedure: HPB-ALL cells were harvested from tissue culture. Cells were incubated at 4°C for 1 hour with various dilutions of test antibody, positive control antibody, or negative control antibody. The cells were washed once and incubated at 4°C for 1 hour with an FITC-labelled goat anti-human IgG (Fc-

specific, mouse absorbed). The cells were washed twice and analysed by cytofluorography. Chimeric OKT3 was used as a positive control for direct binding. Cells incubated with mock- transfected COS cell supernatant, followed by the FITC-labelled goat anti-human IgG, provided the negative control. To test the ability of CDR-grafted OKT3 to block murine OKT3 binding, the HPB-ALL cells were incubated at 4°C for 1 hour with various dilutions of test antibody or control antibody. A fixed saturating amount of FITC OKT3 was added. The samples were incubated for 1 hour at 4°C, washed twice and analysed by cytofluorography. FITC-labelled OKT3 was used as a positive control to determine maximum binding. Unlabelled murine OKT3 served as a reference standard for blocking. Negative controls were unstained cells with or without mock-transfected cell supernatant. The ability of the CDR-grafted OKT3 light chain to bind CD3-positive cells and block the binding of murine OKT3 was initially tested in combination with the chimeric OKT3 heavy chain. The chimeric OKT3 heavy chain is composed of the murine OKT3 variable region and the human IgG4 constant region. The chimeric heavy chain gene is expressed in the same expression vector used for the CDR-grafted genes. The CDR-grafted light chain expression vector and the chimeric heavy chain expression vector were co-transfected into COS cells. The fully chimeric OKT3 antibody (chimeric light chain and chimeric heavy chain) was found to be fully capable of binding to CD3 positive cells and blocking the binding of murine OKT3 to these cells.

3.3 DETERMINATION OF RELATIVE BINDING AFFINITY

The relative binding affinities of CDR-grafted

anti-CD3 monoclonal antibodies were determined by competition binding (ref. 6) using the HPB-ALL human T cell line as a source of CD3 antigen, and fluorescein-conjugated murine OKT3 (Fl-OKT3) of known binding affinity as a tracer antibody. The binding affinity of Fl-OKT3 tracer antibody was determined by a direct binding assay in which increasing amounts of Fl-OKT3 were incubated with HPB-ALL ($5x10^5$) in PBS with 5% foetal calf serum for 60 min. at 4°C. Cells were washed, and the fluorescence intensity was determined on a FACScan flow cytometer calibrated with quantitative microbead standards (Flow Cytometry Standards, Research Triangle Park, NC). Fluorescence intensity per antibody molecule (F/P ratio) was determined by using microbeads which have a predetermined number of mouse IgG antibody binding sites (Simply Cellular beads, Flow Cytometry Standards). F/P equals the fluorescence intensity of beads saturated with Fl-OKT3 divided by the number of binding sites per bead. The amount of bound and free Fl-OKT3 was calculated from the mean fluorescence intensity per cell, and the ratio of bound/free was plotted against the number of moles of antibody bound. A linear fit was used to determine the affinity of binding (absolute value of the slope).

For competitive binding, increasing amounts of competitor antibody were added to a sub-saturating dose of Fl-OKT3 and incubated with $5x10^5$ HPB-ALL in 200 Ml of PBS with 5% foetal calf serum, for 60 min at 4°C. The fluorescence intensities of the cells were measured on a FACScan flow cytometer calibrated with quantitative microbead standards. The concentrations of bound and free Fl-OKT3 were calculated. The affinities of competing anti-

bodies were calculated from the equation [X]-[OKT3] = (1/Kx) - (1/Ka), where Ka is the affinity of murine OKT3, Kx is the affinity of competitor X, [ ] is the concentration of competitor antibody at which bound/free binding is R/2, and R is the maximal bound/free binding.

4. cDNA LIBRARY CONSTRUCTION

4.1. mRNA PREPARATION AND cDNA SYNTHESIS

OKT3 producing cells were grown as described above and 1.2 x 10^9 cells harvested and mRNA extracted using the guanidinium/LiCl extraction procedure. cDNA was prepared by priming from Oligo-dT to generate full length cDNA. The cDNA was methylated and EcoR1 linkers added for cloning.

4.2. LIBRARY CONSTRUCTION

The cDNA library was ligated to pSP65 vector DNA which had been EcoR1 cut and the 5′ phosphate groups removed by calf intestinal phosphatase (EcoR1/CIP). The ligation was used to transform high transformation efficiency Escherichia coli (E.coli) HB101. A cDNA library was prepared. 3600 colonies were screened for the light chain and 10000 colonies were screened for the heavy chain.

5. SCREENING

E.coli colonies positive for either heavy or light chain probes were identified by oligonucleotide screening using the oligonucleotides:

5′ TCCAGATGTTAACTGCTCAC (SEQ ID NO:1) for the light chain, which is complementary to a sequence in the mouse kappa constant region, and 5′ CAGGGGCCAGTGGATGGATAGAC (SEQ ID NO:2) for the heavy chain which is complementary to a sequence in the mouse IgG2a constant CH1 domain region. 12 light chain and 9 heavy chain clones

were identified and taken for second round screening. Positive clones from the second round of screening were grown up and DNA prepared. The sizes of the gene inserts were estimated by gel electrophoresis and inserts of a size capable of containing a full length cDNA were subcloned into M13 for DNA sequencing.

6. DNA SEQUENCING

Clones representing four size classes for both heavy and light chains were obtained in M13. DNA sequence for the 5' untranslated regions, signal sequences, variable regions and 3' untranslated regions of full length cDNAs [Figures 1(a) (SEQ ID NO:4) and 2(a) (SEQ ID NO:6)] were obtained and the corresponding amino acid sequences predicted [(Figures 1(b) (SEQ ID NO:5) and 2(b) (SEQ ID NO:7)]. In Figure 1(a) the untranslated DNA regions are shown in uppercase, and in both Figures 1 and 2 (SEQ ID NO:4+5) the signal sequences are underlined.

7. CONSTRUCTION OF cDNA EXPRESSION VECTORS

Celltech expression vectors are based on the plasmid pEE6hCMV (ref. 14). A polylinker for the insertion of genes to be expressed has been introduced after the major immediate early promoter/enhancer of the human Cytomegalovirus (hCMV). Marker genes for selection of the plasmid in transfected eukaryotic cells can be inserted as BamH1 cassettes in the unique BamH1 site of pEE6 hCMV; for instance, the neo marker to provide pEE6 hCMV neo. It is usual practice to insert the neo and gpt markers prior to insertion of the gene of interest, whereas the GS marker is inserted last because of the presence of internal EcoR1 sites in the cassette.

The selectable markers are expressed from the SV40 late promoter which also provides an origin of replication so that the vectors can be used for expression in the COS cell transient expression system.

The mouse sequences were excised from the M13 based vectors described above as EcoR1 fragments and cloned into either pEE6-hCMV-neo for the heavy chain and into EE6-hCMV-gpt for the light chain to yield vectors pJA136 and pJA135 respectively.

8. EXPRESSION OF cDNAS IN COS CELLS

Plasmids pJA135 and pJA136 were co-transfected into COS cells and supernatant from the transient expression experiment was shown to contain assembled antibody which bound to T-cell enriched lymphocytes. Metabolic labelling experiments using ^{35}S methionine showed expression and assembly of heavy and light chains.

9. CONSTRUCTION OF CHIMERIC GENES

Construction of chimeric genes followed a previously described strategy [Whittle et al (ref. 13)]. A restriction site near the 3′ end of the variable domain sequence is identified and used to attach an oligonucleotide adapter coding for the remainder of the mouse variable region and a suitable restriction site for attachment to the constant region of choice.

9.1. LIGHT CHAIN GENE CONSTRUCTION

The mouse light chain cDNA sequence contains an AvaI site near the 3′ end of the variable region [Fig. 1(a)]. (SEQ ID NO:4) The majority of the sequence of the variable region was isolated as a 396 bp. EcoR1-AvaI fragment. An oligonucleotide adapter was designed to replace the remainder of the 3′