UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

ELI LILLY AND COMPANY,
Petitioner

v.

TEVA PHARMACEUTICALS INTERNATIONAL GMBH,
Patent Owner

Case IPR2018-01422 (Patent 9,340,614 B2)
Case IPR2018-01423 (Patent 9,266,951 B2)
Case IPR2018-01424 (Patent 9,346,881 B2)
Case IPR2018-01425 (Patent 9,890,210 B2)
Case IPR2018-01426 (Patent 9,890,211 B2)
Case IPR2018-01427 (Patent 9,597,649 B2)[1]

DECLARATION OF MICHAEL P. CARNEY

[1] The word-for-word identical paper will be filed in each proceeding identified in the caption.

I, Michael P. Carney, declare:

1. I have prepared this Declaration in connection with Petitioner's Replies in IPR2018-01422 of U.S. Patent No. 9,340,614; IPR2018-01423 of U.S. Patent No. 9,266,951; IPR2018-01424 of U.S. Patent No. 9,346,881; IPR2018-01425 of U.S. Patent No. 9,890,210; IPR2018-01426 of U.S. Patent No. 9,890,211; and IPR2018-01427 of U.S. Patent No. 9,597,649.

2. I am currently a Research Analyst at Finnegan, Henderson, Farabow, Garrett & Dunner, LLP, 3300 Hillview Avenue, Palo Alto, CA 94304.

3. I am over eighteen years of age and am competent to make this Declaration. I make this Declaration based on my own personal knowledge and based on my knowledge and experience of library science practices.

4. I earned a Master's of Library and Information Science from San Jose State University in 1996 and a Bachelor degree in Political Science from George Washington University in 1990. I have worked as a librarian for over twenty years. I have been employed in the Research & Information Services (formerly Library) Department of Finnegan since 2016. Before that, from 1996-2015, I was employed in the Library Department of Weil, Gotshal & Manges LLP.

5. I am currently a member of the American Association of Law Libraries.

I. Standard Library Practices

6. I have knowledge of and experience with standard library practices regarding receipt, cataloging, and shelving of materials. For example, I have knowledge of and experience with the Machine-Readable Cataloging ("MARC") system, including MARC 21, that libraries use to catalogue materials.

7. Based on standard library practice, when a library receives an item, it stamps the item with the library name and often with a date that is within a few days or weeks of receipt. The library will catalogue the item within a matter of a few days or weeks of receiving it.

8. By the mid-1970s, standard library practice involved cataloguing items using the MARC system. The MARC system was developed in the 1960s to standardize bibliographic records so they could be read by computers and shared among libraries. By the mid-1970s, MARC had become the international standard for bibliographic data, and it is still used today.

9. After an item is catalogued, the public may access the item by searching the catalogue and requesting the item from the library. Standard library practice is to then shelve the item within a matter of a few days or weeks of cataloging it.

10. Taking into account the few days or weeks between receiving an item and cataloging it, and the few days or weeks between cataloging an item and

shelving it, the total time between receiving an item and shelving ranges from a couple of weeks to a few months.

II. MARC Records

11. Many libraries provide public access to their MARC records via the Internet and/or their electronic cataloguing system at the library. In a MARC record, each field provides information about the catalogued item. MARC uses a simple three-digit numeric code (from 001-999) to identify each field in the record. For example, field 245 lists the title of the work and field 260 lists publisher information. Field 008 provides date information for items catalogued in the MARC 21 file format.

12. The first six characters of the field 008 (character positions 00-05) are always in the "**YYMMDD**" format, which indicates the date the MARC record was created. Dates of production, publication, distribution, manufacture or copyright may be specified in characters 07-10. Character position 06 may be used to provide information about the type of date or publication status. For example, the code "n" in the 06 character position indicates "Dates unknown," the code "q" signifies "questionable date" and the code "s" means "single known date/probable date." Attached as Exhibit A to this declaration are explanations of the MARC records for field 008 from the Library of Congress.

III. Tan Thesis

13. Attached as Exhibit B is a true and correct copy of "Application of monoclonal antibodies to the investigation of the role of calcitonin gene-related peptide as a vasodilatory neurotransmitter," a dissertation submitted to the University of Cambridge for the Ph.D. Degree by Keith Kwan Cheuk Tan ("Tan Thesis"). Through our Research & Information Services Department, I obtained the Tan Thesis directly from the University of Cambridge Library on August 30, 2019. I understand that Exhibit B is being served as Exhibit 1287A.

14. The title page of the Tan Thesis includes the following University of Cambridge Library stamp.

As discussed above, upon receiving a published book or report, it is standard library practice to stamp a book with the library name and then shelve the book or report within a matter of a few days or weeks.

15. Attached as Exhibit C is a true and correct copy of the current Cambridge University Library ("CUL") catalogue entry for the Tan Thesis, which I accessed at

http://idiscover.lib.cam.ac.uk/permalink/f/t9gok8/44CAM_ALMA2142964848000 3606 on August 27, 2019. As indicated in the CUL catalogue, the entry was created in 1994 and the Tan Thesis was approved on July 29, 1994.

16. Attached as Exhibit D to this declaration is a true and correct copy of the MARC record from the Cambridge University Library Catalog for its copy of Tan Thesis, which I downloaded from http://idiscover.lib.cam.ac.uk/primo-explore/sourceRecord?vid=44CAM_PROD&docId=44CAM_ALMA21429648480 003606 on August 27, 2019.

17. The MARC record for the Tan Thesis, includes a number of fields. The date field 008 lists the first six characters "020506" in "YYMMDD" format, indicating that the MARC record for the Tan Thesis was created on May 6, 2002. This means, at the latest, the Tan Thesis was catalogued by the Cambridge University Library on May 6, 2002. The first six characters are also followed by the code "s" in character position 06 and "1994" in character positions 07-10. As discussed above, this indicates that the Tan Thesis was produced in 1994.

18. Attached as Exhibit E to this declaration is a true and correct copy of correspondence that I received from Louise Clarke, the Superintendent of the Manuscripts Reading Room at the Cambridge University Library in response to an official request for information concerning the date of public availability of the Tan Thesis. According to Exhibit E, theses are delivered to the Cambridge

University Library about one month after they are approved by the Board of Graduate Studies.

19. Together, this information available from the University of Cambridge Library is consistent with the Tan Thesis being publicly available at least as early as 1994, and long before November 2005.

20. I declare that all statements made herein of my knowledge are true, and that all statements made on information and belief are believed to be true, and 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.

21. I declare under penalty of perjury under the laws of the United States of America that the foregoing is true and correct.

Executed on this 1st day of October 2019, in Palo Alto, California.

By:
Michael P. Carney

EXHIBIT A

Indicators and Subfield Codes

This field has no indicators or subfield codes; the data elements are positionally defined.

Character Positions

00-05 - Date entered on file

06 - Type of date/Publication status

b - No dates given; B.C. date involved
c - Continuing resource currently published
d - Continuing resource ceased publication
e - Detailed date
i - Inclusive dates of collection
k - Range of years of bulk of collection
m - Multiple dates
n - Dates unknown
p - Date of distribution/release/issue and production/recording session when different
q - Questionable date
r - Reprint/reissue date and original date
s - Single known date/probable date
t - Publication date and copyright date
u - Continuing resource status unknown
| - No attempt to code

07-10 - Date 1

1-9 - Date digit
- Date element is not applicable
u - Date element is totally or partially unknown
|||| - No attempt to code

11-14 - Date 2

1-9 - Date digit
- Date element is not applicable
u - Date element is totally or partially unknown
|||| - No attempt to code

15-17 - Place of publication, production, or execution

xx# - No place, unknown, or undetermined
vp# - Various places
[aaa] - Three-character alphabetic code
[aa#] - Two-character alphabetic code

18-34 - Material specific coded elements

35-37 - Language

- No information provided
zxx - No linguistic content
mul - Multiple languages
sgn - Sign languages
und - Undetermined
[aaa] - Three-character alphabetic code

38 - Modified record

- Not modified
d - Dashed-on information omitted
o - Completely romanized/printed cards romanized
r - Completely romanized/printed cards in script
s - Shortened
x - Missing characters
| - No attempt to code

39 - Cataloging source

- National bibliographic agency
c - Cooperative cataloging program
d - Other
u - Unknown
| - No attempt to code

FIELD DEFINITION AND SCOPE

Field 008 character positions 00-17 and 35-39 are defined the same for field 008 in the MARC 21 bibliographic format, regardless of record type. The definition of field 008 character positions 18-34 varies according to the Type of record code in Leader/06 and Bibliographic level code in Leader/07. Certain data elements are defined the same in more than one 008 field configuration. When similar data elements are defined for inclusion in a field 008 for different record types/bibliographic level, they generally occupy the same field 008 character positions.

GUIDELINES FOR APPLYING CONTENT DESIGNATORS

■ CHARACTER POSITIONS

00-05 - Date entered on file

Computer-generated, six-character numeric string that indicates the date the MARC record was created. Recorded in the pattern *yymmdd*.

Pattern *yymmdd* is *yy* for the year, *mm* for the month, and *dd* for the day. The date entered on file in 008/00-05 is never changed. The date and time of latest transaction information in field 005 changes each time a transaction is made to the record. The latest transaction information enables an organization handling more than one version of a record to identify the most current version. The fill character (|) is not allowed in any of these positions. Field 008/00-05 is usually system generated.

06 - Type of date/Publication status

One-character alphabetic code that indicates the type of dates given in 008/07-10 (Date 1) and 008/11-14 (Date 2). For continuing resources, the code in 008/06 also indicates the publication status.

The choice of code for 008/06 is made concurrently with a determination of the appropriate dates for 008/07-14. For most records data is derived from information in field 260 (Publication, Distribution, etc. (Imprint)), field 264 (Production, Publication, Distribution, Manufacture, and Copyright Notice), field 362 (Dates of Publication and/or Sequential Designation), or from note fields.

Dates are represented by four digits. Missing digits in the date are represented by the character *u*.

For continuing resources, 008/07-10 contain the beginning date of publication (chronological designation) and 008/11-14 contain the ending date. For reprints of serials and for reproductions of serials being described in the body of the entry, the beginning and ending dates of the original are input in these character positions.

Precedence of codes (monographic items) - When more than one code applies to a bibliographic item, use the table below to determine the appropriate code to use. The codes listed first take precedence over codes listed subsequently. The left column covers single part items and multipart items completed in one year. The right column covers multipart items for which a span of dates is required.

Single part/multipart items complete in one year	**Collections/multipart items complete in more than one year**
b - B.C. date	b - B.C. date
r - Reprint/original date	i - Inclusive date
e - Detailed date	k - Range of dates
s - Single date	r - Reprint/original date
p - Distribution/production date	m - Initial/terminal date
t - Publication date and copyright date	t - Publication date and copyright date
q - Questionable date	n - Unknown date
n - Unknown date	

Legal characters - Date 1 and Date 2 each usually consist of four digits (e.g., 1963). When part of the date is unknown, missing digits are represented by the character *u* (e.g., "19??" would be recorded as *19uu*). If the date is totally unknown, the millennium may be inferred (e.g., *1uuu*). For Common Era (C.E.) dates of the first millennium, the year is right justified and unused positions contain zeros (e.g., "946 A.D." would be recorded as *0946*). When Date 1 or Date 2 is not applicable, such as when Before Common Era (B.C.) dates are involved, blanks are used (e.g., *####*). For active serials (i.e., when the serial has not ceased publication) and incomplete multipart nonserial items, the date in 008/11-14 is represented by *9999* to indicate that the year is not yet available. The fill character (|) may also be used in 008/06-14 when no attempt has been made to code dates, but its use in 008/07-10 is discouraged. Many MARC-based systems rely on non-fill characters in 008/07-10 for retrieval and duplicate detection. When fill is used in either 008/07-10 or 008/11-14, it should be used in all four character positions, thus a combination of fills and any other character in either of these positions should never occur.

b - No dates given; B.C. date involved

One or more dates associated with the item are Before Common Era (B.C.) dates. B.C. date information can be specifically coded in field 046 (Special Coded Dates).

Each character position in fields 008/07-10 and 008/11-14 contains a blank.

008/06 b
008/07-10 ####
008/11-14 ####
260 ##$c[150-100 B.C.]

c - Continuing resource currently published

Currently published is defined as an item for which an issue has been received within the last three years.

008/07-10 contain the beginning date of publication; 008/11-14 contain the characters *9999*.

008/06 c
008/07-10 1984
008/11-14 9999
260 ##$aNew York :$bXerox Films,$c1984-

008/06 c
008/07-10 1953
008/11-14 9999
260 ##$aChicago :$bUniversity of Chicago Press,
362 1#$aBegan with vol. for 1953.

008/06 c
008/07-10 195u
008/11-14 9999
500 ##$aDescription based on: Vol. 2, no. 2 (Feb. 1956).
[Date of first issue is unknown.]

008/06 c
008/07-10 19uu
008/11-14 9999
260 ##$aNew York :$bWiley Interscience,
500 ##$aDescription based on: 1981.
[Date of first issue is unknown but can be estimated.]

008/06 c
008/07-10 1uuu
008/11-14 9999
260 ##$aNew York :$bDoubleday,
500 ##$aDescription based on: 1901.
[Date of first issue is unknown and cannot be estimated.]

d - Continuing resource ceased publication

New issues of a continuing resource have ceased to be published or that a change in author or title has caused a successive entry record to be created. When a new title supersedes a previously existing one, the earlier title is considered *dead* and coded d in field 008/06. An item is considered to have ceased publication only when there is clear evidence that it has. Generally, a period of more than three years during which no new issue of a continuing resource has been published is considered evidence that it has ceased publication.

008/07-10 contain the beginning date of publication; 008/11-14 contain the date the item ceased to be published.

008/06 d
008/07-10 1928
008/11-14 1941
260 ##$aBerlin :$bVZG,$c1928-1941.
362 0#$aVol. 1, no. 1 (Feb. 1928)-v. 14, no. 2 (Feb. 1941).

008/06 d
008/07-10 1uuu
008/11-14 1958
260 ##$aNew York :$bAmerican Statistical Association,$c -1959.
362 0#$a -1958.

008/06 d
008/07-10 19uu
008/11-14 1929
260 ##$aBoston :$bThe Society,$c -1929.
[Beginning date is unknown but can be estimated.]

008/06 d
008/07-10 1945
008/11-14 19uu
260 ##$aChicago :$bThe Association,$c1945-
515 ##$aNo more published?
[Ending date is unknown but can be estimated.]

e - Detailed date
Detailed date which contains the month (and possibly the day) in addition to the year is present.

008/07-10 contain the year and 008/11-14 contain the month and day formatted *mmdd*.

If the day is unknown, *uu* is used; if the detail on the item indicates only the month, blanks are used. For **visual materials**, this code may be used with televised material to give the date of the original broadcast.

008/06 e
008/07-10 1983
008/11-14 0615
260 ##$aWashington, D.C. :$bDept. of Commerce,$cJune 15, 1983.

008/06 e
008/07-10 1977
008/11-14 05##
260 ##$cMay 1977.
[Day is not applicable because the publication is identified by month only.]

008/06 e
008/07-10 1976
008/11-14 11uu
260 ##$aU.S. :$bTriangle Film Corp.,$cNov. 1976.
[Day of month is unknown.]

i - Inclusive dates of collection
008/07-10 and 008/11-14 contain the inclusive dates applicable to a collection. If the inclusive dates are represented by a single year, that date is given in both places. A collection, whether or not it consists of individually published items, is not considered to exist in a published form. Multipart items are not treated as a collection (see code m).

008/06 i
008/07-10 1765
008/11-14 1770
260 ##$c1765-1770.

008/06 i
008/07-10 18uu
008/11-14 1890
260 ##$c18--?-1890.

008/06 i
008/07-10 1988
008/11-14 1988
260 ##$c1988.

k - Range of years of bulk of collection
008/07-10 and 008/11-14 contain the range of years applicable to most of the material in a collection. If the bulk dates are represented by only a single year, that date is given in both places. A collection, whether or not it consists of individually published items, is not considered to exist in a published form. Multipart items are not treated as a collection (see code m).

008/06 k
008/07-10 1796
008/11-14 1896
260 ##$c1796-1896.

008/06 k
008/07-10 1854
008/11-14 1854
260 ##$c1854.

m - Multiple dates
008/07-10 and 008/11-14 contain the range of years of publication of a multipart item. If both dates for a multipart item are represented by a single year, then code s is used. The code is also used for a single part unpublished item that has been executed over a period of time, such as a painting.

008/07-10 usually contain the initial (or beginning) date and 008/11-14 the terminal (or ending) date.

008/06 m
008/07-10 1972
008/11-14 1975
260 ##$aParis :$bÉditions du Cerf,$c1972-1975.

008/06 m
008/07-10 uuuu
008/11-14 1981
260 ##$aBoston :$bMacmillan,$c -[1981]
[Multipart item for which the earliest volume is not held.]

008/06 m
008/07-10 197u
008/11-14 1987
260 ##$aParis :$bHachette,$c[197-]-1987.
[Multipart item for which the publication date of the first volume is uncertain.]

008/06 m
008/07-10 1943
008/11-14 197u
260 ##$aNew York :$bDover,$c1943-[197-?]
[Multipart item for which the publication date of the last volume is uncertain.]

008/06 m
008/07-10 1943
008/11-14 1945
260 ##$aLondon :$bGollancz,$c1943-1945.
[Single part item for which the publication date spans more than one year.]

008/06 m
008/07-10 1998
008/11-14 9999
260 ##$aBrescia : $bLa scuola,$c<c1998- >
[Multipart item for which the publication date is ongoing.]

n - Dates unknown
Dates appropriate for 008/07-10 and 008/11-14 are unknown, (e.g., when no dates are given in field 260).

008/06 n
008/07-10 uuuu
008/11-14 uuuu
260 ##$a[Spain]

008/06 n
008/07-10 uuuu
008/11-14 uuuu
[Naturally occurring object; field 260 is not present in the record.]

p - Date of distribution/release/issue and production/recording session when different
Both a date of distribution/release/issue (008/07-10) and a date of production/recording (008/11-14) are present because there is a difference between the two dates. For **computer files**, code p is used when there is a difference between the date the file first became operational for analysis and processing in machine-readable form (i.e., production date) and the date the file became available to the public, usually through an established agency (i.e., distribution date). For moving images, if a work with identical content but in a different medium has a later release date than the original work, code p is used (e.g., a videorecording released in 1978 that was originally produced as a motion picture in 1965).

008/06 p
008/07-10 1982
008/11-14 1967
260 ##$aWashington :$bU.S. Navy Dept.,$c1967 :$bDistributed by National Audiovisual Center,$c1982.

q - Questionable date
Exact date for a single date item is not known but a range of years for the date can be specified (e.g., between 1824 and 1846).

Earliest possible date is given in 008/07-10; latest possible date in 008/11-14.

008/06 q
008/07-10 1963
008/11-14 1966
260 ##$aNew York :$bHippocrene Books,$c[between 1963 and 1966]

008/06 q
008/07-10 18uu
008/11-14 19uu
260 ##$aAmsterdam :$bElsevier,$c[19th and early 20th century]
[Decade is unknown for both earliest and latest date.]

r - Reprint/reissue date and original date
008/07-10 contain the date of reproduction or reissue; 008/11-14 contain the date of the original, if known. 008/11-14 contain code u ("uuuu"), if unknown.

If multiple dates are available for the original publication, 008/11-14 contain the earlier date. With original photographic material, the work being described would be a later photoprint made from an earlier created photonegative. With original prints, the work being described would be a restrike made from the (usually deceased) artist's earlier-created plate or block.

008/06 r
008/07-10 1983
008/11-14 1857
260 ##$aBoston :$b[s.n.,$c1983?]
500 ##$aOriginal version: Pittsburg, Pa. : W'm Schuchman & Bro. Lith., [1857].

008/06 r
008/07-10 1966
008/11-14 uuuu
500 ##$aReprinted from Green Howard's Gazette.
[Original date of publication is unknown.]

008/06 r
008/07-10 uuuu
008/11-14 1963
260 ##$aNew York :$b[s.n.,$cn.d.]
500 ##$aPreviously published in 1963.

s - Single known date/probable date

Date consists of one known single date of distribution, publication, release, production, execution, writing, or a probable date that can be represented by four digits. The single date associated with the item may be actual, approximate, or conjectural (e.g., if the single date is uncertain). Code s is also used for a single unpublished item such as an original or historical graphic when there is a single date associated with the execution of the item.

008/07-10 contain the date; 008/11-14 contain blanks (####).

008/06 s
008/07-10 1977
008/11-14 ####
260 ##$aWashington :$bDept. of State,$c1977.

008/06 s
008/07-10 1981
008/11-14 ####
260 ##$a[Darmstadt] :$bTetzlaff,$c1980 [i.e. 1981]

008/06 s
008/07-10 1969
008/11-14 ####
260 ##$aLondon :$bHarcourt, World & Brace,$c[1969?]

008/06 s
008/07-10 1983
008/11-14 ####
260 ##$a[Yerushalayim :$bE. Fisher,$c744 i.e. 1983 or 1984]
[Non-Gregorian dates with no single Gregorian equivalent.]

008/06 s
008/07-10 1946
008/11-14 ####
260 ##$aBerlin :$b[s.n.,$cca. 1946]

008/06 s
008/07-10 198u
008/11-14 ####
260 ##$aNew York :$bHaworth,$c[198-]

008/06 s
008/07-10 19uu
008/11-14 ####
260 ##$aNew York :$bS.R.A.,$c[19--]

Span of dates associated with a single item of uncertain date is coded as questionable (code q). Multiple certain dates needed for a single item are coded as multiple dates (code m). Single or multiple dates associated with a collection are coded as either bulk dates (code k) or inclusive dates (code i).

t - Publication date and copyright date

Date of publication/release/production/execution is present in 008/07-10 and a copyright notice date or phonogram copyright notice date is present in 008/11-14. Deposit dates (i.e., those preceded by "D.L." (*Dépot légal*), etc.) may be treated as copyright dates.

008/06 t
008/07-10 1982
008/11-14 1949
260 ##$aLondon :$bMacmillan,$c1982, c1949.

008/06 t
008/07-10 2002
008/11-14 2001
260 ##$aNew York :$bEpic,$c2002, p2001.

008/06	t
008/07-10	198u
008/11-14	1979
260	##$aRio de Janeiro :$bDelta,$c[198-?], c1979.

u - Continuing resource status unknown
Used for continuing resources when there is no clear indication that publication of the item has ceased.

008/07-10 contain a beginning date of publication; 008/11-14 contain the characters *uuuu* since no ending date is known.

008/06	u
008/07-10	1948
008/11-14	uuuu
362	0#$a1948-

008/06	u
008/07-10	19uu
008/11-14	uuuu
500	##$aDescription based on: 1983.

008/06	u
008/07-10	1uuu
008/11-14	uuuu
	[No information is contained in the source record.]

| - No attempt to code

07-10 - Date 1
A date specified by the code in 008/06 (Type of date/Publication status).

Determination of dates for 008/07-10 is made concurrently with the choice of code for 008/06. See the section above on 008/06 for examples and input conventions related to coded date information. The use of fill characters in 008/07-10, although possible, is discouraged since the data in Date 1 is used for retrieval and duplicate detection in many systems. When fill is used in 008/07-10, all four positions must contain the fill character.

1-9 - Date digit

 # - Date element is not applicable

u - Date element is totally or partially unknown

|||| - No attempt to code

11-14 - Date 2
A date specified by the code in 008/06 (Type of date/Publication status).

Determination of dates for 008/11-14 is made concurrently with the choice of code for 008/06. See the section above on 008/06 for examples and input conventions related to coded date information. Four fill characters (||||) are used when no attempt has been made to code these character positions.

1-9 - Date digit

 # - Date element is not applicable

u - Date element is totally or partially unknown

|||| - No attempt to code

15-17 - Place of publication, production, or execution
Two- or three-character alphabetic code that indicates the place of publication, production, or execution. Place code is an authoritative-agency data element. Code from: *MARC Code List for Countries*. Choice of a MARC code is generally related to information in field 260 (Publication, Distribution, etc. (Imprint)). The code recorded in 008/15-17 is used in conjunction with field 044 (Country of Producer Code) when more than one code is appropriate to an item. The first code in subfield $a of field 044 is recorded in 008/15-17. Three fill characters (|||) may be used in place of a valid code, but their use in 008/15-17 is discouraged. The data in this field 008 data element is used for retrieval and duplicate detection in many systems. When fill is used in 008/15-17, all three positions must contain the fill character.

Two-character codes are left justified and the unused position contains a blank (#). For items reprinted in the original print size, the code is based on the jurisdiction where the reprint was published and not on the jurisdiction associated with the original place of publication.

For **sound recordings**, the code represents the place where the recording company is located. For **still images** that are original or historical graphics, if geographic information can be deduced (as with some photographs), a place code is recorded in this character position. For **archival moving images**, the code represents the country of producing entity from field 257. For mass-produced **videorecordings**, the code represents the place of publication in field 260 (Publication, Distribution, etc. (Imprint)).

008/15-17	cau
044	##$acau$afraspagw
260	##$aBurbank, Calif. :$bColumbia Tristar Home Video,$cc1996.
	[Video published in California of a film co-produced in France, Spain and Germany]

008/15-17	nyu
260	##$a[New York] :$bGardner & Co.,$cc1899.

008/15-17	nyu
245	00$a[Portrait of Cyrus Patten] /$cAnson, New York.
260	##$c1852.
	[A single unpublished graphic item.]

008/15-17	xx#
245	00$aVanity Fair /$cF. Depero.
260	##$c1930.
	[An unpublished graphic item where geographic information cannot be deduced.]

008/15-17 ja#
260 ##$aTokyo :$cShobido & Co.,$c1919.
[A collection consisting wholly of published items.]

When the place of publication/production/execution is totally unknown, code *xx#* is used.

008/15-17 xx#
260 ##$aS. l. :$bs.n.,$c1983.
[Place is completely unknown]

008/15-17 xx#
245 00$a[Hope diamond]$h[realia].
[Field 260 is not present in the record]

When more than one place is involved, the first-named jurisdiction is coded in 008/15-17. The code for the first jurisdiction is repeated in field 044 (Country of producer code), followed by the codes for the other jurisdictions.

008/15-17 enk
260 ##$aLondon ;$aNew York :$bAcademic Press,$c1979.
044 ##$aenk$anyu

For **visual materials** and **music**, if the work is a multi-country production, the code for the first country is recorded in 008/15-17. The code for the first country is repeated in field 044 (Country of Producer Code), followed by the codes for countries of other bodies involved in the production. For **serials** and **integrating resources**, the country code reflects the place of publication of the latest issue, part or iteration. If the record is updated at a later time and the place has changed, the place of publication code is updated. For **mixed materials**, the code represents the repository where the material is assembled.

xx# - No place, unknown, or undetermined
No place of publication, production, etc. can be provided; the place is unknown, or it is undetermined. Examples of such items are: 1) naturally occurring objects; 2) ancient manuscripts.

vp# - Various places
Various places are associated with different parts of items, generally a collection.

[aaa] - Three-character alphabetic code

[aa#] - Two-character alphabetic code

18-34 - Material specific coded elements
See one of the material specific 008/18-34 sections

35-37 - Language
Three-character alphabetic code that indicates the language of the item. Code from: *MARC Code List for Languages*. Choice of a MARC code is based on the predominant language of the item. Three fill characters (|||) may also be used if no attempt is made to code the language or if non-MARC language coding is preferred (and coded in field 041 (Language code)).

For **language material** (i.e., books and continuing resources), the language code is based on the text of the item. The term *text* refers to the principle work(s) included within the publication, excluding the preface, introduction, foreword, appendices, etc. For **computer files**, the language associated with the *data* and/or the *user interface* (e.g., textual displays, audible output in a language) determines the code used in 008/35-37, not the programming language. (Accompanying documentation in a language other than that of the data and/or user interface is coded in field 041.) For **maps**, the language of names and text associated with the map or globe determines the code used. For **music**, the predominant language of the sung or spoken text associated with the score or sound recording is recorded in 008/35-37. For **visual materials**, coding depends on the type of material. For moving image materials, the language content is defined as the sound track, the accompanying sound, or sign language. For moving image materials with no sound or sign language content or, if with sound, no narration, use zxx (no linguistic content). For filmstrips and slides, code for the text on the film, the accompanying sound or the accompanying printed script (for works with no sound or, if with sound, no narration). For all other still images, including original or historical graphic material and opaque and non-opaque graphic material, and three-dimensional materials, the language content is that associated with the material, i.e., captions or other text associated with the item or collection that are part of the chief source of information. For **mixed materials** the language code is based on the predominant language of an item or materials in a collection.

When only one language is associated with an item, the code for that language is recorded.

008/35-37 spa
245 00$aRentabilidad bruta del inversionista en bolsa.$pBonos del tesoro.

If more than one language code is applicable, the code for the predominant language is recorded in 008/35-37, and the codes for all of the languages, including the predominant language, are recorded in field 041 (Language Code). The code recorded in 008/35-37 is always the same as the language code recorded in the first occurrence of subfields $a or $d (for sound recordings).

008/35-37 rus
041 0#$arus$aeng
500 ##$aChiefly in Russian; with some contributions in English.

If there is no predominant language, the language codes are recorded in English alphabetical order in field 041 and the first one is recorded in 008/35-37.

008/35-37 eng
041 0#$aeng$aspa
546 ##$aText in English and Spanish.

When formulating a bibliographic record for a **translation**, the code for the language of the translation, not the language of the original, is given in 008/35-37. (The code for the language of the original is recorded in subfield $h of field 041.)

008/35-37 eng
041 1#$aeng$hger
[English translation of a German title]

- No information provided

zxx - No linguistic content
Item has no sung, spoken, or written textual content. Examples of such items are: 1) instrumental or electronic music; 2) sound recordings consisting of nonverbal sounds; 3) moving image materials with no sound or sign language content, or if sound, no narration; 4) visual materials other than moving images with no printed titles, captions, etc.; 5) computer files that consist of no more than the machine language (e.g., COBOL) or character codes (e.g., ASCII) used in source programs.

mul - Multiple languages
Item is multilingual with no predominant language and the cataloging institution has chosen not to specify a language in 008/35-37.

sgn - Sign languages
Primary medium of communication is a sign language, e.g., a book containing pictures of the handshape of each letter of a particular sign system, or a videorecording that is signed. The particular sign language system is stated in field 546 (Language Note), e.g., American Sign Language. The codes for secondary and related languages may be indicated in field 041 (Language Code).

008/35-37 sgn
041 0#$asgn$aeng
546 ##$aAmerican Sign Language, with some text in English.

und - Undetermined
Language of the item cannot be determined. Also used for works having content consisting of arbitrary syllables, humming, or other human-produced sounds for which a language cannot be specified.

[aaa] - Three-character alphabetic code

---

38 - Modified record
One-character code that indicates whether any data in a bibliographic record is a modification of information that appeared on the item being cataloged or that was intended to be included in the MARC record.

Such modifications include: the romanization of data that originally appeared in a non-roman script; substitution of characters available for those characters that could not be converted to machine-readable form (e.g., special symbols, "¤"); shortening of records because the amount of data exceeded a system imposed maximum allowable length. A record is not considered to be modified when romanizing fields in the record that are not transcriptions of data from the item (e.g., headings, complete nonquoted fields, subscription address, etc.). MARC equivalents for certain letters used in lesser known languages using the Roman alphabet are also not considered to be modified.

Precedence of codes - When more than one code applies to a bibliographic item, use the following table to determine the appropriate code to use. The codes are listed in precedence order, with codes listed first taking precedence over codes listed subsequently. Code # is the highest priority.

Not modified
s Shortened
d Dashed-on information omitted
x Missing characters
r Completely romanized/printed cards in script
o Completely romanized/printed cards romanized

Codes are assigned a priority (recorded in the order of the following list) that determines which code is input when more than one code applies to the item.

Default recommended:

 # - Not modified
Record has not been modified in any way (e.g., it is not shortened and it contains no characters that could not be converted to machine-readable form).

d - Dashed-on information omitted
MARC record does not contain "dashed-on" information found on the corresponding manual copy, either because the dashed-on information was input as a separate record, recorded in field 500 (General Note), or because it was omitted. "Dashed-on" information is generally the brief description of material related to a main item being cataloged that is not considered important enough to catalog separately. The "dashed-on" technique has not been used heavily since the introduction of machine-readable bibliographic records.

o - Completely romanized/printed cards romanized
Bibliographic data in the MARC record is completely romanized and any printed cards produced are also in romanized form.

r - Completely romanized/printed cards in script
Bibliographic data in the MARC record is completely romanized but the printed cards are available in the original (vernacular) script.

s - Shortened
Some of the data was omitted because the data exceeded the maximum length allowed by the system used to create or process it.

In systems where the length of data is restricted, it is usually at the field or record level. MARC bibliographic records have a maximum length of 99,999 characters. (For further information, see *MARC 21 Specifications for Record Structure, Character Sets, and Exchange Media*.) Code s is rarely used in current records.

x - Missing characters
Record contained characters that could not be converted to machine-readable form (e.g., incidental nonroman characters on predominantly roman alphabet records, mathematical symbols, etc.).

Note: Many institutions choose to simply romanize any nonroman characters encountered in bibliographic data. The technique of representing special symbols by a descriptive word or phrase is often used as an alternative to omitting the special symbol completely (e.g., "[tree]" included in data to represent the picture of a tree that was meant to be an integral part of the title). It is unlikely that code x will be used in current records.

| - No attempt to code

---

39 - Cataloging source
One-character code that indicates the original cataloging source of the record. If the cataloging source is known, it is identified in subfield $a of field 040 (Cataloging Source).

 # - National bibliographic agency
Creator of the original cataloging data is a national bibliographic agency (e.g., U.S. Library of Congress or Library and Archives Canada).

008/39 #
040 ##$aDLC$cDLC
[Record was created and transcribed by the U.S. Library of Congress.]

008/39 #
040 ##$aDLC$cWvU$dWvU$dCU$dCStRLIN
[Record was created by the Library of Congress, transcribed by West Virginia University, and modified by West Virginia University, University of California, and RLIN.]

008/39 #
040 ##$aCaOONL$beng$cCaOONL
[Record was created and transcribed by National Library of Canada.]

c - Cooperative cataloging program
Creator of the cataloging data is a participant (other than a national bibliographic agency) in a cooperative cataloging program.

008/39 c
040 ##aMHcMH
[Harvard University Library cataloging input online as part of the Program for Cooperative Cataloging.]

008/39 c
040 ##$aCaBVAU$cCaOONL
[Record created by the Univ. of British Columbia and transcribed by NLC.]

d - Other
Source of the cataloging data is an organization that is other than a national bibliographic agency or a participant in a cooperative cataloging program.

008/39 d
040 ##$aWyU$cWyU
[Record was created and transcribed by the University of Wyoming.]

008/39 d
040 ##$aIEN$dCStRLIN$dMiU
[Record was created by Northwestern University and modified by RLIN and the University of Michigan.]

u - Unknown
Creator of the cataloging data is unknown. Used when an organization transcribes manual cataloging data from an unknown source. In this case, field 040 lacks a subfield $a and subfield $c contains the MARC code for the transcribing organization.

008/39 u
040 ##$cWMUW
[The University of Wisconsin--Milwaukee is responsible for the content designation and transcription of cataloging from an unknown source.]

| - No attempt to code

INPUT CONVENTIONS

Capitalization - Alphabetic codes are input in lowercase.

Field length - Field 008 should always consist of forty (40) character positions.

CONTENT DESIGNATOR HISTORY

008/06 - Type of date/Publication status
b - No dates given; B.C. date involved [NEW, 1987]
Prior to the definition of this code and field 046 (Special Coded Dates), data elements for coded information about B.C. dates were not provided in MARC.
c - Actual date and copyright date (BK, CF, MP, MU, VM) [OBSOLETE]
c - Serial item currently published [REDEFINED, 1995]
t - Publication date and copyright date [NEW, 1995]
Prior to its redefinition, code c had a different definition for books, computer files, maps, music, and visual materials than it did for serials. The difference was eliminated by the definition of a new code t (Publication date and copyright date) which is used in place of the obsolete nonserial code c. The definition of the serial code c was retained.
d - Detailed date (BK, VM) [OBSOLETE]
d - Serial item ceased publication [REDEFINED, 1995]
e - Detailed date [NEW, 1995]
Prior to its redefinition, code d had a different definition for books and visual materials than it did for serials. The difference was eliminated by the definition of a new code e (Detailed date) which is used in place of the obsolete nonserial code d. The definition of the serial code d was retained.
i - Date of distribution/release/issue and production/recording session when different (MU) [OBSOLETE, 1978]
Prior to the definition of code p (Date of distribution/release/issue and production/recording session when different) for music in 1978, this type of date was identified by code i. The current definition of code i (Inclusive dates of collection) was approved in 1983.
i - Inclusive dates of collection [NEW, 1983]
Prior to the definition of code p (Date of distribution/release/issue and production/recording session when different) for music in 1978, this type of date was identified by code i.
n - Dates unknown
Prior to the introduction of AACR 2, code n was used with all forms of material when subfield $c (Date of publication, distribution, etc.) in field 260 (Publication, Distribution, etc. (Imprint)) contained the statement "n.d." for "no date". In cataloging formulated according to AACR 2, code n is used only 1) in records for mixed materials when there is no date in field 260 or in field 245, and 2) in visual material records for naturally occurring objects and artifacts when subfield $g (Date of manufacture) is not present in field 260 . Prior to 1995, zeros or blanks were used for unknown portions of Date 1 and Date 2 for some forms of material.

008/15-17 - Place of publication, production, or execution
Technique for indicating a questionable place by modifying the MARC country code was made obsolete in 1972. Prior to that time, the letter *q* was added to a two-character place code (e.g., Paris? was coded as *frq*) or the letters *d, l, s*, or *v* were used instead of the letters *c, k, r,* or *u* as the third character in the three-character codes for jurisdictions within Canada, the Soviet Union, the United Kingdom, and the United States (e.g, Chicago? was coded as *ilv* instead of *ilu*). Prior to 1980, place codes were limited to two character positions (positions 15-16) in the visual materials specifications. The three-character codes for Canada, the U.K., the U.S., and the U.S.S.R. were not used (e.g., London was coded uk).
??q - Questionable place coding [OBSOLETE, 1972]

008/35-37 - Language
Prior to 2006, three blanks were used to indicate that the item has no sung, spoken or written text. Code zxx (No linguistic content) was added at that time to indicate this situation and three blanks were redefined as no information provided.

008/38 - Modified record
u - Unknown [OBSOLETE] [CAN/MARC only]

008/39 - Cataloging source
Code n was made obsolete following the cessation of publication of *New serial titles*.
 # - Library of Congress [REDEFINED, 1997]
a - National Agricultural Library [OBSOLETE, 1997] [USMARC only]
b - National Library of Medicine [OBSOLETE, 1997] [USMARC only]
d - Another national library cataloguing [REDEFINED, 1997] [CAN/MARC only]
l - Library of Congress cataloguing [OBSOLETE, 1997] [CAN/MARC only]
o - Other institution cataloguing [OBSOLETE, 1997] [CAN/MARC only]
n - Report to New serials titles [OBSOLETE, 1997] [USMARC only]
r - Reporting library [OBSOLETE, 1997] [CAN/MARC only]

EXHIBIT B

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Name of requestor:	Michel Carney
DCU Order number:	Judicial-Proceedings-20190905-CARNEY
Item details	
Title of thesis:	Application of monoclonal antibodies to the investigation of the role of calcitonin gene-related peptide as a vasodilatory neurotransmitter
Author:	Keith Kwan Cheuk Tan.
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PhD 1999

Application of monoclonal antibodies to the investigation of the role of calcitonin gene-related peptide as a vasodilatory neurotransmitter

Keith Kwan Cheuk Tan, BPharm, MSc, MRPharmS

Gonville and Caius College, Cambridge

1994

A dissertation submitted to the University of Cambridge for the Ph.D. Degree

Declaration

This dissertation is an account of my original work. However, a number of monoclonal antibodies were produced by others and made available to me as part of a research collaboration. These antibodies have been distinguished from those that I have produced, and their sources have been clearly stated. The characterization and application of these antibodies, reported in this dissertation, was entirely my own work.

I hereby declare that this dissertation entitled "Application of monoclonal antibodies to the investigation of the role of calcitonin gene-related peptide as a vasodilatory neurotransmitter" is not substantially the same as any that I have submitted for a degree, diploma or other qualification at any other University.

I further state that no part of my dissertation has already been or is being concurrently submitted for any such degree, diploma or other qualification.

Date......9 March 1994...... Signed..............................

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Abbreviations

AUC	Area under the flux-time curve attributable to nerve stimulation
Bis	N'N'-Bis-methylene-acrylamide
BSA	Bovine serum albumin
B_{max}	Concentration of binding sites
CDR	Complementarity-determining region
CGRP	Calcitonin gene-related peptide
cpm	Counts per minute
95% C.I.	95% Confidence interval
DAB	3,3' Diaminobenzidine
DMEM	Dulbecco's modified Eagles medium
EC_{50}	Concentration which produces half-maximal effect
EDTA	Ethylene-diamine-tetraacetic acid
ELISA	Enzyme-linked immunoadsorbent assay
E_{max}	Maximum effect
2FD, 10FD, 20FD	Dulbecco's modified Eagles medium containing 2, 10, 20% foetal calf serum
FITC	Fluorescein isothiocyanate
Fmax	Maximum change in skin blood flow attributable to nerve stimulation
HAT	Hypoxanthine, aminopterin and thymidine
HαCGRP	Human α CGRP
$H\alpha CGRP_{8-37}$	C-terminal (8-37) fragment of HαCGRP
HβCGRP	Human β CGRP
HT	Hypoxanthine and thymidine
i.p.	Intraperitoneal
i.v.	Intravenous
KCl	Potassium chloride
Kd	Dissociation constant
MAb	Monoclonal antibody
MAP	Mean arterial pressure
NK_1	$Neurokinin_1$
PAbs	Polyclonal antibodies
PBS	Phosphate-buffered saline
PBSTx	0.1M PBS/0.3% Triton-X 100

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PEG	Polyethylene glycol
PMSF	Phenylmethyl-sulphonyl-fluoride
RαCGRP	Rat α CGRP
RβCGRP	Rat β CGRP
RIA	Radioimmunoassay
rpm	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SFD	Serum-free Dulbecco's modified Eagles medium
SHR	Spontaneously hypertensive rat
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
TSH	Thyroid-stimulating hormone

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Summary

Calcitonin gene-related peptide (CGRP) is produced by alternative mRNA processing of the calcitonin gene. It is a potent vasodilator and is localized in perivascular sensory neurons. The localization of CGRP-immunoreactivity in primary afferent neurons innervating many different tissues and the wide distribution of CGRP binding sites suggest that CGRP may be a physiologically important neurotransmitter. The aim of the project was to investigate whether vasodilatory responses to CGRP released from perivascular sensory nerves could be blocked with anti-CGRP or anti-CGRP receptor monoclonal antibodies (MAbs).

MAbs against CGRP were successfully produced and characterized for their ability to inhibit CGRP receptor binding (immunoblockade). Unsuccessful attempts were made to develop MAbs against the CGRP receptor by *in vivo* and *in vitro* immunization of animals with CGRP receptor-rich membranes and by an auto-anti-idiotypic approach.

Eleven MAbs against CGRP were screened for immunoblocking properties in an isolated porcine coronary artery assay or an electrically-stimulated isolated rat vas deferens assay. MAb C4.19 was identified as a MAb that effectively blocks the effects of exogenous rat CGRP. It was demonstrated that the pharmacological response to CGRP in the presence of MAb C4.19 could be predicted when the dissociation constant and concentration of binding sites of the antibody were known. Capsaicin was used to stimulate the release of endogenous CGRP from primary afferent neurons. Capsaicin-induced inhibition of electrically-stimulated contractions of the isolated rat vas deferens was significantly attenuated by MAb C4.19. The results of the *in vitro* immunoblockade studies suggest that CGRP has a major role as a neurotransmitter at the neuroeffector junction of the rat vas deferens.

The effect of MAb C4.19 on the hypotensive response to exogenous rat αCGRP (RαCGRP) was investigated in the pentobarbitone-anaesthetized rat. The role of CGRP in mediating antidromic vasodilatation was investigated by blockade of the increase in hind paw skin blood flow produced by saphenous nerve stimulation in the pentobarbitone-anaesthetized rat. Change in skin blood flow was measured by laser Doppler flowmetry. The dose-response relationship for the effect of i.v. RαCGRP was similarly shifted rightward by MAb C4.19 IgG (1 mg/rat i.v.) and Fab' fragment (2 mg/rat i.v.). The C-terminal fragment of human αCGRP (HαCGRP$_{8-37}$; 100 nmol/kg i.v.) also blocked the hypotensive effect of RαCGRP significantly. MAb C4.19 Fab' fragment (2 mg/rat i.v.) and HαCGRP$_{8-37}$ (100 nmol/kg i.v.) but not MAb C4.19 IgG (up to 3 mg/rat i.v.) blocked the increased skin blood flow response to antidromic stimulation of the saphenous nerve. Normal mouse Fab' fragment

(2 mg/rat i.v.) had no significant effect on antidromic skin vasodilatation. The mean percentage changes in skin blood flow parameters due to MAb C4.19 Fab' fragment were significantly different from those due to normal mouse Fab' fragment but not from those due to $H\alpha CGRP_{8-37}$.

The results of this project show that immunoblockade with an anti-CGRP MAb may be used to demonstrate the physiological role of endogenous CGRP. However, Fab' fragments should be used for acute *in vivo* pharmacological studies to ensure effective distribution to the site of action. The results of immunoblockade agree with those obtained by receptor blockade with $H\alpha CGRP_{8-37}$ and provide complementary evidence in support of the role of CGRP in mediating skin vasodilatation.

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Correction (page 1, lines 24-25): "A CGRP ......" should read "A form of CGRP ......"

CHAPTER 1

General introduction

1.1. Calcitonin gene-related peptide

Calcitonin gene-related peptide (CGRP) is a 37-amino acid peptide which was discovered after its structure and existence were predicted from molecular cloning studies of the rat calcitonin gene (Amara *et al.*, 1982; Rosenfeld *et al.*, 1983). Alternative processing of the primary mRNA transcript of the calcitonin gene leads to the production of calcitonin or CGRP. Human CGRP was first isolated from medullary thyroid carcinoma tissue (Morris *et al.*, 1984).

A second gene encoding a closely homologous 37-amino acid peptide was subsequently identified in rat and man (Amara *et al.*, 1985; Steenbergh *et al.*, 1985). The second peptide is termed βCGRP to distinguish it from the originally described CGRP, now referred to as αCGRP.

The calcitonin/αCGRP gene is transcribed as a larger precursor mRNA containing 6 exons which are subsequently spliced to form either calcitonin or αCGRP. The fully processed mRNAs have the first 3 exons in common but contain distinct 3' terminal exons. The transcripts from the βCGRP gene, however, are not processed to form multiple mRNAs. Thus the βCGRP gene does not code for a second calcitonin. The calcitonin/αCGRP and βCGRP genes are both located on chromosome 11.

1.1.1. Structure of CGRP

CGRP consists of 37 amino acids with an N-terminal 6-amino acid ring structure linked by a disulphide bridge and an amidated C-terminus. The two forms of CGRP differ in three amino acid positions in man but only one in the rat (Figure 1.1). A CGRP with striking homology to the rat and human CGRPs has also been isolated from the spinal cord of the pig (Kimura *et al.*, 1987).

CGRP shares limited structural similarities with calcitonin. The sequence homology between human αCGRP (HαCGRP) and human calcitonin is only 16%. The different forms of CGRP cross-react weakly with calcitonin receptors and are about 100- to 1000-fold less potent than human calcitonin in lowering plasma calcium levels and inhibiting osteoclastic activity (Zaidi *et al.*, 1988; Raue *et al.*, 1987). Salmon calcitonin at high concentrations can cross-react with CGRP receptors (Goltzman & Mitchell, 1985; Wimalawansa & El-Kholy, 1993).

Perhaps more significantly, there are major structural similarities between

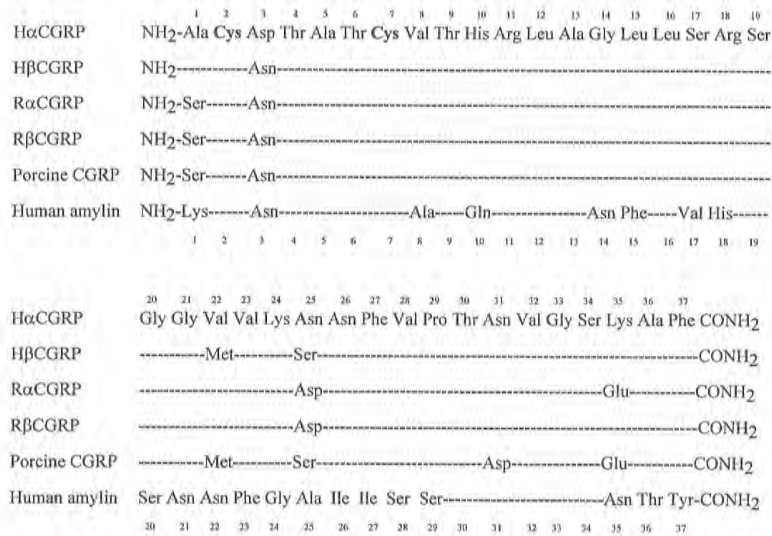

Figure 1.1: Structure of α and β forms of CGRP from man, rat and pig. The structure of human amylin is shown for comparison. Broken horizontal lines indicate regions of sequence identity with HαCGRP. Cysteine residues at positions 2 and 7 (in bold) take part in a disulphide bond.

CGRP and another 37-amino acid peptide called amylin which is secreted from pancreatic islets of Langerhans. Human amylin, also known as islet amyloid polypeptide, was originally isolated from the amyloid deposits of an insulinoma (Westermark *et al.,* 1986) and of Type II diabetic pancreases (Cooper *et al.,* 1987). It has 43% and 46% sequence homology with HαCGRP and human βCGRP (HβCGRP) respectively. Indeed, amylin shares many of the biological properties of CGRP and cross-reacts with CGRP receptors (Section 1.1.9.1.).

More recently, a novel 52 amino acid peptide showing slight sequence homology to CGRP has been isolated from human phaeochromocytoma (Kitamura *et al.,* 1993). This peptide has been named adrenomedullin. Nine amino acid residues in the C-terminal (15-52) end of adrenomedullin are also found in the α and β forms of human CGRP.

1.1.2. Distribution of CGRP

CGRP is widely distributed in the central and peripheral nervous systems (Tschopp *et al.,* 1984; Lee *et al.,* 1985; Wimalawansa, *et al.,* 1987). The presence of CGRP immunoreactivity has been detected primarily by immunocytochemistry and radioimmunoassay with anti-CGRP sera which do not distinguish between the two

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known forms of CGRP. However, the expression of the α and β forms of CGRP has been revealed by hybridization histochemistry using specific RNA probes (Mulderry *et al.*, 1988; Noguchi *et al.*, 1990; Sternini & Anderson, 1992). Both forms of CGRP are localized primarily in neural tissues.

In the brain, CGRP is present in the nuclei of sensory and motor cranial nerves and in cell bodies in distinct regions including the hypothalamus, preoptic area, ventromedial thalamus, medial amygdala and hippocampus (Skofitsch & Jacobowitz, 1985a; Yamamoto & Tohyama, 1989). In the spinal cord, CGRP-immunoreactive fibres are distributed primarily in the dorsal horn which receives sensory input. Dorsal rhizotomy induces a marked loss of CGRP-immunoreactive fibres from the dorsal spinal cord. Thus the CGRP-containing fibres are central projections of afferent neurons originating from the dorsal root ganglion (Gibson *et al.*, 1984). The mRNAs of the α and β forms of CGRP are co-expressed in dorsal root ganglion cells (Noguchi *et al.*, 1990).

CGRP immunoreactivity is found within cells and sensory nerve fibres in diverse peripheral organs including the heart, lung, urogenital tract, tongue, pancreas, skin and gastrointestinal tract (Gibbins *et al.*, 1985; Wimalawansa *et al.*, 1987; Mulderry *et al.*, 1988). Throughout the body, it is localized in perivascular sensory nerve fibres (Rosenfeld *et al.*, 1983; Mulderry *et al.*; 1985, Uddman *et al.*, 1986). The density of fibres around arteries is generally higher than that around veins (Uddman *et al.*, 1986). Combined retrograde tracing and immunocytochemical studies have demonstrated that the CGRP-immunoreactive nerves in the periphery originate from dorsal root ganglia (Alm & Lundberg, 1988; Louis *et al.*, 1989; Sternini & Anderson, 1992). However, the origin of most of the CGRP-immunoreactive cerebrovascular nerve fibres appears to be the trigeminal ganglion. CGRP-immunoreactive fibres are found in the adventitia and the adventitial-medial border of blood vessels (Gulbenkian *et al.*, 1986; Edvinsson *et al.*, 1987; Shoji *et al.*, 1987).

CGRP-immunoreactive fibres are present in all regions of the heart, particularly in association with the coronary arteries, within the papillary muscles, and within the sinoatrial and atrioventricular nodes (Mulderry *et al.*, 1985). Tissue concentrations of immunoreactive CGRP are higher in the atria than the ventricles in rat and guinea pig hearts (Wharton *et al.*, 1986; Wimalawansa & MacIntyre, 1988). In human cardiopulmonary tissue, the highest levels of CGRP immunoreactivity are found in the left anterior descending coronary artery, followed in declining order by the bronchus, right atrium, pulmonary artery, lung and left ventricle (Franco-Cereceda, 1991). CGRP-immunoreactive nerve fibres are very sparse in the proximal region of human epicardial arteries but increases in number distally (Gulbenkian *et al.*, 1993).

CGRP immunoreactivity is also localized in non-nervous tissue. The peptide was originally reported to be absent in the rat thyroid gland (Rosenfeld *et al.*, 1983). However, CGRP immunoreactivity has been subsequently co-localized with calcitonin in thyroid C-cells (Sabate *et al.*, 1985; Lee *et al.*, 1985). Rat thyroid C-cells produce both calcitonin and CGRP mRNAs in a ratio of approximately 95:1. CGRP immunoreactivity is also localized in nerve fibres in the thyroid gland. In the lung, CGRP immunoreactivity is localized in capsaicin-sensitive nerve fibres and in endocrine cells (Cadieux *et al.*, 1986; Shimosegawa & Said, 1991). CGRP immunoreactivity has been localized in subpopulations of endothelial cells of term human umbilical vein and artery (Cai *et al.*, 1993).

CGRP is frequently co-localized with substance P in primary afferent neurons (Lee *et al.*, 1985; Lundberg *et al.*, 1985; Ju *et al.*, 1987; Quartu *et al.*, 1992). The co-existence of the two peptides in trigeminal ganglia, dorsal root ganglia and perivascular nerve fibres has been demonstrated at the ultrastructural (electron microscopical) level (Gulbenkian *et al.*, 1986). However, primary afferent neurons containing only one or other peptide also occur (Lee *et al.*, 1985; Quartu *et al.*, 1992), and CGRP may be co-localized with other peptides such as somatostatin in the sensory axons of human skin (Gibbins *et al.*, 1987).

In addition to its widespread distribution in sensory neurons, CGRP immunoreactivity is localized in motoneurons (Gibson *et al.*, 1984; Mora *et al.*, 1989). Only βCGRP mRNA appears to be expressed in enteric neurons, unlike sensory neurons which express both forms of CGRP mRNA (Mulderry *et al.*, 1988; Sternini & Anderson, 1992). In the autonomic nervous system, CGRP is present pre-ganglionically in sympathetic and parasympathetic nerve fibres (Kruger *et al.*, 1988).

1.1.3. Distribution of CGRP binding sites

Specific binding sites for CGRP are distributed in distinct regions of the brain (Henke *et al.*, 1985; Skofitsch & Jacobowitz, 1985b; Kruger *et al.*, 1988) and the spinal cord (Yashpal *et al.*, 1992). Diverse peripheral tissues contain specific binding sites including blood vessels, heart, liver, thyroid gland, pituitary gland, spleen, lungs, pancreas, penis, adrenal gland and bladder (Goltzman & Mitchell, 1984; Nakamuta *et al.*, 1986; Sigrist *et al.*, 1986, Wimalawansa *et al.*, 1987).

In the cardiovascular system of the rat, the highest concentrations of both CGRP and its binding sites are found in peripheral and mesenteric arteries (Wimalawansa & MacIntyre, 1988). Specific binding sites on blood vessels are distributed in the media and intima (Sigrist *et al.*, 1986). CGRP binding sites have been identified in cultured vascular smooth muscle cells and endothelial cells (Hirata

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Table 1.1: Biological effects attributed to CGRP in the periphery

Effect	Reference
Vasodilatation	Brain *et al.*, 1985
Inotropic effect	Ishikawa *et al.*, 1988
Chronotropic effect	Marshall *et al.*, 1986
Potentiation of inflammatory oedema	Brain & Williams, 1985
Chronic inflammation	Louis *et al.*, 1990
Bronchoconstriction	Palmer *et al.*, 1987
Inhibition of leukotriene release from lung	Di Marzo *et al.*, 1986
Inhibition of insulin secretion	Ahren *et al.*, 1987
Inhibition of insulin-stimulated glycogen synthesis	Leighton & Cooper, 1988
Inhibition of gastrointestinal motility	Forster & Dockray, 1991
Inhibition of gastric acid secretion	Tache, 1992
Stimulation of gastric somatostatin release	Inui *et al.*, 1991
Stimulation of pancreatic amylase release	Seifert *et al.*, 1985
Inhibition of aldosterone secretion	Murakami *et al.*, 1989
Inhibition of urinary tract motility	Maggi *et al.*, 1992
Nicotinic receptor synthesis	New & Mudge, 1986
Nicotinic receptor desensitization	Mulle *et al.*, 1988
Inhibition of T-lymphocyte proliferation	Umeda *et al.*, 1989
T-lymphocyte chemotaxis	Foster *et al.*, 1992
Stimulation of endothelial cell proliferation	Haegerstrand *et al.*, 1990
Peripheral nerve regeneration	Dumoulin *et al.*, 1992
Regulation of calcium metabolism	Zaidi *et al.*, 1988
Intestinal ion transport	Cox *et al.*, 1989

et al., 1988). The density of specific CGRP binding sites may depend on vessel size. Specific binding sites are more abundant in small intramyocardial compared with large epicardial coronary arteries (Sun *et al.*, 1993).

1.1.4. Biological effects of CGRP

The widespread distribution of CGRP and its binding sites in the central and peripheral nervous systems suggests that it is a neuropeptide with multiple physiological roles. Indeed, CGRP has been shown to exhibit biological effects in virtually all organs. A detailed review of the diverse biological effects attributed to CGRP is beyond the scope of this introductory chapter. In accordance with the aim of

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Correction (page 6, line 31):
"... can potent dilate ..." should read "... can potently dilate ..."

Table 1.2: Biological effects attributed to CGRP in the central nervous system

Effect	Reference
Stimulation of noradrenergic outflow	Fisher *et al.*, 1983
Hyperthermia	Dennis *et al.*, 1990
Decrease in food intake	Krahn *et al.*, 1984
Inhibition of gastrointestinal motility	Raybould *et al.*, 1988
Gastric mucosal protection	Tache, 1992
Inhibition of gastric acid secretion	Hughes *et al.*, 1984
Suppression of growth hormone release	Fahim *et al.*, 1990
Central nervous system differentiation	Denis-Donini, 1992
Antinociceptive effect	Pecile *et al.*, 1987
Lowering of nociceptive threshold	Oku *et al.*, 1987

the project (Section 1.3), the role of CGRP as a vasodilator will be reviewed below. However, the peripheral and central effects of CGRP have been summarized in Tables 1.1 and 1.2 respectively.

1.1.5. CGRP as a vasodilator

The first suggestion of the vasodilatory properties of CGRP came from observations by Fisher *et al.* (1983) that intravenous injection of RαCGRP produced dose-dependent hypotension and tachycardia in conscious rats. The vasodilatory activity of rat and human αCGRP was clearly established by Brain *et al.* (1985) in rabbit skin, human skin, isolated rat aorta and the hamster cheek pouch microvasculature. Moreover, HαCGRP appeared to be more potent at lowering blood pressure when infused i.v. to normal volunteers than any other vasodilator (Struthers *et al.*, 1986). Lappe *et al.* (1987) demonstrated that RαCGRP increased cardiac output and reduced total peripheral resistance simultaneously in conscious rats. Therefore, the hypotensive effect of CGRP is mediated through peripheral vasodilatation rather than through reductions in cardiac output. Since these original observations, the potent vasodilatory activity of CGRP has been widely confirmed in man and in all animal species studied, whether in isolated blood vessels (strips or rings), isolated perfused vascular beds or *in vivo*. Examples are shown in Table 1.3. Although regional and species variations exist, it is noteworthy that CGRP can potent dilate both large vessels and arterioles which are more relevant to the regulation of peripheral vascular resistance. Compared with other vasodilators, and substance P in particular, CGRP produces a long-lasting vasodilatation. Following brachial artery infusion in man, its

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Table 1.3: Examples of the vasodilatory response to CGRP observed in various vascular beds and species

Blood vessel/vascular bed	Species	Reference
Renal arterioles	rabbit	Edwards & Trizna, 1990
Skin	human	Brain *et al.*, 1986a
Cerebral artery	human	Edvinsson *et al.*, 1987
Nasal mucosa	pig	Stjarne *et al.*, 1991
Coronary arteries	pig	Franco-Cereceda *et al.*, 1987a
Pulmonary artery	human	McCormack *et al.*, 1989
Mesenteric arterial bed	rat	Kawasaki *et al.*, 1988
Opthalmic artery	pig	Bakken *et al.*, 1992
Uterine artery	human	Nelson *et al.*, 1993a
Gastric submucosa	rat	Chen *et al.*, 1992
Synovial vessels	rat	Cambridge & Brain, 1992
Skeletal muscle arterioles	rabbit	Ohlen *et al.*, 1987
Tracheal artery	dog	Salonen *et al.*, 1988

half-life of biological effect (increased forearm blood flow) was approximately 18 minutes in contrast to a half-life of biological effect of approximately 15 seconds estimated for substance P (McEwan *et al.*, 1988).

The β form of CGRP is also a potent vasodilator (Brain *et al.*, 1986b). Both similar and differential vasodilatory effects have been reported for the two forms of CGRP. Approximately equipotent vasodilatation have been reported in animals and in man *in vitro* (Franco-Cereceda *et al.*, 1987a; Franco-Cereceda, 1991) and *in vivo* (Zaidi *et al.*, 1990; Hughes & Brain, 1991; Williams *et al.*, 1988). On the other hand, studies in conscious rats have suggested that HαCGRP and HβCGRP may differ in some of their regional haemodynamic effects and that these differences may be dose-dependent (Gardiner *et al.*, 1989). HβCGRP has been reported to be a more potent coronary vasodilator than HαCGRP in the rat (Holman *et al.*, 1986) but this does not appear to be the case in man (Franco-Cereceda, 1991). More potent *in vivo* vasodilatory effects have been reported for HαCGRP compared with HβCGRP in man (Beglinger *et al.*, 1991). RαCGRP has been reported to be 3 times more potent than RβCGRP as a dilator of the isolated perfused bed of the rat left gastric artery (Holzer *et al.*, 1993).

The discrepancies in results reported by various workers may reflect species and regional differences in sensitivity to αCGRP and βCGRP. Differences in the metabolic degradation of the two forms of CGRP have been suggested as an

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explanation of the their different potencies *in vivo* but the clearance of HαCGRP and HβCGRP in man have been reported to be similar (Beglinger *et al.*, 1991). On balance, the vasodilatory properties of αCGRP and βCGRP are characterized by their similarity rather than their differences; in general, the differences reported are not large enough (e.g., 10-fold) to be physiologically important.

CGRP may be a potent but selective vasodilator in different vascular beds. The most pronounced increases in blood flow have been detected in the skin and gastric circulations following i.v. administration of CGRP to conscious rats (Ando *et al.*, 1990). Comparison of haemodynamic measurements in different vascular beds indicate that CGRP is a selective dilator in the carotid vascular bed in the rat (Gardiner *et al.*, 1989) and in man (MacDonald *et al.*, 1989; Mulholland *et al.*, 1991). CGRP appears to redistribute blood flow to the skin and carotid artery at the expense of mesenteric blood flow in man (Jager *et al.*, 1990; Mulholland *et al.*, 1991). Differential vasodilatory responses to CGRP have been reported within the coronary arterial bed, depending on the diameter of conduit and resistance vessels studied (Foulkes *et al.*, 1991; Ludman *et al.*, 1991; Sekiguchi *et al.*, 1994). In the kidney, CGRP dilates renal afferent but not efferent arterioles (Edwards & Trizna, 1990).

There is no doubt that CGRP is a potent arterial dilator. However, the evidence for CGRP-induced dilatation of veins is less clear-cut. CGRP relaxes isolated rat femoral (Edvinsson *et al.*, 1989b), human pial (Hardebo *et al.*, 1987) and human pulmonary veins (McCormack *et al.*, 1989) but not isolated human saphenous veins (Marshall *et al.*, 1988). Infusion of CGRP into the brachial artery in man leads to a marked and prolonged increase in forearm blood flow but CGRP does not dilate pre-constricted superficial veins of the hand (McEwan *et al.*, 1988). CGRP is a potent dilator of the rat isolated perfused mesenteric vasculature on the arterial side but is a weak dilator on the venous side (Claing *et al.*, 1992). In the anaesthetized dog, CGRP increases blood flow more potently in the perfused hepatic arterial than the portal vascular bed (Withrington *et al.*, 1992). Thus there is good evidence that CGRP is less potent as a venous than an arterial dilator. The role of CGRP as a physiological venous dilator is, however, supported by the finding in conscious rats that it decreased mean circulatory filling pressure (an index of body venous tone); this was particularly evident when venous tone was elevated (Abdelrahman & Pang, 1992).

1.1.5.1. Mechanisms of vascular relaxation

Several mechanisms have been proposed for the mechanism of CGRP-induced vasodilatation. The vasodilatory effect of CGRP is not mediated by adrenergic, cholinergic or histaminergic mechanisms (Hanko *et al.*, 1985; Franco-Cereceda *et al.*, 1987a). However, both endothelium-dependent and -independent mechanisms have

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been reported. CGRP has also been reported to act as a vasodilator by activation of adenosine 5'-triphosphate (ATP)-sensitive potassium (K_{ATP}) channels (Nelson *et al.*, 1990).

Examples of blood vessels in which vasodilatation is endothelium-independent include the isolated pial artery of the rabbit, cat and man (Hanko *et al.*, 1985), canine cerebral artery (Ikegaki *et al.*, 1989), rabbit hepatic artery (Brizzolora & Burnstock, 1991), human uterine artery (Nelson *et al.*, 1993a) and human coronary artery (Franco-Cereceda, 1991). The endothelium-independent relaxation of isolated porcine coronary artery rings is associated with adenylate cyclase activation and a decrease in intracellular free calcium concentration (Shoji *et al.*, 1987; Kageyama *et al.*, 1993). In contrast, relaxation of the isolated rat aorta is dependent on the presence of an intact endothelium (Brain *et al.*, 1985; Grace *et al.*, 1987). CGRP has been shown to mediate endothelium-dependent relaxation of rat aortic rings by a novel signal transduction mechanism involving activation of both adenylate cyclase and guanylate cyclase (Gray & Marshall, 1992; Wang *et al.*, 1991). The endothelium-derived relaxing factor released by CGRP is probably nitric oxide because the relaxation of aortic rings is blocked by inhibitors of nitric oxide synthase.

CGRP relaxes pre-contracted segments of some human arteries, including radial, gastric and cerebral arteries, in an endothelium-dependent manner (Thom *et al.*, 1987). Whether CGRP-induced vasodilatation is endothelium-dependent may depend on the size of the vessel (Hughes *et al.*, 1988). The relaxation of human resistance arteries by CGRP does not depend on the integrity of the endothelium.

Endothelium-dependent and -independent mechanisms may operate concurrently within a vascular bed. Prieto *et al.* (1991) reported that the relaxation of isolated rat proximal epicardial but not distal intramyocardial coronary artery rings is endothelium-dependent. Both nitric oxide-dependent and -independent mechanisms have been implicated in the hyperaemic action of CGRP in the rat gastric circulation (Holzer *et al.*, 1993).

The hypotensive effect of RαCGRP in conscious rats is attenuated by nitric oxide synthase inhibitors (Abdelrahman *et al.*, 1992). Differential contribution of nitric oxide to the regional vasodilatory effects of CGRP has been demonstrated; CGRP-mediated vasodilatation of the hindquarters vascular bed has a substantial nitric oxide-dependent component (Gardiner *et al.*, 1991c).

In addition to mediating endothelium-dependent CGRP vasorelaxation, there is evidence that nitric oxide may modulate the release of CGRP or act as a vasodilatory neurotransmitter in its own right. The nitric oxide synthase inhibitor L-nitro-arginine methyl ester does not affect the vasodilatory effect of HαCGRP but significantly inhibits capsaicin-induced vasodilatation in rabbit skin (Brain *et al.*,

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1993; Hughes & Brain, 1994). Nitric oxide, rather than CGRP, may mediate relaxation induced by electrical stimulation of bovine basilar artery (Ayajiki *et al.*, 1993).

There is evidence that prostaglandins could mediate the endothelium-dependent vasodilatory effects of CGRP. The peptide is capable of releasing prostacyclin from human umbilical vein endothelial cells (Crossman *et al.*, 1987). The relaxation of rat aortic rings by CGRP has been reported to be partially inhibited by the cyclooxygenase inhibitor indomethacin (Brain *et al.*, 1985). However, further studies failed to demonstrate inhibition of CGRP-induced relaxation of pre-constricted rat aortic rings or cyclic nucleotide responses with ibuprofen or indomethacin. (Gray & Marshall, 1992; Fiscus *et al.*, 1991). The reasons for these contradictory results are unclear. Prostaglandins may play a role in mediating the renal haemodynamic effects of HβCGRP. The increased renal blood flow response to intrarenal infusion of HβCGRP in anaesthetized dogs is markedly attenuated by treatment with indomethacin (Villarreal *et al.*, 1988). The use of indomethacin has also implicated the involvement of prostaglandins in mediating CGRP-induced endothelium-dependent relaxation of rat proximal epicardial coronary artery rings (Prieto *et al.*, 1991).

There is conflicting evidence on the role of K_{ATP} channels in mediating the vasodilatory effects of CGRP. Partial blockade of CGRP vasodilatation and arterial smooth muscle hyperpolarization by the K_{ATP} channel antagonist glibenclamide was originally observed in the isolated rabbit mesenteric artery (Nelson *et al.*, 1990). Some evidence has accumulated in support of the role of K_{ATP} channels including glibenclamide blockade of CGRP-induced vasodilatation of the isolated human uterine artery (Nelson *et al.*, 1993b), the rat basilar artery *in vivo* (Kitazono *et al.*, 1993) and the feline pulmonary vascular bed (Hood *et al.*, 1991). CGRP-induced hypotension is attenuated by glibenclamide in the rabbit (Andersson, 1992) but not in the rat (Abdelrahman *et al.*, 1992). Glibenclamide does not significantly affect the vasodilatory effect of CGRP in the isolated perfused rat lung (Tjen-A-Looi *et al.*, 1992) and the isolated porcine coronary artery (Kageyama *et al.*, 1993), rabbit ophthalmic artery (Zschauer *et al.*, 1992), rat coronary artery (Prieto *et al.*, 1991) and human mammary artery (Boyle & Brown, 1991).

The different mechanisms reported for the vasodilatory action of CGRP is likely to reflect species and regional variation, receptor heterogeneity, the use of different experimental conditions (e.g., *in vitro* or *in vivo*), or a combination of these factors.

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1.1.6. Effects of CGRP on the heart

Positive chronotropic and inotropic effects of CGRP are observed in the isolated rat and guinea pig right atrium (Marshall *et al.,* 1986; Franco-Cereceda & Lundberg, 1985). However, some species differences may exist; CGRP has positive chronotropic effects on rat and guinea pig, but not rabbit, isolated perfused hearts (Holman *et al.,* 1986; Franco-Cereceda & Lundberg, 1985). Rat αCGRP and βCGRP are equipotent chronotropic and inotropic agents in isolated rat atria (Mulderry *et al.,* 1988). The effects of CGRP on isolated atria are dependent on adenylate cyclase activation (Ishikawa *et al.,* 1988), and may be observed in the presence of α- and β-adrenergic, histaminergic and muscarinic receptor antagonists (Franco-Cereceda & Lundberg, 1985; Saito *et al.,* 1987).

It is difficult to unequivocally establish a direct chronotropic effect of CGRP *in vivo* because a tachycardia inevitably accompanies hypotension through the activation of baroreflexes. Gardiner *et al.* (1989) attempted but failed to demonstrate chronotropic effects independently of any change in mean blood pressure in conscious rats. However, tachycardia in the absence of a fall in blood pressure has been reported in healthy volunteers (MacDonald *et al.,* 1989). The effect of β-blockade on the positive chronotropic effect of i.v. CGRP seems to vary between species. The tachycardia accompanying CGRP-induced hypotension has been reported to be abolished by β-adrenoceptor antagonists in conscious dogs (Wang *et al.,* 1989), attenuated in conscious rats (Lappe *et al.,* 1987; Siren & Fuerstein, 1988) and unchanged in healthy man (Gennari *et al.,* 1985).

Removal of baroreflexes by sinoaortic denervation results in enhanced vasodilatory responses but attenuated chronotropic responses in conscious rats (Siren & Fuerstein, 1988). In the anaesthetized rabbit, the chronotropic response to CGRP is markedly attenuated by sinoaortic plus vagal deafferentation and by restoration of arterial blood pressure with phenylephrine (Okamoto *et al.,* 1992). CGRP-induced tachycardia may be markedly attenuated by the ganglion blocker hexamethonium (Abdelrahman & Pang, 1992). In contrast, Haas & Skofitsch (1985) observed CGRP-induced tachycardia in the pithed rat, an animal model devoid of baroreflex mechanisms. The contradictory data have probably arisen from species variation and variable experimental conditions. It is likely that the chronotropic response to CGRP *in vivo* is due in part to baroreceptor-mediated reflexes and in part to a direct action of CGRP on the heart.

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Correction (page 12, lines 11-12):
"...which prevents the interaction between receptor and G-protein ..." should read "...a non-hydrolyzable guanine nucleotide which prevents the formation of high affinity ligand:receptor:G protein ternary complexes ..."

1.1.7. Functional aspects of CGRP receptors

1.1.7.1. Receptor-effector coupling

Stimulation of CGRP receptors leads to activation of adenylate cyclase in various tissues and cells. For example, in the cardiovascular system, CGRP receptors are functionally coupled to adenylate cyclase in the atrium (Sigrist *et al.*, 1986), coronary arteries (Shoji *et al.*, 1987), intracerebral arterioles (Edwards *et al.*, 1991), cultured vascular smooth muscle cells and endothelial cells (Hirata *et al.*, 1988; Crossman *et al.*, 1987). However, CGRP receptors in the rat spinal cord, brainstem and whole brain are not coupled to adenylate cyclase (Goltzman & Mitchell, 1985; Stangl *et al.*, 1993; Semark *et al.*, 1992).

Guanosine 5'-o-(3-thiotriphosphate) (GTP-γ-S), which prevents the interaction between receptor and G-protein, has been reported to decrease the affinity of CGRP binding to rat liver, whole brain, cerebellum, spleen and neonatal cardiac myocyte membrane preparations (Yamaguchi *et al.*, 1988; Semark *et al.*, 1992; Chatterjee & Fisher, 1991; Stangl *et al.*, 1993; Chatterjee *et al.*, 1991). Thus the CGRP receptor in these preparations is probably linked to a G-protein. Chatterjee *et al.* (1993) found that a significant portion of soluble CGRP receptors (from rat cerebellum) could be immunoprecipitated with an antiserum against the amino-terminal region of $G_{s\alpha}$. This is the first evidence that CGRP receptors could be coupled to G_s. Photoaffinity labelling studies indicate that CGRP receptors are likely to be 60 to 70 kDa, glycosylated and with internal disulphide bonds (reviewed by Poyner, 1992)

1.1.7.2. Receptor antagonists

No non-peptide antagonist of CGRP has yet been reported. The most widely used CGRP antagonist is the C-terminal 8-37 fragment of HαCGRP ($H\alpha CGRP_{8-37}$) which was first shown to antagonize CGRP activation of adenylate cyclase in rat liver membranes (Chiba *et al.*, 1989). Shorter C-terminal fragments also behave as CGRP antagonists but the residues in positions 9 to 12 are important for the maintenance of potent antagonistic properties (Mimeault *et al.*, 1991, 1992). The C-terminal 19-37 and 23-37 fragments retain weak but significant antagonistic activity in the guinea pig isolated left atrium assay (Rovero *et al.*, 1992). Antagonistic activity of the tyrosinated C-terminal 28-37 fragment of CGRP has also been reported in the opossum internal anal sphincter (Chakder & Rattan, 1990). However, unlike $H\alpha CGRP_{8-37}$, the 28-37 fragment does not block HαCGRP-induced relaxation of pre-contracted isolated guinea-pig basilar artery segments (Jansen, 1992) nor the carotid vasodilatory effects of HαCGRP in conscious rats (Gardiner *et al.*, 1991a).

Several findings suggest that $H\alpha CGRP_{8-37}$ is not an ideal CGRP antagonist,

apart from its peptide nature. Wide variation in its antagonistic potency has been reported in functional CGRP assays using the same tissue (reviewed by Poyner, 1992). In small diameter rings of the isolated porcine coronary artery that do not show tachyphylaxis to the effects of CGRP, the antagonistic potency of $H\alpha CGRP_{8-37}$ was decreased by repeated exposure of tissues to the fragment. The gradient of the slope of the Schildt plot in this preparation was significantly less than 1 (Foulkes *et al.*, 1991). Non-competitive antagonist behaviour has been reported in the isolated rat aorta (Gray *et al.*, 1991) and intracerebral arterioles (Edwards *et al.*, 1991). $H\alpha CGRP_{8-37}$ is a weak calcitonin receptor agonist (Chiba *et al.*, 1989). Vasodilatory effects occasionally associated with high doses of $H\alpha CGRP_{8-37}$ suggest that it may be a partial agonist of CGRP receptors (Hughes & Brain, 1991). In a photoaffinity labelling study of the CGRP receptor solubilized from human cerebellum, ^{125}I-$H\alpha CGRP_{8-37}$ was crosslinked to a 95 kDa protein in addition to binding proteins labelled by the intact peptide (Stangl *et al.*, 1991). Thus $H\alpha CGRP_{8-37}$ has the potential to cross-react with proteins not involved in CGRP binding.

Some of the discrepancies between early and later studies may be attributable to the quality of $H\alpha CGRP_{8-37}$ used; improvements in synthetic techniques appear to have yielded a more pharmacologically active compound (Escott & Brain, 1993). It is unclear, however, to what extent different sources of supply and variable quality of peptide could explain the inconsistent findings on $H\alpha CGRP_{8-37}$ as a competitive CGRP receptor antagonist.

1.1.7.3. Receptor subtypes

Evidence for the existence of CGRP receptor subtypes has been derived primarily from the differential potencies of the agonist [acetamidomethylcysteine$_{2,7}$]-$H\alpha CGRP$ and the C-terminal fragment antagonists $H\alpha CGRP_{8-37}$ to $H\alpha CGRP_{12-37}$ in a variety of *in vitro* and *in vivo* assays (Dennis *et al.*, 1989). CGRP receptors have been divided into two subclasses: $CGRP_1$ receptors (typically in guinea pig atria) which are sensitive to blockade with $H\alpha CGRP_{8-37}$ and $CGRP_2$ receptors (typically in rat vas deferens) which are markedly less sensitive to blockade with $H\alpha CGRP_{8-37}$ (Dennis *et al.*, 1990). The linear analogue [acetamidomethylcysteine$_{2,7}$]-$H\alpha CGRP$, on the other hand, is a selective agonist at the $CGRP_2$ receptor (Dennis *et al.*, 1989). An additional third class of binding site has also been reported in the nucleus acumbens which can recognize $H\alpha CGRP$, $H\alpha CGRP_{8-37}$, [acetamidomethylcysteine$_{2,7}$]-$H\alpha CGRP$ and salmon calcitonin with high affinities (Sexton *et al.*, 1988; Dennis *et al.*, 1991).

Evidence for CGRP receptor heterogeneity based on differential potency in various tissues from different species must be viewed with caution. However, there is

evidence that receptor heterogeneity may exist within an individual tissue. Foulkes *et al.* (1991) provided evidence for receptor heterogeneity along the length of the porcine coronary arteries by demonstrating greater vasodilatory potency of CGRP, lack of development of tolerance to CGRP and greater antagonistic potency of Hα $CGRP_{8-37}$ in small diameter rings compared with large diameter rings. Gardiner *et al.* (1991a) found that HαCGRP-induced vasodilatation in the hindquarters of conscious rats was more sensitive to $H\alpha CGRP_{8-37}$ antagonism compared with the carotid vascular beds; such differences may be due to receptor heterogeneity.

The differential ability of CGRP to activate adenylate cyclase in membranes from different tissues (Semark *et al.,* 1992; Stangl *et al.,* 1993) also point to the existence of multiple receptors with different second messenger systems. Two populations of binding sites have been demonstrated in some but not all equilibrium radioligand binding studies (reviewed by Poyner, 1992); discrepancies have probably arisen from the wide range of experimental conditions used in different studies.

The binding site identified in human and bovine endothelial cells has an affinity at least 100-fold lower than CGRP receptors in other tissues (Crossman *et al.,* 1987; Hirata *et al.,* 1988; McEwan *et al.,* 1989). It has been suggested to be a distinct receptor subtype (McEwan *et al.,* 1989). However, human calcitonin cross-reacts with this receptor at relatively low concentrations (2 to 3 times molar excess; Hirata *et al.,* 1988) and doubts have been expressed on whether a CGRP "receptor" has been identified (Poyner, 1992).

There are inconsistent reports on the differential ability of $H\alpha CGRP_{8-37}$ to block responses to HαCGRP and HβCGRP. In rabbit skin, $H\alpha CGRP_{8-37}$ was a more potent antagonist of the vasodilatation induced by HαCGRP than HβCGRP (Hughes & Brain, 1991). $H\alpha CGRP_{8-37}$ has been found to block HαCGRP but not HβCGRP-induced dilatation of guinea-pig and human cerebral arteries (Jansen, 1992; Jansen *et al.,* 1992). However, the differential sensitivity of HαCGRP and HβCGRP-mediated responses to $H\alpha CGRP_{8-37}$ antagonism could not be confirmed in the isolated rabbit hepatic and dog basilar arteries (Butler *et al.,* 1993). It is not unclear at present whether HαCGRP and HβCGRP are selective agonists of different CGRP receptor subtypes.

Nuki *et al.* (1994) have recently provided functional evidence for the existence of presynaptic CGRP receptors on perivascular CGRP-containing nerves. In the isolated perfused rat mesenteric arterial bed with active tone produced by methoxamine plus guanethidine, perfusion of CGRP inhibited the vasodilatory response to periarterial nerve stimulation but had no effect on the vasodilatory response to bolus infusion of CGRP. The inhibitory effect of CGRP was antagonized by $H\alpha CGRP_{8-37}$. These findings suggest that CGRP-containing nerves may be

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Correction (page 15, line 4): "Nuke *et al.*," should read "Nuki *et al.*"

endowed with presynaptic receptors that regulate CGRP release from the nerves via a negative feedback mechanism. Unlike postsynaptic $CGRP_1$ receptor-mediated responses, elevation of cyclic AMP does not appear to be responsible for the inhibitory effect of CGRP on neurogenic vasodilatation, and Nuke *et al.* have proposed that the presynaptic CGRP receptor may be a subtype of the $CGRP_1$ receptor.

1.1.8. CGRP as a neurotransmitter

1.1.8.1. Criteria for a neurotransmitter

A number of criteria must be fulfilled before CGRP could be established as an endogenous vasodilatory neurotransmitter. The classical criteria for the identification of a neurotransmitter include: (1) presence of the putative transmitter in terminal axons of appropriate nerves, (2) presence of systems for synthesis and storage, (3) release of putative transmitter by nerve stimulation, (4) mimicry of the response to nerve stimulation by the putative transmitter through receptor occupation, (5) pharmacological modification of the responses to the putative transmitter should have corresponding effects on the responses to nerve stimulation, and (6) presence of mechanisms for terminating the action of the released transmitter (Burnstock, 1986; Bowman & Rand, 1980).

1.1.8.2. Capsaicin

A considerable body of evidence concerning the storage and release of CGRP from nerves and the mimicry of the effects of nerve stimulation by CGRP has been derived from the use of capsaicin as a pharmacological tool. Capsaicin (8-methyl-N-vanillyl-6-noneamide) is the major pungent ingredient of hot peppers of the plant genus *Capsicum*. The neurophysiological and neurochemical effects of capsaicin are highly selective for unmyelinated C-fibres and thinly myelinated Aδ-fibres. Treatment of neonatal, but not adult, rats with the compound leads to degeneration and loss of the majority of primary afferent fibres. Capsaicin is more specific in stimulating C-type fibres than electrical nerve stimulation (Buck & Burks, 1986). The specific action of capsaicin on sensory neurons is mediated by the opening of a membrane cation channel that allows the movement of Ca^{2+} and Na^{+} into cells. A characteristic feature of the specific action of capsaicin on primary afferent neurons is its susceptibility to desensitization.

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1.1.8.2.1. Release of CGRP from capsaicin-sensitive nerves

Capsaicin has been widely used as a pharmacological tool to investigate the "efferent" function of primary afferent neurons (reviewed by Maggi & Meli, 1988). Stimulation of capsaicin-sensitive sensory neurons leads to release of neuropeptides stored in nerve terminals which may, in turn, mediate various efferent functions. The usefulness of capsaicin as a tool for investigating the role of endogenous CGRP is based on evidence that capsaicin releases CGRP from sensory neurons *in vitro* and *in vivo*.

In vitro studies have demonstrated that CGRP is released from perivascular capsaicin-sensitive nerves in the rat and man (Fujimori *et al.*, 1990; Kawasaki *et al.*, 1990a; Del Bianco *et al.*, 1991; Franco-Cereceda, 1991). The release of CGRP from nerve terminals is calcium-dependent (Fujimori *et al.*, 1989; Franco-Cereceda, 1991). A 15-fold rise in plasma CGRP levels has been demonstrated after an intraperitoneal injection of 10 mg/kg capsaicin in rats (Emson & Zaidi, 1989). Neonatal treatment with capsaicin leads to a widespread but variable decrease of CGRP content in central and peripheral tissues of mature rats (Mulderry *et al.*, 1985; Wharton *et al.*, 1986; Diez Guerra *et al.*, 1988; Kashiba *et al.*, 1990; Wimalawansa, 1993). Plasma CGRP levels are significantly lower throughout the lifespan of neonatally treated rats (Wimalawansa, 1993). However, denervation hypersensitivity to the vasodilatory effects of CGRP have not been observed in rats treated neonatally with capsaicin (Bachelard *et al.*, 1992; Ralevic *et al.*, 1992; McEwan *et al.*, 1993).

It is noteworthy that, in addition to capsaicin, a wide range of drugs and endogenous substances could increase (e.g., prostaglandins and bradykinin) or decrease (e.g., adenosine and opioids) the excitability of primary afferent neurons with corresponding effects on CGRP release (reviewed by Maggi & Meli, 1988). Thus the effector functions of endogenous CGRP may be regulated prejunctionally. CGRP itself may have a role in modulating neurotransmission due to other transmitters. For example, CGRP mimics the effect of capsaicin in prejunctionally modulating purinergic transmission in the guinea-pig vas deferens (Ellis & Burnstock, 1989).

1.1.8.2.2. Non-specific effects of capsaicin

In addition to its selective action on primary afferent neurons, capsaicin also has cell non-selective effects that are usually manifested as a transient depression of excitability (Holzer, 1991). The concentrations needed to elicit these cell non-selective effects are typically orders of magnitude higher than those sufficient to stimulate sensory neurons. Cell non-selective effects of capsaicin include inhibition of

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cardiac muscle excitability, inhibition of visceral smooth muscle activity and contraction of vascular smooth muscle. These non-selective effects tend to be sustained, not subject to desensitization, and are readily reproducible on reapplication of capsaicin. Application of capsazepine, the recently discovered competitive capsaicin antagonist, should allow better discrimination of the specific and non-specific actions of capsaicin (Bevan *et al.*, 1992).

The use of capsaicin as a tool for releasing endogenous CGRP is limited by the non-selective release of a number of neuropeptides found in primary afferent neurons which include substance P, neurokinin A, vasoactive intestinal polypeptide, somatostatin, cholecystokinin, corticotropin releasing factor, arginine vasopressin, bombesin, and galanin (Buck & Burks, 1986; Holzer, 1988; Maggi & Meli, 1988). Consideration must therefore be given to the extent to which CGRP, rather than other neuropeptides, mimics responses attributable to nerve stimulation by capsaicin. The problem of capsaicin-induced multiple neuropeptide release also highlights the need for specific blockers of the action of individual neuropeptides.

1.1.8.3. Metabolism of CGRP

The metabolism of CGRP is considered here because a major criterion for a neurotransmitter is that mechanisms for the inactivation of the putative neurotransmitter should exist. Substance P may have a role in regulating the vasodilatory activity of CGRP by stimulation or release of mast cell proteases which degrade CGRP (Brain & Williams, 1988). CGRP is effectively cleaved *in vitro* by human mast cell lysates and purified mast cell tryptase with loss of vasodilatory activity (Walls *et al.*, 1992). The degradation of CGRP by mast cell lysates and tryptase was similarly inhibited by a range of enzyme inhibitors. Thus it has been suggested that tryptase-catalysed CGRP metabolism may be an important mechanism by which the activity of CGRP is regulated *in vivo*.

CGRP has been reported to be metabolized by a substance P degrading endopeptidase in cerebrospinal fluid (Le Greves *et al.*, 1989). However, this enzyme is unlikely to be neutral endopeptidase (E.C.3.4.24.11) which cleaves HαCGRP at different sites. Neutral endopeptidase has been shown to cleave HαCGRP approximately 88 fold less rapidly than substance P (Katayama *et al.*, 1991).

The cleavage of CGRP to fragments with antagonistic activity may be another way of regulating its activity. One CGRP fragment with antagonistic activity, the C-terminal 19-37 fragment (Rovero *et al.*, 1992), has been identified as a major product of CGRP metabolizing peptidases in the rat central nervous system (Sakurada *et al.*, 1991).

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1.1.9. Structurally-related peptides

1.1.9.1. Amylin

Considerable interest has been focused on amylin since its discovery because of its potential pathophysiological role in diabetes mellitus (Johnson *et al.*, 1989). Amylin may counteract the effects of insulin by decreasing second phase insulin secretion, increasing hepatic glucose output, and inhibiting insulin effects on skeletal muscle (Edwards & Morley, 1992). There is clear evidence that amylin cross-reacts with CGRP receptors in rat liver and skeletal muscle membranes (Morishita *et al.*, 1990; Chantry *et al.*, 1991). However, discrepancies in the potency of amylin and CGRP in mediating a range of common effects point to the existence of separate receptors for the two structurally-related peptides (Young *et al.*, 1993). Kreutter *et al.* (1993) showed that, unlike CGRP, the inhibition of insulin-stimulated glucose transport by amylin in isolated soleus muscle was independent of adenylate cyclase stimulation - this effect was not antagonized by $\text{H}\alpha\text{CGRP}_{8\text{-}37}$. Compared with CGRP, amylin has higher potency for adenylate cyclase stimulation in the CHO-K1 cell line (D'Santos *et al.*, 1992) and higher affinity for distinct binding sites in the nucleus accumbens region of rat brain (Beaumont *et al.*, 1993). Comparison of the ability of $\text{H}\alpha\text{CGRP}_{8\text{-}37}$ to antagonize the effects of RαCGRP, RβCGRP and amylin in the isolated guinea-pig left atrium, guinea-pig bladder and rat vas deferens also suggested that amylin is a selective agonist of different CGRP receptor subtypes or its own unique receptor (Giuliani *et al.*, 1992).

Amylin has vasodilatory effects that are significantly weaker than those of CGRP. In rabbit skin, human amylin is about a 100-fold less active as a vasodilator than HαCGRP (Brain *et al.*, 1990). Rat amylin is about 44-fold less potent than RαCGRP as a hypotensive agent in anaesthetized rats (Young *et al.*, 1993). The haemodynamic effects of rat amylin in conscious rats are similar to those of RαCGRP but they are observed at 100-fold higher doses (Gardiner *et al.*, 1991b). It is likely that these effects are mediated via interaction with CGRP receptors since they could be antagonized by $\text{H}\alpha\text{CGRP}_{8\text{-}37}$. However, it is possible that $\text{H}\alpha\text{CGRP}_{8\text{-}37}$ is a non-selective antagonist of separate CGRP and amylin receptors in vascular tissues.

1.1.9.2. Adrenomedullin

Intravenous administration of adrenomedullin to anaesthetized rats produces a potent and long-lasting hypotensive response (Kitamura *et al.*, 1993). It is approximately 10-fold less potent than HαCGRP as a vasodilator in the isolated rat mesenteric arterial bed. The extent to which adrenomedullin binds to CGRP receptors or its own specific receptors is not yet clearly defined. However, the vasodilatory response to

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adrenomedullin in the isolated rat mesenteric arterial bed can be antagonized by HαCGRP$_{8-37}$ which suggests that it interacts, at least in part, with CGRP receptors (Nuki *et al.,* 1993). Adrenomedullin circulates in blood at relatively high concentrations (about 20 fmol/ml in plasma) and has been proposed as a hormone involved in the regulation of blood pressure.

1.1.10. Physiological and pathophysiological roles of CGRP

Intensive research over the 10 years since the discovery of CGRP has steadily unravelled its physiological and pathophysiological roles as a vasodilator. In some conditions, it is not unrealistic to predict that stable non-peptide agonists or antagonists of CGRP might be of therapeutic value. Indeed, CGRP itself has been the subject of clinical trials in man.

1.1.10.1. Neurogenic inflammation

The potent vasodilatory effect of CGRP in the skin suggests that it has a role in thermoregulation. Pathophysiologically, vasodilatation caused by intrademal CGRP potentiates the inflammatory oedema induced by a range of mediators of increased microvascular permeability in rabbit skin (Brain & Williams, 1985; Buckley *et al.,* 1991a). CGRP also potentiates substance P-induced oedema in rat skin (Brain & Williams, 1985; Gamse & Saria, 1985). Neutrophil accumulation is potentiated as a further consequence of its prolonged vasodilatory activity (Buckley *et al.,* 1991b). CGRP potentiates oedema formation induced by histamine in the rat knee joint (Cambridge & Brain, 1992) and active immunization with CGRP reduces the inflammatory response to adjuvant arthritis in the rat (Louis *et al.,* 1990). Thus CGRP may contribute to neurogenic inflammatory responses and a CGRP antagonist may have therapeutic potential as an anti-inflammatory agent.

1.1.10.2. Migraine

There is increasing evidence that CGRP may play a role in neurogenic inflammatory responses associated with migraine (reviewed by Moskowitz, 1992). During a migrainous headache, intracranial blood vessels may become distended and oedematous due to activation of trigeminal sensory nerve terminals and the subsequent release of neuropeptides. The neurogenic and vascular theories of the pathogenesis of migraine are controversial (Humphrey & Feniuk, 1991; Moskowitz, 1992). However, several findings implicate the involvement of CGRP. A substantial elevation of CGRP levels occurs in the external jugular venous blood of patients suffering from classic or common migraine, probably reflecting release from activated

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sensory fibres (Goadsby *et al.*, 1990). Electrical stimulation of the trigeminal ganglion in rats leads to rapid increases in plasma CGRP levels in the superior sagittal sinus (Buzzi *et al.*, 1991). HαCGRP$_{8\text{-}37}$ has been successfully used to antagonize cerebral vasodilatory responses evoked by stimulation of the trigeminally-derived nasociliary nerve in the cat (Goadsby *et al.*, 1993). Thus CGRP may function as a vasodilatory neurotransmitter with pathophysiological roles in the trigeminovascular system.

1.1.10.3. Subarachnoid haemorrhage

In aneurysmal subarachnoid haemorrhage, CGRP may be released from the trigeminovascular system as a vasodilatory defence against vasospasm. The innervation of the cerebral circulation by CGRP-containing fibres is reduced after subarachnoid haemorrhage by up to 50% (Edvinsson *et al.*, 1990), and in patients who die after subarachnoid haemorrhage, there is depletion of CGRP immunoreactivity in brain vessels (Edvinsson *et al.*, 1991). A positive correlation (r=0.7) has been found between CGRP levels in the jugular blood and an index of vasospasm in patients with middle cerebral artery aneurysms (Juul *et al.*, 1990). These findings have prompted clinical trials of CGRP for the prevention of ischaemic deterioration after surgery for aneurysmal subarachnoid haemorrhage. However, clinical benefit has not been clearly demonstrated and there is a need for larger clinical trials to be performed (European CGRP in subarachnoid haemorrhage study group, 1992).

1.1.10.4. Raynaud's phenomenon

Selective vascular supersensitivity to CGRP has been demonstrated in the hands of patients with Raynaud's phenomenon (Shawket, *et al.*, 1989) and a significant reduction in the number of CGRP-immunoreactive neurons has been found in the digital skin of such patients (Bunker *et al.*, 1990). These findings suggest that a deficiency of CGRP is relevant to the pathogenesis of Raynaud's phenomenon. Indeed, Shawket *et al.* (1991) and Bunker *et al.* (1993) have demonstrated the therapeutic benefit of i.v. CGRP infusions in this condition.

1.1.10.5. Hypertension

In normal volunteers, assumption of the upright posture leads to a quick rise in plasma CGRP levels together with expected increases in plasma noradrenaline and aldosterone and plasma renin activity. Intravenous infusion of angiotensin II results in dose-dependent increases of plasma CGRP levels (Portaluppi *et al.*, 1993). These findings suggest that CGRP is released as a physiological response to changes in posture and vasomotor tone. However, there are conflicting reports on circulating

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CGRP levels in hypertensive patients. Significantly lower plasma CGRP levels have been found in patients with severe hypertension (Edvinsson *et al.,* 1989a) and uncomplicated essential hypertension (Portaluppi *et al.,* 1992) compared with normotensive controls. Another study found no significant difference in serum CGRP levels between patients with untreated mild to moderate hypertension compared with age- and sex-matched normotensive controls (Schifter *et al.,* 1991). In contrast, Masuda *et al.* (1992) reported significantly higher plasma CGRP levels in patients with essential and secondary (primary aldosteronism and phaeochromocytoma) hypertension compared with normotensive control subjects. Moreover, a significant positive correlation between systolic and diastolic blood pressures and plasma CGRP levels was demonstrated.

The role of CGRP in the spontaneously hypertensive rat (SHR) model of human essential hypertension is also unclear. Neurogenic vasodilatation and CGRP-immunoreactivity release induced by perivascular nerve stimulation of the perfused mesenteric arterial bed is significantly decreased in SHRs compared with normotensive Wistar-Kyoto controls (Kawasaki *et al.,* 1990b). Both neuronal CGRP levels and CGRP mRNA levels are decreased in SHRs (Supowit *et al.,* 1993; Westlund *et al.,* 1991) with no apparent difference in vascular responses to CGRP (Ando *et al.,* 1990) compared with normotensive Wistar-Kyoto controls. On the other hand, Zaidi *et al.* (1991) found higher neurally-derived plasma CGRP levels in SHRs compared with normotensive controls. Thus, at present, it is difficult to reconcile the competing hypotheses of (1) a pathological impairment of the synthesis or release of CGRP in hypertension and (2) a physiological enhancement of CGRP release as mechanism to compensate for increased peripheral vascular resistance in hypertension.

1.1.10.6. Pregnancy and fluid overload

CGRP may be a physiologically important vasodilator during pregnancy. Plasma volume is expanded by about 40% but changes in peripheral vascular tone usually prevent an increase in blood pressure. Data from two studies show that plasma CGRP levels rise gradually during pregnancy to a peak (about 3-fold increase) in the ninth month and return to baseline 5 days post-partum (Stevenson *et al.,* 1986; Saggese *et al.,* 1990). The tissue content of CGRP in uterine arteries from pregnant women is significantly higher than in non-pregnant women (Nelson *et al.,* 1993a). Moreover, the sensitivity of the isolated uterine artery to CGRP is higher during pregnancy. These findings are consistent with suggestions that CGRP is in part responsible for changes in peripheral vascular tone in response to plasma volume expansion.

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Plasma CGRP levels are also increased in other states of chronic volume expansion such as hepatic cirrhosis (Bendtsen *et al.*, 1991) and in acute fluid overload during haemodialysis (Odar-Cederlof *et al.* 1991). Plasma CGRP levels increases with the severity of cirrhosis and are strongly correlated with fluid excess during haemodialysis.

1.1.10.7. Congestive cardiac failure

Elevated circulating CGRP levels have been detected in congestive cardiac failure (reviewed by Preibisz, 1993). Intravenous infusion of CGRP in patients with severe congestive cardiac failure leads to increased cardiac output, renal blood flow and glomerular filtration rate while systemic and pulmonary vascular resistance are decreased (Gennari *et al.*, 1990; Shekhar *et al.*, 1991; Stevenson *et al.*, 1992). Although no tolerance develops during prolonged infusions, the therapeutic use of CGRP in this condition is limited by the rapid offset of the beneficial effects (within 30 minutes) on cessation of infusion. A selective, orally available, non-peptide CGRP agonist with a long duration of action may be therapeutically useful.

1.1.10.8. Myocardial ischaemia

The effects of CGRP on the coronary circulation has been studied in some detail on the premise that CGRP may regulate coronary tone and participate in reactive hyperaemia following myocardial ischaemia. Circulating CGRP levels are elevated following acute myocardial infarction (reviewed by Preibisz, 1993). Myocardial ischaemia leads to CGRP release from the isolated perfused guinea-pig heart (Franco-Cereceda *et al.*, 1987b) and CGRP-induced relaxation of sheep coronary artery rings is enhanced under simulated ischaemic conditions (Kwan *et al.*, 1990). Intracoronary infusion of CGRP in man leads to dilatation of both normal and artheromatous epicardial coronary arteries showing irregularity at angiography (Ludman *et al.*, 1991). However, studies in dogs suggest that CGRP does not have a significant role in the regulation of microvascular tone during acute myocardial ischaemia (Sekiguchi *et al.*, 1994). $H\alpha CGRP_{8-37}$ blocked the vasodilatory effect of exogenous HαCGRP but had no effect on the diameters of coronary arterial microvessels during acute myocardial ischaemia (induced by occlusion of the left anterior descending coronary artery).

1.1.10.9. Sepsis

Decreased vascular resistance and increased cardiac output occur in the hyperdynamic state of sepsis. Joyce *et al.* (1990) reported that patients with pyrexia, elevated white

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cell counts, bacterial septicaemia and hyperdynamic status had 7.5-fold higher plasma CGRP levels compared with normal volunteers; this finding suggests that CGRP may be a beneficial modulator of the hyperdynamic circulation during sepsis. On the other hand, studies in rats suggest that excessive stimulation of CGRP release by endotoxins could lead to septic shock. Administration of Gram-negative bacterial endotoxin to conscious rats led to a 22-fold increase in plasma CGRP levels followed by cardiovascular collapse (Wang *et al.,* 1992). The tachycardia and hypotension could be transiently reversed by i.v. administration of HαCGRP$_{8-37}$ (Huttemeier *et al.,* 1993).

1.1.10.10. Other possible roles

The above overview of the potential physiological importance of CGRP has been confined to the vasodilatory role of CGRP. Other possible roles of CGRP as a vasodilator include gastric mucosal protection (Holzer *et al.,* 1991; Lambrecht *et al.,* 1993) and the modulation of pulmonary hypertension following chronic hypoxia (Tjen-A-Looi *et al.,* 1992). The non-vasodilatory effects of CGRP are broad (Tables 1.1 and 1.2) and it must be recognized that CGRP may play diverse roles outside the cardiovascular system.

1.2. Monoclonal antibodies as pharmacological tools

1.2.1. Antibodies

Antibodies are host glycoproteins produced in response to the presence of foreign molecules in the body. Humoral immunity due to circulating antibodies in the γ-globulin fraction of plasma proteins is a major defence against bacterial infections. Antibodies are synthesized primarily by plasma cells, a terminally differentiated cell of the B-lymphocyte lineage.

Proteins which have the general structural features of antibodies, but do not have known antigen binding properties, are known as immunoglobulins. Five classes of immunoglobulin antibodies (IgG, IgM, IgE, IgA and IgD) are produced by the lymphocyte-plasma cell system. The major structural features of antibodies may be discussed by considering monomeric IgG which is the most abundant immunoglobulin in serum. The antibody molecule may be visualized as a symmetrical Y-shaped structure consisting of two identical polypeptide heavy chains and two identical polypeptide light chains. The glycosylated heavy chains are joined by disulphide bonds to each other, and each non-glycosylated light chain is joined by a disulphide bond to one heavy chain. There are five types of heavy chains, which

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determine the class of immunoglobulin molecules, and two types of light chains (κ and λ). Over 95% of light chains are κ in the mouse and rat. The molecular weight of the heavy and light chains of IgG are approximately 55 kDa and 25 kDa respectively.

Each heavy and light chain is made up of several homology units of about 110 amino acids, which form globular regions termed domains. Each heavy chain comprises one variable (V_H) and three constant domains (C_H^1 to C_H^3), whereas light chains consist of a single variable (V_L) and a single constant domain (C_L). Within each variable domain lie 3 distinct hypervariable regions which form the majority of contact residues for the binding of the antibody to the antigen. The hypervariable regions are also known as complementary determining regions (CDRs). The two variable domains, V_H and V_L from the heavy and light chains respectively, are folded in such a way that brings the 6 hypervariable regions together to form an antigen-combining site. Each IgG molecule has two identical antigen-combining sites at the tip of the arms of the Y-like structure. The remarkable specificity of antibodies to the vast number of possible antigens is attributable to variability in both the length and amino acid composition of the hypervariable regions.

1.2.2. Monoclonal antibodies

Until 1975, all antibodies were obtained from the serum of immunized animals. The antibodies were polyclonal in nature and suffered from the disadvantage of batch-to-batch variation arising from intra- (between immunization) and inter-animal variability. The availability of polyclonal antibodies (PAbs) was limited by the blood volume and life span of the animal.

The publication of a method for the production of unlimited quantities of monospecific antibodies (monoclonal antibodies; MAbs) by Kohler & Milstein (1975) transformed the field of immunology and, before long, the new MAb technology was exploited in virtually all areas of biology and medicine. Kohler and Milstein reported the successful fusion of proliferating B-lymphocytes from mouse spleen and myeloma cells in culture. The resulting hybrid cells, called hybridomas, inherit the properties of antibody secretion from the parent B lymphocytes and immortality from the parent myeloma cells. Thus potentially unlimited quantities of MAbs may be obtained from cloned hybridomas. The principles and techniques of MAb production are discussed in Chapter 3.

1.2.3. Anti-peptide MAbs: immunoblockade

Immunoblockade refers to the use of antibodies to bind hormones or neurotransmitters in order to prevent their interaction with receptors and thus block

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Correction (page 25, lines 35-36): "Greves, 1984" should read "Greaves, 1984"

their biological effects. The technique has its origins in passive immunization which involves the use of a specific antiserum to neutralize microbial toxins. Passive immunization was first reported in 1890 by Behring and Kitasato who demonstrated that serum from an animal actively immunized against diphtheria toxin could be used to neutralize even a fatal dose of the toxin in another animal (reviewed by Llewelyn *et al.*, 1992).

Examples of the use of immunoblockade to study the physiological role of a hormone can be found in the literature of the 1950's. Following prolonged treatment with insulin, sera from several animal species abolished the hyperglycaemic effects of insulin injected simultaneously into mice (Maloney & Coval, 1955). Armin *et al.* (1960a) showed that hyperglycaemia could be dose-dependently induced by single i.v. injections of an anti-insulin serum whilst prolonged i.v. infusion of the antiserum led to a diabetic syndrome in conscious rats (Armin *et al.*, 1960b). Nevertheless, investigations into the effect of anti-insulin antibodies on insulin receptor binding has continued at least into the 1980's (e.g., de Pirro *et al.*, 1980; Komori *et al.*, 1986).

Immunoblockade offers an alternative to the synthesis of receptor antagonists and their application to the study of the physiological role of endogenous substances. The application of anti-peptide antibodies to assess the physiological role of neuropeptides, as opposed to hormones, is relatively new. There is a lack of information on the optimum conditions required for immunoblockade. Despite the introduction of hybridoma technology, immunoblockade studies have generally involved the use of PAbs. The variable apparent specificity, affinity and binding capacity of PAbs create problems in the study of antibody-antigen interactions. MAbs of defined affinity are an advantage in the quantitative study of immunoblockade. The use of a MAb avoids the potential complication of studying the interaction of antigen with different populations of antibodies found in serum such as high affinity-low capacity and low affinity-high capacity antibody sites commonly encountered in anti-insulin serum (Goldman *et al.*, 1978). Hybridoma technology allows the selection of MAbs with the desired fine specificity, degree of cross-reaction, affinity and physical properties for the purpose of immunoblockade.

1.2.4. Anti-receptor MAbs: receptor antagonism

Immunoblockade with anti-peptide antibodies offers an alternative to the more conventional application of peptide or non-peptide receptor antagonists. However, antibodies could also be developed against receptors for use as receptor antagonists. The applications of MAbs to the study of receptor biology are diverse (reviewed by Greves, 1984). From a pharmacological viewpoint, anti-receptor MAbs would be

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particularly useful as functional receptor antagonists, tools for receptor localization and probes for receptor heterogeneity.

Various strategies have been advocated for the development of MAbs against membrane receptors. The most direct route is to immunize animals with isolated receptor (Strosberg & Schreiber, 1984). Although classic, this procedure is applicable after only considerable progress in the purification of the receptor protein. Immunization with receptor-rich membranes or whole cells may be attempted when purified receptor is unavailable. If the receptor has been cloned or partially sequenced, a synthetic peptide corresponding to the desired portion of the receptor molecule may be used for immunization (e.g., Mestikawy *et al.*, 1990; Azmitia *et al.*, 1992).

Anti-receptor antibodies have also been generated by a technique known as the complementary peptide strategy. It has been postulated that peptide ligands and their receptors can each be encoded, at least in part, by complementary nucleotide sequences. Thus complementary synthetic peptides representing part of a receptor may be predicted from antisense mRNA that is complementary to the mRNA encoding a known peptide. This strategy was first used by Bost *et al.* (1985) who generated an antibody against a synthetic peptide complementary to corticotropin (ACTH) which recognized the adrenal cell ACTH receptor.

A popular strategy for the development of anti-receptor antibodies which does not require immunization with purified receptor is the anti-idiotypic approach. The strategy involves development of anti-ligand antibodies which are then used as surrogate receptors for immunization. This is followed by screening for anti-idiotypic antibodies, a subset of which will also recognize the receptor for the ligand. A one-step auto-anti-idiotypic approach which exploits the presence of a normally functioning idiotypic-anti-idiotypic network is a further, and potentially more effective, method for the generation of anti-receptor MAbs. The anti-idiotypic route to anti-receptor antibodies is reviewed in detail in Chapter 6.

1.3. Aims of the project

The review of the localization, release, receptor binding and biological effects of CGRP in Section 1.1 indicates that it is an endogenous substance with potential autonomic, metabolic, somatosensory, integrative and motor functions. However, the physiological importance of many of the biological effects attributed to CGRP remains to be demonstrated. No CGRP antagonists were available when the project began. Therefore the challenge was, firstly, to develop and validate the pharmacological tools required, and secondly, to use these tools effectively to the

demonstrate the physiological role of CGRP, with particular reference to vasodilatation.

The aim of the project was to investigate whether vasodilatory responses to CGRP released from perivascular sensory nerves could be blocked with anti-CGRP or anti-CGRP receptor MAbs. Thus the project was motivated by both an interest in the role of CGRP as an endogenous vasodilator and the application of MAbs as pharmacological tools for probing the physiological role of a neuropeptide. The main aim was translated into several specific objectives which were:-

1. to develop anti-CGRP MAbs for immunoblockade studies.
2. to develop anti-CGRP receptor MAbs which could be used as specific antagonists of CGRP.
3. to demonstrate the utility of immunoblockade as a pharmacological technique.
4. to demonstrate the neurotransmitter role of CGRP.
5. to demonstrate the role of CGRP as an endogenous vasodilator.

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CHAPTER 2

Introduction to immunoblockade: pharmacokinetic and pharmacodynamic considerations

2.1. Introduction

Immunoblockade may be described as the blockade of the effects of a biological mediator by inhibition of its binding to specific receptors with antibodies directed against the mediator. The process of immunoblockade is illustrated schematically for CGRP in Figure 2.1. Compared with the quantitative study of receptor antagonism, immunoblockade often appears to be more of an art than a science. Only one other research group has made efforts to examine the theoretical aspects of immunoblockade (Tilders *et al.,* 1990; van Oers & Tilders, 1991; van Oers *et al.,* 1992). The efficacy of *in vivo* immunoblockade depends on the distribution of an antibody to the site of action in sufficient concentration, the relative affinity of the antibody and receptor for the endogenous ligand, and the elimination of the antibody. The purpose of this chapter is to consider the pharmacology of immunoblockade in kinetic and dynamic terms.

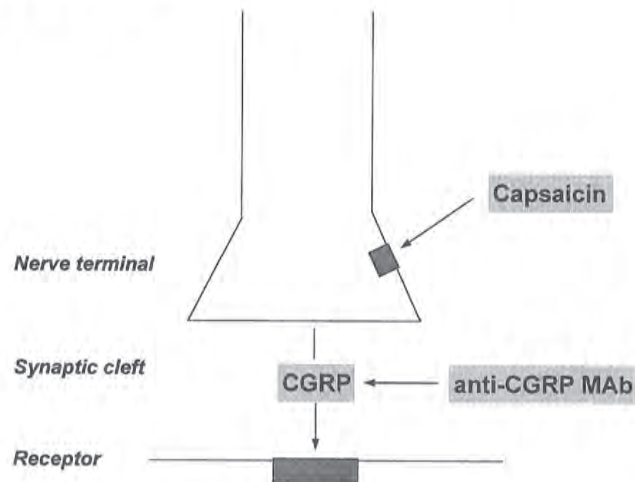

Figure 2.1: Schematic representation of the process of immunoblockade at a neuroeffector junction. Capsaicin acts on a prejunctional site to release CGRP from a sensory neuron terminal. Anti-CGRP MAb binds to CGRP in the synaptic cleft and inhibits receptor binding.

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2.2. Pharmacokinetics

The time course of a drug in the body is dependent on the processes of absorption, distribution and elimination. The dosage regimen and the processes of absorption, distribution and elimination determine the concentration of a drug at its site of action and therefore the intensity of its effects as a function of time. Pharmacokinetics is concerned with the quantification of the relationship between dose and drug concentration over time. The pharmacokinetic properties of immunoglobulins, principally immunoglobulin G (IgG), are considered here. Differences between the pharmacokinetics of IgG, $F(ab')_2$ and Fab' fragments are highlighted[1].

2.2.1. Distribution of antibodies

The distribution of antibody molecules to the site of action is a prerequisite for immunoblockade. For the immunoblockade of CGRP, the site of action of antibodies is primarily the synaptic cleft which lies within the extravascular compartment. The capillary wall is the major barrier to the transport of macromolecules such as IgG to extravascular spaces (Garlick & Renkin, 1970; Arfors *et al.,* 1979). To gain access to extravascular targets, antibody molecules must pass through the endothelial lining of a capillary or postcapillary venule. The ability of intravascular solutes to cross the capillary barrier depends on molecular size. As illustrated in figure 2.2, capillary permeability falls steeply with increasing molecular size of solutes up to 4 nm, beyond which the decline is relatively gradual. During inflammation, however, high molecular weight proteins can escape from microvessels through gaps between adjacent endothelial cells (Arfors *et al.,* 1979).

It should be noted, however, that there are regional variations in the transcapillary transport of IgG. The discontinuous endothelial lining of the sinusoidal circulations of liver, spleen and bone marrow allows free passage of IgG. Thus it could be predicted, for example, that IgG will distribute rapidly to the extravascular spaces in the liver; immunoblockade of CGRP-induced changes in liver blood flow may be readily achieved. This is much less likely in the skin and muscle because the capillary permeability/surface area product (a measure of transcapillary movement) of IgG in the carcass is approximately 100 times lower than that in the liver (Covell *et al.,* 1986). On the basis of greater capillary permeability, Fab' fragments (molecular

[1]The fragmentation of MAbs is described in Chapter 4. Fab' fragments are produced from $F(ab')_2$ fragments by reduction followed by alkylation. Thus Fab' fragments are distinguished from Fab fragments which are univalent antigen binding fragments arising directly from digestion of IgG with papain. Fab' fragments are about 10% larger than Fab fragments but the small difference in molecular size is unlikely to be pharmacokinetically important. Fab' fragments will be considered in this chapter unless studies related directly to Fab fragments.

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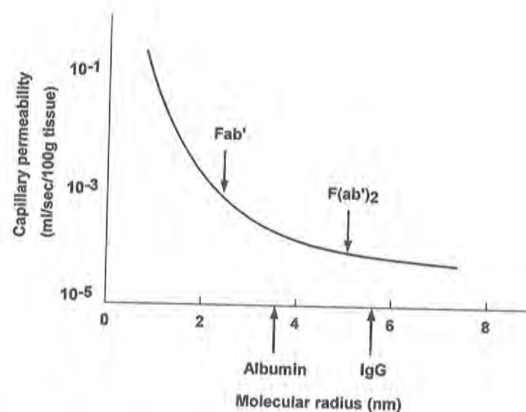

Figure 2.2: Relationship between molecular radius and permeability/surface area product which describes the movement of solutes from capillary plasma to interstitial fluid (Thomas *et al.*, 1989; Garlick & Renkin, 1970; Arfors *et al.*, 1979).

weight 50 kDa) may be predicted to be more accessible to the synaptic cleft within the interstitial space. Aubree-Lecat *et al.* (1993) have demonstrated by mathematical simulation that, for proteins above 100 kDa, the fraction of injected dose gaining access to the pharmacological target is principally limited by capillary permeability. When such a transport limitation exists, little benefit is gained by increasing the affinity of a macromolecule for its target or increasing the rate of uptake by the target.

IgG has a distribution volume that exceeds that of plasma plus interstitial fluid. However, non-specific whole IgG reside for the most part in the plasma compartment of the body (Covell *et al.*, 1986). IgG molecules do not bind to proteins within plasma but their distribution may be attributable in part to binding to Fc receptors present on many cell surfaces, particularly cells from lymphoid tissues and the reticuloendothelial system. Thus IgG may be retained in the liver, spleen and bone marrow through the interaction of its Fc fragment with Fc receptors on resident macrophages. Despite the similar size of Fc and Fab fragments, the pharmacokinetic profile of Fc fragment resembles that of IgG rather than Fab fragment (Arend & Silverblatt, 1975). The lack of Fc receptor interactions partially explains the pharmacokinetic differences between Fab fragments and IgG.

Covell *et al.* (1986) showed that non-specific homologous Fab' fragments have a larger total volume of distribution, distribute more rapidly into this volume, and produce a higher interstitial space to plasma concentration ratio compared with whole IgG. The mean steady-state volume of distribution of human Fab fragments in the rat

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Correction (page 57, lines 2,23,26): "Llyod" should be spelt "Lloyd"

has been estimated as 0.43 l/kg (Pentel *et al.*, 1988) which is larger than extracellular volume in the rat (0.3 l/kg; Bianchi *et al.*, 1981). In the dog, Llyod & Smith (1978) reported an initial "distribution" half-life of 0.54 hour for sheep Fab fragments compared with 2.28 hours for IgG. This is in good agreement with a mean distribution half-life of homologous Fab in the rat of 0.25 hour compared with 2.2 hours for IgG (Arend & Silverblatt, 1975). Pentel *et al.* (1988) estimated a longer distribution half-life of 2.39 hours for human Fab fragments in the rat.

The distribution of $F(ab')_2$ fragments to capillary plasma, interstitial and cell-associated volumes and the rate of distribution into these volumes are comparable to those of IgG (Covell *et al.*, 1986).

2.2.2. Elimination of antibodies

IgG is metabolized primarily in the gut (72.8%) and the liver (20.5%). In contrast, Fab' fragment is metabolized principally by the kidney (73%) and the gut (22.9%) (Covell *et al.*, 1986). The elimination of Fab fragments is delayed in nephrectomized animals (Arend & Silverblatt, 1975) and is markedly impaired in patients with severe renal dysfunction (Ujhelyi *et al.*, 1993). Radioactive-labelled Fab fragments, but not IgG, may be autoradiographically detected in renal proximal tubule cells after i.v. administration (Arend & Silverblatt, 1975). Antibody-antigen complexes are removed from the circulation primarily through phagocytosis by macrophages.

Covell *et al.* (1986) estimated that the mean residence times of homologous non-specific IgG_1, $F(ab')_2$ fragment and Fab' fragment were 8.3 days, 0.5 days and 0.24 days respectively. Reported elimination half-lives of various IgG with specific binding properties range from 2.1 to 7.4 days (Llyod & Smith, 1978; Shockley *et al.*, 1992; Wawrzynczak *et al.*, 1992). The elimination half-life of non-specific human Fab fragments in rats (Pentel *et al.*, 1988) and sheep digoxin-specific Fab fragments in dogs (Llyod & Smith, 1978) have been reported as 16.3 and 17.1 hours respectively. The more rapid elimination of Fab' fragment leads to a lower number of cycles through the interstitial and cell-associated compartments compared with IgG, and therefore, fewer opportunities for binding (Covell *et al.*, 1986). This potential disadvantage may be overcome by the use of higher doses and repeated administration of Fab' fragment when necessary.

Kinetic parameters estimated in some studies should be interpreted cautiously because of inadequate blood sampling periods following antibody administration. For example, the pharmacokinetic parameters of IgG and Fab fragments estimated from blood or plasma concentrations observed up to 3 hours post-dose (Triguero *et al.*, 1991; Johnston *et al.*, 1988) are very different from those estimated from plasma or

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serum concentrations observed up to 80 hours post-dose (Shockley *et al.*, 1992; Pentel *et al.*, 1988). The low volume of distribution of Fab fragment (0.046 l/kg) estimated by Johnston *et al.* (1988) is likely to reflect an initial distribution volume and the short half-life termed an "elimination half-life" (110 to 115 minutes) is more likely to reflect distribution as well as elimination. However, differences in pharmacokinetic parameters reported could also be due to differences in species from which antibodies were derived, the species to which the antibodies were administered and dose-dependent pharmacokinetics.

Repeated administration of heterologous IgG may elicit a strong anti-species immune response that could greatly increase IgG clearance. For example, Madon *et al.* (1991) found that the long-term effect of a sheep antiserum to rat growth hormone in rats was inversely related to its rate of elimination which, in turn, depended on the ability of the animal to mount an effective anti-sheep response. Heterologous Fab' fragment offers the advantage of lower immunogenicity compared with IgG.

2.2.3. Pharmacokinetics of different antibody classes

The various immunoglobulin classes differ in chemical characteristics such as size, charge, amino acid composition and carbohydrate content, and in their biological properties. Within each class, immunoglobulin subclasses exhibit further biological differences. Not surprisingly, the half-lives of immunoglobulins of different classes in serum are variable (Goding, 1986). However, studies which have formally compared the pharmacokinetics of purified immunoglobulins of defined class and subclass are lacking. Such comparisons are difficult because the pharmacokinetics of a particular antibody is likely to depend, in part, on its binding characteristics, the species it was derived from and the host species. Studying a series of mouse anti-melanoma MAbs in mice, Shockley *et al.* (1992) reported shorter terminal half-lives of approximately 85 hours for IgG_1 compared with 110 to 115 hours for IgG_{2a}.

The specific binding of a peptide by antibodies of different subclasses may have variable kinetic consequences *in vivo*. In the case of insulin, ^{125}I-insulin complexed to guinea pig IgG_2 is cleared significantly more rapidly in the rat compared with ^{125}I-insulin complexed to guinea pig IgG_1. This may be due to more rapid sequestration of IgG_2 in the liver through an interaction with Fc receptors on Kupffer cells (Arquila *et al.*, 1987).

2.2.4. Pharmacokinetics of CGRP

Limited information is available on the pharmacokinetics of CGRP. The most detailed pharmacokinetic experiments have been performed in the sheep by Braslis *et*

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Correction (page 59, line 26):
"... it the concentration ..." should read "... it is the concentration ..."

al. (1988). The clearance of HαCGRP (standard error) was 22.6 (2.1) and 15.0 (1.7) ml/kg/min when HαCGRP was infused to steady-state at 1 and 5 pmol/kg/min respectively. The significantly lower clearance at the higher infusion rate suggests that CGRP metabolism may be saturable or that clearance may be altered by haemodynamic changes induced by higher doses. The decline of plasma HαCGRP was biexponential with an initial half-life of 3.6 minutes and a terminal half-life of 13.6 minutes. The liver and kidney were the major organs involved in the clearance of HαCGRP with extraction ratios of 0.14 and 0.25 respectively. However, the clearance of HαCGRP by the liver, kidney, gut, lung and brain accounted for only a third of total body clearance suggesting that more generalized metabolic systems are involved. Using high performance liquid chromatography, Braslis *et al.* (1988) assessed the CGRP species detected by their radioimmunoassay and found that the major immunoreactive peak (which comprised more than 85% of CGRP-immunoreactivity) co-eluted with HαCGRP.

The clearance of HαCGRP in sheep agrees well with the clearance (standard error) of HαCGRP and HβCGRP in man which has been reported as 24.5 (2.9) ml/min/kg and 24.9 (5.6) ml/min/kg respectively following infusion of 4.4 pmol/kg/min to steady-state (Beglinger *et al.*, 1991). Struthers *et al.* (1986) measured plasma HαCGRP levels over 20 minutes following cessation of a constant i.v. infusion in man and estimated a half-life of 9.7 minutes. The pharmacokinetics of Rα CGRP in man was studied by Kraenzlin *et al.* (1985) who observed a biexponential decline of RαCGRP levels with longer initial half-life (6.9 minutes) and terminal half-life (29.4 minutes) than HαCGRP in sheep. Consistent with a longer terminal half-life, a lower clearance of 11 ml/min/kg was estimated for RαCGRP in man.

Although the above studies have provided useful information on the global pharmacokinetics of CGRP, it the concentration of CGRP at the synaptic cleft which is most relevant to immunoblockade studies of CGRP. No specific information is available but it is known that the intra-synaptic concentration of a classical neurotransmitter such as noradrenaline is inversely related to the width of the synaptic cleft (Bevan & Su, 1974). The minimum width of autonomic neuromuscular clefts varies considerably in different tissues, from about 20 nm in the vas deferens to 2 μm in large elastic arteries (Burnstock, 1986). Thus the concentration of CGRP achieved in the synaptic cleft is likely to vary among different tissues. The concentration of antibody required for immunoblockade of endogenous CGRP should be optimized for a particular tissue. For immunoblockade to be observed, antibody binding sites must not be saturated or if saturated, transfer of antibody to the cleft must be sufficiently fast.

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The width of synaptic clefts also affects the time required for a transmitter to traverse the cleft, and therefore, the signal transfer time. The time (t) it takes a randomly jumping particle to move a distance x in one specific direction increases with the square of distance. Einstein showed that:

$$t = \frac{x^2}{2D}$$

where D is the solute diffusion coefficient. For example, the time for a glucose molecule to diffuse across a distance of 100 nm, which is comparable to a tight vascular neuromuscular gap, is 5×10^{-6} second (Levick, 1991). The short time required for synaptic forms of signal transfer has led some to doubt that immunoblockade of a neurotransmitter could really occur (see "Pharmacodynamics"; Section 2.3.3.).

CGRP-immunoreactive nerves are found in the adventitia or adventitial-medial border of blood vessels. At least some vascular smooth muscle cells will be directly innervated and signal transfer is likely to be rapid. However, endothelium-dependent vasodilatation has also been reported and this implies relatively slow diffusion of CGRP through the media to the intima of blood vessels.

A further consideration that is relevant to chronic immunoblockade studies *in vivo* is whether blockade of a neuropeptide would promote positive feedback release such that the concentration of free neuropeptide is returned to normal. This pharmacokinetic question has not been addressed experimentally.

2.3. Pharmacodynamics

Pharmacodynamics is the study of the biochemical and physiological effects of drugs and their mechanisms of action. Defined as such, it is a broad subject that encompasses molecular mechanisms to the quantification of the relationship between drug concentration and the magnitude of drug effect.

2.3.1. Mechanisms of immunoblockade

The mechanism of immunoblockade is generally proposed to be an interference with signal transfer through the formation of an inactive immune complex between antibody and peptide during the intercellular journey of the peptide from the site of secretion to the site of action (Figure 2.1). Several assumptions that are implicit in this proposed mechanism of action should be critically examined. The binding of an

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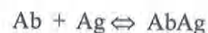
antibody to a peptide may not lead to inhibition of the biological activity of the peptide. Indeed, depending on the epitope on the peptide an antibody is directed against, potentiation of biological activity may occur; this has been reported, for example, for antibodies directed against corticotropin releasing factor, growth hormone and CGRP (Tilders *et al.*, 1990; Shaw *et al.*, 1992). Antibody binding may also potentiate or prolong the biological effect of a peptide by inhibiting its metabolic breakdown.

When an antiserum is used, it is possible that some of the observed effects may be due to an interaction between auto-anti-idiotypic antibodies and receptors (Chapter 7). There is also evidence that antibodies to neuropeptides can be internalized into specific neurons after local administration in the brain. Tilders *et al.* (1990) reviewed the evidence for the uptake of substance P, vasopressin and corticotropin releasing factor into neurons containing the respective peptides and suggested that the internalization of antibodies to peptide-containing cell bodies leads to inhibition of their secretory activity. Although the mechanisms of neuronal uptake of antibodies are unclear, antibody binding to peptides within neurons does challenge the assumption that immunoblockade always occurs through the interaction of antibody and peptide in liquid phase. Even if the neuronal uptake of native antibodies is not a general phenomenon, it may be induced by the cationization of antibodies. Triguero *et al.* (1989, 1991) have demonstrated that cationized antibodies retain antigen binding properties and exhibit enhanced uptake into cells.

2.3.2. Antibody-antigen interaction

The interaction between antibody (Ab) and antigen (Ag) and the antibody-antigen complex (AbAg) may be expressed as:



$$Ab + Ag \Leftrightarrow AbAg$$

The rate of complex formation = $k_1[Ab][Ag]$ and the rate of complex dissociation = $k_2[AbAg]$ where k_1 and k_2 are the on-rate and off-rate constants respectively. At equilibrium, the rate of complex formation = rate the of complex dissociation , and

$$k_1[Ab][Ag] = k_2[AbAg]$$

Therefore,

$$\frac{k_2}{k_1} = Kd = \frac{[Ab][Ag]}{[AbAg]}$$

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where Kd is the equilibrium dissociation constant which is equal to the concentration of antibody producing half-maximal binding. The affinity[2] of Ab for Ag is described by the affinity constant (reciprocal of Kd).

Under the condition that the [Ab] is considerably higher than [Ag] (when most Ab is not involved in binding), the ratio of free over bound Ag will be primarily dependent on [Ab] and Kd:

$$\frac{[Ag]}{[AbAg]} = \frac{Kd}{[Ab]}$$

By increasing [Ab], the ratio of free to bound Ag declines proportionally. Thus the extent of immunoblockade may be assessed by examining the relative values of Kd and [Ab]. For example, when [Ab] is 100 times higher than Kd (i.e. 100Kd),

$$\frac{[Ag]}{[AbAg]} = \frac{1}{100} = 0.01$$

$$[Ag] = 0.01[AbAg]$$

Substituting for [Ag], it can be calculated that 99% of the antigen will be bound:

$$\text{Proportion bound} = \frac{[AbAg]}{[Ag]+[AbAg]} = \frac{1}{1.01} \times 100 = 99.0\%$$

2.3.3. Effect of antibody-ligand interaction on pharmacological response

The considerations above are limited to the assessment of the likelihood and extent of immunoblockade when antibody concentrations are considerably higher than antigen concentration. However, it is more informative to study the effect of an antibody over the entire concentration-response range of a ligand.

---

[2]Affinity is a thermodynamic measurement of the strength of the non-covalent interaction between one site of the antibody and of the antigen. Avidity, on the other hand, is an operational term expressing the ability of an antibody preparation to bind antigens and depends, therefore, not only on affinity but also on multivalency and other non-specific factors. It is common that the multivalent IgM has an avidity (functional affinity) of 10^2 to 10^4 times higher than the affinity of isolated sites (Fab fragments).

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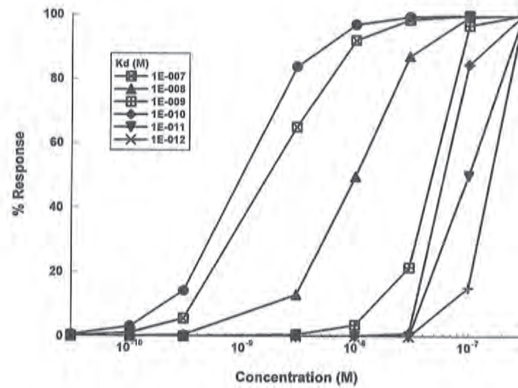

Figure 2.3: Effect of dissociation constant (Kd) of antibody-ligand binding on the concentration-response curve of the ligand. Pharmacological response was simulated with the logistic model using the following parameter values: $E_0 = 0$; $E_{max} = 100\%$; $EC_{50} = 1$ nM; N = 1.5. The concentration-response relationship in the absence of anti-ligand antibody is plotted with closed circles. Kd was varied from 10^{-7} to 10^{-12} M (1E-007 to 1E-012 M) with B_{max} fixed at 100nM.

Given the dissociation constant of antibody binding to ligand (Kd), the concentration of binding sites (B_{max}), and the total concentration of ligand (C), the concentration of ligand bound (B(C)) could be predicted from the following equilibrium binding model:

$$B(C) = \frac{B_{\max} \times C}{Kd + C}$$

The unbound ligand concentration may be obtained by subtraction of bound from total ligand concentration. Assuming that only unbound ligand is active, the pharmacological effect (E) of ligand in the presence of anti-ligand antibody could be simulated with the sigmoidal E_{max} (logistic) model below (Holford & Sheiner, 1981):

$$E = E_0 + \frac{E_{\max} \times C^N}{EC_{50}^N + C^N}$$

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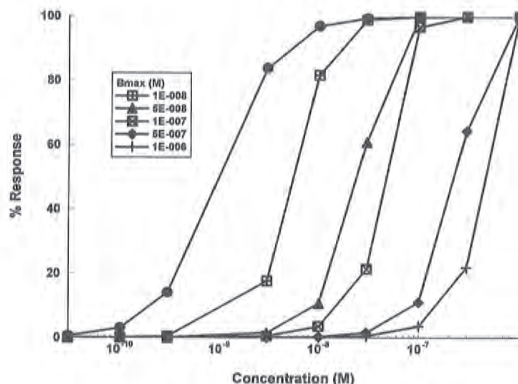

Figure 2.4: Effect of concentration of antibody binding sites (B_{max}) on the concentration-response curve of a drug. Pharmacological response was simulated with the logistic model using the following parameter values: $E_0 = 0$; $E_{max} = 100\%$; $EC_{50} = 1$ nM; N = 1.5. The concentration-response relationship in the absence of antibody directed against the drug is plotted with closed circles. B_{max} of the immunoblocking antibody was varied from 10^{-8} to 10^{-6} M (1E-008 to 1E-006 M) while Kd remained constant at 1nM.

where E_0 is the baseline effect, E_{max} the maximum effect, EC_{50} the concentration which produces half-maximal effect and N the steepness factor (Hill coefficient).

Changing the value of one parameter at a time, the influences of antibody Kd and B_{max} on pharmacological response were simulated for a range of concentrations commonly encountered in pharmacological experiments. Figures 2.3 and 2.4 shows the consequences of using antibodies of different binding affinities and different concentrations of a particular antibody respectively. Due to the non-linearity of the antibody binding and pharmacodynamic models, EC_{50} does not shift proportionally with changes in Kd and the shifts in concentration-response curves are non-parallel. The simulations clearly indicate that a high concentration of a high affinity (low Kd) antibody produces the greatest blockade of pharmacological response.

A major assumption of the above simulations is that equilibrium binding of antibody to ligand is achieved prior to the arrival of ligand at the receptor. This is most valid when the time for signal transfer is long, i.e. an antibody will have a high probability of achieving maximal binding to ligand prior to arrival of ligand at the receptor. van Oers & Tilders (1991) showed that the time to achieve maximal antibody binding is determined by the on-rate constant, Kd and the local antibody concentration. These authors performed simulations based on the theoretical framework they presented, and concluded that immunoblockade of signalling

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processes that take less than 10 milliseconds is unlikely. They expressed doubt if peptidergic synaptic transmission could be blocked and suggested that most positive results on the immunoblockade of neuropeptides probably represent interference with non-synaptic transmission. However, Tilders and co-workers have not considered the time-course of receptor binding. The on- and off-rates of antibody and receptor binding span similar ranges. Provided that an antibody is distributed to the synaptic cleft, the antibody and receptor will compete for binding to neuropeptide released into the cleft. The amount of peptide bound to antibody relative to receptor (and therefore the extent of immunoblockade) will depend on the relative on- and off-rates of antibody and receptor binding and the concentrations of antibody and receptor.

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CHAPTER 3

Development of monoclonal antibodies against CGRP

3.1. Introduction

The clonal selection theory (Burnet, 1959) postulated that each mammalian B lymphocyte has the potential to make only one antibody of a particular specificity. It is this theory that provides the conceptual framework for the development of monoclonal antibodies (MAbs). The revolutionary impact of MAb technology began with the discovery that fusion of a B-lymphocyte with a myeloma cell yields a hybrid cell which has inherited the properties of monospecific antibody secretion and immortality from the parent cells (Kohler & Milstein, 1975). The production of MAbs may be considered as 5 sequential processes:- (1) immunization, (2) fusion, (3) screening for antibodies of interest, (4) cloning, and (5) expansion of hybridoma cell lines.

The generation of a strong immune response to an antigen of low molecular weight usually requires coupling of the antigen to an immunogenic carrier protein. When the antigen is a water-soluble protein, the use of an adjuvant is usually necessary to augment the immune response. The most commonly used adjuvants are Freund's complete adjuvant (a water-in-oil emulsion in which killed and dried *Mycobacterium tuberculosis* are suspended in oil phase) and Freund's incomplete adjuvant (with bacteria omitted). The above considerations are relevant to CGRP since it is a relatively small water-soluble molecule that is recognized as "self" by the animal. The success of the immunization schedule may be assessed by testing the serum of immunized animals for specific binding activity. Spleens from animals with positive antisera are chosen for fusion with myeloma cells.

The original successful fusions were achieved by the use of inactivated Sendai virus but polyethylene glycol (PEG) is now established as the fusing agent of choice. PEG is favoured because it is commercially available, and its use results in a higher fusion frequency and greater reproducibility. Treatment of spleen and myeloma cells with PEG results in fusion of membranes and the formation of binucleate cells called heterokaryons. The nuclei fuse at the next cell division, generating hybrid cells.

The key to the successful selection of hybrid cells is the use of myeloma cells which lack the salvage pathway enzyme hypoxanthine guanine ribosyltransferase (HGPRT) and the culture of cells in medium containing hypoxanthine, aminopterin and thymidine (HAT). When the main synthetic pathways are blocked by the folic acid analogue aminopterin, the cell must depend on the salvage pathway enzymes HGPRT and thymidine kinase. $HGPRT^-$ myeloma cells die in HAT medium because

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both the main and salvage pathways are blocked. Fusion with $HGPRT^+$ spleen cells provides $HGPRT^-$ myeloma cells with the missing enzyme, and the resulting hybrid cells are able to grow in HAT medium. Unfused spleen cells die in culture after a few days.

A rapid, sensitive and specific assay is required for the selection of culture wells containing hybridomas secreting the desired antibodies and for the isolation of individual clones secreting monoclonal antibodies. Cloning by limiting dilution is generally the preferred method. Once cloned, hybridoma cells may be propagated in culture or grown as ascitic tumours in histocompatible animals.

The objective of the work described in this chapter was to develop MAbs against CGRP which could be used in immunoblockade studies.

3.2. Methods

3.2.1. Conjugation procedure

Materials	**Supplier**
RαCGRP	Peninsula
1-ethyl-3(3-dimethylaminopropyl)-carbodiimide HCl	Sigma
Freund's complete and incomplete adjuvant	Behringwerke AG, Germany
Bovine serum albumin	Sigma

RαCGRP was conjugated to bovine serum albumin (BSA) using a water-soluble carbodiimide according to the general procedure described by O'Shaughnessy (1982). The conjugation reaction requires the presence of amino and carboxyl groups (Bauminger & Wilchek, 1980). In general, the amino groups are derived from lysyl residues of the protein carrier and carboxyl groups contributed by the hapten (aspartate and glutamate in RαCGRP).

RαCGRP (0.5 mg; 130 nmol) and BSA (2.3 mg; 32.5 nmol) were dissolved in 325 μl of deionized water pH 7.0, and 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride (16.25 mg; 81.25 nmol) was added to this solution. The mixture was incubated overnight at 4°C, aliquoted, and stored at -70°C.

3.2.2. Immunization protocol

3.2.2.1. Preparation of antigen (RαCGRP:BSA conjugate) in Freund's adjuvant

Fifty μl of the RαCGRP:BSA conjugate was diluted to 625 μl with water and mixed with 1875 μl Freund's adjuvant (3 volumes of the diluted aqueous immunogen) to

obtain a water-in-oil emulsion according to the following procedure. The aqueous immunogen was drawn into a plastic luer lock syringe and Freund's adjuvant into another plastic luer lock syringe. The syringes were connected with a three way tap. The aqueous solution was injected into the oil and the mixture passed rapidly back and forth between the two syringes a few dozen times (Goding, 1986). A water-in-oil emulsion was confirmed by allowing drops of the mixture to fall on to the surface of water in a beaker and observing discrete globules from the second drop onwards.

3.2.2.2. Immunization schedule

Ten 5 to 6 week old female Balb/C mice were immunized. Each mouse was identified by marking the ear with an ear clipper. Freund's complete adjuvant was used for the priming dose and Freund's incomplete adjuvant used in the first and second booster injections. Two hundred μl of the mixture, containing 35 μg of the immunogen, was injected into each mouse by the intraperitoneal (i.p.) route. The first and second booster injections were given at 4-weekly intervals. A final aqueous injection was given 3 days prior to fusion.

3.2.2.3. Screening of serum for anti-CGRP antibodies

Blood was collected from mice 8 days following the second booster injection. Each mouse was placed in a restrainer and blood collected by lightly cutting a tail vein with a scalpel. Two untreated mice were bled to obtain control serum. Blood was left to clot at 4°C and serum separated by centrifugation at 11,000 rpm for 5 minutes in a Biofuge B (Haraeus) centrifuge. Sodium azide (final concentration in serum 0.05%) was added to serum samples to minimize microbiological degradation.

3.2.3. Enzyme-linked immunoadsorbent assay (ELISA)

Buffers and reagents used in ELISA

Phosphate buffered saline (PBS) pH 7.4

NaCl	0.14 M	8.00 g
KCl	2.7 mM	0.20 g
KH_2PO_4 (anhydrous)	1.5 mM	0.20 g
Na_2HPO_4 (anhydrous)	8.1 mM	1.15 g
Deionized water		to 1 litre

(Adjusted to pH 7.4 with sodium hydroxide if necessary)

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Acetate-Citrate Buffer pH 6

Sodium acetate 1 M

Adjusted to pH 6 with citric acid 1 M

Tetramethylbenzidine (10 mg/ml in dimethylsulphoxide; ICN Flow)

Tween 20 (0.05% v/v; Sigma)

Horseradish peroxidase conjugated rabbit anti-mouse antibody
(Heavy and light chains; concentrate diluted 1 in 1000 for use; ICN Flow)

Substrate reagent

1 M acetate-citrate buffer pH 6	1 ml
Deionized water	9 ml
Tetramethylbenzidine	100 µl
6% hydrogen peroxide	10 µl

3.2.3.1. Development of indirect ELISA screening assay

A chequerboard ELISA was used to determine the optimum coating concentration of CGRP (the antigen) and the titre of mouse antiserum. The general procedures are described below. A 96-well microtitre plate was coated with 50 µl serial 1 in 2 dilutions of CGRP (starting from 40 µg/ml) down each column. Serial 1 in 2 dilutions of mouse antiserum (starting from a 1 in 100 dilution; 50 µl) was added along each row of the plate. One control column was not coated with antigen. Serum from a non-immune mouse was added to a control row. It was found that a CGRP coating solution of 1.25 µg/ml produced good signals. For convenience, 1.5 µg/ml (400 nM; 75 ng in 50 µl) CGRP solution was used to coat plates in all subsequent ELISAs.

3.2.3.2. Experimental procedures

Ninety-six well microtitre plates (Linbro EIA plus titration plates; Flow) were coated with 50 µl of 400 nM CGRP solution or PBS vehicle per well, sealed with film, and left overnight at 4°C. Further steps were performed at room temperature. Plates were washed three times with 300 µl PBS/Tween 20 0.05% between each step of the ELISA. Wells were incubated with 100 µl porcine gelatin 0.25% in PBS pH 7.4 (blocking step) for 2 hours (or overnight at 4°C). Fifty µl serum or hybridoma culture supernatant (3 to 4 drops from a Pasteur pipette) was added to each well and incubated for 2 hours. When appropriate, serial dilutions were made in PBS/Tween 20

0.05%/gelatin 0.25%. Controls used were normal mouse immunoglobulin (10 μg/ml), culture medium and Sp2 myeloma cell culture supernatant. Wells were incubated with 50 μl horseradish peroxidase-conjugated rabbit anti-mouse antibody (1 in 1000 in PBS/Tween 20 0.05%/gelatin 0.25%) for 1 hour. One hundred μl substrate reagent (0.1 M acetate-citrate buffer pH 6 containing 0.006% hydrogen peroxide and 100 μg/ml tetramethylbenzidine as chromogen [Bos *et al.*, 1981]) was added to each well. Positive wells developed a blue colour when 25 μl of 2 M sulphuric acid was added to give a yellow colour with greater intensity. Absorbance was measured at 450 nm using an ELISA plate reader (Titertek Multiskan Plus Mk II, Flow Laboratories).

3.2.4. Radioimmunoassay (RIA)

Assay buffer pH 7.4

Na_2HPO_4	7.1 g	50 mM
EDTA	3.72 g	10 mM
BSA	3 g	0.3%
Deionized water		to 1 litre

Radioimmunoassay (RIA) was performed as a secondary screening assay. The RIA incubation mixture consisted of 50 μl hybridoma supernatant, 50 μl 2-[^{125}I]-iodohistidyl^{10}-HαCGRP (40,000 counts per minute) and 400 μl assay buffer. Tissue culture medium, Sp2 myeloma culture supernatant, normal mouse immunoglobulin (10 μg/ml) and a blank without supernatant were used as controls. The assay was performed at 4°C with a 4 day incubation period. Bound and free CGRP were separated by charcoal precipitation. One dextran-coated charcoal tablet (Steranti Separex; Steranti Research) was stirred in 15 ml separation buffer (assay buffer containing 0.25% gelatin) for 30 minutes at 4°C. Two hundred and fifty μl of the dextran/charcoal suspension was added to each assay tube followed by centrifugation at 2000 *g* for 20 minutes (Centra-7R centrifuge, IEC). Supernatant and charcoal were separated and the pellet counted for 3 minutes in a Beckman Gamma 5500 counter.

3.2.5. Preparation of feeder layer cells

One day prior to the day of fusion, MRC-5 human lung diploid fibroblasts (Flow; Long *et al.*, 1986) were trypsinized (Trypsin-EDTA, Sigma), counted and diluted to approximately 5-8 x 10^4 cells/ml. The cells were irradiated in 25 cm^2 flasks with 10,000 rads of high energy X-ray from a 20 MeV linear accelerator set at 16 MeV

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(Department of Radiotherapy, Addenbrooke's Hospital). One hundred µl per well of the irradiated MRC-5 cell suspension were dispensed into five 96-well plates (Costar; low evaporation plates).

3.2.6. Preparation of myeloma cells

NSO (Flow; Kearney *et al.*, 1979) and Sp2 (Flow; Shulman *et al.*, 1978) myeloma cell lines were used in fusions. Both cell lines are non-producers of immunoglobulin heavy and light chains. Myeloma cells were thawed at least 2 weeks before fusion and grown in two 75 cm^2 flasks. Cells were fed daily for at least 3 days prior to the day of fusion. Both NSO and Sp2 cells grew well in Dulbecco's modified Eagles medium containing 2% foetal calf serum (2FD). Myeloma cells in stationary culture grow at approximately 3×10^5 cells/ml. Therefore two 75 cm^2 flasks (30 ml each) could be assumed to yield approximately 1.8×10^7 cells. Fusion was only performed if myeloma cell viability (measured by dye exclusion) was greater than 90%.

3.2.7. Fusion procedure

Equipment

1 x tea strainer/sieve (sterilized by autoclaving)
1 x large petri dish
2 x 100 mm petri dishes
1 x 5 ml syringe plunger
3 x universals containing 10 ml SFD (on ice)
3 x 50 ml tubes
3 x 10 ml sterilin tubes
1 x 10 ml sterilin tube containing 10ml SFD
150 ml SFD
2 x HAT in 10FD (50 ml/fusion)
5 x 96-well plates coated with MRC-5 feeder cells
Pipette tips (200 µl)
1, 10 and 25 ml sterile disposable pipettes
Plugged and unplugged sterile Pasteur pipettes
1 reagent trough
Improved Neubauer haemocytometer
2 pairs of sterilized scissors and forceps for dissection
2 pairs forceps (in laminar flow cabinet)
1 x universal with ethanol (for dissection)

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1 x 2 ml syringe with needle

1 x 500 ml beaker with ice

1 washbottle containing 70% ethanol

Sterile consummables were generally obtained from suppliers. Other equipment was sterilized either in a Gallenkamp hotbox oven (160°C for 2 hours) or in a Denley autoclave (121°C for 20 minutes).

Materials	**Supplier**
Foetal calf serum	GlobePharm
Dulbecco's modified Eagles medium (DMEM) with 3.70 g/L NaHCO3, with phenol red as pH indicator, without glutamine	Flow
Polyethylene glycol (PEG 1500; fusion tested); 1 ml/ fusion	BCL
Hypoxanthine (50x concentrate; 5 mM)	Flow
Aminopterin (50x concentrate; 2×10^{-5} M)	Flow
Thymidine (50x concentrate; 8×10^{-4} M)	Flow
L-glutamine (200 mM stock)	Flow
Penicillin (50,000 iu/ml stock)	Flow
Streptomycin (5000 μg/ml stock)	Flow
Amphotericin B (250 μg/ml stock)	Flow
Trypan blue	Sigma

Stock solutions (5 ml) of penicillin and streptomycin, amphotericin B and L-glutamine were added to 500 ml culture medium just before use.

3.2.7.1. Experimental procedures

The equipment above were assembled in the tissue culture laboratory and a mouse obtained from the animal house. Polyethylene glycol (PEG) and culture medium were allowed to warm up to room temperature. Three 10 ml aliquots of serum-free Dulbecco's modified Eagles medium (SFD) were placed in ice. Hypoxanthine, aminopterin and thymidine mixture (HAT) was thawed and 10% foetal calf serum in Dulbecco's modified Eagles medium (10FD) prepared.

The mouse was killed by cervical dislocation, placed in a large petri dish and soaked with 70% ethanol. The spleen was carefully removed. (Scissors and forceps were flamed before use. One pair of scissors and forceps were used to make incision into skin and a second pair of scissors and forceps used to make incision into the peritoneum and to remove spleen.) The spleen was placed in a 100 mm petri dish and transferred to a vertical-flow laminar flow cabinet (Gelaire BSB4). The mouse was removed from the laboratory. Hands were thoroughly washed and disinfected prior to further manipulations.

The spleen was washed twice by immersing in two 10 ml aliquots of ice-cold SFD and transferred to a tea strainer resting on a petri dish. Five ml cold SFD was added to the tea strainer and the spleen was pushed through the strainer using a 5 ml syringe plunger. Spleen cells were transferred to a 10 ml tube. The above procedure was repeated with another 5 ml cold SFD. The remaining cells were transferred to the 10 ml tube and cells passed up and down a Pasteur pipette several times to ensure adequate suspension. Clumps were allowed to settle for 3 to 5 minutes.

Myeloma cells were harvested from flasks using a 25 ml pipette and transferred to 50 ml tubes. Spleen cells were transferred to another tube with a Pasteur pipette. Care was taken not to take up any clumps. The spleen and myeloma cells were centrifuged at 400 *g* (1500 rpm; Denley centrifuge) for 5 minutes in the same spin. Supernatant was removed and spleen and myeloma cells resuspended in 10 ml SFD. Myeloma cells were counted (1×10^7 cells required total) using an improved Neubauer haemocytometer under a Nikon TMS inverted phase contrast microscope. The cell suspension was diluted 9:1 with 1% trypan blue in 0.9% saline to assess cell viability if required.

Spleen cells were added to myeloma cells in a ratio of 10 to 1 (10^8 spleen cells to 10^7 myeloma cells). The mouse spleen was assumed to produce 10^8 splenocytes in order to save time during the fusion. The cell mixture was made up to 50 ml with SFD, mixed well, and centrifuged at 400 *g* for 5 minutes. Supernatant was removed with the pellet left as dry as possible. The pellet was tapped gently and 1 ml PEG added from a 1 ml pipette over 1 minute with gentle and continuous stirring with the pipette tip. Immediately after the addition of PEG, 1ml SFD was added over 1 minute (very slowly initially), followed by 1 ml additions twice over 30 second intervals, and then at 15 second intervals with continuous stirring until 10 ml had been added. A further 15 ml SFD was added slowly, with continuous stirring. The mixture was centrifuged for 5 minutes at 400 *g*. Fifty ml 2xHAT in 10FD was prepared whilst cells were being centrifuged. The supernatant was removed. The pellet was tapped gently and cells resuspended in 50 ml of 2xHAT (for five 96-well plates). By the use of a multi-channel pipette, 100 μl of the cell suspension was transferred to each well of five 96-well plates containing MRC-5 feeder layer cells. The 96-well plates were kept in a humidified incubator (IR1500 5% CO_2, 37°C; Flow).

3.2.8. Post-fusion management

Plates were left undisturbed for 4 to 5 days after the day of fusion. Cells were fed after 4 to 5 days, and again after 7 to 10 days, with 1xHAT in 10FD. (Half the volume of

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medium was removed from each well using a Pasteur pipette attached to vacuum line and replaced with fresh medium). Feeding was performed at least 3 times before screening to ensure that any antibodies initially secreted by unfused splenocytes were adequately diluted out to avoid false positive results in the screening assay. Hybridoma cells were cultured with HAT for a week or longer, and then with hypoxanthine and thymidine (HT) for at least 1 week. Positive cultures were duplicated, fed with separate medium (HT/10FD) to minimize contamination risks, and expanded in 24-well plates. The original fusion plates were kept for up to 6 weeks with feeding (HAT/10FD) at 7 to 10 day intervals until cell lines were frozen. Supernatants were screened and cell lines frozen and cloned as soon as possible after fusion.

3.2.9. Screening of supernatants

Supernatants from fusions were screened by ELISA (Section 3.2.3) when culture supernatants were turning yellow (acidic) and confluent hybridoma cells were visible under the microscope. After the blocking step, 3 to 4 drops of tissue culture supernatant were added from a Pasteur pipette to microtitre-plate wells coated with RαCGRP or PBS vehicle. Non-immune mouse immunoglobulin (10 μg/ml; Sigma), myeloma culture supernatant and fresh tissue culture medium were added to control wells.

3.2.10. Selection of positive hybridoma cells for cloning

It is important to clone hybridoma cells as soon as possible after positive wells have been identified to reduce the risk of overgrowth by non-producer cells. However, it was impractical to clone cells from the large number of positive wells identified in one of the fusions. Therefore further screening tests were performed as soon as possible to identify supernatants with the most desirable properties. Further assays included ELISAs to assess relative affinity and selectivity for different forms of CGRP, RIA and receptor binding inhibition assays.

3.2.11. Cloning by limiting dilution

MRC-5 feeder layer cells were prepared as described for the fusion procedure (100 μl per well; 5-8 x 10^8 cells/ml) at least 3 days before cloning. Hybridoma cells were maintained in exponential growth by appropriate feeding.

Cells were resuspended thoroughly with a Pasteur pipette and 0.5 to 1 ml

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transferred to a 10 ml tube or universal. Trypan blue 1% solution was diluted 1 in 10 in cell suspension and viable cells counted. Cloning was postponed if there were fewer than 10^5 cells/ml. Cells were diluted 1 in 100 (100 μl + 10 ml HT/20FD). This was followed by serial dilutions in HT/20FD to give cell suspensions of 30 cells/ml (3 cells/well), 10 cells/ml and 3 cells/ml. One hundred μl of the diluted cell suspensions were dispensed into 96-well plates containing MRC-5 feeder cells to give 3 (half plate), 1 (half plate) and 0.3 cells per well (one plate).

Supernatants from wells with discrete clones from the 0.3 cells/well plate were screened by indirect ELISA. Cell lines were cloned twice in order to increase the probability of monoclonality.

3.2.12. Cryopreservation of hybridoma cells

Cell lines were fed regularly for several days to ensure that they were in good condition. Cells from each well of a 24-well plate were resuspended with a Pasteur pipette, transferred to sterile 10 ml tubes, and centrifuged at 400 *g* (1500 rpm; Denley centrifuge) for 5 minutes. Supernatant was removed and cells resuspended in 1 ml freezing medium (10% dimethylsulphoxide [ACS specification; Sigma] in foetal calf serum). The cell suspension was aliquoted to a 1 ml cryovial (Nunc). Cryovials were placed in a polystyrene box and frozen at -70°C for at least 24 hours. The cryovials were then stored in coded racks in a liquid nitrogen container (BT55, L'air Liquide, France). Records of the identity and location of frozen cell lines were kept in a computer database.

3.2.13. Thawing of cryopreserved cells

Cryovials were quickly removed from liquid nitrogen storage and placed in a 37°C water bath (Grant W28). Thawed cells were diluted with 5 ml 20FD (warmed to 37° C) as soon as possible and centrifuged for 5 minutes at 400 *g*. The supernatant was aspirated and discarded. The pellet was resuspended in 2 ml 20FD for seeding into one well of a 24-well plate containing MRC-5 feeder cells (0.5 ml; 5-8 x 10^8 cells/ml). Feeder cells were used since some cell lines were not successfully thawed and cultured in the absence of feeder cells. Alternatively, 20% MRC-5 feeder cell-conditioned medium in 20FD was successfully used for cell culture.

3.2.14. Cryopreservation of spleen cells

Spleens from positive mice which were not used for immediate fusion were frozen 3

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days after a final booster injection of conjugated CGRP in aqueous solution. Spleen cells were resuspended at approximately 2 x 10^7 cells/ml in 5 ml freezing medium and frozen in 0.5 to 1 ml aliquots, as described in Section 3.2.12.

3.2.15. Bulk production of MAbs *in vivo*

MAbs may be produced in bulk by growing hybridoma cells in culture or as ascitic tumours in histocompatible animals (Galfre & Milstein, 1981). Large volumes of cell culture supernatants may be obtained at a concentration range of 2 to 20 μg/ml. However, more concentrated solutions were required for pharmacological studies and this was most readily achieved by growing cell lines *in vivo* and harvesting the resulting ascites with antibody concentration in the range of 2 to 20 mg/ml. There was another reason for the need to produce some MAbs *in vivo*. Some hybridomas would not grow in culture in the absence of feeder layer cells. Bulk production in roller culture was therefore not possible.

Balb/C mice were given a 100 μl i.p. injection of pristane (2,6,10,14-tetramethyl-pentadecane; Sigma) at least 7 days prior to i.p. injection of hybridoma cells. Hybridomas were in exponential growth when they were harvested, centrifuged (1500 rpm for 10 minutes; Denley centrifuge), and resuspended in normal saline. About 10^6 to 10^7 cells in 500 μl normal saline were injected into each animal i.p. using a 21 gauge needle. Mice were monitored daily for abdominal distension. Abdominal distension was apparent from 3 days post-injection, and ascites was generally tapped a week to two weeks post-injection. Ascites fluid was centrifuged at 2000 *g* for 10 minutes (Centra-7R, IEC; 3000 rpm), supernatant recovered, and activity checked by indirect ELISA. Sodium azide (10 mM) was added to ascites as a preservative. Ascites fluid was stored at 4°C for up to 1 month and at -70°C for longer periods.

3.2.16. Bulk production of MAbs *in vitro*

When C4.19 and C4.6 cell lines had been cloned twice (see "Results"), larger volumes of supernatant were obtained by culture of hybridoma cells in a 24-well plate, 25 cm^2 flasks, and eventually in 75 cm^2 flasks when 200 ml of supernatant was obtained after 3 weeks. The cell lines could be expanded further in roller culture if desired.

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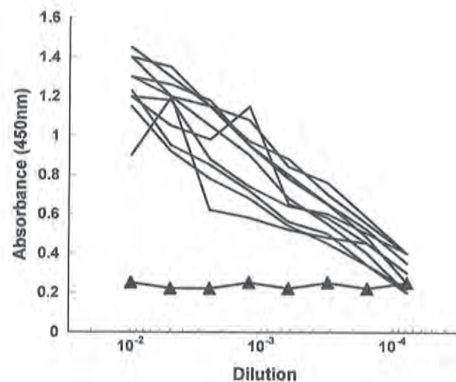

Figure 3.1: Binding of serum from mice immunized with RαCGRP to RαCGRP by indirect ELISA. All 10 mice were positive for anti-CGRP antibodies. Two-fold dilutions were made, starting from a serum dilution of 1 in 100. Symbols have been omitted for clarity except for triangles which show the lack of specific binding by serum from a non-immunized mouse.

3.3. Results

3.3.1. Immunizations

Following the second booster injection, all 10 mice immunized with RαCGRP were found to be positive in the ELISA for antibodies to CGRP with high titre whilst all controls were negative (Figure 3.1). Some of the antisera were also found to inhibit the binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to rat liver membranes in a receptor binding assay (described in Chapter 5). Mice whose serum was active in the binding inhibition assay (Figure 3.2) were chosen for fusions.

3.3.2. Fusions

Two fusions were performed with different results. The first fusion was performed using NSO myeloma cells. Supernatant from only one well was found to be positive by ELISA. The second fusion, performed using Sp2 myeloma cells, was highly successful. Hybridoma cells were detected microscopically in every well of the 96-well plates and supernatants from 100 wells were found to be strongly positive by ELISA. Positive cell lines were transferred to 24-well plates and frozen as soon as possible.

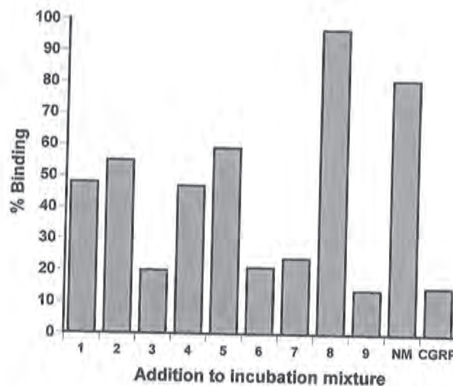

Figure 3.2: Effect of serum, diluted 1 in 100, from mice (1 to 9) immunized with RαCGRP on the binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to rat liver membrane preparation. Serum from a non-immunized mouse (NM) and excess RαCGRP (1 μM) were used as controls. Binding was expressed as a percentage of total binding in the presence of buffer alone. Values are the mean of two observations.

3.3.3. Cloning of selected cell lines

Due to the large number of positive supernatants, three selection criteria was applied in the choice of cell lines for cloning: (1) stability of the cell lines, (2) relative affinity of the antibodies for CGRP, and (3) cross-reactivity with different species of CGRP. An assumption of these selection procedures is that the fusion products had been plated sufficiently sparsely to give a high probability of monoclonality at the outset.

Sixty-four of the 100 cell lines expanded in 24-well plates were found to be positive when re-screened by ELISA, and these hybridomas were considered the more stable cell lines. Figure 3.3 shows that there were apparent differences in the affinity of antibodies from different hybridoma lines. The validity of this simple method of determining relative affinity (van Heyningen *et al.*, 1983) depends on prior knowledge that the variation of immunoglobulin concentration in supernatants from confluent hybridomas is generally less than ten-fold. The supernatants also demonstrated different cross-reactivities with the different forms of rat and human CGRP (Figure 3.4). Six cell lines were chosen for cloning on the basis of high relative affinity and selectivity for different forms of CGRP (Table 3.1).

Two further criteria were applied before cell lines were recloned. Until now, supernatants had been screened with an ELISA in which the antigen was immobilized

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Table 3.1: Summary of first cloning results

Cell line	Number of wells with growth		
	0.3 cell/well	*1 cell/well*	*3 cells/well*
C4.63	13/96	12/48	30/48
C4.56	19/96	34/48	45/48
C4.19	18/96	26/48	45/48
C4.22	16/96	19/48	36/48
C4.5	17/96	29/48	43/48
C4.6	14/96	22/48	38/48

on solid phase. It is known that some antibodies which are active in an ELISA do not bind to antigen in liquid phase, for example, in a RIA. Since it was essential that the MAbs developed could bind to CGRP in liquid phase in immunoblockade experiments, supernatants were tested in a RIA and a receptor binding inhibition assay (described in Chapter 5). Supernatants from hybridoma lines 6 and 19 were strongly active in the RIA (Figure 3.5) and receptor binding assay (Figure 3.6). Thus the cell lines 6 and 19 were cloned again (Table 3.2). The MAbs were coded C4.6 and C4.19.

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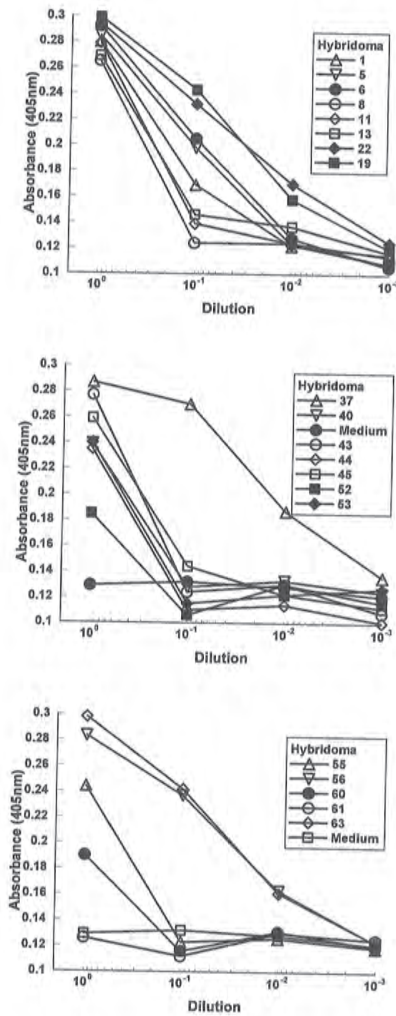

Figure 3.3: Relative affinity of hybridoma supernatants for RαCGRP by indirect ELISA. Tissue culture medium (10FD) was used as a control. Values plotted are single observations.

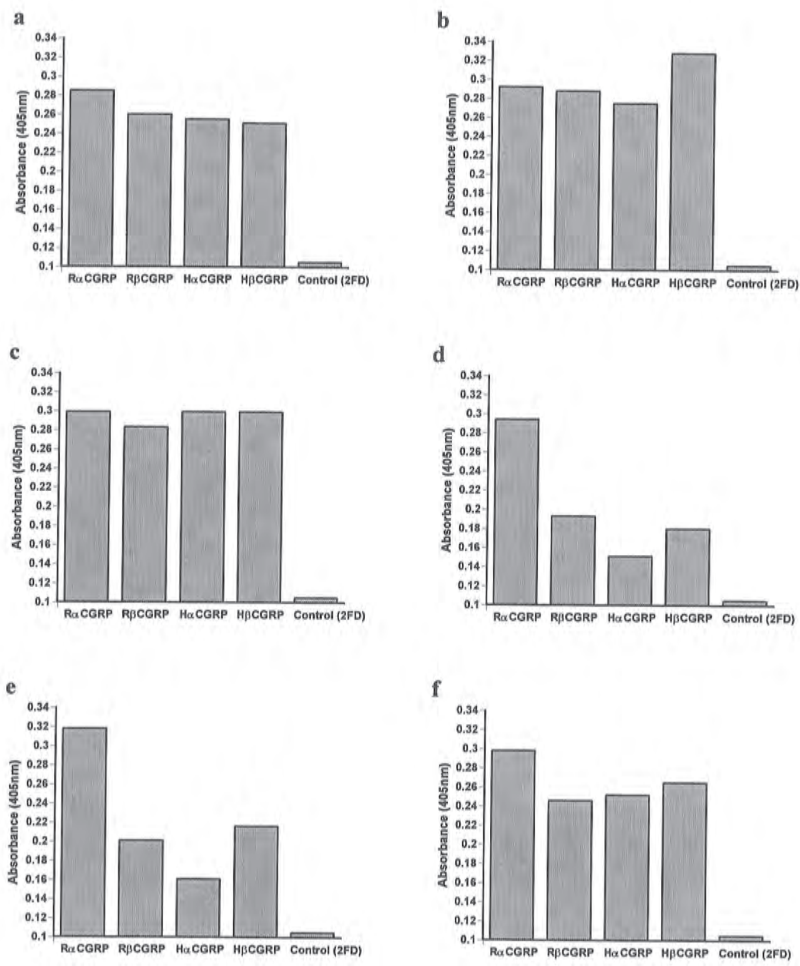

Figure 3.4: Binding of hybridoma supernatants C4.5 (a), C4.6 (b), C4.19 (c) C4.22 (d), C4.56 (e) and C4.63 (f) to different forms of rat and human CGRP by indirect ELISA. Values plotted are single observations.

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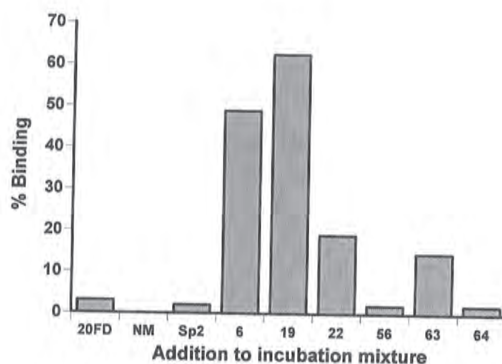

Figure 3.5: Binding of hybridoma supernatants 6, 19, 22, 56, 63 and 64 to 2-[^{125}I]-iodohistidyl^{10}-HαCGRP by RIA. Controls were culture supernatant (20FD), normal mouse immunoglobulin at 10 μg/ml (NM) and Sp2 myeloma cell supernatant. Binding was expressed as percentage of specific binding in the presence of buffer alone. Values are the mean of two observations.

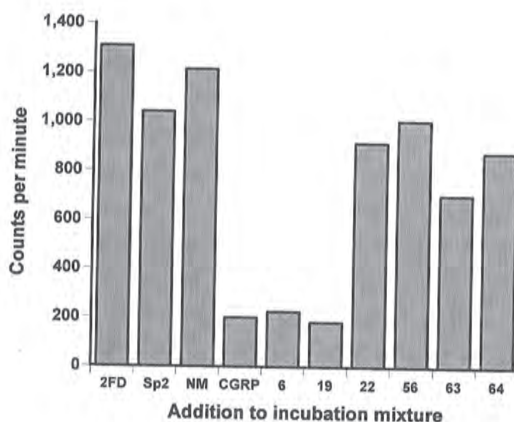

Figure 3.6: Effect of hybridoma supernatants 6, 19, 22, 56, 63 and 64 on the binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to rat liver membrane preparation. Controls were culture supernatant (2FD), Sp2 myeloma cell supernatant, normal mouse immunoglobulin at 10 μg/ml (NM) and excess RαCGRP (1 μM; non-specific binding). Values are the mean of two observations.

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Table 3.2: Summary of second cloning results

Cell line	No. of wells with growth		
	0.3 cell/well	*1 cell/well*	*3 cells/well*
C4.6	8/96	13/48	27/48
C4.19	9/96	11/48	30/48

3.4. Discussion

The present work has led to the successful production of two MAbs (C4.6 and C4.19) which bind to CGRP by ELISA and RIA, and inhibit CGRP binding in a receptor binding assay. The MAbs have broad cross-reactivity with different forms of CGRP as assessed by indirect ELISA. The binding characteristics of MAbs C4.19 and C4.6 suggested that they would be promising candidates as immunoblocking agents.

Since the original report by Kohler & Milstein (1975), the procedure for MAb production has been modified by many groups. Because few formal controlled comparisons have been reported, it was difficult to select the most appropriate protocol from the literature. Nevertheless, it is possible to identify some factors which have most probably contributed to the successful production of anti-CGRP MAbs in this study.

Spleens from mice which had strongly positive serum in both ELISA and receptor binding assay were chosen for fusions. Discrepancies in the potency of different antisera in the receptor binding assay and ELISA could be accounted for by variable titres of antibodies which could inhibit receptor binding by binding to the active site of CGRP. Alternatively, CGRP receptor binding could be displaced by anti-CGRP receptor antibodies arising from a normally functioning anti-idiotypic network; this hypothesis is the basis of the auto-anti-idiotypic approach to anti-receptor antibodies (Chapter 6).

The myeloma cell lines used in this study were non-producers of immunoglobulin heavy and light chains. As a general rule, non-producer myelomas are the best choice since the hybrids will express only the antibody heavy and light chains of the parental spleen. Greater success was achieved with the Sp2 than the NS0 cell line in this study despite claims by others of fastidious growth characteristics and low fusion frequency with the Sp2 cell line (Goding, 1986). Whatever the choice of myeloma, one of the most important factors for the successful derivation of hybrids is the way in which the myeloma culture has been maintained prior to fusion. The aim is

Correction (page 87, line 14):
"The protocol followed ..." should read "The protocol which was followed ..."

to maintain exponential growth for as long as possible. More than a week of exponential growth is recommended (Galfre & Milstein, 1981).

The choice of foetal calf serum is critical since sera from different sources vary greatly. The batch of foetal calf serum used in the current work was tested for capability to support the growth of myeloma cells at 1 cell per well prior to use in fusion. The important variables in PEG-induced fusion are concentration, purity, pH and duration of exposure to PEG. The PEG used for the fusions described here had been tested for fusion capability by the supplier. Fusion frequency is reported to be highest at pH 8.0 (Westerwoudt, 1986). The duration of exposure to PEG may be critical since both fusion frequency and undesirable toxicity increase with time of exposure. Likewise, there is a trade-off between low concentrations (<30%) which give poor fusion frequency and high concentrations (>50%) which result in overwhelming toxicity. PEG may be tolerated for longer times at lower concentrations. The protocol followed attempted to exploit understanding of the above factors by the use of a high concentration of PEG initially, followed by gradual dilution over time.

Fusion products were plated into 96-well plates since monoclonality from the outset would be more likely compared with the use of 24-well plates. Fresh medium and supplements were always used for fusion and the early post-fusion stages of cell culture. Since every tissue culture manipulation increases the risk of contamination, manipulations were kept to a minimum.

The use of cell feeder layers improves fusion frequency and cloning efficiency. The growth characteristics of cells *in vitro* are influenced by the number of cells present in culture. Feeder cells are presumed to condition the medium and optimize the environment for hybridoma survival and growth. The use of feeder layers may be critical when cells are plated sparsely after fusion and during cloning. MRC-5 diploid fibroblasts have been clearly shown to be superior to a variety of other feeder layer cells (Long *et al.*, 1986).

Production of the MAbs was facilitated by the rapid, sensitive and specific ELISA screening assay. Porcine gelatin was used as the blocking agent in the ELISA instead of BSA because the mice were immunized with CGRP conjugated to BSA. The assessment of relative affinity and cross-reactivity by ELISA and secondary screening with the RIA and receptor binding assay provided a rational basis for the identification of hybrids for cloning and expansion. Screening procedures must be oriented as closely as possible towards the final application of the MAbs. The detection of immobilized CGRP in an ELISA was not the end point of the current work. Since the MAbs were being produced for immunoblockade studies, binding characteristics in liquid phase were assessed by RIA and the potential for

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immunoblockade assessed by a simple receptor binding assay. The results of the RIA and receptor binding assay were consistent: only supernatants from hybrids C4.6 and C4.19 which were positive in the RIA could inhibit CGRP receptor binding.

In order to reduce the risk of overgrowth by non-producer cells and to ensure that the antibodies were truly monoclonal, cloning was performed as soon as supernatants with the desired properties were identified. The statistics of limiting dilution have been considered theoretically and verified experimentally (Coller & Coller, 1986). If cells are grown in small numbers, the fraction of wells with growth should follow the Poisson distribution. The Poisson equation assumes a random distribution of elements in the suspension being sampled. Thus it is important that the cell suspension is thoroughly mixed before performing limiting dilution cloning. Using the equations derived by Coller & Coller (1986) on the results of 96-well plates plated at 0.3 cells/well, it was calculated that the probability of monoclonality for the 6 hybrid cell lines on first cloning was between 0.90 and 0.95. The probability of monoclonality of hybridomas C4.6 and C4.19, which were cloned twice, was between 0.95 and 0.99. Apart from ensuring monoclonality, a reason for repeated cloning was to reduce the rate of chromosome loss. After 2 cycles of cloning, rate of chromosome loss is small but the risk of chromosome loss never ceases completely (Goding, 1986).

In summary, two MAbs (C4.6 and C4.19) have been successfully produced as tools for immunoblockade studies. Chapters 4, 7, 8 and 9 describe the further characterization of these and other MAbs and their subsequent application in pharmacological experiments.

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CHAPTER 4

Characterization, purification and fragmentation of monoclonal antibodies against CGRP

4.1. Introduction

The need for further characterization and purification of MAbs depends on their intended use. Since it was an objective of the project to understand the process of immunoblockade quantitatively, at least partial purification of MAbs was desirable. It was also necessary to select the MAb with the most desirable properties for use in pharmacological experiments. The isotype, affinity and specificity of 4 anti-CGRP MAbs were characterized in the present investigations. In addition, the utility of the MAbs for the immunocytochemical localization of CGRP was investigated.

A major objective of the project was to compare the application of whole immunoglobulins with antibody fragments in immunoblockade studies. The potential advantages of the use of antibody fragments have been discussed in Chapter 2. Immunoglobulins may be fragmented by controlled proteolysis. The portion of the polypeptide chain between the C_H1 and C_H2 domains of the heavy chain, known as the "hinge region", is susceptible to proteolysis. When an IgG molecule is subject to proteolytic attack by the enzyme papain in the presence of low concentrations of sulphydryl compounds, one or more peptide bonds in the hinge region are split, leading to the release of two identical Fab fragments (fragment having the antigen binding site) and a homogeneous Fc fragment (fragment that crystallizes).

The enzyme pepsin also cleaves at the hinge region but the cleavage site lies on the carboxyl side of the inter-heavy chain disulphide bonds. The resulting fragment is named $F(ab')_2$ because it contains two antigen binding sites. Fab' fragments may be produced from $F(ab')_2$ by reduction followed by alkylation. Mild conditions of reduction with cysteine are used to lyse the inter-heavy chain disulphide bonds whilst leaving the disulphide bond between the heavy and light chains intact. Fab' fragments are distinguished from Fab fragments which are univalent antigen binding fragments arising directly from digestion of IgG with papain. Fab' fragments are about 10% larger than Fab fragments but the small difference in molecular size is unlikely to be significant in immunoblockade studies.

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4.2. Methods

4.2.1. ELISA, receptor binding assay and RIA

Materials	Supplier
HαCGRP, HβCGRP, RαCGRP, RβCGRP	Bachem or Peninsula
2-[^{125}I]-iodohistidyl^{10}-HαCGRP	Amersham
HαCGRP$_{8\text{-}37}$	Bachem
C-terminal 25-37 and Tyr^0-28-37 fragments of HαCGRP	Celltech
Substance P	Peninsula
Rat amylin	Peninsula

Four anti-CGRP MAbs (C4.6, C4.19, R1.50 and R2.73) from cell lines that had been cloned twice were characterized for their relative affinity for different forms of CGRP by the indirect ELISA described in Chapter 3. Dr. C. Plumpton (Clinical Pharmacology Unit, Addenbrooke's Hospital) provided the MAbs R1.50 and R2.73 which were raised against RαCGRP. The ability of the MAbs to inhibit CGRP receptor binding was compared in the rat liver membrane binding assay described in Chapter 5.

To allow modelling of immunoblockade in tissue bath experiments (Chapter 8), RIA was performed to estimate the dissociation constant (Kd) of MAb C4.19 and the maximum binding capacity (B_{max}) of the purified ascites. The RIA method is found in Chapter 3. The dilution of purified ascites that bound 50% of radiolabelled CGRP was used in the binding displacement experiments. The displacement of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP binding to MAb C4.19 by RαCGRP and RβCGRP was investigated.

The Kd and B_{max} of MAb C4.19 for RαCGRP and RβCGRP were estimated by fitting the following model to binding data from RIA displacement experiments:

$$B(C) = \frac{B_{max} \times C}{Kd \times (1 + D/Kd_D) + C} + NS \times C$$

where B(C) is the binding B of ligand (2-[^{125}I]-iodohistidyl^{10}-HαCGRP) concentration C in the presence of displacer (RαCGRP or RβCGRP) concentration D. Kd and Kd_D are the dissociation constants of antibody binding to ligand and displacer respectively. B_{max} is the concentration of binding sites and NS is the non-specific binding parameter. Parameter estimation was performed using the non-linear extended least-squares regression package MKMODEL version 4.72 (Biosoft, Cambridge).

The CGRP binding site of MAb C4.19 and its potential cross-reactivity with relevant peptides were investigated by RIA and indirect ELISA. The ability of 1 μM $H\alpha CGRP_{8-37}$ or substance P to displace 2-[^{125}I]-iodohistidyl^{10}-HαCGRP binding to MAb C4.19 was tested in the RIA. The potential of MAb C4.19 to bind C-terminal (8-37, 25-37 and Tyr^{0}-28-37) fragments of HαCGRP, substance P and rat amylin was tested by indirect ELISA. The same concentration of HαCGRP or other peptides (400 nM) was used to coat wells of the microtitre plate.

4.2.2. Determination of antibody class

Knowledge of antibody class and subclass is useful for determining the strategy for purification. For example, Protein A binds IgG with high affinity and specificity and Protein A affinity chromatography is a particularly relevant for IgG purification. Moreover, antibodies of different classes may exhibit different pharmacokinetic characteristics *in vivo* (Chapter 2).

MAbs were isotyped by the Ouchterlony immunodiffusion technique (Ouchterlony & Nilsson, 1986) according to the commercial kit supplier's instructions (Serotec). Ouchterlony analysis is an efficient method for the determination of antibody class because the technique is very sensitive and simple, is not antigen-dependent, and does not require enzyme- or radioisotope-labelled antisera. Tissue culture supernatant was used for Ouchterlony analysis because ascites fluid contains normal immunoglobulins which may lead to ambiguous results.

Approximately 75 μl of culture supernatant (from densely grown cloned hybridoma cells) was added to the large central well of the rosette. Ten μl of antisera directed against the 4 mouse IgG subclasses (IgG_1, IgG_{2a}, IgG_{2b}, IgG_3), IgA and IgM were added to each of the outer 6 wells of the same rosette. The plate was then tightly closed and diffusion allowed to proceed at room temperature for 24 to 48 hours until precipitation lines, which indicate the presence of a reaction, were visible.

4.2.3. Determination of protein concentration

Protein concentration was measured by a Coomassie brilliant blue dye binding assay (Bio-Rad) with bovine γ globulin as standard. The automated assay was performed in a 96-well microtitre plate. Ten μl of sample buffer (blank) was added to every well in column 1. Ten μl of protein standard (0.0625 mg/ml to 0.50 mg/ml) was added in triplicate in rows to wells of columns 2 to 4. Ten μl of appropriately diluted sample was added in duplicate to the remaining wells. Two hundred μl of Biorad dye reagent (diluted 1 in 5) was added to each well. The solutions were thoroughly mixed. Optical

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density was measured at 600 nm after 5 to 10 minutes using a microtitre plate reader (Titertek Multiskan Plus Mk II, Flow Laboratories).

4.2.4. Purification of MAbs

4.2.4.1. Ammonium sulphate precipitation

4.2.4.1.1. Principles

Precipitation by salting-out to remove non-specific proteins is highly effective, simple and cheap. Concentration of purified protein may also be achieved by the procedure. Although other salts such as sodium sulphate may be used, the precipitation of antibodies is commonly performed by the use of ammonium sulphate.

Proteins in solution form hydrogen bonds with water through their exposed polar and ionic groups. When high concentrations of small, highly charged ions such as ammonium sulphate are added, these groups compete with proteins for binding to water. This removes water molecules from proteins and decreases their solubility, resulting in precipitation. The factors that will affect the concentration at which a particular protein will precipitate include the number and position of the polar groups, the molecular weight of the protein, the pH of the solution, and the temperature at which the precipitation is performed. The concentration (or percentage saturation) at which antibodies will precipitate varies from species to species. Fifty percent saturation is required for the precipitation of mouse antibodies. Most of the other components of serum and ascites fluid do not precipitate at this degree of saturation (Harlow & Lane, 1988).

Elevated levels of specific monoclonal immunoglobulins, as well as normal immunoglobulins and serum proteins, are present in ascites fluid. Ammonium sulphate precipitation is effective in removing serum proteins, particularly albumin, but normal immunoglobulins remain in ascites purified by this method. Some contamination with other high-molecular weight proteins will remain as well as proteins that are trapped in the large flocculant precipitates .

The required level of antibody purity is dependent on the application in mind, and ammonium sulphate precipitation may be readily combined with chromatographic methods if purer antibody preparations are needed.

4.2.4.1.2. Preparation of saturated ammonium sulphate solution

Saturated ammonium sulphate solution was prepared by adding 1 kg of ammonium sulphate crystals (BDH) to 1 litre of deionized water and stirring for 1 to 2 days at room temperature. Undissolved crystals was observed to be present in the solution.

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The temperature of the solution was lowered to 4°C overnight and the pH adjusted to 7.4 with diluted ammonium hydroxide solution (BDH). The pH of small aliquots which had been diluted 1 in 20 was measured after each addition of ammonium hydroxide. The pH of the saturated solution was not measured directly because salt errors and junction potentials could produce errors of greater than 1 pH unit (Tijssen, 1985).

4.2.4.1.3. Experimental procedures: ascites fluid

Ascites fluid which was confirmed by ELISA to be active was pooled and centrifuged for 30 minutes at 48,000 *g* (20,000 rpm; Sorvall) at 4°C. The supernatant was recovered. Saturated ammonium sulphate was added dropwise to the ascites fluid with gentle stirring until a 50% saturated solution was achieved. A milky suspension was observed and this was stirred for 1 to 2 hours. The suspension was centrifuged for 30 minutes at 48,000 *g* (20,000 rpm; Sorvall) at 4°C. The supernatant was discarded. The pellet was resuspended in 50% saturated ammonium sulphate in PBS and the above spin repeated. The pellet was finally resuspended in the required volume of PBS (30 to 50% of the original ascites fluid volume). The solution was dialysed three times against 1000 volumes of PBS over 48 hours to remove residual ammonium sulphate. The dialysed solution was finally centrifuged for 30 minutes at 48,000 *g* (20,000 rpm; Sorvall) to remove denatured protein. Protein concentration was estimated by the Biorad assay described in Section 4.2.3. The purified ascites fluid was tested for activity by ELISA.

4.2.4.1.4. Experimental procedures: hybridoma culture supernatant

Forty ml of culture supernatant was mixed with 40 ml of saturated ammonium sulphate solution. The mixture stirred for 60 minutes and centrifuged at 48,000 *g* (20,000 rpm; Sorvall). The pellet was resuspended in 800 μl of PBS and the solution dialysed three times against 1000 volumes of PBS over 48 hours. The dialysate was centrifuged (10000 rpm; Hereaus) to remove denatured protein.

4.2.4.2. Protein A sepharose affinity chromatography

Equipment (all from Pharmacia)

Chromatography column (borosilicate glass; Pharmacia C10 column; 1 cm i.d.) packed with Protein A sepharose CL-4B (Pharmacia).
Peristaltic pump P-1
Single path monitor UV1 (optical and control units)
Frac-100 fraction collector

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Correction (page 95): Buffer formulas should read:-

Start Buffer pH 8.9

1.45 M glycine (chromatographically homogenous grade; BDH)

3 M sodium chloride

Adjusted to pH 8.9 with sodium hydroxide

Elution Buffer pH 3.0

100 mM citric acid

20 mM sodium hydroxide

Adjusted to pH 3.0 with sodium hydroxide

Chart recorder

Start Buffer pH 3.0

1.45 M glycine (chromatographically homogeneous grade; BDH)

20 mM sodium chloride

Adjusted to pH 3.0 with sodium hydroxide

Elution Buffer pH 8.9

100 mM citric acid

3 M sodium chloride

Adjusted to pH 8.9 with sodium hydroxide

Protein A-sepharose affinity chromatography was used to purify MAb R1.50 IgG and $F(ab')_2$ and Fab' fragments. Staphylococcal protein A binds to the Fc region of many IgG molecules and provides a method of IgG purification. It is also possible to separate purified IgG from Fab fragment which does not bind Protein A due to the lack of Fc region.

Two column volumes of start buffer (degassed) were run through the column followed by pre-cycling with elution buffer (degassed) until a stable chart recorder baseline was obtained. Start buffer was run through the column again. The sample was diluted with start buffer and loaded at 0.5 ml/min. Absorbance was monitored at 280 nm and 1 ml fractions collected. Following the first peak, IgG was eluted with elution buffer. At the end of the procedure, PBS/thiomersal 0.05% was run through the column for storage, and 70% ethanol was run through the pump and the detector flow cells.

4.2.5. Fragmentation of MAbs

4.2.5.1. Preparation of $F(ab')_2$ by pepsin digestion

Materials	**Supplier**
Pepsin	Boehringer Mannheim
Citric acid (anhydrous)	Sigma
Trisodium citrate	Sigma
MAb R1.50 IgG	C. Plumpton
MAb C4.19 IgG	K. Tan
Normal mouse IgG	Sigma

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100 mM Citrate buffer pH 3.5

100mM citric acid was titrated against 100 mM trisodium citrate (100 mM citric acid: 19.21 g/l; 100 mM trisodium citrate: 29.41 g/l). Ratio is approximately 2:1 citric acid:trisodium citrate.

4.2.5.1.1. Experimental procedures

Pepsin is a non-specific protease which is only active at acid pH, and is irreversibly denatured at neutral or alkaline pH. Preliminary experiments were performed with Protein A affinity purified MAb R1.50 to determine optimum conditions. Two published methods were evaluated. The methods used the same protein:enzyme ratio but digestion was performed in 200 mM acetate buffer at pH 4.2 (Tijssen, 1985) or 100 mM citrate buffer at pH 3.5 (Parham, 1986). Forty-eight hour time course studies were performed. Samples were removed at 4, 8, 12, 24 and 48 hours from the incubation mixture and the reaction stopped. The extent of fragmentation was followed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The activity of the $F(ab')_2$ fragments produced was assessed by indirect ELISA.

The following optimized method was developed and was subsequently followed. IgG (or ammonium sulphate purified ascites fluid) was diluted to 5 mg/ml with 100 mM citrate buffer pH 3.5 (Antibodies in PBS could be diluted 1 in 5 without altering the pH of citrate buffer). Pepsin (1 mg/ml stock) was added to IgG in a ratio 1:100 (pepsin:IgG w/w) and the mixture incubated at 37°C for 4 hours. The reaction was stopped by addition of 1 M Tris HCl pH 9 to the mixture to give pH 7.4.

4.2.5.2. Preparation of Fab' fragments from $F(ab')_2$ fragments

Materials	**Supplier**
Anhydrous L-cysteine	Sigma
Iodoacetamide	Sigma
$F(ab')_2$ fragments prepared as above	

Reagents

1 M cysteine freshly prepared in 0.1 M Tris HCl pH7.4 (r.t.)/0.1 M NaCl

0.5 M iodoacetamide freshly prepared in 0.1 M Tris HCl pH7.4 (r.t.)/0.1 M NaCl (kept in dark).

4.2.5.2.1. Experimental procedures

Preliminary experiments investigated the effect of cysteine concentration on $F(ab')_2$ reduction and the following method was developed. The procedures were scaled up when necessary. Twenty μl 1 M cysteine was added to 2 ml pepsin digest or $F(ab')_2$ in

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PBS (protein concentration 2 to 5 mg/ml), giving a final cysteine concentration of 10 mM. The mixture was incubated for 2 hours at 37°C. The reaction was stopped by addition of 300 µl 0.5 M iodoacetamide (alkylation step). After an incubation period of 1 hour at room temperature in the dark, the mixture was dialysed three times against 1000 volumes of phosphate buffered saline over 2 days. The activity of the anti-CGRP Fab' fragments produced was checked by indirect ELISA based on the avidin-biotin reaction (see Section 4.2.5.4.).

4.2.5.3. Concentration of $F(ab')_2$ and Fab' fragments

$F(ab')_2$ and Fab' fragments were concentrated by use of Centriprep-30 and Centriprep-10 concentrators (Amicon) with 30 kDa or 10 kDa molecular weight cutoffs respectively. Ultracentrifugation was performed according to the manufacturer's instructions. The protein concentration of the concentrated sample and the filtrate was determined. Protein was undetectable in the filtrate.

4.2.5.4. Indirect ELISA of $F(ab')_2$ and Fab' fragments

Materials	**Supplier**
Biotinylated goat anti-mouse (Fab specific) IgG	Sigma
Streptavidin-horseradish peroxidase	Vector Laboratories

The polyclonal rabbit anti-mouse reagent used in the routine ELISA consists of antibodies mainly directed against the Fc region. Therefore a modified ELISA which employed a biotinylated goat anti-mouse (Fab specific) IgG as the second antibody (1 in 1000) and streptavidin-horseradish peroxidase (1 in 500) was used for the detection of $F(ab')_2$ and Fab' fragments.

4.2.6. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Equipment

Mini-PROTEAN II slab cell (Biorad) consisting of lower buffer chamber, casting stand, inner cooling core and sandwich clamp assemblies.

Electrophoresis power supply EPS500/400 (Pharmacia)

Gilson pipettes fitted with Multiflex pipette tips (sample loading)

Biorad Gel Dryer model 543

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Solutions

Acrylamide/Bis (30% total acrylamide; 2.67% cross-linker)

Acrylamide	29.2 g
N'N'-Bis-methylene-acrylamide (Bis)	0.8 g
Deionized water to	100 ml

Solution filtered with Whatman No.1 paper.

Sample buffer

Deionized water	20 ml
0.5 M Tris-HCl, pH 6.8	5 ml
Glycerol	4 ml
10% (w/v) SDS	8 ml
0.05% (w/v) Bromophenol blue	1 ml
2-β-mercaptoethanol	2 ml (or deionized water for non-reducing gel)

Electrode (running) buffer, pH 8.3

Tris base	3 g
Glycine	14.4 g
SDS	1 g
Deionized water to	1000 ml

Resolving (separating; lower) gel (10%)

Deionized water	4 ml
1.5M Tris-HCl, pH 8.8	2.5 ml
10% SDS	100 μl
Acrylamide/Bis solution	3.35 ml
10% ammonium persulphate (fresh)	50 μl
N,N,N',N'-tetramethylethylene-diamine (TEMED)	5 μl

Stacking (upper) gel

Deionized water	6.1 ml
0.5 M Tris-HCl, pH 6.8	2.5ml
10% SDS	100 μl
Acrylamide/Bis stock	1.3 ml
10% ammonium persulphate (fresh)	50 μl
TEMED	10 μl

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Coomassie Blue staining solution

Coomassie Blue R-250	200 mg
Methanol	80 ml
Glacial acetic acid	20 ml
Deionized water to	200 ml

Destain solution

Methanol	400 ml
Glacial acetic acid	100 ml
Glycerol	10 ml
Deionized water to	1000 ml

Pre-stained molecular weight markers

The contents of vial (SDS-70B; Sigma) were dissolved in 0.4 ml twice-concentrated sample buffer. 0.4 ml 48% w/v urea was added and the mixture incubated at 37°C for 2 hours before aliquoting for storage at -20°C. Five μl was used for one lane of each gel. Pre-stained molecular weight markers from Biorad were also used in some experiments.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Laemmli buffer system (Laemmli, 1970) and the Biorad mini-PROTEAN slab cell which allowed analysis of protein samples in miniature gels. The acrylamide concentration used was 10% as this provided the best resolution for the analysis of antibodies and antibody fragments. The lower gel was poured, overlaid with water saturated isobutanol and allowed to polymerize for about 45 minutes. Liquid was removed and the upper gel poured with comb in place. The gel was allowed to polymerize over approximately 45 minutes and the comb removed. The wells were rinsed with deionized water followed by running buffer.

Samples were diluted with an equal volume of sample buffer and heated to 100°C for 2 to 5 minutes. Under such conditions proteins unfold and bind to SDS which imparts a very strong negative charge to the protein, dominating its native charge. The protein-SDS complexes possess essentially identical charge densities and electrophoretic mobility in acrylamide is inversely proportional to the logarithm of molecular weight. Five to 10 μg of protein was loaded into each well. Molecular weight markers were run in conjunction with the samples. The gel was run at 200 mV until the bromophenol blue line reached the bottom. The gel was placed into staining solution for at least 30 minutes, destained, and dried onto cellophane with a gel dryer (Biorad model 543).

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The purity of immunoglobulins and Fab' fragments was determined by densitometric scanning of dried gels (Molecular Dynamics Personal Densitometer; ImageQuant software).

4.2.6.1. Analysis of antibody fragmentation by SDS-PAGE

SDS-PAGE allows the analysis of the cleavage of heavy chains, the cross-linking of polypeptide chains by disulphide bridges and the size of the fragments produced. SDS-PAGE can be performed under reducing or non-reducing conditions by preparing the samples in the presence or absence of the reducing agent β-mercaptoethanol. This permits the analysis disulphide bonds between IgG polypeptide chains.

Under non-reducing conditions, IgG migrates as a single protein band with molecular weight of approximately 150 kDa; under reducing conditions, IgG migrates as two bands corresponding to the heavy chain (~55 kDa) and the light chain (~25 kDa). $F(ab')_2$ under non-reducing condition migrates as a single band of ~ 110 kDa and when reduced is a doublet of bands at ~25 kDa. The lower band of the doublet corresponds to the light chain and the other is the NH_2-terminal half of the cleaved heavy chain (Fd'). Fab' migrates under non-reducing conditions as a single band of ~50 kDa and when reduced yields a doublet identical to that of $F(ab')_2$.

Fc under non-reducing and reducing conditions gives a single band of ~25kDa, migrating slower than the Fab doublet. This is not observed following pepsin digestion which breaks down the Fc fragment into smaller peptides (Goding, 1986).

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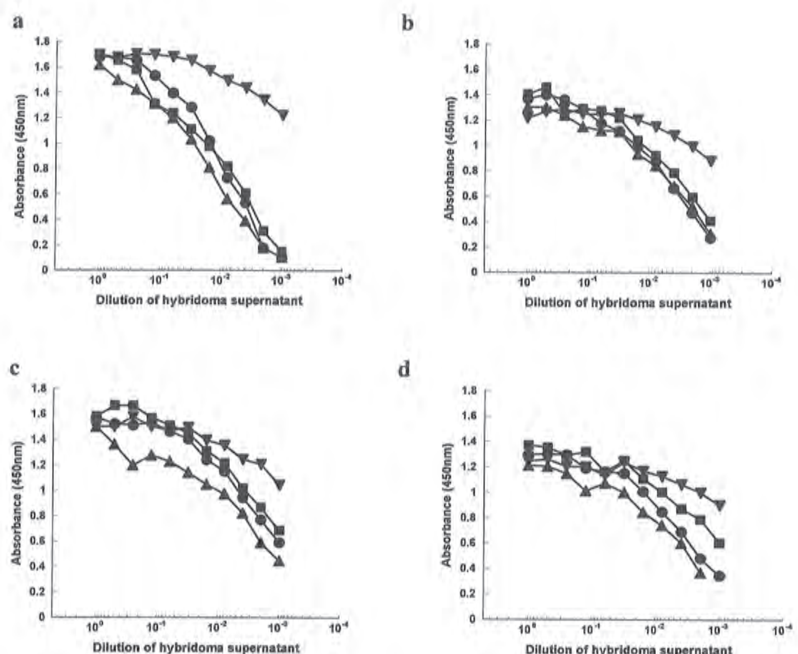

Figure 4.1: Binding of MAb C4.6 (triangles), C4.19 (circles), R1.50 (inverted triangles) and R2.73 (squares) to RαCGRP (a), RβCGRP (b), HαCGRP (c) and HβCGRP (d) by indirect ELISA. The same concentration of each form of CGRP was used to coat microtitre plate wells. Values are the mean of two observations.

4.2.7. Immunocytochemistry

The localization of CGRP-like containing structures in the rat spinal cord by MAbs C4.6, C4.19 and R1.50 was characterized by an immunoperoxidase method based on the avidin-biotin complex (ABC) system and by immunofluorescence. The principles and experimental procedures for immunostaining free-floating rat spinal cord sections using the ABC system are fully described in Chapter 6. Hybridoma supernatants were diluted 1 in 2 for immunostaining. Antibody specificity was tested by pre-absorption of MAbs with excess RαCGRP (2 μM) for 2 hours at room temperature.

Double immunofluorescence for the simultaneous localization of CGRP-like and substance P-like immunoreactivity was performed by the following procedures. Free floating rat spinal cord sections (30 μm) were blocked with 0.1 M PBS containing 3% normal horse serum, 0.1% BSA and 0.3% Triton-X 100 (blocking

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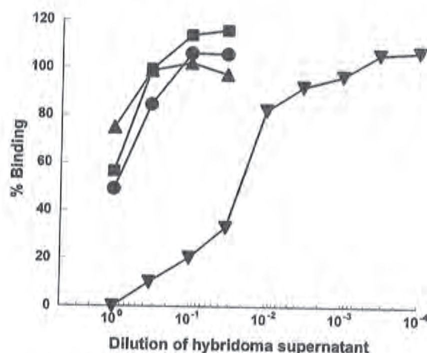

Figure 4.2: Effect of MAbs C4.6 (triangles), C4.19 (circles), R1.50 (inverted triangles) and R2.73 (squares) on the specific binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to rat liver membrane preparation. Binding was expressed as a percentage of total specific binding in the presence of buffer alone. Values are the mean of two observations.

reagent) followed by overnight incubation at 4°C with primary antibodies. The primary antibodies used were MAb C4.19 (hybridoma supernatant diluted 1 in 2 with blocking reagent) and a rabbit anti-substance P serum (Seralab; diluted 1 in 100 with blocking reagent). After washing, tissue sections were incubated for 2 hours with a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody (Seralab; diluted 1 in 80) or a Texas Red-conjugated goat anti-mouse antibody (Biomeda; diluted 1 in 80). Tissue sections were washed, mounted on slides with an aqueous mountant (Vectashield), and viewed under a fluorescence microscope (see Chapter 6).

4.3. Results

4.3.1. ELISA, receptor binding assay and RIA

MAbs C4.6, C4.19, R1.50 and R2.73 cross-reacted with the α and β forms of human and rat CGRP by indirect ELISA (Figure 4.1). MAb R1.50 apparently had the highest relative affinity for all forms of CGRP but this was particularly evident for RαCGRP. MAb R1.50 was also the most effective MAb for inhibition of CGRP receptor binding (Figure 4.2).

The binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to MAb C4.19 was displaced by RαCGRP and RβCGRP in a concentration-dependent manner (Figure 4.3) but not by substance P or HαCGRP$_{8\text{-}37}$ at 1 μM. No difference in binding was

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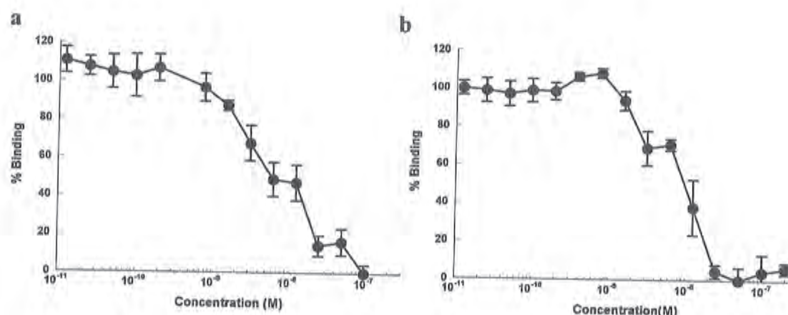

Figure 4.3: Displacement of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP binding to MAb C4.19 by RαCGRP (a) and RβCGRP (b). Binding was expressed as a percentage of total specific binding in the presence of buffer alone. Means and standard errors are plotted (n=4).

found whether assay buffer or Krebs buffer (Chapter 8) was used in the RIA. The dissociation constants (Kd) of MAb C4.19 for RαCGRP and RβCGRP were estimated to be 1.9 and 2.5 nM respectively. The estimated Kd and B_{max} (1.3 nmol/mg) were used for the simulation of *in vitro* immunoblockade (Chapter 8). MAb C4.19 did not bind to C-terminal (8-37, 25-37 and Tyr^0-28-37) fragments of HαCGRP, substance P and rat amylin by indirect ELISA (signal indistinguishable from background). The lack of cross-reactivity of MAb C4.19 with C-terminal fragments of CGRP by RIA and indirect ELISA suggests that it is directed against the N-terminus of CGRP.

4.3.2. Determination of antibody class

MAbs C4.6, C4.19, R1.50, and R2.73 were all found to be of the IgG_1 subclass.

4.3.3. Purification and fragmentation of MAbs

Cleavage of IgG to $F(ab')_2$ was clearly time dependent at pH 4.2, as shown by SDS-PAGE for MAb R1.50 (Figure 4.4). An incubation period of 48 hours was necessary for complete fragmentation at this pH. In contrast, fragmentation to $F(ab')_2$ was complete after 4 hours incubation at pH 3.5 (Figure 4.4) and this shorter procedure was chosen for further work.

The effective reduction of $F(ab')_2$ to Fab' fragments with 10 mM of cysteine was detected by the appearance of a band at approximately 45 kDa in the non-reduced SDS-PAGE gel. MAb R1.50 was successfully purified by Protein A affinity

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chromatography and fragmented to $F(ab')_2$ and Fab' fragments (Figure 4.5). Large-scale fragmentation of MAb R1.50 led to Fab' fragments which were used in tissue bath experiments (Chapter 7).

MAb C4.19 was effectively purified by ammonium sulphate precipitation of ascites fluid (Figure 4.6). The purity of immunoglobulins in the antibody preparation purified by ammonium sulphate precipitation was 55%. Application of the experimental procedures originally optimized for MAb R1.50 to MAb C4.19 and normal mouse IgG also led to the successful production of Fab' fragments (Figures 4.6 and 4.7). The purity of the MAb C4.19 Fab' fragment produced was 92%. MAb C4.19 and normal mouse Fab' fragments were used for *in vivo* immunoblockade studies (Chapter 9).

Similar binding curves were observed when the same protein concentration of MAb R1.50 and MAb C4.19 IgG, $F(ab')_2$ and Fab' were used to detect RαCGRP by indirect ELISA (Figure 4.8).

4.3.4. Immunocytochemistry

CGRP-immunoreactive nerve fibres were detected by MAbs C4.6, C4.19 and R1.50 in the rat spinal cord (Figures 4.9 to 4.13). Specific immunostaining with the lowest background was observed with MAb C4.19. Immunostaining was abolished by pre-absorption with 2 μM CGRP (Figures 4.10). The highest density of CGRP-like immunoreactive nerve fibres was found in laminae I and II (Figure 4.11). Immunoreactive fibres were also detected around the central canal. Motoneurons in the ventral horn were characteristically immunostained (Figure 4.12). No difference in CGRP-like immunostaining was observed in the presence of 10 μM neurotensin or cholecystokinin. Double immunofluorescence showed that CGRP- and substance P-like immunoreactivity were co-localized in laminae I and II of the spinal cord (Figure 4.13).

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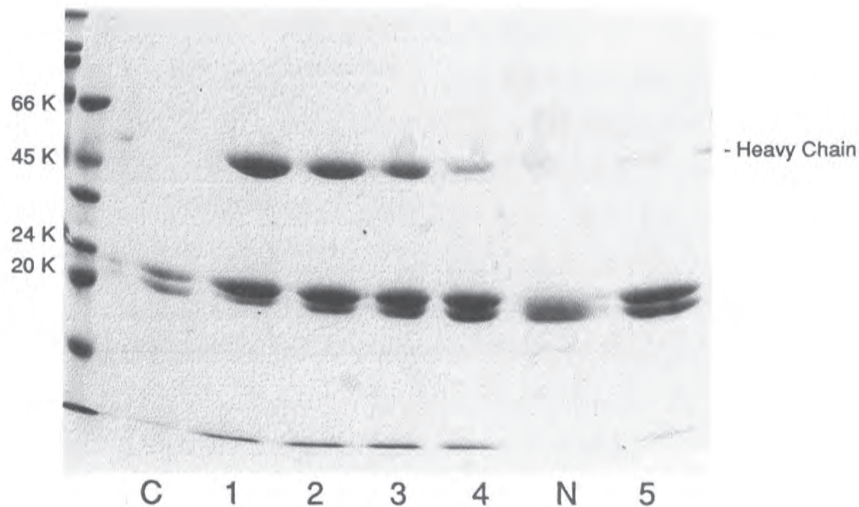

Figure 4.4: SDS-PAGE under reducing conditions of MAb R1.50 following incubation with pepsin in 200mM acetate buffer pH 4.2 for 4 (1), 8 (2), 12 (3), 24 (4) and 48 (5) hours. Like MAb R1.50, normal mouse IgG (N) was effectively fragmented, with disappearance of heavy chain, after 48 hours incubation with pepsin at pH 4.2. Complete fragmentation to $F(ab')_2$ was observed after incubation of MAb R1.50 IgG for only 4 hours with pepsin in 100mM citrate buffer at pH 3.5 (C). The concentration of acrylamide was 10%.

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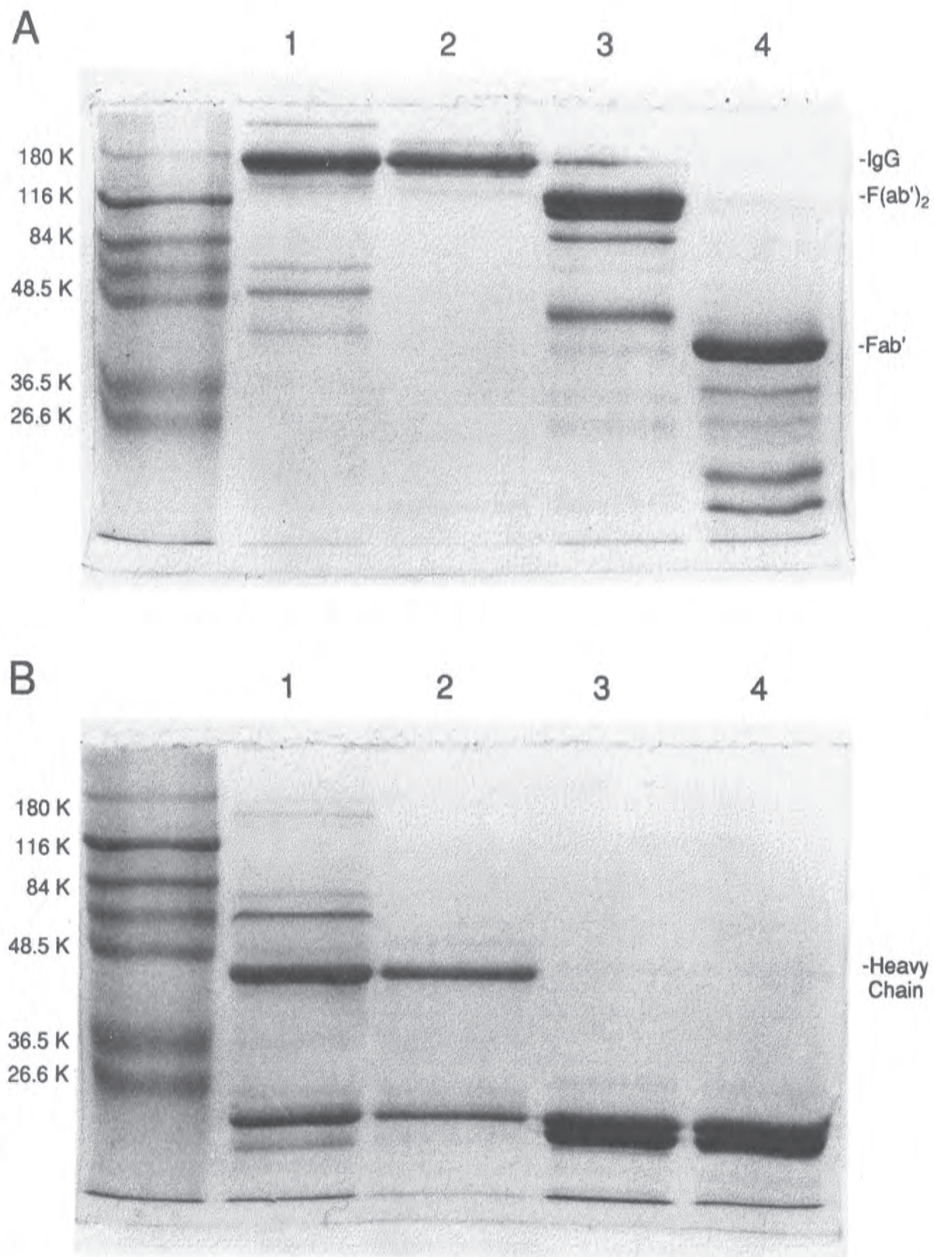

Figure 4.5: SDS-PAGE under non-reducing (A) and reducing (B) conditions of MAb R1.50 IgG purified from ascites fluid by ammonium sulphate precipitation (1), IgG purified by Protein A affinity chromatography (2), F(ab')$_2$ fragment (3) and Fab' fragment (4). The concentration of acrylamide was 10%.

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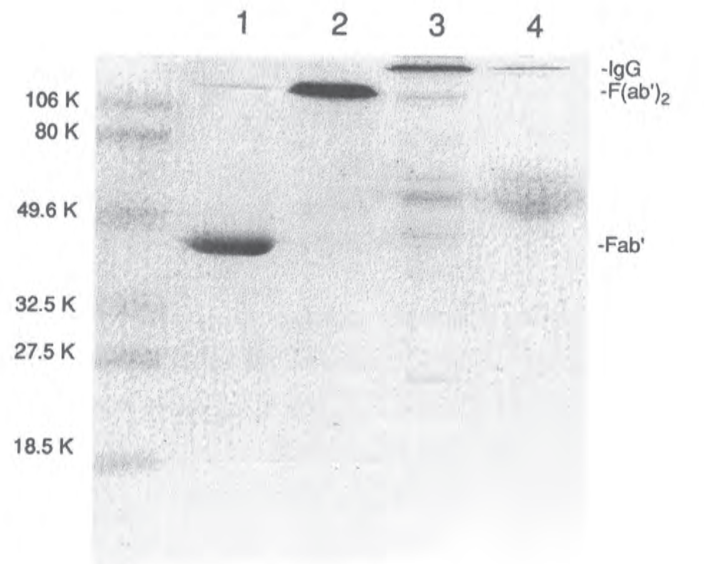

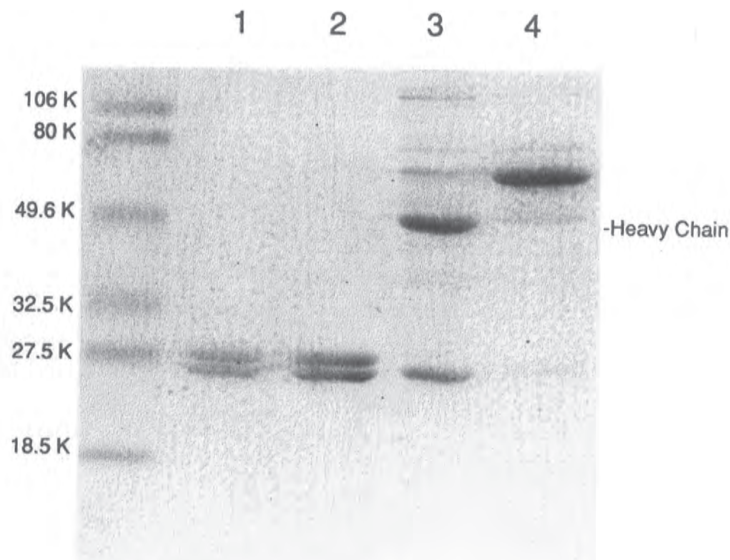

Figure 4.6: SDS-PAGE under non-reducing (top) and reducing (bottom) conditions of (1) MAb C4.19 Fab' fragment, (2) $F(ab')_2$ fragment, (3) IgG purified from ascites fluid by ammonium sulphate precipitation, and (4) neat ascites fluid. The concentration of acrylamide was 10%. The most abundant protein in ascites fluid was albumin (69kDa). The effectiveness of pepsin digestion is indicated by the complete disappearance of the heavy chain of IgG.

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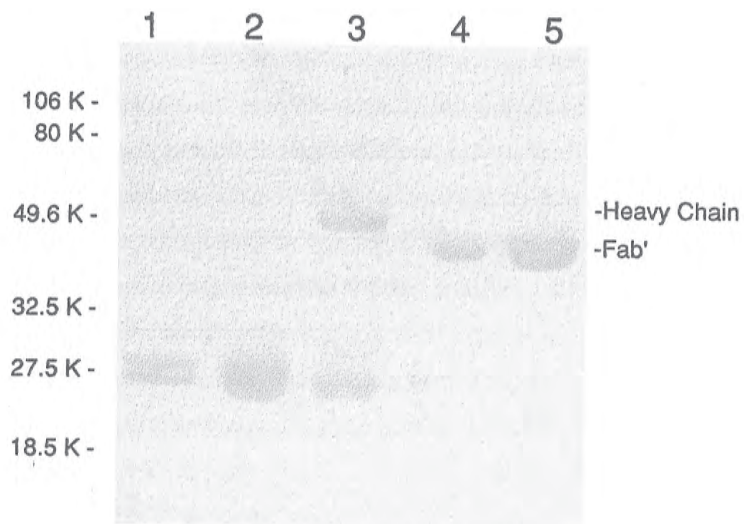

Figure 4.7: SDS-PAGE under reducing (1, 2, 3) and non-reducing (4, 5) conditions of normal mouse IgG (3), normal mouse Fab' fragment (2, 5) and MAb C4.19 Fab' fragment (1, 4). The concentration of acrylamide was 10%.

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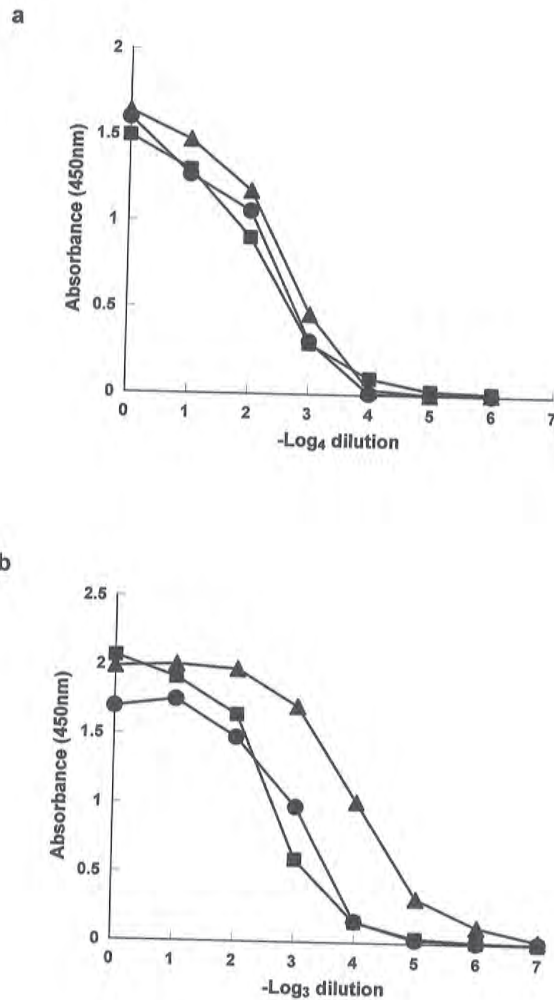

Figure 4.8: Binding of MAb R1.50 (a) and MAb C4.19 (b) IgG (squares), $F(ab')_2$ (triangles) and Fab' (circles) to RαCGRP by indirect ELISA. The starting protein concentration of MAb R1.50 and fragments was 20 μg/ml. The starting protein concentration of MAb C4.19 and fragments was 30 μg/ml. Values are the mean of two observations.

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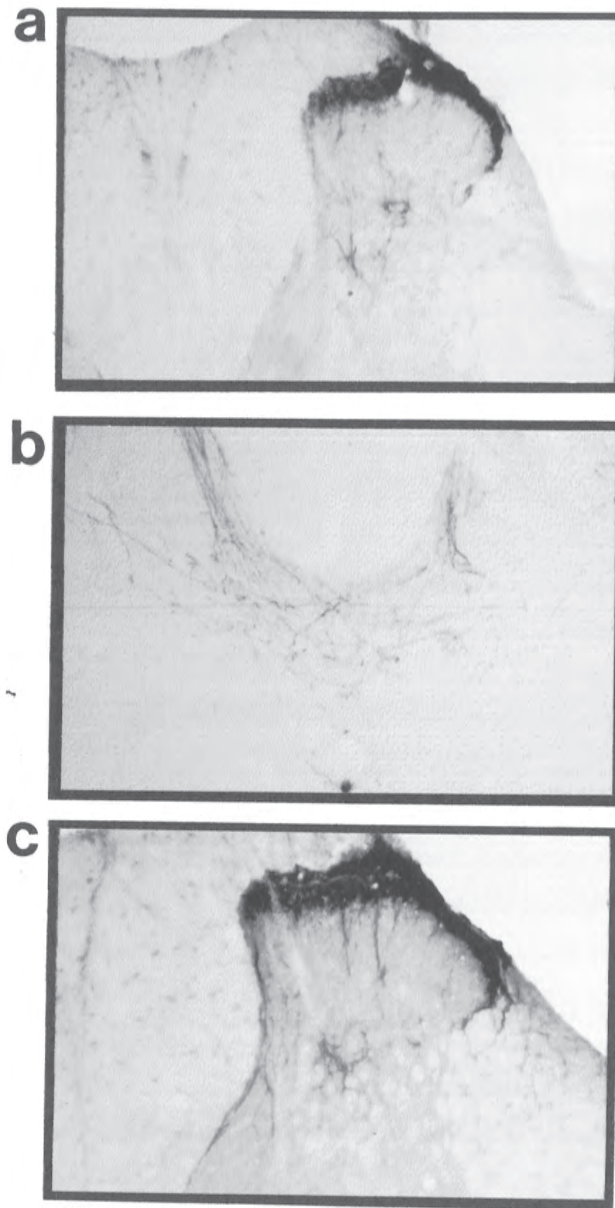

Figure 4.9: Immunostaining of nerve fibres in the dorsal horn and the area dorsal to the central canal of the rat spinal cord by MAb C4.6 (a,b x100). CGRP-immunoreactive fibres were also observed with MAb R1.50 (c; dorsal horn; x 100).

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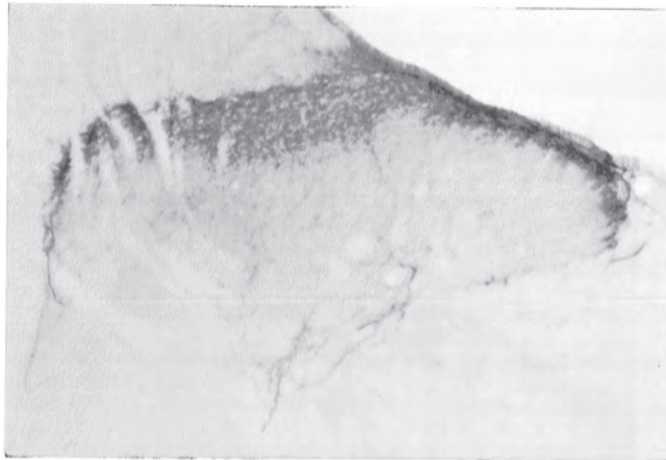

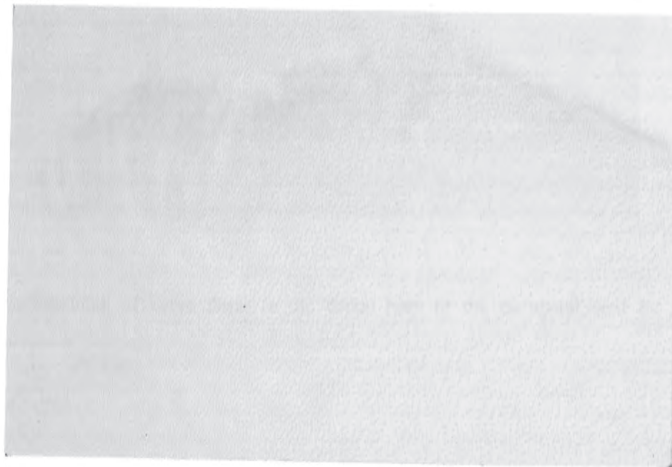

Figure 4.10: Demonstration of the specific immunostaining of nerve fibres by MAb C4.19 (a; x100) which could be abolished by pre-absorption with 2 μM RαCGRP for 2 hours at room temperature (b; x100). The dorsal horn of the rat spinal cord is shown.

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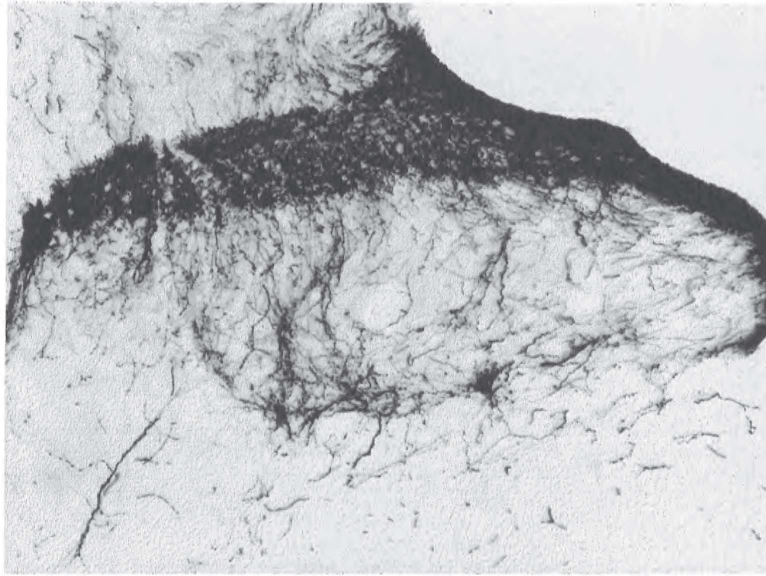

Figure 4.11: Immunostaining of nerve fibres in the dorsal horn of the rat spinal cord by MAb C4.19 (x100).

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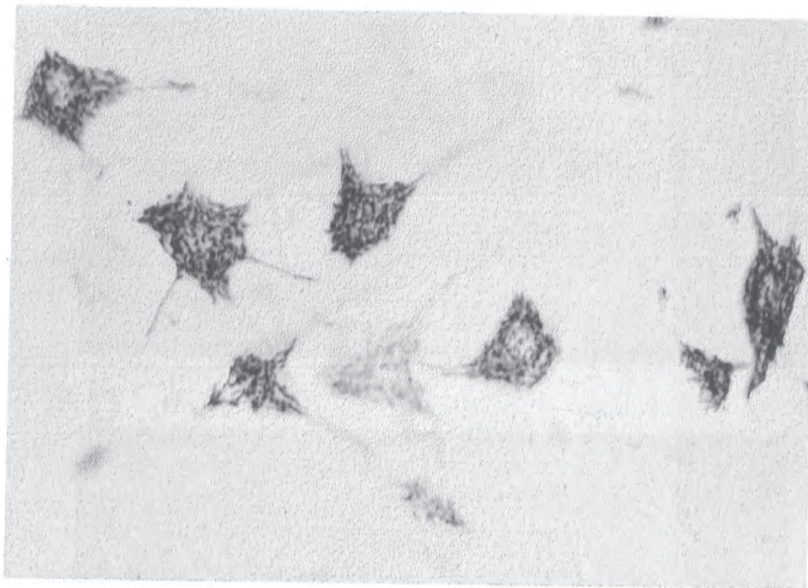

Figure 4.12: Immunostaining of motoneurons in the ventral horn of the rat spinal cord by MAb C4.19 (x100).

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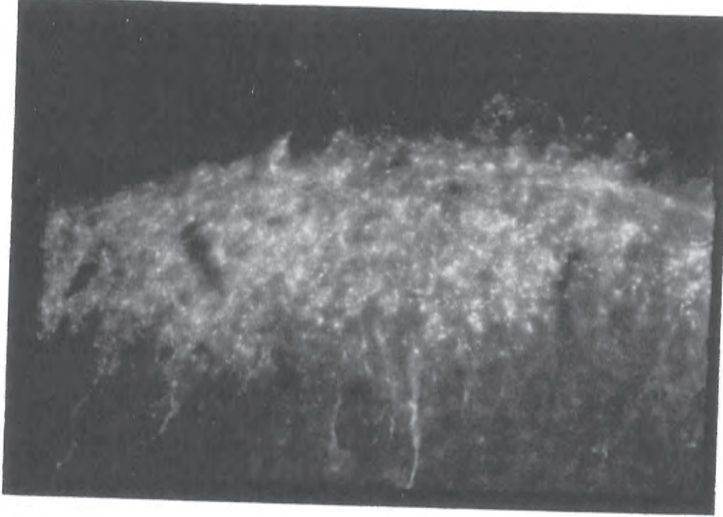

Figure 4.13: Double immunofluorescence staining of substance P- (a; FITC; x250) and CGRP- (b; Texas red; x250) immunoreactive nerves in the dorsal horn of the rat spinal cord by rabbit anti-substance P serum (Seralab) and MAb C4.19 respectively.

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4.4 Discussion

Four MAbs have been purified and fragmented for pharmacological experiments. Biochemical characterization of the 4 candidate MAbs led to the initial choice of MAb R1.50 as the model MAb for immunoblockade studies (Chapter 7). However, all 4 MAbs were further assessed for their utility in immunoblockade experiments (Chapter 8), and MAb C4.19 was eventually used to investigate the physiological role of CGRP (Chapters 8 and 9).

Ammonium sulphate precipitation was particularly useful as a purification method because it also facilitated the concentration of MAbs. Concentrated MAb solutions allowed the addition of small volumes to tissue baths and injection of low volumes *in vivo*. In general, ascites fluid purified by ammonium sulphate precipitation was considered sufficiently pure for pharmacological experiments. Whilst purer antibody preparations may offer advantages, each purification step also potentiates the risk of denaturing antibodies. The concentration of specific antibody in purified ascites fluid may be obtained by estimating B_{max} from RIA experiments, as performed for MAb C4.19.

Protein A affinity chromatography is considered to produce low yields for mouse IgG_1 (Goding, 1986). However, this problem was overcome by the use of high salt concentrations and high pH which improve the binding of IgG_1 to protein A. Protein A affinity chromatography yielded highly pure MAb R1.50 and offered a method for the separation of IgG and $F(ab')_2$ fragments. However, the fragmentation of pure IgG to $F(ab')_2$ under optimized conditions was clearly complete and purification of the pepsin digestion mixture proved unnecessary. Further experience showed that pepsin treatment of ascites fluid purified by ammonium sulphate precipitation yielded pure $F(ab')_2$ as assessed by SDS-PAGE. These observations are in agreement with those of Parham (1983) who reported that pepsin treatment of ascites fluid at pH 3.5 degraded almost all proteins except IgG_1 to small molecular weight peptides. Thus, contrary to the recommendation by Lamoyi (1986), complete purification of IgG prior to pepsin digestion was unnecessary.

The different IgG subclasses vary in their susceptibility to proteolytic cleavage (Parham, 1983). IgG_1 is more resistant to papain than other IgG subclasses (Goding, 1986), and all attempts to produce MAb R1.50 Fab fragments by papain digestion failed. IgG_1 is also resistant to pepsin but the appropriate optimization of time and pH parameters led to excellent yields of MAb R1.50 $F(ab')_2$ fragments. Fab' fragments were successfully produced by reduction of $F(ab')_2$ fragments.

Optimization of the fragmentation procedure for each MAb may be necessary because individual MAbs appear to be unique in their susceptibility to fragmentation

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(Parham, 1986). Indeed, pepsin digestion has been reported to rapidly destroy some MAbs (Lamoyi, 1986). However, application of the procedures optimized for MAb R1.50 to MAb C4.19 and normal mouse IgG, initially on an analytical scale, led to equally successful fragmentation. This could be related to the fact that MAb C4.19 is also an IgG_1 and that IgG_1 is a major IgG subclass in normal mouse serum. MAb R1.50 and MAb C4.19 fragments retained binding activity. The ELISA results usefully indicated the relative binding activity of IgG and the fragments at the same protein concentration. The greater binding activity of MAb C4.19 F(ab')2 fragments at a lower molar concentration than Fab' fragments is probably due to the higher avidity of bivalent molecules.

Specific immunostaining with very low background was found when MAb C4.19 was used for immunocytochemistry. The spinal cord was used as a model tissue for the assessment of the utility of MAbs in immunocytochemistry because patterns of CGRP immunoreactivity has been well characterized in this tissue with CGRP antisera (Gibson *et al.*, 1984). Both αCGRP and βCGRP are found in the rat spinal cord. Although the concentration of αCGRP may be 3 to 6 times higher than βCGRP in the dorsal spinal cord (Mulderry *et al.*, 1988), the CGRP immunoreactivity detected by MAb C4.19 probably relate to both forms of CGRP.

The role of CGRP in the dorsal horn of the spinal cord may include potentiation of synaptic transmission by altering calcium conductance (Ryu *et al.*, 1988; Oku *et al.*, 1988) and by increasing the release of substance P (Oku *et al.*, 1987). The presence of CGRP in motoneurons and its co-localization with choline acetyl transferase has led to suggestions that CGRP may be involved in neuronal regeneration, astroglial gene expression and enhancement of cholinergic transmission at the neuromuscular junction (Takami *et al.*, 1985; Streit *et al.*, 1989; Haas *et al.*, 1991; New & Mudge, 1986; Jinnai *et al.*, 1989).

Double immunofluorescence was shown to be possible using MAb C4.19 and an anti-substance P rabbit antiserum. Double immunostaining with MAb C4.19 is being used to examine physiologically important issues in collaboration with others.

In summary, 4 MAbs against CGRP have been purified, concentrated, fragmented and characterized with immunoblockade experiments in mind. MAb R1.50 emerged as a promising candidate MAb for pharmacological studies but all 4 MAbs were subsequently screened pharmacologically. MAb C4.19 also proved to be a useful tool for immunocytochemistry.

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CHAPTER 5

Development of monoclonal antibodies against the CGRP receptor

5.1. Introduction

A number of approaches to the development of MAbs against receptors have been advocated (reviewed in Chapter 1). The most direct route to anti-receptor antibodies is to immunize with the receptor itself. In practice, animals have been immunized with receptor-rich crude membrane preparations, whole cells which express the relevant receptor, affinity purified receptor preparation, peptide fragments of sequenced receptors or receptor protein bands identified in SDS-PAGE.

Methods which do not require purification of the CGRP receptor were investigated in this project since receptor purification is time-consuming and no published methods were available when the studies were started. This chapter details attempts to develop anti-CGRP receptor MAbs by *in vivo* and *in vitro* immunization with membrane preparations. *In vitro* immunization in culture (Reading, 1982, 1986; Boss, 1984, 1986) was investigated because it offered a number of advantages over conventional *in vivo* immunization, particularly when crude membranes were used as immunogens. An auto-anti-idiotypic approach was also pursued in collaboration. The characterization of MAbs developed with the auto-anti-idiotypic approach is described in Chapter 6.

5.2. Methods

Materials	Supplier
RαCGRP	Peninsula
Ethylene-diamine-tetraacetic acid (EDTA)	Sigma
Tris(hydroxymethyl)aminomethane (Tris)	Sigma
Phenylmethyl-sulphonyl-fluoride (PMSF)	Sigma
Bovine serum albumin (BSA)	Sigma
Bacitracin	Sigma
Aprotinin	Bayer
Magnesium Chloride	Amersham
Polyethyleneimine	Sigma

5.2.1. Preparation of rat liver membranes

Buffers

Buffer A:	0.1 mM PMSF in 50 mM Tris HCl pH 7.4
Buffer B:	0.5 mM EDTA
	0.1 mM PMSF in 50 mM Tris HCl pH 7.4

Wistar rats were killed by stunning and cervical dislocation. Livers were dissected out, immediately placed on ice, and transferred to a liquid nitrogen container as soon as possible for storage. Livers were thawed and their wet weight determined. All procedures during membrane preparation were performed at 4°C as far as possible. Buffer A was added at a volume (ml) equivalent to five times the wet weight (g). The livers were chopped up with scissors and tissue homogenized in short bursts using a Polytron homogenizer (Kinematica, Switzerland). The homogenate was centrifuged at 15000 *g* for 20 minutes at 4°C in a RC5C Sorvall Instruments (Dupont) centrifuge. Supernatant was decanted and centrifuged at 48000 *g* for 60 minutes at 4°C. The pellet was resuspended in Buffer B at a volume (ml) equivalent to five times the wet weight (g) and the suspension centrifuged at 48000 *g* for 60 minutes at 4°C. The pellet was finally resuspended in Buffer A. The membrane preparation was aliquoted and stored at -70°C. Protein concentration was determined as described below.

5.2.2. Preparation of bovine cerebellum membranes

Bovine cerebellum was collected from the local abattoir, transported to the laboratory in ice, and membranes prepared as described above for the rat liver.

5.2.3. Determination of protein concentration of membrane preparations

Protein concentration was measured by the Coomassie brilliant blue dye binding assay (Bio-Rad) described in Chapter 4. Bovine γ-globulin was used as the protein standard.

5.2.4. Receptor binding assay of CGRP using rat liver membrane preparation

Binding buffer

Aprotinin	20 u/ml
Bacitracin	0.1%
PMSF	0.1 mM

Magnesium chloride	5 mM
BSA	0.5%
Tris HCl pH 7.4 (at 4°C)	50 mM

Wash buffer

BSA	0.5%
Tris HCl pH 7.4 (at 4°C)	50 mM

The incubation mixture consisted of 50 μl binding buffer, 50 μl RαCGRP (10^{-12} to 10^{-6} M) or binding buffer (total bindable counts), 50 μl 2-[^{125}I]-iodohistidyl^{10}-HαCGRP (40,000 counts per minute [cpm]) and 100 μl membrane suspension (3 mg/ml). The tubes were gently shaken and then incubated at 4°C for two hours. The incubation mixture was vacuum filtered through GF/C filter paper (Whatman) that had been pre-soaked in polyethyleneimine (250 μl in 200 ml 50 mM Tris HCl pH 7.4). The filter paper was washed three times with 3 ml ice-cold wash buffer. Individual pieces of filter paper were placed in polypropylene tubes and radioactivity counted for 3 minutes in a Beckman Gamma 5500 counter.

5.2.4.1. Reduction of non-specific binding by siliconization and use of BSA

An experiment was designed to investigate the non-specific binding of CGRP. The effect of co-incubation with BSA and the siliconization of glassware and polypropylene materials on non-specific binding was tested. Glass and polypropylene tubes were siliconized by rinsing with Sigmacote® (Sigma) and drying in air. One ml aliquots of a 2-[^{125}I]-iodohistidyl^{10}-HαCGRP solution containing 50,000 cpm with or without 0.5% BSA were added to glass or polypropylene tubes. Samples (80 μl) were taken at timed intervals over 1 hour for radioactivity counting.

5.2.4.2. Estimation of receptor binding parameters

One or two-site binding models were fitted to the binding displacement data by non-linear least squares regression (MKMODEL version 4.72; Biosoft, Cambridge) with weighting factor inversely proportional to the square of the predicted bound concentration. The structural model parameters estimated were Kd (dissociation constant), B_{max} (concentration of binding sites), and the non-specific binding constant (NS). Initial Kd and B_{max} values were estimated by the method of DeBlasi *et al.* (1989). The Kd, B_{max} and NS of the displacer (cold ligand) were fixed at the values of the parameters of the radioactive-labelled ligand.

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5.2.5. Use of receptor binding assay for screening serum and cell culture supernatants

The incubation mixture consisted of 50 μl serum or supernatant, 50 μl binding buffer, 50 μl 2-[^{125}I]-iodohistidyl^{10}-HαCGRP (40,000 cpm) and 100 μl membrane suspension (3 mg/ml). Non-specific binding was assessed by addition of excess RαCGRP (10^{-6}M) to the incubation mixture. Cell culture medium, myeloma supernatant and normal mouse immunoglobulin (or serum) were added to control tubes. The mixture was incubated overnight at 4°C before filtration.

An alternative procedure was used to screen supernatants obtained by the *in vitro* immunization protocol. Supernatants and membrane preparation were co-incubated for 5 days at 4°C prior to addition of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP and further incubation for 2 hours at 4°C.

5.2.6. Receptor binding assay of CGRP using bovine cerebellum membrane preparation

Specific binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to bovine cerebellum membrane was confirmed using the binding assay developed for rat liver membrane preparation.

5.2.7. Dot immunobinding assay for immunoglobulin in supernatants

Reagents

Phosphate buffered saline pH 7.4 (PBS; formula in Chapter 3)

Tween 20 (Sigma; 0.05% v/v)

4-chloro-1-naphthol (Sigma; 3 mg/ml freshly made in methanol)

Horseradish peroxidase conjugated rabbit anti-mouse antibody (ICN Flow; 1 in 1000)

Substrate reagent

Deionized water	20 ml (5 parts)
4-chloro-1-naphthol	4 ml (1 part)
Hydrogen peroxide 6%	40 μl (0.01% final concentration)

A dot immunobinding assay was used to screen for immunoglobulin secretion by hybridomas. The following procedures were adapted from those described by Hawkes (1986). A sheet of 0.45 μm nitrocellulose membrane filter paper (Schleicher and Schuell) was lined with pencil and ruler to outline square areas. Half to one μl of

supernatant from fusion was added to the centre of each square and the blots allowed to dry. Non-immune mouse immunoglobulin (10 μg/ml; Sigma) was used as a positive control. Myeloma culture supernatant and fresh cell culture medium were used as negative controls. The filter paper was washed three times with PBS/Tween 20 0.05%. The filter paper was left to soak for several minutes between each wash. PBS/Tween 20 0.05% was removed and approximately 30 ml of PBS/BSA 1% was added (blocking step). The filter paper was left to soak in this solution for 15 to 30 minutes. PBS/BSA 1% was removed and 30 ml horseradish peroxidase conjugated rabbit anti-mouse antibody (1 in 1000 in PBS/Tween 20 0.05%/BSA 1%) added and left for 1 hour. After washing the filter paper three times with PBS/Tween 20 0.05%, substrate reagent was added and left to soak until dots appeared. The filter paper was rinsed with deionized water, dried and photocopied if required.

5.2.8. *In vivo* immunization protocol and screening for anti-receptor antibodies

Four 5 to 6 week old female Balb/C mice were immunized with rat liver membrane preparation on 4 occasions at monthly intervals with 20, 4, 4 and 14 mg of protein in 200 μl respectively. Another group of 14 Balb/C mice were immunized with 0.7 to 1 mg of rat liver membrane preparation on 5 occasions. Ten Balb/C mice and four 10-week-old female LOU/C rats were immunized with 1 mg of bovine cerebellum membrane preparation 3 times at monthly intervals. The i.p. route was used for all injections. Blood was collected from mice by tail bleed 7 days after booster injections and serum tested in the receptor binding assay.

5.2.9. *In vitro* immunization protocol and fusion

Culture medium

		Stock solution	*Supplier*
50 μM 2-mercaptoethanol	0.5 ml	50 mM	Gibco
20mM HEPES	10 ml	1 M	Northumbria
1mM sodium pyruvate	5 ml	100 mM	Gibco
2% non-essential amino acids	10 ml	100 x	Flow
Foetal calf serum (20%)	100 ml		GlobePharm
Dulbecco's modified Eagles medium*	500 ml		Flow

*containing L-glutamine, penicillin 500 iu/ml, streptomycin 50 μg/ml, Amphotericin B 2.5 μg/ml.

Sterile stock solutions of the above materials were obtained from manufacturers.

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However, adjuvant peptide (N-acetylmuramyl-L-alanyl-D-isoglutamine; Sigma) was not supplied sterile. Five mg was stored at -20°C, thawed, and made up in 5 ml fresh SFD under sterile conditions. This was dispensed as twelve 400 μl aliquots into cryovials and stored at -70°C. The remaining 200 μl was left in a Bijou tube, sealed and left in an incubator but no growth was detected. The rat liver membrane preparation was washed twice in PBS by centrifuging at 48,000 *g* for 1 hour and sterilized by γ-irradiation at 2.5 mRads (Department of Radiotherapy, Addenbrooke's Hospital).

One mg of rat liver membrane preparation was added to 10 ml culture medium in a 75 cm^2 flask. Ten ml culture medium was warmed to 37°C in a water bath. The spleen was removed from a Balb/C mouse into ice cold SFD and washed once in 10 ml SFD. Spleen cells were suspended in 10 ml SFD and centrifuged for 5 minutes at 1500 rpm (Denley). The pellet was resuspended in 10 ml SFD and centrifugation repeated. The pellet was then resuspended in 10 ml warm (37°C) medium (prepared as above) and transferred to the 75 cm^2 flask. An aliquot of adjuvant peptide (400 μl) was added to the flask. The flask was kept with a loose lid in a humidified incubator (IR1500 5% CO_2, 37°C; Flow).

Blast cells were visible under the microscope after two days. Fusion with myeloma cells was performed as described in Chapter 3 four days after immunization.

The strategy was to screen initially for hybridomas producing immunoglobulins using the dot immunobinding assay, expand positive lines in 24-well plates so that cells could be cryopreserved and more supernatants obtained for screening and, finally, to screen supernatants with the receptor binding assay.

5.3. Results

5.3.1. Receptor binding assay

The binding assay was developed by adapting assay methods reported in the literature (Nakamuta *et al.*, 1986) and binding assays for other peptides used in the Unit. Non-specific binding, determined by addition of excess of unlabelled RαCGRP (1μM), was of the order of 20%. The importance of co-incubation with BSA and the siliconization of both glassware and polypropylene material in reducing non-specific binding was clearly demonstrated (Figure 5.1). The combined use of siliconization and BSA was particularly effective for polypropylene material. These findings clearly have implications for experiments other than the binding assay. Efforts were made to use siliconized glass and polypropylene materials and to dissolve CGRP in BSA-containing solutions whenever possible.

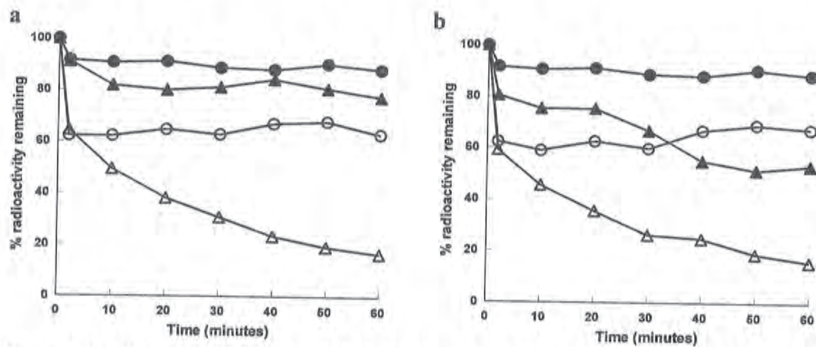

Figure 5.1: Effect of siliconization and BSA (0.5%) co-incubation on the non-specific binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to glass (a) and polypropylene (b) tubes over time. Non-specific binding is indicated by the loss of radioactivity in solution (single observations). Values from non-siliconized and siliconized materials (without BSA) are shown in open triangles and open circles respectively. The effect of BSA co-incubation on non-specific binding to non-siliconized and siliconized tubes is shown with closed triangles and closed circles respectively.

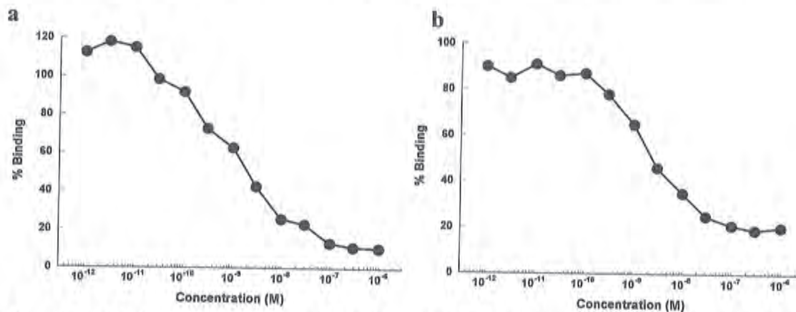

Figure 5.2: Displacement of the specific binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to rat liver (a) and bovine cerebellum (b) membrane preparations. Values are the mean of two observations.

Specific binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP was determined in rat liver and bovine cerebellum membrane preparations used for immunization (Figure 5.2). High concentrations of atrial natriuretic factor (0.1 μM), neuropeptide Y (2 μM), substance P (10 μM) and neurokinin A (2 μM) did not displace the specific binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP in the assay. Cell culture medium containing HAT, penicillin, streptomycin, amphotericin and HEPES-azide did not inhibit binding, thus permitting the use of the assay in screening hybridoma supernatants.

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Table 5.1: Coefficient of variation of binding assay results

Addition to incubation mixture (n=4)	Coefficient of variation (%)
Buffer (total binding)	3.0
Culture medium (20FD/HAT/Azide)	1.2
Normal mouse immunoglobulin (10 μg/ml)	2.1
RαCGRP (1 μM; non-specific binding)	6.5

Coefficient of variation was calculated by division of the standard deviation by the mean and multiplication by 100.

The estimated Kd and B_{max} were 1.7 nM and 400 fmol/mg protein respectively for the binding of CGRP to the rat liver membrane receptor. The one-site model fitted the data better than the two-site model as assessed by examination of residuals, coefficient of the variation of the parameters and the Schwartz information criterion.

Within the same assay, the coefficient of variation (standard deviation divided by mean) was evaluated for total binding and binding in the presence of culture medium, normal mouse immunoglobulin and excess RαCGRP (non-specific binding) using four replicates. The results are summarized in Table 5.1. The low coefficients of variation suggest that the assay is highly reproducible, at least within run.

5.3.2. *In vivo* immunization

Serum from mice immunized up to 5 times with different quantities of rat liver membrane did not inhibit the binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP in the binding assay. Figure 5.3 shows the results of one of the serum screening assays for anti-CGRP receptor antibodies. Similarly serum from rats and mice immunized with bovine cerebellum membrane were negative in the binding assay. Therefore, no fusions were attempted with spleens from these animals.

5.3.3. *In vitro* immunization

High fusion frequency was achieved with 100% of wells in 96-well plates producing hybridoma clones. All 186 supernatants screened in the dot immunobinding assay were positive for immunoglobulin secretion. The intensity of the dots were comparable to that due to normal mouse immunoglobulin at 10 μg/ml. Thus 186 cell lines were expanded in 24-well plates and cryopreserved. The hybridomas were allowed to overgrow prior to testing of the supernatants. Eighteen of the 186 supernatants tested in the binding assay apparently inhibited binding of 2-[^{125}I]-

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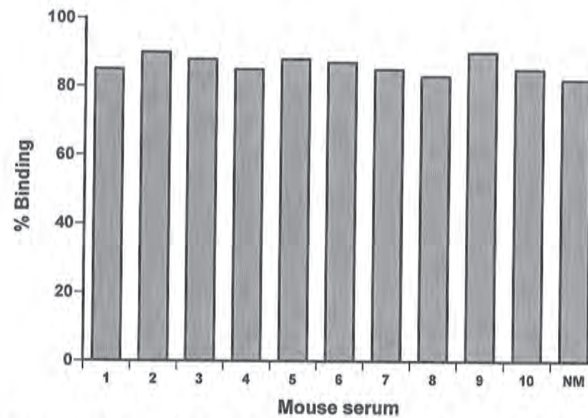

Figure 5.3: Effect of serum from mice (1 to 10) immunized 5 times with 0.7 to 1 mg of rat liver membrane preparation on the specific binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to rat liver membranes. Sera were collected 7 days after the last immunization and diluted 1 in 5 in the binding assay. Non-immune mouse serum (NM) was used as a control. Values are the mean of two observations.

iodohistidyl^{10}-HαCGRP by 30% or greater compared with normal mouse immunoglobulin. The hybridomas were therefore thawed, grown to confluence and their supernatants re-tested in the binding assay. However, inhibition of receptor binding could not be confirmed on re-screening of the supernatants (Figure 5.4).

5.4. Discussion

Since the desired use of anti-receptor MAbs in this project was as receptor antagonists, it was important to screen for inhibition of binding to the ligand binding site. A receptor binding assay was therefore more relevant than screening assays such as immunoprecipitation or Western blotting. A major problem in the use of the receptor binding assay was the lack of positive controls. This is a common problem in the development of screening assays for hybridoma production. In the case of the ELISA for CGRP (Chapter 3), it was possible to optimize the assay using serum from immunized animals. This was not possible for the binding assay since no positive sera was detected using the assay. Some confidence in the assay, however, was gained when the reproducibility of the assay was assessed. The coefficient of variation of binding was low in the presence of relevant additives, and non-specific binding was also reproducible.

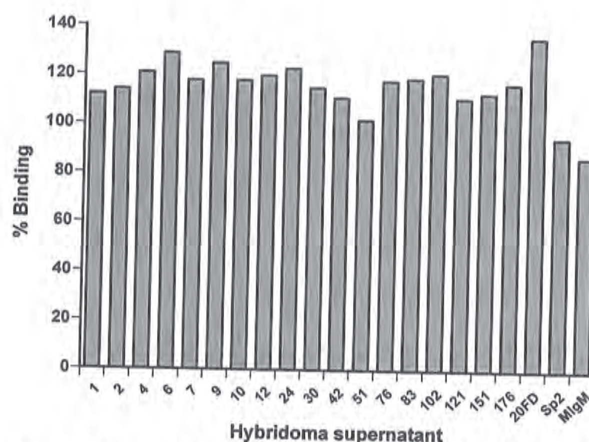

Figure 5.4: Screening of hybridoma supernatants from fusion following *in vitro* immunization with liver membrane preparation. The effect of supernatants on the specific binding of 2-[^{125}I]-iodohistidyl^{10}-HαCGRP to rat liver membranes is plotted. Controls were culture medium (20FD), Sp2 myeloma supernatant and non-specific mouse immunoglobulin (MIgG; 10 μg/ml). Values are the mean of two observations.

The variables investigated in the *in vivo* immunization protocols used in this study were: (1) amount of protein in the immunogen (1 to 20 mg), (2) tissue source of CGRP receptor, (3) species of receptor preparation and (4) species immunized. There are no definitive guidelines on immunization protocols for the generation of antibodies against large protein molecules. Microgram to 50 mg amounts of protein have been used by other workers (Goding, 1986). Assuming a molecular weight of 70 kDa for the CGRP receptor in rat liver (Chantry *et al.*, 1991; Stangl *et al.*, 1993), the amount of receptor (400 fmol/mg) in the crude immunogen used was only 2.8 x 10^{-5} times (0.003%) that of the membrane protein by weight. Nevertheless, MAbs against the insulin receptor have been generated by immunizing animals with whole IM-9 lymphocytes; the insulin receptor is estimated to represent less than 0.01% of a cell's membrane proteins (Roth & Morgan, 1985; Soos *et al.*, 1986). The cerebellum was used as an alternative source of receptors since it has been reported to be particularly rich in CGRP binding sites amongst tissues in the brain (Inagaki *et al.*, 1986; Henke *et al.*, 1987; Wimalawansa *et al.*, 1993). Bovine tissue was used since the potential sequence similarity of the mouse and rat receptor might prevent an effective immune response in the mouse. Due to known inter-animal variability in responses, relatively large numbers of mice and rats were immunized.

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The purity of the immunogen *per se* is irrelevant in the production of MAbs. All that is required is a specific screening assay that would identify the antibody-secreting hybridoma of interest. However, impure material may give weaker specific responses. It is possible that contaminants could affect the response against the antigen of interest. Immunodominant antigens would give strong immune responses even when present in trace amounts. The responsiveness of individual animals to the various components of crude immunogens is reported to be highly variable, involving suppression as well as induction (Galfre & Milstein, 1981). When crude immunogens are used, it is critical that a highly specific and sensitive screening assay is available for screening. Crude membranes were used in this study for immunization under the assumption that the binding assay would serve as an effective screen. Failure to detect any inhibition of receptor binding by serum from mice immunized with the crude membranes suggested that success was unlikely. Therefore, no fusions were attempted.

The probability of raising anti-receptor antibodies would be increased if purified receptor was used for immunization and for the screening assay. However, immunization with purified receptor is not necessarily the perfect solution. Purified receptor may lose the native conformation and sub-components of the receptor moiety. Furthermore, antibodies will be generated in order of frequency according to the immunogenic potency of domains of the isolated receptor; these may not overlap with the functionally important domains *in situ*. Thus, an auto-anti-idiotypic approach (Chapter 6) was pursued in preference to the more conventional approach of immunizing with purified receptor.

In vitro immunization was used following unsuccessful *in vivo* immunization. Although *in vitro* immunization for the production of MAbs was reported as long ago as 1978, the technique has not been widely adopted. Nevertheless, this technique offered a number of theoretical and practical advantages over *in vivo* immunization which were relevant to the present study. *In vivo* immunization could have failed because of antigen-specific non-responsiveness (tolerance) or selective responsiveness to one or a few components of the immunogen preparation (antigen hierarchy response). In contrast, there is good evidence that tolerance and suppression could be broken by *in vitro* immunization and it has been possible to obtain antibodies against self or highly conserved antigens (Reading, 1982). Antibodies have been raised against soluble, membrane-bound or whole cell antigens using this technique (Reading, 1986). Amounts of antigen as low as nanograms have been used successfully. *In vitro* immunization is also rapid and has been claimed to lead to higher fusion frequencies with more hybrids secreting antibodies of interest (Boss, 1984). IgM antibodies tend to be produced by *in vitro* immunization. The size of the

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