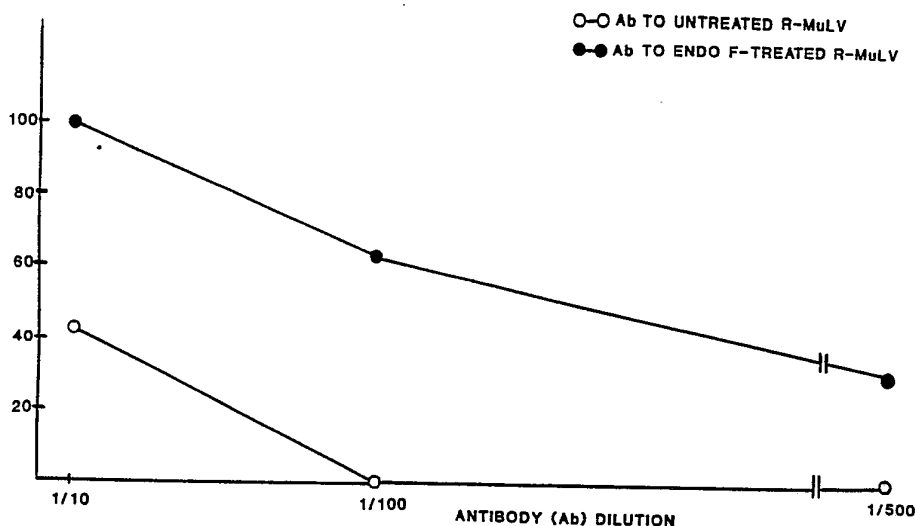




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁴ : C12N 7/00	A1	(11) International Publication Number: WO 86/ 03224 (43) International Publication Date: 5 June 1986 (05.06.86)
<p>(21) International Application Number: PCT/US85/02319</p> <p>(22) International Filing Date: 20 November 1985 (20.11.85)</p> <p>(31) Priority Application Number: 676,008</p> <p>(32) Priority Date: 29 November 1984 (29.11.84)</p> <p>(33) Priority Country: US</p> <p>(71) Applicant: SCRIPPS CLINIC AND RESEARCH FOUNDATION [US/US]; 10666 North Torrey Pines Road, La Jolla, CA 92037 (US).</p> <p>(72) Inventors: ELDER, John, H. ; 1451 MacKinnon, Cardiff, CA 92007 (US). ALEXANDER, Stephen ; 10206 Lipscomb Drive, San Diego, CA 92126 (US).</p>	<p>(74) Agents: GAMSON, Edward, P.; Dressler, Goldsmith, Shore, Sutker & Milnamow, Ltd., 1800 Prudential Plaza, Chicago, IL 60601 (US) et al.</p> <p>(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).</p> <p>Published <i>With international search report.</i></p>	

(54) Title: POLYPEPTIDES AND ANTIBODIES RELATED TO DEGLYCOSYLATED VIRAL GLYCOPROTEINS



(57) Abstract

An N-linked, carbohydrate-free polypeptide comprising an amino acid residue sequence substantially identical to that of a viral envelope N-linked glycoprotein, its related non-virus whose envelope protein is free of N-linked carbohydrate, and methods of producing and utilizing the polypeptide. The polypeptide is substantially free of N-linked glycosylation of the glycoprotein and induces the production of antibody-containing serum that (i) provides improved cross-reactivity among related viral strains and/or (ii) neutralizes the virus of said viral glycoprotein at a greater serum dilution than does antibody-containing serum induced by the viral envelope N-linked glycoprotein when the polypeptide and the viral envelope N-linked glycoprotein are individually utilized to induce antibody production in separate host mammals of the same strain using substantially identical immunization regimens.

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	GA	Gabon	MR	Mauritania
AU	Australia	GB	United Kingdom	MW	Malawi
BB	Barbados	HU	Hungary	NL	Netherlands
BE	Belgium	IT	Italy	NO	Norway
BG	Bulgaria	JP	Japan	RO	Romania
BR	Brazil	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	LI	Liechtenstein	SN	Senegal
CH	Switzerland	LK	Sri Lanka	SU	Soviet Union
CM	Cameroon	LU	Luxembourg	TD	Chad
DE	Germany, Federal Republic of	MC	Monaco	TG	Togo
DK	Denmark	MG	Madagascar	US	United States of America
FI	Finland	ML	Mali		
FR	France				

-1-

POLYPEPTIDES AND ANTIBODIES RELATED
TO DEGLYCOSYLATED VIRAL GLYCOPROTEINS
DESCRIPTION

5 Technical Field

 The present invention relates to
polypeptides comprising an amino acid residue
sequence substantially identical to that of a viral
envelope N-linked glycoprotein, and more particularly
10 to polypeptides and nor-viruses that are
substantially free of the N-linked glycosylation of a
viral envelope N-linked glycoprotein and are capable
of inducing the production of antibody-containing
serum that (i) provides improved cross-reactivity
15 among related viral strains and/or (ii) neutralizes
the virus of said viral glycoprotein at a greater
serum dilution than does antibody-containing serum
induced by the viral envelope N-linked glycoprotein.

Background of the Invention

20 The pathways and mechanisms by which
carbohydrate moieties (glycosyl groups) are added to
glycoproteins are now relatively well defined,
Hubbard et al., Ann. Rev. Biochem., 50, 555 (1981).
However, heretofore little has been known about the
25 role carbohydrates play in the function of this class
of proteins.

 Possible roles posed for glycosylation
include increasing hydrophilicity of certain regions
of a given molecule, protection of the molecule from
proteolytic attack, facilitation of mobilization to
30 the cell surface and dictation of secretion of
certain proteins. See, for example, Gibson et al.,
Trends Biochem. Sci., 5, 290 (1980); Schwarz et al.,
Trends Biochem. Sci., 5, 65 (1980); Heifetz et al.,
35 Biochemistry, 18, 2186 (1979).

-2-

Although examples of each of the above suggestions are evident, generalizations regarding the role of the carbohydrate on glycoproteins as a class are not apparent. See, Sharon et al., The
5 Proteins, Volume V, 3d ed., Neurath et al., eds., Academic Press, New York (1982).

There has been much speculation in the literature regarding the role(s) carbohydrate may play in a host animal's immune response to viruses,
10 in particular for retroviruses and influenza viruses. See, Wunner et al., J. Gen. Virol., 64, 1649 (1983); and Snyder et al., Proc. Natl. Acad. Sci, (USA), 77, 1622 (1980).

Several studies have been reported wherein
15 bovine leukemia virus glycoproteins were treated with glycosidases, exoglycosidases, trypsin, and/or proteases, and then examined for antigenic activity. In each case, it was reported that the carbohydrate moiety of the glycoprotein was essential for
20 stimulation of effective immunogenic activity against the virus in cattle. Bruck et al., Virology, 136, 20 (1984); Miller et al., "Vaccination with Glycosidase-Treated Glycoprotein Antigen Does Not Prevent Bovine Leukemia Virus Infection in Cattle",
25 5th International Symposium of Bovine Leukosis, Tubingen, October 19-21, 1982; Schwerr et al., Virology, 109, 431 (1981); and Portetelle et al., Virology, 105, 223 (1980).

Thus, in order to use a glycoprotein as an
30 immunogen, it has heretofore been thought that the presence of carbohydrate groups was critical for the glycoprotein to elicit an effective immune response. One reason for that conclusion may be that glycosidase preparations have typically been
35

-3-

contaminated with proteases that cleave the glycoproteins into non-immunogenic fragments.

It has also been reported that when the purified glycoprotein called gp71 of Friend murine leukemia virus was treated with endoglycosidase, 70 percent of the carbohydrate was removed without affecting type, group, or interspecies antigenic reaction as detectable in radioimmunoassays or adsorption of cytotoxic antibodies, Schafer et al., J. Virology, 21, 35 (1977); and Bolognesi et al., J. Virology, 16, 1453 (1975). However, while antigenicity was examined for the deglycosylated glycoprotein, the immunogenic effects of the deglycosylated protein were not considered.

The immunogenic contributions of the carbohydrate and polypeptide portions of the major envelope glycoprotein (gp90) of the equine infectious anemia virus called EIAV have been analyzed by Montelaro et al., Virology, 136, 368 (1984). The analysis was made by measuring the effects of specific glycosidase and protease digestions on the reactivity of the glycoprotein with immune sera from infected horses. The results of both direct and competitive radioimmunoassays indicated that immune sera contained antibodies reactive with both carbohydrate and protein moieties of EIAV gp90, with the predominant reactivity apparently being against the gp90 polypeptide epitopes.

Those results may be contrasted with descriptions of retrovirus glycoprotein antigenicity in which antigenic reactivity was attributed exclusively to protein, Bolognesi et al., J. Virology, supra; and Van Eldrik et al., Virology, 86, 193 (1978), or to carbohydrate, Schmerr et al., Virology, supra; and Portetelle et al., Virology,

-4-

supra. See also, Ho-Terry et al., Arch. Virology,
79, 139 (1984); and Salabe et al., Clin. Exp.
Immunol., 25, 234 (1976). However, the usefulness of
5 glycosidase-treated glycoprotein as an immunogen was
again not analyzed in any of the above reports.

The loss of tumor transplantation of L1210
leukemic tumor cells after treatment with
neuraminidase, that liberates sialic
(N-acetylneuraminic) acid moieties by cleaving their
10 O-glycoside links with underlying amino-sugars, has
been reported by Bagshawe et al., Nature (London),
218, 1254 (1968). There, mice were inoculated with
40,000 L1210 tumor cells, receiving all cells treated
with neuraminidase or all untreated cells. All mice
15 that received 40,000 untreated tumor cells died
within 20 days, but mice that received tumor cells
treated with neuraminidase remained in apparent
normal health for more than 100 days. However,
neither the use of the deglycosylated tumor cells as
20 an immunogen nor the use of deglycosylated viruses
was disclosed.

Summary of the Invention

The present invention contemplates a
25 polypeptide free of N-linked carbohydrate having an
amino acid residue sequence substantially identical
to that of a viral envelope N-linked glycoprotein, as
well as methods of producing and utilizing the
polypeptide.

30 In one aspect of the invention, a
polypeptide having an amino acid residue sequence
substantially identical to that of a viral envelope
N-linked glycoprotein is contemplated. The
polypeptide is substantially free of the N-linked
35 glycosylation of the glycoprotein and induces the

-5-

production of antibody-containing serum that (i) exhibits improved cross-reactivity among related viral strains and/or (ii) neutralizes the virus of said viral glycoprotein at a greater serum dilution than does antibody-containing serum induced by the viral envelope N-linked glycoprotein when the polypeptide and the viral envelope N-linked glycoprotein are individually utilized to induce antibody production in separate host mammals of the same strain using substantially identical immunization regimens.

Another aspect of the present invention contemplates a modified virus or virion described herein as a nor-virus or nor-virion whose envelope protein has an amino acid residue sequence substantially identical to that of the unmodified virus envelope protein, the latter envelope protein containing N-linked glycosylation in the unmodified state. The envelope protein of the nor-virus is substantially free of carbohydrate moieties covalently linked to asparagine residues (N-links) and induces the production of antibody-containing serum that (i) exhibits improved cross-reactivity among related viral strains and/or (ii) neutralizes the virus of said viral glycoprotein at a greater serum dilution than does antibody-containing serum induced by the virus when the polypeptide and the virus are individually utilized to induce antibody production in separate host mammals of the same strain using substantially identical immunization regimens.

In yet another aspect of the present invention, an inoculum against infection by a retrovirus or a myxovirus is contemplated. The

-6-

inoculum includes an effective amount of the above-described polypeptide or nor-virus of the invention in a physiologically tolerable diluent. The inoculum, when introduced into a host, is capable of inducing the production of antibodies in the host that immunoreact with the nor-virus, the virus, and with a related viral strain, and neutralize the virus in vitro. Preferably, such inocula are utilized to protect the host from in vivo viral infection.

10 In a further aspect of the present invention, antibodies to a retrovirus or a myxovirus are contemplated. The antibodies are raised in an animal host to the above-described polypeptide or nor-virus of the invention and have the capacity to immunoreact with the virus, the nor-virus, and a related viral strain as well as neutralize the virus in vitro.

In yet another aspect of the present invention, a method for the production of antibodies to a virus is contemplated. The method comprises (i) introducing into an animal host an effective amount of the above-described polypeptide or nor-virus of the invention that is capable of inducing the production of antibodies in the serum of the host; and (ii) collecting induced antibody-containing serum from the host. The antibodies may be recovered in purified form from the induced antibody-containing serum by well known techniques, such as affinity purification. The antibodies and antibody-containing serum so prepared immunoreact with the virus, the nor-virus, a related viral strain and also neutralize the virus in vitro.

30 In a still further aspect of the present invention, a method of improving the immunogenicity of a viral envelope N-linked glycoprotein is

-7-

contemplated. The method comprises (i) providing a viral N-linked envelope glycoprotein or virus having an N-linked envelope glycoprotein; (ii) reacting the glycoprotein with a glycosidase such as an
5 endoglycosidase or exoglycosidase to remove glycosyl groups from the glycoprotein to form the above-described polypeptide or virus, respectively. The deglycosylated polypeptide or virus (the nor-virus) so formed, when utilized in an effective
10 amount in an inoculum, is capable of inducing the production of antibody-containing serum that (i) exhibits improved cross-reactivity among related viral strains and/or (ii) neutralizes the virus of said viral glycoprotein at a greater serum dilution
15 than does antibody-containing serum induced by the corresponding glycoprotein or virus, respectively, when the polypeptide or nor-virus and the respective glycoprotein or virus are individually utilized to induce antibody production in separate host mammals
20 of the same strain using substantially identical immunization regimens.

Still another aspect of the present invention, contemplates a method of immunizing an animal against a virus. The method comprises (i)
25 providing a unit dose of inoculum comprising the above-describe polypeptide or nor-virus of the present invention dispersed in an effective amount in a physiologically tolerable diluent, the polypeptide or nor-virus having the capacity to induce the
30 production of antibodies in the animal that immunoreact with the virus, nor-virus, a related viral strain as well as neutralize the virus in vitro, and preferably protect the animal from the virus; and (ii) introducing the unit dose of the
35

-8-

inoculum into the blood stream of the animal to be immunized.

The present invention provides several benefits and advantages.

5 One benefit of the present invention is that the polypeptide or nor-virus of the invention provides improved immune system susceptibility to viral immunogens.

10 One of the advantages of the present invention is that antibodies raised against the polypeptide or nor-virus of the invention display a greater neutralizing titer than do antibodies raised against a corresponding glycoprotein or a fully glycosylated virus, respectively, when used as
15 immunogens in separate animals of the same strain under similar immunization conditions, and thus provide an improved method of immunizing animals against viruses.

20 A further advantage of the present invention is that antibodies raised against the polypeptide or nor-virus of the invention provide improved cross-reactivity among related viral strains, thereby providing the ability to use a nor-virus or carbohydrate-free viral envelope polypeptide of this
25 invention in an inoculum against another, related virus.

30 Other advantages and benefits of the present invention will become readily apparent to those skilled in the art from the following description of the invention, the drawings and the appended claims.

Brief Description of the Drawings

In drawings forming a portion of the disclosure of this invention:

35

-9-

FIGURE 1 is a photograph of a Western blot analysis visualized by autoradiography showing the reactivity of heteroantisera to Rauscher murine leukemia virus (R-MuLV) glycoprotein gp70 before and after deglycosylation with Endoglycosidase F (Endo F), Elder and Alexander, Proc. Natl. Acad. Sci. (USA), 79, 4540 (1982). Immune reactivity was assessed against R-MuLV either untreated (native) or deglycosylated with Endo F to form a non-virus as described in Elder and Alexander, supra, followed by separation on SDS-PAGE, as in Laemmli, Nature, 227, 680 (1970), and transfer to nitrocellulose [Western blotting as described in Towbin et al., Proc. Natl. Acad. Sci. (USA), 76, 4350 (1979) and modified in Johnson et al., Gene Anal. Techn., 1, 3 (1984)].

Lane A shows reactivity of R-MuLV gp70 to a goat heteroserum prepared against purified R-MuLV gp70, as described in Elder et al., Nature, 267, 23 (1977), before (-) and after (+) treatment with Endo F. Lane B shows reactivity of goat heteroserum made against intact virus (sera obtained from the Resources Branch of the National Cancer Institute) with R-MuLV proteins before (-) and after (+) treatment of those proteins with Endo F. Lane C shows reactivity of a goat heteroserum made against TWEEN-ether disrupted virus (sera obtained from the Resources Branch of the National Cancer Institute) before (-) and after (+) treatment with Endo F. As used herein, the term "TWEEN" is used to indicate polyoxyethylene (20) sorbitan monooleate. Arrows denote bands corresponding to gp70 before and after Endo F treatment, as is appropriate for the lanes.

Radioactive bands were removed and counted to quantitate changes in reactivity following deglycosylation. Reaction with a monoclonal antibody

-10-

that recognized both untreated and deglycosylated gp70 verified that the relative amounts of gp70 remained unchanged after deglycosylation.

FIGURE 2 is a photograph of a Western blot analysis visualized by autoradiography showing the effect of deglycosylation of Rauscher murine leukemia virus (R-MuLV) glycoprotein gp70 on reactivity with monoclonal antibodies. Immunoprecipitations were performed using a panel of 78 monoclonal antibodies made against purified R-MuLV gp70, as described in Niman and Elder, Virology, 123, 189 (1982). R-MuLV disrupted with Nonidet P-40 [polyoxyethylene (9) octyl phenyl ether; Sigma Chemical Co., St. Louis, MO] and radiolabeled with ^{125}I (Amersham, Arlington Heights, IL) was used as antigen. Samples were deglycosylated by treatment with Endo F that removed substantially all N-linked glycans from the glycoproteins, Elder and Alexander, Proc. Natl. Acad. Sci. (USA), supra. The precipitates were then separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), and visualized by autoradiography, as described before. Reactivities before (-) and after (+) deglycosylation are shown.

A complete variety of responses was obtained. Twenty-six of the antibodies gave only background bands in immune precipitation (Lane A); six reacted only after carbohydrate removal (Lane B); eight monoclonals reacted only with the untreated gp70 (Lane C); twenty-nine improved in reactivity to various degrees following deglycosylation (Lanes D-E); and nine reacted equally well before and after deglycosylation (Lane F).

FIGURE 3 is a photograph of a Western blot analysis visualized using peroxidase-coupled goat anti-rabbit IgG, hydrogen peroxide and o-dianisidine as colorant showing the reactivity of anti-

-11-

synthetic peptide antisera to the X47 influenza hemagglutinin (HA) before and after deglycosylation with Endo F.

5 X47 influenza virus (H₃N₂ subtype) was grown in embryonated eggs. The virus was concentrated from allantoic fluid by centrifugation.

One-half of the viral sample was treated with Endo F, while the other portion was used as a buffer control. Samples of the glycosylated and
10 deglycosylated virus representing approximately 10⁶ plaque forming units of virus were run on adjacent lanes by SDS-PAGE, Laemmli, Nature, supra, and subsequently blotted onto nitrocellulose, Towbin et al., Proc. Natl. Acad. Sci. (USA), supra, and Johnson
15 et al., Gene Anal. Techn., supra. Strips containing a lane of each of the glycosylated and deglycosylated samples were incubated with the various rabbit anti-synthetic peptide antisera made against synthetic peptides corresponding to regions of the
20 X47 influenza HA molecule, as in Green et al., Cell, 28, 477 (1982) and Min Jou et al., Cell, 19, 683 (1980). After washing, the blots were incubated with peroxidase-coupled goat anti-rabbit IgG. The blots were developed with hydrogen peroxide and
25 o-dianisidine. Reactivities before (-) and after (+) deglycosylation with Endo F are shown.

Lane A shows that reactivity of rabbit heteroserum against intact X47 influenza virus decreases dramatically with the deglycosylated HA
30 molecule. Lane B shows that anti-synthetic peptide serum 15 (HA sequence positions 140-156 that contain no site of glycosylation) reacts with neither glycosylated nor deglycosylated HA. Lane C shows that anti-synthetic peptide serum 2 (HA sequence
35 positions 1-36 that contain 2 sites of glycosylation

-12-

at positions 8 and 23) improves in reactivity when the HA is deglycosylated. Lane D shows that anti-synthetic peptide 17 (HA sequence positions 174-196 with no site of glycosylation) reacts equally well with either glycosylated or deglycosylated HA. The arrows denote bands corresponding to the influenza HA molecule before and after Endo F treatment, as is appropriate.

FIGURE 4 is a graph showing the adsorption of neutralizing antibodies by deglycosylated Rauscher murine leukemia virus (R-MuLV). The ordinate shows the percent of neutralization and the abscissa shows antibody dilution over an average of five adsorption studies. Antibodies raised to non-deglycosylated R-MuLV (Δ - Δ) were used as a control and adsorption of neutralizing antibodies by untreated R-MuLV (O-O) and by R-MuLV deglycosylated with Endo F (\bullet - \bullet) were compared.

FIGURE 5 is a photograph of a Western blot analysis visualized using peroxidase-coupled goat anti-rabbit antibodies, hydrogen peroxide and 4-chloro-2-naphthol as described in Section III C, and shows the reactivity of antisera raised against R-MuLV or deglycosylated, nor-R-MuLV. The R-MuLV gp70 was deglycosylated (treated) with Endo F thereby providing the deglycosylated protein called as p49. The left panel of lanes shows the reactivity of antisera raised against deglycosylated R-MuLV to native and deglycosylated R-MuLV. The right panel of lanes shows the reactivity of antisera raised against native R-MuLV to native and deglycosylated R-MuLV.

FIGURE 6 is a graph showing the neutralization of R-MuLV by antisera raised against a control and against deglycosylated R-MuLV. The ordinate shows the percent of neutralization and the abscissa shows the antibody dilution. The

-13-

neutralization of untreated R-MuLV (O-O) was compared to that of R-MuLV treated with Endo F (nor-R-MuLV) (●-●).

FIGURE 7 is a graph showing the
5 neutralization of feline leukemia virus (FeLV) by antisera raised against untreated FeLV (O-O) and deglycosylated FeLV (nor-FeLV) (●-●). As above for FIGURE 6, the ordinate shows the percent of
10 neutralization and the abscissa shows the antibody dilution.

FIGURE 8 is a photograph of a Western blot analysis visualized as described in FIGURE 5 showing the relative lack of cross-reactivity of antisera raised against native FeLV with R-MuLV. The left
15 lane shows the reactivity of the antisera to native and deglycosylated R-MuLV. The right lane shows the reactivity of the antisera to native and deglycosylated FeLV.

FIGURE 9 is a photograph of a Western blot analysis visualized as described in FIGURE 5 showing
20 cross-reactivity of antisera raised against deglycosylated, nor-FeLV. The left lane shows the reactivity of the antisera to native and to deglycosylated R-MuLV. The right lane shows the
25 reactivity of the antisera to native and to deglycosylated, nor-FeLV.

Detailed Description of the Invention

The present invention is directed to a
30 polypeptide that is free of N-linked carbohydrate that has an amino acid residue sequence substantially identical to that of a viral envelope N-linked glycoprotein, its related nor-virus whose envelope protein is free of N-linked carbohydrate, and to
35 methods of producing and utilizing same. The polypeptide is substantially free of glycosylation of

-14-

the N-linked glycoprotein and induces the production of antibody-containing serum that (i) provides improved cross-reactivity among related viral strains and/or (ii) neutralizes the virus of said viral glycoprotein at a greater serum dilution than does antibody-containing serum induced by the viral envelope N-linked glycoprotein when the polypeptide and the viral envelope N-linked glycoprotein are individually utilized to induce antibody production in separate host mammals of the same strain using substantially identical immunization regimens.

A polypeptide of this invention is a nor- or deglycosylated form of a native viral envelope glycoprotein that contains a plurality of asparagine-linked (N-linked) carbohydrate moieties in the native form. Such a polypeptide is substantially the same length and has substantially the identical amino acid residue sequence as does the native envelope glycoprotein.

An alternative description of such a polypeptide is that it is a deglycosylation reaction product of an N-linked viral envelope glycoprotein. Particularly preferred polypeptides are reaction products of an endoglycosidase or exoglycosidase, such as neuraminidase, and an N-linked viral envelope glycoprotein. In most preferred practice, the endoglycosidase is the enzyme referred to herein as Endo F and the viral envelope glycoprotein is that of a retrovirus such as a leukemia-inducing retrovirus or a myxovirus such as an influenza virus. In all instances, the polypeptide reaction product has substantially the same amino acid residue length and substantially identical amino acid residue sequence as does the native viral envelope glycoprotein.

35

-15-

By referring to the polypeptide as having substantially the same length as the native glycoprotein, it is meant that the polypeptide may differ by a few residues, e.g., about 5 to about 20 residues, from the native glycoprotein. However, as compared to the native glycoprotein, the polypeptide is relatively free from proteolytic cleavage that reduces its amino acid residue sequence to less than about 90 percent, or more preferably, less than about 95 percent, of the number of residues present in the native glycoprotein. Most preferably, the polypeptide has the same number of amino acid residues as does the native glycoprotein. Thus, deglycosylation of the N-linked carbohydrate moieties does not substantially reduce the length of the native viral envelope protein.

Similarly, by referring to the polypeptide as having a substantially identical amino acid residue sequence to that of the native glycoprotein, it is meant that the amino acid residues present in the polypeptide are also present in the glycoprotein. Thus, for example, if a few amino acid residues have been lost by proteolysis during the deglycosylation reaction, those remaining in the polypeptide are also present in the glycoprotein.

A free, uncombined polypeptide molecule of this invention is most preferably prepared from an N-linked viral envelope glycoprotein of a native virus as contrasted to the possible preparation by recombinant DNA technology of a non-glycosylated or glycosylated protein of the same amino acid residue sequence. It is believed that the three-dimensional structure imposed upon the envelope protein during its synthesis and glycosylation is of some importance to the preparation and presentation of naturally

-16-

occurring epitopes that induce production of neutralizing and/or protective antibodies.

The term polypeptide used in relation to a before-described product is meant to encompass a reaction product as a free molecule as well as a reaction product molecule in combined form such as the envelope protein of an intact virus or partially disrupted virus. Thus, for example, an intact virus having an envelope glycoprotein containing N-linked carbohydrate moieties may be deglycosylated by reaction with a deglycosylase to form a nor-virus that previously contained N-linked carbohydrate moieties and is substantially free of N-linked carbohydrate moieties. Since such a nor-virus includes the before-described polypeptide in a combined form as the viral envelope "protein", that nor-virus is considered herein to be within those materials encompassed by the before-described term "polypeptide". Contrarily, the term "nor-virus" is used more narrowly herein, and excludes the before-described polypeptide as a free molecule. When the phrase "polypeptide or nor-virus" appears herein, it is meant to encompass the free, uncombined polypeptide molecule and the combined viral or disrupted viral form of that molecule, respectively.

Definitions

The following definitions are provided to assist the reader in understanding the following disclosure and discussion.

Virus or virion -- An infectious agent that lacks independent metabolism and is able to replicate only within a living host cell. The individual virus particle (virion) consists of DNA or RNA and a protein shell or envelope.

-17-

Nor-virus or nor-virion -- A virus particle or virion that normally contains asparagine-linked (N-linked) carbohydrate moieties from which such N-linked carbohydrates have been removed.

5 Native -- The word "native" used with respect to a virus or protein refers to the usual chemical state of that virus or protein even though the virus or protein may be made by man's intervention through mutation. Thus, a native virus
10 or protein as used herein contains N-linked carbohydrate moieties.

Heteroserum or Heteroantiserum -- Antibody-containing serum that contains antibodies that immunoreact with a plurality of epitopes from a
15 plurality of antigenic determinants at several places on the immunogenic entity, e.g., virus, protein, or glycoprotein, when that entity is used as an antigen.

Oligoclonal serum -- Antibody-containing serum that contains antibodies that immunoreact with
20 one or more epitopes of a synthetic peptide immunogen that corresponds to an antigenic determinant of a viral, protein or glycoprotein immunogen. Oligoclonal sera typically have a narrower specificity than do heterosera.

25 Monoclonal antibodies -- Antibodies or serum-containing antibodies having specificity for a single epitope of a single antigenic determinant of the immunogenic entity. Monoclonal antibodies may be contaminated with minor amounts of antibodies that
30 are typically unrelated to the immunogenic entity and are typically artifacts of preparation, as where ascites is used.

Sequence substantially identical -- The term "sequence substantially identical" is used herein to
35 mean little if any cleavage of proteins, particularly

-18-

in the middle of the sequence, although a few amide bonds at the ends of the protein may be cleaved.

I. General Discussion

5 The ability of heteroantisera, monoclonal antibodies and anti-synthetic peptide (oligoclonal) antibodies to immunoreact with deglycosylated viral glycoproteins has been examined. The results discussed hereinafter indicate that the reactivities of the majority of antibodies (antibody-containing
10 sera) raised against these glycoproteins were markedly influenced by the attached carbohydrate (glycosyl) moieties.

 All heteroantisera prepared in rabbits or goats to either fully glycosylated retrovirus or
15 influenza virus were virtually unreactive with the deglycosylated viral envelope glycoproteins after carbohydrate removal. Assays using a selected panel of monoclonal antibodies raised against purified Rauscher murine leukemia virus (R-MuLV) envelope
20 glycoprotein gp70 indicated that a large number (35 of 78) of those antibodies exhibited improved reactivity, while the immunoreactivity of other monoclonal antibodies decreased or remained unchanged (17 of 78) after carbohydrate was removed from the
25 gp70 antigen. The remaining monoclonal antibodies (26 of 78) showed only background reactivities. The majority of anti-synthetic peptide (oligoclonal) antibodies to influenza virus hemagglutinin also exhibited improved immunoreactivity after
30 carbohydrate was removed from the hemagglutinin antigen molecule.

 These assays demonstrated that (1) carbohydrate side chains on viral glycoproteins influence the immune response to these antigens; and
35 (2) the more native the glycoprotein immunogen, the

-19-

more prominent the carbohydrate influence observed. The results discussed hereinafter also indicate that the immune response to the glycoproteins is not simply a function of the immunogenicity of certain domains over others, but is rather a direct measure of carbohydrate influences on the host animal's immunological response to the foreign immunogen (antigen).

The assays discussed were facilitated by the use of Endoglycosidase F, Elder and Alexander, Proc. Natl. Acad. Sci. (USA), supra, that efficiently cleaves both N-linked high mannose and complex glycans from glycoproteins, and thus permits direct screening of aspects of the carbohydrate-protein interaction. The polypeptides of the invention were obtained from viral envelope glycoproteins, illustratively, by immunoreaction with Endo F. However, they may also be obtained by immunoreaction with endoglycosidases called Endo H and Endo D, Elder and Alexander, supra, or by immunoreaction with neuraminidase, an exoglycosidase. The deglycosylation of R-MuLV envelope glycoprotein gp70 results in the formation of a protein called p49.

Further assays were undertaken to characterize the relationship between those antibodies influenced by carbohydrate and the antibodies responsible for virus neutralization. The results discussed hereinafter illustrate that carbohydrate directs the immune response to determinants other than those involved in viral infectivity. Surprisingly, it was found that viral antigens exhibited a relatively stronger neutralizing response after carbohydrate removal. This result was directly opposite to those previously reported, Bruck et al., Virology, supra; Miller et al., 5th

-20-

International Symposium of Bovine Leukosis, supra;
Schmerr et al., Virology, supra; Portetelle et al.,
Virology, supra.

5 The initial assay in this regard was
performed based upon the observation that all
heterosera or oligoclonal sera raised against
retroviruses (murine, feline and primate) as well as
influenza virus were virtually unreactive to their
10 respective envelope glycoprotein antigens by immune
precipitation or Western blotting after carbohydrate
was removed from these antigens by treatment with
Endo F. As the surface glycoproteins are the primary
targets of virus neutralization, it was expected that
15 neutralization of deglycosylated virus would be
similarly diminished.

Since complete deglycosylation by Endo F
involves relatively harsh treatment that can diminish
viral infectivity in itself, assays were performed
using native (envelope glycoprotein) and
20 deglycosylated (nor-glycoprotein) antigens to adsorb
antisera, followed by evaluation of the
neutralization titers of the sera against fully
infectious viruses. Surprisingly, it was found that
deglycosylated Rauscher murine leukemia virus
25 (nor-R-MuLV) was able to adsorb neutralizing
antibodies as well as, if not better than, native
viral antigen. Thus, the results discussed
hereinafter illustrate that N-linked envelope
carbohydrates dramatically affect the overall immune
30 response to the viral glycoproteins, but to regions
not involved in virus infectivity.

It was also desired to determine what
influence carbohydrate removal has on the efficacy of
the virus as an immunogen. Two aliquots of Rauscher
35 murine leukemia virus (R-MuLV) were prepared

-21-

identically, except that one sample was treated with Endo F to deglycosylate the surface glycoprotein, gp70 to form p49, while the other aliquot was left in its native form.

5 These immunogens were dispersed in physiologically tolerable diluents and were subsequently introduced by injection in rabbits. The resultantly raised (induced) antisera were examined in both Western blotting and neutralization assays.

10 As expected, untreated, native R-MuLV elicited antibodies that only reacted by Western blotting with non-deglycosylated, native gp70. Deglycosylated R-MuLV (nor-R-MuLV), however, induced antibodies that reacted equally well with the native
15 envelope glycoprotein, gp70, and with the nor-glycoprotein, p49. This was an unexpected result as it would be expected that antibodies raised against deglycosylated immunogens would fail to react or would exhibit diminished reactivity against the
20 native envelope glycoprotein antigen. Furthermore, the overall antibody response to all the viral proteins was markedly enhanced by removal of carbohydrate from the gp70.

 The results discussed below were obtained
25 using polypeptides of the invention comprising amino acid residue sequences substantially identical to those of Rauscher murine leukemia virus, (R-MuLV), feline leukemia virus (FeLV) and influenza virus envelope N-linked glycoproteins. However,
30 polypeptides of the invention also include polypeptides comprising amino acid residue sequences substantially identical to those of any virus generally, and particularly to those of leukemia virus and myxovirus envelope N-linked glycoproteins.

35

-22-

Leukemia viruses (retroviruses) include a group of RNA viruses causing leukemia and/or leucocyte-related tumors in animals. These viruses include avian leukosis virus, Rous sarcoma and murine leukemia viruses, Friend leukemia virus, Rauscher murine leukemia virus and feline leukemia virus, those human leukemia viruses called HTLV I, HTLV II and HTLV III, as well as Mink Cell Focus-Forming Viruses (MCF) and Adult T-Cell Leukemia Virus (ATLV). Myxoviruses include a group of viruses, such as the viruses of influenza, parainfluenza, mumps and Newcastle disease, that characteristically cause agglutination of erythrocytes.

Therefore, it is to be understood that the results discussed hereinbelow are illustrative of embodiments utilizing polypeptides substantially identical in sequence to Rauscher murine leukemia virus, feline leukemia virus and influenza virus envelope N-linked glycoproteins, but the present invention is not intended to be so limited.

II. Results

A. Influence of Glycosylation on Immune Reactivity of Antisera Prepared Against Viral Glycoproteins

The abilities of heteroantisera and monoclonal antibodies to react with viral glycoproteins deglycosylated with Endo F were assayed using Rauscher murine leukemia virus (R-MuLV). FIGURE 1 shows the results of Western blot analyses of untreated (native) and deglycosylated R-MuLV using heterosera prepared to purified R-MuLV glycoprotein (gp70), intact R-MuLV, and TWEEN-ether disrupted R-MuLV.

-23-

All three antisera showed diminished reactivity to gp70 after treatment of the virus with Endo F. The heteroserum prepared against purified gp70 immunoreacted to only 40 of percent control levels after carbohydrate removal (calculated by removing and counting the radioactive bands from nitrocellulose strips) as shown in FIGURE 1A.

Antiserum raised to whole, native unmodified virus, whether prepared against intact (FIGURE 1B) or TWEEN-ether disrupted (FIGURE 1C) viruses, reacted with deglycosylated gp70 at the level of 1-3 percent of the reactivity observed against untreated, native virus. Immunoreactivity of the anti-whole virus antisera to the nonglycosylated core protein p30 (major band at a molecular weight of 30,000 daltons in FIGURES 1B-C) remained unchanged after Endo F treatment, underscoring the glycoprotein specificity of the response. These results indicate that the presence of carbohydrate greatly influenced the immune response when the glycoprotein immunogen was presented in a relatively native conformation (i.e., presented as whole virus).

In FIGURE 2, representative immune precipitations using anti-R-MuLV gp70 monoclonal antibodies, as in Niman and Elder, Virology, supra, versus untreated and deglycosylated R-MuLV are shown. The results of this assay demonstrate that some monoclonal antibodies immunoprecipitated control and Endo-F treated gp70 very poorly (FIGURE 2A), others precipitated gp70 only after carbohydrate was removed (FIGURE 2B), and another group only reacted with untreated R-MuLV gp70 (FIGURE 2C).

The majority of these antibodies (35 of 52 immune precipitating monoclonal antibodies) improved in reactivity to various degrees following

-24-

deglycosylation of gp70 (FIGURES 2D-E). Still others reacted equally well with both treated and untreated gp70 (FIGURE 2F).

5 The above results demonstrate that the majority of the above panel of monoclonal antibodies were markedly influenced by carbohydrate moieties on R-MuLV gp70. Most of this influence was not via actual contribution of glycan to the epitope recognized by the antibody, since the majority of the
10 monoclonal antibodies improved in reactivity after carbohydrate removal. A small proportion (8 of 52 immune precipitating antibodies) failed to react with R-MuLV gp70 after glycans were removed and thus may have recognized carbohydrate as all or part of an
15 epitope. However, the carbohydrate removal could have caused a shift in the conformation of the polypeptide backbone such that the epitope was no longer recognized.

The effects of glycosylation on the immune
20 response to glycoproteins were further assayed by using the X47 influenza virus (H₃N₂ subtype) hemagglutinin (HA) molecule, a molecule for which the entire primary structure, including glycosylation sites, was known, Min Jou et al., Cell, supra, and
25 for which anti-synthetic peptide antisera directed against peptides spanning greater than 75 percent of the entire HA molecule were available, Green et al., Cell, 28, 477 (1982). Representative results obtained by Western blotting are shown in FIGURE 3.

30 When a rabbit heteroantiserum made against X47 influenza virus was immunoreacted with glycosylated virus and deglycosylated virus (nor-virus) (FIGURE 3A), results strikingly similar to those obtained with heteroantisera against the
35 retroviruses were obtained (FIGURE 2B); namely,

-25-

deglycosylation substantially reduced to abolished the reactivity of the anti-influenza virus antiserum.

Five of the twenty anti-synthetic peptide antibodies gave no reaction either with or without deglycosylation, similar to results with normal rabbit serum (FIGURE 3B). Of the ten oligoclonal sera made against defined regions of the influenza HA molecule that contained attached carbohydrate moieties, seven showed improved reactivity (FIGURE 3C) with the deglycosylated molecule, one had unchanged reactivity, and two were unreactive with either the glycosylated or deglycosylated form of the HA molecule.

In addition, ten anti-synthetic peptide antibodies directed against influenza HA sequences were screened that did not contain sites of glycosylation. Of these, three antisera improved in reactivity following deglycosylation of the HA molecule, four antisera were unchanged in reactivity (FIGURE 3D) and three showed no reactivity. Thus, the majority of site-specific antibodies directed at a sequence of the influenza HA molecule containing a site of glycosylation improved in reactivity against the HA molecule after removal of its carbohydrate moiety.

The above results illustrate the direct involvement of the carbohydrate moieties of glycoproteins with the host's immune recognition and response to these molecules. The assays using monoclonal antibodies to the R-MuLV gp70 molecule and the oligoclonal antibodies specifically directed to regions of the influenza HA molecule (containing sites of carbohydrate attachment) demonstrated that the carbohydrate portion of these glycoproteins can

-26-

effectively block the interaction of antibodies with the underlying polypeptide regions.

In the influenza system this was true even with regions of the HA molecule that did not have attached carbohydrate, presumably by some distal effect. By way of contrast, heteroantisera made to either R-MuLV or influenza virus lost virtually all reactivity toward the gp70 and HA molecules, respectively, after deglycosylation. This latter result shows that not only can the carbohydrate moieties block the interaction of antibodies to protein antigenic determinants, but that the carbohydrate either becomes the major immunogenic target of the glycosylated protein or directs the immune response to areas under influences of carbohydrate attachment. Thus, the carbohydrate moieties perform a dual role by masking certain polypeptide sites and by simultaneously directing the immune response so as to act as immune decoys.

20 B. Neutralization Assays

Assays were conducted to determine whether deglycosylated R-MuLV could adsorb out the neutralizing antibodies from heterosera that failed (or were weak) to react by Western blotting with deglycosylated surface glycoprotein gp70. To do so, a quantity of R-MuLV were inactivated and then split into two aliquots, one of which was treated with Endo F to remove carbohydrates to form nor-viruses containing p49 as the envelope "protein". The two resulting samples were then subdivided into five aliquots of equal volume, and the viruses and nor-viruses were pelleted. Goat anti-TWEEN-ether disrupted R-MuLV serum, diluted 1:100 was then admixed with one pellet each of untreated R-MuLV and deglycosylated nor-R-MuLV. Each admixture was

-27-

maintained for one hour at 37°C to form antibody-virus or antibody-nor-virus adsorbates.

The virus and nor-virus were subsequently pelleted from each of the sera, and the respective
5 sera were then sequentially adsorbed four additional times with the remaining viral pellets of each type, following the same general procedures. A portion of the serum was saved at each step so that at the end, antisera samples adsorbed 1x, 2x, 3x and 4x either
10 with control (R-MuLV) or deglycosylated R-MuLV were obtained.

The sera were then assayed for neutralizing ability as described in detail hereinafter. The results are shown in FIGURE 4.

15 Those results indicate that deglycosylated, nor-R-MuLV adsorbed neutralizing antibodies as well as did untreated, native R-MuLV, since the neutralizing antibodies still recognized the deglycosylated virus. This was the case even though
20 approximately 95 percent of the response by Western blotting was lost, as is shown in FIGURE 4.

Further assays were then conducted wherein rabbits were immunized by injection with inocula containing an effective amount of R-MuLV as control
25 or deglycosylated, nor-R-MuLV in physiologically tolerable diluents to compare their immune responses, both in neutralization and by Western blotting techniques. As described before, the control sera preferentially reacted with untreated, rather than
30 deglycosylated gp70, as shown in FIGURE 5. The antisera raised against deglycosylated, nor-R-MuLV, however, reacted equally well with both native and deglycosylated gp70 (p49) (FIGURE 5) and, overall, gave a much stronger antibody response.

35

-28-

These results illustrate that the carbohydrate moiety is not the only immunogenic part of gp70, since strong antibodies were raised in their absence. Additionally, the carbohydrate moieties
5 masked immunogenic epitopes present in the protein backbone that were subsequently exposed upon deglycosylation.

In FIGURE 6, the results of neutralization assays are shown using sera from the before-mentioned rabbits. As can be seen in FIGURE 6, the nor-virus
10 treated with Endo F elicited a much stronger initial neutralizing titer than did control virus when both virus and nor-virus were individually utilized to induce antibody production in separate host mammals
15 of the same strain using substantially identical immunization regimens. With multiple injections, however, the control virus-immunized rabbit eventually began producing more neutralizing antibodies, and its antibody-containing serum
20 approached the titer of the Endo F-treated immunogen after five or six boosts. These results show that removal of carbohydrate enhances exposure of neutralizing sites, rather than abrogating the ability of the nor-virus to induce neutralizing
25 antibodies.

These assays were repeated with FeLV. In that study, the deglycosylated nor-FeLV produced about twice the response in initial injections as control (undeglycosylated) FeLV, as shown in
30 FIGURE 7. The results shown in FIGURE 7 again illustrate viral neutralization at a greater serum dilution by antibody-containing serum induced by a polypeptide of this invention (nor-virus) than was obtained by serum induced by the viral envelope
35 N-linked glycoprotein (native virus) when the

-29-

polypeptide and viral envelope N-linked glycoprotein were individually utilized to induce antibody production in separate host mammals of the same strain using substantially identical immunization regimens.

With respect to X47 influenza virus hemagglutinin, two rabbits have been injected with native X47 and deglycosylated X47, respectively, as described in detail hereinafter in Section III. Sera were collected from the rabbits. Neutralization assays have not yet been completed.

The above results illustrate that immunogenicity of a viral envelope N-linked glycoprotein can be improved according to the present invention by reacting the glycoprotein with an endoglycosidase, Endo F, that removes the asparagine, N-linked carbohydrate moieties (glycosyl groups) from the envelope glycoprotein to form the polypeptides and nor-viruses of the invention.

As the above results further illustrate, the polypeptides and nor-viruses of the invention are capable of inducing the production of antibody-containing serum that neutralizes the native virus at a greater serum dilution than does antibody-containing serum induced by the N-linked envelope glycoprotein or native virus, respectively, when the polypeptide or nor-virus and the N-linked envelope glycoprotein or virus, respectively, are individually utilized to induce antibody production in separate host mammals of the same strain using substantially identical immunization regimens.

C. Cross-Reactivity Studies

The seriological classification and subdivision of antigenically related viruses is known to result from the polymorphism of certain viral

-30-

proteins. Viral polymorphism reflects the response of specific viral genes to selective pressure and influences the dynamics of the host-parasite relationship. Such is the case for the hemagglutinin molecule of the influenza viruses, Fenner et al., "The Biology of Animal Viruses", 2d ed., Academic Press, New York (1973).

Another molecule whose polymorphism dictates host range, interference, and neutralization properties of the virus is the envelope glycoprotein gp70 of the murine and feline leukemia viruses (retroviruses). Friend, Maloney and Rauscher murine leukemia viruses (type C viruses) known as the FMR group have antigenically related gp70 glycoproteins.

For example, Niman and Elder, in "Monoclonal Antibodies and T-Cell Products", Katz ed., CRC Press, Boca Raton, Florida, pp 23-51 (1982), demonstrated FMR group gp70 antigenic cross-reactivities by generating monoclonal antibodies to Rauscher gp70 that immunoreact with the envelope gp70 glycoproteins of all FMR viruses. It is believed that the antigenic relatedness is the result of homologous amino acid residue sequences forming antigenic determinants common to the FMR group gp70 glycoproteins. The envelope glycoproteins of FeLV, HTLV I, HTLV II, HTLV III, MCF and ATLIV are also believed to exhibit several homologous amino acid residue sequences among themselves as well as with the FMR group envelope glycoproteins.

Antigenic cross-reactivity based upon amino acid residue sequence homology is a phenomenon well known in the art. However, sequence homology does not guarantee cross-reactivity because the presentation of antigenic determinants may differ from one viral strain to another. For example,

-31-

R-MuLV and FeLV-B are believed to have gp70 sequence homology because of the known sequence homology between Friend murine leukemia virus (F-MuLV) and FeLV-B and the close serological relationship between F-MuLV and R-MuLV as demonstrated by Niman and Elder, supra. Yet, heteroserum made to native FeLV-B virion does not cross-react with R-MuLV gp70. This result, shown in FIGURE 8, suggests that antigenic determinants shared by R-MuLV and FeLV-B are not presented to their hosts in the same way.

The presentation of antigenic determinants in the R-MuLV gp70 system was also studied by Niman and Elder, supra. They demonstrated differential immunoprecipitation of native and deglycosylated gp70 by several monoclonal antibodies induced by native Rauscher gp70. It is believed that the differential immunoreactivity observed was due to carbohydrate side chains masking (sterically hindering) antigenic determinants.

To examine the possibility that carbohydrate side chains were masking antigenic determinants common to R-MuLV and FeLV-B, deglycosylated FeLV-B was used to induce antibodies in rabbits as described in detail hereinafter in Section III. As illustrated in FIGURE 9, antisera induced by deglycosylated, nor-FeLV-B immunoreacted with both native FeLV-B and nor-FeLV-B, as well as with native R-MuLV and deglycosylated R-MuLV gp70 glycoproteins.

It is thus seen that antibody-containing serum raised by immunization with one nor-virus immunoreacted with not only the envelope glycoprotein and carbohydrate-free envelope protein (polypeptide) of the immunizing nor-virus (nor-FeLV-B), but also immunoreacted with the envelope glycoprotein and carbohydrate-free envelope protein (polypeptide) of a

-32-

related viral strain; i.e., R-MuLV. Those results illustrate the improved cross-reactivity exhibited by the antibody-containing sera of the present invention.

5 Inasmuch as antigenic relatedness has been shown among the so-called FMR group of leukemia viruses, sequence homologies are known to be present among FeLV, HTLV I, HTLV II, HTLV III, MCF, and ATL envelope glycoproteins, and cross-reactivity between
10 serum of a patient with HTLV-II and a recombinant DNA-produced protein encoded by the env gene of HTLV-I has been reported [Samuel et al., Science, 226, 1094 (1984)], the illustration of improved cross-reactivity shown by the above-mentioned results
15 also illustrates an improved cross-reactivity of antibody-containing serum induced by a non-virus or carbohydrate-free envelope polypeptide of this invention among the nine specific, related viral strains named above, and among related leukemia
20 viruses generally. Similar improvements in antibody-containing serum cross-reactivities also result among additional, related groups of virus strains whose native forms contain N-linked carbohydrate moieties on their envelope proteins when
25 the methods disclosed herein are utilized. Exemplary of such additional, related groups of virus strains include strains of myxoviruses, measles viruses, rabies viruses and hepatitis B viruses.

30 D. Immunizations

 The polypeptides or non-viruses of this invention, when introduced into an animal host as a unit dose inoculum having an effective amount of polypeptide or non-virus in a physiologically
35 tolerable diluent, are capable of inducing production

-33-

of antibodies in the host mammal that immunoreact with the related virus, neutralize the virus in vitro, and preferably protect the host animal from in vivo infection caused by that virus.

5 The "effective amount" of polypeptide or nor-virus in a unit dose depends upon a number of factors. Included among those factors are the body weight of the animal immunized and the number of inoculations desired to be used. Individual unit
10 dose inoculations typically contain about 10 micrograms to about 500 milligrams of polypeptide or nor-virus per kilogram body weight of the mammalian host. Inoculation methods and amounts in rabbits for the purpose of raising antibodies are described below.

15 Useful free polypeptides of this invention may be obtained from whole viruses by well-known techniques. See, for example, Niman and Elder, "Monoclonal Antibodies and T-Cell Products", supra; Niman and Elder, Virology, 123, 187 (1982); and Niman
20 and Elder, Proc. Natl. Acad. Sci. (USA), 77, 4524 (1980). Influenza hemagglutinin may be obtained as described in Brown et al., J. Immunol., 125, 1583 (1980) and Aitken et al., Eur. J. Biochem., 107, 51 (1980). The resulting polypeptides are then
25 deglycosylated with Endo F, as described in Section III hereinafter, and may then be utilized in the inocula of the invention.

 Physiologically tolerable diluents are well known in the art, and alone are not part of the
30 present invention. Exemplary of such diluents are distilled or deionized water, normal saline solutions and phosphate-buffered saline (PBS) solutions.

 The immunizing composition or inoculum may be introduced into the host by intravenous injection,
35 or the like, using known methods. Adjuvants such as

-34-

complete Freund's adjuvant (CFA), incomplete
Freund's adjuvant (IFA), alum, tetanus toxoid and
the like as are well known in the immunological arts
may also be included in the inocula as part of the
5 physiologically tolerable diluent. Booster
injections may also be given, as desired, to build a
desired antibody titer in the host's serum.

Exact doses depend on the animal and
polypeptide or nor-virus used, and can be determined
10 using known challenge techniques. Additional
exemplary amounts of immunogen such as the
polypeptide or nor-virus, and specific reaction
conditions for the inoculum preparation may be found
in Bittle et al., Nature, 298, 30-33 (July, 1982).

15 The term "inoculum" is used herein to mean
any immunizing composition. As such, the term also
embraces vaccines that are useful in man and other
mammals for conferring in vivo protection against a
native virus. A given vaccine and inoculum may be
20 identical where non-human mammalian hosts are
involved, but typically differ where humans are the
intended hosts. The reason for that difference is
that adjuvants such as CFA are not utilized in
humans, and another adjuvant must be used if any
25 adjuvant is to be present in a human vaccine.

E. Antibodies

Antibodies to the polypeptides or
nor-viruses of the present invention can be used in
assays or to treat virus infections. The antibodies
30 can be used directly as whole, intact antibodies or
may be processed to provide Fab or F(ab')₂
portions, all of which are biologically active. The
term "antibody" indicates a whole, intact antibody or
the idiotype-containing polyamide portion of the
35 antibody that is biochemically active and is capable

-35-

of immunoreacting with or binding to its antigenic ligand on the native virus, nor-virus or glycoprotein.

To manufacture antibodies, an immunizing inoculum described before is introduced into the host mammal as by injection. The host is maintained for a time sufficient for antibodies to be induced, usually for one to about four months. The desired antibodies raised are thereafter harvested from host fluids. The whole antibodies so induced can be used directly, or they may be cleaved with pepsin or papain as is well known to provide $F(ab')_2$ or Fab portions that may be used. The antibodies produced may also be used as therapeutic agents for passive immunoprophylaxis.

An animal infected by a retrovirus such as a leukemia virus or a myxovirus such as an influenza virus may be treated with antibodies preferably as whole antibodies raised to the polypeptides or nor-viruses of the present invention. The antibodies are administered in a unit dose having an effective amount of antibodies dispersed in a physiologically tolerable diluent such as saline or phosphate buffered saline.

An effective amount of such antibodies varies depending on the reactivity and type of the antibodies, but generally about 1 milligram to about 50 milligrams of antibody per kilogram animal weight is considered effective. In the case of mice and native murine leukemia virus, 1.5 milligrams of IgG antibody in ascites fluid were found to be effective in prolonging survival. See "Monoclonal Antibodies", Kennett et al. ed., Plenum Press (1980). The antibodies may be introduced intravenously or intraperitoneally, with several administrations given

-36-

at three to seven day intervals. The antibodies may also be given in conjunction with surgical treatment.

The antibodies may be obtained from sera of a second animal, different from the first animal to be treated, by raising antibodies to the polypeptides or non-viruses of this invention. The antibodies may also be obtained from monoclonal sources such as ascites fluid by preparing a hybridoma cell line using known techniques. Whole antibodies are preferred as the antibodies since they are capable of activating the complement system when an immune complex is formed.

The term "unit dose" refers to physically discrete units suitable as unitary dosages for animals, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent or vehicle. The specifications for a novel unit dose of this invention are dictated by and are directly dependent on (a) the unique characteristics of the immunogen and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such active material for therapeutic use in animals.

III. Materials and Methods

A. Deglycosylation

Feline leukemia virus B (FeLV-B) and Rauscher murine leukemia virus (R-MuLV) obtained from the Resources Branch of the National Cancer Institute, Bethesda, MD were deglycosylated using endo-beta-N-Acetylglucosaminidase F (Endo F; Elder and Alexander, supra), that removes substantially all N-linked carbohydrate sidechains. A similar preparation of Endo F is also available from New England Nuclear (Boston, MA).

-37-

The removal of carbohydrate sidechains from R-MuLV was performed under three different virus denaturing (disrupting) conditions. Non-denaturing carbohydrate removal was accomplished by

5 deglycosylating R-MuLV in deglycosylation buffer alone 10 millimolar (mM) sodium phosphate (pH 6.1), 5 mM EDTA and 0.15 molar (M) NaCl). Deglycosylation resulting in mild disruption of the nor-virus was performed using deglycosylation buffer containing 1

10 percent Nonidet P40. Carbohydrate removal was also performed under disrupting-reducing conditions using deglycosylation buffer containing 1 percent Nonidet P40 and 1 percent 2-mercaptoethanol. FeLV-B was deglycosylated using only the latter of the above

15 buffer conditions.

Virus deglycosylated using non-denaturing conditions was first inactivated by exposing 2 milliliters (ml) of virus [0.1 milligram (mg)/ml in deglycosylation buffer] in an uncovered petri dish

20 (Falcon model 1007; Falcon, Oxnard, CA) to ultraviolet light (uv) (Sylvania Germicidal model G30T8, GTE-Sylvania, Stamford, CT) for 5 minutes. Complete inactivation was confirmed by the inability to induce virus producing foci when used as a control

25 in the appropriate neutralization assay described hereinafter.

The uv inactivated virus was then split into two 1 ml aliquots, one of which was incubated with 50 units of Endo F at 37°C for 16 hours, the control

30 aliquot being similarly incubated without Endo F.

Deglycosylation under 1) mild disrupting and 2) disrupting-reducing conditions was performed by incubating 10 units of Endo F with 200 micrograms of virus in 200 microliters of the appropriate

35 deglycosylation buffer for 16 hours at 37°C. Control

-38-

virus was similarly prepared without treatment with Endo F.

With respect to the X47 influenza virus, deglycosylation was accomplished by admixing 2×10^8 plaque-forming units (pfu) of X47 influenza virus in 0.1M Tris HCl (pH 7.6), 0.1 percent sodium dodecyl sulfate (SDS), 1 percent 2-mercaptoethanol, and 0.05M EDTA. The resulting admixture was then immersed in boiling water for 2 minutes.

Subsequently, Nonidet P40 was added to provide a concentration of 1 percent. The admixture was cooled to 22°C and 20 units of Endo F were added thereto. The new admixture was then incubated at 22°C for 16 hours, immersed in boiling water for 2 minutes, and then cooled to 22°C. Then, an additional 20 units of Endo F were admixed therein and the resulting admixture was incubated at 22°C for 16 hours. At this point, the deglycosylated influenza virus was suitable for injection into rabbits.

B. Virus Neutralizing Antibody Adsorption

The ability of an antigen to immunoreact with antibodies indicates that the antigen contains determinants that are the same or similar to those immunogenic determinants that induced the antibodies. Therefore, the ability of virus-neutralizing antibodies induced by native virus to immunoreact with deglycosylated virus indicates the presence of virus-neutralizing immunogenic determinants on the deglycosylated virus.

The present assays used a virus-neutralizing antibody adsorption technique to examine whether deglycosylated virus (R-MuLV or FeLV-B) could adsorb out (immunoreact with) the neutralizing antibodies in

-39-

heterosera induced by native virus (R-MuLV or FeLV-B).

Goat anti-TWEEN-ether disrupted virion (R-MuLV or FeLV-B) was serially adsorbed (immunoreacted) four times using 200 micrograms of deglycosylated (uv inactivated, non-denatured) or control virus per adsorption. Illustratively, 1.5 ml of goat anti-gp70 antiserum diluted 1:100 using Minimum Eagle's Medium (MEM) containing 10 percent fetal calf serum was admixed (immunoreacted) with virus for 1 hour at 20°C with agitation. The reaction mixture was centrifuged for 5 minutes in a Fisher model 235A microcentrifuge to pellet the immunoreaction products.

A 200 microliter aliquot was removed from the supernatant and designated the 1x adsorption. The remaining supernatant was adsorbed against fresh virus following the above procedure. Further adsorptions were sequentially repeated until antiserum samples adsorbed 1x, 2x, 3x and 4x were obtained. These serially adsorbed serum samples were then assayed for their ability to neutralize native virus in the neutralization assay described hereinbelow.

25 C. Neutralization Assay

The immunoblotting neutralization assay described in Elder et al., Bio Techniques, 170-172, May-June, 1984 was used to examine, 1) the ability of deglycosylated virus to adsorb out neutralizing antibodies, and 2) the ability of antiserum induced by deglycosylated virus to neutralize native virus. The assay measured in vitro viral neutralization (inactivation) by immunoreaction between antibodies and live (infectious) virus particles. The assay was

35

-40-

reproducible and yielded a percent reduction in virus producing foci between control and experimental sera.

The type of cell line subjected to infection in the assay depended upon the virus under investigation because most viruses have limited host specificity. In the present assays, when the feline virus FeLV-B was being examined, the dog thymus cell line CF2th (ATCC CRL 1430, American Type Culture Collection, Bethesda, MD) was used as viral host. When the murine virus R-MuLV was being investigated, the mouse embryo cell line SC-1 (ATCC CRL 1404, American Type Culture Collection, Bethesda, MD) was used as viral host.

Both cell lines were grown in Minimum Eagle's Medium (MEM) supplemented with 10 percent fetal calf serum, penicillin (100 units/milliliter), streptomycin (100 milligrams/milliliter), 4 mM L-glutamine, and 1.0 mM sodium pyruvate. Following trypsinization with 0.025 percent trypsin in phosphate-buffered saline (PBS) and washing in MEM, 3×10^5 cells were added to tissue culture petri dishes (Falcon #3003, Falcon, Oxnard, CA) and were incubated overnight at 37°C in a humidified atmosphere of 5 percent CO₂.

When the neutralization assay was used to examine the ability of deglycosylated virus to adsorb out neutralizing antibodies, native (untreated and infectious) virus (FeLV-B or R-MuLV) was immunoreacted with sera previously subjected to the virus neutralizing antibody adsorption technique described hereinabove. Non-adsorbed and normal rabbit sera were used as controls in addition to the control sera obtained during the adsorption.

When the neutralization assay was used to examine the ability of antiserum induced by

-41-

deglycosylated virus to neutralize native virus,
native (untreated and infectious) virus (FeLV-B or
R-MuLV) was immunoreacted with sera induced by
nor-virus whose carbohydrate sidechains had been
5 removed. Heteroserum raised against native virus and
normal rabbit serum were used as controls.

Study and control immunoreactions were
performed in separate containers by admixing 5
microliters of antiserum with 100 microliters of MEM
10 containing 400 focus-forming units of virus. Each
mixture was incubated at 37°C in a humidified 5
percent CO₂ atmosphere for 40 minutes. A solution
of 5 milliliters of MEM containing 10 micrograms per
milliliter hexadimethrine bromide (Polybrene P415,
15 Sigma Chemical Co. St. Louis, MO) was then added.
The resulting admixture was then used to inoculate
the appropriate host cell culture.

The host cell cultures were inoculated by
replacing the overnight growth MEM with 5 milliliters
20 of the above admixture. This mixture was replaced
after 24 hours and the cultures were incubated in MEM
for an additional 4 day period at 37°C in a 5 percent
CO₂ atmosphere. The cultures were then terminated
by aspirating off the MEM, washing 3 times with 10
25 milliliters of PBS and allowing the monolayers to dry.

Virus-producing cell foci in the monolayer
were detected by two techniques: (1) a radioimmune
assay (RIA) and (2) an ELISA. Both assays were
performed by pre-wetting a nitrocellulose disc
30 designated BA85 (Schleicher & Schuell, Inc., Keene,
NH) in PBS, placing the wetted disc over the
monolayer and pressing firmly so as to transfer the
cells from the petri dish onto the disc.

The cells were fixed on the disc by placing
35 the disc in Amido Black Dye [0.045 percent Naphthol
Blue Black (Sigma Chemical Co. St. Louis, MO), 45

-42-

percent methanol, 10 percent acetic acid] for 1 minute and then destained (45 percent methanol, 10 percent acetic acid) for 5 minutes. Non-specific binding sites were then blocked by incubating the
5 discs in BLOTTO [Bovine Lacto Transfer Technique Optimizer, 5 percent weight per volume non-fat dry milk, 0.01 percent Antifoam A Emulsion (a 30 percent aqueous emulsion of a silicone polymer containing anionic emulsifiers, Sigma Chemical Co., St. Louis,
10 MO), 0.0001 percent merthiolate (Sigma Chemical Co., St. Louis, MO) in PBS] overnight at 4°C, as in Johnson and Elder, J. Exp. Med., 159, 1751 (1983).

After blocking, each disc was placed in 5 milliliters of fresh BLOTTO with 50 microliters of
15 goat anti-whole virus antiserum, (either anti-FeLV-B or anti-R-MuLV as appropriate) for 3 hours at 20°C while shaking. The discs were then washed with 5 milliliters of BLOTTO three times for 15 minutes each. The washed discs were placed in 5 milliliters
20 of BLOTTO containing 5 microliters of rabbit anti-goat antisera and were incubated at 20°C for 1 hour with shaking. They were then again washed 3 times with BLOTTO.

Goat anti-whole virus antiserum was prepared
25 by injecting either FeLV or R-MuLV virus particles into goats and recovering the sera. The rabbit anti-goat antisera were prepared by injecting rabbits as described above using purified goat gamma-globulin as immogen in a physiologically tolerable diluent.

30 For the RIA, Staphylococcus aureus Protein A was labeled with ¹²⁵I, and 5 milliliters of BLOTTO containing 2.5 microliters of ¹²⁵I-labeled Protein A was incubated with the discs for 1 hour at 20°C with shaking. This allowed the labeled Protein A to
35 bind with the bound rabbit anti-goat antibodies. The

-43-

discs were again washed with lithium chloride buffer [0.5 M LiCl (Fisher Scientific, Pittsburgh, PA), 0.1 M Tris Base (Sigma Chemical Co. St. Louis, MO] for 30 minutes and finally with water for 30 minutes. The washed discs were dried and exposed to Kodak XRP-1 x-ray film available from Eastman Kodak Company, Rochester, NY.

For the ELISA, peroxidase-coupled goat anti-rabbit IgG (Tago, Burlingame CA) was diluted 1:500 in BLOTTO. The BLOTTO-washed discs were then immersed in an amount of the diluted, peroxidase-coupled goat anti-rabbit IgG composition to cover the discs, and maintained for a time period of 1.5 hours to bind the peroxidase-labeled goat antibodies to the disc-bound rabbit antibodies. The discs were thereafter washed in a bath of BLOTTO for 10 minutes followed by a washing in a deionized water bath for 10 minutes.

An ELISA-developing bath was prepared by admixing the following: 8 ml of a solution of 4-chloro-2-naphthol (3 mg/ml in methanol), 4 ml of 1 molar sodium acetate (pH 5.9), 41 ml of water and 30 microliters of 30% hydrogen peroxide. The above, washed discs were immersed in a sufficient amount of developing bath to cover the discs, and maintained in the bath for a period of 2-5 minutes. The color development reaction was stopped by washing the discs with water, and the discs were air dried.

The Western blot analyses of FIGURES 5, 8 and 9 were also visualized following the above ELISA technique.

Results of the assays are shown the graphs of FIGURES 4, 6 and 7. The percent neutralization was calculated from the number of foci on any given

-44-

disc treated with an antiserum compared to the number of foci on a control disc treated with control sera.

D. Western Blotting

The Western Blot technique was done according to published procedures and was used to examine the ability of heteroserum raised against the whole virus to bind deglycosylated gp70. The viral proteins were separated by gradient (5-17.5 percent) SDS-polyacrylamide gel electrophoresis. See Laemmli, Nature, supra, and Towbin, et al., Proc. Natl. Acad Sci. (USA), supra.

Proteins were electrophoretically transferred to nitrocellulose (Schleicher & Schuell, Keene, NH) as described by Towbin et al., Proc. Natl. Acad. Sci. (USA), 76, 4350 (1976), using an electroblot apparatus, (E.C. Apparatus Corp., Jacksonville, FL) with buffer consisting of 25 millimolar Tris Base, 192 millimolar glycine, 20 percent methanol and 0.1 percent sodium dodecyl sulfate (pH 8.3). Following the transfer, the nitrocellulose was blocked in BLOTTO to reduce non-specific binding. The resulting blots were reacted with 100 microliters of heteroserum to R-MuLV or FeLV as appropriate in 10 milliliters of BLOTTO for 3 hours, and were then washed three times for 1 hour with 50 milliliters of fresh BLOTTO.

Antibodies bound to specific control and deglycosylated viral proteins were detected by one of two methods. In the first method, antibodies were detected by reacting the blots with 20 microliters of ¹²⁵I-labeled Protein A in 10 milliliters of BLOTTO for 1 hour. The blots were then washed four times in 50 milliliters of fresh BLOTTO for 15 minutes and then under a continuous flow of water for 20 minutes.

-45-

In the second method, such antibodies were detected by reacting the blots with peroxidase-coupled goat anti-rabbit IgG, hydrogen peroxide and o-dianisidine as described in FIGURE 3.

5 E. Immunizations

 The antisera against deglycosylated, nor-virus used in the neutralization assays described herein were obtained from immunized rabbits. Control sera were obtained from rabbits by bleeding just
10 prior to initial immunization.

 Antiserum to deglycosylated nor-R-MuLV was obtained by immunization with 200 micrograms nor-R-MuLV, deglycosylated under mild disrupting conditions, as described hereinabove, in complete
15 Freund's adjuvant (CFA), in incomplete Freund's adjuvant (IFA) and on alum (5 milligrams/milliliter) on days 0, 14 and 21, respectively. Thereafter, the rabbit was boosted with nor-R-MuLV, deglycosylated under disrupting-reducing conditions, as described
20 hereinabove, or alum on a monthly basis. Rabbits immunized with influenza-related immunogens were injected with inocula containing 2×10^7 plaque-forming units of virus or nor-virus following the above immunization regimen.

25 Each immunization consisted of introducing the inoculum in four subcutaneous injections of 50 micrograms of deglycosylated, nor-virus, one on each shoulder and hip. Rabbits were bled 7 days after the last immunization, and in some cases, the rabbits
30 were further boosted, as described above, with immunogen in alum and bled as necessary. The collected blood was allowed to clot overnight and then was centrifuged to obtain the sera used in the assays discussed hereinabove.

35

-46-

In addition to the adjuvants, the inocula also contained a physiologically tolerable diluent such as water, or phosphate-buffered saline (pH 7.4). Inocula stock solutions were prepared with CFA or IFA as follows: An amount of deglycosylated nor-virus conjugate sufficient to provide the desired amount of deglycosylated nor-virus inoculation was dissolved in phosphate-buffered saline (PBS). Equal volumes of CFA or IFA were then mixed with the deglycosylated nor-virus conjugate solution to provide an inoculum containing deglycosylated nor-virus, water and adjuvant solution in which the water to oil ratio was 1:1. The mixture was thereafter homogenized to provide the inoculum stock solution.

The foregoing is intended as illustrative of the present invention but not limiting. Numerous variations and modifications may be effected without departing from the true spirit and scope of the invention.

25

30

35

-47-

WHAT IS CLAIMED IS:

1. A polypeptide comprising an amino acid residue sequence substantially identical to that of a viral envelope N-linked glycoprotein, said
5 polypeptide being substantially free of the N-linked glycosylation of said glycoprotein and inducing the production of antibody-containing serum that neutralizes the virus of said viral glycoprotein at a greater serum dilution than does antibody-containing
10 serum induced by the viral envelope N-linked glycoprotein when the polypeptide and the viral envelope N-linked glycoprotein are individually utilized to induce antibody production in separate host mammals of the same strain using substantially
15 identical immunization regimens.
2. The polypeptide of claim 1 wherein said glycoprotein is a glycoprotein of feline leukemia virus-B.
3. The polypeptide of claim 1 wherein said
20 glycoprotein is surface glycoprotein gp70 of Rauscher murine leukemia virus.
4. The polypeptide of claim 1 wherein said antibodies induced by said polypeptide provide improved cross-reactivity among related viral
25 strains.
5. A nor-virus whose envelope protein has an amino acid residue sequence substantially identical to that of a viral envelope N-linked glycoprotein, said envelope protein of said nor-virus
30 being substantially free of carbohydrate moieties covalently linked to asparagine residues and inducing the production of antibody-containing serum that neutralizes the virus of said viral glycoprotein at a greater serum dilution than does antibody-containing
35

-48-

serum induced by said virus, when said nor-virus and said virus are individually utilized to induce antibody production in separate host mammals of the same strain using substantially identical immunization regimens.

6. The nor-virus of claim 5 wherein said glycoprotein is a glycoprotein of feline leukemia virus-B.

7. The nor-virus of claim 5 wherein said glycoprotein is surface glycoprotein gp70 of Rauscher murine leukemia virus.

8. The nor-virus of claim 5 wherein said glycoprotein is X47 influenza virus hemagglutinin.

9. A nor-virus whose envelope protein has an amino acid residue sequence substantially identical to that of a viral envelope N-linked glycoprotein, said envelope protein of said nor-virus being substantially free of carbohydrate moieties covalently linked to asparagine residues and inducing the production of antibody-containing serum that provides improved cross-reactivity among related viral strains, when said nor-virus and the virus of said viral glycoprotein are individually utilized to induce antibody production in separate host mammals of the same strain using substantially identical immunization regimens.

10. The nor-virus of claim 9 wherein said glycoprotein is surface glycoprotein gp70 of Rauscher murine leukemia virus.

11. The nor-virus of claim 9 wherein said glycoprotein is X47 influenza virus hemagglutinin.

12. The nor-virus of claim 9 wherein said glycoprotein is a glycoprotein of feline leukemia virus-B.

-49-

13. The nor-virus of claim 9 wherein said virus is a retrovirus.

14. The nor-virus of claim 9 wherein said virus is a myxovirus.

5 15. The nor-virus of claim 9 wherein said antibodies induced by said nor-virus neutralize said virus at a greater serum dilution than do said antibodies induced by said virus.

10 16. An inoculum against infection by a retrovirus comprising an effective amount of (1) a polypeptide comprising an amino acid residue sequence substantially identical to that of a viral envelope N-linked glycoprotein, said polypeptide being substantially free of the glycosylation of said
15 N-linked glycoprotein and inducing the production of antibody-containing serum that (i) provides improved cross-reactivity among related viral strains and (ii) neutralizes the virus of said viral glycoprotein at a greater serum dilution than does
20 antibody-containing serum induced by said viral envelope N-linked glycoprotein when said polypeptide and said viral envelope N-linked glycoprotein are individually utilized to induce antibody production in separate host mammals of the same strain using
25 substantially identical immunization regimens, and (2) a physiologically tolerable diluent, said inoculum, when introduced into a host, being capable of inducing the production of antibodies in the host that immunoreact with said retrovirus, neutralize
30 said retrovirus in vitro, and protect the host from in vivo retroviral infection.

17. The inoculum of claim 16 wherein said glycoprotein is a surface glycoprotein of said retrovirus.

35

-50-

18. An inoculum against infection by a myxovirus having an effective amount of (1) a polypeptide comprising an amino acid residue sequence substantially identical to that of a viral envelope N-linked glycoprotein, said polypeptide being substantially free of the N-linked glycosylation of said glycoprotein and inducing the production of antibody-containing serum that (i) provides improved cross-reactivity among related viral strains and (ii) neutralizes the virus of said viral glycoprotein at a greater serum dilution than does antibody-containing serum induced by said viral envelope N-linked glycoprotein when said polypeptide and said viral envelope N-linked glycoprotein are individually utilized to induce antibody production in separate host mammals of the same strain using substantially identical immunization regimens, and (2) a physiologically tolerable diluent, said inoculum, when introduced into a host, being capable of inducing the production of antibodies in the host that immunoreact with said myxovirus, neutralize said myxovirus in vitro, and protect the host from in vivo myxoviral infection.

19. The inoculum of claim 18 wherein said glycoprotein is an influenza virus hemagglutinin.

20. Antibodies to retrovirus, said antibodies being raised in an animal host to a polypeptide, said polypeptide comprising an amino acid residue sequence substantially identical to that of a viral envelope N-linked glycoprotein, said polypeptide being substantially free of the N-linked glycosylation of said glycoprotein and inducing the production of antibody-containing serum that (i) provides improved cross-reactivity among related viral strains and (ii) neutralizes the virus of said

-51-

viral glycoprotein at a greater serum dilution than does antibody-containing serum induced by said viral envelope N-linked glycoprotein when said polypeptide and said viral envelope N-linked glycoprotein are individually utilized to induce antibody production in separate host mammals of the same strain using substantially identical immunization regimens, said antibodies having the capacity to immunoreact with said retrovirus and neutralize said retrovirus in vitro.

21. The antibodies of claim 20 wherein said glycoprotein is a surface glycoprotein of said retrovirus.

22. The antibodies of claim 20 wherein said retrovirus is Rauscher murine leukemia virus.

23. The antibodies of claim 20 wherein said retrovirus is feline leukemia virus-B.

24. Antibodies to myxovirus, said antibodies being raised in an animal host to a polypeptide, said polypeptide comprising an amino acid residue sequence substantially identical to that of a viral envelope N-linked glycoprotein, said polypeptide being substantially free of the N-linked glycosylation of said glycoprotein and inducing the production of antibody-containing serum that (i) provides improved cross-reactivity among related viral strains and (ii) neutralizes the virus of said viral glycoprotein at a greater serum dilution than does antibody-containing serum induced by said viral envelope N-linked glycoprotein when said polypeptide and said viral envelope N-linked glycoprotein are individually utilized to induce antibody production in separate host mammals of the same strain using substantially identical immunization regimens, said

-52-

antibodies having the capacity to immunoreact with said myxovirus and neutralize said myxovirus in vitro.

25. The antibodies of claim 24 wherein said
5 glycoprotein is an influenza virus hemagglutinin.

26. A method for the production of antibodies that neutralize a virus comprising the steps of:

(a) introducing into an animal host an
10 effective amount of a polypeptide comprising an amino acid residue sequence substantially identical to that of a viral envelope N-linked glycoprotein, said polypeptide being substantially free of the N-linked glycosylation of said glycoprotein and inducing the
15 production of antibody-containing serum that (i) provides improved cross-reactivity among related viral strains and (ii) neutralizes the virus of said viral glycoprotein at a greater serum dilution than does antibody-containing serum induced by said viral
20 envelope N-linked glycoprotein when said polypeptide and said viral envelope N-linked glycoprotein are individually utilized to induce antibody production in separate host mammals of the same strain using substantially identical immunization regimens, said
25 polypeptide being capable of inducing the production of antibodies in the serum of said host; and

(b) maintaining said host for a period of time sufficient for antibodies to be induced; and

(c) collecting said induced
30 antibody-containing serum from said host;
said antibody-containing serum immunoreacting with said virus and neutralizing said virus in vitro.

27. The method of claim 26 wherein said
35 virus is a retrovirus.

-53-

28. The method of claim 26 wherein said virus is a myxovirus.

29. A method of improving the immunogenicity of a viral envelope N-linked glycoprotein comprising the steps of:

5 (a) providing said viral envelope N-linked glycoprotein; and

(b) reacting said glycoprotein with a glycosidase, said glycosidase removing glycosyl groups from said glycoprotein to form a polypeptide, said polypeptide being capable of inducing the production of antibody-containing serum that (i) provides improved cross-reactivity among related viral strains and (ii) neutralizes the virus of said viral glycoprotein at a greater serum dilution than does antibody-containing serum induced by said viral envelope N-linked glycoprotein when said polypeptide and said viral envelope N-linked glycoprotein are individually utilized to induce antibody production in separate host mammals of the same strain using substantially identical immunization regimens.

30. The method of claim 29 wherein said glycosyl cleavage occurs at the asparagine residue on said glycoprotein.

25 31. The method of claim 29 wherein said glycoprotein is surface glycoprotein gp70 of Rauscher murine leukemia virus.

32. The method of claim 29 wherein said glycoprotein is X47 influenza virus hemagglutinin.

30 33. The method of claim 29 wherein said glycoprotein is a glycoprotein of feline leukemia virus-B.

34. The method of claim 29 wherein said native virus is a retrovirus.

35

-54-

35. The method of claim 29 wherein said native virus is a myxovirus.

36. A method of immunizing an animal against a virus comprising the steps of:

5 (a) providing a unit dose of inoculum comprising a polypeptide of claim 1 dispersed in an effective amount in a physiologically tolerable diluent, said polypeptide having the capacity to induce the production of antibodies in said animal
10 that immunoreact with said virus, neutralize said virus in vitro, and protect said animal from said virus; and

(b) injecting the unit dose of said inoculum into the blood stream of the animal to be
15 immunized; and

(c) maintaining said host for a period of time sufficient for protective antibodies to be induced.

37. The method of claim 36 wherein said
20 virus is a retrovirus.

38. The method of claim 36 wherein said virus is a myxovirus.

25

30

35

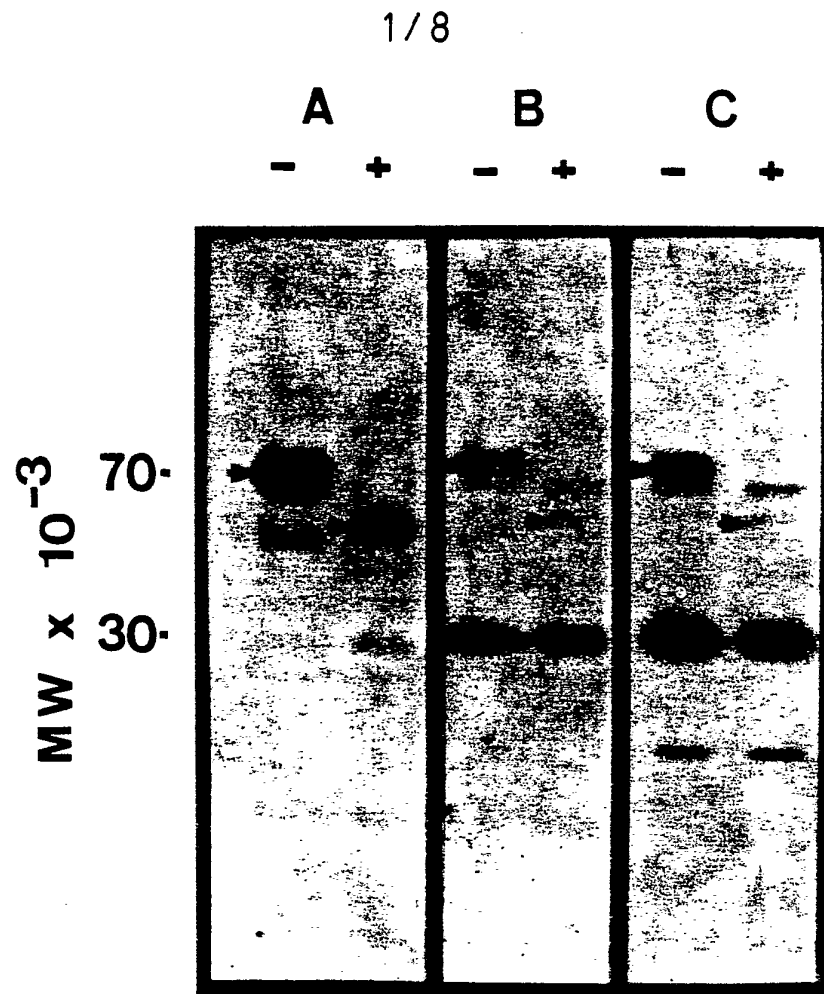
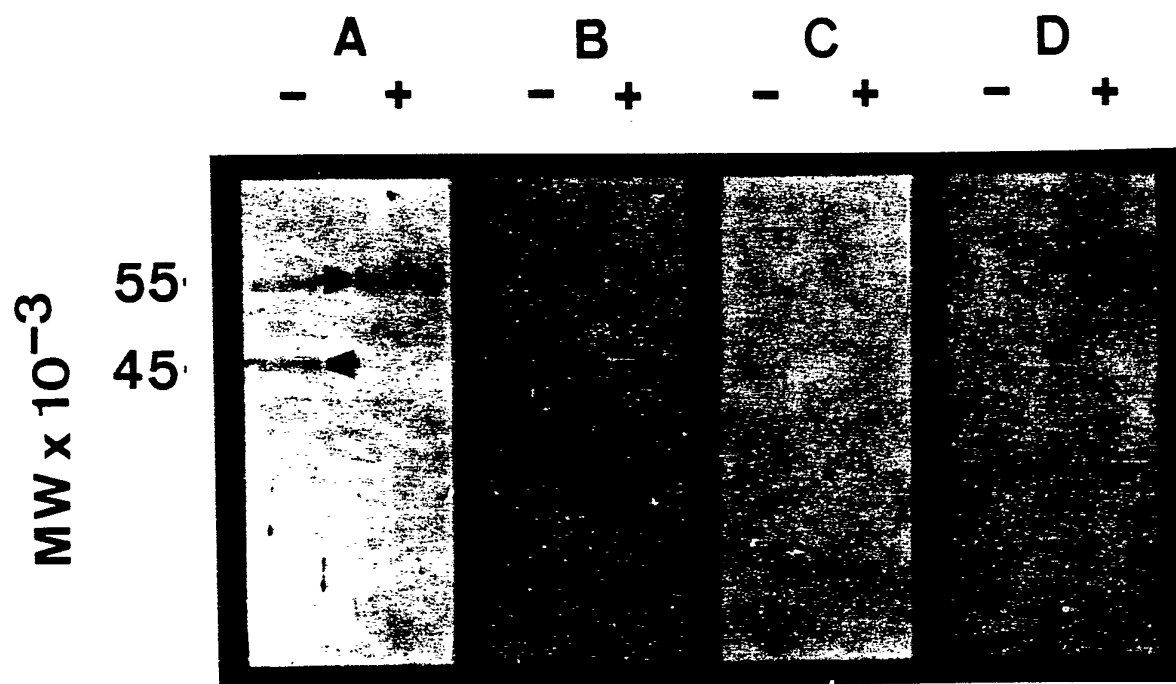


FIG. 1

FIG. 3



2 / 8

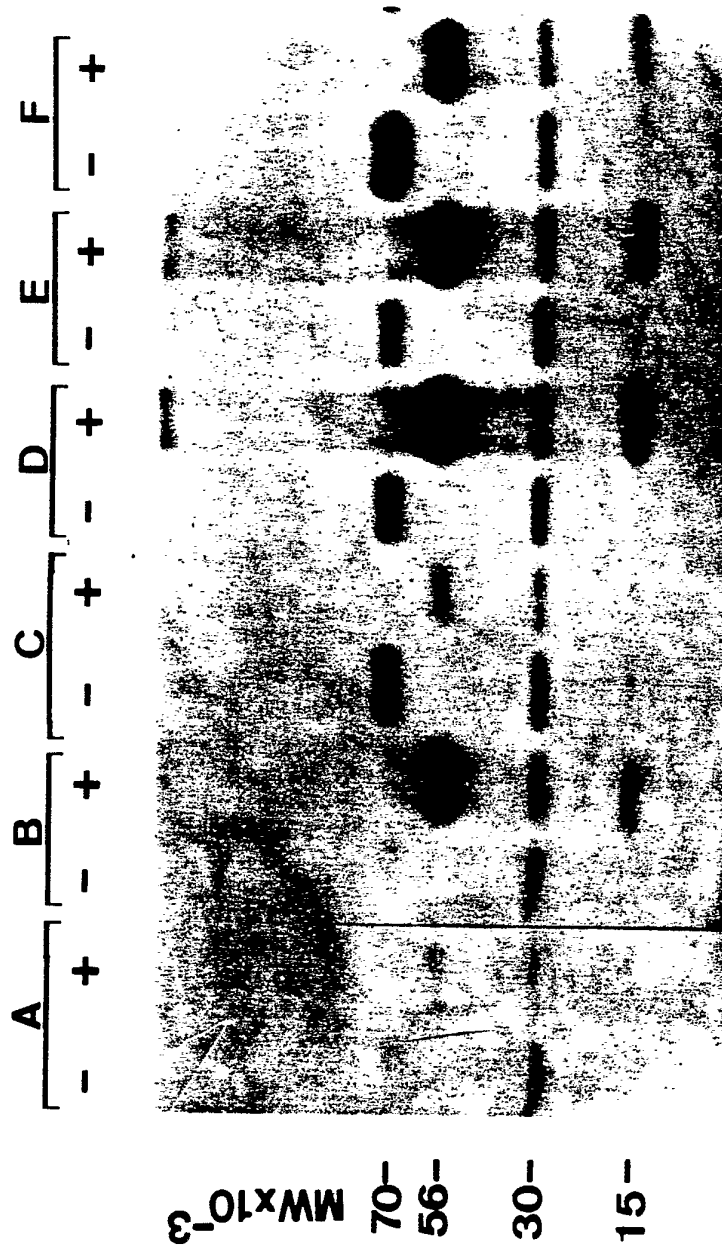
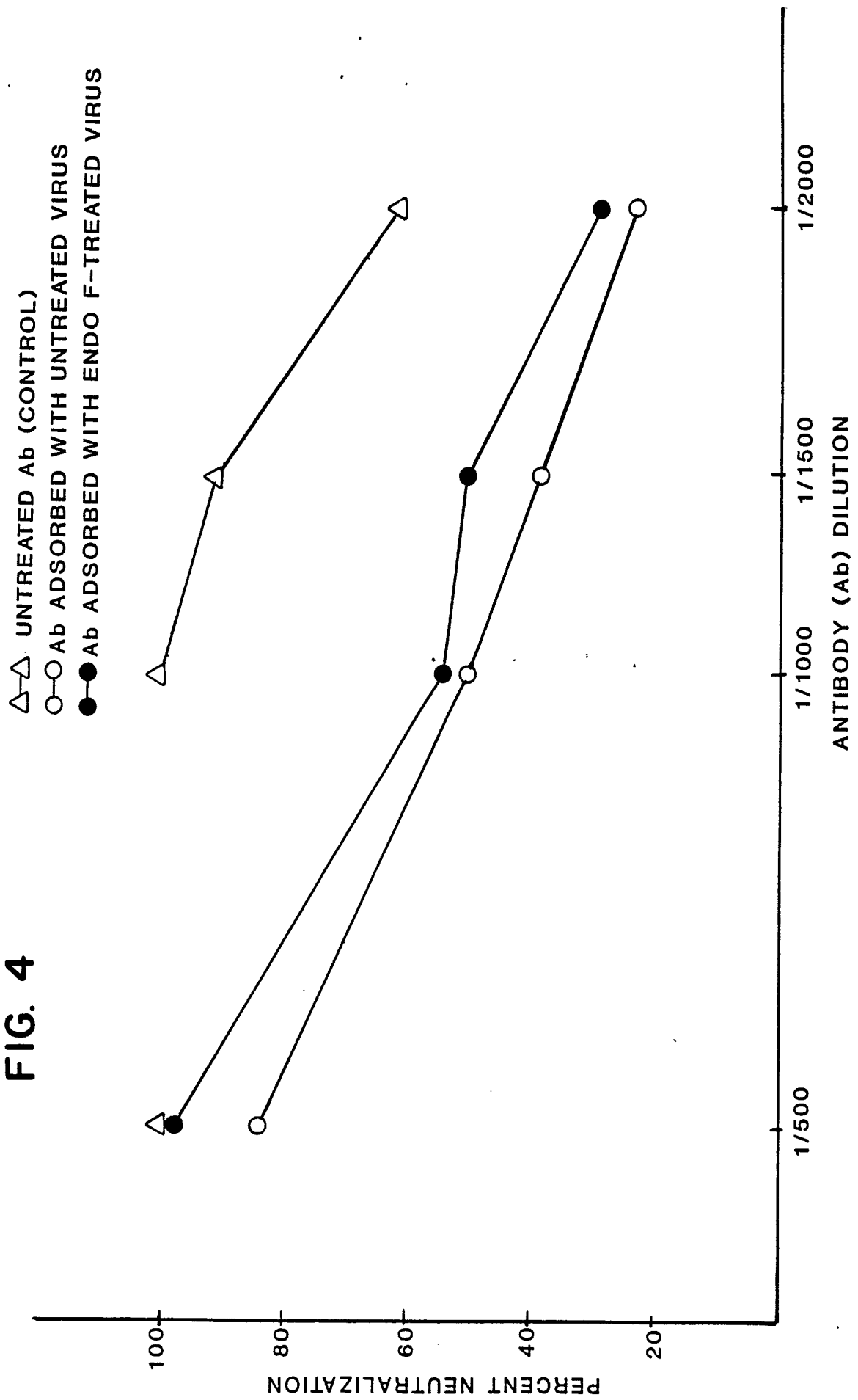


FIG. 2

3 / 8

FIG. 4



LENS ELEVATION SHEET

4 / 8

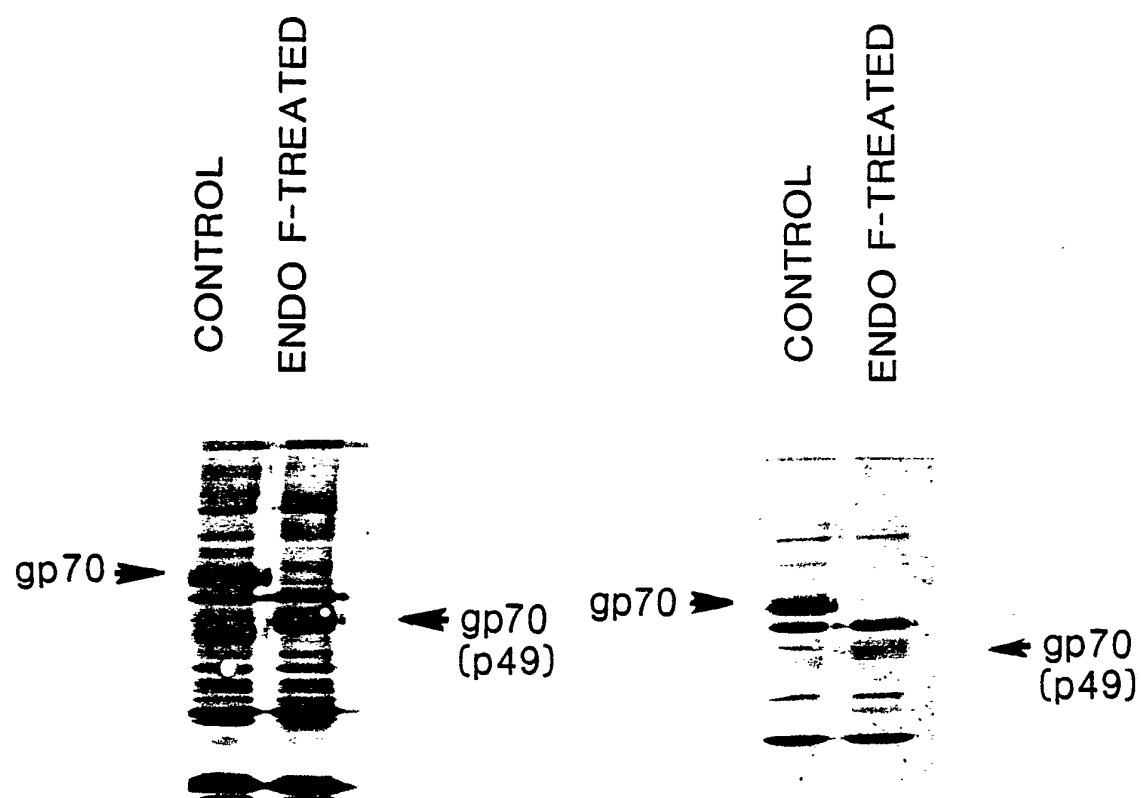
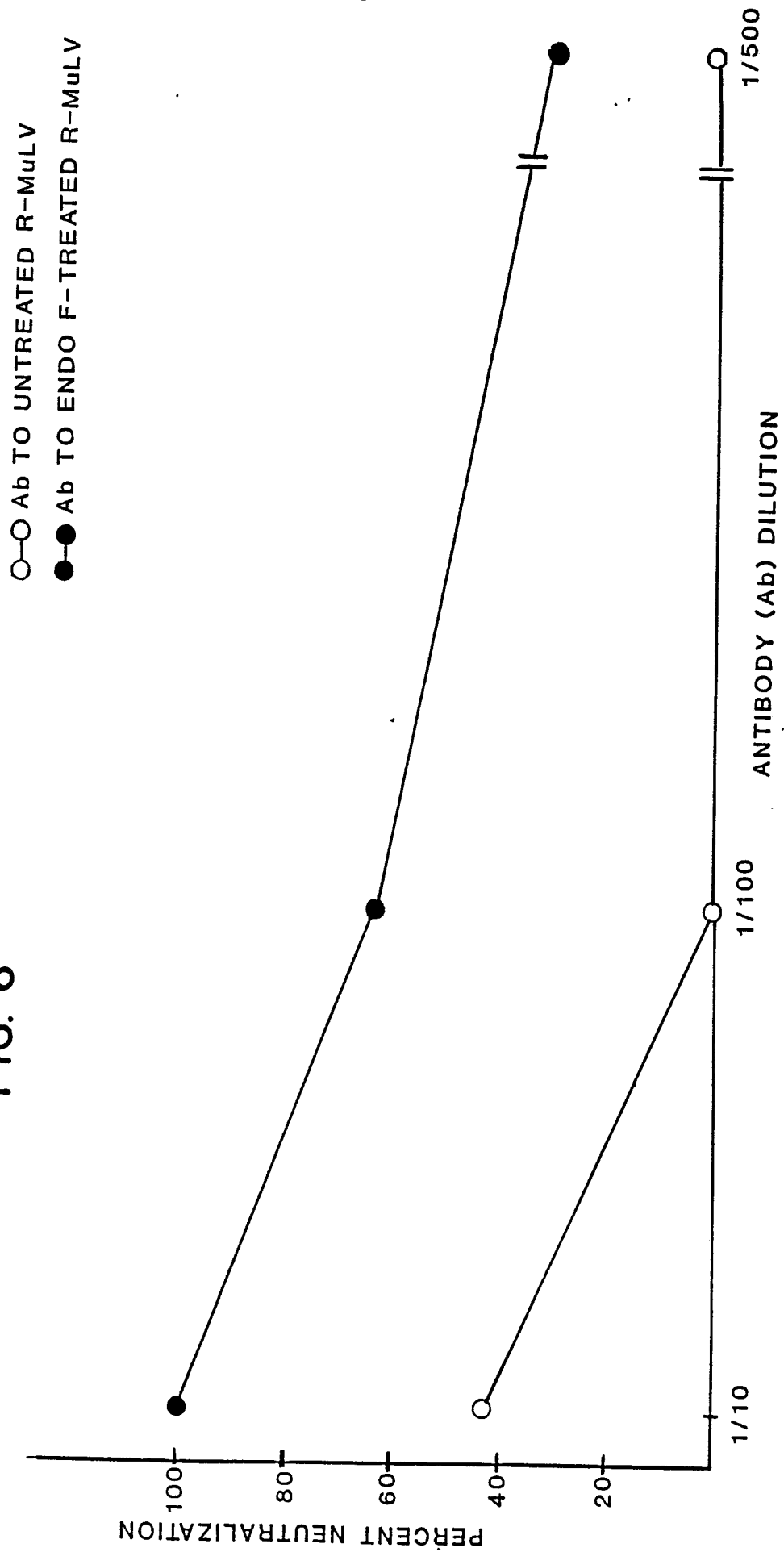


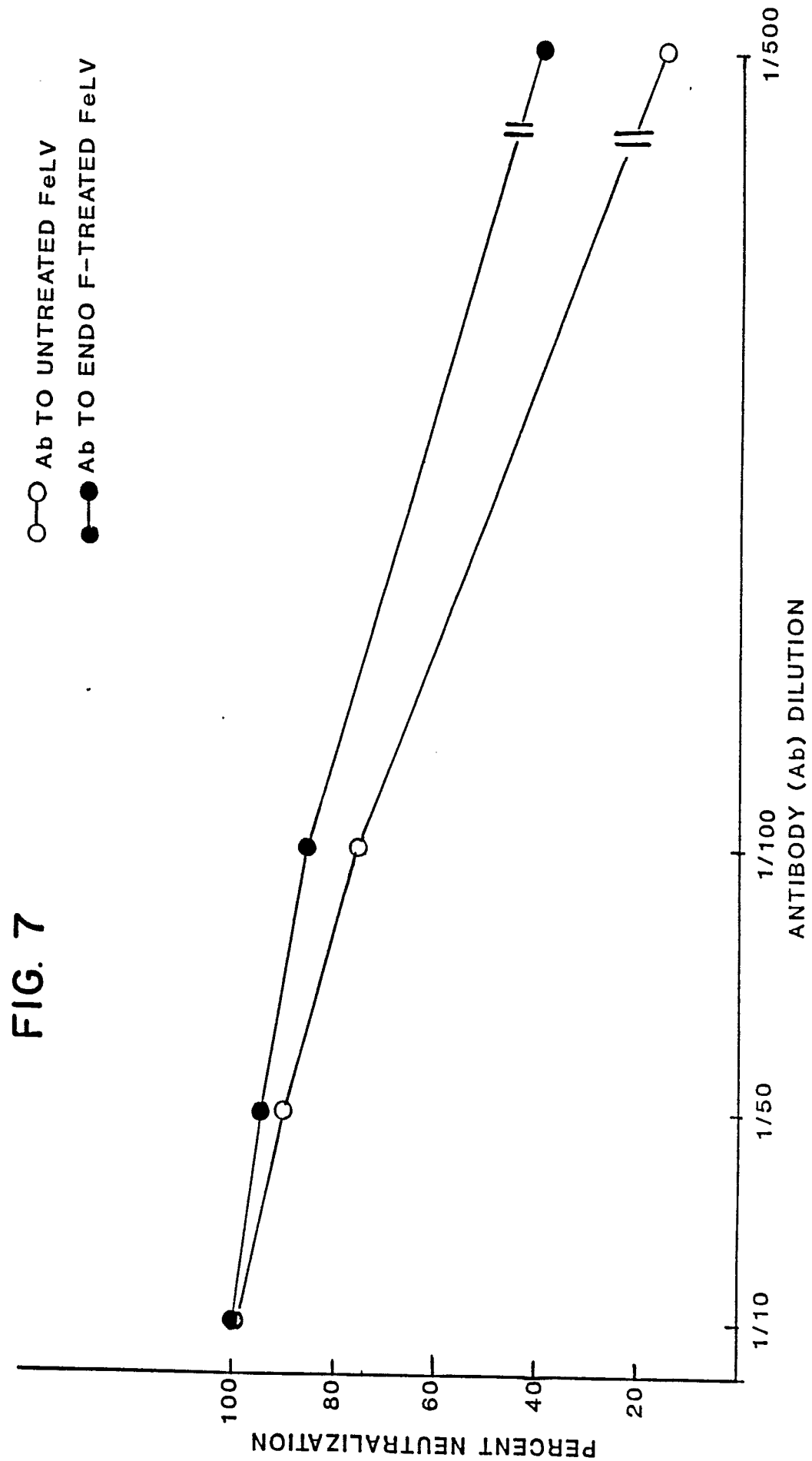
FIG. 5

5 / 8

FIG. 6



6 / 8



7/8



R-MuLV		FeLV-B	
CONTROL	ENDO F-TREATED	CONTROL	ENDO F-TREATED

FIG. 8

8 / 8

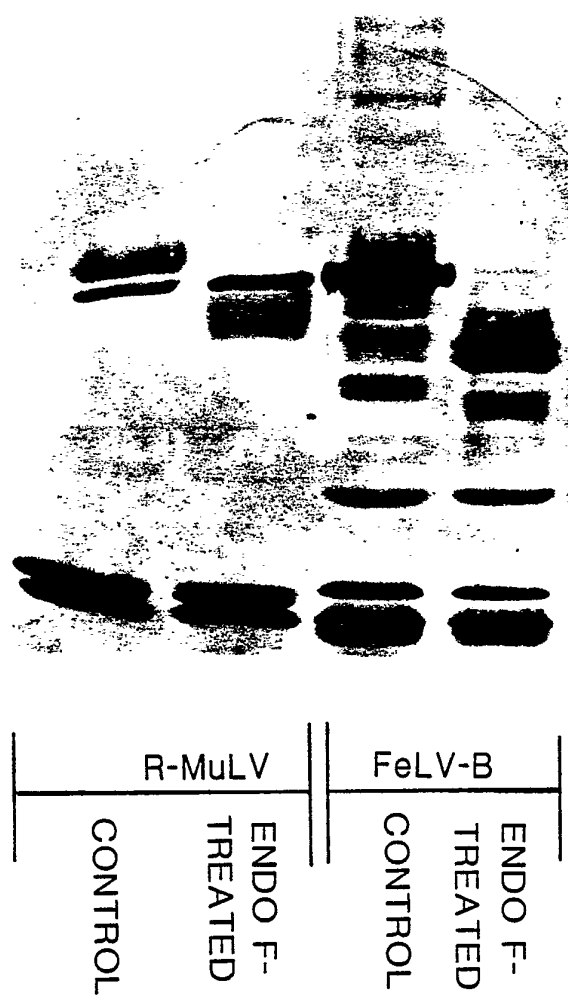


FIG. 9

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US85/02319

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³ According to International Patent Classification (IPC) or to both National Classification and IPC <div style="display: flex; justify-content: space-between; margin-top: 10px;"> U.S. CL. 435/235 INT. CL.⁴ C12N 7/00 </div>														
II. FIELDS SEARCHED <div style="text-align: center; margin-top: 10px;">Minimum Documentation Searched ⁴</div> <table border="1" style="width: 100%; border-collapse: collapse; margin-top: 5px;"> <tr> <th style="width: 20%;">Classification System</th> <th style="width: 80%;">Classification Symbols</th> </tr> <tr> <td style="vertical-align: top; padding: 5px;">U.S.</td> <td style="padding: 5px;">435/68, 235, 236, 238 424/89, 93</td> </tr> </table> <div style="text-align: center; margin-top: 10px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁵</div>			Classification System	Classification Symbols	U.S.	435/68, 235, 236, 238 424/89, 93								
Classification System	Classification Symbols													
U.S.	435/68, 235, 236, 238 424/89, 93													
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴ <table border="1" style="width: 100%; border-collapse: collapse; margin-top: 5px;"> <tr> <th style="width: 10%;">Category *</th> <th style="width: 60%;">Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷</th> <th style="width: 30%;">Relevant to Claim No. ¹⁸</th> </tr> <tr> <td style="vertical-align: top; text-align: center; padding: 5px;">A</td> <td style="padding: 5px;"> <u>Proc. Natl. Acad. Sci. USA Vol. 77, p.1622-1626. 1980.</u> H. Snyder and E. Fleissner. Specificity of human antibodies to oncovirus glycoproteins: Recognition of antigen by natural antibodies directed against carbohydrate structures. </td> <td style="vertical-align: top; text-align: center; padding: 5px;">1-38</td> </tr> <tr> <td style="vertical-align: top; text-align: center; padding: 5px;">Y</td> <td style="padding: 5px;"> <u>Virology, Vol. 109, p.431-434. 1981.</u> M. Schmerr et al. Antigenic Reactivity of a soluble glycoprotein associated with bovine leukemia virus. </td> <td style="vertical-align: top; text-align: center; padding: 5px;">1-38</td> </tr> <tr> <td style="vertical-align: top; text-align: center; padding: 5px;">Y</td> <td style="padding: 5px;"> <u>Proc. Natl. Acad. Sci. USA Vol. 79, p.4540-4544. 1982.</u> J.Elder and S. Alexander. endo-B-N-Acetylglucosaminidase F: Endoglycosidase from <u>Flavobacterium meningosepticum</u> that cleaves both high-mannose and complex glycoproteins. </td> <td style="vertical-align: top; text-align: center; padding: 5px;">1-38</td> </tr> </table>			Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸	A	<u>Proc. Natl. Acad. Sci. USA Vol. 77, p.1622-1626. 1980.</u> H. Snyder and E. Fleissner. Specificity of human antibodies to oncovirus glycoproteins: Recognition of antigen by natural antibodies directed against carbohydrate structures.	1-38	Y	<u>Virology, Vol. 109, p.431-434. 1981.</u> M. Schmerr et al. Antigenic Reactivity of a soluble glycoprotein associated with bovine leukemia virus.	1-38	Y	<u>Proc. Natl. Acad. Sci. USA Vol. 79, p.4540-4544. 1982.</u> J.Elder and S. Alexander. endo-B-N-Acetylglucosaminidase F: Endoglycosidase from <u>Flavobacterium meningosepticum</u> that cleaves both high-mannose and complex glycoproteins.	1-38
Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸												
A	<u>Proc. Natl. Acad. Sci. USA Vol. 77, p.1622-1626. 1980.</u> H. Snyder and E. Fleissner. Specificity of human antibodies to oncovirus glycoproteins: Recognition of antigen by natural antibodies directed against carbohydrate structures.	1-38												
Y	<u>Virology, Vol. 109, p.431-434. 1981.</u> M. Schmerr et al. Antigenic Reactivity of a soluble glycoprotein associated with bovine leukemia virus.	1-38												
Y	<u>Proc. Natl. Acad. Sci. USA Vol. 79, p.4540-4544. 1982.</u> J.Elder and S. Alexander. endo-B-N-Acetylglucosaminidase F: Endoglycosidase from <u>Flavobacterium meningosepticum</u> that cleaves both high-mannose and complex glycoproteins.	1-38												
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁵ * Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"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)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 50%;"> <p>"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</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"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.</p> <p>"&" document member of the same patent family</p> </div> </div>														
IV. CERTIFICATION <table border="1" style="width: 100%; border-collapse: collapse; margin-top: 5px;"> <tr> <td style="width: 50%; padding: 5px;"> Date of the Actual Completion of the International Search ² <div style="text-align: center; font-size: 1.2em; margin-top: 10px;">February 18, 1986</div> </td> <td style="width: 50%; padding: 5px;"> Date of Mailing of this International Search Report ² <div style="text-align: center; font-size: 1.2em; margin-top: 10px;">27 FEB 1986</div> </td> </tr> <tr> <td style="padding: 5px;"> International Searching Authority ¹ <div style="text-align: center; margin-top: 10px;">ISA/US</div> </td> <td style="padding: 5px;"> Signature of Authorized Officer ²⁰ <div style="text-align: center; margin-top: 10px;"> Michael Rabson </div> </td> </tr> </table>			Date of the Actual Completion of the International Search ² <div style="text-align: center; font-size: 1.2em; margin-top: 10px;">February 18, 1986</div>	Date of Mailing of this International Search Report ² <div style="text-align: center; font-size: 1.2em; margin-top: 10px;">27 FEB 1986</div>	International Searching Authority ¹ <div style="text-align: center; margin-top: 10px;">ISA/US</div>	Signature of Authorized Officer ²⁰ <div style="text-align: center; margin-top: 10px;"> Michael Rabson </div>								
Date of the Actual Completion of the International Search ² <div style="text-align: center; font-size: 1.2em; margin-top: 10px;">February 18, 1986</div>	Date of Mailing of this International Search Report ² <div style="text-align: center; font-size: 1.2em; margin-top: 10px;">27 FEB 1986</div>													
International Searching Authority ¹ <div style="text-align: center; margin-top: 10px;">ISA/US</div>	Signature of Authorized Officer ²⁰ <div style="text-align: center; margin-top: 10px;"> Michael Rabson </div>													

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No ¹⁸
Y	Nature Vol. 298, p.30-33. 1982. J. Bittle et al. Protection against foot-and-mouth disease by Immunization with a chemically synthesized peptide predicted from the viral nucleotide sequence.	16-38
Y	Virology Vol. 136, p.20-31. 1984. C. Bruck et al. Biologically active epitopes of bovine leukemia virus glycoprotein GP 51.	1-15
P,X	Science Vol. 226, p.1328-1330. Dec. 1984. S.Alexander and J. Elder. Carbohydrate dramatically influences immune reactivity of antisera to viral glycoproteins antigens.	1-38