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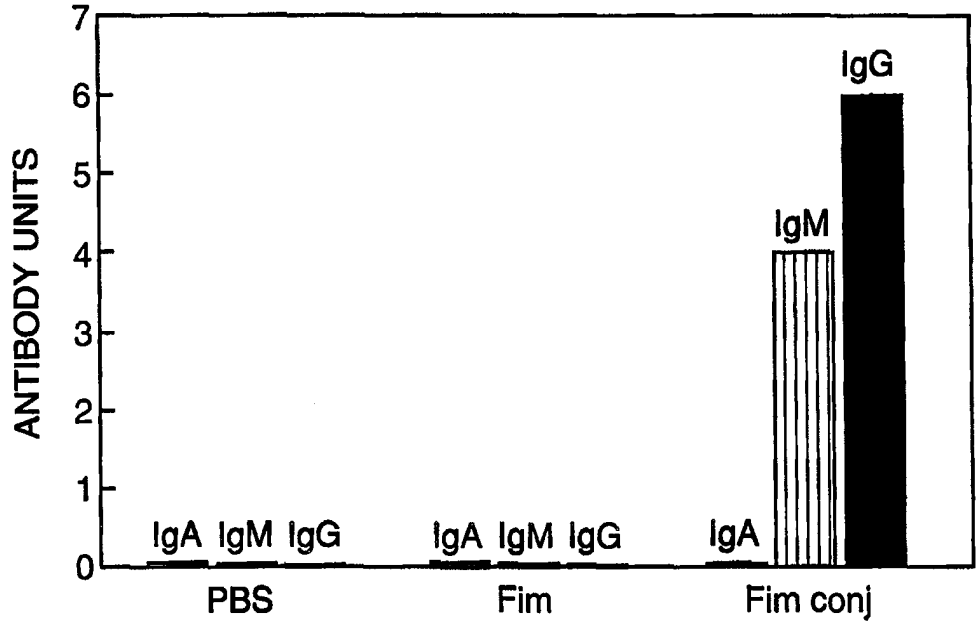
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(57) Abstract

A vaccinating conjugate comprises an antigen conjugated to a carrier selected from *Bordetella pertussis* fimbria, pertussis toxin, pertussis toxoid, and pertussis 69kD protein. The conjugate may also comprise a second antigen, different from the first. An oral vaccinating composition comprises *Bordetella pertussis* fimbria or fimbria–antigen conjugate.

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VACCINES CONTAINING *BORDETELLA PERTUSSIS* ANTIGEN

The present invention relates to vaccines containing *Bordetella pertussis* antigen, to conjugate vaccines, to methods of conjugating carrier and immunising components to form a vaccine conjugate and to use of a conjugate vaccine for vaccination of humans and animals, and also to oral vaccines against pertussis.

Many pathogenic bacteria which cause severe invasive disease possess a carbohydrate capsule which is an essential virulence component. The carbohydrate capsules are potential vaccine components since antibodies directed against them are usually protective by virtue of their complement-mediated bactericidal activity. Antibodies raised against the carbohydrate are specific for the particular serogroup from which the carbohydrate was obtained; there is one major pathogenic serogroup for *Haemophilus influenzae*, four major serogroups for *Neisseria meningitidis* and over 80 serogroups for a *Streptococcus pneumoniae*.

A major disadvantage of capsular vaccines is that carbohydrates are T-cell independent antigens and hence the immune response they raise is low (especially in infants), short-lived, unboostable and has an affinity which does not mature. The antigens can be converted to T-cell dependency by conjugation to proteins which enhance the immune response, including providing a memory response.

Immunisation with *Haemophilus influenzae* type b (Hib) capsular polysaccharide protein conjugate vaccine has been demonstrated to confer protection against Hib disease in young children. This is a persuasive argument in favour of introducing similar paediatric vaccine strategies for the control of childhood infections with, for example, *Neisseria meningitidis* and *Streptococcus pneumoniae.* However, these new vaccines will be complex mixtures of antigens. Adverse antigenic interactions and limitations of formulation technologies were encountered during the introduction of Hib vaccination. It seems these problems will only be exacerbated when the new vaccines are included in the established paediatric immunisation programme.

The problems stem from the fact that infant immunity to polysaccharides will only operate through a T-cell dependent mechanism and, therefore, this type of vaccine requires conjugating to a carrier protein. At present the only carrier proteins available for human use are tetanus toxoid (TT) or a genetically toxoided diphtheria toxin (DT). Increased use of these carriers may not be possible as there is evidence that pre-existing immunity to them, which may arise either through maternal antibodies passively transferred in early life or immunological memory to existing vaccines, can adversely effect the immune responses to the carbohydrate moiety. Clearly such interactions may reduce the effectiveness of current polysaccharide conjugate vaccines for primary immunisation and booster applications respectively.

There is therefore the problem that extended uses of the existing toxoid carriers will result in diphtheria/tetanus overload and reduced immune response to the carbohydrate conjugated to the toxoid. In addition, the toxoids require detoxification which can alter their immunological properties. Outer membrane proteins, which have been proposed as carriers, do not have the above problems, but are complex mixtures which are difficult to characterise and vary in composition from one mixture to the next.

Another difficulty relates to the practicalities of increasing the number and complexity of vaccines for paediatric immunisation. Vaccine manufacturers have been successful in producing combinations of paediatric vaccines which can be delivered simultaneously from one syringe, thus simplifying immunisation programmes. It will now be increasingly difficult technically to extend the range of components in combination vaccines using current formulation and adjuvant technologies. The prospect of re-introducing multiple injections with all of the corresponding problems of increasingly complex vaccination programmes is likely to occur unless suitable alternative delivery systems (e.g. to mucosal surfaces) are introduced.

It is therefore generally recognised that alternative carrier proteins are required for the introduction of novel or second generation conjugate vaccines.

It is an object of the invention to provide a conjugate vaccine for presentation of an immunogenic carbohydrate in a conjugate which can be used in parallel with or subsequently to existing toxoid based vaccines with amelioration or reduction of the problems and potential problems hitherto encountered. A further object is to provide a carrier protein that is an alternative to the existing toxoid carriers, for manufacture of a conjugate vaccine. A still further object is to provide vaccines that can be used for vaccination against more than one pathogen in a single vaccine formulation.

Accordingly, a first aspect of the invention provides a conjugate, for use in a vaccine, comprising an antigen conjugated to a carrier selected from (i) a fimbria of *Bordetella pertussis*, (ii) a pertussis toxin, (iii) a pertussis toxoid, and (iv) pertussis 69kD protein.

The antigen is suitably an antigenic component of a pathogenic bacteria or virus, in which context "antigen" is to be understood to encompass variants, derivatives and fragments of an antigenic component of a pathogenic bacteria or virus such that immunisation with the antigen results in protective immunity against that pathogenic organism.

Bordetella pertussis fimbriae can be purified from culture of *Bordetella pertussis* (for example, EP-A-0231083 describes purification of pertussis antigens), or can be produced by recombinant techniques, and consequently reference to a fimbria of *Bordetella pertussis* is to be understood as a reference to a fimbria whether derived by purification of natural fimbriae or by recombinant expression of DNA encoding fimbriae, and is also to be understood to encompass variants, derivatives and fragments of fimbriae which are nevertheless recognised as being variants, derivatives or fragments of *Bordetella pertussis* fimbriae, as immunisation with such variants, derivatives or fragments results in induction of antibodies that are protective against challenge by *Bordetella pertussis*. An amount of fimbriae in the range 10μg-50μg is a typical vaccinating dose.

Purification and characterisation of fimbriae isolated from *B.pertussis* is also described by Zhang et al in Infection and Immunity, May 1985, pp 422-427, and by Robinson et al in Vaccine, vol. 7, August 1989 pp 312 onwards.

Recombinant fimbriae production is described in Mol. Microbiol., 1990, January, vol 4(1), pp 39-47 and Infect. Immun., May 1991, vol 59(5), pp 1739-1746.

Conjugation of antigen to carrier is achieved by conventional means. In an embodiment of the invention, the carrier is conjugated to the antigen using a C6 spacer, in which the fimbriae are first derivatised and then added to a solution of antigen. It is also an option for the antigen first to be derivatised and this can be of advantage when the antigen is liable to be damaged by the derivatisation conditions, which typically include variations in pH. Typically, for conjugation of antigen to protein, a bifunctional group is introduced so as to link the two together.

In use of the invention, an animal is immunised with a vaccine comprising the immunogenic conjugate and is protected against challenge by the pathogenic organism from which the antigen component of the conjugate has been derived. In this sense, protection is acknowledged by survival against a challenge with a lethal dose of the pathogenic organism, or by extended life expectancy in response to challenge with such a lethal dose. Protection is also acknowledged by a patient being less affected, less ill, following challenge by a sub-lethal dose of pertussis.

The invention is of advantage in that it provides an alternative carrier molecule for preparation of immunogenic conjugates for presentation of an antigen in combination with a T-cell epitope. The immune response to the immunogenic conjugate of the invention is enhanced compared with the immune response against isolated antigen, thus improving the efficiency compared with vaccination by antigen alone. The invention also provides an alternative to the existing toxoid carriers, and therefore overcomes the problem of toxoid overload which can occur with extended and repeated uses of vaccines containing these toxoids.

A further advantage of using *pertussis fimbriae* as a component of the conjugate is that they do not require detoxification prior to incorporation into a vaccine. Detoxification, which is required for the diphtheria and tetanus toxins used in the art, can alter the immunological properties of the protein. A still further advantage is that fimbriae from *Bordetella pertussis* also confer or enhance projective immunity against *Bordetella pertussis*, a paediatrically relevant pathogen, and thus a conjugate comprising fimbriae induces a dual immune response.

The existing paediatric immunisation programme includes immunisation with DTP vaccine, conferring protection against diphtheria, tetanus and pertussis in a single vaccine. The invention opens the possibility of incorporating a fourth component, which fourth component could be, for example, antigen conferring immunity against *Haemophilus influenzae*, into this three-component vaccine, by incorporating a conjugate vaccine according to the invention which confers immunity against both pertussis and a fourth pathogenic organism.

Pertussis toxoid is a further alternative component of the conjugate of the invention and offers the potential to act as an antigen carrier without contributing to the risk of tetanus/diphtheria toxoid overload experienced with prior art carriers. pertussis toxoid also offers vaccination against both the antigen and pertussis itself.

Pertussis toxin, another alternative carrier, is optionally denatured or otherwise treated so as to render it non-toxic prior to administration to a patient. This step may be carried out prior to conjugation to antigen or after conjugation. Alternatively, toxin is used in a conjugate vaccine at a low, non-toxic dose.

69kD protein, another alternative carrier, is optionally produced by purification from culture or by recombinant means, and reference to 69kD protein is understood to encompass variants, derivatives and fragments of the protein that retain the essential immunogenicity of the intact protein.

The conjugate of the invention, as mentioned, comprises an antigen. The source

or nature of the antigen is not limited to any particular sub-group of antigens, and indeed it is possible that the antigen in isolation is not immunogenic, but only becomes immunogenic which incorporated into the conjugate of the invention.

Suitable antigens include carbohydrates, polysaccharides, monosaccharides, oligosaccharides, proteins, peptides, glycopeptides, lipopolysaccharides and similar and related molecules. Typically, the antigen will be, or will be derived from, a component of a bacteria or virus which appears on an outer surface of the bacteria or virus, such as a component of the bacterial cell wall, or a component of a fimbria or cilia or flagella, or a component of the outer envelope of a virus, a specific example of which is the surface antigen of hepatitis B virus. By way of illustration, the antigen can be a component of or derived from *Bordetella bronchiseptica*, *Clostridium tetani*, Cytomegalovirus, Dengue virus, Epstein-Barr virus, Flavivirus, Hepatitis A, B, C, D or E virus, Herpes Simplex virus, Influenza virus, JEV, Measles virus, Mumps virus, *Mycobacteria tuberculosis*, Rotavirus, Rubella virus, TBE, Vibrio cholerae, *Haemophilus Influenzae*, *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Staphylococcus A*, *B.parapertussis*, HIV, HPV, polio virus, *Brucella*, *Y.pestis*, *Helicobacter pylori*, *B. burgdorfeii*, malaria and RSV though the invention is not to be construed as limited just to this sub-group of antigens.

In an embodiment of the invention, the antigenic conjugate comprises a carrier of the invention, such as a fimbria from *Bordetella pertussis*, conjugated to two different antigens. The conjugate is thus of use in conferring or enhancing protective immunity against pertussis, and also against each of the two different pathogenic organisms from which the different antigens conjugated to the fimbria are obtained or derived. An immunogenic conjugate of the invention thus optionally comprises a fimbria of *Bordetella pertussis* to which Meningococcal C polysaccharide and Hib capsular carbohydrate have both been conjugated. This embodiment of the invention can therefore be used to confer protective immunity against three pathogenic organisms. An advantage of this embodiment of the invention is that multiple immunities can be conferred via a single vaccine component, avoiding the need to prepare mixtures of individual vaccines and

reducing the need for repeated and complex vaccination schedules using vaccines conferring immunity against just a single organism. This embodiment of the invention is made possible by the physical structure of fimbriae which are long, multimeric molecules containing multiple locations at which antigens can conveniently be coupled.

The conjugates of the invention are suitable for incorporation into microparticles for delivery via a large variety of routes including oral delivery. The preparation of such microparticles is described in EP-A-0266119, EP-A-0333523 and EP-A-0706792, the contents of which are incorporated herein by reference.

It is also known that there are a number of different types of *Bordetella* fimbriae. One type bears agglutinogen 2 and has a molecular weight of about 22,500 daltons, and the second bears agglutinogen 3 and has a molecular weight of about 22,000 daltons. These tend to be found on different serotypes of pertussis:agg 2 is found on serotypes 1.2.0 and 1.2.3, and agg 3 is found on serotypes 1.0.3 and 1.2.3. A further embodiment of the invention comprises a mixture of two immunogenic conjugates, each conjugate comprising a different pertussis fimbria type conjugated to the same or to a different antigen.

The invention also provides a method of preparing a conjugate of a carrier selected from (i) a fimbria of *Bordetella pertussis*, (ii) a pertussis toxin, (iii) a pertussis toxoid and (iv) pertussis 69kD protein with an antigen, the method comprising the steps of combining a preparation of the carrier with an antigen preparation so as covalently to conjugate the antigen to the carrier and thereafter recovering the conjugate from the mixture. Conjugation is based on primary amine groups in the carrier molecule and thus conjugation of the carrier to the antigen is possible wherever these amine groups are available on the surface of the carrier. Where two or more such groups are available so a conjugate of carrier plus two antigens is possible.

In an embodiment of the method described in a specific example below, lyophilised

fimbriae are dissolved in a solution of antigen, which solution is then maintained at reduced temperature for an extended period of time so as to allow conjugation of the antigen to the fimbriae. Preferably, an amount of fimbriae are dissolved in acidic buffer, stabilised, dialysed and then lyophilised. The antigen is prepared by dissolving the antigen in a suitable buffer and then the lyophilised fimbriae are added to that buffer, the resulting mixture is dialysed for an extended period of time and then the mixture is lyophilised and immunogenic conjugate recovered.

The invention relates also to use of the immunogenic conjugate of the invention, and thus the invention also provides use of the conjugate of the invention in manufacture of a medicament for vaccination of humans or animals against a pathogenic organism from which the antigen is derived or obtained. The invention also provides a method of vaccination of humans or animals comprising administration to the human or animal of an effective immunising amount of the conjugate of the invention.

Vaccines incorporating the immunogenic conjugate of the invention can be formulated according to techniques that are standard in this art, and the vaccines can comprise conventional pharmaceutically acceptable carriers and excipients with which the skilled person will be familiar.

Whilst the invention has provided a method of conjugation of the antigen to the fimbria, the immunogenic conjugates of the present invention may be prepared according to any conventional techniques for the covalent conjugating of antigens to carrier molecules and the invention is not to be construed as limited to the specific methods of conjugation that have been described and which are exemplified below.

It is known to vaccinate infants by injection of DTP vaccine, providing immunisation against diphtheria, tetanus and pertussis. Vaccination by this route is uncomfortable for both infant and parent, and there is the associated problem that formulations for injection must comply with the requirement for strict sterility.

Another object of the invention is to provide an alternative to injected vaccines against pertussis. Accordingly, a second aspect of the invention provides a vaccine against pertussis, comprising an oral formulation of a fimbria of *Bordetella pertussis* in a pharmaceutically acceptable carrier. The meaning and ambit of reference to fimbria is as for the first aspect of the invention.

Surprisingly, it has been found that oral administration of fimbriae can result in production of antibodies protective against challenge by pertussis. This avoids the problems associated with injected vaccines. The invention also provides a method of vaccinating against pertussis by administering fimbriae or fimbriae-antigen conjugate orally, and to use of fimbriae or fimbriae-antigen conjugate in manufacture of a medicament for oral vaccination against pertussis.

In an embodiment of the invention, fimbriae or fimbriae-antigen conjugate are formulated with a particulate carrier, typically being adsorbed onto or conjugated to the outside of the particles. Polymers such as PLG and mineral particles may be used. Preferably, *Bordetella pertussis* fimbriae are adsorbed onto particles of 10 microns or less in diameter. In particular, a suspension of mineral particles of 10 microns or less in diameter is suitable. Following oral administration uptake of these particles onto which have been absorbed fimbriae or fimbriae-antigen conjugate may occur via the Peyer's patches in the intestine. In a specific embodiment of the invention, described in further detail below, an oral vaccinating composition comprises a colloidal suspension of alum onto which has been adsorbed fimbriae according to the invention. In these embodiments of the invention, the oral vaccine can be substantially free of antigenic or immunizing components other than the fimbriae or fimbriae-antigen conjugate.

Alum is a very known vaccine adjuvant, and to date exclusively used by injection. The inventors have found that when alum plus fimbriae are given orally a good immune response is obtained. The antibody response produced is enough to give protection and that protection includes both a serum response and a mucosal response.

It is known in the art that systemic injection of vaccine gives a good serum response, principally IgG. It has been found that oral vaccination according to the second aspect of the invention gives both an IgG response and also an IgA response. This is significant because IgA appears on mucosal surfaces, which are the entry point for most pathogens.

Preferably, prior to or simultaneously with administration of the fimbriae-containing formulation, the stomach of the patient is pre-neutralised, so that acid activated proteases do not destroy the vaccine components. This can be achieved using conventional acid reducing neutralizing medicaments. Optionally, the vaccine of the invention is formulated so as to comprise an effective amount of a compound to neutralize stomach acid. In a specific embodiment of the invention, described below, a vaccinating composition is formulated using bicarbonate buffer, specifically of strength 0.1M, having a pH in the range 8.2-8.5, though other acid-neutralising solutions would also be expected to be suitable for the compositions of the invention. A further option is for an acid-neutralising pharmaceutical to be administered prior to administration of the vaccine formulation.

In use of this aspect of the invention, an animal is administered orally with a formulation of the invention, and optionally is subsequently administered a booster dose of a formulation of the invention, and is thereby protected against pertussis.

It is thought that M cells in the gut take up particulate material, as part of a continuous gut content testing process, and pass their contents to lymph nodes and finally on to macrophages where the immune response is based. It is thought that alum particles pass into the M cells and thus join the chain leading to an immune response; though the applicant does not wish to be bound by this theory.

An oral vaccine of an embodiment of the invention comprises a suspension of a carrier adapted for passage or other transport to antigen presenting cells and fimbriae. Where alum is the carrier a formulation as currently used in the art for injection is suitable. For administration to children a flavouring or sweetening agent

is an optional addition. The vaccine of the second aspect of the invention may also include a preservative, or an excipient which assists in freeze-drying the vaccine for easy, room temperature storage.

There now follows description of specific embodiments of the invention illustrated by drawings in which:

Fig. 1 shows serum anti-polysaccharide antibody responses from mice immunised on day 0, 14 and 28 with phosphate-buffered saline (PBS), fimbriae (Fim) or fimbriae-polysaccharide conjugate (Fim Conj) and tested for specific anti-polysaccharide antibodies on day 42;

Fig. 2 shows the serum anti-fimbriae antibody responses from mice immunised with phosphate-buffered saline (PBS), fimbriae (Fim) or fimbriae-polysaccharide conjugate (Fim Conj) and tested on day 21 for specific anti-fimbriae antibodies;

Fig. 3 shows the percent protection of mice, immunised with phosphate-buffered saline (Naive), fimbriae (Fim) or fimbria-meningococcal C polysaccharide conjugate (Fim Conj) and subsequently challenged with 10^6 cfu dose of *B.pertussis*;

Fig. 4 shows the number of survivors per group of five mice following challenge with *Neisseria meningitidis* on day 35 following immunisation by phosphate-buffered saline (1), fimbriae (2), fimbriae-meningococcal C conjugate (3) or AC vaccine (4), Fig. 4a showing the results following challenge by 10^6 cfu of bacteria and Fig. 4b showing the results following challenge by 10^8 cfu of bacteria;

Fig. 5 shows the numbers of mice surviving following challenge by 10^6 cfu *meningitidis* in control groups (1) and groups immunised with fimbriae (2), fimbriae-polysaccharide conjugate (3) and commercial ACVax (trade mark) vaccine (4);

Fig. 6 shows *in vitro* bactericidal titres following immunisation, legend as for Fig. 5;

Fig. 7 shows graphs of anti-fimbrial responses in external secretions elicited by oral alum-adjuvanted fimbriae: 7a-stools, 7b-vaginal washes, 7c-saliva;

Fig. 8 shows graphs of average protection elicited by orally administered alum-adjuvanted fimbriae; and

Fig. 9 shows graphs of anti-fimbrial responses in serum elicited by intraperitoneal or oral alum-adjuvanted fimbriae.

Example 1

Purification of *Neisseria meningitidis* serogroup C polysaccharide.

The method is essentially as described by Gotschlich, E. (1975). Purification of the group-specific polysaccharide. Monogr. Allergy 9, 245-258. Polysaccharide is purified from strain L91 543 (C2a P1.2R, obtained from Manchester Public Health Laboratory).

Bacteria are grown overnight on blood agar plates and inoculated into 100ml Frantz medium in 250ml conical flasks and incubated with shaking for 7h. Conical flasks containing 750ml Frantz medium are then inoculated with 10ml of the seed culture and incubated overnight with shaking at 37°C.

Purification of polysaccharide.

1. 100ml 10%(w/v) hexadecyltrimethylammonium bromide (CTB) is added to each 1L centrifuge pot.
2. Centrifuge pots are filled to 1L with culture and allowed to stand for 1h at room temperature.
3. The bacteria and precipitated polysaccharide are then harvested by centrifugation (RC3B centrifuge, 5000rpm, 30min) and the supernatant is discarded.
4. The pellets are resuspended in approx. 200ml H_2O, homogenised to prepare a smooth suspension and an equal volume of 2M $CaCl_2$ added. This is stirred for 1h to release the polysaccharide from the CTB complex.
5. Absolute ethanol is added to 25% (v/v) to precipitate DNA and stirred for 90min
6. This is centrifuged 25,000g for 20min and the supernatant retained.
7. The ethanol concentration is raised to 80% (v/v) to precipitate the polysaccharide.

8. The precipitate is then recovered by centrifugation (25,000g, 10min).
9. The precipitate is washed x4 with absolute ethanol to remove the CTB and the pellet resuspended in PBS ready for phenol extraction to remove contaminating protein.

Phenol extraction

1. A 90%(w/v) phenol solution is prepared by dissolving 90g phenol by adding 10ml boiling H_2O and melting in a water bath at 56°.
2. Polysaccharide in PBS and 90% phenol are mixed 1:1 and whirlimixed periodically during 15min at room temperature.
3. The mixture is centrifuged in a bench centrifuge at 4100rpm for about 15min.
4. The top aqueous phase is removed and stored at 4°.
5. The phenol layer is re-extracted with PBS and incubated for 15min at room temperature. Centrifuge as above, remove the top aqueous layer and pool with the previous extract.
6. The aqueous extract is dialysed against 0.1M $CaCl_2$, overnight to remove any remaining phenol.
7. The dialysed polysaccharide is centrifuged at 100,000g for 5h to pellet the lipooligosaccharide.
8. The supernatant is retained and 3 volumes of absolute ethanol added.
9. The precipitate is recovered by centrifugation and washed with absolute ethanol.
10. The final pellet allowed to dry at 35° and the dry weight recorded.

Conjugation of meningococcal polysaccharides to *Bordetella pertussis* fimbriae

Stage 1: derivatisation of protein with adipic acid dihydrazide (ADH)

1. 10mgs of fimbriae were weighed out and dissolved in 1ml of 0.1M citrate buffer, pH 4.7.
2. 35mgs of ADH were weighed out and dissolved in 0.5ml of citrate buffer pH 4.7.
3. 3.9mgs of (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide

hydrochloride)(EDC) were weighed out and dissolved in 0.5 ml of citrate buffer pH 4.7.

4. Soln 2. was added to soln 1., and then soln 3. was added.
5. The mixture was put on rollers at 20°C for 3h.
6. The mixture was dialysed for 72h at 4°C vs several changes of 10mM ammonium bicarbonate buffer and finally lyophilised.

Stage 2: activation of polysaccharide and conjugation to derivatised protein.

1. A 25mg/ml soln of CNBr (cyanogen bromide) in HPLC grade, dry acetonitrile was prepared.
2. 5mgs of purified meningococcal C polysaccharide was weighed out and dissolved in 1ml of 4mM NaOH. The solution was cooled to 4°C for 15 min.
3. 20μL OF CNBr soln was added to the polysaccharide soln, and the mixture gently agitated at 4°C for 6 min.
4. 1 ml of 0.5M $NaHCO_3$ was added to 3., and the mixture was added to 5mg of derivatised fimbriae dissolved in 3ml of 0.5M $NaHCO_3$.
5. The mixture was put on rollers for 16h at 4°C, dialysed extensively vs 10mM ammonium bicarbonate and lyophilised, to yield fimbria-meningococcal polysaccharide C conjugates.

This method of conjugation of fimbriae to polysaccharide was adapted from the method of Schneerson et al (J. Exp. Med., 152, pp 361-376, 1980, "Preparation, Characterisation, and Immunogenicity of *Haemophilus Influenzae* Type b Polysaccharide - Protein Conjugates"), and found to achieve consistent conjugation by derivatising the fimbriae with C6 spacer prior to conjugation.

It is not essential for the fimbriae to be derivatised, and instead the polysaccharide can be derivatised and this can sometimes minimise the exposure of more labile proteins to inclement conditions, such as low pH followed by high pH.

Mouse intra-peritoneal challenge model of meningococcal infection.

Adult mice (6-8 weeks old) are immunised with polysaccharide conjugate vaccine

on days 0,14, 28 and then challenged with serogroup C *N. meningitidis* on day 35. The challenge dose (0.5ml) is given by intra-peritoneal injection and contains 10^6-10^8 bacteria and iron dextran (2mg). A further intra-peritoneal injection of 2mg iron dextran is also given at 24h following challenge. The number of surviving mice in each group is recorded for 72h post infection.

Bactericidal antibody assay.

The method used for *Neisseria meningitidis* serogroup C bactericidal assay is essentially the same as detailed in the Centers for Disease Control and Prevention protocol (*Neisseria meningitidis* serogroup A/C serum bactericidal assay, Maslanka et al., 1995 CDC Atlanta, USA) with the following exceptions:

N. meningitidis strain GN is used for measurement of serogroup C bactericidal antibody.

Hanks balanced salt solution with 0.1 % bovine serum albumin (w/v) is used as buffer.

The agar "tilt" method only is used for enumeration of bacteria, 10μl from each well of a 96 well assay plate is applied to BHI + 1% horse serum plates.

The results of immunisation of mice with phosphate-buffered saline, fimbriae or fimbriae-polysaccharide conjugate are illustrated on Figs 1 and 2. Fig 1 shows no specific anti-polysaccharide responses in mice immunised with phosphate-buffered saline or with fimbriae alone, and significant anti-polysaccharide responses in mice immunised with a conjugate with fimbriae and polysaccharide, though no IgA antibodies were observed. Fig 2 shows the presence of specific anti-fimbriae antibodies in mice immunised both with fimbriae alone and also in mice immunised with the conjugate of fimbriae and polysaccharide. Fig. 3 shows there was effective protection against challenge by *Bordetella pertussis* following immunisation with pertussis fimbriae alone or fimbriae-polysaccharide conjugate.

Fig. 4 shows the number of survivors in a group of five mice immunised with phosphate-buffered saline, fimbriae, fimbriae-meningococcal C polysaccharide or a known vaccine. Fig. 4a shows that 24 hours post-challenge the group immunised with the fimbriae-polysaccharide conjugate all survived, whereas only two survived from the groups immunised with phosphate-buffered saline or with fimbriae alone. In Fig. 4b it is shown that following challenge by an increased number of bacteria, namely 10^8 compared to 10^6 shown in the top table, none of the groups immunised with phosphate ash offered saline or with fimbriae alone survived for the first 24 hours, whereas four of the group immunised with fimbriae polysaccharide survived for the first 24 hours.

Example 2

Meningococcal C bactericidal assay

Mice were immunised with PBS (injected i/p), Fimbriae (10μg injected i/p). Fimbriae-polysaccharide conjugate (10μg protein + 10μg polysaccharide injected i/p) or with a commercial meningococcal vaccine (ACVax, 10μg polysaccharide injected i/m). Animals were immunised on days 0, 14 & 28. Animals were challenged with a lethal dose of 10^6 cfu's of *N.meningitidis* on day 35. Bactericidal assays were performed as below on pre-challenge sera.

Bactericidal assay protocol

On a 96 well plate, make serial dilutions of heat-inactivated serum in bactericidal assay buffer (5% w/v BSA in Geys Balanced Salt Solution) in a final volume of 40μl. Add 10μl of a 8 x 10^4 cfu's suspension of *N.meningitidis* to each well followed by 10μl of heat-inactivated baby rabbit complement. Incubate for 1h at 37°C and then plate out 10μl of suspension from each well onto brain/heart infusion agar plates containing 1% horse blood. Incubate overnight & count colonies. Compare with appropriate controls to ascertain bactericidal titres. These are defined as titres which elicit >50% specific complement mediated kill of bacterial colonies.

Results

By 48h post challenge, all control animals (animals immunised with PBS or fimbriae) were dead. 1/5 mice given the commercial meningococcal vaccine survived at 48h while 3/5 mice immunised with the fimbriae-polysaccharide experimental conjugate vaccine survived (figure 5). The results of the bactericidal assay show that immunisation of mice with the experimental conjugate vaccine elicited *in vitro* bactericidal titres of 512 compared with 128 elicited by the commercial vaccine (figure 6).

Example 3

Formulations of fimbriae were prepared as follows. For oral administration, three volumes of fimbriae is diluted with one volume of commercially available alum adjuvant. A volume equivalent to 10μg of fimbriae (20-100 μl) is diluted with four volumes of 0.1M bicarbonate buffer (to a maximum volume of 500 μl), which has a pH of 8.2-8.5 and is designed to be acid-neutralising for oral administration, and suitable for administration by oral gavage. For I.P. administration, three volumes of antigen is diluted with one volume of commercially available adjuvant and a volume equivalent to 10 μg of fimbriae is injected. Formulations containing fimbriae - polysaccharide conjugate were similarly prepared.

Example 4

Alum formulations as described in Example 3 were used in combination with pertussis fimbriae and administered to mice via i/p or oral routes, and when orally there was prior neutralising of gut acid. The results are illustrated in Fig.s 7, 8 and 9.

Fig. 7 shows that antibodies against fimbriae were found to include antibodies of IgG, IgM and IgA classes in stools (Fig. 7a), vaginal washes (Fig. 7b) and saliva (fig. 7c). Fig. 8 shows that immunisation (using 10μg of fimbriae) via oral administration of fimbriae resulted in protection from challenge by *B.pertussis*, and

comparable protection to that provided by i/p administration of fimbriae. Anti-fimbrial responses in serum are shown in Fig. 9; though reduced in amount, serum response following oral administration was nevertheless significant.

Example 5

A Method for Encapsulation of Fimbriae and Fimbriae-antigen conjugate in microparticles

Equipment:

1) Silverson Laboratory mixer with 3/4" probe fitted with emulsor screen.
2) High speed centrifuge.
3) Normal laboratory glassware, beakers, measuring cylinders, stirrers etc.

Reagents:

1) Poly(lactide-co-glycolide) (PLG) solution - 400 mgs in 3 ml dichloromethane.
2) Solution of fimbriae (1mg/ml) in PBS.
3) Polyvinyl alcohol (PVA) solution (8% w/v in water).

Method:

1) Add 600μl of fimbriae solution to 3 ml PLG solution and emulsify in the Silverson mixer at 4000 rpm for 2½ min.
2) Add this emulsion to 100 ml PVA and emulsify at 4000 rpm for 2½ min at room temperature.
3) Add the double emulsion to 1 litre of water and stir vigorously for 1 min.
4) Distribute the suspension of microparticles in centrifuge containers and centrifuge at 10,000 x g_{av} for 30 mins.
5) Resuspend the microparticle pellet in 25ml of water and homogenise with a hand homogeniser with large clearance (0.5mm) to make a homogeneous suspension. Dilute with 200 ml of water and recentrifuge as above.
6) Repeat steps 5 and 6 four times.
7) Resuspend the microparticle pellet in 25 ml of water as above, transfer to a vessel suitable for freeze drying, shell freeze and lyophilise for 48 h.

Thus were obtained microparticles of about 2-5μm diameter, for oral administration or incorporation into other vaccinating compositions. The method is also suitable for encapsulation of fimbriae-antigen conjugate.

The results thus show protective immunity can be generated against both *Bordetella pertussis* and *Neisseria meningitidis* via a conjugate of pertussis fimbriae and meningococcal C polysaccharide. The invention provides an additional and alternative carrier to known toxoid antigen carriers and is of use in preparing novel conjugate vaccines for a wide range of immunisation applications. Oral administration of fimbriae provides protective immunity against pertussis, and is of application to production of oral vaccines.

CLAIMS

1. A conjugate, comprising:-

   (a) an antigen, conjugated to

   (b) a carrier selected from (i) a fimbria of *Bordetella pertussis* (ii) a pertussis toxin, (iii) a pertussis toxoid, and (iv) pertussis 69kD protein.

2. A conjugate according to Claim 1, comprising:-

   (a) a first antigen; and

   (b) a second antigen, different from the first; both conjugated to

   (c) the carrier.

3. A conjugate according to Claim 1 or 2, wherein the carrier is *Bordetella pertussis* fimbria.

4. A composition comprising a microparticle and a conjugate according to any of Claims 1-3, wherein the conjugate is inside the microparticle and the microparticle is 10 microns or less in diameter.

5. A composition according to Claim 4 wherein the microparticle consists of or comprises a polymer selected from (i) lactide-containing polymers (ii) glycolide-containing polymers, and (iii) polymers containing both lactide and glycolide.

6. A vaccine comprising a conjugate according to any of Claims 1-3 or a composition according to any of Claims 4-5.

7. A method of conjugating an antigen to a carrier, comprising:-

   (a) dissolving *Bordetella pertussis* fimbria in a solution of antigen; and

(b) either (i) derivatising the fimbria or (ii) derivatising the antigen; and

(c) maintaining the resultant solution to allow conjugation of the antigen to the fimbria.

8. A method according to Claim 7, comprising:-

dialysing the resultant solution;
lyophilizing the dialysed solution; and
recovering lyophilized conjugate.

9. Use of a carrier selected from (i) a fimbria of *Bordetella pertussis* (ii) a pertussis toxin, (iii) a pertussis toxoid, and (iv) pertussis 69kD protein, in manufacture of a vaccine.

10. Use according to Claim 9 wherein the carrier is *Bordetella pertussis* fimbria.

11. A method of vaccination comprising administering an effective amount of a conjugate, wherein the conjugate comprises

(a) an antigen, conjugated to

(b) a carrier selected from (i) a fimbria of *Bordetella pertussis* (ii) a pertussis toxin, (iii) a pertussis toxoid, and (iv) pertussis 69kD protein.

12. A method according to Claim 11 wherein the carrier comprises *Bordetella pertussis* fimbria.

13. An oral vaccinating composition, comprising *Bordetella pertussis* fimbria in a pharmaceutically acceptable carrier.

14. An oral vaccinating composition according to Claim 13, wherein the *Bordetella pertussis* fimbria are adsorbed onto particles of 10 microns or less in diameter.

15. An oral vaccinating composition according to Claim 13 or 14 comprising a suspension of mineral particles.

16. An oral vaccinating composition according to any of Claims 13 to 15 comprising alum particles.

17. An oral vaccinating composition according to any of Claims 13 to 16, further comprising a component to neutralise gut acid.

18. An oral vaccinating composition according to any of Claims 13 to 17, comprising a conjugate according to any of Claims 1-3.

FIG. 1

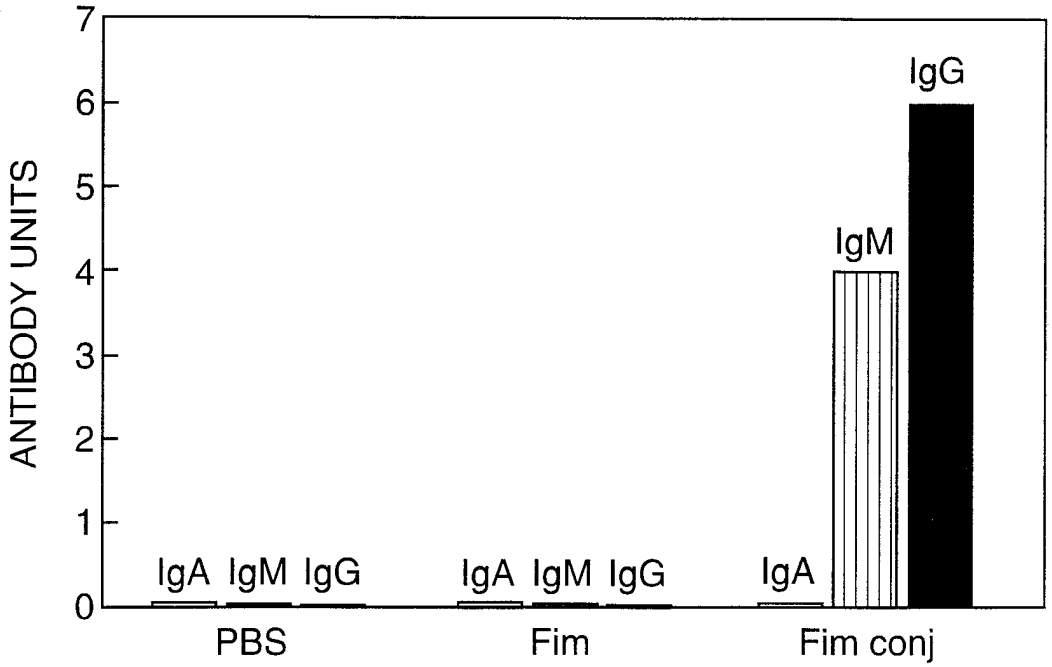

ANTIBODY UNITS
0
1
2
3
4
5
6
7
IgA IgM IgG
IgA IgM IgG
IgA
IgM
IgG
PBS
Fim
Fim conj

FIG. 2

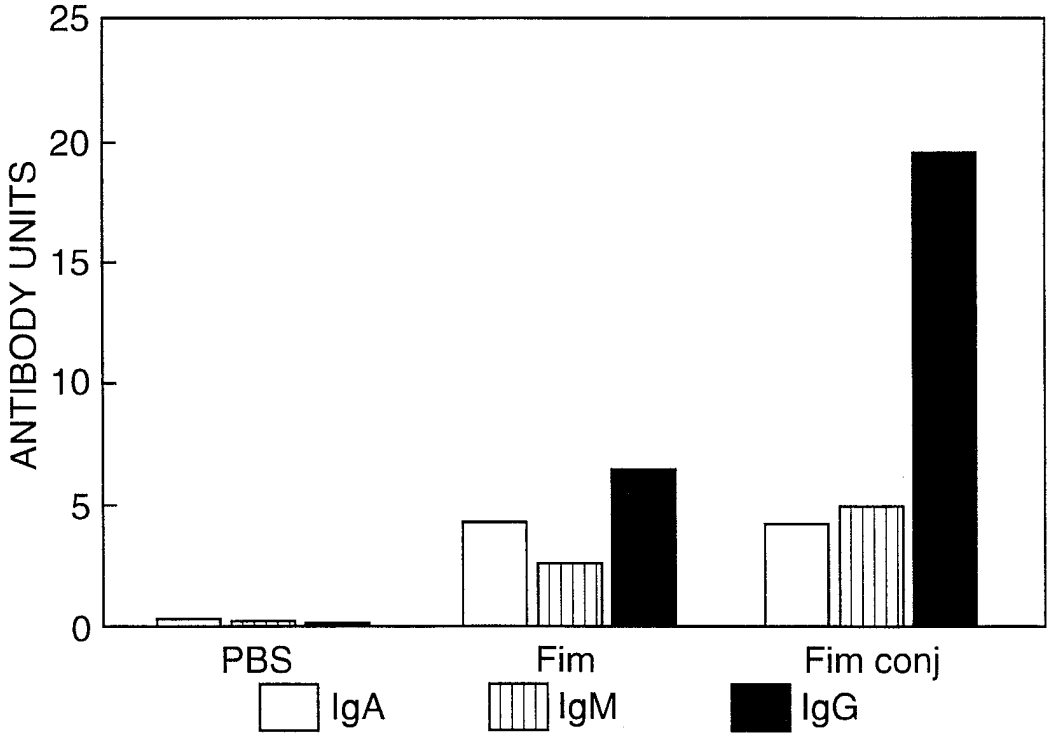

ANTIBODY UNITS
0
5
10
15
20
25
PBS
Fim
Fim conj
IgA
IgM
IgG

FIG. 3

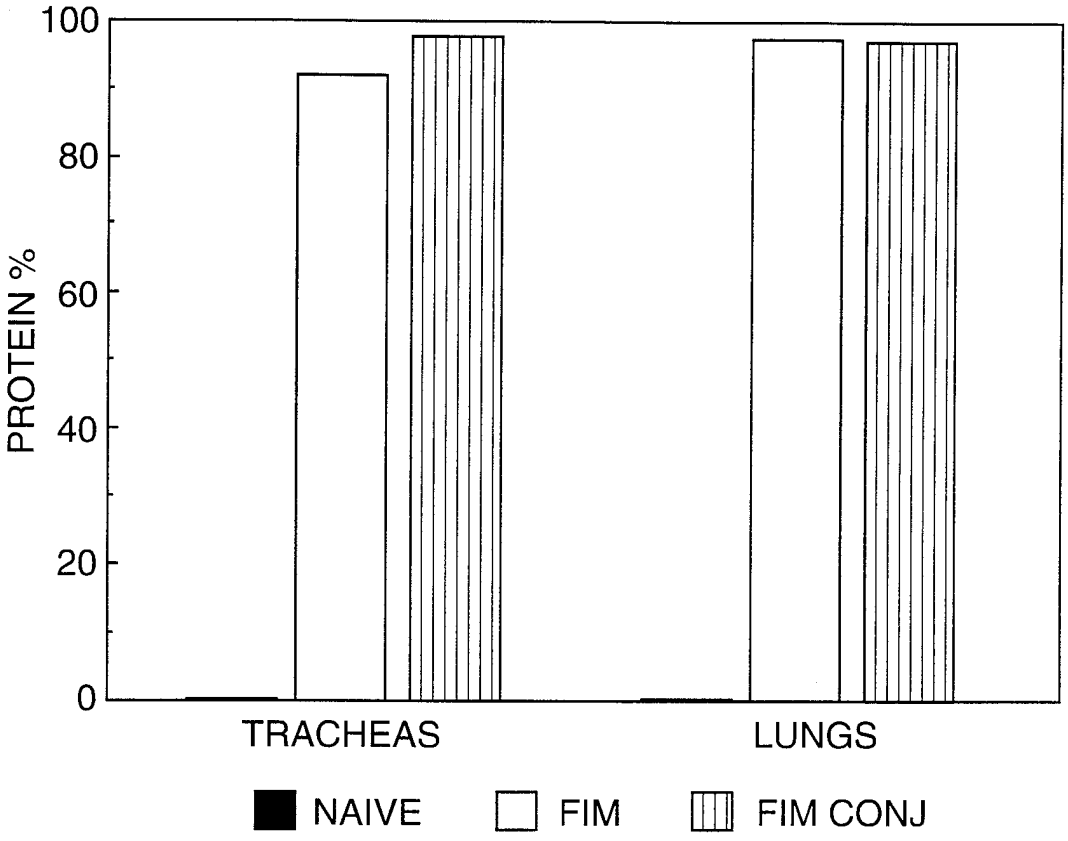

100
80
60
40
20
0
PROTEIN %
TRACHEAS
LUNGS
NAIVE
FIM
FIM CONJ

FIG. 8

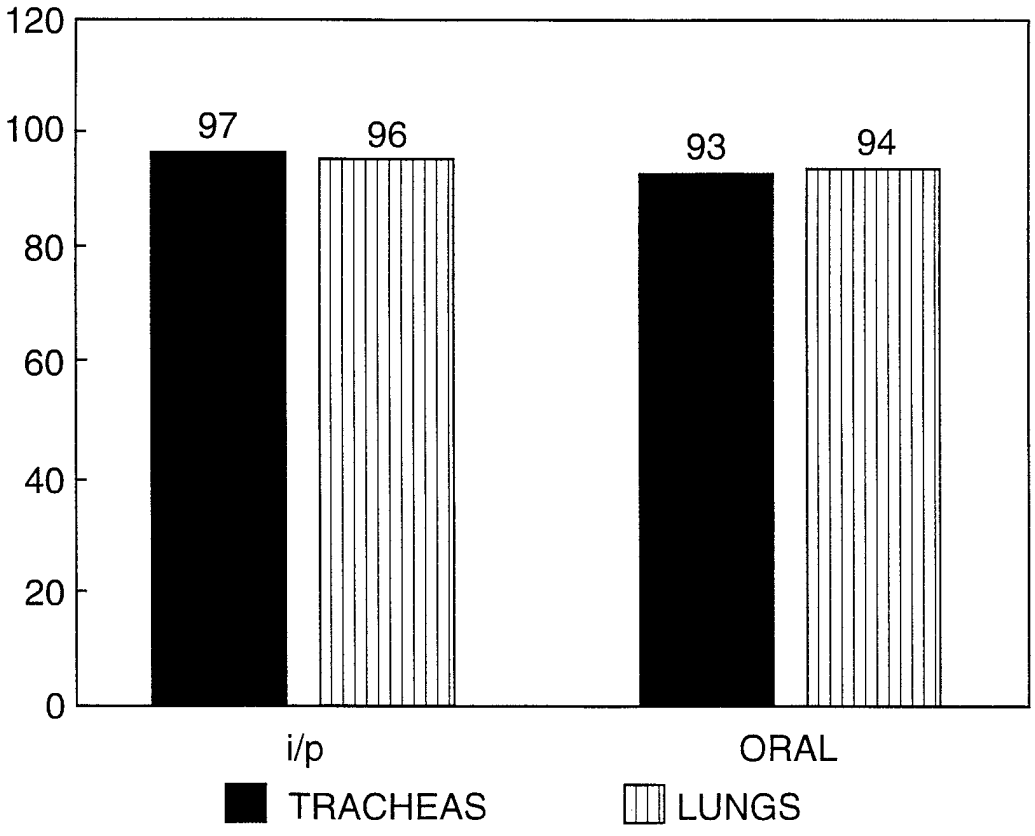

120
100
80
60
40
20
0
97
96
93
94
i/p
ORAL
TRACHEAS
LUNGS

FIG. 4a

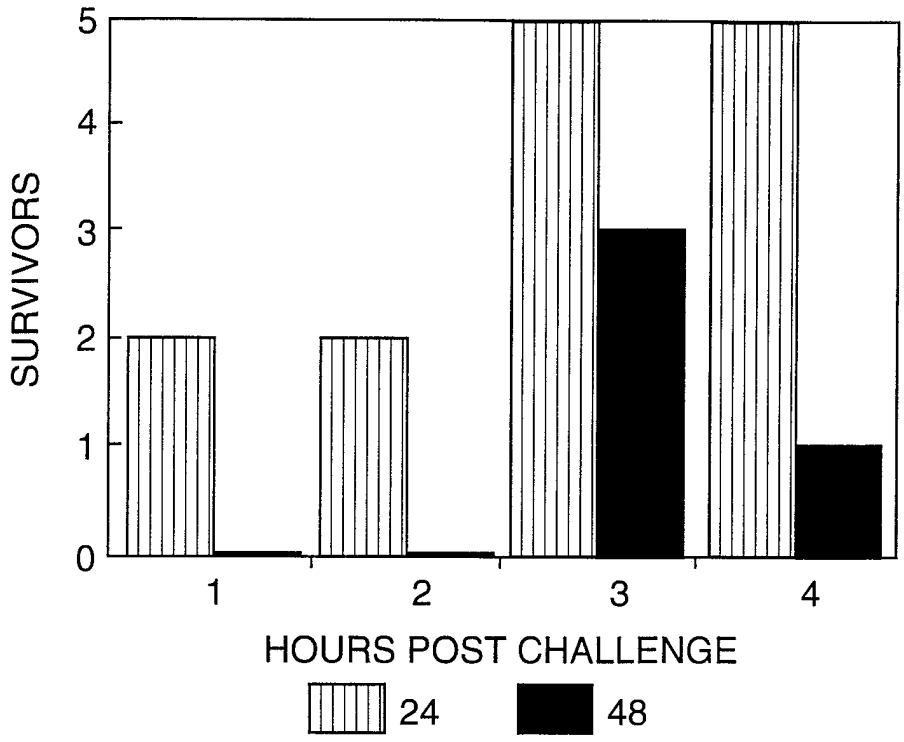

SURVIVORS
0
1
2
3
4
5
1
2
3
4
HOURS POST CHALLENGE
24
48

FIG. 4b

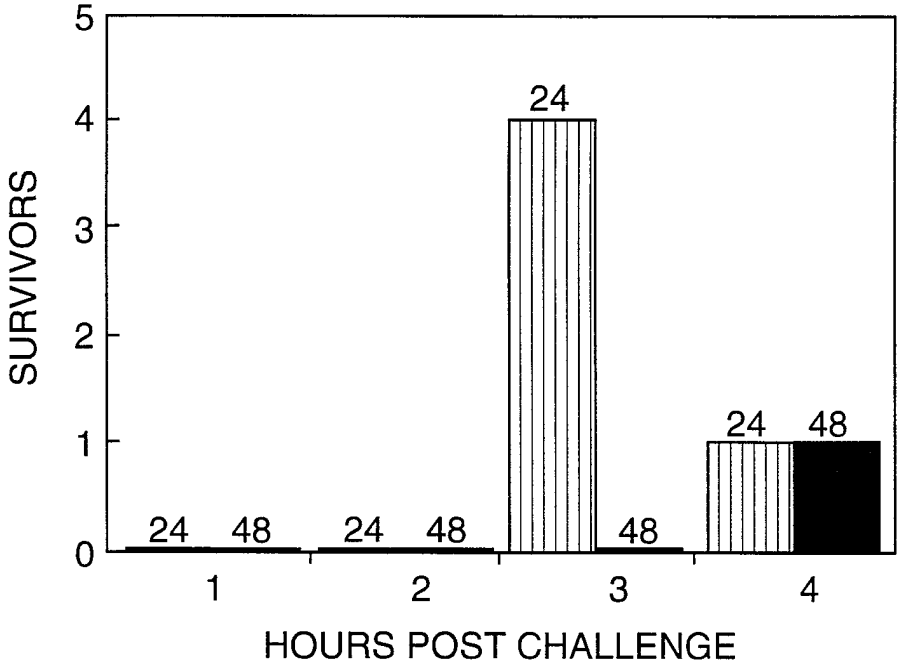

SURVIVORS
0
1
2
3
4
5
24
24
48
24
48
48
24
48
1
2
3
4
HOURS POST CHALLENGE

FIG. 5

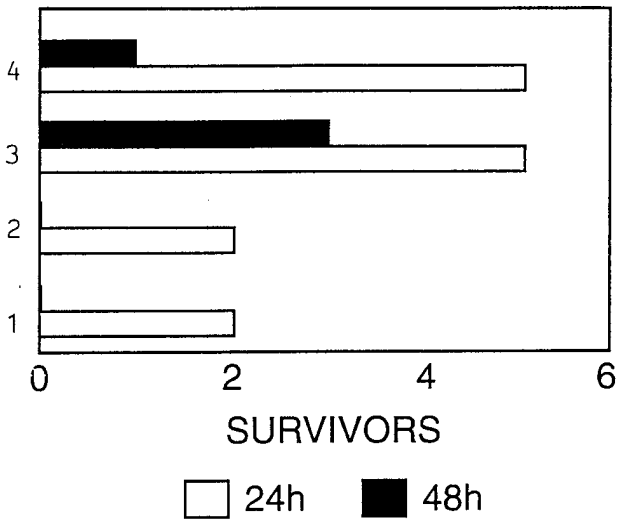


FIG. 6

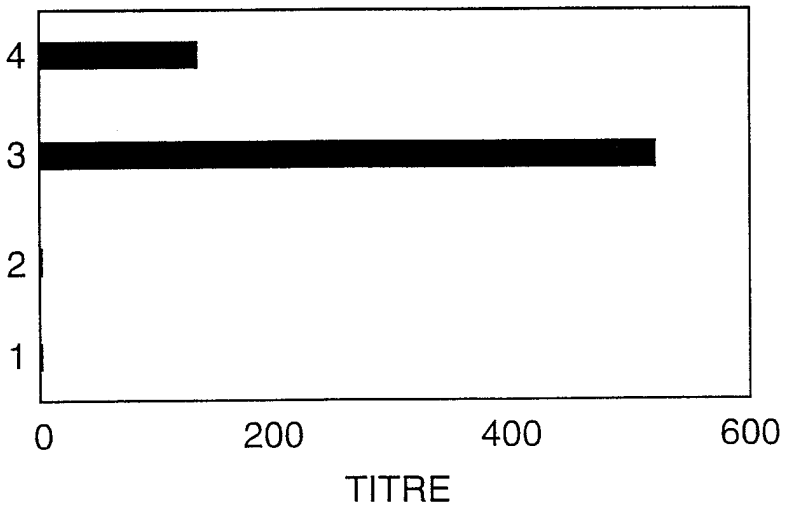


FIG. 7(a)

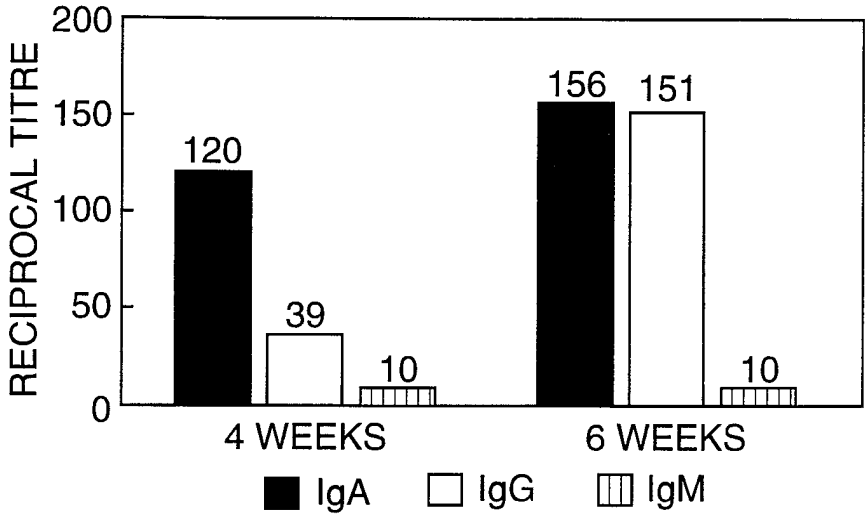


FIG. 7(b)

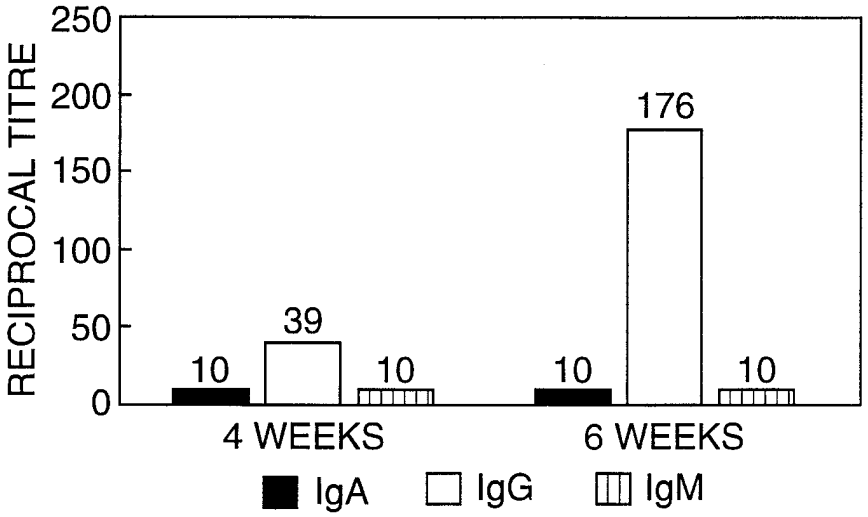
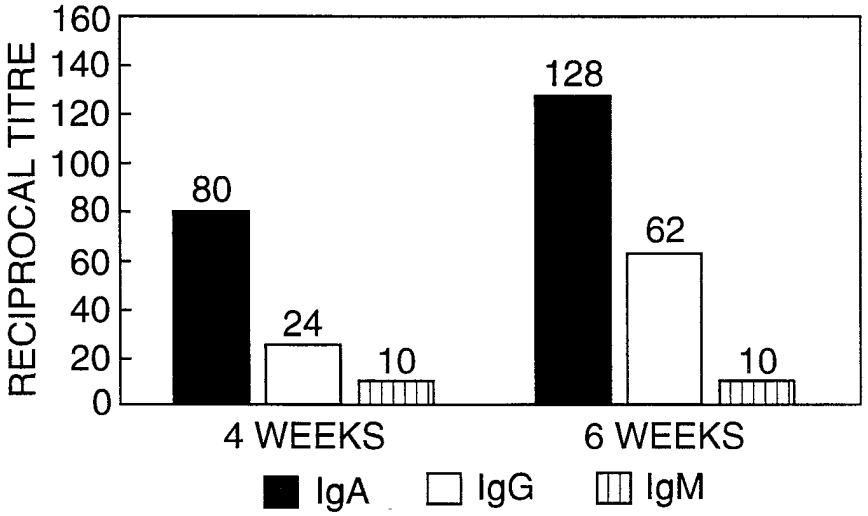


FIG. 7(c)



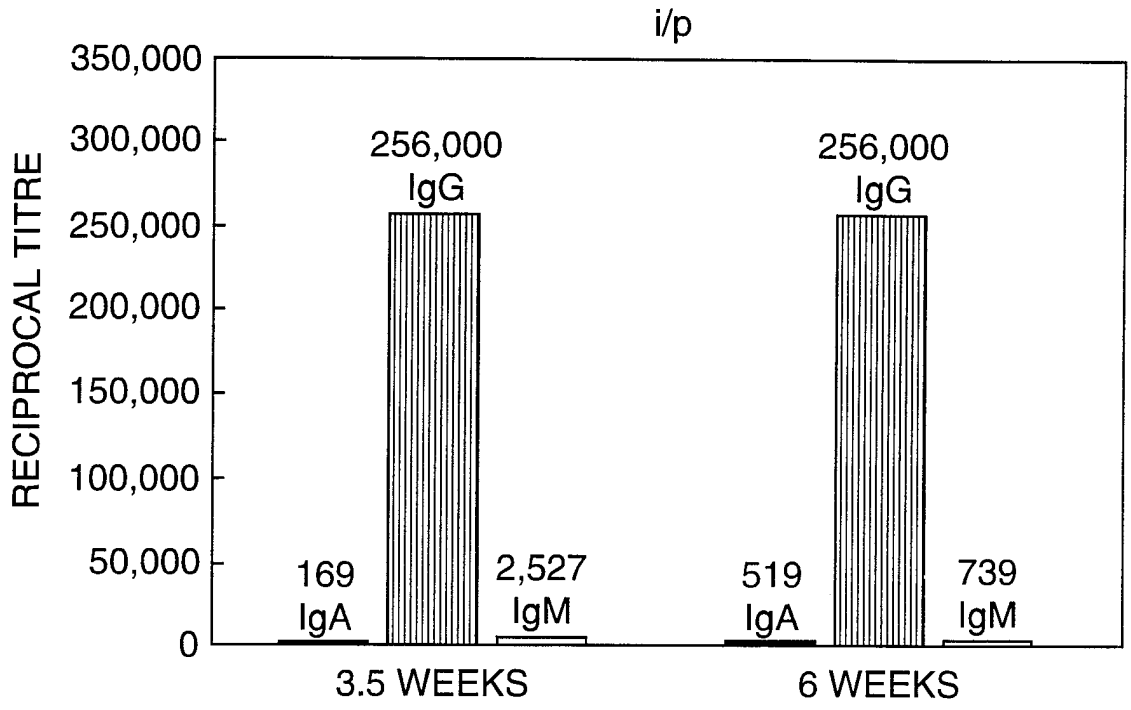
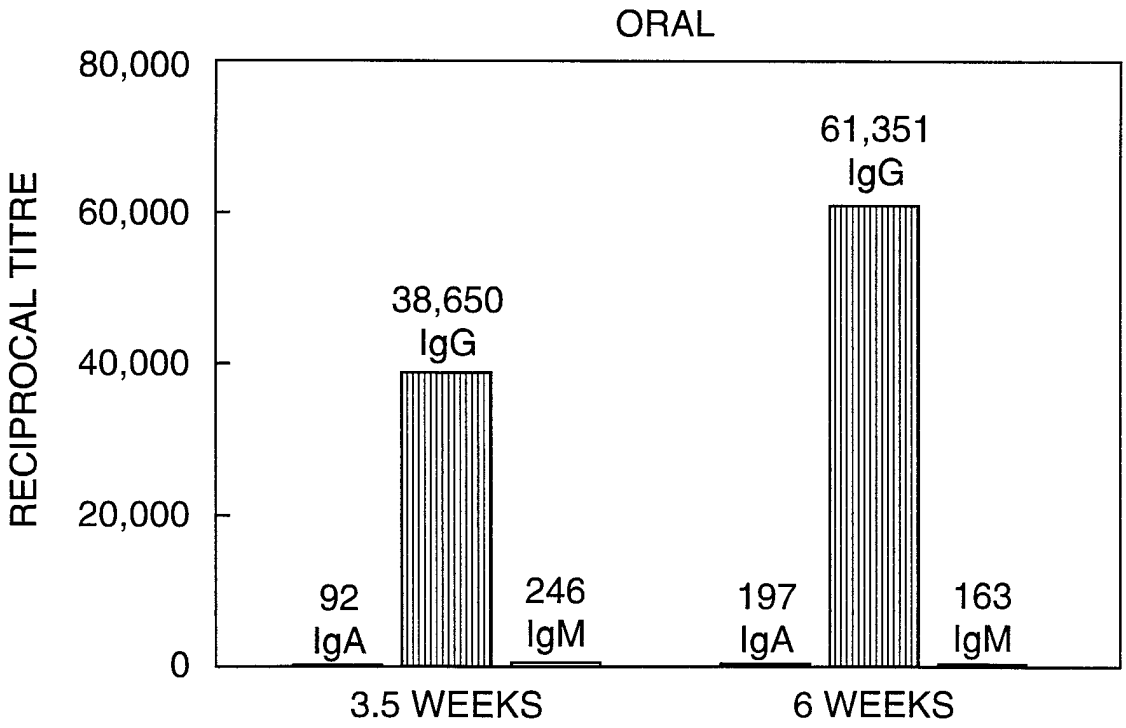

i/p
RECIPROCAL TITRE
350,000
300,000
250,000
200,000
150,000
100,000
50,000
0
256,000
IgG
256,000
IgG
169
IgA
2,527
IgM
519
IgA
739
IgM
3.5 WEEKS
6 WEEKS

FIG. 9


ORAL
RECIPROCAL TITRE
80,000
60,000
40,000
20,000
0
61,351
IgG
38,650
IgG
92
IgA
246
IgM
197
IgA
163
IgM
3.5 WEEKS
6 WEEKS