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(51)    International Patent Classification (Int. Cl.⁶): A61K 39/145, 39/39
(54)    Title:    INFLUENZA VACCINE COMPOSITIONS

(57)    Abstract: The present invention provides vaccine compositions
capable of enhancing a protective response to a selected
influenza antigen, said composition containing at least
the antigen and 3D-MPL, and methods of enhancing an
immune response to influenza using these compositions.

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(56)    Documents cited:    WO 92/16231        WO 92/11291 A        GB 2220211

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# AP 000431

## INFLUENZA VACCINE COMPOSITIONS

### Cross-Reference to Other Applications

This is a continuation-in-part application of co-
5 pending United States patent application serial number
021,535, filed February 19, 1993.

### Field of the Invention

This invention relates to vaccines useful in
10 preventing infection with influenza in humans.

### Background of the Invention

Influenza virus infection causes acute respiratory
disease in man, horses and fowl, sometimes of pandemic
15 proportions. Influenza viruses belong to the
orthomyxovirus family of RNA viruses and, as such, have
enveloped virions of 80 to 120 nanometers in diameter,
with two external glycoprotein spikes, hemagglutinin (HA)
and neuraminidase (NA), and five internal proteins,
20 nucleoprotein, matrix protein and three polymerases.
Influenza viral RNA also codes for two non-structural
proteins (NS1 and NS2) which are produced in infected
cells, but are not incorporated into infectious virions.

Three types of influenza virus, Type A, Type B and
25 Type C, infect humans. Type A viruses have been
responsible for the majority of human epidemics in modern
history, although there are also sporadic outbreaks of
Type B infections. Known swine, equine and fowl viruses
have mostly been Type A, although Type C viruses have
30 also been isolated from swine.

Within a virus, genetic variation in the surface
proteins HA and NA has resulted in three important
subtypes, designated H1N1, H2N2 and H3N2. Within Type A,
subtypes H1 ("swine flu"), H2 ("asian flu") and H3 ("Hong
35 Kong flu") are predominant in human infections.

Influenza viruses continually undergo genetic change
in their surface glycoproteins which affects antigenic
variation. This is most pronounced within the A virus
type, where major genetic changes in the HA or NA

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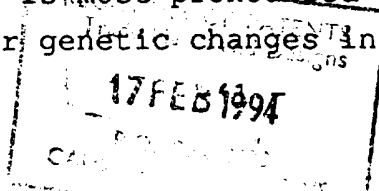
proteins have already occurred ("antigenic shifts"). The emergence of these new virus subtypes have caused a pandemic spread of infection resulting in significant mortality and morbidity. For example, the H1N1 viruses,

5   prevalent before 1957, were replaced by the H2N2 virus subtype which remained predominant until 1968, when they were, in turn, replaced by the H3N2 subtype. Currently, H3N2 strains are still circulating, but since 1977, H1N1 viruses have re-emerged. The HAs within a given subtype

10  also undergo smaller genetic changes (point mutations) every year or two ("antigenic drift"). These are largely restricted to antigenic determinants clustered around the sialic acid binding site in the HA1 and result in the emergence of new virus strains. Although this antigenic

15  drift does not cause serious mortality and morbidity to the extent caused by antigenic shift, it is responsible for yearly influenza epidemics.

Influenza vaccines are classified into three types, whole-virion, split, and subunit. Whole-virion vaccines,

20  based on intact viral particles, although generally more immunogenic, tend to be more reactogenic and are therefore being replaced by split and subunit vaccines which are prepared from purified viral components obtained after disruption of the virus by treatment with

25  various chemical agents. The distinction between split and subunit vaccines resides in the fact that subunit vaccine contain almost exclusively haemagglutinin and neuraminidase, the surface antigens of the virus, whereas split vaccines contain in addition variable amounts of

30  internal components of the virus such as the ribonucleoprotein and the matrix protein.

Currently available commercial influenza vaccines are based on the principle that antibody to HA or NA confers protection. They consist of non-adjuvanted,

35  inactivated, whole, or split virus products utilizing virus grown in embryonated hen's eggs. All influenza vaccines currently contain preparations from H1N1, H3N2, and type B virus strains. Due to the annual antigenic variation, specific virus strains are updated on a yearly

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basis according to WHO recommendations, which are based
on epidemiological surveillance of prelevent circulating
virus strains.

There is no "universal" influenza virus vaccine,
5  i.e., a non-strain specific vaccine.  Recently, attempts
have been made to prepare such universal, or semi-
universal, vaccines from reassortant viruses prepared by
crossing different strains.  More recently, such attempts
have involved recombinant DNA techniques focusing
10  primarily on the HA protein.

Influenza vaccines are under utilized for a variety
of reasons including doubts about efficacy, fear of side
effects, need for annual revaccination, and lack of
interest among providers.  Present vaccines have
15  demonstrated efficacy ranging from approximately 60-80%
against infection with influenza viruses that are
antigenically closely related to the virus strains used
in the vaccine.  This rate of protective efficacy tends
to decrease when the HA antigen of the epidemic strain HA
20  "drifted" away from the vaccine strain and would fall to
zero if a "shift" in subtype occurs.  In addition,
protection appears to be diminished in some
immunocompromised groups, such as the elderly living in
nursing homes.
25  Thus the major drawbacks of currently available
vaccines result from the fact that frequent antigenic
drift dictates that the component virus strains be
changed annually and individuals must undergo
revaccination.
30  There remains a need in the art for vaccine
formulations and compositions capable of inducing
protective responses in animals for a wide variety of
pathogens.

35  Summary of the Invention

In one aspect, the present invention provides a
vaccine composition capable of stimulating an enhanced
immune and protective response in a vaccinated animal
against influenza, the composition comprising a selected

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influenza antigen or antigenic polypeptide and an
effective amount of 3-o-deacylated monophosphoryl lipid A
(3D-MPL).

5          In another aspect, the invention provides a vaccine
composition comprising a selected influenza antigen or
antigenic polypeptide, an effective amount of 3D-MPL and
a liposome preparation.  The liposome preparation is
defined herein and, in addition to acting as a carrier,
acts as an adjuvant and offers significant manufacturing
10    and formulation advantages.

In a further aspect, the invention provides a method
for enhancing a vaccinee's immune response to a selected
influenza antigen.  This method involves administering to
a mammal, preferably a human, a vaccine composition
15    described above.

Other aspects and advantages of the present
invention are described further in the following detailed
description of the preferred embodiments thereof.


20    Brief Description of the Drawings
Fig. 1 is a bar graph illustrating cross-protection
for H1N1 and H2N2 subtype influenza viruses in mice
immunized with Flu D protein (SK&F 106160) in aluminum
plus 3D-MPL, as described in Example 18.

25         Fig. 2A is a bar graph illustrating splenic
proliferative responses pre-challenge in mice vaccinated
with flu D formulations and controls.  See  Example 20.

Fig. 2B is a bar graph illustrating splenic
proliferative responses post-challenge in mice vaccinated
30    with flu D formulations and controls.  See Example 20.

Fig. 3A is a bar graph illustrating lymph node
proliferative responses obtained on day 4 in mice
vaccinated with 20 µg flu D in aluminum (open bars) or
aluminum and 3D-MPL (cross-hatched bars).  See Example
35    21.

Fig. 3B is a bar graph illustrating lymph node
proliferative responses obtained on day 4 in mice
vaccinated with 5 µg flu D in aluminum (open bars) or
aluminum and 3D-MPL (cross-hatched bars).  See Example

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21.

Fig. 3C is a bar graph illustrating lymph node proliferative responses obtained on day 4 in mice vaccinated with 1 μg flu D in aluminum (open bars) or

5   aluminum and 3D-MPL (cross-hatched bars). See Example 21.

Fig. 4A is a bar graph illustrating proliferation on day 2 by immune lymph nodes in mice immunized with 1 μg D protein vaccine formulation. See Example 21.

10   Fig. 4B is a bar graph illustrating proliferation on day 3 by immune lymph nodes in mice immunized with 1 μg D protein vaccine formulation. See Example 21.

Fig. 4C is a bar graph illustrating proliferation on day 4 by immune lymph nodes in mice immunized with 1 μg D

15   protein vaccine formulation. See Example 21.

Fig. 4D is a bar graph illustrating IL-2 production on day 2 by immune lymph nodes in mice immunized with 1 μg D protein vaccine formulation. See Example 21.

Fig. 4E is a bar graph illustrating IL-2 production

20   on day 3 by immune lymph nodes in mice immunized with 1 μg D protein vaccine formulation. See Example 21.

Fig. 4F is a bar graph illustrating IL-2 production on day 4 by immune lymph nodes in mice immunized with 1 μg D protein vaccine formulation. See Example 21.

25   Fig. 5A is a graph demonstrating interferon levels in antigen-stimulated cultures from mice immunized as described in Example 24 below.

Fig. 5B is a graph demonstrating IL-2 levels in antigen-stimulated cultures obtained from mice immunized

30   as described in Example 24 below.

Fig. 6A is graph showing virus titers determined in the nose by MDCK microassay on days 1, 3, 5, 7 and 9 post-challenge (5 mice per group) for a control containing alum and 3D-MPL (--square--), influenza

35   monovalent split vaccine containing A/PR/8 strain with no adjuvant (--triangle--), and a strain A/PR/8 adjuvanted with 3D-MPL (solid line and circle). See Example 28.

Fig. 6B is graph showing virus titers determined in the nose by MDCK microassay on days 1, 3, 5, 7 and 9

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post-challenge (5 mice per group) for a control
containing alum and 3D-MPL (--square--), influenza
monovalent split vaccine containing Singapore strain with
no adjuvant (--triangle--), and the Singapore strain
5　　adjuvanted with 3D-MPL (solid line and circle). See
Example 28.

　　　Fig. 6C is graph showing virus titers determined in
the trachea by MDCK microassay on days 1, 3, 5, 7 and 9
post-challenge (5 mice per group) for the same three
10　vaccine formulations as in Fig. 6A.

　　　Fig. 6D is graph showing virus titers determined in
the trachea by MDCK microassay on days 1, 3, 5, 7 and 9
post-challenge (5 mice per group) for the same three
vaccine formulations as in Fig. 6B.

15　　　Fig. 6E is graph showing virus titers determined in
the lung by MDCK microassay on days 1, 3, 5, 7 and 9
post-challenge (5 mice per group) for the same three
vaccine formulations as in Fig. 6A.

　　　Fig. 6F is graph showing virus titers determined in
20　the lung by MDCK microassay on days 1, 3, 5, 7 and 9
post-challenge (5 mice per group) for the same three
vaccine formulations as in Fig. 6B.


Detailed Description of the Invention

25　　　The present invention provides vaccine compositions
capable of eliciting an enhanced immune response in
vaccinated hosts, including humans, as well as methods
for preparing and using such vaccine compositions. A
vaccine composition of this invention is characterized by
30　containing an effective amount of a selected influenza
antigen or antigenic polypeptide and 3-o-deacylated
monophosphoryl lipid A (3D-MPL). Optionally, a liposome
preparation may also be a component of the vaccine
compositions of this invention.

35　　　The inventors have discovered that the combination
of 3D-MPL and certain influenza antigens are effective in
achieving protective responses against influenza, which
are not achieved by the influenza antigen alone. For
example, with the antigenic polypeptide known as Flu D,

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described below, this response is such that a lower amount of antigen is required to obtain the same results as are achieved with purified Flu D and Complete Freunds Adjuvant (CFA), a known strong adjuvant which is toxic to

5    animals.

Further, when the selected influenza antigen and 3D-MPL are entrapped within a liposome as described herein, a protective response which exceeds that achieved by the antigen and any other adjuvant combination is obtained.

10   By the term "enhanced immune response" as used herein, is meant that the vaccinated host produces a stronger cellular immune response (protective T lymphocyte production) to the vaccine composition of the invention than is or would be produced by the host in

15   response to the selected antigen when not adjuvanted, or when adjuvanted with other conventional adjuvants suitable for internal administration. An increased antibody (B cell) response is also anticipated by this enhanced response.

20   By the term "immunologically effective amount" or "effective amount" as used herein is meant that amount of antigen which induces a protective immune response.

By the terms "selected antigen", "antigenic polypeptide or protein" or "immunogen" as used herein is

25   meant a whole or inactivated pathogen, an immunogenic protein, peptide or fragment from the pathogen, which is optionally fused to another peptide or protein which is of homologous or heterologous origin. These terms also include a split virus, defined below. These terms may

30   also include non-proteinaceous biological materials from the pathogen. The pathogens are preferably disease-causing organisms which infect humans, although animal pathogens may also be employed in these vaccines, where desired for veterinary purposes. These terms refer to

35   the ability of the whole pathogen, split virus, peptide or fusion protein to elicit a protective immune response in a vaccinated host.

By the term "monovalent vaccine" is meant a vaccine containing antigens from a single type or subtype of

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influenza virus, e.g., H1N1, H2N2, H3N2 of Type A, Type B and Type C.

By the term "multivalent vaccine" is meant a vaccine containing antigens from more than a single type or
5   subtype of influenza virus, i.e., a trivalent vaccine may contain antigens from any three influenza types or subtypes, e.g., H1N1, H2N2, H3N2 of Type A, Type B and Type C.

By the term "split virus" is meant an influenza
10   virus suspension, obtained from embryonated hens' eggs inoculated with seed lot material, which is partially purified and concentrated. The concentrated virus suspension is treated with a detergent, such as sodium-desoxylcholate, to disrupt the virus particles. Removal
15   of viral phospholipids during this splitting process produces an inactivated influenza antigen for which the reactogenicity potential is greatly reduced. The split virus suspension is completely inactivated by the combined effect of detergent and formaldehyde.
20   The following disclosure of the compositions and methods of this invention specifically describes vaccine compositions for prophylactic use against influenza virus.

In one preferred embodiment of this invention, a
25   vaccine composition capable of eliciting an enhanced immune response protective against infection with influenza virus contains at least a selected influenza antigenic polypeptide, such as $NS1_{1-81}HA2_{65-222}$ (referred to herein as Flu D, the D protein or the Flu D protein),
30   adjuvanted with 3D-MPL.

Currently, flu D protein is one preferred influenza antigenic polypeptide for use in the vaccine compositions of this invention because it is the most easily purified of the influenza fusion proteins which contain the entire
35   carboxy-terminal region of HA2 portion of the hemagglutinin region. D protein comprises the first 81 amino acids of NS1 fused to amino acid 65 of the truncated HA2 subunit (amino acids 65-222). Optionally, as is the case with the other NS1-HA2 fusions proteins

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disclosed herein, a linker sequence may be inserted between the two fused sequences.

In a presently preferred embodiment, the DNA coding sequence for flu D protein is prepared by as described in
5   EP 0366238 by restricting the HA2 coding sequence with PvuII and ligating the C-terminal region of the NcoI site between amino acids 81 and 82 in the NS1 coding sequence via a synthetic oligonucleotide linker. This linker sequence codes for glutamine-isoleucine-proline. D
10  protein, for which a 90-95% purity has been achieved, requires application of the purification methods described herein to substantially remove the host cell (*E. coli*) proteins and other contaminants.

The flu D protein and the recombinant expression and
15  purification thereof are disclosed in detail in co-pending U. S. Patent Application SN 07/751,899 and in its corresponding European Patent Application No. 366,238, published May 2, 1990 and U. S. Patent Application Sn 07/387,558 and in European Patent Application No.
20  366,239, published May 2, 1990. These applications are incorporated by reference for the purpose of describing this protein, its expression and purification.

Other suitable influenza antigenic polypeptides in addition to flu D, may be used in the vaccine
25  compositions of this invention including those described in European Patent Applications 366,238 and EP 366,239, both published May 2, 1990 and in co-pending U. S. Patent Applications SN 07/751,898, 07/751,896 and 07/837,773. Such proteins include $\Delta M$, $\Delta M+$, A, C, C13, C13 short, and
30  $\Delta D$. Other suitable include Cys-less D, $HA2_{66-222}$, and NS1H3HA2 constructs, such as those described in co-pending U.S. Patent Applications Ser. No. 07/751,898, 07/751,896, and 07/837,773 and WO 93/15763 incorporated by reference herein. Particularly desirable are the
35  H3HA2 constructs referenced above. Recent studies by the inventors have shown that mice immunized with a "cocktail" containing both NS1-H1HA2 and NS1-H3HA2 fusion proteins were protected from lethal challenge with both H1 and H3-virus subtypes.

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Coding sequences for the HA2, NS1 and other viral
proteins of influenza virus can be prepared synthetically
or can be derived from viral RNA by known techniques, or
from available cDNA-containing plasmids as described in
5    the above-incorporated published European applications.
For example, in addition to the above-cited references, a
DNA coding sequence for HA from the A/Japan/305/57 strain
was cloned, sequenced and reported by Gething et al,
Nature, 287:301-306 (1980); an HA coding sequence for
10    strain A/NT/60/68 was cloned as reported by Sleigh et al,
and by Both et al, both in Developments in Cell Biology,
Elsevier Science Publishing Co., pages 69-79 and 81-89,
(1980); an HA coding sequence for strain A/WSN/33 was
cloned as reported by Davis et al, Gene, 10:205-218
15    (1980); and by Hiti et al, Virology, 111:113-124 (1981).
An HA coding sequence for fowl plague virus was cloned as
reported by Porter et al and by Emtage et al, both in
Developments in Cell Biology, cited above, at pages 39-49
and 157-168.
20        Systems for cloning and expressing the vaccinal
polypeptide in various microorganisms and cells,
including, for example, *E. coli*, *Bacillus*, *Streptomyces*,
*Saccharomyces*, mammalian and insect cells, are known and
available from private and public laboratories and
25    depositories and from commercial vendors.
The D protein can be purified by the process
described below in Example 13. Various conventional
procedures can be employed in connection with the
purification of the proteins of the compositions of the
30    present invention, although such other procedures are not
necessary to achieve a highly purified, pharmaceutical
grade product. Such procedures can be employed between,
before or after the above described process steps. One
such optional step is diafiltration, a form of continuous
35    dialysis which is extremely effective in achieving many
buffer exchanges. Diafiltration is preferably carried
out across a cellulosic membrane or ultrafilter.
Suitable membranes/filters are those having from about a
1000 molecular weight (MW) cut-off to those having pore

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size up to 2.4 μm diameter. A number of different
systems adaptable to diafiltration are commercially
available, such as the 10K Amicon dual spiral cartridge
system. In the process of the present invention,

5    diafiltration using a 20 mM Tris buffer at about pH 8 can
be effectively employed in the purification and
subsequent concentration of the polypeptide to be
employed in the vaccine composition of this invention.

In another preferred embodiment, the antigen used in

10   a vaccine composition of the invention is a whole
inactivated pathogen, such as a split virus, described in
detail in Example 25. A monovalent split influenza
vaccine containing a split virus or a multivalent (e.g.
trivalent) split influenza vaccine containing more than

15   one split virus, may also be adjuvanted with 3D-MPL. In
one formulation, the vaccine contains the split virus
prepared from an H1N1 strain, such as Singapore/6/86
[Sachsisches Serumwerkl GmbH (SSW), Dresden, German and
the National Institute for Biological Standards and

20   Control (NIBSC, London, England)] which is currently used
in conventional flu vaccines. Alternatively, other H1N1
split viruses may be prepared from A/PR/8/34 (also called
A/PR/8) described in T. Francis, Proc. Soc. Exp. Biol.
Med., 32:1172 (1935) and available from the American Type

25   Culture Collection, Rockville, Maryland, USA under ATCC
No. VR-95. The monovalent vaccine contains antigens from
one strain of influenza type virus. Alternatively, the
vaccine may be multivalent, containing more than one
influenza antigen, e.g., two or three split viruses, to

30   increase the reactivity against more than one influenza
type virus. An example of a trivalent vaccine includes,
for example, the H1N1 Singapore/6/86 strain, with an H3N2
strain Beijing/32/92 [SSW, Dresden, Germany], and a Type
B strain, Panama/45/90 [SSW, Dresden, Germany].

35   Influenza viruses, which can be prepared as split
viruses by known means as described in Example 25 include
any strains, subtypes and types, particularly those
recommended by WHO, many of which are available from
clinical specimens and from public depositories, such as

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the American Type Culture Collection, 12301 Parklawn
Drive, Rockville, Maryland, 20852, USA (ATCC) and NIBSC.
For example, other suitable H3N2 virus strains include,
without limitation, A/Victoria/H3/75 described in Fiers

5    et al, *Cell*, *19*: 683-696 (1980); A/Udorn described in C.
J. Lai et al, *Proc. Natl. Acad. Sci., USA*, *77*:210-214
(1980); Palese and Schulman, *Virol.*, *57*:227-237 (1974);
and A/HK/8/68, described in WHO Weekly Epid. Record, vol.
43:401 (1968) and available from the ATCC as No. VR-544

10   as well as Beijing/32/92.  Other suitable Type B strains
include, without limitation, those known as B/Lee/40
described by Krystal et al, *Proc. Natl. Acad. Sci., USA*,
*79*:4804-4900 (1982) and B/Taiwan, available from the ATCC
as No. VR-295, Panama/45/90, and B/Yamaghta strains.

15   H2N2 viruses may also be useful in these vaccines.

It should be understood that in addition to other
influenza viruses or inactivated viral preparation, other
antigenic materials from other pathogens are anticipated
to be employed in addition to the exemplified antigens

20   following the teachings contained herein.

Another component of a vaccine composition of this
invention is the adjuvant, 3D-MPL.  3D-MPL is described
in detail in U. S. Patent No. 4,912,094, incorporated by
reference herein, and is commercially available from RIBI

25   Immunochem Research Inc., Hamilton, Montana.  Briefly,
3D-MPL is a derivative of the endotoxin, mono-phosphoryl
Lipid A (MPL), a Lipid A analog isolated from a
heptoseless, RE mutant of a Gram-negative bacteria, such
as *Salmonella minnesota*.  MPL lacks a phosphate group at

30   the C-1 position of glucosamine.  Treatment of the MPL
molecule to remove the acyl chain at position 3 of
glucosamine, yields 3D-MPL.  3D-MPL is non-toxic, in
contrast to other enterobacterial lipopolysaccharides,
but retains the antigenic activity of the parent

35   endotoxin.  This molecule is useful in preventing Gram-
negative sepsis and endotoxemia.

3D-MPL can be dissolved in water to yield solutions
of vesicular aggregates, which are presumably composed of
lipid bilayer membranes.  Thus, it is not likely that 3D-

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MPL is seen as individual molecules by the immune system,
but rather interacts by membrane-membrane contacts,
involving cell surfaces and vesicle surfaces.

5    In a preferred embodiment, the particle size of the
MPL is 'small' and in general does not exceed 120nm.

To make 3 deacylated monophosphoryl lipid A with a
small particle size, in general not exceeding 120nm the
procedure described in GB 2 220 211 may be followed (or
commercial MPL of larger particle size may be purchased
10   from Ribi Immunochem.) and the product may then be
sonicated until the suspension is clear.  The size of the
particles may be estimated using dynamic light scattering
as described hereinbelow.

Preferably the size of the particles is in the range
15   60-120nm.

Most advantageously the particle size is below
100nm.

According to the present invention, the inventors
have determined that an influenza antigenic polypeptide,
20   e.g., flu D protein, when highly purified, is neither
immunogenic nor protective in the absence of adjuvant.
However, when adjuvanted with 3D-MPL, as described herein
and illustrated in Examples 14-24 below, the protein is
capable of inducing protection against influenza
25   infection.  When the selected antigen is a split virus in
a monovalent or trivalent composition, a composition of
the present invention demonstrates an increase in
immunogenicity and cross-reactivity.

Further, the inventors have discovered that when an
30   influenza antigenic polypeptide, for example, flu D
protein, is adjuvanted with 3D-MPL, a lower dose of flu D
protein is required to achieve the same level of immune
response obtained when the protein is adjuvanted with an
aluminum adjuvant only.  When the selected antigen is a
35   split influenza virus in a monovalent or trivalent
composition, it is anticipated that reactogenicity of the
composition will be decreased by the use of less antigen
in this embodiment of the invention.

Additional adjuvants may also be included in the

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vaccine compositions of the invention.  One desirable
additional adjuvant is alum, or aluminum hydroxide or
aluminum phosphate.  When flu D protein is adjuvanted
with a combination of aluminum and 3D-MPL, a level of
5   potency is reached equivalent to that seen with Complete
Freunds Adjuvant (CFA), the classic adjuvant for
supporting T cell responses but which is not suitable for
internal administration in humans.

       To prepare a preferred vaccine composition of this
10   invention containing an antigenic polypeptide, e.g., flu
D protein and 3D-MPL, a desired amount of the flu D
protein in admixed with a suitable amount of the 3D-MPL,
as described in more detail below.  Optionally,
lyophilized lipid A is admixed with the pre-liposome gel
15   described in detail below prior to the antigen.  Most
preferably, a molar ratio of phosphatides to lipid A is
66:1.  However, the density of the agent may be varied to
the desired level.

       Other suitable agents for addition to the vaccine
20   composition include, for example, IL-2, QS21 [C. R.
Kensil et al, J. Immunol., 146(2):431-437 (1991)], and
muramyl dipeptides (MDP).  In addition, other water
soluble or insoluble chemicals or drugs and/or adjuvants
described above can be incorporated into the vaccine
25   compositions of this invention.  For example, muramyl
dipeptides may also be used at similar ratios as
described above or as desired.  Other drugs which may
form part of this vaccine may include any substance that
when taken into the vaccinee modifies one or more of its
30   functions, for example as recited in an official
pharmacopeia, or a substance used in the treatment or
prevention of an infection.

       The vaccine compositions of this invention may
further contain suitable carriers which are well known to
35   those of skill in the vaccine art and can be readily
selected.  Exemplary carriers include sterile saline,
lactose, sucrose, calcium phosphate, gelatin, dextrin,
agar, pectin, peanut oil, olive oil, sesame oil, squalene
and water.  Additionally, the carrier or diluent may

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include a time delay material, such as glyceryl
monostearate or glyceryl distearate alone or with a wax.
Optionally, suitable chemical stabilizers may be used to
improve the stability of the pharmaceutical preparation.

5 Suitable chemical stabilizers are well known to those of
skill in the art and include, for example, citric acid
and other agents to adjust pH, chelating or sequestering
agents, and antioxidants. Alternatively, when a
liposomal delivery system is part of the vaccine

10 composition, no carriers are necessary.

Another preferred vaccine composition of this
invention comprises a selected antigen, e.g., the flu D
protein as described above, and 3D-MPL, which components
are entrapped or intercalated in a liposome preparation.

15 Optionally, the liposome, flu D protein, and 3D-MPL-
containing vaccine composition may also contain one or
more additional influenza antigens or other antigens or
desirable adjuvants and agents as described above. In
one preferred formulation, the vaccine contains the

20 antigenic polypeptide Flu D protein and 3D-MPL placed
into a carrier liposome. In another formulation, the
vaccine contains a mono- or multi-valent influenza
antigen similarly involved with a carrier liposome.

The inventors have discovered that the liposome

25 preparations described herein are capable of functioning
not only as carriers, but also as adjuvants, and are
particularly advantageous because making them does not
require organic solvents or high shear fields which is a
significant advantage for protein drugs, and all

30 ingredients for the liposomes are considered safe for
internal administration. Because of the simplicity and
flexibility of the preparative method, these liposome
preparations are suitable for large scale manufacturing.

The inventors have also discovered that 3D-MPL can

35 be readily incorporated into the liposomal structures
described herein in combination with influenza antigens
to obtain results superior to that found when influenza
antigens are combined with known conventional adjuvants.
Significantly, a vaccine composition containing D protein

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in a 3D-MPL and liposome formulation has even greater potency than D protein in CFA. See, e.g., Examples 20 and 22 below.

The liposome preparations useful in the vaccine compositions and methods of this invention are described in co-pending United States Patent Application Ser. No. 07/714,984, (US 5230899) incorporated herein by reference. Generally, the word "liposome" has been proposed and accepted as the term to be used in the scientific literature to describe synthetic, oligolamellar lipid vesicles. Such vesicles are usually comprised of one or more concentric natural or synthetic lipid bilayers surrounding an internal aqueous phase.

Specifically as defined herein and according to the incorporated reference, the liposome preparations useful in the vaccine composition of the invention are prepared by dispersing in an aqueous medium in a manner adequate to form liposomes, a composition comprising a liposome-forming material containing a long chain aliphatic or aromatic-based acid or amine; a hydrating agent of charge opposite to that of the acid or amine, which agent is present in a molar ratio of between 1:20 and 1:0.05 relative to the acid or amine; and water in an amount up to 300 moles relative to the solids.

The preparation of the liposomal adjuvant carriers useful in the invention follows. Examples of liposome-forming materials include saponifiable and non-saponifiable lipids, e.g., the acyl glycerols, the phosphoglycerides, the sphingolipids, the glycolipids, etc. The fatty acids include saturated or unsaturated alkyl $(C_8\text{-}C_{24})$ carboxylic acids, mono-alkyl $(C_8\text{-}C_{27})$ esters of $C_4\text{-}C_{10}$ dicarboxylic acids (e.g., cholesterol hemi-succinic acid and fatty acid derivatives of amino acids in which any N-acyl carboxylic acids also are included (e.g., N-oleoyl threonine, N-linoleoyl serine, etc.). Mono- or di-alkyl $(C_8\text{-}C_{24})$ sulfonate esters and mono- or di-alkyl $(C_8\text{-}C_{24})$ phosphate esters can be substituted for the fatty acids. Furthermore, mono- or di-acyl $(C_8\text{-}C_{24})$ glycerol derivatives of phosphoric acids and mono- or di-

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acyl ($C_8$~$C_{24}$) glycerol derivatives of sulfuric acids can be
used in place of the fatty acids.

     Additionally, the fatty acids also can be replaced
by amines (e.g., $C_8$~$C_{24}$ $NH_2$), $C_8$~$C_{24}$ fatty acid derivatives

5    of amines (e.g., $C_8$~$C_{24}$ CONH~$NH_2$), $C_8$~$C_{24}$ fatty alcohol
derivatives of amino acids (e.g., $C_8$~$C_{24}$ OOC~$NH_2$), and
$C_8$~$C_{24}$ fatty acid esters of amines (e.g., $C_8$~$C_{24}$ COO~$NH_2$).

     Photopolymerizable lipids and/or fatty acids (or
amines) (e.g., diacetylenic fatty acids) also can be

10   included, which can provide a sealed liposome with cross-
linked membrane bilayers upon photo-initiation of
polymerization.

     A long chain aliphatic and/or aromatic-based acid or
amine includes an acid or amine having an open chain

15   structure and consisting of paraffin, olefin and
acetylene hydrocarbons and their derivatives, i.e.,
saturated and unsaturated hydrocarbons or the backbone of
such chain contains an where aromatic substituent. Such
acids and amines may have more than one such function.

20   The term "long chain" means that the backbone of the
aliphatic chain of the acid or amine has ten or more
carbon atoms. If the chain contains an aromatic group,
such as phenyl, the chain will comprise at least a five
carbon backbone in conjunction with that aromatic group.

25   The chain of carbon atoms comprising the backbone may be
variously substituted with saturated or unsaturated
aliphatic or aromatic functions.

     The terms "acid" or "amine" are conventionally
defined chemical functionalities. For example, an acid

30   function may be a carboxylate acid, or a phosphorous or
sulfur derived acid function such as phosphate, phosphite
or pyrophosphate or sulfate, sulfite, thiosulfate, or
similarly constituted phosphorous or sulfur-based acid.
Amines must be sufficiently basic so as to have an

35   ionizable hydrogen or be capable of forming quaternary
salts which have an ionization constant such that they
are capable of forming the requisite hydrate complex.

     As used in the vaccine composition and relative to
the amount of water employed in the liposome composition,

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the term "solids" refers to the liposome-forming
materials and the acid or amine component, hydrating
agents, the 3D-MPL, alum, and the selected antigenic
material to be encapsulated.

5　　　　For the purpose of this invention, a hydrating agent
means a compound having at least two ionizable groups,
preferably of opposite charge, one of which is capable of
forming an easily dissociative ionic salt, which salt can
complex with the ionic functionality of the acid or amine

10　in the liposome-forming material.  The hydrating agent
inherently does not form liposomes in and of itself.
Such an agent will also be physiologically acceptable,
i.e., it will not have any untoward or deleterious
physiological effect on the host to which it is

15　administered in the context of its use in this invention.
　　　　The preferred hydrating agents are alpha amino acids
having an ionizable omega substitution such as a
carboxylate, amino, and guanidino function and those
compounds represented by the formula:
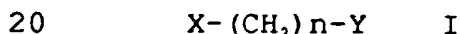
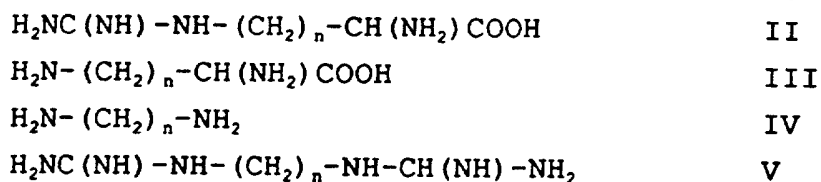20　　　　$X-(CH_2)n-Y$　　　I
wherein

　　　　X is $H_2N-C(NH)-NH-$, $H_2N-$, $ZO_3S-$, $Z_2O_3P-$, or $ZO_2C-$
wherein Z is H or an inorganic or organic cation;
　　　　Y is $-CH(NH_2)-CO_2H$, $-NH_2$, $-NH-C(NH)-NH_2-COOH$,

25　$CH(NH_2)SO_3Z$ or $ZH(NH_2)PO_3Z_2$ wherein Z is defined above; and
　　　　　　n is the integer 1-10; or
　　　　　　a pharmaceutically acceptable salt thereof.
Also included in the list of preferred compounds are the
N,N'-dialkyl substituted arginine compounds and similar

30　compounds where the alkyl chain length is varied.
　　　　More preferred hydrating agents are the omega-
substituted, alpha amino acids such as arginine, its N-
acyl derivatives, homoarginine, gamma-aminobutyric acid,
asparagine, lysine, ornithine, glutamic acid, aspartic

35　acid or a compound represented by the following formulas:
　　　　　　$H_2NC(NH)-NH-(CH_2)_n-CH(NH_2)COOH$　　　　　　II
　　　　　　$H_2N-(CH_2)_n-CH(NH_2)COOH$　　　　　　　　　　III
　　　　　　$H_2N-(CH_2)_n-NH_2$　　　　　　　　　　　　　　IV
　　　　　　$H_2NC(NH)-NH-(CH_2)_n-NH-CH(NH)-NH_2$　　　V

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$$HOOC-(CH_2)_n-CH(NH_2)COOH \qquad VI$$

$$HOOC-(CH_2)_n-COOH \qquad VII$$

$$HO_3S-(CH_2)_n-CH(NH_2)COOH \qquad VIII$$

$$H_2O_3S-(CH_2)_n-CH(NH_2)COOH \qquad IX$$

$$HO_3S-(CH_2)_n-CH(NH_2)SO_3H \qquad X$$

$$H_2O_3S-(CH_2)_n-CH(NH_2)PO_3H_2 \qquad XI$$

wherein n is 2-4.

The most preferred compounds are arginine, homoarginine, gamma-aminobutyric acid, lysine, ornithine, glutamic acid or aspartic acid. The hydrating agents of this invention may be used alone or as a mixture. No limitation is implied or intended in the use of mixtures of these hydrating materials.

The hydrating agents of this invention are listed in the catalogue of many chemical producers, can be custom manufactured by such producers, or can be made by means known in the art. Arginine, homoarginine, lysine, glutamic acid, aspartic acid, and other naturally occurring amino acids may be obtained by the hydrolysis of protein and separation of the individual amino acids or from bacterial sources.

The compounds of formula II can be made by the method of Eisele, K. et al, _Justusliebigs. Ann. Chem._, p. 2033 (1975). Further information on several representative examples of these compounds is available through their respective Chemical Abstracts Service numbers as follows: nonarginine, CAS #14191-90-3; arginine, CAS #74-79-3; and homoarginine, CAS #151-86-5. For representative examples of formula III, see for 2,4-diaminobutonic acid CAS #305-62-4 and for lysine CAS #56-87-1. Methods for making representative compounds of formula IV are available from Chemical Abstracts as follows: ethane diamine, CAS #305-62-4; propane diamine - 54618-94-9; and 1,4-diaminobutane, CAS #52165-57-8. See specifically Johnson, T.B., _J. Am. Chem. Soc._, 38:1854 (1916).

Of the compounds of formula VI, glutamic acid is well known in the art and is available from many commercial sources. Descriptions of how to make other

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representative compounds is contained in the literature,
for example: 2-aminohexandioc acid - CAS # 62787-49-9 and
2-aminoheptandioc acid - CAS # 32224-51-0.  Glutamic
acid, the compound of formula VII where n is 2 is well

5      known in the art and can be made by the method of Maryel
and Tuley, Org. Syn., 5:69 (1925).  Other representative
compounds in this group can be made according to the art
as referenced by the following CAS numbers: hexadioic
acid, CAS # 123-04-9 and heptadioic acid, CAS # 111-16-0.

10     Homocysteic acid is known in the art referenced by CAS #
56892-03-6.  The compound 3-sulfovaline is described in
the literature referenced by CAS #23405-34-2.

Mixtures of the hydrating agent, liposome-forming
materials and discrete amounts of water form a gel-like

15     mass.  When in this gel form, the hydrating agent and
acid or amine, in conjunction with all liposome
forming materials, arrange into a "hydrate complex" which
is a highly ordered liquid crystal.  Hydrate complex
means the complex formed between the hydrating agent and

20     the acid or amine in the liposome-forming material.
Complexing in this context denotes the formation of
dissociative ionic salts where one functionality
associates with the ionic functionality of the liposome-
forming material and the other functionality has

25     hydrophilic properties which impart water-solubility to
the resulting complex.  While the liquid crystal
structure of the hydrate complex varies with pH and
amount of hydrating agent, the liquid crystal structure
remains.  NMR spectroscopy confirms that this crystal

30     structure consists of multilamellar lipid bilayers and
hydrophilic layers stacked together in alternating
fashion.  The $^{31}$P-NMR spectrum exhibits an anisotropic
peak, confirming the existence of multilamellar bilayers.

Although the primary components of these liposomes

35     will be lipids, phospholipids, other fatty acids, there
may also be added various other components to modify the
liposomes' permeability.  There may be added, for
example, non-ionic lipid components such as polyoxy
alcohol compounds, polyglyceral compounds or esters of

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polyoles, polyoxyalcolinolated alcohols; the esters of
polyoles and synthetic lipolipids, such as cerebrosides.
Other materials, such as long chain alcohols and diols,
sterols, long chain amines and their quaternary ammonium
5    derivatives; polyoxyethylenated fatty amines, esters of
long chain amino alcohols and their salts and quaternary
ammonium derivatives; phosphoric esters of fatty
alcohols, polypeptides and proteins.
The composition of the liposome can be made of more than
10   one component of the various kinds of lipids, the fatty
acids, alkyl amines, or the like, and the hydrating
agents.

     If the lipid component itself or antigenic material
and any other materials to be added to the vaccine
15   composition to be encapsulated possess the aforementioned
properties, the lipid composition may not require the
inclusion of the fatty acids (or the amines) or the
hydrating agents to form the "pre-liposome gel" or
liposomes.  For example, the mixture of
20   dipalmitoylphosphatidylcholine (DPPC) and distearoyl
phosphatidylethanolamine forms the "pre-liposome gel" or
liposomes with aqueous glutamic acid solution and the
mixture of DPPC and oleic acid with aqueous epinephrine
solution forms the "pre-liposome gel" and liposomes.

25     For use in the vaccine composition as an adjuvanting
material, the liposome preparation preferably includes
phospholipids, oleic acid (or phosphatidyl-ethanolamine)
and arginine or lysine (or glutamic acid and/or aspartic
acid).

30     About 1:20 molar ratio of hydrating agent relative
to the liposome-forming material will provide the
salutory effects of this invention with an upper limit of
about 1:0.05.  The preferred concentration range for the
hydrating agent is between a 1:2 to 1:0.5 molar ratio of
35   the hydrating relative to the liposome-forming material.

     As a practical matter, thus a matter of preference,
if liposomes are prepared with a long chain aliphatic or
aromatic-based acid, it is preferred to use hydrating
agents which contain at least one ionizable nitrogen,

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such as arginine, homoarginine, lysine, ornithine, and
the like. Conversely, if the amphipatic materials used
to form the liposomes contain a long chain aliphatic or
aromatic-based amine, it is preferred to use a di-acid
5    such as glutamic acid, aspartic acid; any of the alkyl
di-acids such as the simple di-acids such as valeric
acid, caprylic, caproic, capric or the like; or those di-
acids having two phosphate, or sulfate functionalities;
or those di-acids having mixed $-COOH/-SO_3H$ or $-COOH/-PO_3H_2$
10   functions.

Certain aliphatic and aromatic-based acids and
amines are preferred in the practice of this invention.
Such compounds can have multiple functions such as having
two or more acid or amine groups or combinations thereof.
15   For example, one could use a di-acid, a di-amine or a
compound having an acid and an amine function. The
preferred compounds are those with one or two acid or
amine functions. More preferred are the fatty mono-acids
of 10-20 carbons, saturated and unsaturated. Most
20   preferred are the alkyl and alkenyl acids of 10 to 20
carbon atoms, particularly oleic acid.

Mixtures of liposome-forming materials, a long chain
aliphatic or aromatic-based acid or amine, one or more
hydrating agents with up to 300 moles of water relative
25   to the total solids, with or without a selected amount of
antigenic material, produces a gel which forms liposomes
directly therefrom upon addition of an aqueous solution.
This gel can be labelled a pre-liposome gel because its
structural characteristics are essentially those of
30   liposomes and, the gel has the facility for being
converted into liposomes upon dilution with an aqueous
solution. Aqueous solution in excess of about 300 molar
equivalents cause the beginning of liposome formation.

The structure of this gel is a highly ordered liquid
35   crystal which forms an optically clear solution. The X,
Y, and Z dimensions of the liquid crystal vary with the
concentrations of hydrating agent at constant pH as well
as with the pH of the solution. By varying the hydrating
agent concentration at constant pH or changing the pH

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while maintaining the percentage of hydrating agent, the size and number of lamellae structures of the lipid bilayers of the subsequent liposome vesicles can be controlled.

5        The gel structure itself can accommodate up to approximately 300 moles of water per mole of solid without disturbing the stability of the gel structure. The structure of the gel as determined by proton nuclear magnetic resonance (NMR) spectroscopy is comprised of

10    multilamellae lipid bilayers and hydrophilic layers stacked together in an alternating fashion.  The $^{31}$P-NMR spectrum of the same gel exhibits an anisotropic peak further confirming that the gel consists of a multilamellar bilayer.  This gel can be autoclaved, a

15    convenient means of sterilization.  Furthermore, the gel shows no discoloration and remains clear at room temperature for at least one year after being autoclaved. If desirable and feasible, the gel can optionally further be sterilized by filtration through an appropriate

20    sterilization filter.  Upon dispersion of the gel into an aqueous solution, liposomes are efficiently and spontaneously produced.

         The pre-liposome gel, with or without the 3D-MPL and antigenic material to be encapsulated, also can be

25    dehydrated (e.g. lyophilized) and the powder rehydrated to form liposomes spontaneously, even after a long period of storage.  This capability provides the vaccine compositions particularly useful for administering water-sensitive antigenic materials where long term pre-use

30    storage is needed.

         The pre-liposomal gel is prepared as follows.  A semisolid liquid crystalline gel referred to as the pre-liposomal gel is prepared by combining three basic ingredients: a phospholipid, a fatty acid, and a

35    hydrating agent in water.  Depending upon the lipid composition desired, a variety of phospholipids or mixtures thereof ranging in gel-liquid transition temperatures (Tm) may be employed.  The fatty acid can be chosen based on degree of saturation or chain length and

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is usually mixed with the phospholipid into a thick
paste. Cholesterol may be added to the lipid mix to
control the bilayer character. (Cholesterol in some
instances increases the Tm of the membrane thereby
5     influencing its permeability to entrapped agents).

The hydrating agent, preferably an alpha amino acid,
such as arginine, is added to the lipid mix as a solution
in water at a slow rate until a homogeneous paste is
achieved. One desirable formulation employs egg
10     phosphatidyl choline:oleic acid at a 1:1.2 molar ratio.
The arginine is added in water at approximately 1:1.2
phospholipid to arginine molar ratio.

The concentration of L-arginine in the aqueous phase
component of the gel governs the diameter of the stable
15     liposome particle which eventually forms. The size of
the particle depends on the route of administration and
whether or not it is desired to target macrophage cells.
For example, for oral administration, a liposome particle
size of between about 1 to about 5 micrometers is
20     desired. To target to lymphocytes, a particle size of
between about 200 to 500 nm is preferred. For a depot
effect, a liposome particle size between about 5 to about
10 micrometers is desired. Various size particles may be
easily tested and selected by one of skill in the art.

25     The final pre-liposomal gel can contain up to about
65 to about 70% water by weight, has the consistency of
an ointment, and has the appearance of a typical liquid
crystal when observed under a polarizing light
microscope. The pre-liposomal gel can be stored under an
30     inert atmosphere, or lyophilized to a dry powder for long
term storage.

In the formation of the vaccine compositions of this
invention, the same steps of liposome preparation are
followed with the addition of an immunologically
35     effective amount of the selected antigen, e.g., an
antigenic polypeptide, particularly the flu D protein,
and 3D-MPL, and optionally, one or more additional
immunogenic proteins, peptides or fragments from a
selected pathogen mixed with the liposome preparation.

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To prepare such vaccine compositions, the selected antigen is encapsulated or intercalated within the liposome preparation by mixing therewith.

There are two methods by which the 3D-MPL and the selected antigen can be incorporated into the liposome composition to prepare a vaccine composition according to this invention. One method involves the incorporation of the antigen with liposomes by hydration of the pre-liposomal gel, or the hydrated lyophilized powder, with a solution of the antigen in an appropriate buffer. The other method involves the physical mixing of the pre-liposomal gel with a lyophilized preparation of the antigen. This mixture is subsequently hydrated which causes the spontaneous formation of liposomes.

The method selected is the one which results in the largest level of antigen-liposome association with the smallest loss of antigen as unassociated fraction and is dependent upon the physico-chemical properties of the antigen in the presence of the lipid. Generally, however, the ratios of the components in such a vaccine mixture are 20 mg antigenic protein: 2 mg 3D-MPL: 300 mg liposomal gel. For instance, when the antigen is flu D, the second method is employed because the antigen has significant hydrophobic character and a low aqueous solubility and, as below described, to form a flu-D composition according to this invention, 300 mg of pre-liposomal gel was mixed with 20 to 30 mg flu-D protein and 0.5 to 2 mg 3D-MPL.

It is considered that one of skill in the art given the teachings herein can readily determine the selected amount of the antigenic material to use in the practice of this invention.

Optionally, other agents may be added co-entrapped with the selected antigen, e.g., flu D protein, and 3D-MPL in a liposome composition. Suitable other agents are described above.

Once the liposomes are formed in the presence of the 3D-MPL, selected antigen, and any optionally additional components, any unassociated antigen may be removed by

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various means.  Typically, the liposomes are centrifuged
serially at approximately 100,000 x g and the
supernatants are discarded.  The final liposome pellet is
reconstituted in an acceptable liquid, for example, 5%

5    dextrose, normal saline, or buffered solution, for
injection at a desired lipid or antigen concentration.
Other methods known to those of skill in the art, such as
gel filtration or dialysis, may be employed for removal
of unassociated antigen.

10    The description of the selected antigen, 3D-MPL and
liposome formulation also encompasses the use of the
split viruses described herein in place of, or in
addition to, Flu D.

The present invention also provides a method of

15    enhancing an immune response, particularly a T-cell
mediated response, in a human or other mammal, to the
selected antigen in the vaccine composition.  This method
involves administering to the human or other mammal a
vaccine composition of the invention containing 3D-MPL

20    and the selected antigen.  Optionally a liposome
preparation may be part of the composition as described
above.  This method is not limited to any particular
antigen exemplified herein.  In a preferred embodiment,
the method is useful in eliciting an enhanced protection

25    effective against influenza infection.  In such an
embodiment, the vaccine composition comprises an
effective amount of flu D and 3D-MPL, as described above.
In another embodiment the vaccine composition comprises
an effective amount of one or more split influenza

30    viruses and 3D-MPL, as described above.  Other vaccines
may be prepared and administered in effective amounts in
a similar manner.

As indicated by the examples, particularly Examples
25-30, the vaccine composition employing split viruses

35    adjuvanted with 3D-MPL results in superior virus
clearance particularly in the lung, the stimulation of
higher neutralizing antibody titers; the occurrence of
heterosubtypic ($H_1N_1$) cross-reactivity in the absence of
neutralizing activity; and altered progression of disease

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from upper to lower respiratory track (via trachea).

The dosage and administration protocols can be optimized in accordance with standard vaccination practices for these vaccine compositions. Typically, the

5  vaccines will be administered intramuscularly, although other routes of administration may be used, such as the subcutaneous, colonic, oral, pulmonary, intradermal, intraperitoneal, or intravenous routes. The route chosen may be dictated by the type of immune response desired.

10 For example, a subcutaneous route may provide the classical "depot effect" or more prolonged stimulation than others. It is also possible that a given route of administration may generate a stronger antibody response than a cellular response, or vice versa. For example,

15 the oral route of administration may permit generation of an enhanced IgA response that is useful for local immunity.

Based on what is known about other polypeptide vaccines, it is expected that a useful single dosage for

20 average adult humans of an influenza antigenic polypeptide vaccine, such as the flu D protein containing vaccine of this invention is in the range of between about 1 to about 1000 micrograms of D protein, preferably about 50 to about 500 micrograms protein, and most

25 preferably about 100 µg, in admixture with suitable amounts of 3D-MPL adjuvant. When these doses of flu D protein are used, the amounts of 3D-MPL in the vaccine composition are between about 1 to about 500 micrograms 3D-MPL/µg viral protein, more preferably about 10 to

30 about 50 µg 3D-MPL/µg viral protein, and most preferably about 50 µg 3D-MPL.

When flu D and 3D-MPL are administered via a liposome carrier as described herein, the preferred amount of D protein in the vaccine composition is between

35 about 50 to about 500 µg and the amount of 3D-MPL is between about 10 to about 50 µg. Such a 3D-MPL adjuvanted flu D vaccine composition will contain about 1 to about 10 mg, and preferably about 3 mg, liposome material per about 0.2 mg antigenic protein.

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When the flu D and 3D-MPL vaccine formulation optionally contains another adjuvant, such as aluminum or aluminum hydroxide, the preferred dosage of D protein is between about 10 to about 500 µg protein; the preferred

5    amount of aluminum or aluminum hydroxide is between about 10 to about 500 µg.

Similarly, based on what is known about other split virus vaccines, it is expected that a useful single dosage for average adult humans of an influenza

10   monovalent or multivalent split virus vaccine, such as those described herein, is about 15 micrograms of hemagglutinin (HA)  *per strain*  with total protein ranging from about 80-300 µg/ml, in admixture with suitable amounts of 3D-MPL adjuvant.  When these doses of split virus are used,

15   the amount of 3D-MPL in the vaccine composition is *preferably* about 50 micrograms 3D-MPL *per dose*.                    .  Of course, these amounts of virus protein and 3D-MPL may be altered by one of skill in the art.

When one or more split virus and 3D-MPL are

20   administered via a liposome carrier as described herein, the preferred amount of each split virus in the vaccine composition is likely to be less than about 15 µg HA/strain and the amount of 3D-MPL is between about 10 to about 50 µg.  Such a 3D-MPL adjuvanted influenza split

25   virus vaccine composition will contain about 1 to about 10 mg, and preferably about 3 mg, liposome material per about 0.2 mg viral protein.

When the split virus and 3D-MPL vaccine formulation optionally contains another adjuvant, such as aluminum or

30   aluminum hydroxide, the preferred dosage of each split virus in the vaccine composition may be adjusted downward.  A preferred amount of aluminum or aluminum
·  hydroxide is similar to that described above for the antigenic polypeptide.

35   Any of the vaccines described by this invention can be administered (preferably in 0.5 mls dosage units) initially in late summer or early fall and can be readministered two to six weeks later, if desirable, or periodically as immunity wanes, for example, every two to

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five years.

The following examples illustrate the preferred
methods for preparing vaccine compositions of the
invention. These examples are illustrative only and do
5    not limit the scope of the invention.

## Example 1 - Gel Preparation

Dipalmitoylphosphatidylcholine, 3.0 grams, was
weighed into a 50 ml beaker. Oleic acid 1.2 grams was
10   added and mixed together to form a uniform paste.

Arginine 0.72 grams in 30 ml of distilled deionized
water was added to the dipalmitoylphosphatidyl-
cholineoleic acid paste and heated to 45°C. With mixing
by hand, the mixture formed a clear stable gel. The gel
15   was stored and liposomes later formed by diluting the get
with phosphate buffered saline.

## Example 2 - Preparation of Liposomes

Dipalmitoylphosphatidylcholine, 120 mg, and 24 mg of
20   oleic acid were added together and mixed thoroughly until
a white homogeneous paste was observed.

Then 20 mg of arginine was dissolved into 60 ml of
phosphate buffered saline (ionic strength = 0.15, pH =
7.4). The arginine-saline solution was added to the
25   paste and heated to 40°C for ½ hour, or until a slightly
turbid solution was observed.

## Example 3 - Large Scale Gel and Liposome Preparation

i)    Gel Manufacture: To 50 grams of egg phosphatide
30   powder type 20 (Asahi Chemicals) was added 20 grams of
oleic acid N.F. Mixing gave a white paste which was
cooled to 4°C and ground into a fine powder. This powder
was added to an aqueous solution containing 20 grams of
arginine and 500 grams of distilled deionized water. The
35   mixture was mixed with a spatula as the solution was
heated to about 35°C to help hydrate phospholipids. A
homogeneous, slightly yellow get was formed. This gel
can be autoclaved and stored at 4°C or can be frozen and
later reconstituted.

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ii) <u>Manufacture of Liposomes:</u> The gel prepared in the
preceding paragraph was taken from cold storage and
returned to room temperature. It was then mixed with 2
liters of phosphate buffered saline, pH 7.4. A white
5    opaque liposome solution was formed.

<u>Example 4 - Liposome Formation from the Gel</u>

A homogenous paste of 1.0 gram of
dipalmitoylphosphatidycholine (DPPC) and 400 mg of oleic
10   acid was formed. Then 300 mg arginine was mixed in 10 mL
of phosphate buffered saline, heated to 45°C and added to
the DPPC/oleic acid paste to form liposomes.

<u>Example 5 - Pre-Liposome Gel</u>

15   One gram of dipalmitoylphosphatidylcholine (DPPL)
was mixed with 400 mg of oleic acid to form a homogeneous
paste. 300 mg of arginine was mixed with 2 ml of water
at 45°C until dissolved. The arginine solution was mixed
with the DPPC/oleic acid paste at about 45°C to give a
20   thick gel. Liposomes formed when this gel was diluted
with phosphate buffered saline.

<u>Example 6 - Various Liposome Formulations</u>
A. <u>Cholesterol Containing Liposomes</u>
25   Cholesterol, 15 mg, was mixed with 100 mg
dipalmitoylphosphatidylcholine (DPPC) to form a
homogeneous powder. Then 23 mg of oleic acid was added
to the powder and thoroughly mixed to form a homogeneous
paste. To make liposomes, 30 mg of arginine was added to
30   10 ml of phosphate buffered saline, heated to 40°C and
added to the DPPC/cholesterol/oleic acid paste. The
combination was mixed at 40°C to obtain liposomes.
B. <u>Palmitic Acid-Containing Liposomes</u>
Dipalmitoylphosphatidylcholine (DPPC) 250 mg was
35   mixed with 25 mg of palmitic acid to form a uniform
powder. Then 80 mg of oleic acid was mixed with this
powder and heated to 45°C with constant stirring until a
uniform paste was formed. Arginine 100 mg was dissolved
in 25 ml of distilled deionized water and heated to 45°C.

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This arginine solution was added to the paste at 45°C and
mixed until a uniform homogeneous gel was formed. The
gel was diluted ten fold with phosphate buffered saline
to form liposomes.

5   C.   Isostearic Acid-Containing Liposomes

      Dipalmitoylphosphatidylcholine 100 mg was mixed with
50 mg of isostearic acid to form a uniform homogeneous
paste. An arginine solution of 50 mg of arginine in 2.0
ml of distilled deionized water was made and added to the

10 isostearic acid paste and heated to 45°C. This mixture
was mixed until a clear gel was formed. Liposomes are
formed upon dilution with phosphate-buffered saline.

  D.   Oleoyl Threonine Containing Liposomes

      Dipalmitoylphosphatidylcholine 125 mg and 75 mg of

15 oleoyl threonine were added together and heated to 40°C to
form a paste. Then 2 ml of distilled deionized water was
added with constant mixing at 40°C.
A clear gel was formed which can be diluted with
phosphate buffer saline at pH 5 to form liposomes.

20   E.   Myristyl Amine containing Liposomes

      Dipalmitoylphosphatidylcholine 192 mg was added to
72 mg of myristyl amine and heated with constant mixing
until a uniform paste was formed. Glutamic acid 65 mg in
5 ml of distilled deionized water was added to the paste

25 and heated until a gel was formed. Phosphate buffered
saline was added to the get to form liposomes.

  F.   DLPC Containing Liposomes

      Dilaurylphosphatidylcholine (DLPC) 50 mg was mixed
with 20 mg oleic acid to form a homogeneous paste.

30 Arginine 20 mg was added to 10 ml of phosphate buffered
saline, added to the paste and hand mixed until a turbid
liposome solution formed.

  G.   Phosphatidylethanolamine-Glutamic Acid Liposomes

      L-glutamic acid 32 mg was dissolved in 2.0 ml of

35 distilled deionized water and the pH adjusted to 5.2 with
1.0 N sodium hydroxide. This solution was heated to 60°C,
and 100 mg of phosphatidylethanolamine added. The
solution was kept to 60°C with constant mixing until a
uniform viscous gel was observed.

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The phosphatidylethanolamine-glutamic acid gel was diluted 1/10 by phosphate buffered saline. Vesicular-like structures are observed under phase contrast light microscopy.

5

## Example 7 - Effect of Arginine Concentration on Liposome Size

To 502 mg of dipalmitoylphosphatidylcholine (DPPC) was added 10 microliters of (2-palmitoyl-1-$C_{14}$) (0.1

10　mCi/ml) dipalmitoylphosphatidylcholine. Chloroform was added to effect complete mixing of the radioactivity and then evaporated. Oleic acid (OA), 195 mg, was then mixed into the lipid to form a paste. Five ml of distilled water containing 119 mg of arginine was added

15　and mixed at 45°C to form a clear gel.

One gram of the gel was weighed into four different vials and arginine was added as indicated in the following Table 1.

### Table 1

20

| Sample ID | Sample Composition DPPC:OA:Arg |
|---|---|
| Vial 1 + 1 ml water | (1:1:1) |
| Vial 2 + 1 ml of | |
| 50 mg/ml Arg in $H_2O$ | (1:1:3) |
| Vial 3 + 1 ml of | |
| 84 mg/ml Arg in $H_2O$ | (1:1:5) |
| Vial 4 + 1 ml of | |
| 192 mg/ml Arg in $H_2O$ | (1:1:10) |

One-half gram of each solution was diluted in 50 ml of phosphate buffered saline of pH 7.8.

35　The estimated average diameter was obtained from a Sephracyl S-1000 column chromatographic analysis employing $_{14}$C-isotope labelled DPPC (i.e. diameters were determined based on intensity of scattering using photon correlation spectroscopy (PCS)). The effects are given

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in the following Table 2.

## Table 2
### Effects of Arginine Concentration on Vesicle Size

| System | Ratio | pH | Estimated Diameter (nm) |
|--------|-------|-----|-------------------------|
| DPPC:OA:Arg | 1:1:1 | 7.8 | ~220 |
| DPPC:OA:Arg | 1:1:3 | 7.8 | ~140 |
| DPPC:OA:Arg | 1:1:5 | 7.8 | ~90 |
| DPPC:OA:Arg | 1:1:10 | 7.8 | ~20 |

## Example 8 - pH Effect on Vesicle Size

Additionally, the vesicle size can be varied by varying the pH of the aqueous buffer solution.

To 100 mg of dipalmitoylphosphatidylcholine (DPPC) was added 25 microliters of (2-palmitoyl-1-$C_{14}$) (0.1 mCi/ml) dipalmitoylphosphatidylcholine. Chloroform was added to effect complete mixing of the radioactivity and then evaporated. Oleic acid (OA), 40.1 mg, was then mixed into the lipid to form a paste. One ml of a solution containing 24 mg/ml arginine in water was added to the lipid mixture and mixed at 45°C to form a clear gel.

Two 100 mg aliquots of this gel were diluted in 10 ml of phosphate buffer at pH 9.0 and 7.4 respectively.

Again, the estimated average diameter was obtained from the Sephracyl S-1000 column chromatographic analysis employing $^{14}C$-isotope labelled dipalmitoyl-phosphatidylcholine. Results are given in the following Table 3.

## Table 3

### pH Effects on Vesicle Size

| System | Ratio | pH | Estimated Diameter (nm) |
|---|---|---|---|
| DPPC:OA:Arg | 1:1:1 | 7.4 | ~<300 |
| DPPC:OA:Arg | 1:1:1 | 7.8 | ~220 |
| DPPC:OA:Arg | 1:1:1 | 9.0 | ~25.4 |

Thus, a desired size of the liposomal vesicles can be prepared by varying the arginine concentration or the pH of the aqueous buffer solution.

### Example 9 - Liposome Stability

Sterile liposomes may be prepared from the heat sterilized pre-liposome gel. Alternatively, the liposome gel or the liposomes may be sterile filtered through an appropriate sterilizing filter.

Liposomes prepared from DPPC:OA:Arg (1:1:2) at pH 8.0 were heat sterilized and stored at room temperature for approximately one year without adding antimicrobial agents and anti-oxidants. No bacterial growth, discoloration and precipitation were observed. Negative stain electron microscopic examination of the one year old liposomes revealed that the liposomal vesicles are stable.

### Example 10 - Sucrose Latency

Encapsulated sucrose latency was measured using $C_{14}$-sucrose encapsulated with the DPPC:OA:Arg (1:1:1) liposome system in aqueous phosphate buffer solution at pH 7.8. The result was presented in the following Table 4.

Table 4

| Days | % Sucrose Latency |
|------|-------------------|
| 0 | 100 |
| 1 | 97.4 |
| 3 | 93.4 |
| 7 | 91.4 |

Thus, the present liposome system has an excellent
latency for drug delivery.

Example 11 - Lyophilized Liposomes

Oleic acid, 30.0 gm, and 7.5 gm of cholesterol
U.S.P. were confected.  Then 75.0 gm of phosphatide type
20 powder (Asahi Chemical Co.) was mixed with the oleic
acid/cholesterol mixture until an homogeneous paste was
formed.

Then 15.0 gm of arginine (free base) was dissolved
in 183 gm of distilled, deionized water.  This arginine
solution was mixed slowly with the lipid paste to form a
homogeneous gel.  The gel pH was adjusted to 7.4 using
5.0 N HCl.

A 10.0 gm, aliquot of this pre-liposome gel was
transferred to a 10 ml vial and lyophilized.  The
resulting powder formed liposomes when diluted with 5 ml
of phosphate buffered saline.

Example 12 -  Construction of Flu D Expression Plasmids

Plasmid pAPR701 is a pBR322-derived cloning vector
which carries coding regions for the M1 and M2 influenza
virus proteins (A/PR/8/34).  It is described by Young et
al, in The Origin of Pandemic Influenza Viruses, 1983,
edited by W. G. Laver, Elsevier Science Publishing Co.

Plasmid pAPR801 is a pBR322-derived cloning vector
which carries the NS1 coding region (A/PR/8/34).  It is
described by Young et al, cited above.

Plasmid pAS1 is a pBR322-derived expression vector

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which contains the $P_L$ promoter, an N utilization site (to relieve transcriptional polarity effects in the presence of N protein) and the cII ribosome binding site including the cII translation initiation codon followed immediately by a BamHI site.  It is described by Rosenberg et al, _Methods Enzymol._, 101:123-138 (1983).

Plasmid pAS1deltaEH was prepared by deleting a nonessential EcoRI-HindIII region of pBR322 origin from pAS1.  A 1236 base pair BamHI fragment of pAPR801, containing the NS1 coding region in 861 base pairs of viral origin and 375 base pairs of pBR322 origin, was inserted into the BamHI site of pAS1deltaEH.  The resulting plasmid, pAS1deltaEH/801 expresses authentic NS1 (230 amino acids).  This plasmid has an NcoI site between the codons for amino acids 81 and 82 and an NruI site 3' to the NS sequences.  The BamHI site between amino acids 1 and 2 is retained.

Plasmid pAS1ΔEH/801 was cut with BglII, end-filled with DNA polymerase I (DNApolI; Klenow) and ligated closed, thus eliminating the BglII site.  The resulting plasmid pBgl⁻ was digested with NcoI, end-filled with DNApolI (Klenow) and ligated to a BglII linker.  The resulting plasmid, pB4, contains a BglII site within the NS1 coding region.  Plasmid pB4 was digested with BglII and ligated to a synthetic DNA linker as described in Example 4 of EP 0366238.

The resulting plasmid, pB4+, permits insertion of DNA fragments within the linker following the coding region for the first 81 amino acids of NS1 followed by termination codons in all three reading frames.  Plasmid pB4+ was digested with XmaI (cuts within linker), end-filled (Klenow) and ligated to a 520 base pair PvuII/HindIII, end-filled fragment derived from the HA2 coding region.  The resulting plasmid, pD, codes for a protein comprised of the first 81 amino acids of NS1, three amino acids derived from the synthetic DNA linker (Gln-Ile-Pro), followed by amino acids 65-222 of the HA2, as shown in Fig. 2 of published European Patent Application No. 366,238.

To facilitate plasmid selection in production
fermentations, a BamHI fragment derived from the pD
expression plasmid encoding the recombinant flu D
protein, was ligated into the BamHI site of a pAS1

5    plasmid derivative containing a kanamycin resistance gene
from Tn903 for selection [Berg et al, Microbiology, ed.
D. Schlessinger, pp. 13-15, American Society for
Microbiology (Washington, DC 1978); Nomura et al, The
Single-Stranded DNA Phages, ed. D. Denhardt et al,

10   pp.467-472, Cold Spring Harbor Laboratory (New York
1978); Castellazzi et al, Molecul. Gen. Genet., 117:211-
218 (1982)]. This results in the vector, $pC_{13}(H_{65-222})Kn$.

As described in Shatzman and Rosenberg, Meth.
Enzymol., 152:661-673 (1987), pOTS207 is a pAS derived

15   cloning vector which carries the kanamycin resistance
gene from Tn903 [Berg, cited above; Nomura, cited above;
Castellazzi, cited above]. It was constructed by
digesting plasmid pUC8 [Yanisch-Perron et al, Gene,
33:103-119 (1985)], with BamHI and ligated to a BclI

20   fragment containing the kanamycin gene from Tn903. The
resulting plasmid, pUC8-Kan, was digested with EcoRI and
PstI, and the fragment containing the kanamycin gene was
inserted between the EcoRI and PstI sites of pOTSV
[Shatzman and Rosenberg, cited above]. The resulting

25   plasmid is pOTS207.

A 520 bp fragment encoding the HA2 coding sequence
was isolated from pJZ102 [a pBR322-derived cloning vector
which carries a coding region for the entire HA protein
(A/PR/8/34) (described by Young et al, The Origin of

30   Pandemic Influenza Viruses, ed. W.G. Laver, Elsevier
Science Publishing Co. (1983)] and inserted into pB4+
which had been cut with XmaI and end filled. The
resulting plasmid, $pC_{13}H_{65-222}$ was digested with BamH1 and
the fragment encoding the flu D protein isolated from

35   this fragment was then ligated into the BamH1 site of
pOTS207 to produce the plasmid $pC_{13}(H_{65-222})Kn$ [SmithKline
Beecham].

The sequence of the kanamycin resistant C13(H65-
222)Kn plasmid was derived as follows: nucleotide

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positions 1-31, 3136-3964, 4021-4343, 4533-7166 were
derived from pBR322 [Young et al, cited above];
nucleotide positions 32-1879, 4344-4532 were derived from
λ phage, nucleotide positions 1880-2122, 2682-3135 were

5    derived from the NS1 gene, nucleotide positions 2123-
2132, 2660-2681 were derived from a synthetic linker,
nucleotide positions 2133-2659 were derived from the HA2
gene, nucleotide positions 3965-4020 were derived from
the $pCV_1$ polylinker, and nucleotide positions 7167-8601

10   were derived from the pUCKan12 (Tn903:Kn$^r$).  The DNA
sequence of the coding region for the flu D protein
derivative was confirmed by the dideoxy chain termination
method of sequencing DNA [Sanger et al, <u>Proc. Natl. Acad.
Sci. USA</u>, <u>74</u>:5463-5467 (1977)].

15       This $C_{13}(H_{65-222})$Kn plasmid is essentially the same as
the plasmids which are described in co-pending U. S.
Patent Application Serial No. 07/387,200, its
corresponding published EPA No. 366,238, and co-pending
U. S. Patent Application Serial No. 07/387,558, its

20   corresponding published EPA No. 366,239, with the
exception that the β-lactam marker has been removed and
replaced with a Kanamycin marker.  The above applications
are incorporated for reference for their description of
other appropriate vectors.

25       The $pC_{13}(H_{65-222})$Kn plasmid was transformed into *E. coli*
expression strain AR58 [SmithKline Beecham]; and
production of the flu D protein was confirmed by
immunoblot analysis [Towbin et al, <u>Proc. Natl. Acad. Sci.
USA</u>, <u>76</u>:4350 (1979)] which revealed a major

30   immunoreactive species at the predicted molecular weight
of 27.7 kD.


<u>Example 13 - Purification of D Protein</u>

The $NS1_{1-81}$-$HA2_{65-222}$ or D protein (MW 27.7 kD), was

35   purified as described in detail in published European
Patent Application NO. 366,239, corresponding to U.S.
Patent Application Ser. No. 07/387,558.

Following the induction of synthesis of D protein by
any *E. coli* host strain AR58 [SmithKline Beecham] using

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the pC13(H$_{65-222}$)Kn system described in Example 12, the
bacterial cells were collected by centrifugation and
frozen at -70°C until used.  This pellet was thawed and
resuspended in 50 mM Tris, 2 mM EDTA, 0.1 mM

5  dithiothreitol (DTT), 5% glycerol, at pH 8.  The
resulting suspension was treated with lysozyme (final
concentration of at least about 0.2 mg/ml) for about 1
hour under ambient conditions.

This suspension was then lysed on a Manton Gaulin

10  homogenizer [APV Gaulin, Inc., Everett, Massachusetts] at
8,000 psi in two passes.  The resulting suspension was
treated with Triton X-100 (1% final concentration) and
deoxycholate (DOC; 0.1% final concentration).  The pellet
containing D protein was suspended in 50 mM Gly-NaOH

15  buffer containing 2 mM EDTA and 5% glycerol at pH 10.5
using a Turrax homogenizer.  The resulting suspension
after addition of Triton X-100 (1% final concentration)
was stirred for about 1 hour in a 4°C cold room, then
centrifuged.  The pellet containing D protein was

20  dissolved in 8 M urea, 50 mM Tris at pH 8.0, overnight at
4°C.  The supernatant contained the D protein.

This supernatant, after the addition of DTT (to 50
mM final concentration), was stirred under ambient
conditions for about 1 hour then loaded onto a DEAE Fast

25  Flow Sepharose [Pharmacia] column equilibrated with 8M
urea and 50 mM Tris buffer at pH 8.  D protein was eluted
with a 0 to 0.3 M NaCl gradient (over five column volumes
or greater) in equilibration buffer.

Fractions containing D protein were concentrated on

30  a Minisette tangential flow apparatus [Pharmacia],
treated with 10 mg SDS per mg of protein, DTT [Sigma] (to
a final concentration of 50 mM) and stirred at ambient
temperature for about 1.5 hour.

The resulting solution was loaded onto a Superose-12

35  column [Pharmacia], equilibrated with 25 mM Tris-Glycine
buffer at pH 8,6, containing 1% SDS.  The D protein was
eluted at its predicted monomer MW in an isocratic
gradient of equilibration buffer.  Fractions containing D
protein of adequate purity were pooled, concentrated,

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treated with 10 mg SDS per mg of protein and DTT (to 50 mM final concentration) and then chromatographed on a Superose-12 column under the same conditions described immediately above.

5         Fractions containing D protein of the highest purity were pooled, concentrated and loaded onto a G25 Sephadex fine column [Pharmacia], equilibrated with 50 mM Tris buffer at pH 8.0 containing 8 M urea. D protein was eluted isocratically with equilibration buffer and
10    fractions containing D protein free of SDS were pooled and concentrated. This highly purified sample of D protein was then concentrated, dialyzed against 20 mM Tris, 1 mM EDTA, pH 8.0 and sterile filtered.


15    Example 14 -  Evaluation of Flu D Vaccine Compositions
          Detailed descriptions of the $D_u$ protein, and methods used for in vitro T cell assays and protection studies are described in S. B. Dillon et al, Vaccine, 10:309 (1992), incorporated by reference herein.
20        CTL and proliferation assays were performed as described previously [S. B. Dillon et al, cited above]. IL-2 was measured on an IL-2 dependent CTL line (CTLL) and IFNγ was measured by a commercial ELISA kit.
          Vaccine compositions according to this invention
25    containing superose purified D protein (Example 13) in aluminum hydroxide adjuvant, with or without 3D-MPL, were prepared as follows: 3D-MPL (RIBI Immunochemical, Hamilton, MT) was reconstituted in sterile water for injection to a working concentration of 1 mg/ml. This
30    stock solution was sonicated for 30 minutes, and subsequent dilutions, made in 5% dextrose, were sonicated for an additional 10 minutes prior to addition to mixtures of the D protein in aluminum hydroxide, prepared as described previously [Dillon et al, Vaccine,
35    10(5):309-318 (1992)].
          The antigen doses were titrated from 0.01, 0.1, 1.0, 10, 20, to 100 µg/dose of injection; and the 3D-MPL doses were titrated from 0.025, 0.25, 2.5, 25, to 50 µg/dose of injection. The ratio of 3D-MPL:antigen was maintained at

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2.5:1 (w/w) for all antigen doses except the 100 μg dose, as shown in Table 5 below. Aluminum hydroxide was 100 μg per dose in all cases. A control vaccine composition contained 20 μg antigen mixed with CFA, as described in

5    Dillon et al, Vaccine, 10(5):309-318 (1992). The final injection volumes were 0.2 ml per mouse.

For these protection studies, mice (CB6F$_1$; H-2$^{dxb}$) were injected subcutaneously with a selected vaccine dose, at weeks 0 and 3, and challenged intranasally

10   (under metofane anesthesia) at week 7 with 2 to 5 LD$_{50}$ doses of A/Puerto Rico/8/34 [A/PR/8/34 (influenza type A, H1N1)] virus.

Detailed descriptions of the methods used for protection studies are described in S. Dillon et al,

15   Vaccine, 10(5): 309-318 (1992), incorporated by reference herein. Percent survival at day 21 post-challenge was compared between groups by the Fischers exact probability test.

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## Table 5

| Antigen Dose (μg) | 3D-MPL Dose (μg) | %Survival (Day 21) |
|---|---|---|
| 100 | 50 | 27 |
| 100 | 0 | 53[*] |
| | | |
| 20 | 50 | 86[*,++] |
| 20 | 0 | 40[**] |
| | | |
| 10 | 25 | 67[*] |
| 10 | 0 | 40[**] |
| | | |
| 1.0 | 2.5 | 80[*,++] |
| 1.0 | 0 | 7 |
| | | |
| 0.1 | 0.25 | 20 |
| 0.1 | 0 | 20 |
| | | |
| 0.01 | 0.025 | 20 |
| 0.01 | 0 | 13 |
| | | |
| 0.0 | 50 | 13 |
| 0.0 | 0 | 0 |

[*] $p \leq 0.003$ vs adjuvant control.

[**] $p \leq 0.01$ vs adjuvant control.

[++] $p \leq 0.01$ vs aluminum formulation at same antigen dose.

The results of Table 5 show that the minimum dose of flu D antigen required for significant protection from lethal challenge was reduced from 10 μg (with aluminum only) to 1 μg when 3D-MPL was included in the vaccine composition. Also, the percentage of mice surviving challenge was increased when the 20 μg ($p \leq 0.01$), 10 μg ($p \geq 0.05$; NS) and 1 μg ($p \leq 0.01$) antigen doses were administered with 3D-MPL as well as aluminum vs. aluminum only in the vaccine composition. Survival in a CFA control group at 20 μg antigen was 73% in this

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experiment.

## Example 15 - Evaluation of Flu D Vaccine Compositions

This experiment was performed essentially as
described in Example 14 above except that the dose of 3D-
MPL was titrated and administered either alone, with the
minimal effective dose of flu D antigen (1 µg) and the
aluminum adjuvant (100 µg), or with flu D antigen (10µg)
and the aluminum adjuvant (100 µg). The results reported
in Table 6 identify 2 µg 3D-MPL as the minimal effective
dose.

### Table 6

| Antigen Dose (µg) | 3D-MPL Dose (µg) | % Survival (Day 21) |
|---|---|---|
| 10 | 10 | 80[+] |
| 10 | 2.0 | 73[+] |
| 10 | 0.4 | 53[+] |
| 10 | 0.0 | 53[*] |
| | | |
| 1 | 10 | 80[+,++] |
| 1 | 2.0 | 87[+,++] |
| 1 | 0.4 | 33[*] |
| 1 | 0.0 | 33 |
| | | |
| 0 | 10 | 0 |
| 0 | 2.0 | 0 |
| 0 | 0.4 | 0 |
| 0 | 0.0 | 7 |

[+] $p \leq 0.001$ vs adjuvant control.

[*] $p \leq 0.02$ vs adjuvant control.

[++] $p \leq 0.01$ vs aluminum formulation at same antigen dose.

## Example 16 - Evaluation of Flu D Vaccine Compositions

In this experiment, the potency of liposomes plus
3D-MPL was compared to liposomes (Lipo) without 3D-MPL

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[Ribi Immunochem], and to Al(OH), or CFA, by determining
the level of protection seen after challenge with 2, 10
or 50 $LD_{50}$ doses of virus.   The vaccine used was the same
as in Table 9 below.

5        Mice were injected at weeks 0 and 3 and challenged
with A/PR/8/34 at week 7.   Survival is shown in Table 7
below through day 21.

## Table 7

| | | | % Survival | | |
| | Dose | | Challenge Dose ($LD_{50}$) | | |
| Antigen | (μg) | Adjuvant | 2 | 10 | 50 |
|---|---|---|---|---|---|
| D protein | 50 | Lipo | 53 | 53[b] | 7 |
| | 0 | Lipo | 27 | 0 | 0 |
| D protein | 50 | Lipo/3D-MPL[a] | 100[b] | 73[b,c] | 47[b,c] |
| | 0 | Lipo/3D-MPL | 13 | 0 | 0 |
| D protein | 50 | $Al^{3+}$ | 86[b] | 20 | 13 |
| | 0 | $Al^{3+}$ | 7 | 13 | 0 |
| D protein | 50 | CFA | 86[b] | 80[b,c] | 13 |
| | 0 | CFA | 33 | 0 | 0 |

[a] The amount of 3D-MPL per liposome was not determined.
[b] $p \leq 0.003$ vs. adjuvant control.
[c] $p \leq 0.003$ vs. aluminum hydroxide formulation at same
antigen dose.

30        The results showed that significant protection
against a 50 $LD_{50}$ challenge was achieved with the
liposome/3D-MPL formulation but not with any of the other
adjuvant formulations tested (Table 7).   Significant
protection was achieved against the 2 $LD_{50}$ challenge with
35   all formulations, and all formulations except aluminum
were protective against the 10 $LD_{50}$ challenge dose.
Therefore, the D protein in the 3D-MPL/liposome
formulation constitutes a vaccine with even greater

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potency than the D protein in CFA, which is the classic
adjuvant for supporting T cell response, but is not
suitable for use in humans.

5    Example 17 - Evaluation of Flu D Vaccine Compositions

         In this experiment, a vaccine composition of the
invention containing flu D protein, 3D-MPL, and liposomes
was evaluated in comparison to a vaccine composition
containing flu D, 3D-MPL and aluminum adjuvant.  The
10   actual amount of 3D-MPL incorporated into liposomes was
measured.

         The flu D protein (superose purified) in 25 mM
Tris/1 mM EDTA, pH 8, at a total protein concentration of
2.49 mg/mL was dialyzed against 20 mM ammonium
15   bicarbonate pH 8 and lyophilized to a powder in 20 mg
aliquots.

         Egg phosphatides (Asahi Type 5) and oleic acid
(Sigma) were admixed at approximately a 1:1.2 molar
ratio.  L-arginine (free base), 0.38 M in water was added
20   (1:1 molar ratio phosphatide:arginine) to the
phosphatide/fatty acid mixture and mixed until a smooth
and homogeneous gel is formed.  This gel was the pre-
liposomal gel.

         The lyophilized protein was combined with the pre-
25   liposomal gel (0.06-0.08 protein/gel (w/w) ratio) and
mixed at room temperature until a homogeneous paste is
formed.  Monophosphoryl lipid A (Ribi/Immunochem) was
added at a ratio of 1 μmole of lipid A to 66 μmole of
phosphatides.  This was a transparent colorless system
30   composed of nanoparticulate (submicron in diameter)
structures of aggregated 3D-MPL monomers.  The resulting
liposome suspension was further diluted with 25 mM Tris/1
mM EDTA, pH 8.0 buffer and homogenized.  The liposome
preparation was then serially centrifuged (3x) to remove
35   unincorporated protein and 3D-MPL.  Each time the pellet
was resuspended with the Tris/EDTA buffer.  The final
liposome suspension was analyzed and kept stored under $N_2$
at 5°C while not in use.

         The liposomes were subsequently assayed to determine

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protein content by the modified Lowery colorimetric
assay, the phospholipid concentration by the Barlett
assay [Bartlett, G. R., _J. Biol. Chem._, _234_:466-468
(1959)] and liposome size by photon correlation
5    spectroscopy [Malvern Model 4700]. All assays were
performed by standard techniques. Liposomes were not
analyzed for lipid A content, however, it is likely that
all of the lipid A remains in the final liposome
fraction.

10       In the following Table 8, D protein LA and control
LA-I was prepared by incorporating solid lipid A into the
lipid phase. Control LA-II was prepared by hydrating
with a solution of lipid A for incorporation.


15                          Table 8

| Liposome Sample | Phospholipid (μmole/mL) | Protein (mg/mL) | 3D-MPL (μg/mL) | Diameter Z-Avg Mean (nm) |
|---|---|---|---|---|
| Control | 4.37 | not detected | not determined | 200.8 |
| Control+ 3D-MPL | 7.87 | 0.02 | not determined | 201.3 |
| Flu D | 21.8 | 4.89 | not detected | 487.0 |
| FluD+ 3D-MPL | 14.8 | 4.06 | 270 | 502.4 |

      Mice were immunized s.c. with 0.2 ml of the
35   antigen/carrier/3D-MPL formulation at weeks 0 and 3, and
challenged intranasally on week 7 with 5 $LD_{50}$ of A/PR/8/34
virus. The "carrier" was either aluminum hydroxide (Al)
or liposomes (Lipo). Survival was monitored for 21 days.
These results are illustrated in Table 9 below. Unless

otherwise indicated, the p Value is measure vs. a 3D-MPL control.

## Table 9

| Antigen Dose | Carrier | Dose 3D-MPL | % Survival | p Value |
|---|---|---|---|---|
| 1.0 µg | Al | - | 33 | 0.084 |
| 1.0 µg | | 2.5 µg | 80 | 0.000/0.001[a] |
| 0.1 µg | Al | - | 33 | 0.084 |
| 0.1 µg | | 2.5 µg | 93 | 0.000/0.001[a] |
| 0 µg | Al | 2.5 µg | 7 | - |
| 1.0 µg | Lipo | - | 73 | 0.001 |
| 1.0 µg | | 0.05 µg | 80 | 0.000 |
| 0.1 µg | Lipo | - | 46 | 0.054 |
| 0.1 µg | | 0.005 µg | 87 | 0.000/0.020[a] |
| 0 µg | Lipo | 0.05 µg | 13 | - |

[a]    p value vs. homologous dose without 3D-MPL.

This data indicates that by using liposomes as a carrier, the required dose of 3D-MPL (0.005 - 0.05 µg) can be dramatically reduced vs. the amount required with aluminum (about 2 µg). (See also Table 6 above).

## Example 18 - Evaluation of Vaccine Composition

CB6F$_1$ mice (12 per group) were injected sc at weeks 0 and 3 with a vaccine composition comprising 100 µg flu D in aluminum (100 µg) plus 3D-MPL (10 µg). At week 7, the mice were challenged with 3-5 LD$_{50}$ doses of the viruses shown in Fig. 1. Survival was monitored for 21 days post-challenge.

The results of Fig. 1 demonstrate that the antigenic specificity of the vaccine was equivalent to that

demonstrated earlier with the $Al^{+3}$ adjuvant [S. B. Dillon
et al, cited above] (i.e., cross-protective for both H1
and H2 subtypes, but lack of efficacy for H3 or Type B).
Survival was also greater in the vaccinated group
5     challenged with the heterologous H1N1 virus, A/Tai/86,
although the results were not significant, $p < 0.09$. (See
Fig. 1).

**Example 19 - Evaluation of Flu D Vaccine Composition**

10     CB6F$_1$ mice were injected subcutaneously (sc) with a
vaccine composition comprising 1 μg D protein in aluminum
(100 μg) plus 3D-MPL (2.5 μg) or with aluminum only at 0
and 3 weeks. Eighteen mice were challenged at week 7
with 5 $LD_{50}$ doses of A/PR/8/34 virus. Five mice were
15     sacrificed at day 7 post-challenge for lung titers.
(Death in control groups at day 7 was 60% for Al/3D-MPL
and 40% for CFA). Spleens were removed from 2 mice per
group pre-challenge and 3 mice per group at day 6 post-
challenge for proliferation and cytokine assays (Table 15
20     below). Table 10 reports survival (n=10/group) and lung
virus clearance (n=5/group) after 5 $LD_{50}$ challenge. Lung
virus titer was recorded on day 7 in the fourth column of
Table 10.

The results in Table 10 show that the reduction in
25     lung virus titers in mice given a 5 $LD_{50}$ challenge was of
greater magnitude with 3D-MPL vs. CFA (2.4 $log_{10}$ vs. 0.9
$log_{10}$), although survival was equivalent in these groups.

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Table 10

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| Antigen (μg) | Adjuvant | Survival | log$_{10}$ TCID$_{50}$/lung |
|---|---|---|---|
| 1.0 | Al/3D-MPL | 86%* | 4.41 ± 0.83* |
| 0 | Al/3D-MPL | 0% | 6.80 ± 0.45 |
| 1.0 | Al | 33% | 6.39 ± 0.41⁺ |
| 0 | Al | 0% | 7.39 ± 0.41 |
| 1.0 | CFA | 86%** | 6.00 ± 0.50⁺⁺ |
| 0 | CFA | 26% | 6.90 ± 0.72 |

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* p ≤ 0.001 vs. adjuvant control group.

** p ≤ 0.01 vs. adjuvant control group.

⁺ p ≤ 0.003 vs. adjuvant control group.

⁺⁺ p ≤ 0.03 vs. adjuvant control group.

## Example 20 - Evaluation of Flu D Vaccine Composition

To determine if splenic T cell proliferation, γIFN production were correlated with protection, these responses were monitored in the spleens of mice immunized with 1 μg of D protein in Al vs Al/3D-MPL (2.5 ng 3D-MPL) adjuvants (from the study shown in Table 10) pre- and post-challenge. At week 7, two mice were sacrificed 6 days after virus challenge. Spleens were co-cultured with D protein and pulsed with $^3$H-thymidine on day 3. Culture supernatants were harvested 8 hours later.

Overall, splenic proliferative responses were similar pre- vs. post-challenge for groups vaccinated with either D protein in the Al or Al/3D-MPL formulation (maximal cpm in unstimulated cultures equalled 2750 cpm) (Fig. 3). The magnitude of the proliferative response was also similar for either adjuvant group with the maximum stimulation index equally 3.0 or lower (Fig. 2). Pre-challenge gamma-interferon levels in antigen-stimulated culture supernatants were only modestly (about 2 fold) elevated and the two adjuvant groups were

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equivalent as shown in Table 11.

However, in contrast, gamma-interferon production
was increased greater than 7 fold in antigen-stimulated
cultures from the Al/3D-MPL group post-challenge; whereas
5 post-challenge levels did not rise in the group
vaccinated with Al$^{+3}$ only (Table 11). These results thus
provide evidence that interferon-gamma production is
correlated with reduced lung titers and survival post-
challenge, and further show that T cell proliferation per
10 se does not reflect the difference between the two
adjuvant groups.

## Table 11

| | | | | ng/ml IFN-gamma | |
|---|---|---|---|---|---|
| Antigen | Injection Dose | Adjuvant | μg/ml in vitro | Pre-chllnge | Post-chllnge |
| D | 1 μg | Al$^{+3}$/3D-MPL | 0 | <0.9 | <0.9 |
| | | | 1 | 1.0 | >7.0 |
| | | | 10 | 1.8 | >7.0 |
| – | – | Al$^{+3}$/3D-MPL | 0 | <0.9 | <0.9 |
| | | | 1 | <0.9 | 1.0 |
| | | | 10 | 0.94 | <0.9 |
| D | 1 μg | Al | 0 | <0.9 | <0.9 |
| | | | 1 | 1.9 | 1.0 |
| | | | 10 | 1.9 | 1.0 |
| – | – | Al | 0 | <0.9 | 1.2 |
| | | | 1 | <0.9 | 1.7 |
| | | | 10 | 1.1 | 1.0 |

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<u>Example 21 -   Evaluation of Flu D Vaccine Composition</u>

To further investigate a role for CD4$^+$ cells,
antigen-specific proliferation and cytokine production
were compared in mice vaccinated with the vaccine

5    compositions containing flu D and aluminum adjuvant (Al)
vs. vaccine compositions containing flu D and Al/3D-MPL
formulations.

Lymph nodes from mice given a single injection of 1,
5 or 20 µg D protein in Al/3D-MPL (ratio of 3D-

10   MPL:antigen at 2.5:1 w/w) or Al$^+$ adjuvant (100 µg) 7 days
earlier were cultured with 0-30 µg/ml purified D protein.

The results are illustrated in Figs. 3 and 4.
Proliferation was clearly increased in the groups that
received Al/3D-MPL adjuvant, and the difference was

15   greatest at the lowest *in vivo* antigen dose (1 µg).  In
Fig. 3 maximal cpm in unstimulated cultures equalled 1368
cpm.  In Fig. 4 maximal cpm in unstimulated cultures
equalled 1600 cpm; and maximal cpm of CTLL (an IL-2
dependent CTL line) cells cultured with supernatants from

20   unstimulated cultures equalled 195 cpm).  Peak (48 hours)
IL-2 activity was greater in the groups vaccinated with
the Al/3D-MPL formulations, although the levels were
generally low in all cultures (Fig. 4).

Table 12 provides the results of the analysis of

25   interferon-gamma in supernatants from the same antigen-
stimulated cultures.  Interferon levels in supernatants
from adjuvant control cultures were all <1 ng/ml.
Interferon-gamma was measured with Gibco-BRL ELISA kit.
This cytokine, which was highest at day 4 of culture, was

30   up to 5-fold greater in the Al/3D-MPL group.

### Table 12

| µg antigen (in vivo) | Adjuvant (µg/mL) | antigen in vitro | IFN-gamma (ng/ml) | | |
|---|---|---|---|---|---|
| | | | day 2 | day 3 | day 4 |
| 1.0 | Al* | 0 | <1 | <1 | <1 |
| | | 1 | <1 | <1 | <1 |
| | | 10 | <1 | <1 | 1.1 |
| 1.0 | Al/3D-MPL | 0 | <1 | <1 | <1 |
| | | 1 | <1 | <1 | 1.9 |
| | | 10 | 1.1 | 2.9 | 4.9 |

The results from Table 11 and 12 collectively support a role for interferon gamma in the mechanism of action of MPL adjuvant.

### Example 22 - Evaluation of Flu D Vaccine Composition

Since the above examples indicate that 3D-MPL improves potency of the vaccine, a study was done to determine if the booster injection was necessary for protection. Mice were vaccinated with a single sc injection of 50 µg D and challenged 7 weeks later with 2 $LD_{50}$ A/PR/8/34. Alternatively, a second group was given a booster injection with the same antigen dose at 3 weeks. The 3D-MPL dose was 125 µg. The flu D was adjuvanted with either aluminum hydroxide, aluminum plus 3D-MPL, or CFA. Controls for each adjuvant were performed.

The results in Table 13 below (shown in Example 23) show that the incorporation of 3D-MPL (125 µg) into a vaccine formulation with 50 µg of antigen significantly increased survival in mice given either one or two injections when compared to the same dose of the vaccine protein adsorbed to aluminum. Again, survival in the Al/MPL group was comparable to that seen in the CFA.

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## Example 23 - Cytotoxic T Lymphocyte Assays

Detailed descriptions of the methods used for *in vitro* T cell assays are described in S. Dillon *et al*,
5   cited above. Assays to detect memory CTL were performed after secondary *in vitro* stimulation with virus as described previously [See, F. Ennis et al, Lancet, II:887-891 (1981); A. Yamada et al, J. Exp. Med., 162:663-674 (1985); and K. Kuwono et al, J. Exp. Med.,
10   169:1361-1371 (1989)]. Briefly, spleen or lymph node cells from immune or control mice were cultured at a ratio of 6:1 with influenza virus-infected, syngeneic spleen cells for 5 days. Culture medium was RPMI 1640 supplemented with 10% fetal calf serum [Hyclone
15   Laboratories, Logan, UT], 2 mM glutamine, $5 \times 10^{-5}$ M 2-ME, 10 mM HEPES, penicillin and streptomycin.

The spleens from mice given one or two sc injections of 50 µg D protein (0 and 3 weeks), were removed at week seven and restimulated *in vitro* as
20   described above. One lytic unit ($LU_{35}$) is defined as the number of effector cells in the chromium release assay required for 35% lysis (determined by regression analysis). Table 13 reports the results of H1N1-specific CTL activity in these spleens of mice
25   immunized with SK&F 106160 (D Protein) in aluminum hydroxide and 3D-MPL.

## Table 13

### LU$_{35}$ per culture

| # Injections: | Control Targets | | A/PR/8/34 Targets | | A/Taiwan/86 Targets | | Percent Survival | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 2 |
| **Formulation** | | | | | | | | |
| D/Al$^{+3}$* | <1 | 19 | 53 | 56 | 51 | 69 | 7 | 33 |
| D/Al$^{+3}$/3D-MPL* | <1 | <1 | 41 | 43 | 64 | 53 | 40[+,++] | 73[+,++] |
| D/CFA* | <1 | <1 | 129 | 88 | 98 | 131 | 53[+,++] | 93[+,++] |
| Al$^{+3}$ | <1 | <1 | <1 | <1 | 35 | 35 | N.D. | 7 |
| Al$^{+3}$/3D-MPL | 18 | 18 | <1 | <1 | 29 | 29 | N.D. | 0 |
| CFA | <1 | <1 | <1 | <1 | <1 | <1 | N.D. | 0 |

* = Antigenic dose is 50 μg flu D protein.

+ = p < 0.05 vs. adjuvant control group

++ = p < 0.05 vs. Al$^{3+}$ adjuvant group

In the experiments of this example, 3D-MPL did not
potentiate class I restricted memory CD8+ CTL relative to
the response generated with aluminum hydroxide (indicated
as Al⁻) as reported in Table 13.  In addition, the
5    results in Table 13 show that splenic CTL were comparable
in mice given 1 vs. 2 sc injections, whereas protection
was clearly improved in mice given a second injection.
Therefore it appears that a mechanism other than
CD8+ CTL must be responsible for the improved immunity
10    seen when 3D-MPL is included in the vaccine composition
containing the antigenic peptide and aluminum adjuvant,
or when mice are given a booster injection.


Example 24 - Depletion Studies
15    To further determine which T cell subset is best
correlated with the activity of the 3D-MPL adjuvant,
depletion studies were done with anti-CD-4 or anti-CD-8
monoclonal antibodies (Mabs).  An initial study was
performed using a post-vaccination protocol for T cell
20    subset depletion as follows.  Mice (15/group) were
immunized with two subcutaneous injections of 50 ug flu D
(SK&F 106160) in Al/3D-MPL at 0 and 21 days, and
challenged with 5 $LD_{50}$ doses of A/PR/8/34 on day 42.
Antibodies (300 μg/mouse) were administered ip post-
25    vaccination on days 32, 33, 34, 39, 40 and 41 pre-
challenge and on days 43, 48 and 53 post-challenge.  In
another study, mice were treated with Mabs to deplete T
cell subsets prior to the first injection of vaccine
(pre-vaccination protocol) on days -3, -2, -1.  After the
30    vaccination on day 0, mice were further treated with Mabs
on days, 7, 14, 18, 19 and 20, and then boosted with
vaccine on day 21.  Mice were further treated with Mabs
on days 28, 35, 39, 40 and 41 prior to virus challenge on
day 42.  Mice were further treated with Mabs post-
35    challenge on days 43, 49 and 54.  The results of these T
cell subset depletion experiments are shown in Table 14
below.  The results show that the effectiveness of
vaccination was reduced when mice were depleted of either
the CD4⁺ or CD8⁺ T cell subsets, the effect was more

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pronounced when Mab treatment was initiated prior to
vaccination. Therefore, these results definitively show
that the mechanism of action of the flu D vaccine in
Al/MPL formulation is T cell mediated, and both T cell
5   subsets are required for activity.

Since production of gamma interferon correlated with
protection (Table 11), a study was performed to determine
the phenotype of T cell responsible for producing this
cytokine in vaccinated mice. Mice were depleted of CD4$^+$
10   or CD8$^+$ T cell subsets by injecting anti-CD4 or anti-CD8
Mabs ip daily for 3 days (300 µg/injection). Four days
after the last Mab injection, mice were immunized with 20
µg flu D protein in the aluminum hydroxide (100 µg) and
3D-MPL (20 µg) formulation, and lymph nodes were removed
15   7 days later. The lymph node cells were restimulated *in
vitro* with 0, 1 or 10 µg/ml D protein, and supernatants
collected on days 1-4 for interferon and IL-2 assays.
The results in Fig. 5 show that IL-2 production was
completely eliminated by anti-CD4 treatment, but was also
20   partially reduced in anti-CD-8 treated mice (Fig. 5B).
Peak IFNγ production (day 4 supernatants) was reduced by
approximately 50% in anti-CD4 or anti-CD8 treated mice
(Fig. 5C). Therefore, both CD4$^+$ and CD8$^+$ T cell subsets
produced IL-2 and IFNγ.

Table 14

| | Antigen (μg) | 3D-MPL (μg) | Mab Treatment | % Survival (Mabs Post-Vaccination) | % Survival (Mabs Pre-Vaccination) |
|---|---|---|---|---|---|
| 5 | | | | | |
| | 50 | 10 | none | 70[*] | 80[*] |
| 10 | 50 | 10 | anti-CD4 | 30 | 20^{+} |
| | 50 | 10 | anti-CD8 | 50[**] | 0^{+} |
| 15 | 50 | 10 | anti-CD4[*] anti-CD8 | 30 | 10^{+} |
| | 0 | 10 | none | 0 | 0 |

20   [*]  $p \leq 0.002$ vs. adjuvant control (group 5)
   [**]  $p \leq 0.02$ vs. adjuvant control (group 5)
   [+]  $p \leq 0.01$ vs. untreated control (group 1)

The examples presented above demonstrate that a
25   recombinant influenza H1N1 vaccine formulated with
aluminum plus 3D-MPL facilitated virus clearance and
survival, and reduced the antigen dose required for
significant protection against lethal challenge, reaching
a level of potency equivalent to that seen with CFA. The
30   data collected to date provides evidence that the
mechanism by which 3D-MPL acts to potentiate the activity
of this recombinant influenza vaccine appears to be via
the selective potentiation of CD4^{+} T cell responses, and
may be restricted to TH1-type cells which produce IL-2
35   and IFNγ.

Example 25 - Preparation of Split Virus
Split viruses, such as those produced by Sachsisches
Serumwerkl GmbH (SSW) (Dresden, Germany), may be prepared

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by well known methods, such as those documented in, for
example, European Pharmacopoeia PA/PH/Exp 3 (1992); DAB
10 "Vaccines Influenzae ex virorum fragmentis
praeparatum" and the World Health Organization draft
5   revised requirements for Influenza vaccines (Inactivated)
(1990). Such split viruses are prepared using one or
more influenza virus strains recommended by WHO and the
EEC, such as A/Singapore/6/86, A/Beijing/32/92 and
B/Panama/45/90. These strains can change depending on
10  the strains popular in a particular year.

As described in more detail elsewhere, influenza
viruses are obtained from embryonated hens' eggs
inoculated with seed lot material. These virus
suspensions are partially purified and concentrated. The
15  concentrated virus suspension is treated with a
detergent, sodium desoxylcholate, to disrupt (or "split")
the virus particles. Following the removal of viral
phospholipids during the splitting process, the
reactogenicity potential is greatly reduced. The split
20  virus suspension is completely inactivated by the
combined effect of the detergent and formaldehyde.

More specifically, the process for producing a split
virus of this invention is as follows.

A.    Preparation of monovalent whole virus inoculum
25      On the day of inoculation of embryonated eggs a
fresh inoculum is prepared by mixing the influenza
working seed lot with a phosphate buffer containing
gentamycin sulphate at 0.5 mg/ml and hydrocortisone at 25
µg/ml. The virus inoculum is kept at 2-8°C.
30      Nine to eleven day old embryonated eggs are used for
virus replication. The eggs are incubated at the farms
before arrival at the manufacturing plant and enter into
the production rooms after decontamination of the shells.
The eggs are inoculated with 0.2 ml of the virus inoculum
35  on an automatic egg inoculation apparatus. The inoculum
is injected at a pressure of ± 0.03 MPa. The inoculated
eggs are incubated at the appropriate temperature (virus
strain-dependent) for 50 to 96 hours. At the end of the
incubation period, the embryos are killed by cooling the

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eggs and storage for 12-60 hours at 2-8°C.

The allantoic fluid from the chilled embryonated
eggs is harvested by appropriate egg harvesting machines.
Usually, 8 to 10 ml of crude allantoic fluid can be
5　collected per egg.   To the crude monovalent virus bulk is
add 0.100 mg/ml thiomersal.

The harvested allantoic fluid is clarified by flow
through centrifugation at a volume of 100-200 L/hour and
a centrifugal force of 10-17,000 g.   This preclarified
10　liquid can be further clarified on a 6-8 μm membrane
filter.

To obtain a $CaHPO_4$ gel in the clarified virus pool,
0.5 M $Na_2HPO_4$ and 0.5 M $CaCl_2$ solutions are added to reach
a final concentration of $CaHPO_4$ of 1.5 g to 3.5 g
15　$CaHPO_4$/liter depending on the virus strain.   After
sedimentation for at least 10 hours, the supernatant is
removed and the sediment containing the Influenza virus
is resolubilized by addition of a 0.26 M EDTA solution.
The concentration of EDTA varies between 4.5 and 10
20　g/liter of the original harvest volume.

The resuspended sediment is filtered on a 6 to 8 μm
filter membrane.

The Influenza virus is concentrated by isopycnic
centrifugation in a linear sucrose gradient (0-55%) at
25　90,000 g.   The flow through volume is from 8-12
liters/hour.   At the end of the centrifugation, the
content of the rotor is recovered by four different
fractions (the sucrose is measured in a refractometer):

- fraction 1   55-52% sucrose
30　　　　　- fraction 2   52-38% sucrose
- fraction 3   38-20% sucrose
- fraction 4   20-0% sucrose

For further vaccine preparation, only fractions 2
and 3 are used.   Fraction 3 is diluted in order to reduce
35　the sucrose content to approximately 6%.   The Influenza
virus present in this diluted fraction is pelleted at
53,000 g to remove soluble contaminants.   The pellet is
resuspended and thoroughly mixed to obtain a homogenous
suspension.   Fraction 2 and the resuspended pellet of

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fraction 3 are pooled and phosphate buffer is added to obtain a volume of 30 liters. At this stage, the product is called "monovalent whole virus concentrate".

B. Split virus monovalent bulk

5     The selected Influenza virus, preferably the monovalent whole virus concentrate of Part A above, is disrupted by centrifugation at 70,000 g through a Nadoc linear sucrose gradient of 0-55% containing a linear distribution of sodiumdesoxycholate from 1,2-1,5%. Tween

10   80 is present at 0.1% in the gradient. The virus concentrate is pumped at the rate of 5 liters/hour. At the end of the centrifugation, the content of the rotor is collected in 3 different fractions:

                 Fraction 1:  55-40% sucrose

15             Fraction 2:  40-13% sucrose

                 Fraction 3:  13-0% sucrose

The haemagglutinin is concentrated in fraction 2. Phosphate buffer containing thiomersal at 0.01% and Tween 80 at 0.01% is added to dilute the fraction four times (±

20   5 liters).

     The diluted fraction 2 is filtered on filter membranes ending with a 0.2 µm membrane. At the end of the filtration, the filters are washed with phosphate buffer containing 0.01% thiomersal and 0.01% Tween 80.

25   As a result, the final volume of the filtered fraction 2 is 5 times the original fraction volume. Depending on the virus strain, a brief sonication of the split virus material can be introduced to facilitate the sterile filtration.

30     The filtered monovalent split material is incubated at 22 ± 2°C for at least 84 hours to allow inactivation of viruses and mycoplasma by the effect of sodium desoxycholate. After this incubation time, phosphate buffer containing 0.01% thiomersal and 0.01% Tween 80 is

35   added in order to bring the total protein content down to maximum of 250 µg/ml. Formaldehyde is added at the rate of 50 µg/ml and the inactivation takes place at 4°C ± 2°C for at least 72 hours.

     The inactivated split virus material is

ultrafiltered on membranes having a mean pore size of
20,000 daltons.  During ultrafiltration, the content of
formaldehyde, NaDOC and saccharose is greatly reduced.
The volume remains constant during ultrafiltration
5    (diafiltration) by adding phosphate buffer containing
0.01% thiomersal and 0.01% Tween 80.

The ultrafiltered split material is filtered on
membranes ending with a 0.2 µm, depending on the virus
strain the last filtration membrane can be 0.8 µm.  At
10    this stage, the product is called:  "monovalent final
bulk".  The monovalent final bulk is stored at 2-8°C for a
maximum of 18 months.


Example 26 - Split Virus Vaccine Composition

15    (a)    Preparation of MPL with a particle size of 60 - 120
nm

Water for injection is injected in vials containing
lyophilised 3 deacylated monophosphoryl lipid A (MPL)
from Ribi Immunochem, Montana using a syringe to reach a
20    concentration of 1 to 2 mg per ml.  A preliminary
suspension is obtained by mixing using a vortex.  The
content of the vials is then transferred into 25 ml Corex
tubes with round bottoms (10 ml suspension per tube) and
the suspension is sonicated using a water bath sonicator.
25    When the suspension has become clear, the size of the
particles is estimated using dynamic light scattering
(Malvern Zetasizer 3).  The treatment is continued until
the size of the MPL particles is in the range 60 - 120
nm, and preferably below 100nm.

30    Suspensions can in some cases be stored at 4 degrees
C without significant aggregation up to 5 months.
Isotonic NaCl (0.15M) or isotonic NaCl plus 10mM
phosphate induces a rapid aggregation (size >3-5µm).


35    (b)    The split virus vaccine composition of this
invention is prepared as follows.

A final bulk-buffer is prepared by adding to water
for injection the concentrated salt solutions: NaCl 4 mg;
$Na_2HPO_4$ 0.52 mg; $KH_2PO_4$ 0.19 mg; KCl 0.1 mg; and $MgCl_2$ 0.05

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mg, and Thiomersal 50 μg. The resulting solution is
stirred 15 minutes before further use.

Monovalent split virus bulk (15 μg HA) is then mixed
with 3D-MPL 50 μg and the resulting mixture is stirred
5   for about 1 hour at room (or ambient) temperature.

The buffer mixture and the virus bulk mixture are
then mixed together. After 30 minutes stirring at room
temperature, the pH is brought to 7.15 ± 0.1. The
resulting final vaccine is stored at +2 - +8°C.

10   To prepare a multivalent split virus vaccine, such
as a trivalent vaccine, monovalent split virus pools of
the selected strains prepared as described above are
mixed to constitute the final multivalent vaccine. The
resulting pooled material is stirred for 30 minutes at
15   room temperature (pH 7.15 ± 0.1). The resulting final
vaccine may be stored at temperatures between about 2°C to
about 8°C. The vaccine is a colourless, light opalescent
aqueous suspension of purified split influenza virus.

For purposes of comparison in the tests of the
20   following examples, a vaccine preparation was prepared
without the adjuvant. For this control vaccine, a final
monovalent bulk-buffer is prepared by adding to water for
injection the concentrated salt solutions: NaCl 4 mg;
$Na_2HPO_4$ 0.52 mg; $KH_2PO_4$ 0.19 mg; KCl 0.1 mg; $MgCl_2$ 0.05 mg;
25   and Thiomersal 50 μg. The resulting solution is stirred
15 minutes before further use. The mixture is stirred
for 15 minutes at room temperature. Monovalent bulk (15
μg HA) is added, followed by 30 minutes stirring at room
temperature (pH 7.15 ± 0.1). The resulting final vaccine
30   is stored at temperatures between +2°C - +8°C.

Two monovalent vaccine compositions of this
invention and monovalent control vaccines were prepared
for the following examples using the H1N1 strains, A/PR/8
and Singapore mixed with 3D-MPL 50μg (particle size <
35   100nm as obtained from Example 26 (a)).


Example 27 - Lethal Challenge in Mice
The immunogenicity of the monovalent vaccine
formulation of this invention has been evaluated and

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compared to the immunogenicity of the monovalent non-
adjuvanted formula and to the adjuvant 3D-MPL without
antigen in a lethal influenza challenge in mice.

5        For each vaccine, CB6F$_1$ mice (30 per group) were
immunized subcutaneously with 2 injections given 3 weeks
apart with a preparation that contains 5 μg of indicated
strain ± 5 μg 3D-MPL. Seven weeks after the first
injection, mice were intranasally challenged under
metofane anaesthesia with 5 LD$_{50}$ of A/PR/8. Survival and
10     clinical signs of illness were monitored for 15 mice up
to 3 weeks after challenge. Lung virus titers were
determined by MDCK microassay [A. L. Frank et al, J.
Clin. Microbiol., 12:426-432 (1980)] on the remaining
animals (5 mice per group).

15        The results are reported in Table 15 below.

Table 15

Mice Lethal Challenge: Survival Rate
and Lung Virus Titers

| Vaccine Group | Survival (%) | Virus titer* | | |
|---|---|---|---|---|
| | | day 3 | day 5 | day 7 |
| Alum + 3D-MPL | 13 | 8.90±0.10 | 8.44±0.55 | 8.69±0.32 |
| Singapore | 80 | 8.10±0.17 | 7.15±0.23 | 3.09±0.10 |
| Singapore + 3D-MPL | 100# | <1 | <1 | <1 |
| A/PR/8 | 100# | 8.01±0.029 | 3.93±0.70 | <1 |
| A/PR/8 + 3D-MPL | 100# | <1 | <1 | <1 |

     *  log TCID/lung (geometric mean ± S.E.)
     #  no visible clinical symptoms

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     The two strains (A/PR/8 and Singapore) induce a
certain level of protection. The survival rate for both
groups was markedly higher than that of the control

preparation devoid of antigen (Alum + 3D-MPL), and lung
virus clearance was faster. Not surprisingly, A/PR/8-
vaccinated group (homologous with respect to challenge)
performed better than the Singapore-vaccinated group
5   (heterologous with respect to challenge). Furthermore,
both strains supplemented with 3D-MPL induced 100%
survival, practically no lung viral titer, and no
clinical symptoms. Clearly the 3D-MPL adjuvanted vaccine
of the invention induced superior protection against
10   lower respiratory track infection and therefore serious
clinical manifestations such as viral pneumonia. From
this experiment it therefore appears that 3D-MPL
adjuvantation improves homosubtypic and heterosubtypic
activity of the split vaccine and is therefore
15   anticipated to provide broader protection against
antigenic shifts and drifts than unadjuvanted vaccine.


## Example 28 - Non-Lethal Challenge in Mice

The protocol was the same as Example 25 above,
20   except that mice were not anesthesized before intranasal
challenge. Virus titration in the respiratory tract,
virus neutralization assays and scanning electron
microscopy of trachea were performed.

Viral neutralization titration against A/PR/8 virus
25   was carried out on days 1, 5 and 9 post-challenge (Table
16).

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Table 16
Viral Neutralization Assay

| Vaccine Group | Neutralizing titer* | | |
|---|---|---|---|
| | day 1 | day 5 | day 9 |
| Alum + 3D-MPL | <1 | <1 | <1 |
| Singapore | <1 | <1 | <1 |
| Singapore +3D-MPL | <1 | <1 | <1 |
| A/PR/8 | 1.6 | 2.3 | 2.2 |
| A/PR/8 + 3D-MPL | 3.5 | 3.6 | 3.3 |

\* mean of $\log_{10}$ (5 mice)

Both A/PR/8 groups produced neutralizing antibodies, with a titer consistently higher in the presence of 3D-MPL. In contrast, the vaccination with Singapore did not induce neutralization antibodies, even with the addition of 3D-MPL. This indicated that the heterosubtypic activity observed previously with Singapore and 3D-MPL was not due to antibody and provides strong evidence that cell-mediated immunity was responsible.

Scanning Electron Micrographs (SEM) of trachea were performed on samples harvested on days 3 and 5. The tissues were evaluated for histophathological changes in the serious and cilliated epithelial cells lining the trachea particularly desqumation. These changes are indicative of the severity of an ongoing influenza infection or the recovery from infection (see Table 17 below).

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Table 17

Non Lethal Challenge in Mice: SEM of Trachea

| Vaccine Grp | Mean severity score* | | Overall Score** |
|---|---|---|---|
| | day 1 | day 5 | |
| Alum + 3D-MPL | 1.7 | 1.0 | 0 |
| Singapore | 2.0 | 1.7 | 2+ |
| Singapore+MPL | 2.4 | 2.4 | 3+ |
| A/PR/8 | 2.0 | 2.0 | 2+ |
| A/PR/8+3D-MPL | 2.0 | 2.4 | 3+ |

\*      semi quantitative, done blind.
        Regenerating:   1:   severe lesion
                       2:   partial lesion
                       3:   normal tissue

\*\*    overall score, from 0 (no protection) to
       4+ (complete protection)

The results of SEM suggest that vaccination with either A/PR/8 or Singapore strain alone gave protection; in both cases this positive effect is enhanced by 3D-MPL addition, thereby indicating that adjuvanted split vaccine may alter the progression of influenza disease.

Virus titers were determined in the nose, trachea and lungs by MDCK microassay on days 1, 3, 5, 7 and 9 post-challenge (5 mice per group). Results are shown in Figs. 6A through 6F below. Not surprisingly, nasal titers were higher than tracheal or pulmonary titers. However, all the treatments assayed did not result in clear differences in nasal or tracheal titers. In contrast, lung titers were differentiated: A/PR/8 + 3D-MPL and Singapore + 3D-MPL titers were always lower or equal to the values of the antigen alone, or of 3D-MPL alone (Figs. 6A through 6F). This indicates that 3D-MPL

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adjuvantation confers a better lung protection in mice.

Both sets of mice experiments (lethal and non lethal challenge) indicate that influenza vaccination with two subtypes of $H_1N_1$ strain (Singapore or A/PR/8) and

5  subsequent homotypic challenge is improved by the incorporation of 3D-MPL as an adjuvant according to this invention.

Example 29 - Immunogenicity of the Vaccine

10  For each vaccine, CB6F$_1$ mice (15 per group) were immunized subcutaneously with 2 injections given 3 weeks apart of a preparation that contains one tenth of a human dose of conventional unadjuvanted Singapore monovalent split vaccine or a vaccine composition of this invention

15  containing the Singapore strain with 3D-MPL. A human dose is defined as a 0.5 ml injection containing 15 μg HA of each viral strain. The formulation used herein contained a hemaglutinin (HA) to 3D-MPL ratio of 1.5 μg HA per 5 μg 3D-MPL to 5 μg HA per 5 μg 3D-MPL. A control

20  group received only 3D-MPL (5 μg). Three weeks after the second injection, mice were bled and sera antibodies were individually assayed by inhibition of haemagglutination. Titers were calculated against a calibrated reference. Mice were considered responders if they have antibody

25  titers greater than the cut-off value.

The results are reported in Table 18. Both types of formulations induce anti-haemagglutination antibodies in all animals, thus the seroresponse rate is maximal. However, the immune response is significantly enhanced by

30  the vaccine formulation of the present invention containing 3D-MPL, as indicated by a geometric mean titer more than 5 times higher than that of the vaccine containing the Singapore strain alone.

Table 18

| Vaccine Group | Individual titers* | | | | | Geometric mean titer | Seroresponse |
|---|---|---|---|---|---|---|---|
| 3D-MPL (5 µg) | <25; | <25; | <25; | <25; | <25; | <25 | 0/15 |
| | <25; | <25; | <25; | <25; | <25; | | |
| | <25; | <25; | <25; | <25; | <25; | | |
| Singapore (1.5 µg HA) | 100; | 200; | 200 | 75; | 200; | 150 | 15/15 |
| | 100 | 25 | 800 | 300 | 50 | | |
| | 200 | 200 | 600 | 200 | 50 | | |
| Singapore (1.5 µg HA) + 3D-MPL 5 µg | 1200 | 600 | 400 | 400 | 1600 | 828 | 15/15 |
| | 800 | 200 | 1600 | 1600 | 400 | | |
| | 400 | 3200 | 400 | 3200 | 1200 | | |

*   cut-off value is lower than 25


    This experiment indicates that antibody response in
mice is improved by the use of the 3D-MPL adjuvanted
vaccine of this invention.


Example 30 - Hypersensitivity Study in Guinea Pig

    A hypersensitivity study was conducted in guinea
pigs to determine whether the addition of 3D-MPL (particle size < 100nm, Example 26) to a
trivalent split vaccine modifies hypersensitivity.  The
trivalent vaccine, designated Trivalent Influsplit, was
prepared as described in Example 25 and contained an H1N1
strain Singapore/6/86, an H3N2 strain Beijing/353/84 and
a Type B strain, B/Yamaghta/16/88.

    The sensitizing agent [allantoic fluid 0.5 or  2.5
mg ± 3D-MPL 50 µg; one human dose (0.5 ml injection
containing 15 µg HA for each of these strains of
influenza virus) Trivalent Influsplit ± 3D-MPL 50 µg] was
given intraperitoneally by six injections, at days 0, 3,
5, 7, 10, and 12.  Guinea pigs were allowed to rest for 4
weeks and then challenged intravenously, under
anesthesia, with the challenging agent (allantoic fluid
0.45 mg; one human dose Trivalent Influsplit ± 3D-MPL 50

μg). The animals were observed 30 minutes after challenge, and again after 2 or 3 hours. The observed symptoms (scratching, breathing problems, convulsions, death) were recorded. Where no symptoms arose from the

5    first challenge, the animals were re-challenged with allantoic fluid (1.3 mg) 24 hours later.

The results of this assay are shown in Tables 19A and 19B below.

Table 19A - Hypersensitivity Studies of Influsplit ± 3D-MPL

| Group | Sensitization Agent | Challenge | | |
| --- | --- | --- | --- | --- |
| | | Agent | Mortality | Other Findings |
| 1 (negative ctrl) | none | allantoic fluid | 0/5 | none |
| 2 (negative ctrl) | none | Influsplit | 0/5 | none |
| 3 (negative ctrl) | none | Influsplit/ 3D-MPL | 0/5 | none |
| 4 (positive ctrl allantoic | allantoic fluid | allantoic fluid | 4/5 | itching, dizziness convulsion |
| 5 (test split) | Influsplit | allantoic fluid | 0/5 | none |
| 6 (test split) | allantoic fluid | Influsplit | 0/5 | none |
| 7 (positive ctrl allantoic/3D-MPL) | allantoic fluid 3D-MPL | allantoic fluid | 4/5 | itching, dizziness convulsion |
| 8 (test split/ 3D-MPL) | - Influsplit - 3D-MPL | allantoic fluid | 0/5 | none |
| 9 (test split/ 3D-MPL) | allantoic fluid | Influsplit/ 3D-MPL | 0/5 | none |

Table 19B - Hypersensitivity Studies of Influsplit ± 3D-MPL

| Group | Sensitization Agent | Re-challenge | | |
|---|---|---|---|---|
| | | Agent | Mortality | Other Findings |
| 1 (negative ctrl) | none | | | |
| 2 (negative ctrl) | none | | | |
| 3 (negative ctrl) | none | | | |
| 4 (positive ctrl allantoic | allantoic fluid | allantoic fluid | 1/5 | itching, dizziness convulsion |
| 5 (test split) | Influsplit | allantoic fluid | 0/5 | none |
| 6 (test split) | allantoic fluid | allantoic fluid | 5/5 | itching, dizziness convulsion |
| 7 (positive ctrl allantoic/3D-MPL) | allantoic fluid 3D-MPL | allantoic fluid | 1/1 | itching, dizziness convulsion |
| 8 (test split/ 3D-MPL) | - Influsplit - 3D-MPL | allantoic fluid | 0/5 | none |
| 9 (test split/ 3D-MPL) | allantoic fluid | allantoic fluid | 5/5 | itching, dizziness convulsion |

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The absence of effect for groups 1 to 3 (negative controls) indicates that hypersensitivity requires prior intraperitoneal sensitization. The absence of hypersensitization in group 5 indicates that Influsplit

5 cannot sensitize to allantoic fluid. A similar conclusion can be drawn from group 6 (sensitization to allantoic fluid and challenge with Influsplit). In fact, results from groups 5 and 6 also indicate that the vaccine does not contain residues of allantoic fluid

10 proteins. The addition of 3D-MPL either to the sensitizing agent (groups 7 and 8) or to the challenging agent (group 9) did not change the response (compare group 7 vs 4; group 8 vs 5; group 9 vs 6).

This experiment demonstrated that the trivalent

15 Influsplit vaccine given as sensitizing agent with or without 3D-MPL is not able to induce hypersensitivity. The trivalent Influsplit vaccine given as challenging agent with or without 3D-MPL is not able to trigger any hypersensivity reaction in animals previously

20 hypersensitized with allantoic fluid; and within the experimental conditions, 3D-MPL does not have any noticeable effect on hypersensitivity reactions. Thus, the vaccine compositions of this invention are safe for administration to mammals.

25 Numerous modifications and variations of the present invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. Such modifications and alterations to the compositions and processes of the present invention

30 are believed to be encompassed in the scope of the claims appended hereto.

A/C

CLAIMS

1. A vaccine composition capable of eliciting an
enhanced immune response to an influenza antigen
comprising an effective amount of said influenza antigen
and 3D-MPL.

2. The vaccine composition according to claim 1 wherein
said influenza antigen is an antigenic polypeptide.

3. The vaccine composition according to claim 1 wherein
said antigenic polypeptide is selected from the group
consisting of D protein $NS1_{1-81}HA2_{65-222}$, $NS1_{1-81}HA2_{1-222}$, $HA2_{66-222}$, $\Delta M$, $\Delta M+$, A, C, C13, C13 short, $\Delta D$, Cys-less D, $HA2_{66-222}$, and NS1H3HA2 constructs.

4. The vaccine composition according to claim 1 wherein
said composition comprises at least one split influenza
virus.

5. The vaccine composition according to claim 4 wherein
said composition comprises three split viruses having
reactivity against at least three strains of influenza
virus.

6. The vaccine composition according to claim 4 wherein
said strains are selected from H1N1, H3N2, H2N2, and Type
B influenza strains.

7. The vaccine composition according to claim 6 wherein
said split virus is derived from the group of influenza
strains consisting of A/PR/8, A/Singapore, A/Udorn,
A/Victoria, A/Texas, A/Beijing, A/Puerto Rico, B/Panama,
B/Yamaghta, B/Lee/40, and B/Taiwan.

8. The vaccine composition according to claim 1 further
comprising an aluminum-containing adjuvant.

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9.    The vaccine composition according to claim 8 wherein
said adjuvant is aluminum hydroxide or aluminum
phosphate.

5    10.    The vaccine composition according to claim 9 wherein
the influenza antigen is $NS1_{1-81}HA2_{65-222}$, the D protein.

11.    The vaccine composition according to claim 10
consisting of between about 1 µg to about 1000 µg D
10    protein and between about 1 µg to about 50 µg 3D-MPL.

12.    The vaccine composition according to claim 11
wherein said amount of D protein is about 2 µg and said
amount of 3D-MPL is about 20 µg.

15

13.    The vaccine composition according to claim 3 wherein
said antigen is the D protein, said composition
comprising between about 50 µg to about 500 µg D protein,
between about 10 µg to about 50 µg 3D-MPL, and between
20    about 100 µg to about 500 µg aluminum adjuvant.

14.    A vaccine composition according to any of claims 1
to 13 additionally comprising a liposome preparation,
wherein said liposome preparation comprises liposome-
25    forming material containing a long chain aliphatic or
aromatic-based acid or amine; a hydrating agent of charge
opposite to that of the acid or amine, which agent is
present in a molar ratio of between 1:20 and 1:0.05
relative to the acid or amine; and water in an amount up
30    to 300 moles relative to the solids present in the
composition.

15.    The vaccine composition according to claim 14
consisting of between about 50 µg to about 500 µg D
35    protein, between about 10 µg to about 50 µg 3D-MPL, and
between about 1 mg to about 10 mg liposome preparation.

16.    The vaccine composition according to claim 14
wherein said antigen is flu D, consisting of between

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about 50 µg to about 500 µg D protein, between about 10 µ
g to about 50 µg 3D-MPL, to about 1 mg to about 10 mg
liposome preparation and between about 100 µg to about
500 µg aluminum adjuvant.

17.  The vaccine composition according to any of claims
14 to 16 wherein the hydrating agent is an alpha amino
acid having an omega substitution which is a carboxylate,
amino, or guanido function or a pharmaceutically
acceptable salt thereof, or a compound of the formula:
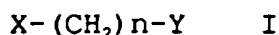
$$X-(CH_2)n-Y \qquad I$$

wherein

X is $H_2N-C(NH)-NH-$, $H_2N-$, $ZO_3S-$, $Z_2O_3P-$, or $ZO_2C-$
wherein Z is H or an inorganic or organic cation;

Y is $-CH(NH_2)-CO_2H$, $-NH_2$, $-NH-C(NH)-NH_2-COOH$,
$CH(NH_2)SO_3Z$ or $ZH(NH_2)PO_3Z_2$ wherein Z is defined above; and
n is the integer 1-10; or

a pharmaceutically acceptable salt thereof and
the acid or amine is an alkyl or alkenyl acid or amine of
10 to 20 carbon atoms.

18.  The vaccine composition according to claim 17
wherein said hydrating agent is arginine, homoarginine,
their N-acyl derivatives, gamma-aminobutyric acid,
asparagine, lysine, ornithine, glutamic acid, aspartic
acid or a compound of the formula:

| | |
|---|---|
| $H_2NC(NH)-NH-(CH_2)_n-CH(NH_2)COOH$ | II |
| $H_2N-(CH_2)_n-CH(NH_2)COOH$ | III |
| $H_2N-(CH_2)_n-NH_2$ | IV |
| $H_2NC(NH)-NH-(CH_2)_n-NH-CH(NH)-NH_2$ | V |
| $HOOC-(CH_2)_n-CH(NH_2)COOH$ | VI |
| $HOOC-(CH_2)_n-COOH$ | VII |
| $HO_3S-(CH_2)_n-CH(NH_2)COOH$ | VIII |
| $H_2O_3S-(CH_2)_n-CH(NH_2)COOH$ | IX |
| $HO_3S-(CH_2)_n-CH(NH_2)SO_3H$ | X, or |
| $H_2O_3S-(CH_2)_n-CH(NH_2)PO_3H_2$ | XI |

wherein n is 2-4, or a pharmaceutically acceptable salt
thereof.

19. A vaccine composition according to any preceding claim wherein the particle size of the 3D-MPL does not exceed 120nm.

5　20. A vaccine composition according to claim 19 wherein said particle size is in the range 60-120nm.

21. A vaccine composition according to claim 19 or 20 wherein said particle size is below 100nm.

10

22. A method for enhancing an immune response to an influenza antigen comprising the step of internally administering to the mammal a vaccine composition of claim 1.

15

23. Use of a vaccine composition according to claim 1 in the manufacture of a medicament for enhancing an immune response to an influenza antigen.

20　24. A method for preparing a vaccine composition according to claim 1 which comprises admixing said antigen and 3D-MPL.

## ABSTRACT

### INFLUENZA VACCINE COMPOSITIONS

5      The present invention provides vaccine compositions capable of enhancing a protective response to a selected influenza antigen, said composition containing at least the antigen and 3D-MPL, and methods of enhancing an immune response to influenza using these compositions.

10

Dated this ........ 17 ........ ...... ...... 19 94

PATENT AGENT FOR THE APPLICANT

Cross Protection in Mice Immunized
with SK&F106160 in Al+3D-MPL
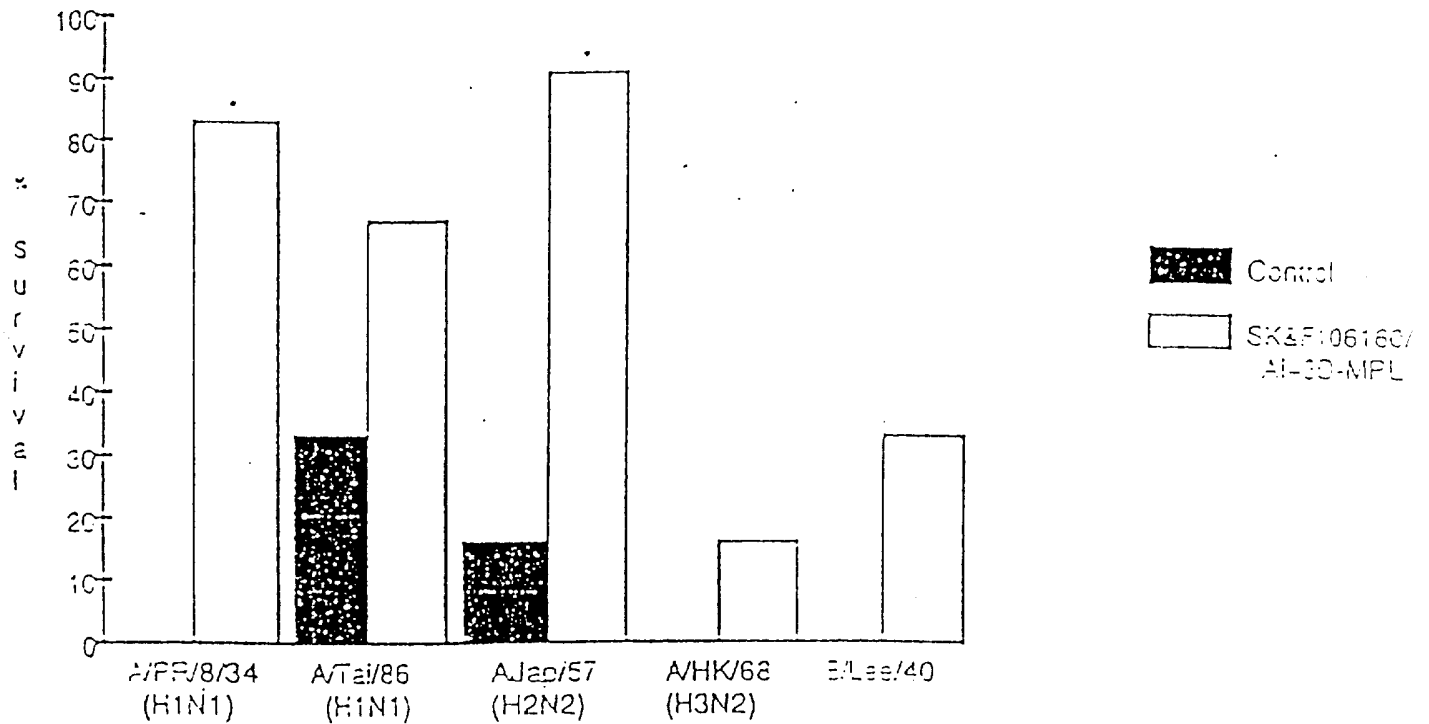


Protocol - SKF106160 10ug/Al+3D-MPL 10ug
        sc(0) > sc(3) > challenge (7)
* - p < 0.05

FIGURE  1

Pre-Challenge
Day 3

Post-Challenge
Day 3
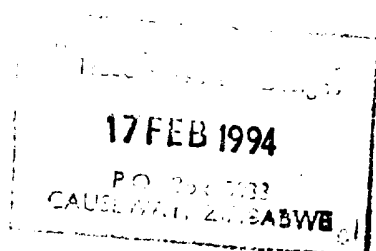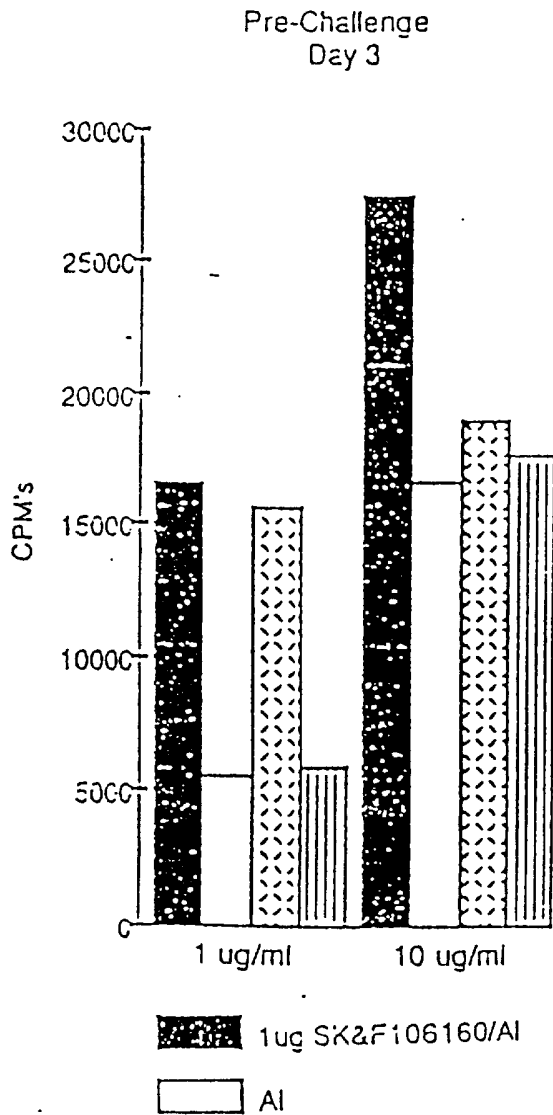


FIGURE 2A

FIGURE 2B

PATENT AGENT FOR THE APPLICANT
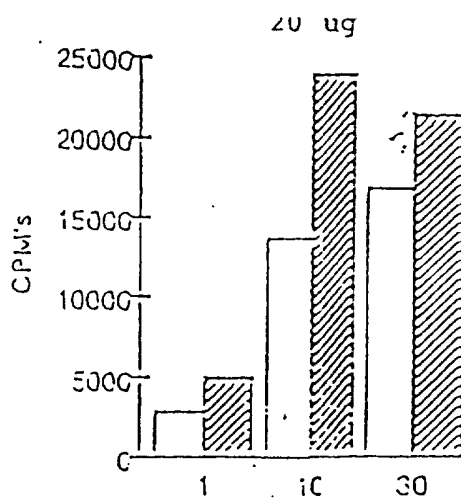
FIGURE   3A
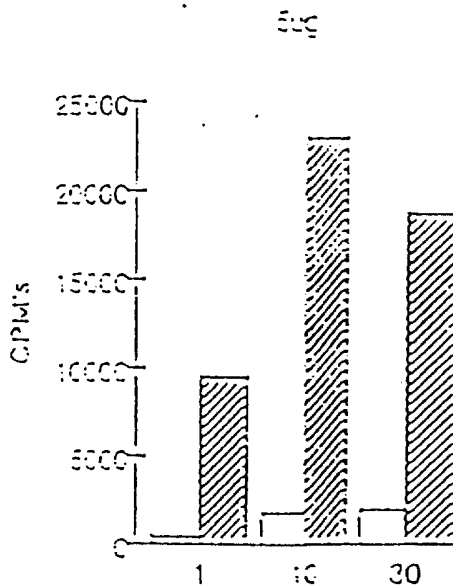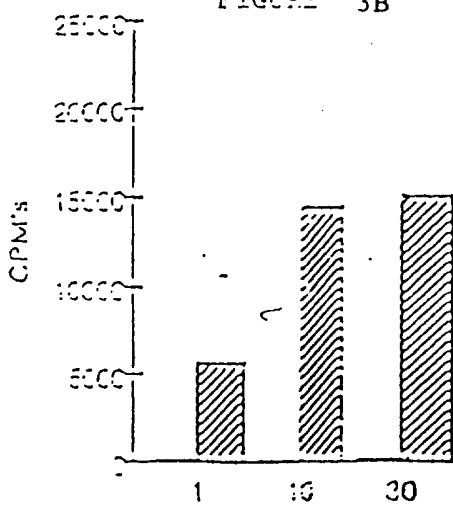
FIGURE   3B

FIGURE   3C
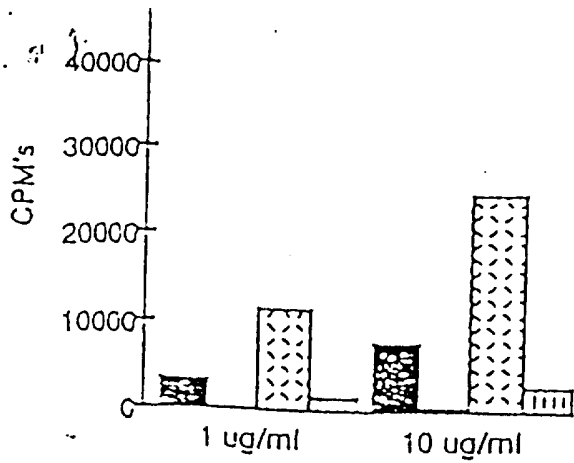
FIGURE 4A

Day 3
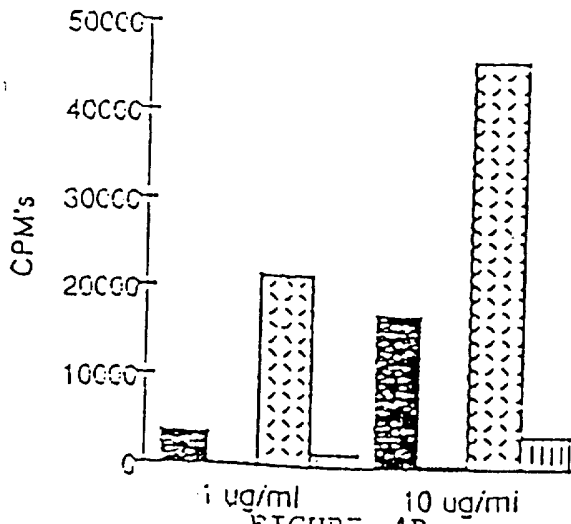
FIGURE 4B

Day 4

FIGURE 4C
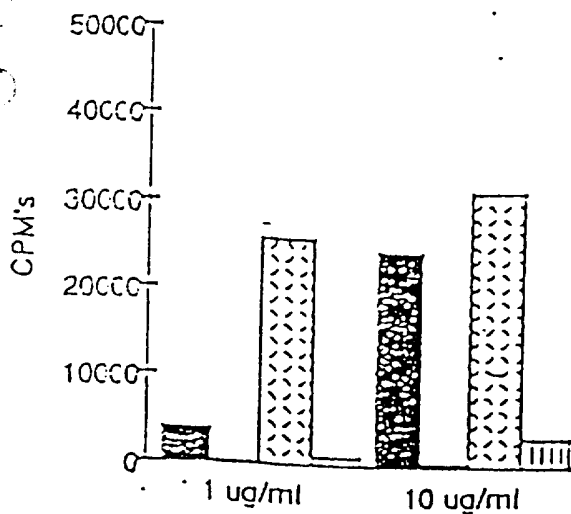


FIGURE 4D

Day 3

FIGURE 4E

Day 4

FIGURE 4F
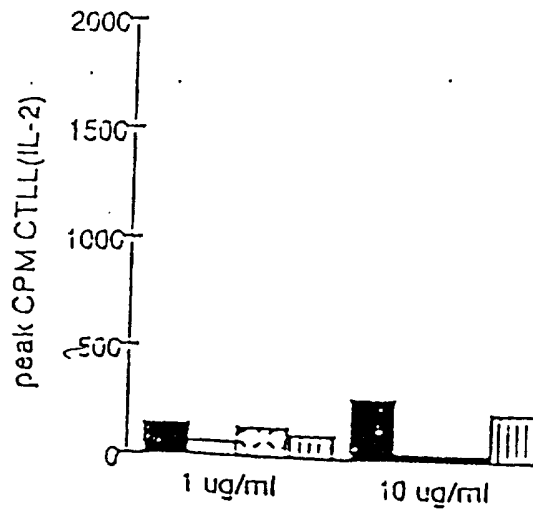
1ug SK&F106160/Al

Al

1 ug SK&F106160/Al+3D-MPL
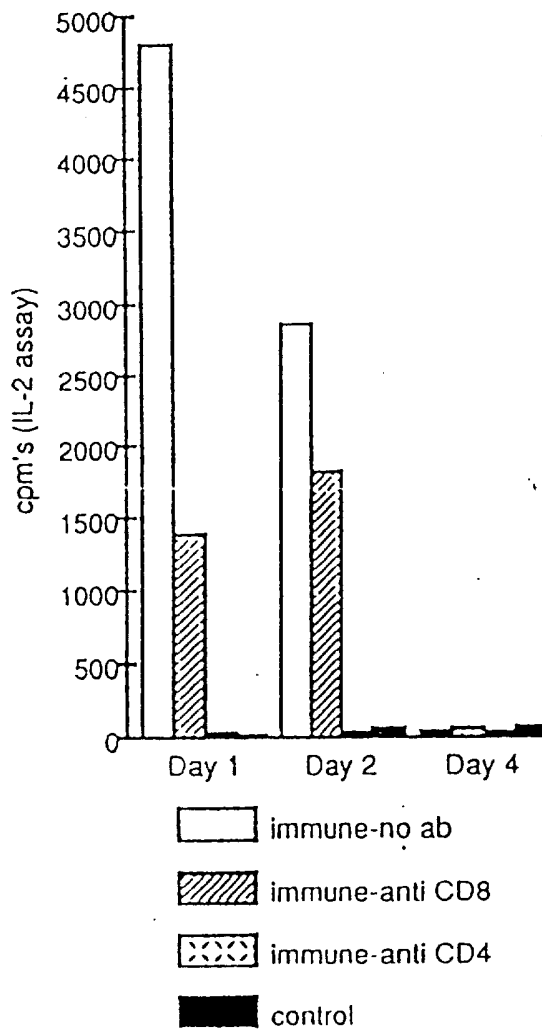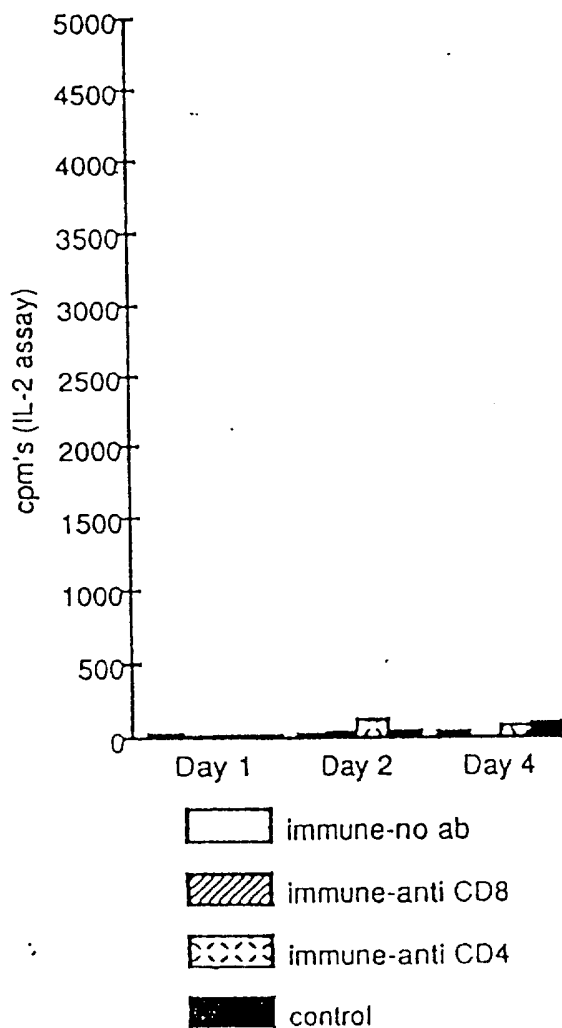
Al/3D-MPL

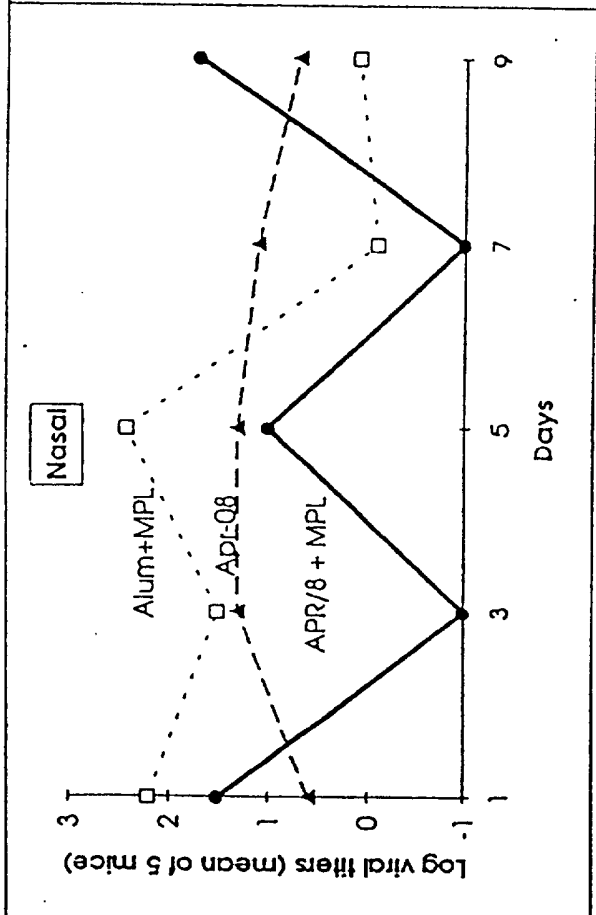PATENT AGENT FOR THE APPLICANT
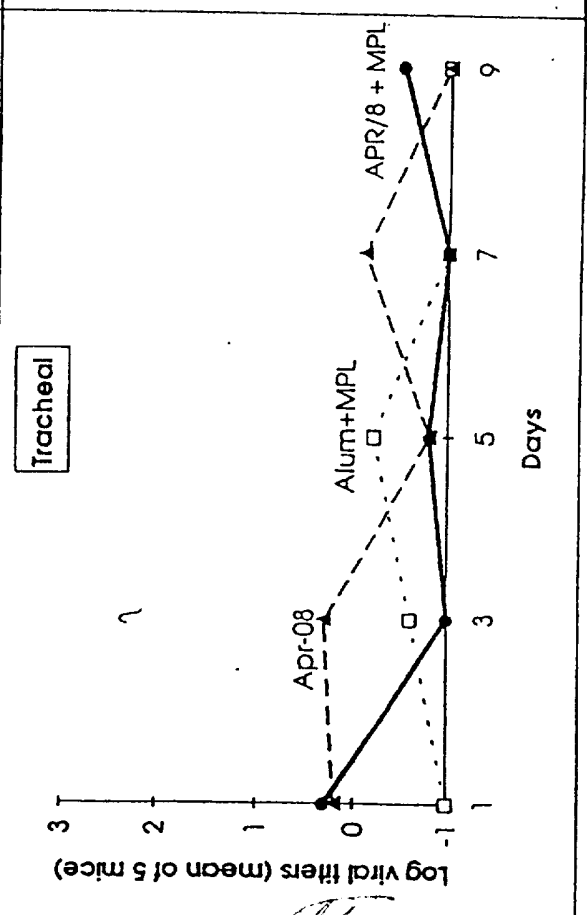
0 ug/ml

10ug/ml



FIGURE 5A

FIGURE 5B

FIGURE 6A

FIGURE 6B

FIGURE 6C

FIGURE 6D

FIGURE 6F

FIGURE 6E