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(54) **ANTIGENE OMP26 DE LA BACTERIE HAEMOPHILUS INFLUENZAE**

(54) **OMP26 ANTIGEN FROM HAEMOPHILUS INFLUENZAE**

(57) L'invention concerne une nouvelle protéine antigénique dérivée de la membrane externe de la bactérie H.influenzae. L'invention concerne également des séquences D'ADN codant une telle protéine, ainsi que des vaccins comprenant ladite protéine et des procédés permettant d'immuniser un sujet contre l'infection par H.influenzae. Des procédés de prévention ou de traitement des infections des voies respiratoires ou de l'otite moyenne, des procédés de détection de la bactérie H.influenzae, et des nécessaires utilisés pour mettre en oeuvre de tels procédés sont également décrits.

(57) A novel antigenic protein derived from the outer membrane of H.influenzae is provided. DNA sequences encoding such a protein are also provided as are vaccines comprising the protein and methods of immunising a subject against H.influenzae infection. The invention also includes methods for the prophylaxis or treatment of respiratory tract infections or otitis media, as well as methods for the detection of H.influenzae, and kits for use in such methods.

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(54) Title: OMP26 ANTIGEN FROM HAEMOPHILUS INFLUENZAE

(57) Abstract

A novel antigenic protein derived from the outer membrane of *H.influenzae* is provided. DNA sequences encoding such a protein are also provided as are vaccines comprising the protein and methods of immunising a subject against *H.influenzae* infection. The invention also includes methods for the prophylaxis or treatment of respiratory tract infections or otitis media, as well as methods for the detection of *H.influenzae*, and kits for use in such methods.

OMP26 ANTIGEN FROM HAEMOPHILUS INFLUENZAE

The present invention relates to a novel antigen of *Haemophilus influenzae*, vaccines comprising it and its use in therapy and diagnosis.

H. influenzae is a Gram-negative aerobic heterotrophic bacteria with the form of rods (Krieg and Holt (ed), *Bergey's Manual of Systemic Bacteriology*, pp 563 (1984). It is a pathogen in acute respiratory infections and is also found in patients with chronic bronchitis and otitis media.

We have now identified, and purified, a unique 26 kDa outer membrane protein (called OMP26) from NTHI, and have surprisingly found that this protein can, when used as an immunogen, induce protective immune responses against infection with homologous and heterologous strains of NTHi. This protein has a molecular mass on SDS-PAGE similar to P5, but has been found to be distinctly different from the protein.

The outer membrane protein P5 is one of two lower molecular mass bands on SDS-PAGE gels used to subtype *H.influenzae* strains, and has an apparent molecular mass of 25-27 kDa. the P5 protein is heat-modifiable, demonstrating an apparent mass of 35 kDa after heating for 30 min at 100°C in the presence of β-mercaptoethanol. Recently, another protein expressed by NTHi, called a fimbrin protein, has been characterised and shown to have similar molecular mass properties, heat modifiability and a 92% sequence homology to the previously described P5. the protein, OMP26, does not demonstrate either sequence homology or heat-modifiable characteristics as defined for either P5 or the fimbrin protein.

Thus, in a first aspect, the present invention provides a protein having a molecular weight of 26 kDa, as determined by SDS-PAGE, which protein is an outer membrane protein of *H. influenzae*. This protein is designated OMP26.

In particular, the protein of the invention has the amino acid sequence shown in figure 1, or one substantially homologous thereto. In a separate embodiment, the protein of the invention has the amino acid sequence shown in figure 1 commencing from amino acid no. 24, or one substantially homologous thereto. The first 23 amino acids constitute a "signal" sequence and it will be appreciated that a protein minus this sequence will be equally apllicable. The protein of the invention is an immunogen and is thus capable of inducing an immune response which will protect against infection with *H. influenzae*.

In the context of the present invent proteins which are "substantially homologous" to OMP26 may be 40%, 50%, 60%, 70%, 80%, 90%, 95% or even 99% homologous. Preferably, the protein will be at least 70% homologous, more preferably 80% homologous, even more preferably 90% homologous and most preferably 95% homologous. The skilled man will appreciate that the percentage degree of homology is one factor only. What is important is that the protein retains its antigenic effect. Thus, it is reasonable to have a protein having a relatively low degree of homology, for instance 40%, while retaining the antigenic activity discussed herein.

In addition, it is known in the art that "conservative" or indeed "semi-conservative" changes can be made to the amino acid sequence of a protein which will not alter its fundamental activity. For example, amino acids such as glycine, valine, leucine and isoleucine, which all have aliphatic side chains, may often be substituted for each other without substantially altering the biological activity of the protein. Similarly, amino acids such as phenylalanine, tyrosine and tryptophan, which all have aromatic side chains, may be substituted for each other. Such proteins which retain the antigenic effect described herein are within the scope of the present invention.

It is also possible that antigenic parts or regions of OMP26 can be employed to induce the protective effect against *H.influenzae*. Such antigenic parts or regions are also within the scope of the present invention.

In a second aspect, the present invention provides a nucleic acid sequence, preferably DNA, which codes for a protein of the invention, variants thereof as described above or indeed antigenic parts or regions. In particular, the invention provides a DNA sequence as shown in figure 1 which codes for OMP26. The skilled man will appreciate that due to the degeneracy of the genetic code it is possible to make conservative changes to the DNA sequence which will not result in changes to the amino acid sequence of the protein. Thus, such DNA sequences are also within the scope of the present invention. Suitably, nucleic acid of the invention can form part of a vector such as a plasmid.

As discussed herein, the proteins of the invention stimulate an immune response against *H.influenzae* and thus, in a third aspect, the present invention provides a vaccine formulation comprising a protein of the invention, as defined herein, optionally together with one or more carriers and/or adjuvants.

In a fourth aspect, the invention provides the use of the protein of the invention, as defined herein, in the preparation of a vaccine against *H.influenzae*.

The vaccine composition of the invention can be used to immunise a subject against *H. influenzae* infection. Therefore, the invention provides, in a fifth aspect, a method of immunising a subject against infection by *H. influenzae*, which comprises administering to the subject a vaccine composition of the invention. The vaccine compositions of the invention can be used to produce both systemic immunity and/or mucosal immunity.

In a sixth aspect, the present invention provides a method for the prophylaxis or treatment of respiratory tract infections or otitis media which comprises the step of administering to a subject a vaccine composition of the invention.

In other aspects the invention provides:

(a) The use of a protein of the invention, as defined herein, in the diagnosis of *H. influenzae* infection;and

(b) A kit for use in the diagnosis of *H. influenzae* infection comprising a protein of the invention, as defined herein.

Preferred features of each aspect of the invention are equally preferred for each other aspect *mutatis mutandis*.

The invention will now be described with reference to the following examples, which should not be construed as in any way limiting the invention.

The examples refer to the figures in which:

FIGURE 1: shows the DNA sequence coding for OMP26 and the amino acid sequence derived from that DNA sequence;

FIGURE 2: shows SDS-PAGE analysis of OMP26 and two other proteins with higher molecular mass;

FIGURE 3: shows the N-terminal sequence of the first 25 amino acids residues of OMP26 (a) and also a comparison of this with proteins from *P. multocida* and *Y. pseudotuberculosis*;

FIGURE 4: shows NTHI-I bacteria recovered in bronchial washings 4 hr post-challenge with live bacteria;

FIGURE 5: shows OMP26-specific levels of IgG subclasses in serum of rats immunised with OMP26;

FIGURE 6: shows an Immunoblot for the detection of OMP26-specific antibody in serum; and

FIGURE 7: shows Antigen-specific proliferation of lymphocytes isolated from the MLN of OMP26 immunised

and non-immune rats.

In the following example, the data have been expressed as the means +/- standard errors of the means. The pulmonary clearance data, total numbers of phagocytic cells, and differential cell count data were compared for statistical significance between groups by one-way analysis of variance, followed by Tuckey's test for multiple-comparison analysis (Macintosh Systat). Antibody data was assessed for between group significance by an unpaired t-test, and lymphocyte proliferation data assessed by a fully factorial analysis of variance (Macintosh Systat). Linear correlation between two variables was determined using the Pearson correlation coefficient (Macintosh Systat).

EXAMPLE 1

(i) Protein purification.

A 26 kDa protein (OMP26) was purified from strain NTHI-I by preparative electrophoresis. Bacteria from overnight culture of 100 agar plates were harvested by scraping the plates, and washed twice by centrifugation at 10,000 x g for 10 min at 4°C. A crude outer membrane preparation was obtained by extraction of the outer membrane component with buffered Zwittergent 3-14 detergent and ethanol precipitation. The outer membrane extract was lyophilised, resuspended in a minimal amount of distilled water and further dissolved in 4 times the volume of sodium dodecyl sulfate (SDS) reducing buffer (62.5 mM Tris, [pH 6.8], 10% [vol/vol] glycerol, 2% [wt/vol] SDS, 5% [vol/vol] β-mercaptoethanol, 1.2 x 10^{-3}% [wt/vol] bromopheno blue). The SDS-preparation was incubated at 37°C for at least 30 min prior to being located onto the stacking gel of the electrophoresis column. OMP26 was purified using preparative polyacrylamide electrophoresis (PAGE). Preparative SDS-PAGE to purify OMP26 was performed using the Bio-Rad Model 491 Prep Cell using a 60 ml 14 T-1.42% C acrylamide/BIS (N,N'-methylene-bis acrylamide) separating gel with a 10 ml 4% T-0.36% C acrylamide/BIS stacking gel polymerised in a 37 mm (internal diameter [i.d.]) column. Fractions eluted from the column were concentrated by lyophilisation, and analysed for protein content by analytical SDS-PAGE. OMP26, isolated using these conditions, contained SDS which was subsequently removed by potassium phosphate and precipitation. Fractions containing OMP26 were pooled and dialysed prior to determination of protein concentration.

Analytical identification of the protein was performed by analytical SDS-PAGE using either gradient 10-15% or homogenous 12.5% acrylamide gels, and silver stained. Protein concentration was determined using the Pierce micro BCA assay. The presence of LOS was assessed by both silver staining of SDS-PAGE mini-gels and assaying with the E-TOXATE *Limulus* lysate test.

RESULTS

OMP26 was successfully separated from a group of three proteins with molecular masses between 26 and 30 kDa. Figure 2 shows the position of this protein in relation to the other two, and the silver stained gel indicates the high degree of purity of the resulting preparation. Assessment of the heat-modifiable characteristic of this protein was performed by heating the protein sample at

100°C for 30 min in the presence of β-mercaptoethanol. It was found that after 30 min the boiled protein sample still migrated with the same molecular mass (Figure 2). To determine whether one of the other neighbouring protein bands may have been the heat-modifiable P5, all three proteins within this mass range were boiled for 30 min in the presence of β-mercaptomethanol, with none of the proteins demonstrating heat-modifiable characteristics (Figure 2). Assessment of the protein for the presence of LOS contamination was performed using the E-TOXATE assay kit and found to be less than 0.6 μg endotoxin per mg protein.

(ii) Preparation of OMP26 for N-terminal amino acid sequencing.

OMP26 was prepared for N-terminal amino acid sequence analysis by transferring the protein band from an SDS-PAGE gel to PVDF membrane. This protein sample was sent to Cortecs Diagnostic, Techbase 1, Newtech Square, Deeside, Clwyd, United Kingdom, for sequence analysis.

Amino acid sequence identification.

An N-terminal amino acid sequence was obtained from the protein band transferred to PVDF. Amino acid sequence analysis for the first twenty-five peptides is shown in Figure 3. The sequence analysis indicates no sequence homology with the N-terminal sequence of either Hib P5 or the fimbrin protein. The N-terminal amino acid sequence did show a 56% homology with a 21.4 kDa protein from *Pasteurella multocida* and a 44% sequence homology with a 19 kDa outer membrane protein from *Yersinia pseudotuberculosis*.

(iii) Immunisation and bacterial challenge.
Specific pathogen-free male rats received an intra-Peyer's patch (IPP) immunisation on day 1, an intra-tracheal (IT) boost on day 14, and the final live bacterial challenge on day 21. The animals were sedated with halothane to facilitate intravenous anaesthesia with chloral hydrate via the tail vein. The small intestine was exposed through a mid-line abdominal incision and the antigen injected subserosal to each Peyer's patch using a 27-guage needle. The immunisation protein (OMP26) was prepared by emulsification of 200 or 800 μg of protein per ml in a 1:1 ratio of Incomplete Freund's adjuvant (IFA) and phosphate buffered saline (PBS), and a total inoculum of 10 or 40 μg protein respectively was administered to each animal. Two control groups of rats consisted of (i) a mixture of untreated and sham-immunised groups (immunised with IFA and PBS), and (ii) a positive group immunised with killed bacteria of the homologous NTHI strain. Rats received an IT boost on day 14, post-IPP immunisation. OMP26-immunised rats received an IT boost of 10 μg of OMP26. The non-immune group received 50 μl PBS, while the killed bacteria-immunised group received 50 μl of killed bacteria (bacteria count of 10^{10} per ml). Animals were challenged for 4 hours with live bacteria (bacteria count 5 x 10^8) 21 days after the first immunisation. A heterologous strain, NTHI-II, was also used for bacterial challenge. Bacteria were grown overnight at 37°C in 5% CO_2 on brain heart infusion agar plates supplemented with 50 ml defibrinated horse blood per litre of agar, recovered, washed and resuspended in PBS to the required concentration. Bacteria were introduced into the lungs via an intra-tracheal cannula and 4 hours later the rats were euthanised. Blood was collected and aliquots of serum stored at -20°C for

antibody analysis. Lungs were lavaged by flushing with 5 x 2 ml of PBS, and the pooled lavage (BAL) assessed for bacteria numbers. Following lung lavage, the lungs were removed, homogenised and assessed for numbers of bacteria. Cytospin slides were prepared for determination of differential cell counts in the lung lavage. Total cell numbers present in the lung lavage were calculated by staining with methylene blue and counting using a haemocytometer.

RESULTS

Rats immunised with OMP26 and challenged with live bacteria of the NTHI-I homologous strain on day 21 showed significant bacterial clearance ($P<0.005$). Rats immunised and boosted with 10 μg OMP26 had 92% fewer bacteria in the lung than the non-immune group after 4 h, whereas rats receiving 40 μg OMP26 in the IPP immunisation, and boosted with 10 μg OMP26, had 96% fewer bacteria and were equivalent to the 95% clearance observed for killed bacteria immunised rats (Figure 4).

Rats immunised with OMP26 were also challenged with live bacteria from a heterologous nontypeable strain, NTHI-II. The results in Table 1 show that OMP26 immunisation also significantly ($P<0.005$) cleared bacteria in a pulmonary challenge by a different strain. The immunised group had 93% fewer bacteria than the non-immune group in the BAL after 4h, demonstrating a rate of bacterial clearance comparable to that for homologous challenge. OMP26 immunisation also reduced the numbers of bacteria present in the lung homogenates of the immunised groups compared with the non-immune groups. The lung homogenates from the rats challenged with NTHI-II (heterologous strain) had significantly less bacteria than the non-immune lungs. However, the magnitude of the difference was 80% in the lungs, with 93% clearance in the BAL as compared with 89% clearance in the lungs, and 87% clearance in the BAL for groups challenged with NTHI-I (the homologous strain). The percentage clearance in this experiment for NTHI-I differed from previous experiments due to the live bacterial inoculum containing considerably more bacteria than usual (usual inoculum ranged between 0.6 and 1.4 x 10^{10} CFU per ml).

Greater numbers of phagocytic cells were present in the BAL of OMP26-immunised animals, and correlated with the enhanced bacterial clearance in these animals (Table 2). The increase in cell recruitment in immunised groups was the same for both homologous and non-homologous bacterial challenge. However, at 4h post-challenge, the differential cell counts were not significantly different between immune and non-immune groups (Table 2), with both groups showing similar ratios of PMNs to macrophage:-

TABLE 1: Pulmonary clearance following OMP-26 immunisation with challenge by homologous and heterologous non-typeable *H. influenzae* bacteria.

Rat group[c]		*H. influenzae* recovered 4 h post-challenge ($\log_{10}$CFU)[a] (% clearance) — Challenge strain of *H. influenzae*[b]: NTHI-I (homologous)	NTHI-II (heterologous)
Non-immune	BAL	7.01 ± 0.11	6.28 ± 0.07
	Lung	7.76 ± 0.05	6.69 ± 0.05
OMP26-immunised	BAL	6.15 ± 0.15 (87%)*	5.15 ± 0.16 (93%)*
	Lung	6.81 ± 0.17 (89%)*	6.01 ± 0.19 (80%)*

a Values represent mean ± SEM in either the BAL or lung homogeneic for rats challenged for 4 h with live bacteria from either NTHI-I or NTHI-II strains on day 21 post-PP immunisation with 10 μg OMP26. All rats received an IT-boost (10 μg) on day 14. Non-immune rats were a combination of sham-treated and untreated animals.

b Concentration of live bacterial in challenge inoculum (as determined by plating of serial dilutions) was 10.38 ($\log_{10}$) CFU per ml for NTHI-I and 10.17 ($\log_{10}$) CFU per ml for NTHI-II.

c n = 4 per group for challenge with NTHI-I
n = 4 for non-immune and n=6 for OMP26-immunised groups for challenge with NTHI-II

* $P<0.001$ compared to non-immune group.

TABLE 2: **Phagocytic cell counts in the BAL 4 h post-pulmonary challenge with five non-typeable *H. influenzae*.**

Rat group	Challenged with NTHI-I (homologous)	Challenged with NTHI-II (heterologous)	Differential cell count from cytospin slide preparation of BAL (%)		
	Total number of cells in BAL (x 10^6)		PMNs	Macrophages	Others
Non-immune	15.9 ± 1.1	17.5 ± 3.0	95.8	2.2	2
OMP26-immunised	26.1 ± 1.1[n]	28.2 ± 1.6[n]	95	2.7	2.1

[n] $P<0.001$ compared to non-immune group.

(iv) **OMPS26-specific ELISA.**

Polysorb microtiter wells were coated with purified OMP26 at a concentration of 1 μg per ml for assay of IgG, IgG_{2a}, IgA, and IgM; and 10 μg per ml for IgG_1, IgG_{2b}, OgG_{2c} and IgE. The plates were washed five times in PBS containing 0.05% Tween 20 between incubation steps. The wells were blocked with 5% skim milk in PBS-0.05% Tween 20 for 60 min. Wells were incubated for 90 min with serum (1/25 to 1/3200), or BAL (1/2 to 1/16) samples were serially diluted in blocking buffer for analysis. Conjugated immunoglobulin used were goat anti-rat IgG (1/2000), IgA (1/1000), and IgM (1/4000) (Fc specific); mouse anti-rat IgG_1 (1/500), IgG_{2a} (1/1000), IgG_{2b} (1/500), and IgG_{2c} (1/500), and wells were incubated with conjugated immunoglobulin for 90 min. The plates were then developed.

RESULTS

Antibody specific to OPM26 was measured in the serum and BAL samples of rats immunised with OMP26, as well as from rats that had been immunised with killed bacteria from four different strains of *H. influenzae*. High OMP26-specific antibody titers for IgG, IgA and IgM were found in the serum, and IgG and IgA in the BAL of rats immunised with OMP26, with the highest levels observed for the group receiving the higher immunisation dosage of 40 μg (Table 3). Detectable levels of OMP26-specific IgG, IgA and IgM in the serum, and IgG and IgA in the BAL, were also found in rats that had been immunised with different strains of *H. influenzae* (Table 3), although the levels observed for these groups were significantly less than those in the OMP26-immunised groups. IgE

ELISAs were also carried out on serum from OMP26 rat groups, however, levels of OMP26-specific IgE could not be detected (data not shown).

Measurement of OMP26-specific IgG subclasses found that OMP26-specific IgG_1 was only detectable following the 40 µg immunisation, whereas significant levels of IgG_{2a} and IgG_{2b} subclasses were found for both 10 µg and 40 µg OMP26 immunisation groups (Figure 5). Levels of both IgG_{2a} and IgG_{2b} increased significantly ($P<0.05$) with the increase in concentration of OMP26 from 10 µg to 40 µg in the IPP inoculum. IgG_{2c} was also measured, however, significant levels of OMP26-specific antibody from this subclass could not be detected (data not shown).

TABLE 3: **Comparison of OMP26-specific antibodies in serum and bronchoalveolar lavage following immunsation with either OMP26 or killed bacteria from *H. influenzae*.**

		Antibody to OMP26 (ELISA titer[a])					
		Serum			Bronchoalveolar Lavage		
Group[b]	n	IgG	IgA	IgM	IgG	IgA	IgM
Non-immune	7	n.d.[d]	n.d.	10.5 ± 1.6	n.d.	n.d.	n.d.
10 μg OMP26	5	1209 ± 255*	936 ± 161*	30.5 ± 4*	4.3 ± 1.0*	6.1 ± 1.9*	n.d.
40 μg OMP26	5	2806 ± 405*	2440 ± 410*	142.5 ± 30.6*	8.3 ± 1.9*	29.1 ± 7.9*	n.d.
NTHI-I[c]	4	58 ± 17*	37 ± 4*	39.1 ± 0.8*	0.5 ± 0.1	1.2 ± 0.5	n.d.
NTHI-II[c]	4	22 ± 4*	26 ± 5*	39.3 ± 5.0*	0.6 ± 0.1	0.4 ± 0.1	n.d.
HI-CD[c]	4	18 ± 1*	20 ± 1*	42.2 ± 3.1*	0.6 ± 0.1	0.8 ± 0.3	n.d.
Hib-II[c]	4	27 ± 4*	38 ± 3*	34.4 ± 2.3*	0.6 ± 0.1	1.6 ± 0.4	n.d.

a Antibody titers calculated as described in the materials and methods.

b Rats were immunised via PP on day 0, received an IT boost on day 14 and were challenged with live bacteria on day 21. Serum and BAL samples were prepared as described in the materials and methods.

c Rats were immunised with killed bacteria from the strain of *H. influenzae* indicated and had received a live challenge with bacteria from the homologous strain.

d n.d. indicates that P5-specific antibody could not be deleted at the lowest sample dilution.

* $P<0.05$ compared to non-immune group.

(v) Immunoblot.
Proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose (0.2 μm pore size). Rat serum from OMP26, NTHI-I, NTHI-II, as well as strains HI-CD, and Hib-II-immunised groups was diluted 10-fold in TTBS-5% (w/v) skim milk powder and was used as the primary antibody. A 500-fold dilution of horseradish peroxidase conjugated goat anti-rat IgG (Fc specific) in TTBS-5% skim milk was used as the second antibody.

RESULTS

Immunoblot analysis of recognition of OMP26 by antibodies present in the serum of non-immune, OMP26-immunised and *H. influenzae*- (four strains) immunised rats has shown recognition of this protein by antibodies present in the serum from each of the immunised groups, but not the non-immune group (Figure 6). This demonstrates the cross-reactivity of antibody-responses generated by immunisation with the *H. influenzae* strains used in this study with the OMP26 purified from the NTHI-I strain.

(vi) Antigen-specific lymphocyte assay.
Lymphocytes obtained from the mesenteric lymph nodes (MLN) were cultured at a concentration of 10^6 cells per ml. The antigen (OMP26) was suspended in culture medium in a 10-fold dilution series and sterile filtered. The cell suspension and antigen were added in triplicate to flat-bottomed multiwell plates to give a final volume of 0.2 ml per well. Lymphocyte proliferation was estimated by [^{3}H]thymidine incorporation for the last 8 h of a 4-day culture. Results were calculated by subtraction of background from the geometric means of triplicate wells, then the geometric mean +/- standard error of the entire

treatment group.

RESULTS

Lymphocytes from the MLN of OMP26-immunised and non-immunised rats were assessed for antigen-specific proliferative responses. Cells from the OMP26-immunised group responded significantly to OMP26 in culture *in vitro*, whereas cells from the non-immunised rats did not show significant proliferation (Figure 7A). The lymphocytes from rats immunised with OMP26 were also cultured with OMP extracts from four *H. influenzae* strains to assess cross-reactive responses. Significant proliferative responses were found in the lymphocytes from the OMP26-immunised group for the OMP extracts from strains NTHI-I, NTHI-II and HI-CD, but no significant proliferation was observed for the extract from the Hib-II strain (Figures 7B-E).

EXAMPLE 2: CLONING AND SEQUENCING OF OMP26

DNA was extracted from NTHi. The region of DNA encoding OMP26 was identified and amplified by standard PCR methods using primers designed to recognise the gene (synthesised at Biomolecular Resource Facility, John Curtin School of Medical Research, Canberra, ACT, Australia). After analysis to determine successful recognition of the correct product, the PCR DNA product was extracted.

Two plasmids were prepared. One DNA product contained the region encoding both the signal peptide and the mature OMP26 product and the second encoded the final mature OMP26 (without the leader signal peptide). The PCR DNA products were digested with the endonucleases Hindiii for

OMP26 plus signal peptide and NspBII plus HindIII for mature OMP26. The digested DNA was recovered and ligated at the SmaI and HindIII sites into the plasmids pQE30 or pQE31 (Giagen GmbH, Hilden, Germany) for OMP26 plus signal peptide or OMP26 mature protein respectively. The plasmids were then purified and precipitated. sequencing was performed by the dye deoxy-terminator procedure at the biomolecular resource Facility, John Curtin School of Medical Research, Canberra, Australia.

RESULTS

The sequence shown in figure 1 represents both the mature OMP26 plus the signal peptide. The signal peptide sequence encompasses the first twenty-three amino acids. The final product expressed by NTHi on the outer membrane commences at amino acid twenty-four.

CLAIMS:

1. A protein having a molecular weight of 26 kDa, as determined by SDS-PAGE, which protein is an outer membrane protein of *H. influenzae*.

2. A protein as claimed in claim 1 which has the amino acid sequence shown in figure 1, or a sequence substantially homologous thereto.

3. A protein as claimed in claim 1 which has an amino acid sequence as shown in figure 1 commencing with amino acid twenty-four as the N-terminal amino acid, or a sequence substantially homologous thereto..

4. A protein as claimed in claim 2 or claim 3 which has an amino acid sequence which is at least 70% homologous to the amino acid sequence shown in figure 1.

5. A protein or peptide which is an antigenic part or region of a protein as defined in any one of claims 1 to 4.

6. A protein which comprises an antigenic part or region of a protein as defined in any one of claims 1 to 4.

7. A protein as claimed in claim 6 which is a fusion protein.

8. A nucleic acid sequence which codes for a protein as defined in any one of claims 1 to 7.

9. A nucleic acid sequence as claimed im claim 8 which

is a DNA sequence.

10. A DNA sequence as claimed in claim 9 which is that shown in figure 1.

11. A vaccine formulation comprising a protein as defined in any one of claims 1 to 7, optionally together with one or more carriers and/or adjuvants.

12. The use of a protein as defined in any one of claims 1 to 7 in the preparation of a vaccine against *H.influenzae*.

13. A method of immunising a subject against infection by *H. influenzae*, which comprises administering to the subject a vaccine as defined in claim 11.

14. A method for the prophylaxis or treatment of respiratory tract infections or otitis media which comprises the step of administering to a subject a vaccine as defined in claim 11.

15. The use of a protein as defined in any one of claims 1 to 7 in the diagnosis of *H. influenzae* infection.

16. A kit for use in the diagnosis of *H. influenzae* infection comprising a protein as defined in any one of claims 1 to 7.

```
        10         20         30         40         50
1234567890 1234567890 1234567890 1234567890 1234567890
ATGAAAAACA TCGCAAAAGT AACCGCACTT GCTTTAGGTA TTGCACTTGC    50
M  K  N  I   A  K  V   T  A  L   A  L  G  I   A  L  A

TTCAGGCTAT GCTTCCGCTG AAGAAAAAAT TGCTTTCATT AATGCAGGTT   100
 S  G  Y   A  S  A  E   E  K  I   A  F  I   N  A  G  Y

ATATTTTTCA ACATCACCCA GATCGCCAAG CGGTAGCAGA TAAACTTGAT   150
 I  F  O   H  H  P   D  R  Q  A   V  A  D   K  L  D

GCTGAATTTA AACCTGTAGC TGAGAAATTA GCAGCAAGCA AAAAAGAAGT   200
A  E  F  K   P  V  A   E  K  L   A  A  S  K   K  E  V

TGATGATAAA ATTGCTGCTG CTCGTAAAAA AGTAGAAGCA AAAGTTGCGG   250
 D  D  K   I  A  A  A   R  K  K   V  E  A   K  V  A  A

CTTTAGAAAA AGATGCACCT CGCTTACGTC AAGCTGATAT TCAAAAACGC   300
 L  E  K   D  A  P   R  L  R  Q   A  D  I   Q  K  R

CAAGAAGAAA TTAATAAATT AGGTGCGGCT GAAGATGCTG AATTACAAAA   350
Q  E  E  I   N  K  L   G  A  A   E  D  A  E   L  Q  K

ATTAATGCAA GAACAAGATA AAAAAGTTCA AGAATTCCAA GCTCAAAATG   400
 L  M  Q   E  Q  D  K   K  V  Q   E  F  Q   A  Q  N  E

AAAAACGTCA AGCTGAAGAA CGTGGTAAAT TATTAGATAG CATTCAAACT   450
 K  R  Q   A  E  E   R  G  K  L   L  D  S   I  Q  T

GCGACAAATA ATTTAGCAAG AGCAAAAGGT TATACTTATG TGCTTGATGC   500
A  T  N  N   L  A  R   A  K  G   Y  T  Y  V   L  D  A

AAATTCAGTT GTATTTGCGG TAGAGGGTAA AGATATTACT GAAGAAGTAT   550
 N  S  V   V  F  A  V   E  G  K   D  I  T   E  E  V  L

TAAAATCTAT CCCTGCTTCT GAAAAAGCAC AAGAGAAAAA ATAATAGGTT   600
 K  S  I   P  A  S   E  K  A  Q   E  K  K   X  X  V

C                                                        601
```

FIG. 1

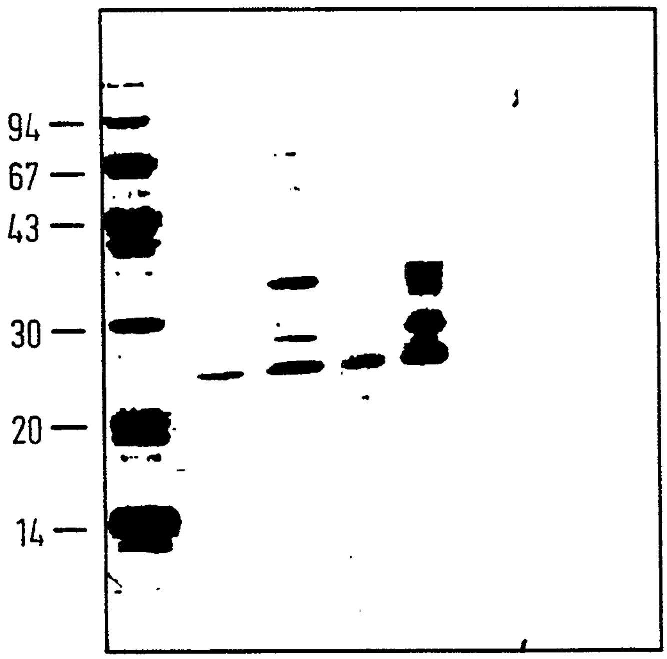

FIG. 2

A.

1	2	3	4	5	6	7	8	9	10	11	12	13
Glu	Glu	Lys	Ile	Ala	Phe	Ile	Asn	Ala	Gly	Tyr	Ile	Phe
E	E	K	I	A	F	I	N	A	G	Y	I	F

14	15	16	17	18	19	20	21	22	23	24	25
Cln	His	His	Pro	Asp	Arg	-	Ala	Val	-	-	Lys?
Q	H	H	P	D	R		A	V			K

B.

OMP26	E E K I A F I N A G Y I F Q H H P D R - A V - - K
P. muisocida skp protein	A M A T E N I A F I S G D Y L F Q N H P Q R K M V A E K L
Y. pseudotuberculosis OMPH	A G K I A I V N V S S I F Q Q L P A R E A V A K Q L

FIG. 3

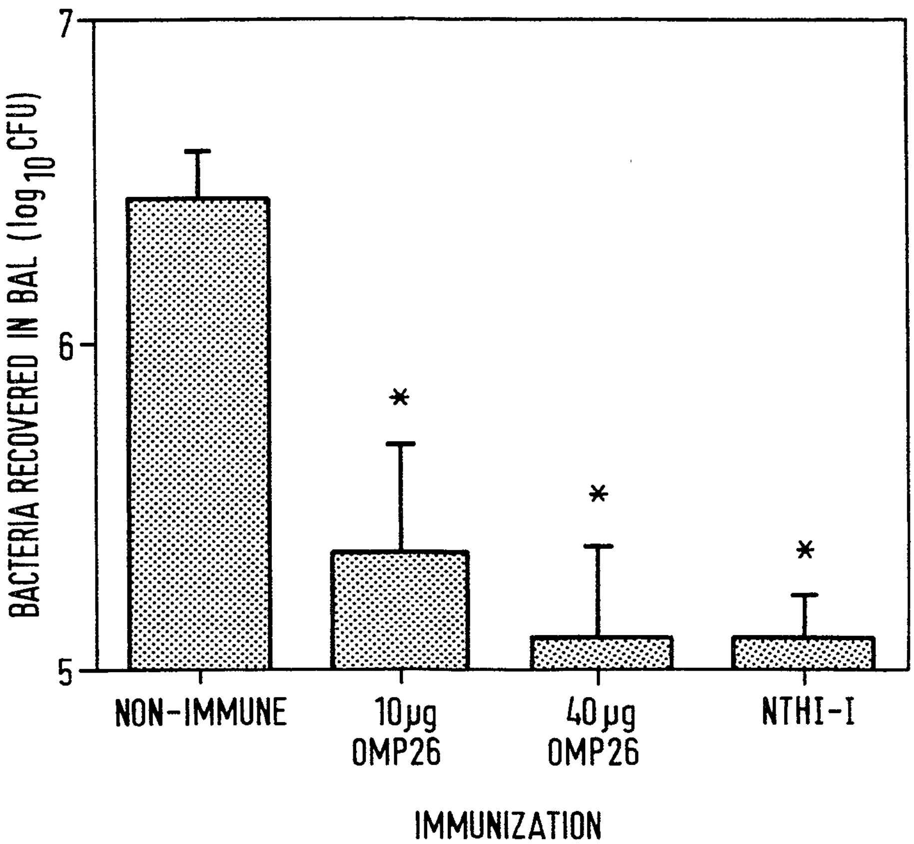
7
6
5
BACTERIA RECOVERED IN BAL (log_{10}CFU)
*
*
*
NON-IMMUNE
10µg OMP26
40µg OMP26
NTHI-I
IMMUNIZATION

FIG. 4

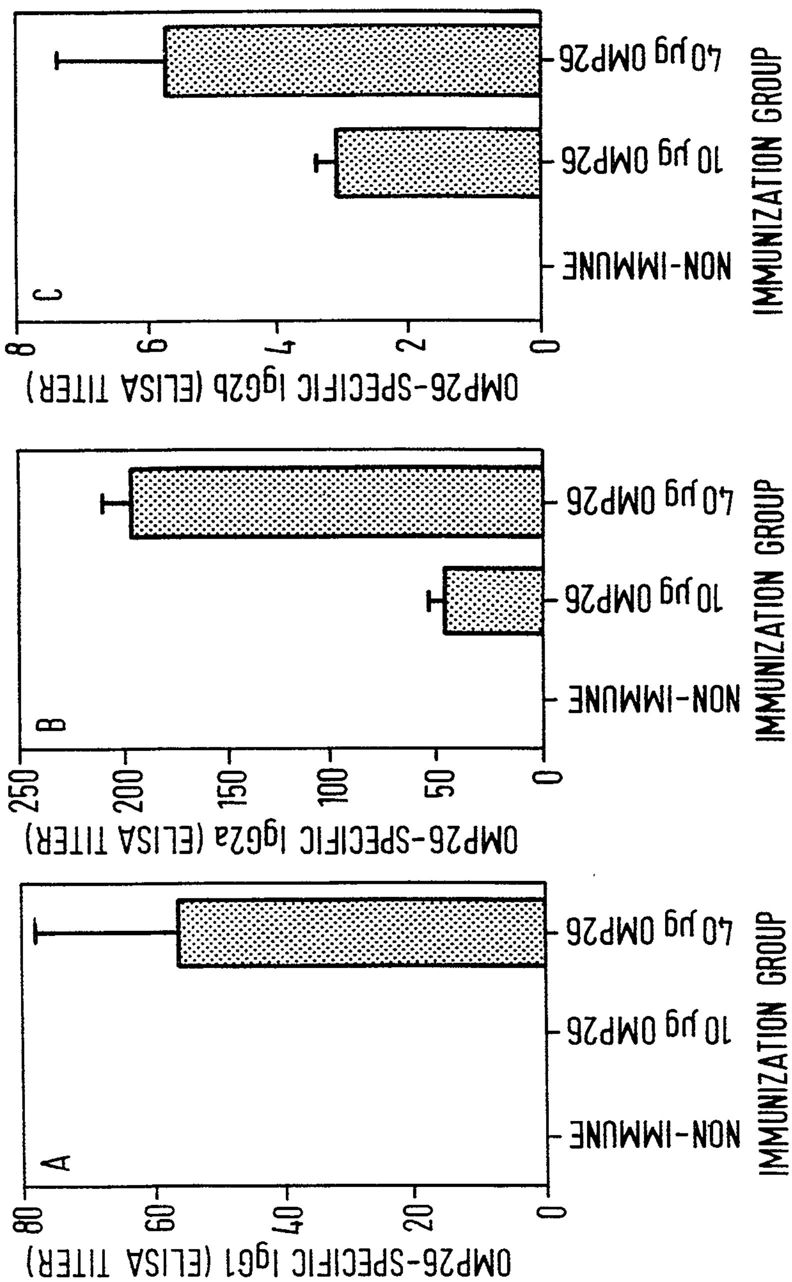
A
OMP26-SPECIFIC IgG1 (ELISA TITER)
0
20
40
60
80
NON-IMMUNE
10 µg OMP26
40 µg OMP26
IMMUNIZATION GROUP
B
OMP26-SPECIFIC IgG2a (ELISA TITER)
0
50
100
150
200
250
NON-IMMUNE
10 µg OMP26
40 µg OMP26
IMMUNIZATION GROUP
C
OMP26-SPECIFIC IgG2b (ELISA TITER)
0
2
4
6
8
NON-IMMUNE
10 µg OMP26
40 µg OMP26
IMMUNIZATION GROUP

FIG. 5

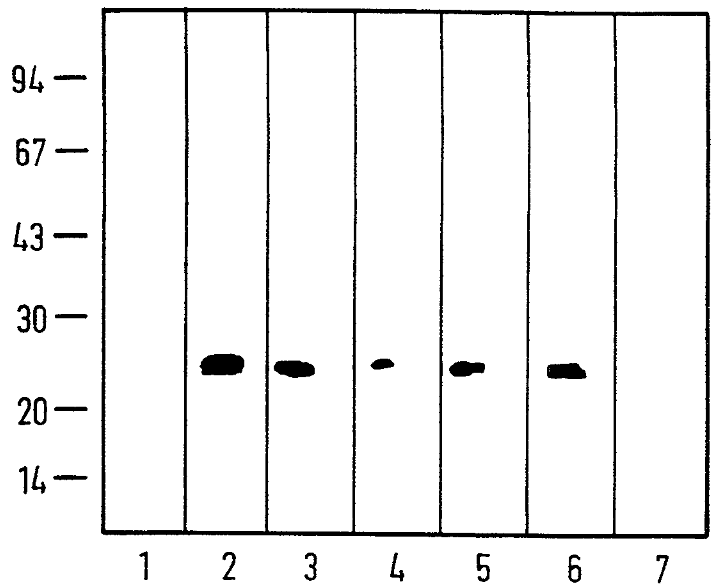

FIG. 6

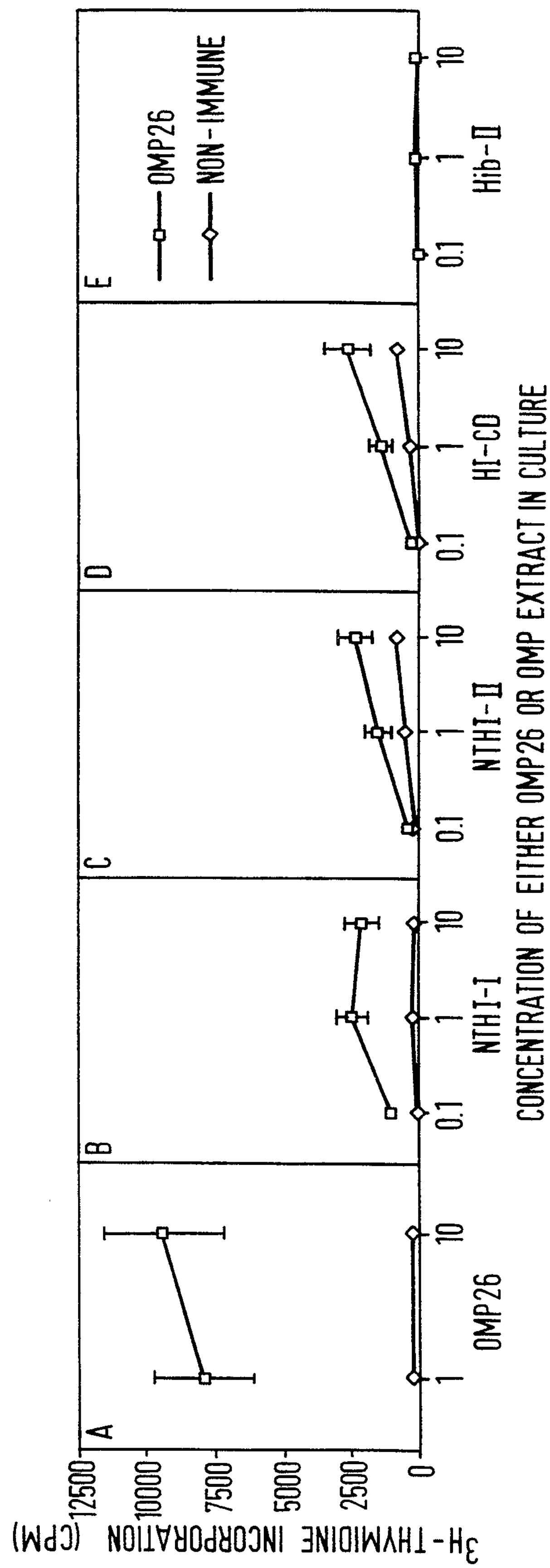
A
B
C
D
E
OMP26
NON-IMMUNE
12500
10000
7500
5000
2500
0
3H-THYMIDINE INCORPORATION (CPM)
1
10
0.1
OMP26
NTHI-I
NTHI-II
HI-CD
Hib-II
CONCENTRATION OF EITHER OMP26 OR OMP EXTRACT IN CULTURE
(μg PROTEIN/ml)

FIG. 7