PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :		(11) International Publication Number:
C12N 15/62, 15/13, C07K 13/00 A61K 39/42, C12P 21/08	A1	WO 93/20210
		(43) International Publication Date: 14 October 1993 (14.10.93)

(21) International Application Number: PCT/GB93/00725

(22) International Filing Date: 6 April 1993 (06.04.93)

(30) Priority data:
9207479.8 6 April 1992 (06.04.92) GB

(71) Applicant *(for all designated States except US):* SCOTGEN LIMITED [GB/GB]; Queen's House, 2 Holly Road, Twickenham, Middlesex TW1 4EG (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only) :* TAYLOR, Geraldine [GB/GB]; Robingrove, Wallingford Road, Compton, Berkshire RG16 0PT (GB). STOTT, Edward, James [GB/GB]; New Henrietta Farm, Mophetts Lane, Down End, Chievely, Berkshire RG16 8TN (GB).

(74) Agent: VALENTINE, Jill, Barbara; SmithKline Beecham, Corporate Patents, Great Burgh, Yew Tree Bottom Road, Epsom, Surrey KT18 5XQ (GB).

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

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

(54) Title: ANTIBODIES FOR TREATMENT AND PREVENTION OF RESPIRATORY SYNCYTIAL VIRUS INFECTION

(57) Abstract

The present invention provides variable light chain and variable heavy chain sequences derived from bovine anti-RSV F protein monoclonal antibodies (mAbs), B4 and B13/B14, and CDR peptides therefrom, which may be employed in the design of fusion proteins (including altered antibodies) which are charaterized by the antigen binding specificity of these mAbs. Also described is a humanized antibody containing bovine variable chain sequences. Methods for producing and using these compositions, including pharmaceutical compositions are disclosed.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SK	Slovak Republic
CI	Côte d'Ivoire	KZ	Kazakhstan	SN	Senegal
CM	Cameroon	LI	Liechtenstein	SU	Soviet Union
CS	Czechoslovakia	LK	Sri Lanka	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	UA	Ukraine
DK	Denmark	MG	Madagascar	US	United States of America
ES	Spain	ML	Mali	VN	Viet Nam
FI	Finland	MN	Mongolia		

ANTIBODIES FOR TREATMENT AND PREVENTION OF RESPIRATORY SYNCYTIAL VIRUS INFECTION

Field of the Invention

This invention relates generally to the field of monoclonal, and recombinant, humanized antibodies, and specifically, to antibodies directed to epitopes on Respiratory Syncytial Virus.

Background of the Invention

Respiratory syncytial virus (RSV) is a pneumovirus of the family *Paramyxoviridae* and is the major cause of severe lower respiratory tract infections in children and calves during the first year of life [Kim *et al.*, Amer. J. Epidemiol., 98:216-225 (1973); Stott *et al.*, J. Hygiene, 85:257-270 (1980); McIntosh and Chanock, in B. N. Fields *et al.* (eds), Virology, Raven Press, New York (1990)]. Human and bovine strains of RSV are antigenically distinct, but closely related, and two subgroups (A and B) of both human and bovine strains have been identified [Lerch *et al.*, J. Virol., 63:833-840 (1989); Anderson *et al.*, J. Infect. Dis., 151:626-633 (1985)].

The use of anti-RSV antibodies for treatment of RSV in murine and bovine species has been suggested. However, the treatment of non-murine or non-bovine species is potentially limited by the immune response of these species to the foreign murine or bovine antibodies. For example, immune responses in humans against murine antibodies have been shown to both immunoglobulin constant and variable regions.

There remains a need in the art to identify specifically the protective epitopes on RSV proteins and the immune effector mechanisms that protect against infection, and to produce and characterize RSV antigens, epitopes and antibodies thereto for use in safe, effective RSV vaccines.

Summary of the Invention

The present invention provides a variety of anti-RSV antibodies, functional fragments thereof including CDRs. These antibodies and fragments are useful in the construction of fusion proteins, particularly chimeric and humanized antibodies, which are characterized by the binding specificity and/or neutralizing activity of an anti-RSV monoclonal antibody (mAb). Also provided is a novel humanized antibody containing bovine antibody variable sequences in association with human immunoglobulin framework and constant regions. Methods for producing these products, which further include therapeutic and pharmaceutical compositions for treating RSV are also disclosed.

Other aspects and advantages of the present invention are described in the following detailed description.

Brief Description of the Drawings

Fig. 1 is a graph illustrating the isolation of recombinant LF1/1298, which contains the RSV Long strain F glycoprotein cDNA with a single transversion C to A at nucleotide 1298, cloned in the polylinker of pGEM4. This recombinant permits expression of the F protein in selected host cells.

Fig. 2 is a diagram of the F glycoprotein primary structure denoting the hydrophobic regions (▌), the site of proteolytic processing (↓), the potential sites for N-glycosylation (⅄), the cysteine residues (●) and the amino acid residues which are changed in neutralization escape mutants (_). The locations of the trypsin fragments recognized by different mAbs are shown below the diagram.

Figs. 3A and 3B compare partial B4 and B13/B14 antibody variable light (VL) chain amino acid sequences [SEQ ID NOS: 1 and 2]. The B4 sequence is reported above the B13/B14 sequence to more readily illustrate comparison between the sequences. In the sequences, the symbol "-" represents a gap in the sequence introduced to improve the alignment

between the sequences. The CDRs are boxed. The underlined sequences correspond to the sequences of the polymerase chain reaction (PCR) oligonucleotide primers used in amplifying these antibody sequences.

Figs. 4A and 4B compare partial B4 and B13/B14 antibody variable heavy (VH) chain amino acid sequences [SEQ ID NOS: 3 and 4] with the B4 sequence reported above the B13/B14 sequence. The symbol "-", CDRs and PCR oligonucleotide primers sequences are defined and illustrated as in Figs. 3A and 3B.

Fig. 5 is a bar diagram showing the competitive binding of 10 anti-F bovine mAbs, labelled with ^{125}I, to the A2 strain of RSV in the presence of increasing amounts of unlabelled antibodies. "Neut" represents the ability of the mAb to neutralize the RSV in a plaque neutralization assay. "FI" refers to the ability of the antibody to inhibit fusion of multinucleated giant cells in an assay. "Protection" refers to whether the mAb was able to protect mice against RSV infection in an *in vivo* assay. Symbols: less than 10% (■), 11 to 80% (cross-hatched box), or greater than 80% (□) remaining bound at the highest amount of competing antibody tested.

Fig. 6 is a bar diagram showing the competitive binding of anti-F murine mAbs. "Neut", "FI", "Protection" and the symbols are defined as in Fig. 5.

Fig. 7 is a bar diagram showing the binding of anti-F mAbs to the RSV A2 strain and antibody-escape mutant RSVs. The antibodies were tested in an ELISA using the purified viruses indicated at the top of the figure to coat microtitre plates. Symbols: less than 20% (■), 20 to 80% (cross-hatched box), greater than 80% (□) of the absorbance values obtained with the A2 strain.

Fig. 8 is a bar diagram showing the binding of anti-F mAbs to RSV Long strain and antibody-escape mutant RSVs.

The antibodies were tested as described in Fig. 7. Symbols: less than 25% (open box), 25 to 50% (cross-hatched box), greater than 50% (■) of the absorbance values obtained with the Long strain.

Fig. 9 is a series of 8 bar diagrams showing the binding of mAb B4 to synthetic octomeric peptides, bound to polyethylene pins, where each amino acid in the sequence corresponding to amino acid #266 through 273 of the RSV F protein [SEQ ID NO: 19] has been replaced in turn with other amino acids (indicated on the abscissa). The sequence of amino acids beneath each bar diagram shows which amino acid has been replaced (indicated by a box around the letter). The antibody binding was tested in an ELISA and the black bars represent the absorbance values obtained with the native sequence of the peptide and the grey bars represent the absorbance obtained with the peptides containing the substituted amino acids.

Fig. 10 is a predicted humanized VH region sequence B4HuVH wherein bovine mAb B4 is the donor antibody [SEQ ID NO: 5]. CDRs are boxed. Underlined residues in the framework regions are murine residues which have been retained.

Fig. 11 is a predicted humanized constant heavy region sequence B4HuVK for use in constructing an altered antibody, wherein B4 is the donor antibody [SEQ ID NO: 6]. CDRs are boxed.

Figs. 12A and 12B provide a contiguous predicted humanized VH region sequence B13/B14HuVH [SEQ ID NO: 7] for use in constructing an altered antibody, wherein B13/B14 is the donor antibody. CDRs are boxed and retained murine residues are underlined.

Fig. 13 is a predicted humanized constant heavy region sequence B13/B14HuVK [SEQ ID NO: 8] wherein B13/B14 is the donor antibody. CDRs are boxed.

Figs. 14A and 14B provide a contiguous DNA sequence and corresponding amino acid sequence [SEQ ID NOS: 9 and 10] for the VH region of RSV19. CDRs are boxed. Underlined sequences correspond to the primers used.

Figs. 15A and 15B provide a contiguous DNA sequence and corresponding amino acid sequence of the RSV19 VL region [SEQ ID NOS: 11 and 12]. CDRs are boxed. Primers are underlined.

Fig. 16 shows the plasmid pHuRSV19VH comprising a human Ig VH region framework and CDRs from murine RSV19.

Fig. 17 shows the plasmid pHuRSV19VK comprising a human Ig VL framework and CDRs derived from RSV19.

Fig. 18 shows the derived Ig variable region amino acid sequences encoded by murine RSV19VH [SEQ ID NO: 13].

Fig. 19 shows the derived Ig variable region amino acid sequences encoded by pHuRSV19VH [SEQ ID NO: 14].

Fig. 20 shows the derived Ig variable region amino acid sequences encoded by pHuRSV19VHFNS [SEQ ID NO: 15].

Fig. 21 shows the derived Ig variable region amino acid sequences encoded by pHuRSV19VHNIK [SEQ ID NO: 16].

Fig. 22 shows the derived Ig variable region amino acid sequences encoded by pHuRSV19VK [SEQ ID NO: 17].

Fig. 23 is the DNA and amino acid encoding the HuVL framework 4, [SEQ ID NOS: 20 and 21] showing the potential splice site. The underlined bases were changed to provide the genuine J1 gene sequence [SEQ ID NO: 22].

Detailed Description of the Invention

I. Definitions.

As used herein, the term "first fusion partner" refers to a nucleic acid sequence encoding an amino acid sequence, which can be all or part of a heavy chain variable region, light chain variable region, CDR, functional fragment or analog thereof, having the antigen binding specificity of a selected antibody, preferably an anti-RSV antibody.

As used herein the term "second fusion partner" refers to another nucleotide sequence encoding a protein or peptide to which the first fusion partner is fused in frame or by means of an optional conventional linker sequence. Such second fusion partners may be heterologous to the first fusion partner. A second fusion partner may include a nucleic acid sequence encoding a second antibody region of interest, e.g., an appropriate human constant region or framework region.

The term "fusion molecule" refers to the product of a first fusion partner operatively linked to a second fusion partner. "Operative linkage" of the fusion partners is defined as an association which permits expression of the antigen specificity of the anti-RSV sequence (the first fusion partner) from the donor antibody as well as the desired characteristics of the second fusion partner. For example, a nucleic acid sequence encoding an amino acid linker may be optionally used, or linkage may be via fusion in frame to the second fusion partner.

The term "fusion protein" refers to the result of the expression of a fusion molecule. Such fusion proteins may be altered antibodies, e.g., chimeric antibodies, humanized antibodies, or any of the antibody regions identified herein fused to immunoglobulin or non-immunoglobulin proteins and the like.

As used herein, the term "donor antibody" refers to an antibody (polyclonal, monoclonal, or recombinant) which contributes the nucleic acid sequences of its naturally-occurring or modified variable light and/or heavy chains, CDRs thereof or other functional fragments or analogs thereof to a first fusion partner, so as to provide the fusion molecule and resulting expressed fusion protein with the antigenic specificity or neutralizing activity characteristic of the donor antibody. An example of a donor

antibody suitable for use in this invention is bovine mAb B4 or B13/14.

As used herein the term "acceptor antibody" refers to an antibody (polyclonal, monoclonal, or recombinant) heterologous to the donor antibody, but homologous to the patient (human or animal) to be treated, which contributes all or a substantial portion of the nucleic acid sequences encoding its variable heavy and/or light chain framework regions and/or its heavy and/or light chain constant regions to a second fusion partner. Preferably a human antibody is an acceptor antibody.

"CDRs" are defined as the complementarity determining region amino acid sequences of an antibody which are the hypervariable regions of immunoglobulin heavy and light chains which provide the majority of contact residues for the binding of the antibody to the antigen or epitope. CDRs of interest in this invention are derived from donor antibody variable heavy and light chain sequences, and include functional fragments and analogs of the naturally occurring CDRs, which fragments and analogs also share or retain the same antigen binding specificity and/or neutralizing ability as the donor antibody from which they were derived. See, e.g., the CDRs indicated by boxes in Figs. 3A, 3B, 4A, 4B, and 10 through 13. By 'sharing the antigen binding specificity or neutralizing ability' is meant, for example, that although mAb B13/B14 may be characterized by a certain level of antigen affinity, and a CDR encoded by a nucleic acid sequence of B13/B14 in an appropriate structural environment may have a lower affinity, it is expected that CDRs of B13/B14 in such environments will nevertheless recognize the same epitope(s) as B13/B14.

A "functional fragment" is a partial CDR sequence or partial heavy or light chain variable sequence which retains

the same antigen binding specificity and/or neutralizing ability as the antibody from which the fragment was derived.

An "analog" is an amino acid or peptide sequence modified by replacement of at least one amino acid, modification or chemical substitution of an amino acid, which modification permits the amino acid sequence to retain the biological characteristics, e.g., antigen specificity, of the unmodified sequence.

An "allelic variation or modification" is an alteration in the nucleic acid sequence encoding the amino acid or peptide sequences of the invention. Such variations or modifications may be due to degeneracies in the genetic code or may be deliberately engineered to provide desired characteristics. These variations or modifications may or may not result in alterations in any encoded amino acid sequence.

As used herein, an "altered antibody" describes a type of fusion protein, i.e., a synthetic antibody (e.g., a chimeric or humanized antibody) in which a portion of the light and/or heavy chain variable domains of a selected acceptor antibody are replaced by analogous parts of CDRs from one or more donor mAbs which have specificity for the selected epitope. These altered antibodies may also be characterized by minimal alteration of the nucleic acid sequences encoding the acceptor mAb light and/or heavy variable domain framework regions in order to retain donor mAb binding specificity. These antibodies can comprise immunoglobulin (Ig) constant regions and variable framework regions from the acceptor mAb, and one or more CDRs from the anti-RSV donor antibodies described herein.

A "chimeric antibody" refers to a type of altered antibody which contains naturally-occurring variable region light chain and heavy chains (both CDR and framework regions) derived from a non-human donor antibody in

association with light and heavy chain constant regions derived from a human acceptor antibody.

A "humanized antibody" refers to an altered antibody having its CDRs and/or other portions of its light and/or heavy variable domain framework regions derived from a non-human donor immunoglobulin, the remaining immunoglobulin-derived parts of the molecule being derived from one or more human immunoglobulins. Such antibodies can also include altered antibodies characterized by a humanized heavy chain associated with a donor or acceptor unmodified light chain or a chimeric light chain, or vice versa.

The term "effector agents" refers to non-protein carrier molecules to which the fusion proteins, and/or natural or synthetic light or heavy chain of the donor antibody or other fragments of the donor antibody may be associated by conventional means. Such non-protein carriers can include conventional carriers used in the diagnostic field, e.g., polystyrene or other plastic beads, or other non-protein substances useful in the medical field and safe for administration to humans and animals. Other effector agents may include a macrocycle, for chelating a heavy metal atom, or a toxin, such as ricin. Such effector agents are useful to increase the half-life of the anti-RSV derived amino acid sequences.

II. Anti-RSV Antibodies

For use in constructing the antibodies, fragments and fusion proteins of this invention, a non-human species may be employed to generate a desirable immunoglobulin upon presentment with the respiratory syncytial virus (RSV) F protein or a peptide epitope therefrom. Conventional hybridoma techniques are employed to provide a hybridoma cell line secreting a non-human mAb to the RSV peptide.

For example, several neutralizing, fusion-inhibiting (FI) and highly protective bovine and murine anti-RSV

monoclonal antibodies (mAbs) are provided by this invention. The production and characterization of the bovine antibodies capable of binding to the F protein, B13 and B14, and other suitable bovine mAbs designated herein as B4, B7 through B10, and murine mAbs, designated herein as 16 through 21, are described in detail in Examples 1 and 2, and in Figs. 5 and 6.

The resulting B13 and B14 anti-RSV antibodies are characterized by the ability to neutralize RSV in a plaque reduction neutralization test. Both B13 and B14 are potent in fusion inhibition assays and are protective in mice. Competition studies, together with studies of antibody-escape mutants, binding to F protein fragments and synthetic peptides suggest that the epitope recognized by mAbs B13 and B14 may be similar to, but not identical to, the epitope recognized by mAb RSV19 (also known as mAb 19 or RSMU19), the IgG_{2a} murine mAb specific for F protein amino acid 417-438 of and described in Example 11 below and in PCT patent application No. PCT/GB91/01554. After sequencing, B13 and B14 have been determined to be substantially identical are referred to as a single mAb called B13/B14 in certain instances. Where the mAbs were tested separately, reference is made to mAb B13 or B14.

The inventors have determined that a previously disclosed anti-RSV mAb, B4, is effective in protecting calves against infection with bovine RSV, as well as protecting mice against infection with human RSV. The ability of bovine mAb B4, administered to calves by the i.t. route, to protect against lower respiratory tract infection with RSV and against the development of pneumonic lesions, indicates that bovine mAbs are potentially effective prophylactic and therapeutic agents in the control of calf respiratory disease. B4 is also potent in fusion inhibition and virus neutralization assays (Examples 16 and 17).

These three bovine mAbs B4, B13, and B14 have been identified as desirable antibodies which may be altered for pharmaceutical or prophylactic use. However, this invention is not limited to the use of these three mAbs or their hypervariable sequences. These mAbs illustrate the products and methods of this invention; wherever in the following description the donor mAb is identified as B4, B13 or B14, it should be understood that any other appropriate anti-RSV neutralizing antibodies and corresponding anti-RSV CDRs may be substituted therefor.

It is anticipated that other antibodies, bovine as well as other species, which are developed against the RSV F protein epitope spanning amino acid 266 through 273 as well as other RSV epitopes of interest described herein, may be useful in compositions of this invention for treating RSV in mice, cattle and humans. Other anti-RSV antibodies may be developed by screening an antibody library including hybridoma products or libraries derived from any species immunoglobulin repertoires in a conventional competition assay, such as described in the examples below, with one or more bovine antibodies or RSV epitopes described herein. Particularly desirable for screening for additional antibodies are the neutralizing and protective mAbs, B4 and B13/B14.

Thus, the invention may provide an antibody, other than B4 or B13/14, which is capable of binding to the RSV peptide spanning amino acid #266 through #273, ITNDQKKL, of the F protein [SEQ ID NO: 19] or other relevant RSV epitopes. This antibody may be a mAb or an altered antibody, an analog of such antibodies, a Fab fragment thereof, or an $F(ab')_2$ fragment thereof. Such other mAbs generated against a desired RSV epitope and produced by conventional techniques, include without limitation, genes encoding murine mAbs, human mAbs, and combinatorial antibodies.

These anti-RSV antibodies may be useful in pharmaceutical and therapeutic compositions for treating RSV in humans and other animals.

III. Antibody Fragments

The anti-RSV antibodies described above may be useful as donors of desirable functional fragments, including the antibody light and heavy chain variable sequences and CDR peptides.

The present invention also includes the use of Fab fragments or $F(ab')_2$ fragments derived from mAbs directed against an epitope of RSV as agents protective *in vivo* against RSV infection and disease. A Fab fragment is the amino terminal half of the heavy chain and one light chain, and an $F(ab')_2$ fragment is the fragment formed by two Fab fragments bound by disulfide bonds. MAb B13/14 or other suitable RSV binding antibodies, provide a source of these fragments, which can be obtained by conventional means, e.g., cleavage of the mAb with the appropriate proteolytic enzymes, papain and/or pepsin.

These Fab and $F(ab')_2$ fragments are also useful themselves as therapeutic, prophylactic or diagnostic agents for RSV in humans and other animals, and are also useful as donors of variable chain sequences, CDRs and other functional fragments useful in this invention.

IV. RSV F Protein Epitopes of Interest

The above-described mAbs recognize certain protective epitopes on the fusion (F) protein of RSV which are recognized by a natural host of RSV. The nucleotide sequence of the F mRNA and the predicted protein sequence of the F protein [SEQ ID NO: 19] have been previously reported in Collins *et al.*, Proc. Nat'l. Acad. Sci. USA, 81:7683-7687 (1984). The amino acid numbering referred to herein is identical to the numbering in this latter reference. The inventors identified an eight amino acid sequence spanning

amino acids 266 through 273 of the F protein [SEQ ID NO: 19], as a suitable target for screening for neutralizing antibodies, as an antigen useful in therapeutic agents against RSV, and in particular, for producing monoclonal antibodies against RSV. Other epitopes of interest include epitopes at around amino acid #429 which are recognized by neutralizing antibodies, B13 and B14.

The regions of the F protein [SEQ ID NO: 19] which react with the neutralizing, fusion-inhibiting, and highly protective bovine and murine mAbs of the invention were mapped by competitive binding assays (Example 6); isolation and sequencing of antibody neutralization escape mutants (Examples 7 and 8); and synthesis of peptides with sequences containing the amino acids changed in the escape mutants and the assessment of the reactivity of these peptides with the mAbs (Examples 9-11).

Sequence analysis of the F protein [SEQ ID NO: 19] of the antibody-escape mutants permits identification of the amino acid residues important in the binding of the highly protective mAbs. Similarly, information on the binding of the protective mAbs to synthetic peptides permits the location of the epitopes that they recognize.

Briefly described, most of the bovine mAbs recognized epitopes similar to those recognized by the murine mAbs, and one of the protective antigenic areas (site B; site II of Fig. 8) is recognized both by cattle, which are a natural host for RSV, and mice. The epitope(s) recognized by the protective bovine mAbs B13 and B14 do not appear to be identical to any recognized by murine mAbs. B13 and B14 bind to a region of the F protein around amino acid 429. This epitope is similar, but distinct from the epitope recognized by murine mAb RSV19 (PCT patent application No. PCT/GB91/01554 and Example 11). For example, mAb B13/B14 does not recognize the peptides spanning F protein amino

acids #417-438, #417-432, and #422-438 all of SEQ ID NO: 19, which are recognized by mAb RSV19. A second antigenic site (area C, Figs 5 and 6; area IV, Fig. 8) on the F protein identified by neutralizing, protective murine mAbs RSV19 and 20 has been located towards the carboxy end of the F1 subunit and has also been described in the above-referenced PCT patent application.

The RSV epitope recognized by B4 is reproduced by the RSV F peptide at the amino acid sequence spanning #255-275 of SEQ ID NO: 19. The inventors have determined using the Geysen pepscan technique, that B4 recognizes an epitope spanning amino acid 266 to 273 of the F protein [SEQ ID NO: 19]. Altered antibodies directed against functional fragments or analogs of this epitope may be designed to elicit enhanced binding with the same antibody. mAbs which are directed against this epitope have been shown to protect mice and/or bovines from *in vivo* RSV infection.

Replacement of each amino acid in the sequence has enabled the discovery that enhanced binding of B4 occurs in mutant epitopes. Changes in amino acids 266, 279 and 273 did not affect binding of mAb B4. Changes in amino acid 267 resulted in reduced binding of mAb B4. Changes in amino acids 268, 269, and 272 resulted in total loss of binding. Substitution at amino acid 271 resulted in significantly enhanced binding (See Example 10).

The epitopes of these antibodies are useful in the screening and development of additional anti-RSV antibodies as described above. Knowledge of these epitopes enables one of skill in the art to define synthetic peptides and identify naturally-occurring peptides which would be suitable as vaccines against RSV and to produce mAbs useful in the treatment, therapeutic and/or prophylactic, of RSV infection in humans or other animals.

IV. Anti-RSV Nucleotide Sequences of Interest

The mAbs B4 and B13/14 or other anti-RSV murine, human and bovine, antibodies described herein may donate desirable nucleic acid sequences encoding variable heavy and/or light chain amino acid sequences and CDRs, functional fragments, and analogs thereof useful in the development of the first fusion partners, fusion molecules and resulting expressed fusion proteins according to this invention, including chimeric and humanized antibodies.

The present invention provides isolated naturally-occurring or synthetic variable light chain and variable heavy chain sequences derived from the anti-RSV antibodies, which are characterized by the antigen binding specificity of the donor antibody. Exemplary nucleotide sequences of interest include the heavy and light chain variable chain sequences of the mAbs B4, B13 and B14, as described below in the examples. Based on this variable region sequence data, B13 and B14 appear to be substantially identical.

The naturally occurring variable light chain of B13/14 is characterized by the amino acid sequence of Figs. 3A and 3B [SEQ ID NO: 2] labelled B13/B14VL. The naturally-occurring variable heavy chain of B13/14 is characterized by the amino acid sequence illustrated in Figs. 4A and 4B [SEQ ID NO: 4] labelled B13VH. These heavy and light chains are described in Example 18.

As described above for B13/B14, the amino acid sequences of the B4VL and VH chains are reported in Figs. 4A and 4B [SEQ ID NO: 4] and 3A and 3B [SEQ ID NO: 2], respectively, with the putative CDR peptides boxed. In both VH chains of B13/B14 and B4, the CDR3 peptides are unusually long, having 25 and 21 amino acids, respectively, in contrast to the vast majority of human and rodent CDR3s which have less than 20 amino acids.

The nucleic acid sequences encoding the variable heavy and/or light chains, CDRs or functional fragments thereof,

are used in unmodified form or are synthesized to introduce desirable modifications. These sequences may optionally contain restriction sites to facilitate their insertion or ligation to a second fusion partner, e.g., a suitable nucleic acid sequence encoding a suitable antibody framework region or the second fusion partners defined above.

Taking into account the degeneracy of the genetic code, various coding sequences may be constructed which encode the VH and VL chain amino acid sequences, and CDR sequences (e.g., Figs. 3A, 3B, 4A, 4B, and 10 through 13) and functional fragments and analogs thereof which share the antigen specificity of the donor antibody.

Thus, these isolated or synthetic nucleic acid sequences, or fragments thereof are first fusion partners, which, when operatively combined with a second fusion partner, can be used to produce the fusion molecules and the expressed fusion proteins, including altered antibodies of this invention. These nucleotide sequences are also useful for mutagenic insertion of specific changes within the nucleic acid sequences encoding the CDRs or framework regions, and for incorporation of the resulting modified or nucleic acid sequence into a vector for expression.

VI. Fusion Molecules, Fusion Proteins and Other Proteins of this Invention

A fusion molecule may contain as a first fusion partner a nucleotide sequence from an anti-RSV donor mAb, fragment or analog which sequence encodes an amino acid sequence for the naturally occurring or synthetic VH or VL chain sequences, a functional fragment or an analog thereof. When the first fusion partner is operatively linked to a second fusion partner, the resulting fusion molecule and expressed fusion protein is characterized by desirable therapeutic or prophylactic characteristics.

The fusion molecule, upon expression, can produce a fusion protein which is an altered antibody, a chimeric, humanized or partially humanized antibody. Altered antibodies directed against functional fragments or analogs of RSV may be designed to elicit enhanced binding in comparison to the donor antibody.

An exemplary fusion molecule may contain a synthetic VH and/or VL chain nucleotide sequence from the donor mAb encoding a peptide or protein having the antigen specificity of mAb B4 or B13/14. Still another desirable fusion molecule may contain a nucleotide sequence encoding the amino acid sequence containing at least one, and preferably all of the CDRs of the VH and/or VL chains of the bovine mAbs B4 or B13/14 or a functional fragment or analog thereof. The second fusion partners with which the anti-RSV sequences first fusion partners are associated in the fusion molecule are defined in detail above.

Where the second fusion partner is a nucleic acid sequence encoding a peptide, protein or fragment thereof heterologous to the nucleic acid sequence having anti-RSV antigen specificity, the resulting fusion molecule may express both anti-RSV antigen specificity and the characteristic of the second fusion partner. Typical characteristics of second fusion partners can be, e.g., a functional characteristic such as secretion from a recombinant host, or a therapeutic characteristic if the fusion partner is itself a therapeutic protein, or additional antigenic characteristics, if the second fusion partner has its own antigen specificity.

If the second fusion partner is derived from another antibody, e.g., any isotype or class of immunoglobulin framework or constant region (preferably human), or the like, the resulting fusion molecule of this invention provides, upon expression, an altered antibody. Thus a

fusion molecule which on expression produces an altered antibody can comprise a nucleotide sequence encoding a complete antibody molecule, having full length heavy and light chains, or any fragment thereof, such as the Fab or $F(ab')_2$ fragment, a light chain or heavy chain dimer, or any minimal recombinant fragment thereof such as an F_v or a single-chain antibody (SCA) or any other molecule with the same specificity as the donor mAb.

As one example, a fusion molecule which on expression produces an altered antibody may contain a nucleic acid sequence encoding an amino acid sequence having the antigen specificity of an anti-RSV antibody directed against the F protein amino acid sequence spanning amino acid #266 through #273 of SEQ ID NO: 19, ITNDQKKL and analogs thereof, operatively linked to a selected second fusion partner. Analogs of that epitope include those identified in the examples, such as SEQ ID NO: 56 when amino acid #266 is replaced with Ala, Cys, Asp, Glu, Phe, Gly, His, Leu, Pro, Gln, Arg, Ser, Thr, Val, Trp, and Tyr; or SEQ ID NO: 57 when amino acid #269 is replaced with Glu, Phe, Ile, Leu, Met, Arg, Ser, Thr, Val, Trp, and Tyr; or SEQ ID NO: 58 when amino acid #271 is replaced with Asp, Glu, Phe, Ile, Leu, Met, Arg, Ser, Thr, Val, Trp, Tyr and Gln; or SEQ ID NO: 59 when amino acid #273 is replaced with Ala, Cys, Asp and Glu. Desirably the source of the nucleic acid sequences is mAb B4.

Another fusion molecule which on expression produces an altered antibody may contain a nucleic acid sequence encoding the variable heavy chain sequence of Figs. 4A and 4B, a functional fragment or analog thereof, the variable light chain sequence of Figs. 3A and 3B, a functional fragment or analog thereof, or one or more B4 CDR peptides.

Another exemplary fusion molecule may contain a nucleic acid sequence encoding an amino acid sequence having the

antigen specificity of the anti-RSV antibody B13/B14, operatively linked to a selected second fusion partner. For example, the nucleic acid sequence may encode the VH chain sequence of Figs. 4A and 4B [SEQ ID NO: 4], a functional fragment or analog thereof, the VL chain sequence of Figs. 3A and 3B [SEQ ID NO: 2], a functional fragment or analog thereof, or one or more B13/B14 CDR peptides.

When the fusion protein which is obtained upon expression of the fusion molecule is an altered antibody, the antibody contains at least fragments of the VH and/or VL domains of an acceptor mAb which have been replaced by analogous parts of the variable light and/or heavy chains from one or more donor monoclonal antibodies. These altered antibodies can comprise immunoglobulin (Ig) constant regions and variable framework regions from one source, e.g., the acceptor antibody, and one or more CDRs from the donor antibody, e.g., the anti-RSV antibodies described herein.

An altered antibody may be further modified by changes in variable domain amino acids without necessarily affecting the specificity of the donor antibody. It is anticipated that heavy and light chain amino acids (e.g., as many as 25% thereof) may be substituted by other amino acids either in the variable domain frameworks or CDRs or both. Such altered antibodies may or may not also include minimal alteration of the acceptor mAb VH and/or VL domain framework region in order to retain donor mAb binding specificity.

In addition, these altered antibodies may also be characterized by minimal alteration, e.g., deletions, substitutions, or additions, of the acceptor mAb VL and/or VH domain framework region at the nucleic acid or amino acid levels may be made in order to retain donor antibody antigen binding specificity.

Such altered antibodies are designed to employ one or both of the VH or VL chains of a selected anti-RSV mAb

(optionally modified as described) or one or more of the above identified heavy and/or light chain CDR amino acid and encoding nucleic acid sequences. As another example, an altered antibody may be produced by expression of a fusion molecule containing a synthetic nucleic acid sequence encoding three CDRs of the VL chain region of the selected anti-RSV antibody or a functional fragment thereof in place of at least a part of the nucleic acid sequence encoding the VL region of an acceptor mAb, and a nucleic acid sequence encoding three CDRs of the VH chain region of a selected anti-RSV antibody, e.g., the bovine mAb B13/14, or a functional fragment thereof in place of at least a part of the nucleic acid sequence encoding the VH region of an acceptor mAb, such as a human antibody.

The altered antibodies can be directed against a specific protein epitope of RSV spanning amino acid #266-273 of SEQ ID NO: 19. It has been demonstrated that monoclonal antibodies which are directed against this epitope protect mice and/or bovines from *in vivo* RSV infection.

A suitable acceptor antibody, for supplying nucleic acid sequences as second fusion partners, may be a human (or other animal) antibody selected from a conventional database, e.g., the Kabat database, Los Alamos database, and Swiss Protein database, by homology to the nucleotide and amino acid sequences of the donor antibody. Desirably the acceptor antibody is selected from human IgG subtypes, such as IgG_1 or IgG_2, although other Ig types may also be employed, e.g., IgM and IgA. For example, a human antibody characterized by a homology to the framework regions of the donor antibody (on an amino acid basis) may be suitable to provide a heavy chain constant region and/or a heavy chain variable framework region for the insertion of the donor CDRs. A suitable acceptor antibody capable of donating light chain constant or variable framework regions may be

selected in a similar manner. It should be noted that the acceptor antibody heavy and light chains are not required to originate from the same acceptor antibody.

The acceptor antibody need not contribute only human immunoglobulin nucleotide sequences to the desired fusion molecule, and resulting expressed fusion protein. For instance a fusion molecule may be constructed in which a DNA sequence encoding part of a human immunoglobulin chain is fused to a DNA sequence encoding the amino acid sequence of a polypeptide effector or reporter molecule.

Similarly rather than a human immunoglobin, a bovine or another species' immunoglobulin may be used, e.g., to create a 'bovinized' or other species' altered antibody.

One example of a particularly desirable fusion protein is a humanized antibody. As used herein, the term "humanized antibody" refers to a molecule having its CDR regions and/or other portions of its VL and/or VH domain framework regions derived from an immunoglobulin from a non-human species, the remaining immunoglobulin-derived parts of the molecule being derived from a human immunoglobulin.

Suitably, in these humanized antibodies one, two or preferably three CDRs from the anti-RSV antibody VH and/or VL regions are inserted into the framework regions of a selected human antibody, replacing the native CDRs of that latter antibody. Preferably, the variable domains in both human heavy and light chains have been altered by one or more CDR replacements. However, it is possible to replace the CDRs only in the human heavy chain, using as the light chain an unmodified light chain from the bovine donor antibody. Alternatively, a compatible light chain may be selected from a human acceptor antibody as described above. A chimeric light chain may also be employed. The remainder of the altered antibody may be derived from any suitable acceptor human immunoglobulin.

Such altered antibodies according to this invention include a humanized antibody containing the framework regions of a human IgG subtype into which are inserted one or more of the CDR regions of a bovine antibody. Such a humanized antibody can contain the VH CDR peptides of the bovine mAb inserted into the heavy chain framework region of a human antibody and in association with the bovine light chain, or a bovine/human chimeric light chain. Such an exemplary humanized antibody is described in Example 20. Alternatively, such an altered antibody may be associated with a desired human light chain. Similarly, a chimeric antibody can contain the human heavy chain constant regions (preferably IgG) fused to the anti-RSV antibody, preferably bovine mAb, Fab regions. An exemplary chimeric antibody is described in Example 19.

The altered antibody preferably has the structure of a natural antibody or a fragment thereof and possesses the combination of properties required for effective prevention and treatment of a desired condition in animals or man depending on the antigenicity supplied by the donor antibody. The altered humanized antibody thus preferably has the structure of a natural human antibody or a fragment thereof, and possesses the combination of properties required for effective therapeutic use. Such "humanized" antibodies are effective in the prevention and treatment of RSV infection in an appropriate animal model for RSV infection in humans, and recognize a large variety of human clinical isolates of RSV. Because of their above-denoted characteristics, nucleic acids encoding the bovine mAbs B4, B13 and B14 provide desirable RSV epitope specific donor sequences (first fusion partners) for the construction of a fusion molecule, which upon expression produces a humanized antibody according to this invention which can elicit a minimal immune response in humans. See, for example, the

variable heavy and light chain sequences of Figs. 4A, 4B, 3A, 3B and 10 through 13.

A fusion protein which is a chimeric antibody, as defined above, differs from the humanized antibodies by providing the entire non-human donor antibody heavy chain and light chain variable regions, including framework regions in association with human (or other heterologous animal, where desired) IgG constant regions for both chains. It is anticipated that chimeric antibodies which retain additional non-human sequence in comparison to humanized antibodies of this invention, may also prove likely to elicit some desirable immune response in the human.

A preferred altered antibody is one directed against respiratory syncytial virus (RSV), preferably one specific for the fusion (F) protein of RSV. A particularly preferred antibody of this kind has all or a portion of the variable domain amino acid sequences of B4 or B13/B14 reported in Figs. 3A, 3B, 4A and 4B in its light and heavy chains, respectively. Figs. 10 through 13 illustrate predicted amino acid regions suitable for use in a "humanized" antibody and are described in the Brief Description of the Drawings section above. Additionally, an altered antibody of this invention may be characterized by the presence of one or more of the CDR peptides identified in the above figures.

As one example, an altered antibody may contain a the VL chain region of Fig. 11 or a functional fragment thereof in place of at least a part of the VL region of an acceptor mAb, and a VH chain region of Fig. 10 or a functional fragment thereof in place of at least a part of the VH region of an acceptor mAb, such as a human antibody. The resulting humanized antibody is characterized by the antigen binding specificity of mAb B4.

Still another preferred altered antibody may contain a VL chain region of Fig. 13 or a functional fragment thereof in place of at least a part of the VL region of an acceptor mAb, and the VH chain region of Figs. 12A and 12B or a functional fragment thereof in place of at least a part of the VH region of the acceptor mAb. The altered antibody is thus characterized by the antigen binding specificity of mAb B13/B14.

Alternatively, functional fragments of the variable sequences, such as the B4 CDR peptides, including:

SYSVS (amino acids 31-35 of SEQ ID NO: 3);

DASNGGIIYYNPALKS (amino acids 50-65 of SEQ ID NO: 3);

CSVGDSGSYACTXaaGXaaRKGEYVDA, wherein Xaa is any or no amino acid (amino acids 100-122 of SEQ ID NO: 3);

SGSS(S or D)NIG(R or I)(W or F)(G or A)V(N or G) (amino acids 22-34 of SEQ ID NO: 1);

YESSRPS (amino acids 50-56 of SEQ ID NO: 1);

ATGDYNIA (amino acids 89-96 of SEQ ID NO: 1);

ATGDYNIAV (amino acids 89-97 of SEQ ID NO: 1);

or the B13/B14 CDR peptides, including

GNTKRPS (amino acids 50-56 of SEQ ID NO: 2);

VCGESKSATPV (amino acids 89-99 of SEQ ID NO: 2);

DHNVG (amino acids 31-35 of SEQ ID NO: 4);

VIYKEGDKDYNPALKS (amino acids 50-65 of SEQ ID NO: 4);

LGCYPVEGVGYDCTYGLQHTTFXaaDA, wherein Xaa is any amino acid (amino acids 98-122 of SEQ ID NO: 4),

may be used in place of the larger variable region sequences of the figures.

Such altered antibodies can be effective in prevention and treatment of respiratory syncytial virus (RSV) infection in animals and man.

Another species of therapeutic, diagnostic or pharmaceutical protein of this invention is provided by the proteins or peptides encoded by the first fusion partner

which are associated with above-described effector agents.

One example of such a protein provides an anti-RSV amino acid sequence of the invention associated with a non-protein carrier molecule. Another example contains a desired anti-RSV sequence of the invention to which is attached an non-protein reporter molecule. Additionally, the entire fusion proteins described above may be associated with an effector agent.

The procedure of recombinant DNA technology may be used to produce a protein of the invention in which the F_c fragment or CH3 domain of a complete anti-RSV antibody molecule has been replaced by an enzyme or toxin molecule.

Another example of a protein of this invention contains an anti-RSV amino acid sequence of the invention with a macrocycle, for chelating a heavy metal atom, or a toxin, such as ricin, attached to it by a covalent bridging structure.

In general, fusion or linkage between the anti-RSV antibody nucleotide sequences sequences and the second fusion partner in the fusion molecule or association of the peptides encoded by the first fusion partner and an effector agent, may be by way of any suitable conventional means. Such conventional means can include conventional covalent or ionic bonds, protein fusions, or hetero-bifunctional cross-linkers, e.g., carbodiimide, glutaraldehyde, and the like. For association of the non-proteinaceous effector agents, conventional chemical linking agents may be used to fuse or join to the anti-RSV amino acid sequences.

Additionally, conventional inert linker sequences which simply provide for a desired amount of space between the first and second fusion partners in the fusion molecule may also be constructed into the molecule. The design of such linkers is well known. Such techniques and products are

known and readily described in conventional chemistry and biochemistry texts.

VII. Production of Fusion Proteins and Altered Antibodies

Preferably the fusion proteins and altered antibodies of the invention will be produced by recombinant DNA technology using genetic engineering techniques. The same or similar techniques may also be employed to generate other embodiments of this invention, e.g., to construct the chimeric or humanized antibodies, the synthetic light and heavy chains, the CDRs, and the nucleic acid sequences encoding them, as above mentioned.

Briefly described, a hybridoma producing the anti-RSV antibody, e.g., the bovine mAb B4, is conventionally cloned, and the cDNA of its heavy and light chain variable regions obtained by techniques known to one of skill in the art, e.g., the techniques described in Sambrook *et al.*, Molecular Cloning (A Laboratory Manual), 2nd edition, Cold Spring Harbor Laboratory (1989). The variable regions of the mAb B4 are obtained using PCR primers, and the CDRs identified using a known computer database, e.g, Kabat, for comparison to other antibodies.

Homologous framework regions of a heavy chain variable region from a human antibody are identified using the same databases, e.g., Kabat, and a human (or other desired animal) antibody having homology to the anti-RSV donor antibody is selected as the acceptor antibody. The sequences of synthetic VH regions containing the CDRs within the human antibody frameworks are defined in writing with optional nucleotide replacements in the framework regions for restriction sites. This plotted sequence is then synthesized by overlapping oligonucleotides, amplified by polymerase chain reaction (PCR), and corrected for errors. A suitable light chain variable framework region may be designed in a similar manner or selected from the donor or

acceptor antibodies. As stated above, the source of the light chain is not a limiting factor of this invention.

These synthetic VL and/or VH chain sequences and the CDRs of the anti-RSV mAbs and their encoding nucleic acid sequences, are employed in the construction of fusion proteins and altered antibodies, preferably humanized antibodies, of this invention, by the following process. By conventional techniques, a DNA sequence is obtained which encodes the non-human donor antibody (e.g., B4, B13/B14) VH or VL chain regions. In such a donor antibody at least the CDRs and those minimal portions of the acceptor mAb light and/or heavy variable domain framework region required in order to retain donor mAb binding specificity as well as the remaining immunoglobulin-derived parts of the antibody chain are derived from a human immunoglobulin.

A first conventional expression vector is produced by placing these sequences in operative association with conventional regulatory control sequences capable of controlling the replication and expression thereof in a host cell. Similarly, a second expression vector is produced having a DNA sequence which encodes the complementary antibody light or heavy chain, wherein at least the CDRs (and those minimal portions of the acceptor monoclonal antibody light and/or heavy variable domain framework region required in order to retain donor monoclonal antibody binding specificity) of the variable domain are derived from a non-human immunoglobulin. Preferably this second vector expression vector is identical to the first except in so far as the coding sequences and selectable markers are concerned so to ensure as far as possible that each polypeptide chain is equally expressed. Alternatively, a single vector of the invention may be used, the vector including the sequence encoding both light chain and heavy chain-derived

polypeptides. The DNA in the coding sequences for the light and heavy chains may comprise cDNA or genomic DNA or both.

A selected host cell is co-transfected by conventional techniques with both the first and second vectors to create the transfected host cell of the invention comprising both the recombinant or synthetic light and heavy chains. The transfected cell is then cultured by conventional techniques to produce the altered or humanized antibody of the invention. The humanized antibody which includes the association of both the recombinant heavy chain and/or light chain is screened from culture by appropriate assay, such as an ELISA assay. Similar conventional techniques may be employed to construct other fusion molecules of this invention.

Thus, the invention also includes a recombinant plasmid containing a fusion molecule, which upon expression produces an altered antibody of the invention. Such a vector is prepared by conventional techniques and suitably comprises the above described DNA sequences encoding the altered antibody and a suitable promoter operatively linked thereto. The invention includes a recombinant plasmid containing the coding sequence of a mAb generated against the F protein 266-273 epitope.

Suitable vectors for the cloning and subcloning steps employed in the methods and construction of the compositions of this invention may be selected by one of skill in the art. For example, the conventional pUC series of cloning vectors commercially available from supply houses, such as Amersham (Buckinghamshire, United Kingdom) or Pharmacia (Uppsala, Sweden), may be used. Additionally, any vector which is capable of replicating readily, has an abundance of cloning sites and marker genes, and is easily manipulated may be used for cloning. Thus, the selection of the cloning vector is not a limiting factor in this invention.

Similarly, the vectors employed for expression of the altered antibodies according to this invention may be selected by one of skill in the art from any conventional vector. The expression vectors also contain selected regulatory sequences which are in operative association with the DNA coding sequences of the immunoglobulin regions and capable of directing the replication and expression of heterologous DNA sequences in selected host cells, such as CMV promoters. These vectors contain the above described DNA sequences which code for the altered antibody or fusion protein. Alternatively, the vectors may incorporate the selected immunoglobulin sequences modified by the insertion of desirable restriction sites for ready manipulation.

The expression vectors may also be characterized by marker genes suitable for amplifying expression of the heterologous DNA sequences, e.g., the mammalian dihydrofolate reductase gene (DHFR) or neomycin resistance gene (neo^R). Other preferable vector sequences include a poly A signal sequence, such as from bovine growth hormone (BGH) and the betaglobin promoter sequence (betaglupro). The expression vectors useful herein may be synthesized by techniques well known to those skilled in this art.

The components of such vectors, e.g. replicons, selection genes, enhancers, promoters, and the like, may be obtained from natural sources or synthesized by known procedures for use in directing the expression of the recombinant DNA in a selected host. Other appropriate expression vectors of which numerous types are known in the art for mammalian, bacterial, insect, yeast, and fungal expression may also be selected for this purpose.

Such a vector is transfected into a mammalian cell or other suitable cell lines via conventional techniques. The present invention also encompasses a cell-line transfected with these described recombinant plasmids. The host cell

used to express the altered antibody or molecule is preferably a eukaryotic cell, most preferably a mammalian cell, such as a CHO cell or a myeloid cell. Other primate cells may be used as host cells, including human cells which enable the molecule to be modified with human glycosylation patterns. The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. See, e.g., Sambrook *et al.*, cited above.

Bacterial cells may prove useful as host cells suitable for the expression of the recombinant mAbs of the present invention. However, due to the tendency of proteins expressed in bacterial cells to be in an unfolded or improperly folded form or in a non-glycosylated form, any recombinant mAb produced in a bacterial cell would have to be screened for retention of antigen binding ability. For example, various strains of *E. coli*, *B. subtilis*, *Streptomyces*, other bacilli and the like may also be employed in this method.

Where desired, strains of yeast cells known to those skilled in the art are also available as host cells, as well as insect cells and viral expression systems. See, e.g. Miller *et al.*, Genetic Engineering, 8:277-298, Plenum Press (1986) and references cited therein.

The general methods by which the vectors of the invention may be constructed, transfection methods required to produce the host cells of the invention, and culture methods necessary to produce the fusion protein or altered antibody of the invention from such host cell are all conventional. Likewise, once produced, the fusion proteins or altered antibodies of the invention may be purified from the cell culture contents according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis

and the like. Such techniques are within the skill of the art and do not limit this invention.

Yet another method of expression of the humanized antibodies may utilize expression in a transgenic animal. For example, a method of expression of the humanized antibodies of the invention may be by expression in the milk of a female transgenic animal, such as described in U.S. Patent No. 4,873,316, which is incorporated herein by reference. For example, a DNA sequence for a selected humanized antibody of the invention may be operatively linked in an expression system to a milk-specific protein promoter, or any promoter sequence specifically activated in mammary tissue, through a DNA sequence coding for a signal peptide that permits secretion (and maturation, if necessary) of the desired protein in the mammary tissue. Suitable promoters and signal peptides may be readily selected by one of skill in the art.

The expression system is transgenically introduced into a host genome using standard transgenic techniques, for example by microinjection into the pronuclei of fertilized mammalian eggs. See, e.g. B. Hogan et al, "Manipulating The Mouse Embryo: A Laboratory Manual" Cold Spring Harbor Laboratory (1986); R.L. Brinster *et al*, Cell, 27:223-231 (1991).] As a result, one or more copies of the construct or system are incorporated into the genome of the transgenic mammal. The presence of the expression system permits the female of the mammalian species to produce and secrete the recombinant humanized antibody into its milk. This system allows for high level production of the humanized antibodies of the invention.

This latter method of expression may be particularly suitable for a humanized antibody containing bovine CDRs, and especially suitable for the oral administration of this

antibody to bovines as well as human infants. Other transgenic systems may also be employed.

Once expressed by the desired method, the altered antibody is then examined for *in vitro* activity by use of an appropriate assay. Presently, conventional enzyme linked immunosorbent assay (ELISA) formats are employed to assess qualitative and quantitative binding of the altered antibody to the RSV epitope (see Example 3). Other assays may also be used to verify efficacy prior to subsequent human clinical studies performed to evaluate the persistence of the altered antibody in the body despite the usual clearance mechanisms.

Example 11 below demonstrates the method of constructing the altered humanized antibodies derived from the murine monoclonal antibody RSV19, such as HuRSV19VH/VK and HuRSV19VHFNS/HuRSV19VK which are described in copending PCT patent application No. PCT/GB91/01554. Following the procedures described for humanized antibodies prepared from the murine RSV19, one of skill in the art may also construct humanized antibodies from the bovine antibodies, variable region sequences and CDR peptides described herein (see Examples 19 and 20). Altered antibodies can be produced with variable region frameworks potentially recognized as "self" by recipients of the altered antibody. Minor modifications to the variable region frameworks can be implemented to effect large increases in antigen binding without appreciable increased immunogenicity for the recipient. Such altered antibodies can effectively prevent and eradicate infection. Of particular interest for such humanized antibodies are the antibodies B4, B13 and B14 described herein. Such antibodies are useful in treating, therapeutically or prophylactically, a human against human RSV infection. Such antibodies may also be useful as diagnostic agents.

VII. Therapeutic/Prophylactic Uses of the Invention

This invention also relates to a method of treating, therapeutically or prophylactically, human RSV infection in a human in need thereof which comprises administering an effective, human RSV infection-treating dose of antibodies including one or more of the mAbs described herein, or fragments thereof, or an altered antibody as described herein, or another fusion protein, to such human. This invention also relates to a method of treating, therapeutically or prophylactically, bovine or other species' RSV infection in a bovine or other animal in need thereof which comprises administering an effective, RSV infection-treating dose of antibodies or molecules including one or more of the mAbs described herein, or fragments thereof, or an altered antibody as described herein, to such animal.

The fusion proteins, antibodies, altered antibodies or fragments thereof of this invention may also be used in conjunction with other antibodies, particularly human monoclonal antibodies reactive with other markers (epitopes) responsible for the disease against which the altered antibody of the invention is directed. Similarly monoclonal antibodies reactive with other markers (epitopes) responsible for the disease in a selected animal against which the antibody of the invention is directed may also be employed in veterinary compositions.

The fusion proteins or fragments thereof described by this invention may also be used as separately administered compositions given in conjunction with chemotherapeutic or immunosuppressive agents. The appropriate combination of agents to utilized can readily be determined by one of skill in the art using conventional techniques. As an example of one such combination, the altered antibody HuRSV19VHFNS/HuRSV19VK described in Example 11, or a

similarly altered B4, B13 or B14 antibody, may be given in conjunction with the antiviral agent ribavirin in order to facilitate the treatment of RSV infection in a human.

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

A variety of cytotoxic agents are suitable for use in immunotoxins, and may include, among others, radionuclides, chemotherapeutic drugs such as methotrexate, and cytotoxic proteins such as ribosomal inhibiting proteins (e.g., ricin).

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

The mode of administration of the therapeutic agent of the invention may be any suitable route which delivers the

agent to the host. The fusion proteins, antibodies, altered antibodies, and fragments thereof, and pharmaceutical compositions of the invention are particularly useful for parenteral administration, i.e., subcutaneously, intramuscularly or intravenously. The compositions for parenteral administration will commonly comprise a solution of the altered antibody of the invention or a cocktail thereof dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be employed, e.g., water, buffered water, 0.4% saline, 0.3% glycine, and the like. These solutions are sterile and generally free of particulate matter. These solutions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, etc. The concentration of the antibody of the invention in such pharmaceutical formulation can vary widely, i.e., from less than about 0.5%, usually at or at least about 1% to as much as 15 or 20% by weight and will be selected primarily based on fluid volumes, viscosities, etc., according to the particular mode of administration selected.

Thus, a pharmaceutical composition of the invention for intramuscular injection could be prepared to contain 1 mL sterile buffered water, and 50 mg of an altered antibody of the invention. Similarly, a pharmaceutical composition of the invention for intravenous infusion could be made up to contain 250 ml of sterile Ringer's solution, and 150 mg of an altered antibody of the invention. Actual methods for preparing parenterally administrable compositions are well known or will be apparent to those skilled in the art and are described in more detail in, for example, Remington's

Pharmaceutical Science, 15th ed., Mack Publishing Company, Easton, Pennsylvania.

To effectively prevent RSV infection in a human or other animal, one dose of approximately 1 mg/kg to approximately 20 mg/kg of a molecule or an antibody of this invention should be administered parenterally, preferably i.v. (intravenously) or i.m. (intramuscularly); or one dose of approximately 20 ug/kg to approximately 2 mg/kg of such antibody should be administered i.n. (intranasally). Preferably, such dose should be repeated every six (6) weeks starting at the beginning of the RSV season (October-November) until the end of the RSV season (March-April). Alternatively, at the beginning of the RSV season, one dose of approximately 5 mg/kg to approximately 100 mg/kg of an antibody of this invention should be administered i.v. or i.m. or one dose of approximately 0.5 mg/kg to approximately 10 mg/kg of such antibody should be administered i.n.

To effectively therapeutically treat RSV infection in a human or other animal, one dose of approximately 2 mg/kg to approximately 20 mg/kg of an antibody of this invention should be administered parenterally., preferably i.v. or i.m.; or approximately 200 ug/kg to approximately 2 mg/kg of such antibody should be administered i.n. Such dose may, if necessary, be repeated at appropriate time intervals until the RSV infection has been eradicated.

For example, in Example 16, the dose of B4 required to protect calves when administered by the i.t. route was 300 μg/kg body weight. This is 300 to 1000-fold less than the amount of human IgG, containing high titres of RSV-neutralizing antibody, required to reduce RSV infection in cotton-rats and owl monkeys, passively immunized by the i.t. route [Hemming and Prince, Reviews of Infectious Diseases, 12:S470-S475 (1990)]. It has been shown that about 10-fold less antibody is required to reduce virus shedding when

given by the topical route when compared with intravenous administration [Prince & Hemming, (1990)]. Therefore, it is estimated that a dose of approximately 3 mg/kg of mAb B4 given i.v. would be needed to significantly reduce RSV shedding in calves. This is similar to the amount of murine or "humanized" mAb required to protect mice against RSV infection [Tempest *et al.*, (1991)].

The compositions of the invention may also be administered by inhalation. By "inhalation" is meant intranasal and oral inhalation administration. Appropriate dosage forms for such administration, such as an aerosol formulation or a metered dose inhaler, may be prepared by conventional techniques. For example, to prepare a composition for administration by inhalation, for an aerosol container with a capacity of 15-20 ml: Mix 10 mg of an antibody of this invention with 0.2-0.2% of a lubricating agent, such as polysorbate 85 or oleic acid, and disperse such mixture in a propellant, such as freon, preferably in a combination of (1,2 dichlorotetrafluoroethane) and difluorochloromethane and put into an appropriate aerosol container adapted for either intranasal or oral inhalation administration. As a further example, for a composition for administration by inhalation, for an aerosol container with a capacity of 15-20 ml: Dissolve 10 mg of an antibody of this invention in ethanol (6-8 ml), add 0.1-0.2% of a lubricating agent, such as polysorbate 85 or oleic acid; and disperse such in a propellant, such as freon, preferably a combination of (1.2 dichlorotetrafluoroethane) and difluorochloromethane, and put into an appropriate aerosol container adapted for either intranasal or oral inhalation administration.

The antibodies, altered antibodies or fragments thereof described herein can be lyophilized for storage and reconstituted in a suitable carrier prior to use. This

technique has been shown to be effective with conventional immune globulins and art-known lyophilization and reconstitution techniques can be employed.

Depending on the intended result, the pharmaceutical composition of the invention can be administered for prophylactic and/or therapeutic treatments. In therapeutic application, compositions are administered to a patient already suffering from a disease, in an amount sufficient to cure or at least partially arrest the disease and its complications. In prophylactic applications, compositions containing the present antibodies or a cocktail thereof are administered to a patient not already in a disease state to enhance the patient's resistance.

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

It should also be noted that the fusion proteins, antibodies, variable sequences, CDR peptides and epitopes of this invention may be used for the design and synthesis of either peptide or non-peptide compounds (mimetics) which would be useful in the same therapy as the antibody. See, e.g., Saragovi *et al.*, Science, 253:792-795 (1991).

Natural RSV infections have also been reported in cattle, goats, sheep and chimpanzees. Thus, for example, utilizing the methodology described above, an appropriate mouse antibody could be "bovinized", and appropriate framework region residue alterations could be effected, if necessary, to restore specific binding affinity. Once the appropriate mouse antibody has been created one of skill in the art, using conventional dosage determination techniques,

can readily determine the appropriate dose levels and regimens required to effectively treat, prophylactically or therapeutically, RSV infection in the selected animal.

The following examples illustrate various aspects of this invention and are not to be construed as limiting the scope of this invention. All amino acids are identified by conventional three letter codes, single letter codes or by full name, unless otherwise indicated. All necessary restriction enzymes, plasmids, and other reagents and materials were obtained from commercial sources unless otherwise indicated. All general cloning ligation and other recombinant DNA methodology were as described in "Molecular Cloning, A Laboratory Manual." (1982), eds. T. Maniatis *et al.*, published by Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, ("Maniatis et al") or the second edition thereof (1989), eds. Sambrook *et al.*, by the same publisher ("Sambrook *et al.*").

The following examples illustrate the construction of exemplary altered antibodies and expression thereof in suitable vectors and host cells.

Example 1 - Preparation of Monoclonal Antibodies

Murine monoclonal antibodies 1 to 14 were described in Taylor *et al.*, (1984) cited above and incorporated herein by reference. Several of these antibodies were produced by immunizing BALB/c mice with bovine RSV, strain 127. The bovine RSV, strain 127 was isolated at Compton in 1973 from a calf with respiratory disease. Others of these antibodies were produced with cells persistently infected with the Long strain of human RSV [Fernie *et al.*, Proc. Soc. Exp. Biol. Medic., 167:83-86 (1981)]. Murine monoclonal antibodies 16 to 21 were produced from BALB/c mice inoculated intranasally (i.n.) on two occasions, three weeks apart, with $1X10^4$ pfu of the human RSV strain A2, grown in Hep-2 cells. Human RSV, strain A2, subtype A was isolated from a child in

Australia [Lewis *et al.*, Med. J. Austr., 48:932-933 (1961)]. After an interval of four months, the mice were inoculated intraperitoneally (i.p.) with $2X10^7$ pfu of the bovine 127 strain. Three days after the booster inoculation, the immune splenocytes were fused with NS-1 myeloma cells [American Type Culture Collection, designation TIB18]. The resulting hybridomas were screened for antibody to RSV by radioimmunoassay and immunofluorescence, cloned twice on soft agar and cloned cells inoculated into BALB/c mice to produce ascitic fluid as described in Taylor *et al.*, cited above.

Bovine monoclonal antibodies B1 to B6 were produced as described in Kennedy *et al.*, J. Gen. Virol., 69:3023-3032 (1988), incorporated herein by reference. At the same time, bovine mAbs B7 to B10, B13 and B14 were produced from bovine lymphocytes obtained from the same calf, but the lymphocytes were stored in liquid nitrogen and fused with NS1 cells at later dates. The resulting heterohybridomas were screened for bovine antibody to RSV by ELISA and in some cases also by the fusion inhibition assay [essentially as described in Kennedy *et al.* (1988), cited above], but adapted to microtitre plates. Cloned heterohybridoma cells secreting bovine mAbs to RSV were inoculated into pristane-primed nude BALB/c mice to produce ascitic fluid or grown in serum-free, DCCM-1 medium [Biological Industries, Ltd., Glasgow, U.K.]. Antibody was purified from cell culture supernatant using Protein G Sepharose 4 Fast Flow [Pharmacia LKB]. Bound antibody was eluted with 0.1M glycine, pH2.7, neutralized with 1M Tris-HCl (pH9.0) and dialyzed against phosphate buffered saline (PBS).

The antibody AK13A2 raised against the Long F protein was a generous gift of Dr. P. Coppe, Centre d'Economie Rurale, Marloie, Belgium. The mAbs 1BC11 (a negative control antibody), 47F and 49F have been described by

Garcia-Barreno *et al.*, J. Virol., 63:925-932 (1989). MAb 7C2 is described in Trudel *et al.*, (1987), cited above. The antibodies, 47F, AK13A2 and 49F, were purified from ascitic fluids by protein A-Sepharose chromatography and peroxidase labelled [Garcia-Barreno *et al.*, (1989), cited above]

All of the murine and bovine mAbs and hybridoma cell lines producing them described herein, except mAbs 1BC11, 47F, 49F, AK13A2 and 7C2, are available from the laboratory of Dr. Geraldine Taylor, Institute for Animal Health, Compton Laboratory, Compton, Near Newbury, Berks, RG16ONN, England.

Example 2 - Characterization of Monoclonal Antibodies

The specificities of the mAbs for F protein viral polypeptides were determined by radioimmune precipitation of (^{35}S)-methionine or (^{3}H)-glucosamine labelled RSV infected cell lysates performed as described by Kennedy *et al.*, J. Gen. Virol., 69:3023-3032 (1988). The specificity was confirmed by Western blots (immunoblotting) of non-reduced and reduced RSV-infected cell lysates performed as described by Taketa *et al.*, Electrophor., 6:492-497 (1985). The antigens used in immunoblotting were either Hep-2 cells infected with the human RSV A2 strain or calf kidney (CK) cells infected with the bovine RSV strain 127. Uninfected Hep-2 or CK cells were used as control antigens.

Only mAbs B1, B4, B5 [Kennedy *et al.*, cited above] and mAbs RSV19, B13 and B14 reacted with F protein denatured by boiling in dithiothreitol. Whereas mAbs B1, B4 and B5 recognized 46K and 22K fragments of denatured F1 protein in Western blotting, mAbs RSV19, B13 and B14 only recognized 46K fragments. The properties of mAbs 16 to 18, 20 and 21, RSV19, B7 to B10, B13 and B14, not previously described, for the assays described below are shown in Table 1 below. The properties of all the other mAbs in these assays are summarized in Figs. 5 and 6.

The ability of the mAbs to inhibit multinucleated giant cell formation was assayed in MA104 cells [American Type Culture Collection, Rockville, MD] 24 hours after infection with the RSV A2 strain [Kennedy *et al.*, cited above, incorporated herein by reference]. The results of this assay are reported in Table 1 under the column "Fusion Inhibition", and in Figs. 5 and 6 as "FI". A "-" indication means that the mAb did not inhibit the giant cell formation. A "+" indication means that the mAb inhibited the formation of multinucleated giant cells.

Four murine mAbs (11, 13, RSV19 and 20) and four bovine mAbs (B4, B5, B13 and B14) inhibited the formation of multinucleated giant cells.

The ability of mAbs to neutralize RSV was assayed by a plaque reduction neutralization test performed as described in Kennedy *et al.*, cited above. The results of this assay are reported in Table 1 under the column "Neut. titre", and in Figs. 5 and 6 as "Neut". In the figures, a "-" indication means no neutralization occurred; a "+" indication means that the antibody was neutralizing. Seven of the murine mAbs and four of the twelve bovine mAbs, i.e., B4, B5, B13 and B14, neutralized RSV.

The ability of mAbs to protect against RSV infection was studied in BALB/c mice as follows. 100 µl of ascitic fluid containing the mAbs was injected intra-peritoneally into groups of five mice. One day later, the mice were inoculated i.n. with 10^4 pfu of the A2 RSV strain. On day 5 of the infection, the mice were killed and their lungs assayed for RSV on secondary CK monolayers, according to the procedure described in Taylor *et al.*, Infect. Immun., 43:649-655 (1984).

The results of this assay are also reported in Table 1 under the column "Prot. of Mice", and in Figs. 5 and 6 under "Protection". In the figures, a "-" indication means that

the mAb did not protect the immunized mice against RSV infection. A "+" or "+++" indication means that the mAb did protect the animals to a lesser or greater degree, respectively. The eight mAbs that were effective in the fusion inhibition assay (i.e., murine mAbs 11, 13, RSV19 and 20, and bovine mAbs B4, B5, B13, and B14) were highly effective in preventing RSV infection in BALB/c mice when administered i.p. 24 hours prior to i.n. challenge with the A2 strain of RSV.

All antibodies, except murine mAbs 9 and 10 [Taylor *et al.*, (1984)] and bovine mAb B8, which were specific for bovine RSV, reacted with both the A2 and the human B subtype (8/60) [Common Cold Unit, Salisbury, England] strains of human RSV (both grown in Hep-2 cells) and with bovine strains of RSV [Taylor *et al.*, (1984), cited above; Kennedy *et al.*, cited above]. These results indicate that the epitopes recognized by the highly protective, fusion-inhibiting mAbs were highly conserved among strains of RSV.

Table 1

Properties of mAbs to the F protein of RSV

mAb	Ig class	ELISA titre (log_{10}) A2	8/60	BRSV	Neut. titre[1]	Fusion Inhib.	%C lysis[2]	Prot of mice[3]
16	G1	6.8	6.6	6.8	2.0	-	0	0.6
17	G2b	6.1	6.3	6.1	<1.0	-	59	0.6
18	G2a	7.0	6.8	6.2	3.4	-	43	1.6
RSV19	G2a	6.4	6.7	6.7	3.4	+	2	>3.8
20	G2a	>6.0	8.6	7.5	4.3	+	76	>3.8
21	G2a	8.9	7.4	6.8	<1.0	-	68	1.0
B7	G1	3.0	4.9	4.9	<1.0	-	6	0
B8	G1	<2.0	<2.0	4.0	<1.0	-	8	0.2
B9	G1	5.1	5.4	4.9	<1.0	-	2	0.4
B10	G1	5.1	5.4	6.0	<1.0	-	9	0.5
B13	G1	6.0	5.1	5.4	5.8	+	0	2.2
B14	G1	5.6	5.2	5.6	5.4	+	0	>2.2

[1] 50% plaque reduction titre expressed as log_{10}

2 Percent specific release with 1/100 dilution of mAb and rabbit complement

3 Log_{10} reduction in titre of RSV in the lungs of passively immunized mice compared with control animals

Example 3 - Enzyme Linked Immunosorbent Assay (ELISA)

RSV antigens to be tested in the ELISA were each prepared from Hep-2 cells, 3 to 4 days after infection. Cells were scraped into medium, spun at 500 g for 5 minutes, resuspended in distilled water and treated with 0.5% (w/v) NP40 detergent to yield a cell lysate. Control antigen was made in a similar way using uninfected Hep-2 cells.

The ELISA was performed as follows: Microtitre plates were coated with RSV or control antigen, diluted in distilled water, overnight at 37°C, incubated with blocking buffer consisting of 5% normal pig serum in PBS and 0.05% Tween 20 for 1 hour at room temperature and washed 5x with PBS/Tween. Serial 3-fold dilutions of mAb were added to the wells and the plates incubated for 1 hour at room temperature. After washing 5x with PBS/Tween, HRP-conjugated rabbit anti-bovine IgG (Sigma) diluted 1:4000 or HRP-conjugated goat anti-mouse IgG (Kpl, Maryland, USA) diluted 1:2000, was added to each well. After a final wash, bound conjugate was detected using the substrate 3,3',5,5'-tetramethylbenzidine (TMB, ICN, Immunobiologicals, Illinois).

Example 4 - Purification of the F glycoprotein and trypsin treatment

The F protein was purified by immunoaffinity chromatography from extracts of Hep-2 cells infected with the Long strain [See, Walsh *et al.*, J. Gen. Virol., 66:409-415 (1985); and Garcia-Barreno *et al.*, (1989), cited above]. Several aliquots of the purified protein (15 µg each) were incubated and digested with either 2µg, 4µg, 8µg or 16µg of trypsin for 4 hours at 37°C. The digestion was terminated

by the addition of electrophoresis sample buffer [Studier, J. Mol. Biol., 79:237-248 (1972)] and boiling of the samples for 3 minutes. SDS-PAGE separated the samples. The samples were electrotransferred to Immobilon membranes.

Example 5 - Epitopes on F protein.

As an initial step to locate the epitopes recognized by the antibodies, AK13A2, 47F, 7C2, RSV19, 20 and B4, used in the selection of mutant viruses in Example 7, below, the binding of mAbs to the trypsin fragments of purified F protein were tested by Western blot [Towbin *et al.*, Proc. Nat'l. Acad. Sci. USA, 76:4350-4354 (1979)]. The protein fragments were either stained with Coomassie blue or developed with antibodies AK13A2 or 19.

Increasing amounts of trypsin generated smaller fragments of the F1 subunit which were stained by Coomassie blue. Four F1 fragments of 30, 20.5, 19 and 15 K were recognized by mAb AK13A2. The 20.5 and 19 K fragments had been mapped previously [Lopez *et al.*, J. Gen. Virol., 64:927-930 (1990)] at the NH_2 terminal end of the F1 subunit. Antibodies B4, 47F, and 7C2 recognized the same set of fragments as AK13A2. Thus, the epitope recognized by these mAbs can be ascribed to amino acid sequences included within the NH_2 terminal third of the F1 subunit.

In contrast, RSV19 reacts with a different set of F1 fragments. Only large size fragments (26 and 22 K), generated with low trypsin amounts, reacted with RSV19 (mAb 20 reacted less efficiently with the same set of fragments). Thus, epitopes 19 and 20 contain trypsin sensitive amino acid sequences which were tentatively located within the carboxy terminal two thirds of the F1 subunit (Fig. 2), outside the region covered by the fragments recognized by antibody B4. The NH_2 terminal end of the 26 and 22 K fragments could not be determined by direct protein sequencing because their low yield after trypsin treatment.

The diagram of Fig. 2 shows the F glycoprotein primary structure denoting the hydrophobic regions, the site of proteolytic processing, the potential sites for N-glycosylation, the cysteine residues and the amino acid residues which are changed in the neutralization escape mutants (see Table 3A-3C below). The locations of the trypsin fragments recognized by different mAbs are shown below Fig. 2.

The region on the F protein recognized by mAbs B13 and B14 were identified by examining their binding to F protein fragments, expressed in *E. coli*. Recombinant C protein (rC, $F_{377-524}$) of SEQ ID NO: 19 and recombinant D protein (rD, $F_{371-550}$) of SEQ ID NO: 19 were used as antigens in ELISA as described in Example 3. These peptide sequences of the F protein were fused to an influenza non-structural protein fragment containing amino acids 1 - 81 of the influenza nonstructural protein 1 (NS-1) at their amino termini, inserted into an expression plasmid and expressed in *E. coli*. The production of these fusion peptides involved conventional procedures. MAbs B13, B14 and RSV19, but not B4, bound to these protein fragments. Table 2 below illustrates the binding of anti-F mAbs to recombinant F protein fragments in ELISA. These findings suggest that the region of the F protein recognized by B13 and B14 is similar to that recognized by RSV19 and is within the carboxy terminal third of the F1 subunit.

Table 2

mAb	rC	rD	RSV
B13	5.7*	>3.0	5.6
B14	6.2	>3.0	5.6
RSV19	5.3	>3.0	7.4
B4	<1.5	<1.5	5.9

*Log_{10} titer by ELISA, rC at 2 µg/well and rD at 1 µg/well used as antigens.

Example 6 - Identification of antigenic areas in the F protein.

The epitope specificity of the 16 murine and 12 bovine mAbs to the F protein were analyzed by a competitive binding assay using purified and labelled mAbs. In summary, these competitive binding assays identified twelve antigenic sites on the F protein, many of which overlapped extensively. Three epitopic sites were recognized by both the neutralizing mAbs and the highly protective FI mAbs, e.g., B4, B5, B13 and B14. These findings are similar to those of others who have identified three antigenic sites on the F protein involved in neutralization using murine mAbs, two of which are involved in FI activity [Walsh *et al.*, J. Gen. Virol., 68:505-513 (1986) and Beeler *et al.*, J. Virol., 63:2941-2950 (1989)]. These findings suggest that virus neutralization can occur by a mechanism independent of preventing the fusion of the virus with the cell membrane, e.g. steric hindrance of virus attachment.

A. Purification and labelling of mAbs

The IgG from ascitic fluid containing either murine or bovine mAbs was purified on either Protein A-sepharose or Protein G-sepharose Fast-Flow [Pharmacia LKB]. The ascitic fluids were mixed with equal volumes of 0.1M phosphate buffer (pH 8), and passed through a Protein A-sepharose column with the same buffer. Bound antibodies were eluted with 0.1 M citrate buffer (pH 6.0 to 3.5). Fractions eluted with low pH buffers were collected in 1M Tris-HCl (pH 9.0). IgG from tissue culture supernatants was purified on Protein G-sepharose Fast-Flow and eluted with 0.1M glycine as described above. Purified IgG was dialyzed

against PBS and labelled with ^{125}I using chloramine T or coupled to biotin.

B. Competitive Binding Assay

A dilution of ^{125}I-labelled, or biotinylated, mAbs, determined to give approximately 10,000 cpm at 90% of maximum binding to RSV antigen in a radioimmunoassay, was allowed to react with RSV antigen in the presence of increasing amounts of various unlabelled mAbs to the F protein. For mAbs B13 and B14, a dilution of biotinylated mAbs, determined to give 90% of maximum binding to RSV-infected cell lysate, was allowed to bind to RSV antigen in the presence of increasing amounts of unlabelled antibody. An unlabelled mAb to the nucleoprotein (N) was used as a control.

The results of this assay are illustrated in Figs. 5 and 6. Some mAbs inhibited the binding in a dose-dependant manner; other mAbs, however, did not interfere with the binding of the test antibody. Unlabelled mAb to the N protein of RSV did not interfere with the binding of any of the mAbs to the F protein. These studies identified groups of mAbs that competed for simultaneous binding to antigen. Epitopes recognized by competing mAbs were considered to be operationally within the same antigenic area of the F protein. The competition profiles of the mAbs overlapped extensively (Figs. 5 and 6).

Therefore the clustering of epitopes was done on the basis of partial similarities and was analyzed using the Leucocyte typing database IV [Gilks, "Leukocyte typing database IV" Oxford University Press (1990)]. These studies showed that the 16 murine mAbs recognized 7 antigenic areas on the F protein [SEQ ID NO: 19] (Fig. 6). mAbs 2 and 5 competed with nearly all of the other murine mAbs. Two highly protective mAbs, 11 and 13, appeared to recognize the same epitope (site B), whereas two other mAbs, RSV19 and 20,

which were also highly protective, were similar to each other but different from mAbs 11 and 13, and mapped to site C.

Most of the 12 bovine mAbs mapped to the same sites as the murine mAbs (Figs. 5 and 6). Murine mAbs 2 and 5 competed with only 4 of the bovine mAbs (B2, B3, B4 and B6). A neutralizing murine mAb, 14, which mapped to site G in competition studies with the murine mAbs (Fig. 6), showed a competition profile that was similar to the bovine mAbs B2, B3 and B6 and was therefore placed in group H (Fig. 5). The binding of bovine mAbs B1 and B7 were not inhibited by any of the murine mAbs and, indeed, B7 appeared to recognize a distinct epitope. The epitopes recognized by 2 highly protective bovine mAbs B4 and B5, were similar to each other and to 2 of the highly protective murine mAbs, 11 and 13. mAb 18, which is partially protective in mice, and B10, which is not protective, also map in this area (site B).

The binding of the protective bovine mAbs B13 and B14 was inhibited to various degrees by protective murine mAbs, RSV19 and 20, and the protective bovine mAbs B4 and B5. However, the competition profiles of mAbs B13 and B14 were different from those antibodies mapping to sites B and C, suggesting that they recognize a different site on the F protein.

Taken together, the murine and bovine mAbs recognized 12 antigenic areas, most of which overlapped extensively. The highly protective, fusion-inhibiting (FI), neutralizing mAbs mapped to 2 or possibly 3 sites (areas B, C and L in Fig. 5) on the F protein. mAbs that neutralized virus but did not have FI activity mapped to 3 sites (areas B, D and H). However, mAbs which have neither neutralizing nor FI activity also map to these sites.

Example 7 - Antibody escape mutants

The pattern of reactivity of antibody-escape mutants with the mAbs confirmed the mapping of the protective epitopes deduced from competitive binding assays. In summary, two regions of the F primary structure were identified where the epitopes recognized by neutralizing mAbs were located. The first region mapped within the trypsin resistant amino terminal third of the large F1 subunit. This region contained the overlapping epitopes recognized by mAbs 47F, 49F, 7C2, AK13A2, 11 and B4, included in antigenic area II (Fig. 8) and area B (Figs. 5 and 7). Antigenic areas II and B are identical. Most amino acid changes found in mutants selected with these antibodies were clustered around amino acids 262-272 of SEQ ID NO: 19. Since these antibodies reacted in Western blots with proteolytic fragments of the F1 subunit, it was originally thought that they recognized "linear" epitopes determined by sequences of consecutive amino acids.

However, it seems that some conformations are needed for the integrity of certain epitopes, because only some of them were reproduced by synthetic peptides and amino acid substitutions located at a distant site influenced the binding of some antibodies. For example, the change at amino acid 216 (Asn to Asp), in the mutant 4/4 that conferred resistance to mAb AK13A2, also eliminated the reactivity with antibodies 7C2 and B4 (resistance to which are also conferred by selected changes at position 272). The change at 216 is distantly located from the peptide 255-275, which faithfully reproduced the epitope B4. Consequently, some long range effect of amino acid 216 in the structure adopted by epitope B4 in the F1 subunit is likely to occur.

Although the competition profiles in Figs. 5 and 6 of the mAbs overlapped extensively, protective mAbs 11, 13, B4

and B5 mapped to the same area (site B in Figs. 5 and 7; site II in Fig. 8) and mutants resistant to these antibodies failed to bind only those mAbs recognizing site B. MAbs 7C2 and 47F also mapped to this area. Although there was inhibition of binding of mAbs RSV19 and 20 to RSV antigen by antibodies mapping to site B (site II), and vice versa, cluster analysis suggested that they recognized a different site (site C). This was confirmed by the finding that mutants selected for resistance to mAbs RSV19 and 20 still reacted with mAbs recognizing site B. Similarly, the binding of mAbs B13 and B14 to RSV was inhibited by mAbs mapping to sites B (II) and C. However, B13 and B14 appeared to map to a different region (site L) and this was confirmed by the observation that B13 and B14 bound to all the mutants selected with mAbs mapping to sites B (II) and C.

The neutralization of RSV by the mAbs used to select the escape mutants is theorized to be related to their capacity to inhibit the membrane fusion of the F glycoprotein [Garcia-Barreno *et al.* (1989), cited above; Taylor *et al.*, (1984), cited above]. By analogy with other paramyxoviruses [see, e.g., Morrison, Virus Res., 10:113-136 (1988)], it is assumed that the fusion activity of RSV depends upon the proteolytic processing of the F protein precursor. This modification generates the new NH_2-terminal end of the F1 subunit, proposed to interact with lipid membranes through a short hydrophobic peptide. The antigenic areas of the F glycoprotein identified herein are distantly located from the fusion peptide in a linear map; however, it is possible that other regions of the F protein influence the activity of the fusion peptide. In this respect, mutants altered in the fusogenic activity of the influenza virus hemagglutinin [Daniels *et al.*, Cell, 40:431-

439 (1985)] have been mapped outside the fusion peptide of the HA2 subunit.

The escape mutants were developed and evaluated as follows. The wild type and neutralization escape mutant viruses were grown in Hep-2 cells and purified from culture supernatants as previously described [Garcia-Barreno *et al.*, Virus Res., 9:307-322 (1988)]. The Long and A2 strains of human RSV were plaque purified before being used to select viruses which escaped neutralization (mAb resistant mutants) by mAbs 47F, AK13A2, 7C2, 11, B4, B5, 19 or 20, and other mAbs directed against the F glycoprotein as described herein. These were selected in two different ways:

A. A2 Strain Escape Mutants

Antibody escape mutant viruses of the RSV A2 strain, which are refractory to neutralization by one of the highly protective mAbs, 11, B4, B5, RSV19 and 20, were produced using plaque reduction techniques. For mAbs RSV19, 20, B4 and B5, confluent monolayers of primary CK cells were infected with the A2 strain at a multiplicity of infection (MOI) of 0.2. Starting 24 hours after infection and continuing for 3 to 5 days, the culture medium was replaced daily with fresh medium containing 10% mAb. Virus was harvested when a cytopathic effect (CPE) was apparent.

Virus prepared in this way was mixed with an equal volume of the mAb under test for 1 hour at room temperature and inoculated onto CK monolayers in 35 mm multi-well plates [Nunc]. After 1 hour incubation at 37°C, the plates were overlaid with medium containing 0.25% agarose incorporating a 1 in 10 dilution of the same mAb. Plates were then incubated at 37°C in 5% CO_2 in air for 7 days before adding the vital stain, 0.3% 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide in 0.15M NaCl, to the overlay to visualize virus plaques.

Putative mutant viruses were removed from plates in agar plugs containing single plaques, diluted in medium, mixed with an equal volume of mAb and inoculated onto CK monolayers as before. Mutant viruses were plaque picked again and inoculated into tubes containing coverslips of calf testes cells or Hep-2 cells. After 4 to 6 days incubation, the coverslips were removed and stained with the mAb under test followed by FITC-labelled rabbit anti-mouse IgG [Sigma] or FITC-labelled rabbit anti-bovine IgG [Sigma]. A polyclonal bovine antibody to RSV followed by FITC-labelled rabbit anti-bovine IgG was used as a positive control. RSVs that failed to react by immunofluorescence to the mAb under test were classed as mutants and were used to produce antigen for the ELISA described in Example 3 above.

Mutant viruses refractory to mAb 11 were selected essentially as described above, but without prior culture of the virus in cells containing 10% mAb in the supernatant.

Five mutant viruses were independently isolated from the A2 strain of RSV after plaquing in the presence of mAb 11. Eight mutants were independently isolated after culture in the presence of RSV19, 3 mutants after culture in the presence on mAb 20, 6 after culture in the presence of B5 and 10 after culture in the presence of B4.

After cloning, each escape mutant was used as antigen in the ELISA described in Example 3 to test its reactivity with a panel of anti-F mAbs (Figs. 7 and 8).

Mutant viruses selected for resistance to mAb 11 lost the capacity to bind not only mAb 11 but also mAbs 13, B4, and B5, and had reduced binding to mAb 7C2, when compared with the parent A2 strain of RSV (Fig. 7). All mutant viruses selected for resistance to either B4 or B5 lost the capacity to bind not only B4 or B5 but also 11 and 13. However, some mutants selected with B4 (e.g. C4947/5)

still bound to B5 but at a greatly reduced level when compared with the A2 strain.

As seen for mutants selected for resistance to mAb 11, some B4 and B5 mutants showed reduced binding to 7C2, however others failed to react with 7C2 (e.g. C4947/5). In contrast to mutants selected with mAb 11, some mutants selected with B4 or B5 still reacted with mAb 18 (e.g. C4947/5, 61:19, 61:16, 63:27 and C5014/7). B4 and B5 mutants showed either the same, reduced or no binding to mAb B10 when compared with the parent A2 strain.

All mutant viruses selected with mAbs RSV19 or 20 failed to react only with mAbs RSV19 and 20 (Fig. 7). The binding of mAbs B13 and B14 (Fig. 7) and all other mAbs, described in Fig. 5, to all the mutants was the same as to the parent A2 strain of RSV, i.e., the mutant viruses retained the binding of mAbs from other antigenic areas.

B. Long Strain Escape Mutants

Escape mutants of the Long strain were isolated as previously described [Garcia-Barreno *et al.* (1989), cited above]. Briefly, virus stocks were enriched in mutant viruses by 4-5 consecutive passages in the presence of the selecting antibody, 47F, 7C2 or AK13A2.

Then, the viruses were plaque purified in antibody containing agar plates. Several viral plaques were isolated, and their resistance to antibody neutralization was confirmed. A single plaque originated from each aliquot of the virus stock was chosen for further analysis.

The epitopes recognized by the mAbs B4, 7C2 and AK13A2 were included in antigenic area II previously described by Garcia-Barreno *et al.* (1989), cited above, based solely on their reactivity with antibody-escape mutants. Similarly the epitopes recognized by mAbs RSV19 and 20 were included in antigenic area IV by the same criteria (Fig. 8).

The mutations selected in the escape viruses affected only epitopes from the antigenic area which included the selective antibody. For instance, mutant 4/4 did not react with any of the antibodies grouped in area II, whereas other mutants selected with the same antibody (11/3, 4, 5 and 7) reacted with mAbs 7C2 and B4 but not with 47F, 49F or AK13A2. Similarly, the mutants selected with mAbs 19 or 20 did not bind the antibodies grouped in the antigenic area IV, except mAb 52F. However, in all cases the mutant viruses retained the binding of mAbs from other antigenic areas.

The different reactivities of the antibodies from antigenic area II with the escape mutants indicated that their epitopes might overlap on the F molecule but were not identical. To further differentiate these epitopes, it was determined whether or not the corresponding mAbs would compete for simultaneous binding to the virus using a peroxidase labelled antibody in the ELISA of Example 3 mixed with increasing, non-saturating amounts of each unlabeled antibody previously titrated against the Long strain.

The capacity of an anti-idiotype rabbit antiserum raised against mAb 47F to inhibit the binding of mAbs to RSV was also tested by ELISA [Palomo *et al.*, J. Virol., 64:4199-4206 (1990)].

The results obtained indicated extensive competition between these antibodies for virus binding; however, antibody AK13A2 inhibited the binding of mAbs 47F and 49F in a non-reciprocal manner. In addition, the anti-idiotype antiserum inhibited only the virus binding of mAbs 47F and 49F but not AK13A2, 7C2 and B4.

Thus, the epitopes included in antigenic area II could be distinguished by at least one of the following criteria: i) the reactivity of mAbs with escape mutants, ii) the competition of mAbs for virus binding and iii) the

inhibition of virus binding by an anti-Id antiserum. Only the epitopes 47F and 49F could not be distinguished by the above criteria, but they differ in both neutralizing capacity and susceptibility to denaturing agents.

Example 8 - Location of amino acid changes selected in neutralization escape mutants

In order to identify the amino acid changes selected in the escape mutants, the F protein mRNAs obtained from cells infected with the different viruses were sequenced as follows. Hep-2 cells were infected with the different viruses and harvested 30-40 hours post-infection, when cytopathic effect was evident by the formation of syncytia. Total RNA was isolated by the isothiocyanate-CsCl method [Chirgwin *et al.*, Biochem., 18:5294-5299 (1979)] and poly A+ RNA was selected by oligo dT-cellulose chromatography. These mRNA preparations were used for sequencing by the dideoxy method [Sanger *et al.*, Proc. Nat'l. Acad. Sci., USA, 74:5463-5467 (1977)] using reverse transcriptase and 5'-^{32}P-labelled oligonucleotides followed by a chase with terminal deoxynucleotidyl transferase [DeBorde *et al.*, Anal. Biochem., 157:275-282 (1986)]. The primers used for sequencing were synthesized according to the reported sequence of the Long F protein gene [Lopez *et al.*, Virus Res., 10:249-262 (1988)].

The oligonucleotide primers used for sequencing mutants selected with mAbs RSV19 and 20 were, in anti-RNA sense:

SEQ ID NO: 23	F1216:	5'-ATCTGTTTTTGAAGTCAT
SEQ ID NO: 24	F1300:	5'-ACGATTTTATTGGATGC
SEQ ID NO: 25	F1339:	5'-TGCATAATCACACCCGT
SEQ ID NO: 26	F1478:	5'-CAAATCATCAGAGGGG
SEQ ID NO: 27	F1548:	5'-AATTCATCGGATTTACGA
SEQ ID NO: 28	F1707:	5'-CTCAGTTGATCCTTGCTTAG.

The F mRNA of viruses selected with mAbs AK13A2, AK13A2, 7C2 and B4 were sequenced between nucleotides 420

and 920, which encode the trypsin resistant fragments recognized by those antibodies (Fig. 2). The F mRNA of viruses selected with mAb 11 were sequenced only between nucleotides 893 and 906. Similarly, the F mRNAs of viruses selected with mAbs 19 and 20 were sequenced between nucleotides 1100 and 1680, which encode the region of the tentatively located 26 kDa trypsin resistant fragment recognized by those antibodies.

Table 3 illustrates sequence changes selected in different neutralization escape mutants, including two previously reported mutants selected with mAb 47F [Lopez *et al.*, (1990), cited above]. Only nucleotide (mRNA sense) and amino acid changes at the indicated positions, as compared to the Long and A2 strain sequences, are shown. ND means not done.

This table, parts 3A, 3B, and 3C should be read across for each antibody. For example, for antibody 47F, virus 4, note that a nucleotide change from A to U at position 797 (Table 3A) results in an amino acid change from Asn to Tyr at position 262 (Table 3B), and a loss of antibody binding at 47F, 49F and AK13A2 (Table 3C).

TABLE 3A

Ab used for Selection	Viruses	Nucleotide at position 583	659	786	797	816	827	828	1298
-	Long and A2	C	A	U	A	A	A	A	C
11						U			
47F	4				U				
	7					U			
AK13A2	4/4		G		U				
	11/3				U				
	4				U				
	5				U				
	7						G		
	4'								
7C2	1						G		
	4	A	C						
	11							C	
	12							C	
B4	61:16/7							C	
	61:16/8							C	
19	C484f								A
	C4909/5								A
	C4909/6								A
20	C4902Wa								A
	C4902Wb								A
	C4902Wc								A

TABLE 3B

Ab used for Selection	Viruses	Amino Acid at position 190	216	258	262	268	272	429
-	Long and A2	Ser	Asn	Leu	Asn	Asn	Lys	Arg
11						Ile		
47F	4				Tyr			
	7					Ile		
AK13A2	4/4		Asp		Tyr			
	11/3				Tyr			
	4				Tyr			
	5				Tyr			
	7						Glu	
	4'							
7C2	1						Glu	
	4	Arg		Ser				
	11						Thr	
	12						Thr	
B4	61:16/7						Thr	
	61:16/8						Thr	
19	C484f							Ser
	C4909/5							Ser
	C4909/6							Ser
20	C4902Wa							Ser
	C4902Wb							Ser
					C4902Wc			
								Ser

TABLE 3C

Ab used for Selection	Viruses	Loss of binding with Antibodies
-	Long and A2	-
11		Not determined
47F	4	47F, 49F, AK13A2
	7	47F, 49F, AK13A2, 7C2, B4
AK13A2	4/4	47F, 49F, AK13A2, 7C2, B4
	11/3	47F, 49F, AK13A2
	4	47F, 49F, AK13A2
	5	47F, 49F, AK13A2
	7	47F, 49F, AK13A2
	4'	47F, 49F, AK13A2, 7C2, B4
7C2	1	47F, 49F, AK13A2, 7C2, B4
	4	7C2
	11	47F, 49F, AK13A2, 7C2, B4
	12	47F, 49F, AK13A2, 7C2, B4
B4	61:16/7	47F, 49F, AK13A2, 7C2, B4
	61:16/8	47F, 49F, AK13A2, 7C2, B4
19	C484f	56F, 57F, 19, 20
	C4909/5	56F, 57F, 19, 20
	C4909/6	56F, 57F, 19, 20
20	C4902Wa	56F, 57F, 19, 20
	C4902Wb	56F, 57F, 19, 20
	C4902Wc	56F, 57F, 19, 20

MAb 11 selected mutants which had a single transversion (A to U) at nucleotide 816, which changed Asn-262 to Ile. This change also led to the loss of the epitopes recognized by mAbs 13, B4 and B5, which are included in antigenic area B in Fig. 5 and is identical to that found in mutant 7 selected with mAb 47F which led to the loss of all the epitopes included in antigenic area II of Fig. 8.

Four viruses selected with mAb AK13A2 (11/3, 4, 5 and 7) has a single transversion (A to U) at nucleotide 797 which changed Asn-262 to Tyr. This change eliminated the binding sites for antibodies 47F, 49F and AK13A2 (see also Fig. 8) and it is identical to the change observed in mutant 4 selected with mAb 47F. A fifth virus selected with mAb AK13A2 (4/4) had, in addition, a transition (A to G) at nucleotide 659 which Asn-216 to Asp. This second amino acid change led to the loss of all the epitopes from antigenic area II (Fig. 8). The last mutant selected with mAb AK13A2 (4') had a single transition A to G at nucleotide 827, leading to the replacement of Lys-272 by Glu and the loss of all the epitopes from area II.

All mutants selected with mAb 7C2, except mutant 4, contained single nucleotide changes (A to G or A to C) at positions 827 or 828 which changed Lys-272 to Glu or Thr, respectively. These changes eliminated the reactivity with all the mAbs from antigenic area II. Mutant 4 had two nucleotide substitutions at position 583 (C to A) and 786 (U to C) which changed amino acids 190 (Ser to Arg) and 258 (Leu to Ser). The last mutant had only lost the binding site for mAb 7C2 but retained its reactivity with all the other anti-F antibodies (Fig. 8).

The two mutants selected with mAb B4 had a single nucleotide transversion at position 828 (A to C) which changed Lys-272 to Thr. Thus, all the amino acid changes

selected with mAbs from antigenic area II were clustered in a small segment of the F protein, between amino acids 262 and 272, except the changes at amino acids 258, 216 and 190 which were detected only in viruses with two amino acid substitutions.

All mutants selected with antibodies RSV19 or 20 contained a single C to A transversion at nucleotide 1298 which changed Arg-429 to Ser. This amino acid change, located towards the carboxy terminal end of the cysteine rich region of the F1 subunit (Fig. 2), eliminated the reactivity of all the mAbs grouped in the antigenic area IV, except antibody 52F. Amino acid 429 (Ser) is therefore important for the binding of mAbs RSV19 and 20 to the F protein. The synthetic peptides 417-432 and 422-438 of the F protein [SEQ ID NO: 19] reproduce at least part of the epitope recognized by mAb RSV19. The sequence results confirm the findings shown in Figs. 7 and 8 that antigenic areas II and IV do not overlap.

Example 9 - Reactivity of antibodies with synthetic peptides

Since the antibodies used to select the escape mutants reacted in Western blot with trypsin fragments of the F1 subunit, whether or not synthetic peptides could reproduce the epitopes recognized by these antibodies was determined.

In summary, the results obtained with the synthetic peptides were also indicative of conformational constraints in the epitopes of antigenic area II. Epitope B4 was reproduced by the peptide 255-275 of SEQ ID NO: 55; however, other closely related peptides failed to react with that antibody. In addition, none of the peptides tested reproduced other epitopes of antigenic area II (B). The region of the F1 subunit containing these epitopes is resistant to high doses of trypsin, indicative of a particular three-dimensional conformation which might be preserved in Western blots but not in synthetic peptides.

The peptides shown in Table 4 were synthesized in an Applied Biosystem 430 instrument, using the solid phase technology and t-Boc chemistry [Merrifield, Science, 232:341-347 (1986)]. The peptides were cleaved off the resin with trifluoromethyl sulfonic acid and purified from protecting groups and scavengers by Sephadex G-25 chromatography. The amino acid sequence of each peptide was confirmed by automated Edman degradation in an Applied Biosystem 477 protein sequencer.

Three peptides were synthesized with sequences corresponding to amino acids 250-273, 255-275 or 258-271 of the F1 subunit [SEQ ID NO: 55], which surrounded the positions changed in the mutants selected with mAbs from antigenic area II.

The binding of mAbs to synthetic peptides was tested by ELISA of Example 3 in polyvinylchloride microtitre plates

coated overnight with 1-2 µg of peptide. PBS containing 5% pig serum was used as blocking reagent to eliminate spurious cross-reactions. The results are reported in Table 4 below.

Only antibody B4 and another bovine antibody, B5, reacted with the peptide 255-275 of SEQ ID NO: 55. The B4 titre with this peptide was similar to that obtained against purified virus. However, this antibody did not react with peptides 250-273 nor 258-271 of SEQ ID NO: 55, which contained almost the entire amino acid sequence included in peptide 255-275 of SEQ ID NO: 55. All other antibodies from area II failed to react with any of the peptides.

Three other peptides, corresponding to the sequences 417-432, 422-438 and 435-450 of the F1 subunit [SEQ ID NO: 55] which surrounded the position 429 changed in the escape mutants selected with mAbs RSV19 or 20, were also tested by ELISA (Table 4). Only antibodies RSV19, B13 and B14 reacted with the first two peptides (417-432 and 422-438 of SEQ ID NO: 55).

Thus, two antigenic sites recognized by neutralizing, protective mAbs directed against the F protein have been identified. The first site contains several overlapping epitopes located within the trypsin resistant amino terminal third of the F1 subunit, clustered around amino acids 262-272 of SEQ ID NO: 55. Only one of these epitopes, that recognized by B4, was faithfully reproduced by a short synthetic peptide corresponding to amino acids 255-275 of the F protein [SEQ ID NO: 19]. The second antigenic site was located within the carboxy terminal third of the F1 subunit and the epitope recognized by mAb RSV19 and that recognized by B13 and B14 was reproduced by synthetic peptides corresponding to amino acids 417 to 432 and 422 to 438 of SEQ ID NO: 55. However, the epitopes recognized by

mAbs RSV19, B13, and B14 do not appear to be identical since mAbs B13 and B14 react with antibody-escape mutants selected with mAb RSV19 which have a substitution at amino acid 429-Arg (Fig. 7), indicating that amino acid 429-Arg is not essential for the binding of mAbs B13 and B14 to the F protein. The peptide fragments of the following Table 4 are taken from SEQ ID NO: 55, the F1 subunit.

Table 4

Reactivity of monoclonal antibodies with synthetic peptides

	Monoclonal antibody								
Peptide	7C2	47F	AK13A2	11	B4	RSV19	20	B13	B14
250-273	<2.0*	<2.0	<2.0	<2.0		<2.0	<2.0	ND	ND
255-275	<2.0	<2.0	<2.0	<2.0	6.7	<2.0	<2.0	<2.0	<2.0
258-271	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	ND	ND
417-432	<2.0	<2.0	<2.0	<2.0	<2.0	2.8	<2.0	4.5	3.2
422-438	<2.0	<2.0	<2.0	<2.0	<2.0	6.0	<2.0	5.0	4.3
435-450	<2.0	<2.0	<2.0	<2.0	<2.0	2.3	<2.0	ND	ND
RSV strain A2	8.4	6.1	4.9	6.4	5.3	6.4	6.3	5.6	5.6

* Log_{10} titre of antibody binding to synthetic peptides dried onto wells or RSV antigen tested in an ELISA.

Example 10 - Pepscan Analysis of Epitope Recognized by mAb B4

Overlapping peptides corresponding to amino acids 255 to 275 of the F protein [SEQ ID NO: 19] were synthesized in duplicate as a series of octamers overlapping by seven amino acids and offset by one amino acid, bound to polyethylene pins using F-Moc chemistry following the method of Geyson et al, J. Immunol. Meth., 102:259-274 (1987). The software package, polyethylene pins and amino acids used to produce the peptides were obtained from Cambridge Research Biochemicals, Cheshire, England.

The pins to which the peptides are bound were incubated with blocking buffer in 96 well microtitre plates (PBS containing 0.05% Tween 20 and 2% Marvel) on a rotary shaker. After one hour incubation at room temperature, the pins were incubated with mAb B4, and diluted 1:600 in blocking buffer at 4°C with shaking. After being washed 10 times for 5 minutes in PBS containing 0.05% Tween 20 (PBS/Tw), the pins were incubated with horseradish peroxidase (HRP)-rabbit anti-bovine IgG [Sigma], diluted 1:4000 in blocking buffer. After one hour and 45 minutes, the pins were washed ten times for 5 minutes and incubated, in the dark with agitation, in microtiter plates containing 150µl/well of 50 mg of azino-di-3-ethyl-benzthiazodisulpho-nate [Sigma] dissolved in 100 ml of substrate buffer (0.1M disodium hydrogen orthophosphate; 0.08M citric acid) containing 0.3 µl/ml of 120 volume hydrogen peroxide. When sufficient color had developed, the O.D. was read at 405 nm on a Titertek Multiscan MCC 340 plate reader. MAb B4 recognized a single peptide extending from amino acid #266-273 of SEQ ID NO: 19 and having the sequence I T N D Q K K L bound to the pins.

The binding of B4 to this octomer was studied further using peptides, bound to pins, which represented the above sequence, but where every amino acid in this sequence was replaced in turn with each of the 20 naturally occurring amino acids. Duplicate peptides were synthesized as described above and the binding of mAb B4 to the peptides was determined by ELISA and is shown in Fig. 9. B4 bound to all peptides where amino acid 266-Ile was replaced in turn with all other amino acids, indicating that amino acid 266-Ile was not essential for the binding of B4 to the peptide 266-273 of SEQ ID NO: 19. Similarly, replacement of amino acids 270-Glu and 273-Lys did not affect the binding of B4

to a significant extent. In contrast, substitution of amino acids 268-Asn, 269-Asp and 272-Lys resulted in the total loss of binding of B4, indicating that these amino acids are essential for the binding of B4 to peptide 266-273 of SEQ ID NO: 19. These studies confirm the findings from the sequence analysis of antibody escape mutants (Example 8) which also showed that amino acids 268-Asn and 272-Lys were critical for the binding of B4 to the F protein. Substitution of amino acid 267-Thr resulted in reduced binding of B4 and replacement of amino acid 271-Lys resulted in significantly enhanced binding to the peptide. Maximum binding to the peptide 266-273 of SEQ ID NO: 19 was detected when amino acid 271-Lys was replaced by Ile.

Example 11 - A Humanized Anti-RSV Antibody

The following example describes the preparation of an exemplary altered antibody utilizing the murine IgG_{2a} mAb called RSV19 or RSMU19, described in co-pending PCT application No. PCT/GB91/01554 as the source of the donor variable chain sequences and CDRs. Similar procedures may be followed for the development of altered antibodies, using other anti-RSV antibodies described herein.

RSV19 is specific for the fusion (F) protein of RSV. The RSV19 hybridoma cell line was obtained from Dr. Geraldine Taylor. Methodology for the isolation of hybridoma cell lines secreting monoclonal antibodies specific for RSV is described by Taylor *et al.*, Immunology, 52:137-142 (1984).

As described in the preceding example, cytoplasmic RNA was prepared by the method of Favaloro *et al.*, (1980) cited above from the RSV19 hybridoma cell line, and cDNA was synthesized using Ig variable region primers as follows. For the Ig heavy chain variable region, RSV19VH (see Figs.

15A, 15B and 19), the primer [SEQ ID NO: 33] VH1FOR 5'TGAGGAGACGGTGACCGTGGTCCCTTGGCCCCAG3' was used, and for the Ig light chain variable region, RSV19VK (see Figs. 16A and 16B), the primer [SEQ ID NO: 34] VK1FOR 5'GTTAGATCTCCAGCTTGGTCCC3' was used.

cDNA synthesis reactions consisted of 20µg RNA, 0.4µM VH1FOR or VK1FOR, 250µM each of dATP, dCTP, dGTP and dTTP, 50mM Tris-HCl pH 7.5, 75mM KCl, 10mM DTT, 3mM $MgCl_2$ and 27 units RNase inhibitor in a total volume of 50µl. Samples were heated at 70°C for 10 minutes and slowly cooled to 42°C over a period of 30 minutes. Then, 100µ MMLV reverse transcriptase was added and incubation at 42°C continued for 1 hour.

VH and VK cDNAs were then amplified using PCR. For PCR, the primers used were: VH1FOR; VK1FOR; VH1BACK (described in Example 18), and
[SEQ ID NO: 35] VK1BACK 5'GACATTCAGCTGACCCAGTCTCCA 3'.

Primers VH1FOR, VK1FOR, VH1BACK and VK1BACK, and their use for PCR-amplification of mouse Ig DNA, are described by Orlandi *et al.*, (1989), cited above.

For PCR amplification of VH, DNA/primer mixtures consisted of 5µl RNA/cDNA hybrid, and 0.5µM VH1FOR and VH1BACK primers. For PCR amplifications of VK, DNA/primer mixtures consisted of 5µl RNA/cDNA hybrid, and 0.5µM VK1FOR and VK1BACK primers. To these mixtures was added 200 µM each of dATP, dCTP, dGTP and dTTP, 10mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.01% (w/v) gelatin, 0.01% (v/v) Tween 20, 0.01% (v/v) Nonidet P40 and 2 units Taq DNA polymerase [United States Biochemicals-Cleveland, Ohio, USA]. Samples were subjected to 25 thermal cycles of PCR at 94°C, 1 minute; 60°C, 1 minute; 72°C, 2 minutes; ending with 5 minutes at 72°C. For cloning and sequencing, amplified VH

DNA was purified on a low melting point agarose gel and by Elutip-d column chromatography and cloned into phage M13. The general cloning and ligation methodology was as described in Maniatis *et al.*, cited above.

VH DNA was either directly ligated into the SmaI site of M13 mp 18/19 or, following digestion with PstI, into the PstI site of M13tg131 [Amersham International-Little Chalfont, UK]. Amplified VK was similarly gel purified and cloned by the following alternatives: (1) PvuII digest into M13mp19 (SmaI site); (2) PvuII and BglII digest into M13mp18/19 (SmaI-BamHI site); (3) PvuII and BglII digest into M13tg131 (EcoRV-BglII site); (4) BglII digest into M13tg131 (SmaI-BglII site). The resultant collections of overlapping clones were sequenced by the dideoxy method [Sanger *et al.*, cited above] using Sequenase [United States Biochemicals-Cleveland, Ohio, USA].

From the sequence of RSV19 VH and VK domains, as shown in Figs. 14A and 14B, and 15A, and 15B, respectively, the CDR sequences were elucidated in accordance with the methodology of Kabat *et al.*, in "Sequences of Proteins of Immunological Interest", US Dept of Health and Human Services, US Government Printing Office, (1987) utilizing computer assisted alignment with other VH and VK sequences.

The murine RSV19 CDRs were transferred to human frameworks by site directed mutagenesis. The primers used were:

[SEQ ID NO: 36] VHCDR1 5'CTGTCTCACCCAGTGCATATAGTAGTCG CTGAAGGTGAAGCCAGACACGGT 3'

[SEQ ID NO: 37] VHCDR2 5' CATTGTCACTCTGCCCTGGAACTTCGGGG CATATGGAACATCATCATTCTCAGGATCAATCCA 3'

[SEQ ID NO: 38] VHCDR3 5' CCCTTGGCCCCAGTGGTCAAAGTCACTCCC CCATCTTGCACAATA 3'

[SEQ ID NO: 39] VKCDR1 5' CTGCTGGTACCATTCTAAATAGGTGTTTCCA TCAGTATGTACAAGGGTCTGACTAGATCTACAGGTGATGGTCA 3'

[SEQ ID NO: 40] VKCDR2 5' GCTTGGCACACCAGAAAATCGGTTGGAAACTC TGTAGATCAGCAG 3'

[SEQ ID NO: 41] VKCDR3 5' CCCTTGGCCGAACGTCCGAGGAAGATGT GAACCTTGAAAGCAGTAGTAGGT 3'

The DNA templates for mutagenesis comprised human framework regions derived from the crystallographically solved proteins, NEW [Saul, *et al.*, J. Biol. Chem., 53:585-597 (1978)] with a substitution of amino acid 27 from serine to phenylalanine [See, Riechmann *et al.*, loc.cit.] and REI [Epp *et al.*, Eur J. Biochem. 45:513-524 (1974)] for VH and VK domains, respectively. M13 based templates comprising human frameworks with irrelevant CDRs were prepared as described by Riechmann *et al.*, Nature, 332 (1988).

Oligonucleotide site directed mutagenesis of the human VH and VK genes was based on the method of Nakamaye *et al.*, Nucl. Acid Res., 14:9679-9698 (1986). To 5µg of VH or VK single-stranded DNA in M13 was added a two-fold molar excess of each of the three VH or VK phosphorylated oligonucleotides encoding the three mouse CDR (complementarity determining region) sequences. Primers were annealed to the template by heating to 70°C and slowly cooled to 37°C. To the annealed DNA was added 6 units T4DNA ligase [Life Technologies, Paisley, UK]; 0.5 mM of each of the following nucleoside triphosphates (dATP, dGTP, dTTP and 2'-deoxycytidine 5'-0-)1-thiotriphosphate) (thiodCTP); 60mM Tris-HCl (pH 8.0); 6mM $MgCl_2$; 5mM DTT [Sigma, Poole, UK]; and 10mM ATP in a reaction volume of 50µl. This mixture was incubated at 16°C for 15 hours. The DNA was then ethanol precipitated and digested with 5 units NciI [Life Technologies, Paisley, UK] which nicks the parental strand

but leaves the newly synthesized strand containing thiodCTP intact. The parental strand was then removed by digesting for 30 minutes with 100 units exonuclease III [Pharmacia, Milton Keynes, United Kingdom] in 50 µl of 60mM Tris-HCl (pH 8.0), 0.66mM $MgCl_2$, and 1mM DTT. The DNA was then repaired through addition of 3 units of DNA polymerase I [Life Technologies, Paisley, UK], 2 units T4 DNA ligase in 50 µl of 60 mM Tris-HCl (pH 8.0), 6mM $MgCl_2$, 5mM DTT, 10mM ATP and 0.5 mM each of dATP, dCTP, dGTP and dTTP. The DNA was transformed into competent *E. coli* TG1 cells [Amersham International, Little Chalfont, UK] by the method of Maniatis *et al.*, cited above.

Single-stranded DNA was prepared from individual plaques and sequenced by the method of Messing, Methods in Enzymology, 101:20-78 (1983). If only single or double mutants were obtained, then these were subjected to further rounds of mutagenesis (utilizing the methodology described above) by using the appropriate oligonucleotides until the triple CDR mutants were obtained.

The CDR replaced VH and VK genes were cloned in expression vectors (by the method of Maniatis *et al.*) to yield the plasmids pHuRSV19VH and pHuRSV19VK. The plasmids are shown in Figs. 16 and 17, respectively. For pHuRSV19VH, the CDR replaced VH gene together with the Ig heavy chain promoter, appropriate splice sites and signal peptide sequences were excised from M13 by digestion with HindIII and BamHI, and cloned into an expression vector containing the murine Ig heavy chain enhancer, the SV40 promoter, the gpt gene for selection in mammalian cells and genes for replication and selection in *E. coli*. The variable region amino acid sequence is shown in Fig. 19. A human IgG1 constant region was then added as a BamHI fragment.

The construction of the pHuRSV19VK plasmid was essentially the same except that the gpt gene was replaced by the hygromycin resistance gene and a human kappa chain constant region was added (see Figs. 17 and 22)..

10µg of pHuRSV19VH and 20µg of pHuRSV19VK were digested with PvuI utilizing conventional techniques. The DNAs were mixed together, ethanol precipitated and dissolved in 25µl water. Approximately 10^7 YB2/0 cells [American Type Culture Collection, Rockville, Maryland, USA] were grown to semi-confluency, harvested by centrifugation and resuspended in 0.5ml DMEM [Gibco, Paisley, UK] together with the digested DNA in a cuvette. After 5 minutes on ice, the cells were given a single pulse of 170V at 960uF (Gene-Pulser, Bio-Rad-Richmond, California, USA) and left in ice for a further 20 minute. The cells were then put into 20 ml DMEM plus 10% foetal calf serum and allowed to recover for 48 hours. After this time, the cells were distributed into a 24-well plate and selective medium applied (DMEM, 10% foetal calf serum, 0.8µg/ml mycophenolic acid, and 250µg/ml xanthine). After 3-4 days, the medium and dead cells were removed and replaced with fresh selective medium. Transfected clones were visible with the naked eye 10-12 days later.

The presence of human antibody in the medium of wells containing transfected clones was measured by conventional ELISA techniques. Micro-titre plates were coated overnight at 4°C with goat anti-human IgG (gamma chain specific) antibodies [Sera-Lab-Ltd., Crawley Down, UK] at 1 µg per well. After washing with PBST (phosphate buffered saline containing 0.02% Tween 20x (pH7.5)), 100µl of culture medium from the wells containing transfectants was added to each microtitre well for 1 hour at 37°C. The wells were then emptied, washed with PBST and either peroxidase-conjugated

goat anti-human IgG or peroxidase-conjugated goat anti-human kappa constant region antibodies [both obtained from Sera-Lab Ltd., Crawley Down, UK] were added at 100 ng per well. Plates were then incubated at 37°C for 1 hour. The wells were then emptied and washed with PBST. 340 µg/ml q-phenylenediamine in 50mM sodium citrate, 50mM sodium phosphate (pH 5.0) and 0.003% (v/v) H_2O_2 were added at 200µl per well. Reactions were stopped after 1 to 5 minutes by the addition of 12.5% sulphuric acid at 50µl per well. The absorbance at 492 nm was then measured spectrophotometrically.

The resulting humanized antibody HuRSV19VH/VK (also called RSH200), secreted from cell lines cotransfected with pHuRSV19VH and pHuRSV19VK, was purified on Protein-A agarose columns [Boehringer Mannheim, Lewes, UK] and tested for binding to RSV virus in an ELISA assay. Antigen consisted of calf kidney (CK) cells infected with RSV A2 strain [Lewis *et al.*, Med. J. Australia, 48:932-933 (1961)] and treated with 0.5% (v/v) NP40 detergent to yield a cell lysate. A control cell lysate was similarly prepared using uninfected CK cells. Microtitre plate wells were coated with either infected or control cell lysate. Antigen coated plates were blocked with PBST for 1 hour at 37°C, washed with PBST, and thereafter humanized antibody was applied (i.e., HuRSV19VH/VK). After 1 hour at 37°C, the wells were emptied, washed with PBST and 200 ng goat anti-human IgG antibodies [Sera Lab-Ltd., Crawley Down, UK] added per well. After 1 hour at 37°C, the wells were emptied, washed with PBST and 200µl of a 1:1000 dilution of HRP-conjugated rabbit anti-goat IgG antibodies [Sigma-Poole, UK] were added.

After 1 hour at 37°C, the wells were emptied and washed with PBST. To each well was added 200µl substrate buffer

(340µg/ml q-phenylenediamine in 50mM sodium citrate, 50mM sodium phosphate (pH 5.0) and 0.003% (v/v) H_2O_2). Reactions were stopped by the addition of 50µl 12.5% sulphuric acid. The absorbance at 492 nm was then measured.

This humanized antibody HuRSV19VH/VK (RSHZ00), generated by the straight replacement of the RSV19 heavy and light chain CDRs into the human heavy chain framework regions (variable and constant regions REI and kappa, respectively) bound to whole RSV preparations, although with an affinity less than the donor murine RSV19 antibody.

Example 12 - Production of High Affinity Anti-RSV Antibodies

High affinity antibodies specific for RSV were developed by a method designed to achieve minimal variable region framework modifications giving rise to high affinity binding. The method involves the following order of steps of alteration and testing:

1. Individual framework amino acid residues which are known to be critical for interaction with CDRs are compared in the primary antibody and the altered CDR-replacement antibody. For example, heavy chain amino acid residue 94 (Kabat numbering-see Kabat *et al.*, cited above) is compared in the primary (donor) and altered antibodies. An Arg residue at this position is thought to interact with the invariant heavy chain CDR Asp residue at position 101.

If amino acid 94 comprises Arg in the framework of the primary antibody but not in the framework of the altered antibody, then an alternative heavy chain gene comprising Arg 94 in the altered antibody is produced. In the reverse situation whereby the altered antibody framework comprises an Arg residue at position 94 but the primary antibody does not, then an alternative heavy chain gene comprising the original amino acid at position 94 is produced. Prior to

any further analysis, alternative plasmids produced on this basis are tested for production of high affinity altered antibodies.

2. Framework amino acids within 4 residues of the CDRs as defined according to Kabat (see Kabat *et al.*, cited above) are compared in the primary antibody and altered CDR-replacement antibody. Where differences are present, then for each region (e.g., upstream of VHCDR1) the specific amino acids of that region are substituted for those in the corresponding region of the altered antibody to provide a small number of altered genes. Alternative plasmids produced on this basis are then tested for production of high affinity antibodies.

3. Framework residues in the primary and altered CDR-replacement antibodies are compared and residues with major differences in charge, size or hydrophobicity are highlighted. Alternative plasmids are produced on this basis with the individual highlighted amino acids represented by the corresponding amino acids of the primary antibody and such alternative plasmids are tested for production of high affinity antibodies.

The method is exemplified by the production of a high affinity altered antibody derivative of HuRSV19VH/VK specific for RSV. Comparison of VH gene sequences between RSV19VH and pHuRSV19VH (Figs. 18-22) indicates that 3 out of 4 amino acid differences occur between amino acids 91 to 94 of the F protein of SEQ ID NO: 19, which defines a framework sequence adjacent to heavy chain CDR3.

Thus, plasmid pHuRSV19VHFNS (Fig. 20) was produced by inserting the RSV19 heavy chain CDRs and the four amino acid framework sequence amino acids 91 to 94 into the human framework described in the preceding example. Using

oligonucleotide site directed mutagenesis, the following oligonucleotide was used for mutagenesis of the HuRSV19VH gene in M13:

[SEQ ID NO: 42] HuRSV19VHFNS - 5'CTCCCCCATGAATTACAGAAATAG ACCG 3'.

The cell line cotransfected with pHuRSV19VHFNS and pHuRSV19VK (Fig. 22) produced a second humanized antibody, HuRSV19VHFNS/HuRSV19VK (abbreviated hereafter as RSHZ19). This antibody was tested in an ELISA assay for analysis of binding to RSV antigen prepared from detergent-extracted, virus-infected cells. The substitution of VH residues 91 to 94 in HuRSV19VH/VK with VH residues from mouse RSV19VH partially restored antigen binding levels. Additional analysis of HuFNS binding properties was performed using an ELISA assay in which intact Type A RSV (Long strain) was used as the antigen. The data from such additional analysis show that there is little if any difference between the ability of the murine mAb RSV19 and the humanized RSHZ19 antibodies to bind to intact, non-denatured RSV. This additional analysis also showed detectable binding of HuRSV19VH/VK to intact virus, although of a much lower magnitude than was seen with either RSV19 or RSHZ19.

Thus, the data from this additional analysis suggests that the affinity for the native antigen was restored in the RSHZ19 mAb. Specificity of RSHZ19 for RSV F protein was shown by conventional Western blot analysis using a truncated soluble F protein construct expressed in CHO cells.

Example 13 - Immunofluorescence Analysis of Humanized Antibodies

In order to ascertain the potential clinical usefulness of a humanized antibody specific for RSV, an immuno-

fluorescence analysis of binding to 24 RSV clinical isolates was undertaken. The isolates were obtained from children during the winter of 1983-84 by the Bristol Public Health Laboratory (Bristol, England) and represented both of the major subgroups of RSV. Thirteen isolates were serotyped as subgroup A and 11 isolates as subgroup B. HeLa or MA 104 cells infected with RSV isolates were grown in tissue culture. When the cells showed evidence of cytopathic effect, 20 ml of 0.02% (w/v) disodium ethylenediaminetetra-acetic acid (EDTA) [BDH Chemicals Ltd., Poole, UK] in PBS and 3ml of 0.25% (w/v) trypsin in PBS were added and the cell suspension spotted into wells of PTFE-coated slides (polytetrafluoroethylene coated slides) [Hendley, Essex, UK]. After 3 hours at 37°C, the slides were dried and fixed in 80% acetone. Cells were overlaid with monoclonal antibody (i.e., either humanized antibody, RSHZ19 or the murine antibody RSV19) for 1 hour at room temperature. After extensive washing, either fluorescein-conjugated rabbit anti-mouse IgG [Nordic Laboratories-Tilburg, The Netherlands] or fluorescein-conjugated goat anti-human IgG1 [Southern Biotechnology, Birmingham, Alabama, USA] was added, and the incubation was repeated. After further washing, cells were mounted in glycerol and examined under UV light.

The results of comparative immunofluorescence for the humanized antibody, RSHZ19, and the murine antibody RSV19 indicated that 100% of clinical isolates are recognized by both the humanized and murine antibodies. Such data demonstrated that the humanized antibody has the potential for recognition of most clinical isolates comprising both of the major RSV subgroups.

The humanized antibody, RSHZ19, was next tested for biological activity *in vitro* in a fusion inhibition assay. A suspension of MA104 cells was infected with RSV at an m.o.i. (multiplicity of infection) of 0.01 PFU (plaque forming units) per cell. After 1 hour at 37°C, 2 ml of cells at 10^5/ml were distributed to glass coverslips in tubes. After a further 24 hours at 37°C, the culture medium was replaced by medium containing dilutions of humanized antibody, RSHZ19. Twenty-four hours later, coverslip cultures were fixed in methanol for 10 minutes and stained with May Grunwald stain [BDH Chemicals Ltd., Poole, UK]. The effect of increasing concentrations of RSHZ19 in inhibiting the frequency of giant cells demonstrates the biological activity of the humanized antibody RSHZ19 in inhibiting Type A RSV induced cell fusion. Additional studies showed that the fusion inhibition titres for RSV19 versus RSHZ19 were comparable, providing additional evidence that affinity for the native viral antigen was fully restored in the humanized RSHZ19. The humanized antibody RSHZ19 has also been shown, using methodology analogous to that utilized above for showing inhibition of Type A RSV induced cell fusion, to exhibit a dose dependent inhibition of Type B RSV (strain 8/60) induced giant cell fusion.

The humanized antibody, RSHZ19, was next tested for biological activity *in vitro* in an RSV-mouse infection model. BALB/c mice [Charles Rivers: specific pathogen free category 4 standard] were challenged intranasally with 10_4 PFU of the A2 strain of human RSV [Taylor *et al.*, Infect. Immun., 43:649-655 (1984)]. Groups of mice were administered with 25µg of humanized antibody either one day prior to virus infection or 4 days following infection.

Administration of antibody was either by the intranasal (i.n.) or intraperitoneal (i.p.) routes. 5 days after RSV infection, mice were sacrificed and lungs were assayed for RSV PFU [see, Taylor *et al.*, cited above]. The data showed that RSHZ19 at a single dose of 25 µg per mouse is extremely effective in prevention and treatment of RSV infection.

RSHZ19 was also shown to be active *in vivo* when administered prophylactically to mice challenged with Type B RSV (strain 8/60) using methodology similar to that described above. In addition, the humanized antibody HuRSV19VH/VK was also shown to be active *in vivo* when administered prophylactically to mice challenged with Type B RSV (strain 8/60) using methodology similar to that described above.

Example 14 - Comparison of blood levels of RSHZ19 after i.v. or i.p. Inoculation of Mice

Five female BALB/c mice (weighing approximately 20g) were inoculated i.p. with 50 µg RSHZ19 (CHO) and another 5 were inoculated intravenously (i.v.) with 50 µg RSHZ19 (CHO). Mice were bled from the tail 2 hours, 1, 4, 7, 14, 21 and 46 days later and the levels of RSHZ19 in the sera were determined using two different ELISAs as follows.

(i) Plates were coated with a lysate of either RSV (strain) A2-infected or uninfected Hep-2 cells, followed by dilutions of mouse sera and HRP-anti human IgG.

(ii) Plates were coated with 200ng of anti-idiotypic mAb B12, followed by mouse sera and HRP-anti human IgG.

Both assays gave essentially the same results, although the B12 ELISA appeared to be more sensitive. Two hours after inoculation the serum level of RSHZ19 was 5-fold

greater in mice inoculated i.v. compared with those inoculated i.p.. However, titres of RSHZ19 were equivalent in both groups of mice by 24 hours after inoculation. The level of RSHZ19 remained constant for at least 4 days after inoculation and was beginning to decline at 7 days. After this time, there was a rapid decline in serum levels of RSHZ19 in mice inoculated i.v., whereas the level of RSHZ19 declined more slowly in mice inoculated i.p. These results are summarized in Table 5.

Table 5

Comparison of Serum Levels of RSHZ19 (CHO) After IV or IP Inoculation of Mice

	$\log_{10}$ ELISA titre in mice inoculated			
	IV		IP	
Days	RS ELISA	B12 ELISA	RS ELISA	B12 ELISA
0.1	3.5 ± 0.2	4.6 ± 0.2	3.2 ± 0.1	4.2 ± 0.1
1	3.1 ± 0.2	4.2 ± 0.04	3.3 ± 0.04	4.3 ± 0.1
4	3.2 ± 0.1	4.2 ± 0.1	3.3 ± 0.2	4.2 ± 0.04
7	3.1 ± 0.2	3.7 ± 0.3	3.6 ± 0.2	3.9 ± 0.1
14	< 1.5	< 1.5	3.1 ± 0.2	3.8 ± 0.1
21	< 1.5	< 1.5	2.3 ± 1.3	3.5 ± 0.2
46	ND	< 1.5	ND	3.3 ± 0.1

To investigate if the rapid decline in RSHZ19 between days 7 and 14 in mice inoculated i.v. was due to an immune response to RSHZ19, the sera were tested for antibody to RSHZ19 in an ELISA. Plates were coated with 50ng of RSHZ19, followed by D21 mouse sera and HRP-anti mouse IgG. As seen

in Table 6, mice inoculated i.v. developed antibody to RSHZ19 at day 21, whereas mice inoculated i.p. had no detectable antibody to RSHZ19. These results suggest that tolerance to RSHZ19 developed following i.p., but not i.v., inoculation of mice with this antibody. Mice are inoculated i.p. or i.v. with RSHZ19 produced from CHO or myeloma cells to further confirm these results.

Table 6

Antibody Response to RSHZ19 in Sera of Mice Inoculated i.v. or i.p. with 50 µg RSHZ19 (CHO)

Mice Inoculated	$\log_{10}$ ELISA titre*
i.v.	2.5 + 0.2
i.p.	< 1.5

* Plates coated with 50ng RSHZ19 (CHO)

Example 15 - Recognition of Clinical Isolates

Preliminary experiments using biotin-labeled, RSHZ19 (BO1, 2.5 µg/ml; 9/29/92 from SmithKline Beecham) and FITC-streptavidin (Sigma) on RSV-infected and uninfected calf testes cells showed that biotin-RSHZ19 at 1/40 with FITC-streptavidin at 1/80 gave specific fluorescence of RSV-infected cells.

Nine slides of nasopharyngeal aspirates from children hospitalized with RSV infection were obtained from the WHO Collaborating Centre for Reference and Research on Rapid Laboratory Viral Diagnosis, the Royal Victoria Infirmary,

Newcastle-upon-Tyne, England. Each slide consisted of 3 replicate samples in separate chambers. One sample was stained with Imagen™ RSV, (Novo Nordisk Diagnostics Ltd, Cambridge CB4 4WS, UK) as instructed in the technical data. Another sample was stained with a 1:40 dilution of biotinylated RSHZ19 for 1h at room temperature, washed 3x with PBS, and incubated with FITC-Streptavidin for 1h at room temperature. The third sample was stained with FITC-Streptavidin only. After washing 3x with PBS, the samples stained with FITC-Streptavidin were counterstained with 0.01% Evans blue for 5 min. washed and mounted in 80% glycerol. RSV-infected cells in the nasopharyngeal aspirate samples stained using IMAGEN™ RSV showed discrete fluorescent intracellular cytoplasmic inclusions typical of infected cells stained with mAb to the N protein of RSV. In contrast, nasopharyngeal aspirate cells stained with biotinylated-RSHZ19 and FITC-Streptavidin showed more generalized granular cytoplasmic staining, typical for the F protein. There was no fluorescence of samples stained with FITC-Streptavidin alone.

The results are illustrated in Table 7. Biotinylated RSHZ19 recognized RSV in all the nasopharyngeal aspirates studied. The intensity of fluorescence in samples stained with biotinylated RSHZ19 was less than in those stained with IMAGEN™ RSV; however, the numbers of stained cells appeared to be similar in both samples.

Table 7

Binding of RSHZ19 to RSV in Nasopharyngeal Aspirates

Spec. No.	Date Specimen Received	Sub-type	Fluorescence Imagen™ RSV	Fluorescence FITC-Strept.	Fluorescence Biot. RSHZ19 + FITC-Strept.
6513	02/02/88	A	++++	-	+++
7430	15/03/88	A	+++	-	++
9997	16/07/85	B	++++	-	++
7920	22/03/85	B	++	-	+
8195	20/11/91	ND	++++	-	+++
8818	13/12/91	ND	++++	-	+++
8845	14/12/91	ND	++	-	+
9495	16/01/92	ND	+++	-	++
9575	08/01/92	ND	+++	-	+++

These studies indicate that RSHV19 recognizes all clinical isolates of RSV examined so far.

Example 16 - Prophylactic effect of bovine mAb B4 on RSV infection in calves

Three 1 to 2 week old gnotobiotic calves, weighing 43 to 55 kg, were inoculated intratracheally (i.t.) with 15 mg of purified bovine mAb, B4, and three were inoculated i.t. with PBS. Twenty-four hours later, all calves were challenged i.n. and i.t. with approximately 10^5 pfu of the Snook strain of bovine RSV. The Snook strain of bovine RSV was isolated from the lung of a calf which died of pneumonia [Thomas *et al.*, Brit. J. Exp. Pathol., 65:19-28 (1984)], and grown in secondary CK cells. Nasopharyngeal swabs were

obtained daily after infection and calves were killed on day 7 of infection. Lung washings were obtained at post-mortem by filling the lungs with 800 ml of PBS. Lung washings were centrifuged at 1300 g and the cell pellet resuspended in 5 ml of medium. All samples were assayed for RSV on secondary CK monolayers.

Treatment of calves with mAb B4 24 hours prior to challenge with the bovine strain of RSV had no effect on virus shedding from the nasopharynx throughout the 7 days of infection. However, as reported in Table 8 below, little or no virus was recovered from the lungs of the calves treated with B4, 7 days after RSV challenge. In contrast, between 10^3 and 10^4 pfu/ml was recovered from the lungs of the control calves. Calves given mAb B4 did not develop pneumonic lesions, whereas the lungs of the control animals were pneumonic.

Table 8

Prophylactic effects of bovine mAb B4 on RSV infection in calves

Treatment	Calf No.	D7 Virus Titre (log_{10} PFU/ml) Nose	Lung Wash	% Pneumonic Lesions
B4 d-1*	1097	2.4	<0.7	<1
	1230	3.5	0.7	0
	1242	3.6	<0.7	<1
None	1098	2.2	3.2	9
	1231	<0.7	3.2	6
	1245	2.1	4.2	6

Example 17 - Prophylactic Effects of Bovine mAbs on RSV infection in Calves

Calves were also treated i.t. with 15 mg B13 or 15 mg B1 24 hours prior to challenge with bovine RSV (BRSV). MAb B1 is an anti-F antibody that is non-neutralizing, non-protective in mice but fixes complement (Kennedy et al, (1988)). Although there was a reduction in the titre of virus in the lungs of calves given B13, the difference in titre of virus compared with control calves given PBS was not statistically significant ($p = 0.07$) (Table 8). However, there was a statistically significant reduction in the severity of pneumonic lesions in calves given B13 when compared with controls. There were no significant differences in either the level of virus in the lungs or the severity of pneumonia in calves given B1 when compared with controls (Table 9).

These studies indicate that B4 is more protective against BRSV infection in the calf than B13. Further, a non-protective, complement fixing mAb, whilst not protective in the calf, does not exacerbate pneumonic lesions.

Table 9

Prophylactic Effects of Bovine mAbs on RSV infection in Calves

Treatmt	No. calves	Nasal Shedding Duration (days)	Nasal Shedding Mean peak titre[a]	Lung Virus No. Infec.	Lung Virus Wash titre[a]	Lung % Pneumonic lesions
B4 d-1	3	5.0 ± 0	3.9 ± 0.7	1	<0.7[b]	<1[c]
B13 d-1	4	4.5 ± 0.6	2.9 ± 0.2	2	1.3±1.5[c]	2±2.6[c]
B1 d-1	4	4.8 ± 0.5	3.2 ± 0.7	4	2.6±1.8[d]	5.5±2.4[d]
PBS d-1	9	4.4 ± 1.2	3.0 ± 0.5	9	3.1±1.5	10.5±7.0

[a] $\log_{IU}$ PFU/ml

[b] Probability that passively immunized animals are significantly different from controls. p<0.01;

[c] p=0.07; [d] NS; [a] p<0.05

Example 18 - Cloning and Sequencing of B4, B13 and B14

Cytoplasmic RNA was prepared by the method of Favaloro *et al.*, Meth. Enzymol., 65:718-749 (1980) from B4, B13 and B14 hybridoma cell lines. The primers BCG1FOR: 5'TTGAATTCAGACTTTCGGGGCTGTGGTGGAGG 3' [SEQ ID NO: 29], which is based on sequence complementary to the 5' end of bovine γ-1 and γ-2 constant region genes, and BCL1FOR: 5'CCGAATTCGACCGAGGGTGGGGACTTGGGCTG 3' [SEQ ID NO: 30], which is complementary to the 5' end of the bovine lambda constant region gene, were used in the synthesis of Ig heavy (VH) and light (VL) chain variable region cDNAs, respectively.

cDNA synthesis reactions consisted of 20μg RNA, 0.4μM BCG1FOR or BCL1FOR, 250μM each of dATP, dCTP, dGTP and dTTP,

50mM Tris-HCl pH 7.5, 75mM KCl, 10mM DTT, 3mM $MgCl_2$ and 27 units RNase inhibitor [Pharmacia, Milton Keynes, United Kingdom] in a total volume of 50µl. Samples were heated at 70°C for 10 minutes and slowly cooled to 42°C over a period of 30 minutes. Then, 100µ MMLV reverse transcriptase [Life Technologies, Paisley, United Kingdom] was added and incubation at 42°C continued for 1 hour.

VH and VK cDNAs were then amplified using the polymerase chain reaction (PCR) as described by Saiki *et al.*, Science, 239:487-491 (1988). For the PCR, the primers used were BCG1FOR, BCL1FOR,

[SEQ ID NO: 31] VH1BACK:

5'AGGT(S)(M)(R)CTGCAG(S)AGTC(W)GG 3'

[SEQ ID NO: 32] VL2BACK:

5'TTGACGCTCAGTCTGTGGTGAC(K)CAG(S)(M)GCCCTC 3'

VH1BACK is described by Orlandi *et al.*, Proc. Nat'l. Acad. Sci., USA, 86:3833-3937 (1989). The sequence of VL2BACK was based on nucleotide sequences listed for the 5' end of human lambda variable regions [Kabat *et al.*, (1987), cited above].

For PCR amplification of VH, DNA/primer mixtures consisted of 5µl RNA/cDNA hybrid and 0.5µM BCG1FOR and VH1BACK primers. For PCR amplifications of VL, DNA/primer mixtures consisted of 5µl RNA/cDNA hybrid and 0.5µM BCL1FOR and VL2BACK primers. To these mixtures was added 250µM each of dATP, dCTP, dGTP and dTTP, 10mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.01% (w/v) gelatin, 0.01% (v/v) Tween 20, 0.01% (v/v) Nonidet P40 and 5 units AmpliTaq [Cetus]. Samples were subjected to 25 thermal cycles of PCR at 94°C, 30 seconds; 55°C, 30 seconds; 72°C, 45 seconds; ending with 5 minutes at 72°C. For cloning and sequencing, amplified VH DNA was purified on a low melting point agarose gel and by

Elutip-d column chromatography [Schleicher and Schuell-Dussel, Germany] and cloned into phage M13 [Pharmacia-Milton Keynes, United Kingdom]. The general cloning and ligation methodology was as described in Maniatis *et al.*, cited above.

VH DNA was cloned as PstI-EcoRI fragments into similarly-digested M13mp18/19 [Pharmacia-Milton Keynes, UK]. VL DNA was cloned as SstI-EcoRI fragments into M13mp18/19 digested with the same enzymes. Representative clones were sequenced by the dideoxy method [Sanger *et al.*, Proc. Nat'l. Acad. Sci., USA, 74:5463-5467 (1977)] using T7 DNA polymerase [Pharmacia].

The amino acid sequences obtained by translation of the variable region gene inserts were aligned with known VH and VL sequences to allow identification of the CDRs.

The VL and VH amino acid sequences of B4 and the apparently substantially identical B13 and B14 antibodies are reported in Figs. 3A and 3B (VL), and 4A and 4B (VH). The B4 sequences are reported above the B13/B14 sequences to demonstrate the homologies therebetween.

Example 19 - Chimeric B4 Antibody

To construct the B4 chimeric heavy chain expression vector, the B4VH gene was amplified from an M13 clone (Example 18) by PCR with oligonucleotides VH1BACK (described in Example 18) and VH1FOR (5' TGAGGAGACGGTGACCGTGGTCCCT TGGCCCCAG 3' [SEQ ID NO: 43] described by Orlandi et al, Proc. Nat'l. Acad. Sci. USA, 86:3833-3937 (1989)). The PCR mixture consisted of 0.5 µl M13 phage supernatant 0.5 uM each of the above primers, 250 uM each of dATP, dCTP, dGTP and dTTP, 10 mM KCl, 20 mM Tris-HCl pH 8.8, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100 and 1 unit Vent DNA polymerase (New England Biolabs) in a volume of 50 ul. Samples were

subjected to 15 rounds of amplification at 94°C, 30 seconds; 50°C, 30 seconds; 75°C, 1 minute; ending with 5 minutes at 75°C. Amplified DNA was purified on a low melting point agarose gel and by Elutip-d column chromatography (Schleicher and Schuell-Dussel, Germany). The DNA was cloned as PstI-BstEII fragments into similarly-digested M13VHPCR1 (Orlandi et la, 1989, cited above). The integrity of a chosen clone was confirmed by nucleotide sequencing.

The B4VH was cloned into an expression vector as described in Example 11 except that the human IgG1 constant region was already present in the vector. The plasmid was termed pSVgptB4BoVHHuIgG1.

To create B4 chimeric light chain expression vector, the vector M13VKPCR1 (Orlandi et al, 1989, cited above) was first modified to allow it to accept a lambda, rather than kappa, chain variable region. This was achieved by mutating the 5' end of the existing VK gene using the oligonucleotide 5' TGGGCTCTGGGTTAACACGGACTGGGAGTGGACACC 3'[SEQ ID NO: 44] and the 3' end using the oligonucleotide 5' ATTCTACTCACGACCCATGGCCACCACCTTGGT 3' [SEQ ID NO: 45], introducing HpaI and NcoI restriction sites respectively. The existing NcoI site in the vector was deleted using the oligonucleotide 5' CTCCATCCCATGCTGAGGTCCTGTG 3' [SEQ ID NO: 46].

M13VKPCR1 was grown in *E. coli* RZ1032 (dut^-ung^-) to give single-stranded template DNA containing uracil in place of thymine. 0.5 ug DNA was mixed with 1 pmol each of the three phosphorylated oligonucleotides above and 1 pmol of an oligonucleotide VKPCRFOR (5' GCGGGCCTCTTCGCTATTACGC 3') [SEQ ID NO: 47] which anneals to the M13 template downstream of the insert DNA. The oligonucleotides were annealed to the template in 20 ul of 50 mM Tris-HCl pH 7.5, 25 mM $MgCl_2$, 63

mM NaCl by heating to 80°C for 5 minutes and cooling slowly to room temperature. dATP, dCTP, dGTP and dTTP were added to a 250 µM final concentration, DTT to 7 mM, ATP to 1 mM with 0.5 unit T7 DNA polymerase (USB) and 0.5 unit T4 DNA ligase (BRL) in the same buffer. The 30 µl reaction was incubated at room temperature for one hour and the DNA ethanol precipitated.

In order to nick the parental strand the DNA was dissolved in 50 µl of 60 mM Tris.HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA containing 1 unit uracil DNA glycosylase and incubated at 37°C for one hour before NaOH was added to 0.2 M and incubation continued at room temperature for 5 minutes. The DNA was ethanol precipitated, dissolved in 20 µl TE and the insert fragment amplified by PCR. The reaction mixture contained 2 µl mutant DNA, 0.5 µM each VKPCRFOR and VKPCRBACK (5' CTGTCTCAGGGCCAGGCGGTGA 3') [SEQ ID NO: 48], 250 µM each of dATP, dCTP, dGTP and dTTP, 10 mM Tris.HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% Tween-20, 0.01% gelatin, 0.01% NP40 and 2 units Thermalase (IBI) in 50 ul. Amplification was achieved with 15 cycles of 94°C, 30 seconds; 50°C, 30 seconds; 72°C, 1 minute; ending with 72°C, 5 minutes.

The product DNA was cloned into M13mp19 as a HindIII-BamHI fragment. Representative clones were sequenced and a clone mutant in all three areas was chosen and named M13VLPCR1.

HpaI and NcoI restriction sites were introduced at the ends of the B4VL by amplifying the DNA from an M13 clone (Example 18) using oligonucleotides VL3BACK (5' TCTGTGTTAACGCAGGCGCCCTCCGTG 3') [SEQ ID NO: 49] and VL1FOR (5' GGCTGACCCATGGCGATCAGTGTGGTC 3') [SEQ ID NO: 50] and Vent DNA polymerase as described above for the B4VH above. The

product DNA was purified, digested with HpaI and NcoI and cloned into similarly-digested M13VLPCR1 RF DNA. Clones containing the B4VL were identified by sequencing and the HindIII-BamHI insert of one such clone used to construct an expression vector, pSVhygB4BoVLHuVK, as described in Example 11.

The expression vectors were co-transfected into YB2/0 myeloma cells, transfectomas secreting antibody identified and a chimeric antibody B4BoVH/BoVL purified as described in Example 11. The chimeric antibody was compared to the B4 bovine antibody for binding to RSV-infected cell lysate in an ELISA. The method was essentially as described in Example 11 except that RSV-infected and uninfected Hep2 cell lysates were used. The reporter antibodies were goat anti-human IgG antibodies, HRPO-conjugated (Sera-Lab Ltd, Crawley Down, UK) and rabbit anti-bovine IgG antibodies, HRPO-conjugated (Sigma, Poole, UK), used as 1 in 1000 dilutions.

The bovine and chimeric (BoVH/BoVL) B4 antibodies bound to the infected cell lysate whereas an irrelevant humanized antibody did not. None of the antibodies reacted against the control lysate. It is not possible to draw a direct comparison between the bovine and chimaeric antibodies from this experiment as different reporter antibodies were used.

In a separate experiment comparing the conjugates, about 2.5 fold more bovine antibody than human antibody was required to obtain the same OD reading. Thus the bovine and chimeric antibodies are approximately equivalent in binding.

Example 20 - Humanized B4

A. B4 Humanized Heavy Chain

The B4VH was humanized by transferring the bovine CDRs onto human NEWM VH frameworks (Saul et al, 1978, cited above) using site-directed mutagenesis. The following

bovine framework residues (numbering as Kabat et al, (1987), cited above) were incorporated into the humanized VH alongside the CDRs (see Figure 10).

Phe27, Ser28, Leu29 - while not being part of the hypervariable region, these residues are part of the structural loop of CDR1 (Chothia and Lesk, J. Mol. Biol., 196:901-917 (1987)).

Leu48 - adjacent to CDR2, this residue has affected the binding of other reshaped antibodies.

Arg71 - this residue has been shown to be important in other reshaped examples and is involved in the packing of CDRs 1 and 2 (Tramontano *et al.*, J. Mol. Biol. 215:175-182 (1990)).

Lys94 - the amino acid at this position can affect the conformation of CDR3 by formation of a salt bridge (Chothia and Lesk (1987), cited above).

The template DNA was M13mp19-based and contained a VH gene comprising NEWM frameworks and irrelevant CDRs, similar to that described by Riechmann *et al.*, Nature, 332:323-327 (1988). The mutagenesis was carried out as described above for the construction of M13VLPCR1. The oligonucleotides employed were:

VHCDR1: 5' CTGTCTCACCCAGCTTACAGAATAGCTGCTCAATGAGAAG CCAGACAC 3' [SEQ ID NO: 51]

VHCDR2: 5' CATTGTCACTCTGGATTTCAGGGCTGGGTTATAATATATGATT CCGCCATTGCTTGCGTCTCCAAGCCACTCAAGACC 3' [SEQ ID NO: 52]

VHCDR3: 5' CAAGGACCCTTGGCCCCAGGCGTCGACATACTCGCCCTTGC GTCCAGTACAAGCATAACTTCCACTATCACCAACAGAACACTTTGCACAATA ATAGACCGC 3' [SEQ ID NO: 53]

and the universal M13/pUC-20 primer, 5' GTAAAACGACGGCCAGT 3' [SEQ ID NO: 54].

DNA encoding a VH containing all three B4 CDRs was subsequently excised from the M13 and cloned into the expression vector described for the chimeric VH in Examples 11 and 19 and resulting in pSVgptB4HuVHHuIgG1.

pSVgptB4HuVHHuIgG1 was co-transfected with the chimeric light chain vector, pSVhygB4BoVLHuVK as described in Example 11. The resulting partially humanized antibody B4HuVH/BoVL therefore contains a humanized B4 heavy chain (B4HuVH) with a B4 light chain chimeric B4BoVLHuVK. Cells secreting B4HuVH/BoVL antibody were expanded and antibody purified from 400ml conditioned medium.

The B4HuVH/BoVL antibody was compared to the chimeric antibody B4BoVH/BoVL in binding to RSV strain A2-infected cell lysate in an ELISA. This allowed assessment of the relative binding abilities of the chimeric and humanized heavy chains.

The humanized heavy chain HuVH binds to RSV-infected cell lysate, but is 2-3 fold deficient in binding relative to the chimeric heavy chain BoVH.

Additional murine residues were included to attempt to increase binding. The HuVH gene was mutated to encode the following changes: T at position 73, N at position 76 and F at position 78 to NSV. These residues are part of a ß-turn which forms a fourth loop at the antigen binding surface.

A HuVHNSV/BoVL antibody was produced and tested for binding to a lysate of cells infected with the Snook strain of RSV by ELISA. Inclusion of the RSV residues gave no advantage over the original HuVH.

One other difference between the BoVH and HuVH which might affect binding is the region spanning amino acids #67-70. It is anticipated that the inclusion of the bovine B4 residues L at position 67 and I at position 69 are more

likely to influence the antigen interaction as their side chains pack inside the domain. Additionally the block change to L at position 67, G at position 68, I at position 69 and T at position 70 is also anticipated to be advantageous.

B. B4 Humanized Light Chain

A humanized version of the B4 light chain B4HuVL was constructed by site-directed mutagenesis of the bovine B4VL frameworks to give frameworks of the human KOL lambda variable region (see Figure 11). Cells were selected for the presence of the gpt gene which is found on the heavy chain expression vector.

Northern blotting was used to determine if the HuVL RNA was of the correct size. Total RNA was prepared from BoVH/HuVL and BoVH/HuVL FR4 transfectomas and from BoVH/BoVL transfectomas and untransfected YB2/0 as positive and negative controls. Initial results using BoVL and HuVL probes show bands of approximately the same size for all three species of light chain. In a similar investigation cDNA was prepared from each cell line and PCRs carried out using a constant region primer and VL3BACK. Again the same sized product was obtained for all three species of light chain, indicating no major splicing problem.

Two more humanized light chain constructs - a human REI kappa framework-based version of the light chain and a CDR-grafted light chain with frameworks of the human KIM46L lambda chain, may be made using the actual nucleotide sequence of the KIM46L VL gene (Cairns et al, J. Immunol., 143:685-691 (1989)).

This is believed to be the first example of a bovine antibody being humanized. The lack of bovine variable region sequences in the databases meant that it was

difficult to design primers for PCR amplification and thus to isolate DNA for the initial cloning and sequencing.

Example 21 - Effect of RSHZ19 and RSBV04 administered therapeutically to RSV infected mice

Groups of five mice were inoculated intranasally with approximately 10^5 PFU of the A2 strain of RSV and were treated on day 4 of infection with different amounts of RSBV04 administered intraperitoneally either alone or with 0.5 mg/kg RSHZ19, as shown in Table 10 below. Mice were killed five days after RSV challenge, and the level of virus in the lungs determined on CK cells. The results are shown in Table 10 and indicated that the effect of combined therapy with RSHZ19 and RSBV04 is additive rather than synergistic.

TABLE 10

Group	Dose (mg/kg)[1] RSHZ19	RSBV04	RSV titer in lungs log10 PFU/g
A	0.5	--	3.6 ± 0.7
B	0.5	0.5	2.2 ± 0.6
C	0.5	0.25	2.3 ± 0.8
D	0.5	0.125	2.6 ± 0.9
E	--	1.0	2.1 ± 0.8
F	--	0.75	2.3 ± 0.6
G	--	0.625	2.6 ± 0.7
H	--	--	4.8 ± 0.1

[1]mAbs administered IP on day 4 of infection.

Numerous modifications and variations of the present invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. Such modification and alterations are believed to be encompassed in the scope of the claims appended hereto.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Taylor, Geraldine
Stott, Edward J.

(ii) TITLE OF INVENTION: Novel Antibodies for Treatment and Prevention of Respiratory Syncytial Virus Infection in Animals and Man

(iii) NUMBER OF SEQUENCES: 59

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: SmithKline Beecham Corporation - Corporate Patents
(B) STREET: 709 Swedeland Road
(C) CITY: King of Prussia
(D) STATE: PA
(E) COUNTRY: USA
(F) ZIP: 19406-2799

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: WO
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: GB 9207479.8
(B) FILING DATE: 06-APR-1992

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Jervis, Herbert H.
(B) REGISTRATION NUMBER: 31,171
(C) REFERENCE/DOCKET NUMBER: P50153

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 215-270-5019
(B) TELEFAX: 215-270-5090

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 112 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

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

Ser Val Val Thr Gln Glu Pro Ser Val Ser Gly Ser Leu Gly Gln
1 5 10 15
Arg Val Ser Ile Thr Cys Ser Gly Ser Ser Ser Asn Ile Gly Arg
20 25 30
Trp Gly Val Asn Trp Tyr Gln Gln Val Pro Gly Ser Gly Leu Arg
35 40 45
Thr Ile Ile Tyr Tyr Glu Ser Ser Arg Pro Ser Gly Val Pro Asp
50 55 60
Arg Phe Ser Gly Ser Lys Ser Gly Asn Thr Ala Thr Leu Thr Ile
65 70 75
Ser Ser Leu Gln Ala Glu Asp Glu Ala Asp Tyr Phe Cys Ala Thr
80 85 90
Gly Asp Tyr Asn Ile Ala Val Phe Gly Ser Gly Thr Thr Leu Ile
95 100 105
Val Met Gly Gln Pro Lys Ser
110

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 116 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

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

Ser Val Val Thr Gln Gln Pro Ser Val Ser Gly Ser Leu Gly Gln
1 5 10 15
Arg Val Ser Ile Thr Cys Ser Gly Ser Ser Asp Asn Ile Gly Ile
20 25 30
Phe Ala Val Gly Trp Tyr Gln Gln Val Pro Gly Ser Gly Leu Arg
35 40 45
Thr Ile Ile Tyr Gly Asn Thr Lys Arg Pro Ser Gly Val Pro Asp
50 55 60
Arg Phe Ser Gly Ser Lys Ser Gly Asn Thr Ala Thr Leu Thr Ile
65 70 75
Asn Ser Leu Gln Ala Glu Asp Glu Ala Asp Tyr Phe Cys Val Cys
80 85 90
Gly Glu Ser Lys Ser Ala Thr Pro Val Phe Gly Gly Gly Thr Thr
95 100 105
Leu Thr Val Leu Ser Gln Pro Lys Ser Pro Pro
110 115

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 137 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

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

Gln Val Xaa Leu Gln Glu Ser Gly Pro Ser Leu Val Lys Pro Ser
1 5 10 15
Gln Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Phe Ser Leu Ser
20 25 30
Ser Tyr Ser Val Ser Trp Val Arg Gln Ala Pro Gly Lys Thr Leu
35 40 45
Glu Trp Leu Gly Asp Ala Ser Asn Gly Gly Ile Ile Tyr Tyr Asn
50 55 60
Pro Ala Leu Lys Ser Arg Leu Gly Ile Thr Arg Asp Asn Ser Lys
65 70 75
Ser Gln Val Ser Leu Ser Leu Asn Thr Ile Thr Pro Glu Asp Thr
85 85 90
Ala Thr Tyr Tyr Cys Ala Lys Cys Ser Val Gly Asp Ser Gly Ser
95 100 105
Tyr Ala Cys Thr Gly Arg Lys Gly Glu Tyr Val Asp Ala Trp Gly
110 115 120
Gln Gly Leu Leu Val Thr Val Ser Ser Ala Ser Thr Thr Ala Pro
125 130 135

100

Lys Val

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 141 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

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

```
Gln Val Xaa Leu Gln Gln Ser Gly Pro Ser Leu Val Lys Pro Ser
 1               5                  10                  15
Gln Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Leu Ser Leu Ser
            20                  25                  30
Asp His Asn Val Gly Trp Ile Arg Gln Ala Pro Gly Lys Ala Leu
        35                  40                  45
Glu Trp Leu Gly Val Ile Tyr Lys Glu Gly Asp Lys Asp Tyr Asn
    50                  55                  60
Pro Ala Leu Lys Ser Arg Leu Ser Ile Thr Lys Asp Asn Ser Lys
65                  70                  75
Ser Gln Val Ser Leu Ser Leu Ser Ser Val Thr Thr Glu Asp Thr
                80                  85                  90
Ala Thr Tyr Tyr Cys Ala Thr Leu Gly Cys Tyr Phe Val Glu Gly
            95                  100                 105
Val Gly Tyr Asp Cys Thr Tyr Gly Leu Gln His Thr Thr Phe Xaa
        110                 115                 120
Asp Ala Trp Gly Gln Gly Leu Leu Val Thr Val Ser Ser Ala Ser
    125                 130                 135
Thr Thr Ala Pro Lys Val
140
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 129 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

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

```
Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Arg Pro Ser
 1               5                  10                  15
```

101

Gln Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Phe Ser Leu Ser
20 25 30
Ser Tyr Ser Val Ser Trp Val Arg Gln Pro Pro Gly Arg Gly Leu
35 40 45
Glu Trp Leu Gly Asp Ala Ser Asn Gly Gly Ile Ile Tyr Tyr Asn
50 55 60
Pro Ala Leu Lys Ser Arg Val Thr Met Leu Arg Asp Thr Ser Lys
65 70 75
Asn Gln Phe Ser Leu Arg Leu Ser Ser Val Thr Ala Ala Asp Thr
80 85 90
Ala Val Tyr Tyr Cys Ala Lys Cys Ser Val Gly Asp Ser Gly Ser
95 100 105
Tyr Ala Cys Thr Gly Arg Lys Gly Glu Tyr Val Asp Ala Trp Gly
110 115 120
Gln Gly Thr Thr Val Thr Val Ser Ser
125

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 109 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

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

Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
1 5 10 15
Gly Asp Arg Val Thr Ile Thr Cys Ser Gly Ser Ser Ser Asn Ile
20 25 30
Gly Arg Trp Gly Val Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala
35 40 45

Pro Lys Leu Leu Ile Tyr Tyr Glu Ser Ser Arg Pro Ser Gly Val
50 55 60
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Phe
65 70 75
Thr Ile Ser Ser Leu Gln Pro Glu Asp Ile Ala Thr Tyr Tyr Cys
80 85 90
Ala Thr Gly Asp Tyr Asn Ile Ala Val Phe Gly Gln Gly Thr Lys
95 100 105
Leu Glu Ile Lys

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 133 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

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

Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Arg Pro Ser
1 5 10 15
Gln Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Leu Ser Leu Ser
20 25 30
Asp His Asn Val Gly Trp Val Arg Gln Pro Pro Gly Arg Gly Leu
35 40 45
Glu Trp Leu Gly Val Ile Tyr Lys Glu Gly Asp Lys Asp Tyr Asn
50 55 60
Pro Ala Leu Lys Ser Arg Val Thr Met Leu Lys Asp Thr Ser Lys
65 70 75
Asn Gln Phe Ser Leu Arg Leu Ser Ser Val Thr Ala Ala Asp Thr
80 85 90
Ala Val Tyr Tyr Cys Ala Thr Leu Gly Cys Tyr Phe Val Glu Gly
95 100 105
Val Gly Tyr Asp Cys Thr Tyr Gly Leu Gln His Thr Thr Phe Xaa
110 115 120
Asp Ala Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
125 130

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 111 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

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

Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val
1 5 10 15
Gly Asp Arg Val Thr Ile Thr Cys Ser Gly Ser Ser Asp Asn Ile
20 25 30
Gly Ile Phe Ala Val Gly Trp Tyr Gln Gln Lys Pro Gly Lys Ala
35 40 45

Pro Lys Leu Leu Ile Tyr Gly Asn Thr Lys Arg Pro Ser Gly Val
50 55 60
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Phe
65 70 75
Thr Ile Ser Ser Leu Gln Pro Glu Asp Ile Ala Thr Tyr Tyr Cys
80 85 90
Val Cys Gly Glu Ser Lys Ser Ala Thr Pro Val Phe Gly Gln Gly
95 100 105
Thr Lys Leu Glu Ile Lys
110

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 348 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

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

CAGGTCCAGC TGCAGSAGTC WGGGACAGAG CTTGAGAGGT CAGGGGCCTC 50

AGTCAAGTTG TCCTGCACAG CTTCTGGCTT CAACATTAAA GACTACTATA 100

TGCACTGGAT GAAGCAGAGG CCTGACCAGG GCCTGGAGTG GATTGGATGG 150

ATTGATCCTG AGAATGATGA TGTTCAATAT GCCCCGAAGT TCCAGGGCAA 200

GGCCACTATG ACTGCAGACA CGTCCTCCAA CACAGCCTAC CTGCAGCTCA 250

CCAGCCTGAC ATTTGAGGAC ACTGCCGTCT ATTTCTGTAA TTCATGGGGG 300

AGTGACTTTG ACCACTGGGG CCAAGGGACC ACGGTCACCG TCTCCTCA 348

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 116 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 6
(D) OTHER INFORMATION: /note= "amino acid at position 6 can be either glu or gln"

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

```
Gln Val Gln Leu Gln Xaa Ser Gly Thr Glu Leu Glu Arg Ser Gly
 1               5                  10                  15
Ala Ser Val Lys Leu Ser Cys Thr Ala Ser Gly Phe Asn Ile Lys
            20                  25                  30
Asp Tyr Tyr Met His Trp Met Lys Gln Arg Pro Asp Gln Gly Leu
        35                  40                  45
Glu Trp Ile Gly Trp Ile Asp Pro Glu Asn Asp Asp Val Gln Tyr
    50                  55                  60
Ala Pro Lys Phe Gln Gly Lys Ala Thr Met Thr Ala Asp Thr Ser
65                  70                  75
Ser Asn Thr Ala Tyr Leu Gln Leu Thr Ser Leu Thr Phe Glu Asp
            80                  85                  90
Thr Ala Val Tyr Phe Cys Asn Ser Trp Gly Ser Asp Phe Asp His
        95                  100                 105
Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
    110                 115
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 337 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..333

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

```
GAC ATT CAG CTG ACC CAG TCT CCA CTC TCC CTG CCT GTC ACT   42
Asp Ile Gln Leu Thr Gln Ser Pro Leu Ser Leu Pro Val Thr
 1               5                  10

CTT GGA GAT CAA GCC TCC ATC TCT TGC AGA TCT AGT CAG ACC   84
Leu Gly Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Thr
 15                  20                  25
```

```
                                                    105
CTT GTA CAT ACT GAT GGA AAC ACC TAT TTA GAA TGG TTT CTG  126
Leu Val His Thr Asp Gly Asn Thr Tyr Leu Glu Trp Phe Leu
     30                  35                  40
CAG AAA CCA GGC CAG TCT CCA AAG CTC CTG ATC TAC AGA GTT  168
Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile Tyr Arg Val
          45                  50                  55
TCC AAC CGA TTT TCT GGG GTC CCA GAC AGG TTC AGT GGC AGT  210
Ser Asn Arg Phe Ser Gly Val Pro Asp Arg Phe Ser Gly Ser
               60                  65                  70
GGA TCA GGG ACA GAT TTC ACA CTC AAG ATC AGC AGA GTG GAG  252
Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile Ser Arg Val Glu
                    75                  80

GCT GAG GAT CTG GGA GTT TAT TTC TGC TTT CAA GGT TCA CAT  294
Ala Glu Asp Leu Gly Val Tyr Phe Cys Phe Gln Gly Ser His
 85                  90                  95

CTT CCT CGG ACG TTC GGT GGA GGG ACC AAG CTG GAG ATC TAAC 337
Leu Pro Arg Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile
     100                 105                 110
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 111 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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

```
Asp Ile Gln Leu Thr Gln Ser Pro Leu Ser Leu Pro Val Thr Leu
 1               5                  10                  15
Gly Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Thr Leu Val
            20                  25                  30
```

His Thr Asp Gly Asn Thr Tyr Leu Glu Trp Phe Leu Gln Lys Pro
35 40 45
Gly Gln Ser Pro Lys Leu Leu Ile Tyr Arg Val Ser Asn Arg Phe
50 55 60
Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp
65 70 75
Phe Thr Leu Lys Ile Ser Arg Val Glu Ala Glu Asp Leu Gly Val
80 85 90
Tyr Phe Cys Phe Gln Gly Ser His Leu Pro Arg Thr Phe Gly Gly
95 100 105
Gly Thr Lys Leu Glu Ile
110

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 116 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

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

Gln Val Gln Leu Gln Glu Ser Gly Thr Glu Leu Glu Arg Ser Gly
1 5 10 15
Ala Ser Val Lys Leu Ser Cys Thr Ala Ser Gly Phe Asn Ile Lys
20 25 30

Asp Tyr Tyr Met His Trp Met Lys Gln Arg Pro Asp Gln Gly Leu
35 40 45
Glu Trp Ile Gly Trp Ile Asp Pro Glu Asn Asp Asp Val Gln Tyr
50 55 60
Ala Pro Lys Phe Gln Gly Lys Ala Thr Met Thr Ala Asp Thr Ser
65 70 75
Ser Asn Thr Ala Tyr Leu Gln Leu Thr Ser Leu Thr Phe Glu Asp
80 85 90
Thr Ala Val Tyr Phe Cys Asn Ser Trp Gly Ser Asp Phe Asp His
95 100 105
Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
110 115

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 116 amino acids

(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

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

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

Thr Ala Val Tyr Tyr Cys Ala Arg Trp Gly Ser Asp Phe Asp His
            95                  100                 105
Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
        110                 115
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 116 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

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

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

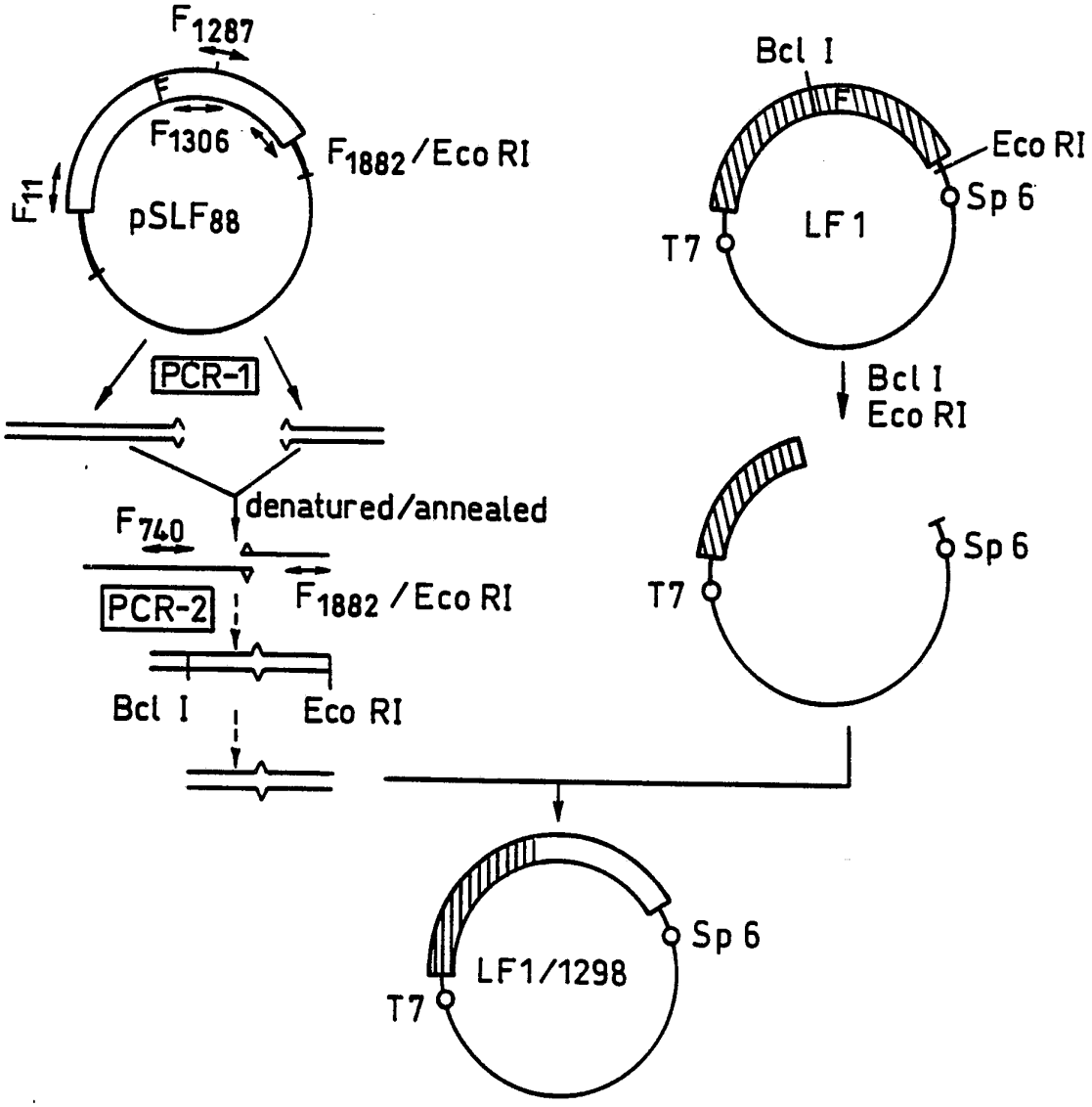

F1287
F1306
F1882/Eco RI
F11
pSLF88
PCR-1
denatured/annealed
F740
F1882/Eco RI
PCR-2
Bcl I
Eco RI
Bcl I
Eco RI
Sp 6
T7
LF 1
Bcl I
Eco RI
T7
Sp 6
LF1/1298
Sp 6
T7

Fig. 1

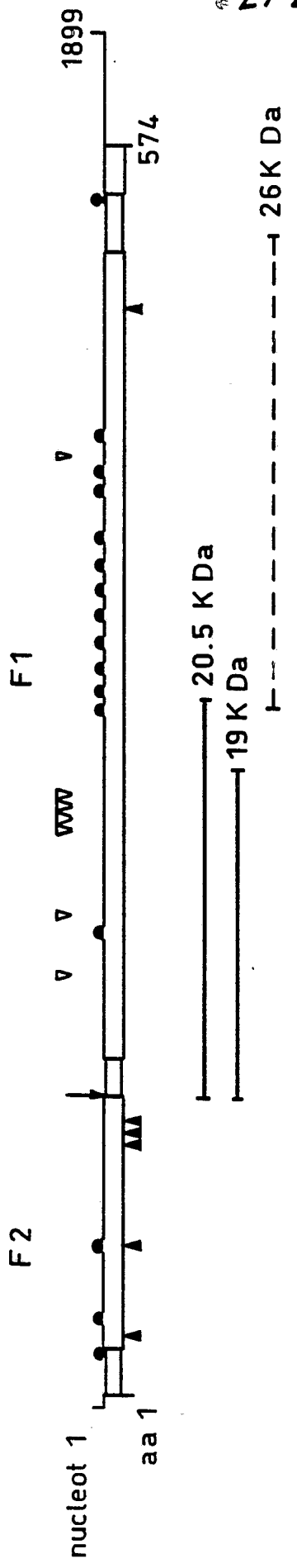


Fig.2

FIGURE 3A

partial B4 antibody variable light chain
amino acid sequence SEQ ID NO: 1

partial B13/B14 antibody variable light chain
amino acid sequence SEQ ID NO: 2

```
          1               5                  10
B4VH:   Ser Val Val Thr Gln Glu Pro Ser Val Ser Gly Ser
B13VH:  Ser Val Val Thr Gln Gln Pro Ser Val Ser Gly Ser

                15                  20
B4VH:   Leu Gly Gln Arg Val Ser Ile Thr Cys Ser Gly Ser
B13VH:  Leu Gly Gln Arg Val Ser Ile Thr Cys Ser Gly Ser

          25                  30                  35
B4VH:   Ser Ser Asn Ile Gly Arg Trp Gly Val Asn Trp Tyr
B13VH:  Ser Asp Asn Ile Gly Ile Phe Ala Val Gly Trp Tyr

                    40                  45
B4VH:   Gln Gln Val Pro Gly Ser Gly Leu Arg Thr Ile Ile
B13VH:  Gln Gln Val Pro Gly Ser Gly Leu Arg Thr Ile Ile

              50                  55                  60
B4VH:   Tyr Tyr Glu Ser Ser Arg Pro Ser Gly Val Pro Asp
B13VH:  Tyr Gly Asn Thr Lys Arg Pro Ser Gly Val Pro Asp
```

FIGURE 3B

```
                                65                  70
B4VH:   Arg Phe Ser Gly Ser Lys Ser Gly Asn Thr Ala Thr
B13VH:  Arg Phe Ser Gly Ser Lys Ser Gly Asn Thr Ala Thr

                      75                  80
B4VH:   Leu Thr Ile Ser Ser Leu Gln Ala Glu Asp Glu Ala
B13VH:  Leu Thr Ile Asn Ser Leu Gln Ala Glu Asp Glu Ala

          85                  90                  95
B4VH:   Asp Tyr Phe Cys Ala Thr Gly Asp Tyr Asn Ile Ala
B13VH:  Asp Tyr Phe Cys Val Cys Gly Glu Ser Lys Ser Ala

                     100                 105
B4VH:    -   -  Val Phe Gly Ser Gly Thr Thr Leu Ile Val
B13VH:  Thr Pro Val Phe Gly Gly Gly Thr Thr Leu Thr Val

            110
B4VH:   Met Gly Gln Pro Lys Ser
B13VH:  Leu Ser Gln Pro Lys Ser Pro Pro
```

FIGURE 4A

partial B4 antibody variable heavy chain
amino acid sequence SEQ ID NO: 3

partial B13/B14 antibody variable heavy chain
amino acid sequence SEQ ID NO: 4

```
          1               5                  10
B4VH:   Gln Val Xaa Leu Gln Glu Ser Gly Pro Ser Leu Val Lys Pro
B13VH:  Gln Val Xaa Leu Gln Gln Ser Gly Pro Ser Leu Val Lys Pro

          15                  20                  25
B4VH:   Ser Gln Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Phe Ser
B13VH:  Ser Gln Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Leu Ser

             30                  35                  40
B4VH:   Leu Ser Ser Tyr Ser Val Ser Trp Val Arg Gln Ala Pro Gly
B13VH:  Leu Ser Asp His Asn Val Gly Trp Ile Arg Gln Ala Pro Gly

                45                  50                  55
B4VH:   Lys Thr Leu Glu Trp Leu Gly Asp Ala Ser Asn Gly Gly Ile
B13VH:  Lys Ala Leu Glu Trp Leu Gly Val Ile Tyr Lys Glu Gly Asp

                   60                  65                  70
B4VH:   Ile Tyr Tyr Asn Pro Ala Leu Lys Ser Arg Leu Gly Ile Thr
B13VH:  Lys Asp Tyr Asn Pro Ala Leu Lys Ser Arg Leu Ser Ile Thr
```

FIGURE 4B

```
                                75                  80
B4VH:   Arg Asp Asn Ser Lys Ser Gln Val Ser Leu Ser Leu Asn Thr
B13VH:  Lys Asp Asn Ser Lys Ser Gln Val Ser Leu Ser Leu Ser Ser

          85                  90                  95
B4VH:   Ile Thr Pro Glu Asp Thr Ala Thr Tyr Tyr Cys Ala Lys -
B13VH:  Val Thr Thr Glu Asp Thr Ala Thr Tyr Tyr Cys Ala Thr Leu

             100                 105                 110
B4VH:    - Cys Ser Val Gly Asp Ser Gly Ser Tyr Ala Cys Thr -
B13VH:  Gly Cys Tyr Phe Val Glu Gly Val Gly Tyr Asp Cys Thr Tyr

                115                 120                 125
B4VH:   Gly - Arg Lys Gly Glu Tyr Val Asp Ala Trp Gly Gln Gly
B13VH:  Gly Leu Gln His Thr Thr Phe Xaa Asp Ala Trp Gly Gln Gly

                    130                 135                 140
B4VH:   Leu Leu Val Thr Val Ser Ser Ala Ser Thr Thr Ala Pro Lys
B13VH:  Leu Leu Val Thr Val Ser Ser Ala Ser Thr Thr Ala Pro Lys

B4VH:   Val
B13VH:  Val
```

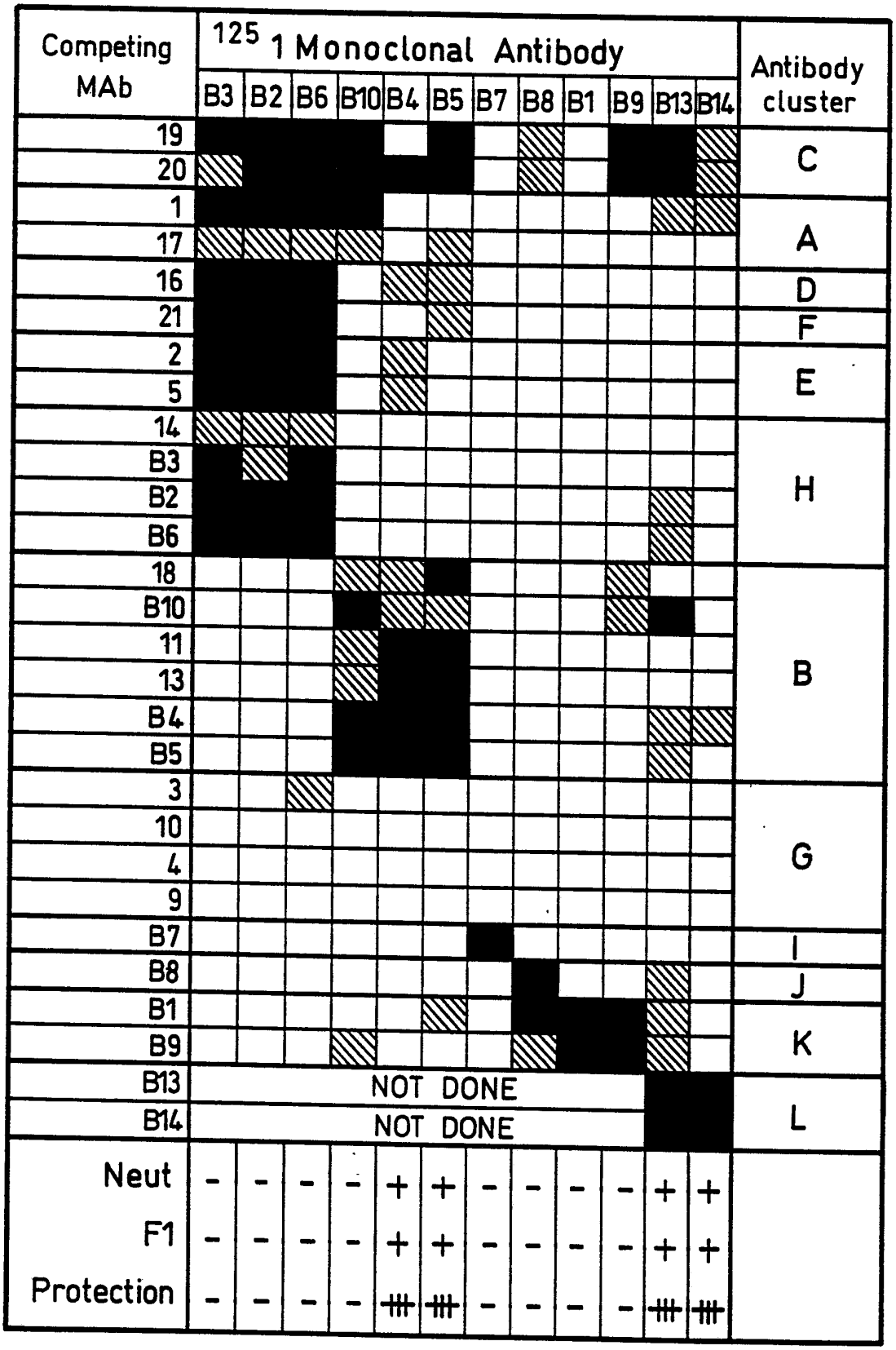


Fig. 5 Competitive binding of anti-F mAbs

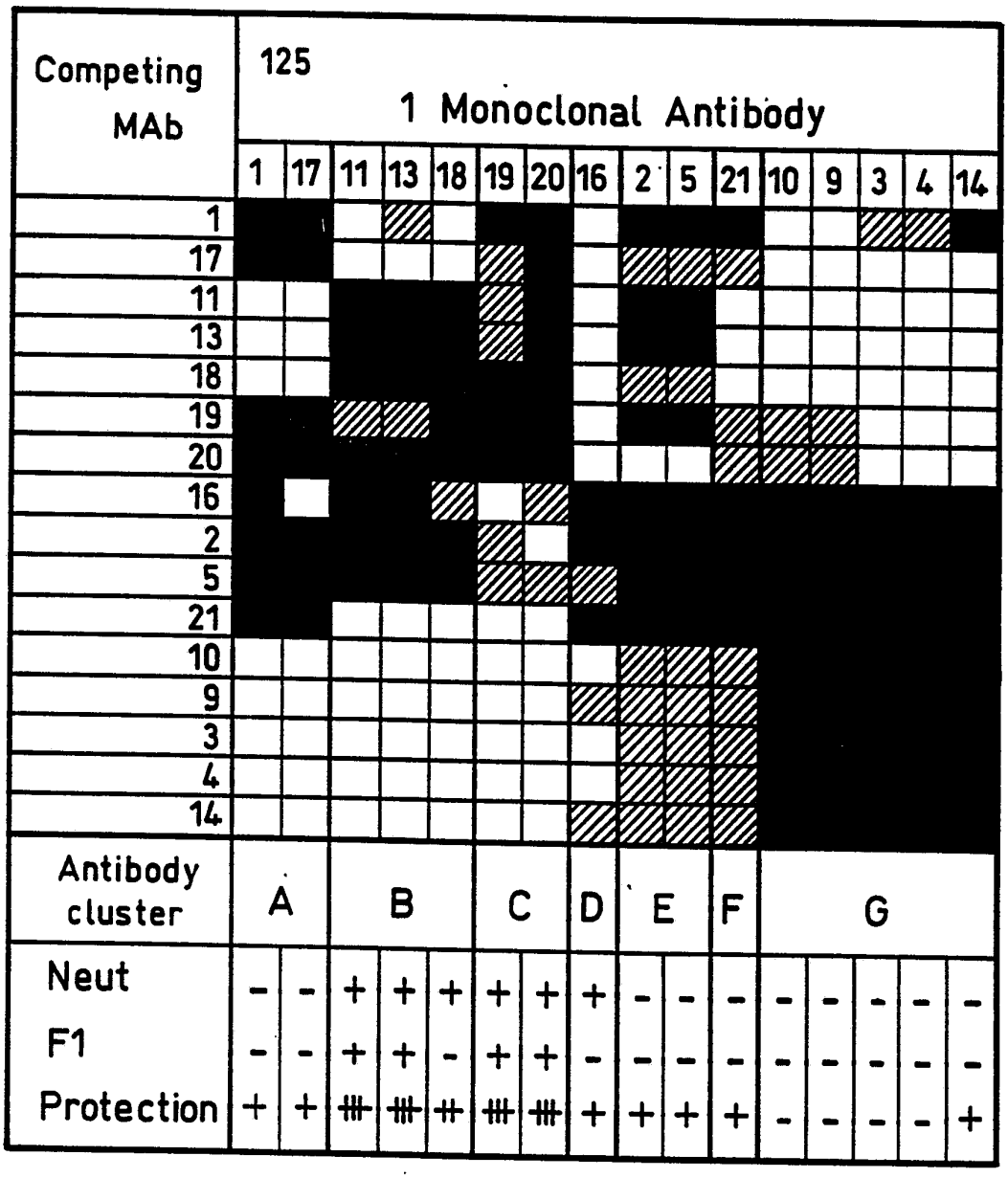


Fig. 6 Competitive binding of anti-F mAbs

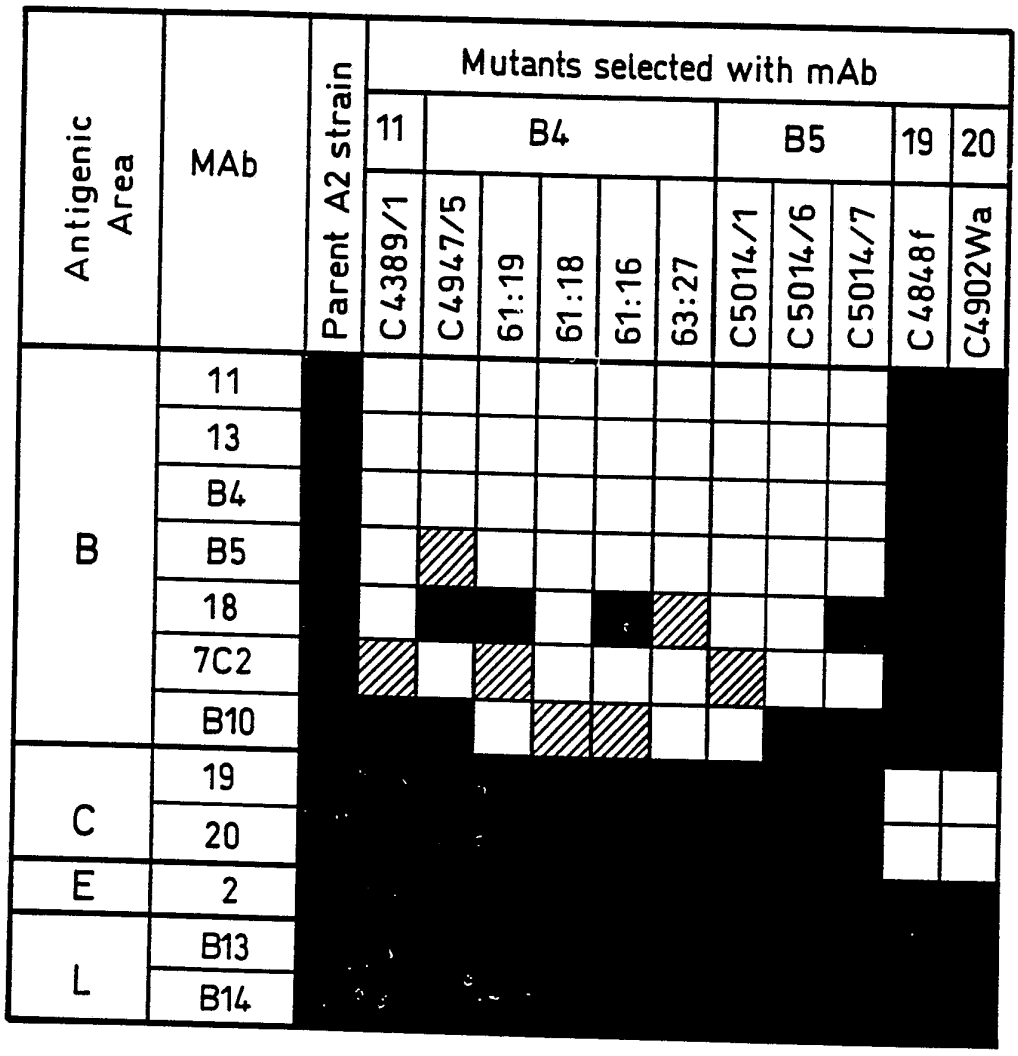


Fig. 7 Binding of F mAbs to antibody-escape mutants of RSV

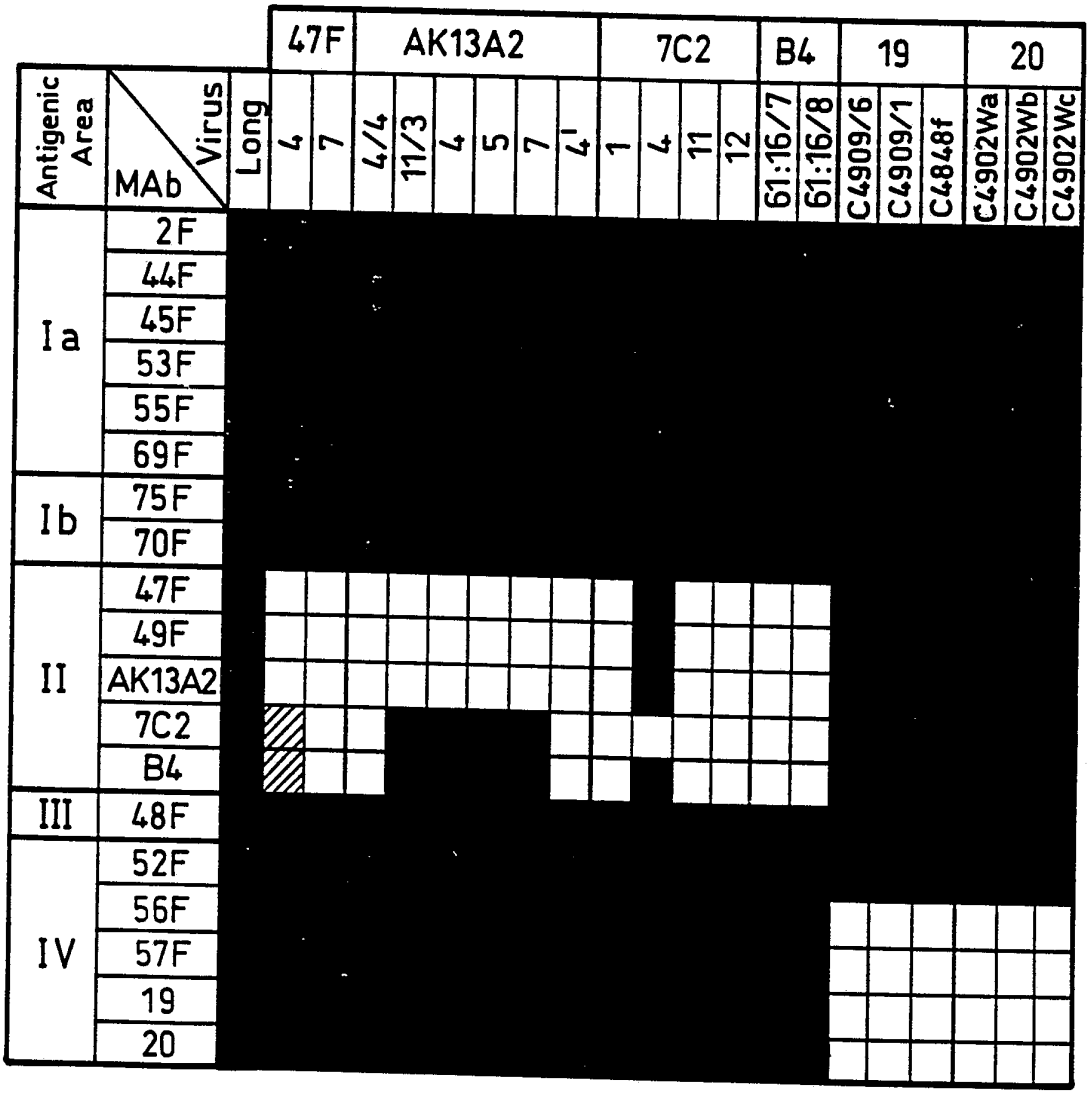

47F
AK13A2
7C2
B4
19
20
Antigenic Area
MAb
Virus
Long
4
7
4/4
11/3
4
5
7
4'
1
4
11
12
61:16/7
61:16/8
C4909/6
C4909/1
C4848f
C4902Wa
C4902Wb
C4902Wc
Ia
2F
44F
45F
53F
55F
69F
Ib
75F
70F
II
47F
49F
AK13A2
7C2
B4
III
48F
IV
52F
56F
57F
19
20

Fig. 8

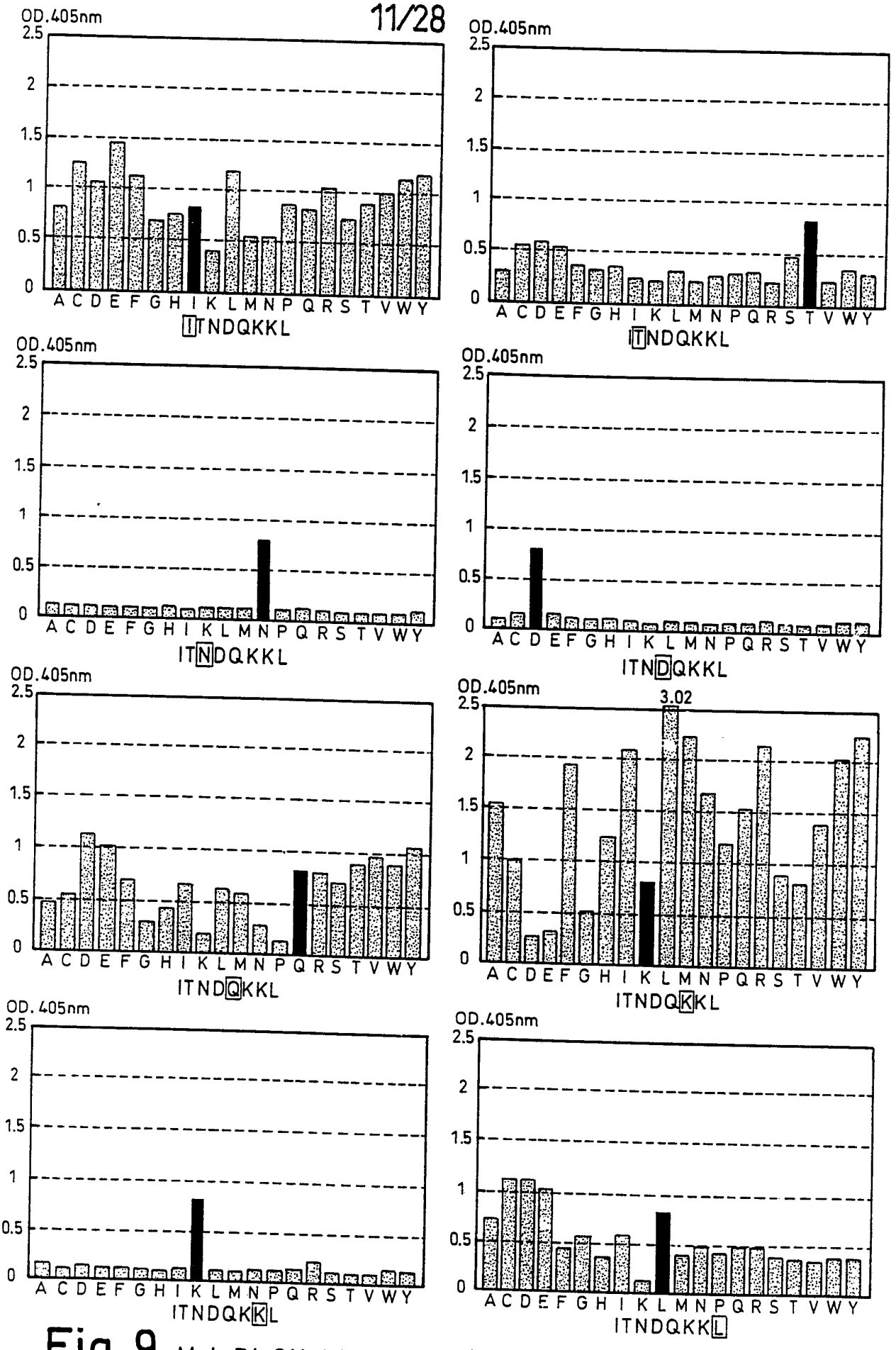


Fig.9 Mab B4 ON REPLACEMENT NET 266-273

FIGURE 10

B4 HuVH SEQ ID NO: 5

```
Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Arg Pro Ser Gln
 1               5                  10                  15

Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Phe Ser Leu Ser Ser Tyr
            20                  25                  30

Ser Val Ser Trp Val Arg Gln Pro Pro Gly Arg Gly Leu Glu Trp Leu
        35                  40                  45

Gly Asp Ala Ser Asn Gly Gly Ile Ile Tyr Tyr Asn Pro Ala Leu Lys
    50                  55                  60

Ser Arg Val Thr Met Leu Arg Asp Thr Ser Lys Asn Gln Phe Ser Leu
65                  70                  75                  80

Arg Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr Cys Ala
                85                  90                  95

Lys Cys Ser Val Gly Asp Ser Gly Ser Tyr Ala Cys Thr Gly Arg Lys
            100                 105                 110

Gly Glu Tyr Val Asp Ala Trp Gly Gln Gly Thr Thr Val Thr Val Ser
        115                 120                 125

Ser
```

FIGURE 11

B4 HuVK SEQ ID NO: 6

```
Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
 1               5                  10                  15

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

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

Leu Ile Tyr Tyr Glu Ser Ser Arg Pro Ser Gly Val Pro Ser Arg Phe
    50                  55                  60

Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu
65                  70                  75                  80

Gln Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Ala Thr Gly Asp Tyr Asn
                85                  90                  95

Ile Ala Val Phe Gly Gln Gly Thr Lys Leu Glu Ile Lys
            100                 105
```

FIGURE 12A

B13/B14 HuVH SEQ ID NO: 7

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

FIGURE 12B

Thr Leu Gly Cys Tyr Phe Val Glu Gly Val Gly Tyr Asp Cys Thr Tyr
100 105 110

Gly Leu Gln His Thr Thr Phe Xaa Asp Ala Trp Gly Gln Gly Thr Thr
115 120 125

Val Thr Val Ser Ser
130

FIGURE 13

B13/B14 HuVK SEQ ID NO: 8

Asp	Ile	Gln	Leu	Thr	Gln	Ser	Pro	Ser	Ser	Leu	Ser	Ala	Ser	Val	Gly
1				5				10						15	
Asp	Arg	Val	Thr	Ile	Thr	Cys	Ser	Gly	Ser	Ser	Asp	Asn	Ile	Gly	Ile
			20				25					30			
Phe	Ala	Val	Gly	Trp	Tyr	Gln	Gln	Lys	Pro	Gly	Lys	Ala	Pro	Lys	Leu
		35					40					45			
Leu	Ile	Tyr	Gly	Asn	Thr	Lys	Arg	Pro	Ser	Gly	Val	Pro	Ser	Arg	Phe
	50					55					60				
Ser	Gly	Ser	Gly	Ser	Gly	Thr	Asp	Phe	Thr	Phe	Thr	Ile	Ser	Ser	Leu
65				70					75					80	
Gln	Pro	Glu	Asp	Ile	Ala	Thr	Tyr	Tyr	Cys	Val	Cys	Gly	Glu	Ser	Lys
				85				90					95		
Ser	Ala	Thr	Pro	Val	Phe	Gly	Gln	Gly	Thr	Lys	Leu	Glu	Ile	Lys	
			100				105					110			

FIGURE 14A

DNA sequence of the RSV19 heavy chain variable region SEQ ID NO: 9

amino acid sequence of the RSV19 heavy chain variable region SEQ ID NO: 10

```
CAG GTC CAG CTG CAG SAG TCW GGG ACA GAG CTT GAG AGG TCA GGG
Gln Val Gln Leu Gln Xaa Ser Gly Thr Glu Leu Glu Arg Ser Gly

GCC TCA GTC AAG TTG TCC TGC ACA GCT TCT GGC TTC AAC ATT AAA
Ala Ser Val Lys Leu Ser Cys Thr Ala Ser Gly Phe Asn Ile Lys

          1
GAC TAC TAT ATG CAC TGG ATG AAG CAG AGG CCT GAC CAG GGC CTG
Asp Tyr Tyr Met His Trp Met Lys Gln Arg Pro Asp Gln Gly Leu

                                                2
GAG TGG ATT GGA TGG ATT GAT CCT GAG AAT GAT GAT GTT CAA TAT
Glu Trp Ile Gly Trp Ile Asp Pro Glu Asn Asp Asp Val Gln Tyr

GCC CCG AAG TTC CAG GGC AAG GCC ACT ATG ACT GCA GAC ACG TCC
Ala Pro Lys Phe Gln Gly Lys Ala Thr Met Thr Ala Asp Thr Ser
```

FIGURE 14B

TCC AAC ACA GCC TAC CTG CAG CTC ACC AGC CTG ACA TTT GAG GAC
Ser Asn Thr Ala Tyr Leu Gln Leu Thr Ser Leu Thr Phe Glu Asp

3

ACT GCC GTC TAT TTC TGT AAT TCA TGG GGG AGT GAC TTT GAC CAC
Thr Ala Val Tyr Phe Cys Asn Ser Trp Gly Ser Asp Phe Asp His

TGG GGC CAA GGG ACC ACG GTC ACC GTC TCC TCA
Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser

FIGURE 15A

DNA sequence of the RSV19 light chain variable region SEQ ID NO: 11

amino acid sequence of the RSV19 light chain variable region SEQ ID NO: 12

GAC ATT CAG CTG ACC CAG TCT CCA CTC TCC CTG CCT GTC ACT CTT GGA
Asp Ile Gln Leu Thr Gln Ser Pro Leu Ser Leu Pro Val Thr Leu Gly

1

GAT CAA GCC TCC ATC TCT TGC AGA TCT AGT CAG ACC CTT GTA CAT ACT
Asp Gln Ala Ser Ile Ser Cys Arg Ser Ser Gln Thr Leu Val His Thr

GAT GGA AAC ACC TAT TTA GAA TGG TTT CTG CAG AAA CCA GGC CAG TCT
Asp Gly Asn Thr Tyr Leu Glu Trp Phe Leu Gln Lys Pro Gly Gln Ser

2

CCA AAG CTC CTG ATC TAC AGA GTT TCC AAC CGA TTT TCT GGG GTC CCA
Pro Lys Leu Leu Ile Tyr Arg Val Ser Asn Arg Phe Ser Gly Val Pro

GAC AGG TTC AGT GGC AGT GGA TCA GGG ACA GAT TTC ACA CTC AAG ATC
Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Lys Ile

FIGURE 15B

AGC AGA GTG GAG GCT GAG GAT CTG GGA GTT TAT TTC TGC TTT CAA GGT
Ser Arg Val Glu Ala Glu Asp Leu Gly Val Tyr Phe Cys Phe Gln Gly

3

TCA CAT CTT CCT CGG ACG TTC GGT GGA GGG ACC AAG CTG GAG ATC
Ser His Leu Pro Arg Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile

TAAC

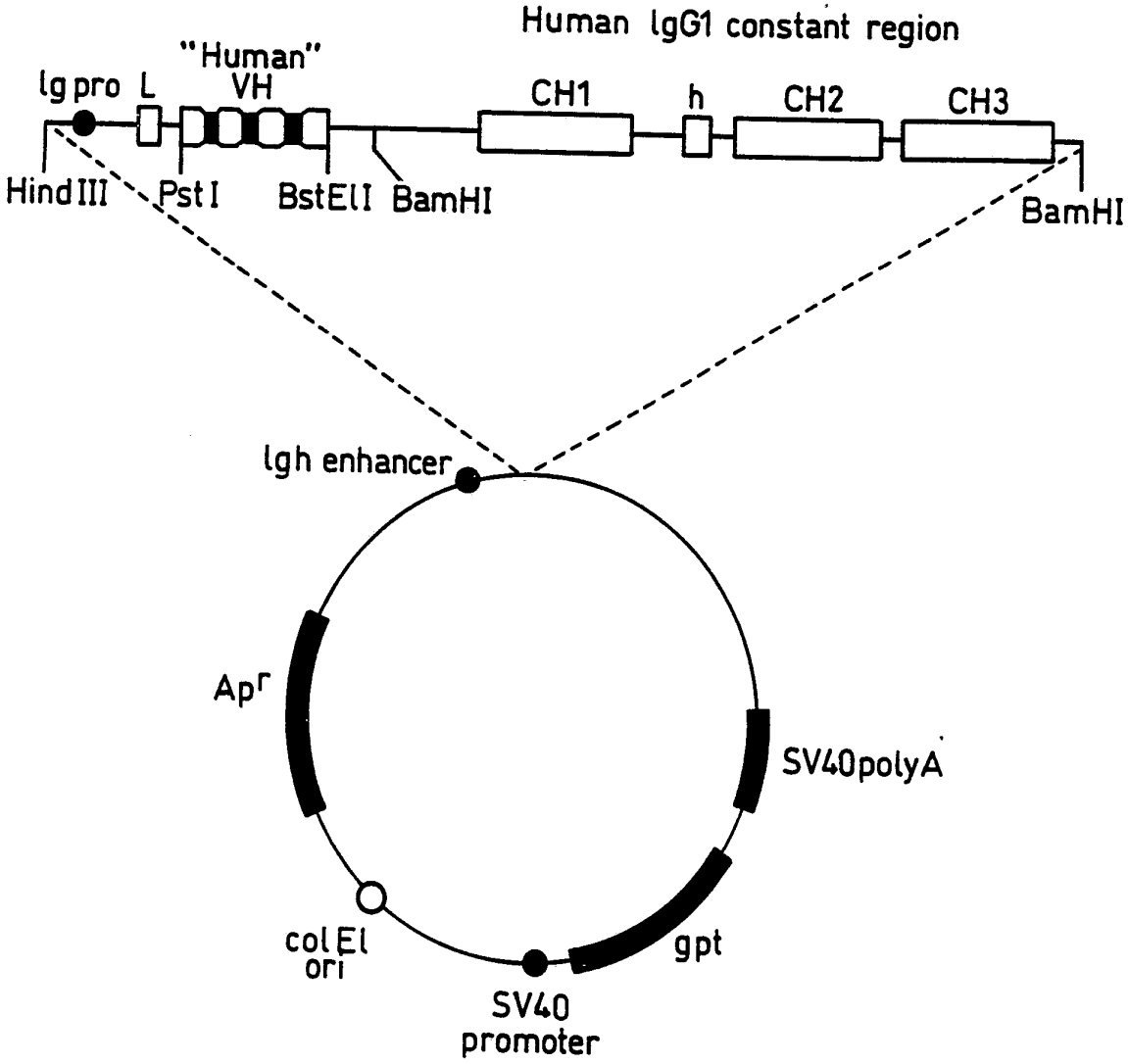

Human IgG1 constant region
"Human"
lg pro
L
VH
CH1
h
CH2
CH3
HindIII
PstI
BstEII
BamHI
BamHI
lgh enhancer
Apr
SV40polyA
colEI
ori
gpt
SV40
promoter

Fig.16

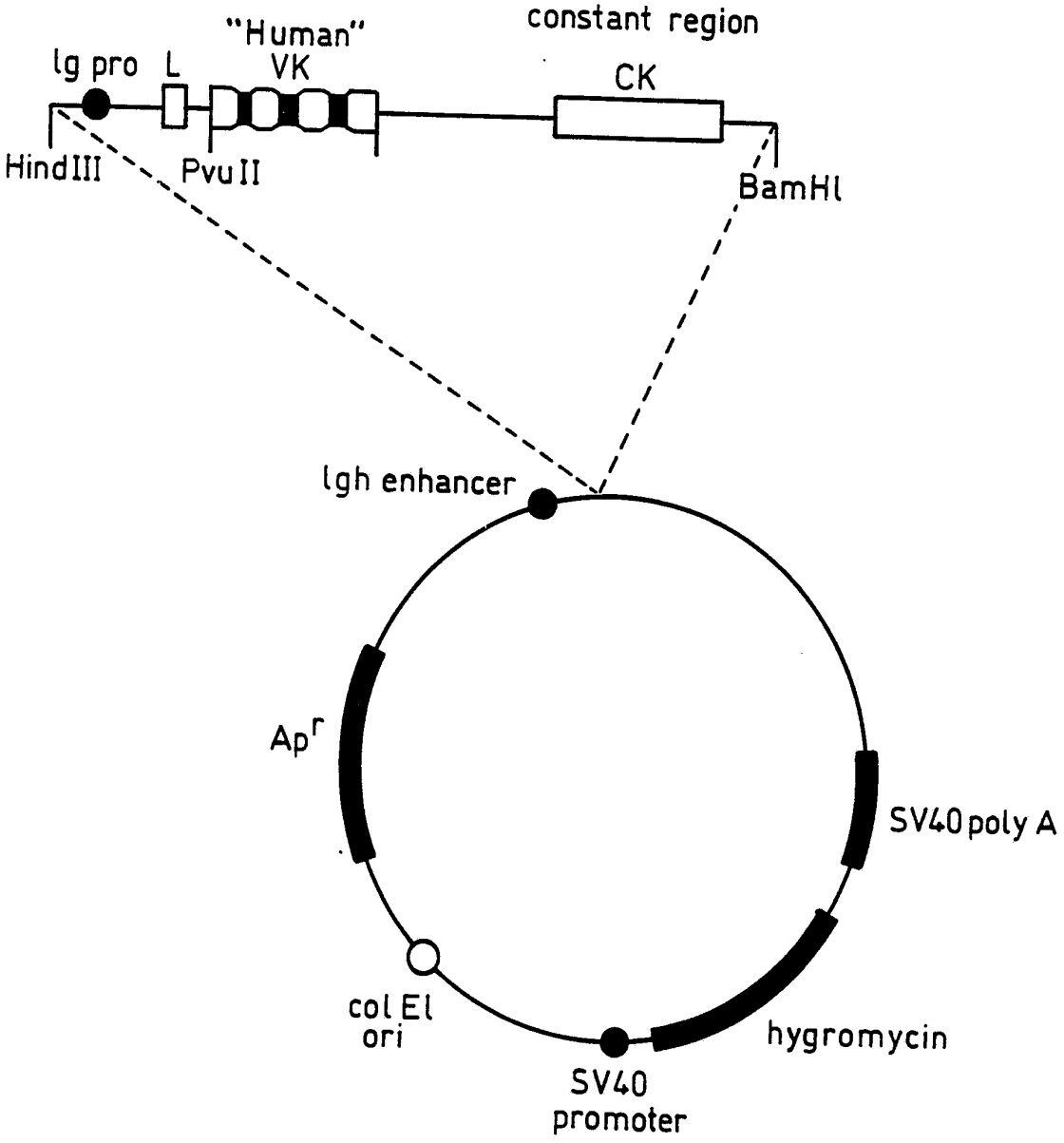

"Human"
constant region
lg pro
L
VK
CK
HindIII
PvuII
BamHl
lgh enhancer
Ap^r
SV40 poly A
col El
ori
hygromycin
SV40
promoter

Fig. 17

FIGURE 18

RSV19VH SEQ ID NO: 13

1

Gln Val Gln Leu Gln Glu Ser Gly Thr Glu Leu Glu Arg Ser Gly

Ala Ser Val Lys Leu Ser Cys Thr Ala Ser Gly Phe Asn Ile Lys

Asp Tyr Tyr Met His Trp Met Lys Gln Arg Pro Asp Gln Gly Leu

Glu Trp Ile Gly Trp Ile Asp Pro Glu Asn Asp Asp Val Gln Tyr

Ala Pro Lys Phe Gln Gly Lys Ala Thr Met Thr Ala Asp Thr Ser

Ser Asn Thr Ala Tyr Leu Gln Leu Thr Ser Leu Thr Phe Glu Asp

Thr Ala Val Tyr Phe Cys Asn Ser Trp Gly Ser Asp Phe Asp His

115

Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser

FIGURE 19

pHuRSV19VH SEQ ID NO: 14

1

Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Arg Pro Ser Gln

Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Phe Thr Phe Ser Asp Tyr

Tyr Met His Trp Val Arg Gln Pro Pro Gly Arg Gly Leu Glu Trp Ile

Gly Trp Ile Asp Pro Glu Asn Asp Asp Val Gln Tyr Ala Pro Lys Phe

Gln Gly Arg Val Thr Asn Leu Val Asp Thr Ser Lys Asn Gln Phe Ser

Leu Arg Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr Cys

Ala Arg Trp Gly Ser Asp Phe Asp His Trp Gly Gln Gly Thr Thr Val

115

Thr Val Ser Ser

FIGURE 20

pHuRSV19VHFNS SEQ ID NO: 15

1

Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Arg Pro Ser Gln

Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Phe Thr Phe Ser Asp Tyr

Tyr Met His Trp Val Arg Gln Pro Pro Gly Arg Gly Leu Glu Trp Ile

Gly Trp Ile Asp Pro Glu Asn Asp Asp Val Gln Tyr Ala Pro Lys Phe

Gln Gly Arg Val Thr Met Leu Val Asp Thr Ser Lys Asn Gln Phe Ser

Leu Arg Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Phe Cys

Asn Ser Trp Gly Ser Asp Phe Asp His Trp Gly Gln Gly Thr Thr Val

115

Thr Val Ser Ser

FIGURE 21

pHuRSV19VHNIK SEQ ID NO: 16

1

Gln Val Gln Leu Gln Glu Ser Gly Pro Gly Leu Val Arg Pro Ser Gln

Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Phe Asn Ile Lys Asp Tyr

Tyr Met His Trp Val Arg Gln Pro Pro Gly Arg Gly Leu Glu Trp Ile

Gly Trp Ile Asp Pro Glu Asn Asp Asp Val Gln Tyr Ala Pro Lys Phe

Gln Gly Arg Val Thr Met Leu Val Asp Thr Ser Lys Asn Gln Phe Ser

Leu Arg Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Phe Cys

Asn Ser Trp Gly Ser Asp Phe Asp His Trp Gly Gln Gly Thr Thr Val

115

Thr Val Ser Ser

FIGURE 22

pHuRSV19VK SEQ ID NO: 17

1
Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly

Asp Arg Val Thr Ile Thr Cys Arg Ser Ser Gln Thr Leu Val His Thr

Asp Gly Asn Thr Tyr Leu Glu Trp Tyr Gln Gln Lys Pro Gly Lys Ala

Pro Lys Leu Leu Ile Tyr Arg Val Ser Asn Arg Phe Ser Gly Val Pro

Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile

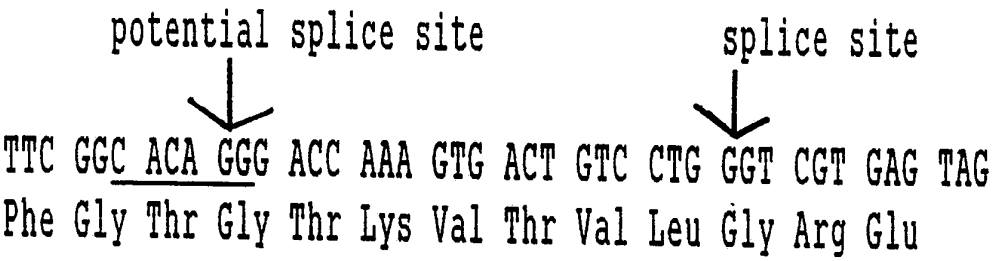
Ser Ser Leu Gln Pro Glu Asp Ile Ala Thr Tyr Tyr Cys Phe Gln Gly

110
Ser His Leu Pro Arg Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys

FIGURE 23

HuVL framework 4

original nucleotide sequence SEQ ID NO: 20



human lambda J1 gene sequence SEQ ID NO: 22

TTC GGA ACT GGG ACC AAG GTC ACC GTC CTA GGT --- AAG TGG

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 93/00725

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/62; C12N15/13; C07K13/00; A61K39/42
C12P21/08

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; C07K ; C12P ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	WO,A,9 204 381 (SCOTGEN LTD) 19 March 1992	1,2,6, 10, 16-20, 28-33
Y	see the whole document	3-5,7-9, 11-15, 21-25,27

Y	J. GEN. VIROL. vol. 69, no. 12, 1988, pages 3023 - 3032 H. KENNEDY ET AL. 'Production and characterisation of bovine monoclonal antibodies to respiratory syncytial virus' see the whole document	3-5,7-9, 11-15, 21-25,27

	-/--	

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
12 AUGUST 1993	27 -08- 1993
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	SKELLY J.M.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	J. VIROL. vol. 64, no. 2, 1990, pages 927 - 930 J. LOPEZ ET AL. 'Location of a highly conserved neutralisation epitope on the F glycoprotein of respiratory syncytial virus' see the whole document	26
X	WO,A,9 201 473 (THE UNITED STATES OF AMERICA) 6 February 1992 see the whole document	1,2,6, 16-20, 26-32
A	J. VIROL. vol. 63, no. 7, 1989, pages 2941 - 2950 J. BEELER ET AL. 'Neutralisation epitopes of the F glycoprotein of respiratory syncytial virus'	
A	J. GEN. VIROL. vol. 72, 1991, pages 1051 - 1058 C. BOURGEOIS ET AL. 'Use of synthetic peptides to locate neutralising antigenic domains in the fusion protein of respiratory syncytial virus'	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB93/00725

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

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

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim 29 is directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

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

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

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

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

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9300725 SA 73109

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 12/08/93

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9204381	19-03-92	AU-A-	8505891	30-03-92
		CA-A-	2091335	12-03-92
		EP-A-	0548190	30-06-93
WO-A-9201473	06-02-92	AU-A-	8403091	18-02-92
		EP-A-	0539522	05-05-93