(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
31 January 2002 (31.01.2002)

PCT

(10) International Publication Number
WO 02/07764 A1

(51) International Patent Classification*: A61K 39/385, 39/10, C07K 17/00

(21) International Application Number: PCT/GB01/03273

(22) International Filing Date: 20 July 2001 (20.07.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0018031.5 21 July 2000 (21.07.2000) GB

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(54) Title: IMPROVEMENTS RELATING TO VACCINES CONTAINING BORDETELLA PERTUSSIS ANTIGEN

Serum IgG response to menCPs in mice

![Graph showing serum IgG response to menCPs in mice](image_url)

(57) Abstract: Dissociated B. pertussis fimbriae are described. A conjugate of an antigen and a B. pertussis fimbrial protein is prepared by dissociating B. pertussis fimbria into fimbrial protein subunits and conjugating the antigen to the fimbrial protein subunits. The subunits are optionally denatured as well. The conjugates are used in vaccines.
SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— with international search report
— before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.
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IMPROVEMENTS RELATING TO 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 *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, can not be boosted and has an affinity which does not mature. These antigens can, however, 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.
Known carrier proteins available for human use are tetanus toxoid (TT) and 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 affect the immune responses to the carbohydrate moiety - giving rise e.g. to carrier induced epitopic suppression. 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.

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 thus generally recognised that alternative carrier proteins are required for the introduction of novel or second generation conjugate vaccines. A new carrier for antigen delivery, including polysaccharides, has been described in WO 98/58668, using *Bordetella pertussis* fimbriae. Nevertheless, there exists a need for further and alternative carrier proteins for vaccine delivery.

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 or other vaccines with amelioration or reduction of the problems and potential problems hitherto encountered. A further object is to provide a carrier
protein that is a further 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, comprising an antigen conjugated to a carrier selected from (i) dissociated \textit{B. pertussis} fimbriae, (ii) denatured \textit{B. pertussis} fimbriae, and (iii) mixtures of (i) and (ii).

Fimbriae for use in the invention are dissociated using a dissociating agent so that the fimbrial structure of many tens and hundreds of repeat units is broken down, yielding individual protein subunits, and it is those individual subunits that are used in the conjugates of the invention. Once fimbriae have been split, the subunits lose some of their structure and whilst this is partially reversible they generally can not and do not reassociate into fimbriae. It is also optional for the dissociation to continue until the fimbrial protein is partly or completely denatured, and this may be achieved using higher concentrations of the dissociating agent or by addition of a reducing agent. In specific examples of the invention, dissociation of fimbriae into subunits is carried out using 6M guanidine hydrochloride, though other dissociating agents are also suitable.

\textit{B. pertussis} fimbriae can be purified from culture of \textit{B. pertussis} (for example, EP-A-0231083 describes purification of pertussis antigens), or can be produced by recombinant techniques, and consequently reference to a fimbria or fimbriae or fimbrial protein of \textit{B. pertussis} is to be understood as a reference to such protein whether derived by purification of natural fimbriae or by recombinant expression of DNA encoding fimbriae or fimbrial protein, and includes references to pure preparations and mixtures including serotype 2 fimbrial subunits, serotype 3 fimbrial subunits, FimX and FimD, as well as other isolated fimbrial subunits and fimbrial components, 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 \textit{B. pertussis} fimbriae, as evidenced by immunisation with such variants, derivatives or fragments resulting in induction of antibodies that are protective against challenge by \textit{B. pertussis}. 

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.

Conjugation of antigen to carrier can be achieved by conventional means. In an embodiment of the invention, the carrier is conjugated to the antigen using a C6 spacer, in which the fimbrial protein subunits are first derivatized and then added to a solution of antigen. It is also an option for the antigen first to be derivatized 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 dose of bacteria sufficient to cause disease.

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.

5 Whilst in the present application there is reference to and discussion of
conjugates of the invention, it is intended that the conjugates of the
invention can be produced by chemical means or alternatively by
recombinant means, for example by expression of a single coding sequence
that contains sequences coding for, respectively, the carrier and the antigen
of the invention. Therefore, references to conjugates herein embrace
references to equivalent polypeptides obtained by recombinant means and
also include references to polypeptides containing a carrier domain and an
antigen domain corresponding to the carrier and the antigen of the conjugate,
obtained recombinantly. Livey et al, Molecular Microbiology (1987), 1(2), pp
203-209 describe cloning and nucleotide sequence analysis of the serotype
2 fimbrial subunit gene of *B. pertussis*. Mooi et al, FEMS Microbiology
Letters 66 (1990), pp 327-332 describe the structure of the *B. pertussis*
gene coding for the sero type 3 fimbrial subunit. Pedroni et al, Molecular
Microbiology (1988), 2 (4), pp 539-543 describe the cloning of a novel pilin-
like gene from *B. pertussis* (FimX). Willems et al, Molecular Microbiology
(1993), 9 (3), pp 623-634 describe isolation of FimD from *B. pertussis*. The
contents of all of these papers are incorporated herein by reference.

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 *B. pertussis* also confer or
enhance protective immunity against *B. pertussis*, a paediatrically relevant
pathogen, and thus a conjugate comprising fimbrial protein subunits can
induce a dual immune response.

A yet further advantage is that by first dissociating the fimbriae, conjugation
of the antigen to the carrier can be carried out more easily. This has benefits
in the process for production of the conjugates, as the process is more
reliable and produces a higher percent of useable conjugate with less
wastage of materials.

In addition, the use of denaturing agents in specific embodiments of the invention confers the advantage that any residual toxin or other contamination is rendered inactive by the denaturing process. The resulting product consequently has better prospects for a vaccine for human use.

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 when 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 *B. 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, *Mycobacterium tuberculosis*, Rotavirus, Rubella virus, TBE, *Vibrio cholerae*, *Haemophilus influenzae*, *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Staphylococcus*, *B. parapertussis*, HIV, HPV, polio virus, *Brucella*, *Yersinia pestis*, *Helicobacter pylori*, *Borrelia burgdorferi*, malaria parasites and RSV, though the invention is not to be construed as limited just to this sub-group of antigens.

The antigenic conjugate of the invention can also comprise a carrier of the invention 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 fimbrial subunit are obtained or derived. An immunogenic conjugate of the invention thus optionally comprises a
fimbrial protein subunit of *B. pertussis* to which Meningococcal C polysaccharide and Hib capsular polysaccharide (polyribosylribitol phosphate, PRP) have both been conjugated. This embodiment of the invention can therefore be used to confer protective immunity against three pathogenic organisms. Multiple immunities can thus 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.

It is a feature of the present invention that conjugates of the invention can typically be of relatively low molecular weight, the carrier part of the conjugate being of low molecular weight compared to, for example, toxoided proteins. There is an advantage in this, as it easier to characterise the conjugate, to confirm that conjugate has been formed and to separate successfully formed conjugate from carrier that has not formed a conjugate with an antigen.

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 *B. pertussis* fimbriae. One type bears Fim 2 and has a molecular weight of about 22,500 daltons, and the second bears Fim 3 and has a molecular weight of about 22,000 daltons. These tend to be found on different serotypes of *B. pertussis*: Fim 2 is found on serotypes 1F2 and 1F2+3, and Fim 3 is found on serotypes 1F3 and 1F2+3. A further embodiment of the invention comprises a mixture of two immunogenic conjugates, each conjugate comprising a different pertussis fimbrial protein subunit 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) dissociated *B. pertussis* fimbriae, (ii) denatured *B. pertussis* fimbriae, and (iii) mixtures of (i) and (ii) 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 typically 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, fimbrial subunits 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 fimbrial protein.

Preferably, an amount of fimbrial subunit protein is dissolved in acidic buffer and derivatized. Antigen is prepared by dissolving it in buffer and then the antigen preparation and the fimbrial protein subunit preparation are combined and allowed to conjugate. Alternatively, the antigen is derivatized prior to combining with fimbrial subunits.

One example of coupling chemistry found particularly suitable is carbodiimide coupling, in which fimbrial protein is derivatized and combined with activated antigen. Another suitable coupling method employs reductive amination.

Native fimbriae may commonly comprise 1000 or more repeat subunits of fimbrial protein. In use of the invention, fimbriae are subjected to a dissociation agent and the first stage of dissociation typically opens up the structure of the fimbriae, splitting it into subunits, each subunit being around 22kDa in size. If the subunits are then denatured, there is a further stage during which the individual subunits lose their structure, and this loss is usually at least partially reversible. After extreme denaturation the loss of structure may become irreversible, though this extent of denaturation is not preferable. Following dissociation, optionally with subsequent denaturation, the next stage is to carry out conjugation to an antigen, and the conjugation reaction is found to be more readily carried out than conjugation of intact fimbriae to antigen, for example the conjugation chemistry requires less stringent conditions.

In use of the conjugate of the invention, protection against *B. pertussis* has
been demonstrated, confirming that the conjugate is useful both as a carrier and also for protection against pertussis. The embodiments of the invention have thus confirmed also that epitopes present on fimbriae, essential to eliciting an immune response that is protective against pertussis have been retained in the dissociated fimbrial protein.

Dissociated and denatured fimbrial protein subunits of the invention are found to have an antigenicity that is altered compared to intact fimbriae, and it is also an option in the invention to adjust the relative amount of denaturation. Where it is desirable to use a carrier that is less antigenic, for example so that repeat uses of the carrier do not stimulate an excessive immune response against the carrier the denaturation level is higher, the carrier nevertheless being sufficiently antigenic to invoke an immune response against the antigen of the conjugate. Where it is desired also to stimulate anti-pertussis immune responses the level of denaturation can be less or dissociated, but substantially non-denatured fimbrial protein subunits can be used.

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 a formulation of a conjugate of the invention in a pharmaceutically acceptable carrier and being for mucosal delivery.

By reference to mucosal delivery it is intended to include intranasal, oral, rectal and vaginal delivery. In particular, mucosal delivery can be achieved intranasally using aerosol administration of a solution of the conjugate.

In preparation of a composition for administration of a conjugate of the invention via mucosal surfaces, the size of particles that may be prepared comprising conjugates of the invention may vary, though it is generally preferred to use particles that are of a size from 0.1 to 10 microns in diameter, with particles from 0.1 to 50 microns in diameter, more particularly less than 30 microns in diameter also being suitable.

Surprisingly, it has been found that mucosal administration of the conjugate can result in production of antibodies against pertussis. This avoids the problems associated with injected vaccines. The invention also provides a method of vaccinating against pertussis by administering the conjugate mucosally, and to use of the conjugate in manufacture of a medicament for mucosal vaccination against pertussis and/or other diseases and organisms.

In an embodiment of the invention, conjugates are formulated with a particulate carrier, typically being adsorbed onto or conjugated to the outside of particles or encapsulated within particles. Polymers such as PLGA (poly(DL-lactic-co-glycolic acid)) and mineral particles may be used. Conjugates can be adsorbed onto particles of 10 microns or less in diameter. In particular, a suspension of particles of 10 microns or less in diameter is suitable. Following mucosal administration uptake of these particles may
occur via the Peyer’s patches in the intestine or via nasopharanx tissue. In
a specific embodiment of the invention, described in further detail below, a
vaccinating composition comprises a colloidal suspension of alum onto which
has been adsorbed conjugate 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 conjugate.

Alum is a very known vaccine adjuvant, and to date exclusively used by
injection. The inventors have found that when alum plus conjugate is given
mucosally a good immune response is obtained.

It is known in the art that systemic injection of vaccine gives a good serum
response, principally IgG. It has been found that mucosal 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.

It is thought that M cells in the gut and nasopharanx take up particulate
material 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 these cells and thus join the chain leading to an immune
response; though the applicant does not wish to be bound by this theory.

In specific embodiments of the invention, described below, the efficacy of
conjugates of the invention in stimulating an immune response to menCPs
in Balb/c mice has been investigated, this model system being acknowledged
as one which closely parallels the immune response in humans.

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

Fig. 1 shows serum IgG response to meningococcal serogroup C
polysaccharide in mice immunised with polysaccharide conjugated to
dissociated fimbriae according to the invention;

Fig. 2 shows serum IgG response to native fimbriae in mice immunised
with meningococcal serogroup C polysaccharide conjugated to dissociated
fimbriae according to the invention;

Fig. 3 shows serum bactericidal antibody titre against serogroup C N.
meningitidis in murine sera following immunisation with the conjugate of the invention;

Fig. 4 shows inhibition of binding to meningococcal serogroup C polysaccharide by antibody raised against the conjugate of the invention;

Fig. 5 shows protective efficacy of the conjugate of the invention against challenge by Neisseria meningitidis serogroup C strain L91543;

Fig. 6 shows protection by dissociated fimbriae of the invention against intranasal colonisation with B. pertussis; and

Fig. 7 shows anti-PRP and anti-fimbrial serum IgG responses in rabbits to conjugate vaccines of the invention.

Example 1
Preparation of Dissociated Fimbriae

Fimbriae were dissociated with the following protocol.

1. A 10mg aliquot of Bordetella pertussis fimbriae, stored at -20 degrees C, is thawed in phosphate buffer pH 7.2 and kept on ice at 4 degrees C.
2. Two volumes of ice-cold acetone are added to the thawed fimbriae and stirred for 15 - 20 minutes, generating a precipitate.
3. The precipitate is recovered by precipitation at 4000rpm for 15 - 20 minutes.
4. The acetone is allowed to evaporate from the precipitated pellet and the pellet is resuspended in 4ml 6M Guanidine-HCl, 0.125M ethanolamine, pH10.5 and left overnight at 4 degrees C, resulting in dissociated fimbriae.
5. The dissociated fimbriae are dialysed against 3 changes of 0.1M ammonium bicarbonate buffer at pH 7.2.
6. The dialysed dissociated fimbriae are freeze dried and are now ready for conjugation chemistry.

Example 2
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.

5 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 H₂O, homogenised to prepare a smooth suspension and an equal volume of 2M CaCl₂ 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.

20 Phenol extraction
1. A 90%(w/v) phenol solution is prepared by dissolving 90g phenol by adding 10ml boiling H₂O and melting in a water bath at 56°C.
2. Polysaccharide in PBS and 90% phenol are mixed 1:1 and whirlmixed 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°C.
5. The phenol layer is re-extracted with PBS and incubated for 15min at room temperature then centrifuged as above. The top aqueous layer is removed and pooled with the previous extract.
6. The aqueous extract is dialysed against 0.1M CaCl₂ overnight to
remove any remaining phenol.
7. The dialysed polysaccharide is centrifuged at 100,000g for 5h to pellet the lipoooligosaccharide.
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 is allowed to dry at 35°C and the dry weight recorded.

Example 3
10 Preparation of protein-polysaccharide conjugates using reductive amination
This method uses reductive amination as the conjugation chemistry

Reagents
50mM Hepes buffer pH8.5
15 Lysine (2mg/ml) made up in 5ml Hepes buffer
DMS (dimethyl suberimidate - 2HCl) (25mg/ml) made up in 2ml Hepes buffer immediately prior to use
PBS (made using HPLC quality water)
Dialysis tubing (12400 MW cut off)
20 Polyethylene glycol (MW 8000)
100mM sodium hydrogen carbonate pH9.0, this is made up as required for dialysis
1mM sodium periodate, prepared immediately prior to use in HPLC quality water cooled to 4°C
25 Ethylene glycol
Sodium borohydride (50mg/ml) made up in HPLC quality water immediately prior to use

Method
30
Day 1

Derivatisation of protein

35 Dialyse protein against 20mM ammonium bicarbonate buffer, pH7.0 using 3 changes of buffer and freeze dry prior to concentrate.
Dissolve 10mg protein in 18ml 50mM Hepes pH8.5.

Add 225μl of lysine solution to the protein solution in a glass bottle.

5 Prepare 2ml of a 25mg/ml solution of DMS and immediately add 1ml to the protein/lysine mixture. This is done in a glass bottle because DMS reacts with metal and plastic. Mix this solution for 1h at room temperature.

Add a further 2ml of lysine solution to stop the reaction.

10 Dialyse overnight against PBS (3 changes).

Remove 100μl for TNBS assay and SDS-PAGE.

15 **Day 2**

Concentrate the dialysed, derivatised protein to 1-2ml with polyethylene glycol (PEG). To do this the dialysed, derivative is left in the dialysis tubing and placed into a container and covered with PEG. This is left at room temperature and checked periodically until the volume has reduced to 1-2ml. This solution is stable at 4°C below pH 8.0.

Dialyse the concentrated protein derivative against 100mM sodium bicarbonate, pH9.0 overnight at 4°C (3 changes).

25 Remove 100μl for TNBS assay and SDS-PAGE.

**Day 3**

30 **Periodate oxidation of polysaccharide**

Prepare a solution of 1mM sodium periodate in HPLC quality water (cooled to 4°C).

35 Dissolve the appropriate amount of polysaccharide in 1ml of this periodate solution and mix in the dark for 30min at 4°C. The amount of polysaccharide used is based on molar ratio and will depend on the number of moles of
protein.

Add 1 drop of ethylene glycol.

Immediately add this to the derivatised protein solution and incubate at 4°C for 2h.

Freshly prepare a 50mg/ml solution of sodium borohydride in HPLC quality water and immediately add 200μl of this solution to the reaction mixture.

Incubate at 4°C overnight.

Remove 200μl for assessment on small Superdex 200 column.

**Day 4**

Dialyse the product against 3 changes of PBS (prepared in HPLC quality water). This will remove any unconjugated, hydrolysed polysaccharide.

The conjugate can be stored at 4°C until required for purification by Superdex 200 FPLC.

**Example 4**

**Preparation of protein-polysaccharide conjugates using carbodiimide mediated coupling**

This method uses Carbodiimide-mediated coupling to conjugate the protein and polysaccharide.

**Derivatisation of carrier protein**

Essentially the method used was described by Schneerson *et al.*, 1980. Briefly, adipic acid dihydrazide (ADH) derivatives of proteins were prepared by reacting protein solutions (1mg ml⁻¹) and ADH (3.45mg/mg protein) with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl (EDAC) (0.3mg/mg protein). The pH of the reaction mixture was maintained at 4.7±0.2 by careful addition of 0.1 M HCl. The reaction was allowed to proceed at room temperature for 3 h and the reaction mixtures terminated by dialysing at 3-8°C against of 5L of 20 mM ammonium bicarbonate. The protein derivatives were then freeze dried.
Activation of polysaccharide

PRP was activated with cyanogen bromide (CNBr). A solution of polysaccharide (45 mg ml⁻¹), equilibrated on ice at 4°C, was brought to 250 mM Na₂CO₃, pH 10.5. CNBr (500 mg ml⁻¹) was added to a final concentration of 0.4 mg/mg polysaccharide and the pH maintained at 10.5 for 6 min. The reaction mixture was lowered to pH 8.5 with careful addition of 1 M HCl. The derivatised DH-protein was added (based on a molar ratio of protein:polysaccharide of 1:10) to the CNBr-activated polysaccharide. The reaction mixture was mixed gently overnight at 3-8°C, after which the mixture was dialysed against 5 L of 50 mM ammonium bicarbonate, 150 mM NaCl before freeze-drying.

Example 5

Immunogenicity and Protection Data for dFim-menCPs conjugate

Sera from immunised Balb/c mice were assayed by ELISA to determine the antibody response to native fimbriae and menCPs. The ELISA data demonstrates specific anti-CPs antibodies. In mice immunised with dfim-CPs(RA) conjugate, high antibody titres were observed after a boost dose (see figure 1). The antibodies were also of high avidity. High anti-fimbrial serum IgG antibody levels were elicited by all vaccines except by menCPs, as expected (see figure 2).

After boosting, the ratio of IgG to IgM was increased with the conjugates but not with polysaccharide alone or an unconjugated mixture, indicating that the response to the conjugate is thymus-dependent (TD), in contrast with the thymus-independent (TI) response elicited by the polysaccharide and the unconjugated mixture. This observation was noted when sera were assayed against the conjugate or the polysaccharide (tables 1 and 2 respectively). The greatest increase in ratio was observed in the sera raised against the dFim-CPs (RA) conjugate sera.

Serum bactericidal antibody (SBA) was determined in the sera of immunised mice after two or three doses of conjugate. The highest bactericidal response was found in mice immunised with dFim-menCPs(RA) conjugate - see figure 3. After 3 doses of this conjugate, the serum bactericidal titre increased to 1:2048.
The ability of the antibody in sera raised against the dFim-CPs (RA) conjugate to specifically inhibit binding to menCPs was determined by inhibition ELISA. Antibodies to the conjugate specifically inhibited binding to menCPs. This was dependent upon the concentration of menCPs, binding to the polysaccharide on the ELISA plate was not completely inhibited by the conjugate because of the high titre of specific antibody in the sera - see figure 4.

From the immunogenicity and SBA data, the protective efficacy of this conjugate was assessed. 100% protection was afforded against the homologous Neisseria meningitidis serogroup C strain at both challenge doses. This was not observed in mice immunised with menCPs alone. Figure 5a shows the results using a challenge dose of $2.4 \times 10^6$ CFU and figure 5b shows the results using a challenge dose of $7.5 \times 10^6$ CFU.

The ability of denatured fimbriae to protect against intranasal colonisation with B. pertussis was determined, and the results are shown in figure 6. Unimmunised animals were highly susceptible to infection with B. pertussis, while all four groups of immunised animals were protected. Although there is variability in the levels of colonisation of lungs and trachea, it is clear that denatured fimbriae induced similar protection against respiratory colonisation to native fimbriae.

**Example 6**

**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, 10 microlitres from each well of a 96 well assay plate is applied to BHI + 1% horse serum plates.

5 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 10⁴ cfu's suspension of N.meningitidis to each well followed by 10μl of heat-inactivated baby rabbit complement. Incubate for 1h at 37 degrees C and then plate out 10μl of suspension from each well onto brain/heart infusion agar plates containing 1% horse serum. 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.

Example 7
A Method for Encapsulation of Dissociated Fimbriae and Dissociated 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 in ethyl acetate.
2) Solution of dissociated fimbrial conjugate or dissociated fimbriae (1mg/ml) in PBS.
3) Polyvinyl alcohol (PVA) solution (8% w/v in water).

Method:
1. A 10% (w/v) 50:50 low iv (60kD) PLG solution in ethyl acetate (dried over sodium carbonate) is prepared by heating the mixture to around 37 degrees C and rolling until dissolution is complete.
2. The PLG ethyl acetate solution (4ml) is homogenised at 3000 rpm using a Silverson homogeniser and the dissociated fimbrial or fimbrial-conjugate solution added. The resultant mixture is homogenised for a further 2.5 minutes.

3. An 8% (w/v) aqueous polyvinyl alcohol solution (92ml) is homogenised in a round bottom flask at around 3000 rpm the first emulsion is added and the resultant mixture homogenised for a further 2.5 minutes.

4. The emulsion is poured into doubly distilled water (100ml-1L) at 37 degrees C and stirred for at least 20 minutes.

5. The microparticles are recovered by centrifuging at 10,000rpm for 25 minutes at 25 degrees C.

6. The microparticles are washed by resuspending in doubly distilled water and centrifuging. This wash step is repeated such that a total of 5 spins are performed, to facilitate removal of the solvent and the emulsion stabilizer.

7. Following the 5th spin the pelleted microparticles are recovered as a slurry in a minimal quantity of doubly distilled water and freeze dried.

Microparticles of about 2-5μm diameter are obtained, for oral administration or incorporation into other vaccinating compositions.

Example 8
Rabbit sera antibody ELISA

Sera from immunised rabbits was assayed by ELISA to determine the antibody response to native/denatured fimbriae and PRP. The summary ELISA data in Figure 7a demonstrates anti-PRP specific antibodies. In rabbits immunised with denatured fimbriae-PRP(EDC), high antibody titres were observed after a boost dose of antigen. High anti-fimbrial specific serum IgG antibody levels were elicited by all vaccines except by PRP as expected (Figure 7b). Denatured fimbriae-PRP and denatured fimbriae elicited equally high anti-fimbrial serum IgG titres, which confirms that fimbrial subunit
structure is sufficiently conserved to maintain important epitopes. Anti-
denatured fimbriae antibodies were observed in all vaccines except PRP (data
not shown).

Higher IgG:IgM ratios were observed in the day 35 sera of rabbits immunised
with the conjugate prepared by carbodiimide coupling, indicating that the
observed boost response is T-cell dependent (table 3).

The results thus show protective immunity can be generated against both B.
pertussis and Neisseria meningitidis via a conjugate of dissociated pertussis
fimbrial protein 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 conjugate provides
protective immunity against pertussis, and is of application to production of
oral vaccines.
Table 1.
Ratio of IgG to IgM in pre-boost and post-boost murine sera tested against menCPs

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<tr>
<th>Sera</th>
<th>Immunogen</th>
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<tbody>
<tr>
<td></td>
<td>DFim-CPs (EDC)</td>
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<tr>
<td>Day 21 (pre-boost)</td>
<td>1:1</td>
</tr>
<tr>
<td>Day 35 (post boost)</td>
<td>2:1</td>
</tr>
<tr>
<td>Increase in Ratio</td>
<td>2-fold</td>
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</table>

Table 2.
Ratio of IgG to IgM in pre-boost and post-boost murine sera tested against conjugate

<table>
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<th>Sera</th>
<th>Immunogen</th>
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<tr>
<td></td>
<td>DFim-CPs (EDC)</td>
</tr>
<tr>
<td>Day 14 (pre-boost)</td>
<td>34:1</td>
</tr>
<tr>
<td>Day 21 (pre-boost)</td>
<td>182:1</td>
</tr>
<tr>
<td>Day 35 (post boost)</td>
<td>2686:1</td>
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<tr>
<td>Increase in Ratio</td>
<td>15-fold</td>
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Table 3.

Ratio of IgG:IgM titres in day 35 rabbit sera

<table>
<thead>
<tr>
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<th>DFim-PRP conjugate</th>
<th>dFim/Prp mixture</th>
<th>PRP alone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio IgG:IgM</td>
<td>33:1</td>
<td>3:1</td>
<td>2:1</td>
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CLAIMS

1. A method of preparing a conjugate of (i) an antigen, and (ii) a *B. pertussis* fimbrial protein, comprising:-

   separating *B. pertussis* fimbriae into fimbrial protein subunits; and conjugating the antigen to the fimbrial protein subunits.

2. A method according to Claim 1, comprising dissociating *B. pertussis* fimbriae so as to separate fimbriae containing many repeat fimbrial protein subunits into individual subunits.

3. A method according to claim 2 wherein the fimbriae are dissociated using guanidine-hydrochloride.

4. A method according to claim 2 or 3 further comprising denaturing the fimbrial protein subunits.

5. A method according to Claim 4 wherein the fimbriae are denatured using guanidine hydrochloride, with optional addition of a reducing agent.

6. A method according to any preceding claim, wherein conjugating the fimbrial protein subunit to the antigen comprises:-

   (a) combining a fimbrial protein subunit and an antigen in solution; and

   (b) either (i) derivatising the fimbrial protein subunit or (ii) derivatising the antigen; and

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

7. A method according to Claim 6, comprising:-
- 25 -
dialysing the resultant solution;
lyophilizing the dialysed solution; and
recovering lyophilized conjugate.

8. A method according to any of Claims 1 to 7, comprising
treating the fimbriae so as to dissociate the fimbriae into fimbrial protein subunits,
further treating the fimbriae so as to denature the dissociated fimbrial protein subunits,
conjugating the denatured, dissociated fimbrial protein subunits with antigen, and
allowing the denatured, dissociated fimbrial protein subunits to renature at least partially and to form aggregates of fimbrial protein-antigen conjugates.

9. A method according to Claim 8, wherein denaturing of the dissociated fimbrial protein subunits is carried out under harsh denaturing conditions, such as by using a high concentration of denaturing agent, and the denaturing conditions are then relaxed, such as by reducing the concentration of denaturing agent, so as to allow at least partial renaturating of the fimbrial protein subunits.

10. A method according to any preceding claim, comprising conjugating a first antigen to a fimbrial protein subunit to make a first conjugate preparation, conjugating a second antigen to a fimbrial protein subunit to make a second conjugate preparation, and admixing the first and second preparations.

11. A conjugate, comprising:-

   (a) an antigen, conjugated to
   (b) a carrier selected from (i) dissociated B. pertussis fimbriae (ii)
denatured *B. pertussis* fimbriae, and (iii) mixtures of (i) and (ii).

12. A composition comprising conjugates according to Claim 12, and comprising at least:

(a) a first antigen; and

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

13. A composition comprising a microparticle and a conjugate according to Claim 11, wherein the conjugate is inside the microparticle and the microparticle is 10 microns or less in diameter.

14. A composition according to Claim 13 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.

15. A vaccine comprising a conjugate according to Claim 11 or a composition according to any of Claims 12 to 14.

16. Use of a carrier selected from (i) dissociated *B. pertussis* fimbriae (ii) denatured *B. pertussis* fimbriae, and (iii) mixtures of (i) and (ii) in manufacture of a vaccine.

17. 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) dissociated *B. pertussis* fimbriae (ii) denatured *B. pertussis* fimbriae, and (iii) mixtures of (i) and (ii).
18. A vaccinating composition, comprising dissociated and/or denatured *B. pertussis* fimbria in a pharmaceutically acceptable carrier.

19. A vaccinating composition according to Claim 18, wherein fimbrial protein subunits are adsorbed onto particles of 10 microns or less in diameter.

20. An oral vaccinating composition according to Claim 18 or 19 comprising a suspension of mineral particles.

21. An oral vaccinating composition according to any of Claims 18 to 20 comprising alum particles.

22. An oral vaccinating composition according to any of Claims 18 to 21, further comprising a component to neutralise gut acid.

23. An oral vaccinating composition according to any of Claims 18 to 22, comprising a conjugate according to any of Claims 11 to 12.

24. Dissociated *B. pertussis* fimbriae.

25. Denatured and dissociated *B. pertussis* fimbriae.

26. Use of dissociated *B. pertussis* fimbriae in manufacture of a vaccine against meningococcal disease.

27. Use of dissociated *B. pertussis* fimbriae in manufacture of a vaccine against Hib disease.
FIG. 1 Serum IgG response to menCPs in mice

- Pre
- D14
- D21
- D35
- D35 avidity
FIG. 2 Serum IgG response to native fimbriae in murine sera

PRE  D14  D21  D35

500000  400000  300000  200000  100000  0

Titre

dFim
dFim/CPS mix
dFim-CPS(EDC)
dFim-CPS(RA)
FIG. 3 Serum bactericidal antibody titre in murine sera comparing two different conjugates

2500  2000  1500  1000  500  0
SBA Titre

CAMR conjugate
CPs
dFlm
Vaccine
FIG. 4 Inhibition ELISA of D35 murine sera after 3 doses of vaccine

Absorbance at 450nm, % inhibition vs. Conc. menCPs (ug/ml)
5/8

**FIG. 5** Protective efficacy of dFim-menCPs(RA) conjugate

a. Challenge dose: $2.4 \times 10^6$ cfu

b. Challenge dose: $7.5 \times 10^8$ cfu
FIG. 7a Anti-PRP serum IgG responses in rabbits to conjugate vaccines
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/385 A61K39/10 C07K17/00

According to International Patent Classification (IPC), or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, EPO-Internal, PAJ, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X Further documents are listed in the continuation of box C.  X Patient family members are listed in annex.

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Date of the actual completion of the international search 13 November 2001

Date of mailing of the international search report 23/11/2001

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Authorized officer Montrone, M

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<td>WO 98 58668 A (FARRAR GRAHAM HENRY ;MICROBIOLOGICAL RES AUTHORITY (GB); JONES DAV) 30 December 1998 (1998-12-30) cited in the application abstract page 1, line 1-4 page 2, paragraph 4 page 3, paragraphs 2,4 page 4, paragraph 5 -page 5, paragraph 1 page 6, paragraph 2 -page 9, paragraph 4 page 14, paragraph 3</td>
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<td>JONES D H ET AL: &quot;Protection of mice from Bordetella pertussis respiratory infection using microencapsulated pertussis fimbriae&quot; VACCINE, BUTTERWORTH SCIENTIFIC. GUILDFORD, GB, vol. 13, no. 7, 1995, pages 675-681, XP004057593 ISSN: 0264-410X abstract page 675, column 1, paragraph 1 -column 2, paragraph 2 page 677, column 2, paragraph 2; figure 2 page 678; figure 3</td>
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<td>CROWLEY-LUKE ANNETTE ET AL: &quot;Formulation and characterisation of Bordetella pertussis fimbriae as novel carrier proteins for Hib conjugate vaccines.&quot; VACCINE, vol. 19, no. 25-26, 2001, pages 3399-3407, XP004238935 ISSN: 0264-410X abstract page 3400, column 1, paragraphs 1,3,4 page 3402, column 2, paragraphs 3,4 page 3405, column 2, paragraph 2 -page 3406, column 1, paragraph 4</td>
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