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Production, characterization and control of a *Neisseria meningitidis* hexavalent class 1 outer membrane protein containing vesicle vaccine

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An experimental serogroup B meningococcal vaccine was prepared from two genetically engineered strains, each expressing three different class 1 outer membrane proteins (OMPs) (PorA). The two strains expressed the subtypes P1 7 16 P1 5 2 P1 19 15 and P1 5^c 10 P1 12 13 P1 7^a 4 respectively. Outer membrane vesicles (OMV) were prepared from these strains by deoxycholate extraction mixed with aluminumphosphate as adjuvant and formulated to final vaccines. The class 1 OMPs represent ca 90% of the protein in the vaccine. The vaccine was found safe for human use and induced a bactericidal immune response in mice against five of the six wild type strains which served as donors for the various porA genes. Copyright © 1996 Elsevier Science Ltd

Neisseria meningitidis serogroup B strains are responsible for a large number of clinical cases of bacterial meningitis in the Netherlands and worldwide^{1,2}. General immunization of the population appears to be the only means to reduce the mortality and morbidity due to meningococcal disease beyond antibiotic therapy.

The B capsular polysaccharide being the first choice as a vaccine component was found to be nonimmunogenic³. After chemical modification and conjugation to a carrier protein the polysaccharide was found to be immunogenic in animals⁴. Results with volunteers are not yet described. The issue of safety, however, needs to be addressed. The B polysaccharide is structurally identical to saccharide containing components such as N-CAM in man and mammals. Currently more experience has been obtained with vaccines which are based on meningococcal outer membrane proteins (OMPs). A number of large scale efficacy trials have been carried out with meningococcal OMPs either purified⁵ or in an outer membrane vesicle (OMV) formulation⁶. In each

instance the OMPs were derived from wild type strains and the vaccines were designed to combat occurring epidemics. These vaccines, contain multiple OMPs derived from one meningococcus. These OMPs are mainly the class 1, 2 or 3, 4 and 5 proteins. Such first generation OMP vaccines demonstrated efficacies around 50–80% except in infants where their efficacy is negligible⁷.

In vitro bactericidal antibody assays were found to be partly strain specific revealing a particular importance of the class 1 OMP⁸. These findings are in line with immunogenicity and protection data in mice and in rats⁹. The extent of heterogeneity of the class 1 OMP is limited¹⁰ which motivated us to develop multivalent class 1 OMP containing meningococcal OMV vaccines. We constructed a number of vaccine strains which are able to express three class 1 OMPs^{11,12}. The expression of Class 3 OMP was eliminated because this protein contributes little to the induction of bactericidal antibodies in mice and man^{8,9}. The B-polysaccharide and lacto-N-tetraose structures are deleted since those are shared with host structures and immune responses against them are poor and may even be deleterious. The expression of *Opc* is left untouched, since this protein may induce cross reactive bactericidal antibodies⁸, although the expression is poor by many case isolates¹³. The heterogeneity and variability of *Opa* proteins appears very high¹⁴ so we did not attempt to realise a high expression, although some *Opa* is left in our vaccine. The six *PorA* proteins chosen, which constitute about 90% of the vaccine protein were chosen on the basis of epidemiological data from the Netherlands¹ but also cover the majority of a global strain collection¹⁰. A major issue to be resolved remains the occurrence

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Table 1 The MoAbs and the meningococcal antigens that they recognize

MoAb	Protein specificity
MN14C11 6	P1 7
MN5C11G	P1 16
MN22C2.55	P1 5
MN18C13F4	P1.2
MN3C5C	P1 15
MN20F4 17	P1 10
MN20B9.34	P1 4
MN20A7 10	P1 12
MN24H10 75	P1 13
MN15A14H6	Class 3 OMP
MN3B9F	Class 4 OMP
15-1P5.5	Class 5 OMP specificity 5 5
S3141	Opc
4A8B2	L3 LPS
MN31D8 51	galE LPS

These MoAbs have been used for ELISA, immunoblotting analyses and immunogold EM

type specific MoAb (Table 1) or sera derived from mice and Protein A-HRP conjugate (Amersham UK)

Identity test by ELISA

The identity of the vaccine was determined by identification of characteristic epitopes using ELISA. Starting at a concentration of 0.3 µg class 1 OMP per well ²log dilutions of the vesicles were coated in PBS 0.01 M pH 7.2 on PVC 96-well plates (Greiner) overnight at room temperature. The coated plates were incubated at 37°C for 1 h with subtype specific monoclonal antibodies in PBS 0.01 M pH 7.2+0.05% (v/v) Tween80+0.5% (w/v) BSA (Table 1). The binding of MoAbs was demonstrated using biotinylated goat anti mouse IgG (Amersham UK) in PBS 0.01 M pH 7.2+0.05% (v/v) Tween80+0.5% (w/v) BSA at 37°C for 1 h and streptavidin horseradish peroxidase (Amersham UK) in PBS 0.01 M pH 7.2+0.05% (v/v) Tween80+0.5% (w/v) BSA at 37°C for 30 min. Binding of the conjugate was visualized using TMB/ethanol substrate. Binding of specific monoclonal antibody should be demonstrated whereas binding of non-specific antibody (directed against subtypes of the other strain) should be absent.

LPS determination

The LPS content was determined by SDS-PAGE²⁴. Vesicles were dissolved in a buffer containing 2% (v/v) β-mercapto-ethanol and 2% (w/v) SDS and boiled for 5 min followed by proteinase K (2.5 mg ml⁻¹, Boehringer) treatment for 1 h at 60°C. The treated samples were separated by SDS-PAGE with a 14% polyacrylamide gel. LPS was oxidized with 0.4% (w/v) periodic acid and visualized by silver staining. Unknown LPS samples were compared with an LPS standard in the same gel. LPS concentration was also determined by gaschromatography.²⁵

Endotoxin determination

The biological activity of the endotoxin was determined in the limulus amoebocyte lysate (LAL) assay. The lipid A part of the endotoxin molecule can activate the gelation of the limulus lysate. The OMV vaccine was incubated in a fivefold dilution with a fixed concentration of limulus lysate (Heamachem Inc. St. Louis

USA) at 37°C for 45 min. The endotoxin activity was visualized using Bromthymol Blue. Unknown activity of the endotoxin was compared with *Escherichia coli* standard endotoxin (FDA Bethesda USA).

Inactivation and sterility control

Trivalent bulk products were tested for absence of live organisms by incubation of the product on gonococcal agar and Tryptone Soy Broth (TSB) medium for 3 days at 37°C in a 5% CO₂ atmosphere.

To test sterility on final lot 5 ml was added to 180 ml Clausen and thioglycolate and incubated for a minimum of 13 days at 20–25°C for Clausen and 30–32°C for thioglycolate. No growth should be observed.

Immunological responses

Immunogenicity testing was performed in NIH mice. Eight week old female NIH mice were immunized subcutaneously with 10 µg total protein (1/10th human dose) of OMV vaccine with 2 mg ml⁻¹ AlPO₄. After 28 days mice were euthanized and blood samples were collected.

Sera were tested for the presence of subtype specific antibodies using an OMV based ELISA by immunoblotting and with a subtype specific bactericidal assay. Reference sera for all experiments were obtained from mice which were hyperimmunized with killed wild type bacteria.

For OMV based ELISA plates were coated with a fixed concentration (0.025–0.1 µg total protein per well) of OMV derived from a single subtype [H44/76 (P1 7 16), 2996 (P1 5 2), MC51 (P1 19 15), 870227 (P1 5^c 10), 870446 (P1 12,13), 892257 (P1 7^h 4)] in PBS 0.01 M pH 7.2 overnight at room temperature. After washing serum samples were titrated in a ²log dilution in PBS 0.01 M pH 7.2+0.05% (v/v) Tween80+0.5% (w/v) BSA and incubated at 37°C for 1 h. The binding of antibodies was demonstrated using biotinylated goat-anti mouse-IgG (Amersham, UK) in PBS 0.01 M pH 7.2+0.05% (v/v) Tween80+0.5% (w/v) BSA at 37°C for 1 h and streptavidin-horseradish peroxidase (Amersham, UK) in PBS 0.01 M pH 7.2+0.05% (v/v) Tween80+0.5% (w/v) BSA at 37°C for 30 min. Binding of the conjugate was visualized using TMB/ethanol substrate.

Bactericidal antibodies were determined in subtype specific bactericidal assays. Bacterial strains as mentioned above were used to determine subtype specific antibodies except for P1 7^h 4 in which case 880049 was used as target for bactericidal antibodies. Briefly 25 µl of bacterial suspension (10⁵ ml⁻¹) were incubated with baby rabbit complement (Pel Freez Chemical Systems, Brown Deer Wisconsin USA) and ²log dilutions of immune serum at 37°C for 1 h in a volume of 100 µl. Seven microlitres of this mixture was pipetted onto gonococcal agar plates in triplicate and incubated overnight at 37°C in a 5% CO₂ atmosphere. The serum bactericidal titre was reported as the highest reciprocal serum dilution yielding 50% killing of bacteria.

RESULTS

Production

Over a period of 2 years six independent OMV production runs were carried out. Three productions

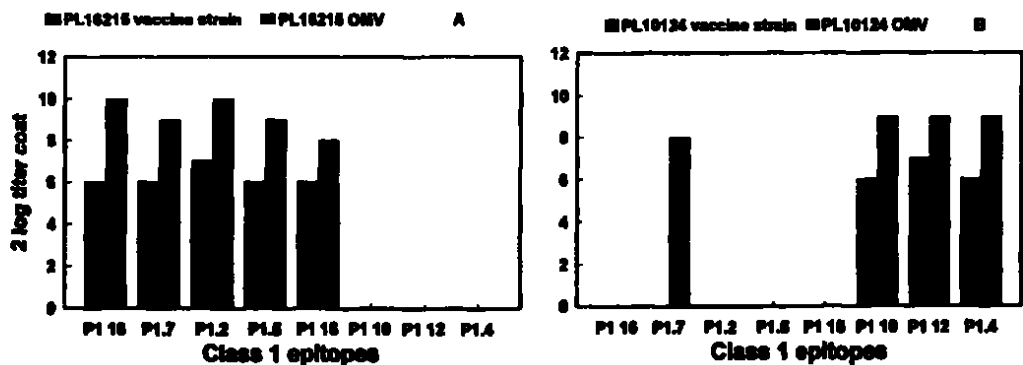


Figure 2 Presence of subtype specific epitopes in the two vaccine strains PL16215 and PL10124 and vesicles derived thereof as measured in ELISA. (A) Results obtained with PL16215 bacteria and OMV (B) the results obtained with PL10124 bacteria and OMV



Figure 3 An electronmicrograph of an immunolabelled vesicle preparation of PL16215 (A) The P1.2 epitope is visualized on the surface of the vesicles by immunogold labelling using the monoclonal antibody MN16C13F4 (anti P1.2). Numerous 6 nm gold particles surround the vesicles (B) Control incubation with the monoclonal MN24H10.75 (anti P1.13) showed no gold particles. Bars indicate 100 nm

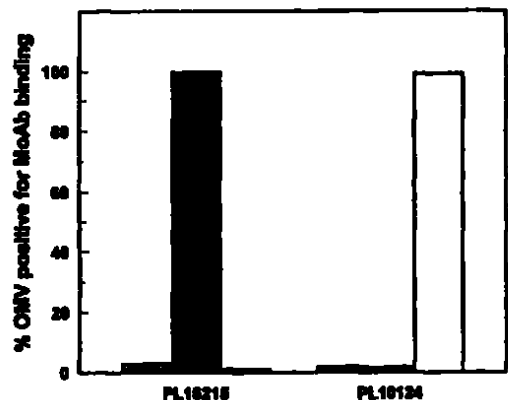


Figure 4 Vesicles of the PL16215 and PL10124 strains were labelled for P1.2 and P1.13 with the immunogold method. The percentage of OMV positive for the two class 1 epitopes is given. Approximately 300 vesicles were analysed per sample. ■ Control incubation without subtype specific MoAb □ a-P1.2 incubation and ■ a-P1.13 incubation. Almost 100% of the vesicles of each strain were positive for the correct class 1 epitope

is at the same time being studied as a possible vaccine adjuvant. Therefore we have carefully monitored the LPS content and the biological activity of the LPS in the vesicles. LPS free vesicles cannot be prepared since the presence of LPS in the OMV is necessary for the stability of the OMV vaccine. The LPS/protein ratio is lowered during the production process by partly replacing LPS by DOC. The LPS content was determined on the intermediate products by SDS-PAGE and silver-

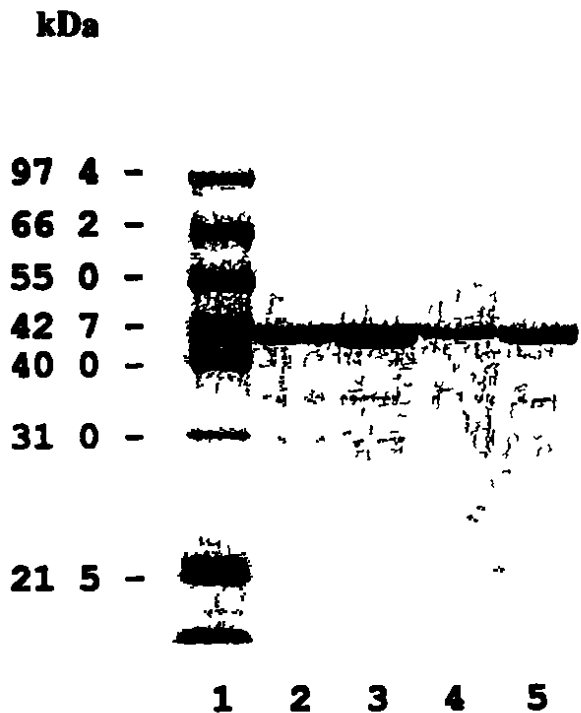


Figure 5 10% SDS-PAGE pattern of different vaccine bulk products. Class 1 OMP are ca 40 kDa. Lane 1 low molecular weight markers, lane 2 OMV PL16215 2 µg total protein, lane 3, OMV PL16215 4 µg total protein, lane 4 OMV PL10124 2 µg total protein; lane 5, OMV PL10124 4 µg total protein

Table 2 The composition of the hexavalent vaccine lot containing 100 µg of total protein. One human dose contains 0.5 ml

Substance	Concentration
Protein	200 µg ml ⁻¹
Class 1 (6 subtypes)	89%
Class 4	7%
Class 5 (5c and 5.5)	3%
82 kDa	<1%
pH	6.9
DOC	22 µg ml ⁻¹
B-PS	Absent
LPS (GalE)	20 µg ml ⁻¹
AlPO ₄	2 mg ml ⁻¹
Sucrose	30 mg ml ⁻¹
Thiomersal	0.7 mg ml ⁻¹

staining²⁴. Results ranged from 2.5 to 10% LPS relative to the protein content. Both European Pharmacopoeia and WHO failed to provide requirements

90% of the protein class 4 and class 5 OMPs and some 82 kDa OMP are present in the vaccine. Each strain expressed three different class 1 OMPs. The advantages of combining three different class 1 OMP in one strain as compared to mixing OMV derived from three individual strains are obvious. The relative amount of class 1 protein is enhanced and thus it is possible to make a vaccine with a relatively low protein content (100 µg per dose) that contains a high amount of target protein. Moreover the LPS content in a vaccine that is a combination of six OMV preparations would be much higher than in the vaccine described.

The OMVs derived from these strains, were found to be stable over time (18 months) and after thermodegradation experiments (data not shown). In each strain class 1 OMPs are synthesized in equimolar amounts and OMVs of the two individual strains are mixed to contain equal amounts of all class 1 OMPs. The choice for the class 1 OMP was driven by findings in man and animals which demonstrate this protein to be a major target for bactericidal antibodies²⁹. The heterogeneity of the class 1 OMP appears to be limited¹⁰. The P1 19 15 subtype in the trivalent OMV was non immunogenic in the animal model tested for bactericidal antibody induction. Immunization of NIH mice with OMV containing only the P1 19,15 subtype and no other class 1 OMP did induce bactericidal antibodies (data not shown). The decreased immunogenicity of the P1 19 15 epitope in the trivalent vaccine in mice is now studied in our laboratory; however an anti-P1 19,15 response could be demonstrated in some human adult volunteers: a fourfold rise in bactericidal antibody titre¹⁸. Whether it is immunogenic in children after a first immunization remains to be investigated.

The vaccines were also found to be safe for human use although LPS is present in a 10% w/w ratio as compared to OMP. It is known that the OMV structure has a shielding effect on the endotoxin properties of LPS²⁰. Taken together the results with regard to safety of the vaccine do not deviate much from the data obtained with the Folkehelse vaccine²⁰. Since this vaccine proved to be safe in adolescents we do not expect adverse reactions on the basis of these observations.

A phase I study in adults with this vaccine has been carried out¹⁸ and phase II studies in children and infants have started.

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Construction of *Neisseria meningitidis* strains carrying multiple chromosomal copies of the *porA* gene for use in the production of a multivalent outer membrane vesicle vaccine

Peter van der Ley* Jenny van der Biezen and Jan T Poolman

Starting with *Neisseria meningitidis* strain H44176 a set of strains was constructed for use in production of a multivalent outer membrane vesicle vaccine. The aim was to remove unwanted outer membrane components and at the same time to improve the range of protection. This was accomplished through transformation with plasmid constructs made in *Escherichia coli* and their homologous recombination into the meningococcal chromosome. Deletion of the *cps* locus resulted in loss of expression of the group B capsular polysaccharide as well as the lacto N-neotetraose structure in lipopolysaccharide. Deletion of the *porB* gene abolished expression of the class 3 outer membrane protein. Additional copies of the *porA* gene encoding the immunodominant class 1 outer membrane protein were inserted into one of the *opa* genes and into the *rmpM* gene encoding the class 4 outer membrane protein. This construction was done with three sets of *porA* alleles resulting in three trivalent strains each of which expressed a different combination of class 1 epitopes.

Keywords: *Neisseria meningitidis*; *porA* gene; outer membrane vesicle vaccine

Neisseria meningitidis is a human pathogen of worldwide significance. Current vaccines consist of the group A C W 135 and Y capsular polysaccharides and provide short term protection against strains of these serogroups in people above the age of 2 years. They are ineffective in infants although the group A polysaccharide induces some response starting from the age of 6 months. At present there is no vaccine available against group B organisms, which are the predominant cause of meningococcal disease in many countries. Development of a safe and effective vaccine based on the serogroup B capsular polysaccharide is complicated by its poor immunogenicity and the existence of identical structures in the human host. Therefore, vaccines based on meningococcal outer membranes are currently under investigation. Recent field trials in humans with such vaccines have demonstrated at least partial protection against group B infection^{1,2}. These vaccines consist of outer membrane vesicles prepared from one particular strain since many of the major outer membrane proteins

(OMPs) show a high degree of variation amongst different strains. A large proportion of the protective antibodies induced is expected to be strain-specific. Mixing vaccines derived from a number of different strains has limitations, such as high amounts of toxic lipopolysaccharide (LPS) and non protective OMPs.

Not all outer membrane components are needed or wanted in a vaccine. Among the major OMPs, the porins are important targets for bactericidal antibodies. They consist of class 1 protein encoded by the *porA* gene, and class 2/3 proteins encoded by the *porB* gene. Variation in the cell surface-exposed parts of these proteins forms the basis for the division of meningococcal strains in different subtypes and serotypes, respectively³. Monoclonal antibodies against class 1 protein are particularly effective in bactericidal assays, and in conferring protection in an animal model^{4,5}. After immunization of mice with outer membranes, the bactericidal activity is also mainly directed against this protein⁶. Comparison of *porA* genes from different subtypes has shown that the sequence variation is largely confined to two short regions which determine the subtype-specific epitopes and are targets for bactericidal antibodies⁷⁻⁹. Among the other major OMPs the highly conserved class 4 protein (encoded by the *rmpM* gene) might possibly induce blocking antibodies¹⁰ and the class 5 proteins (encoded

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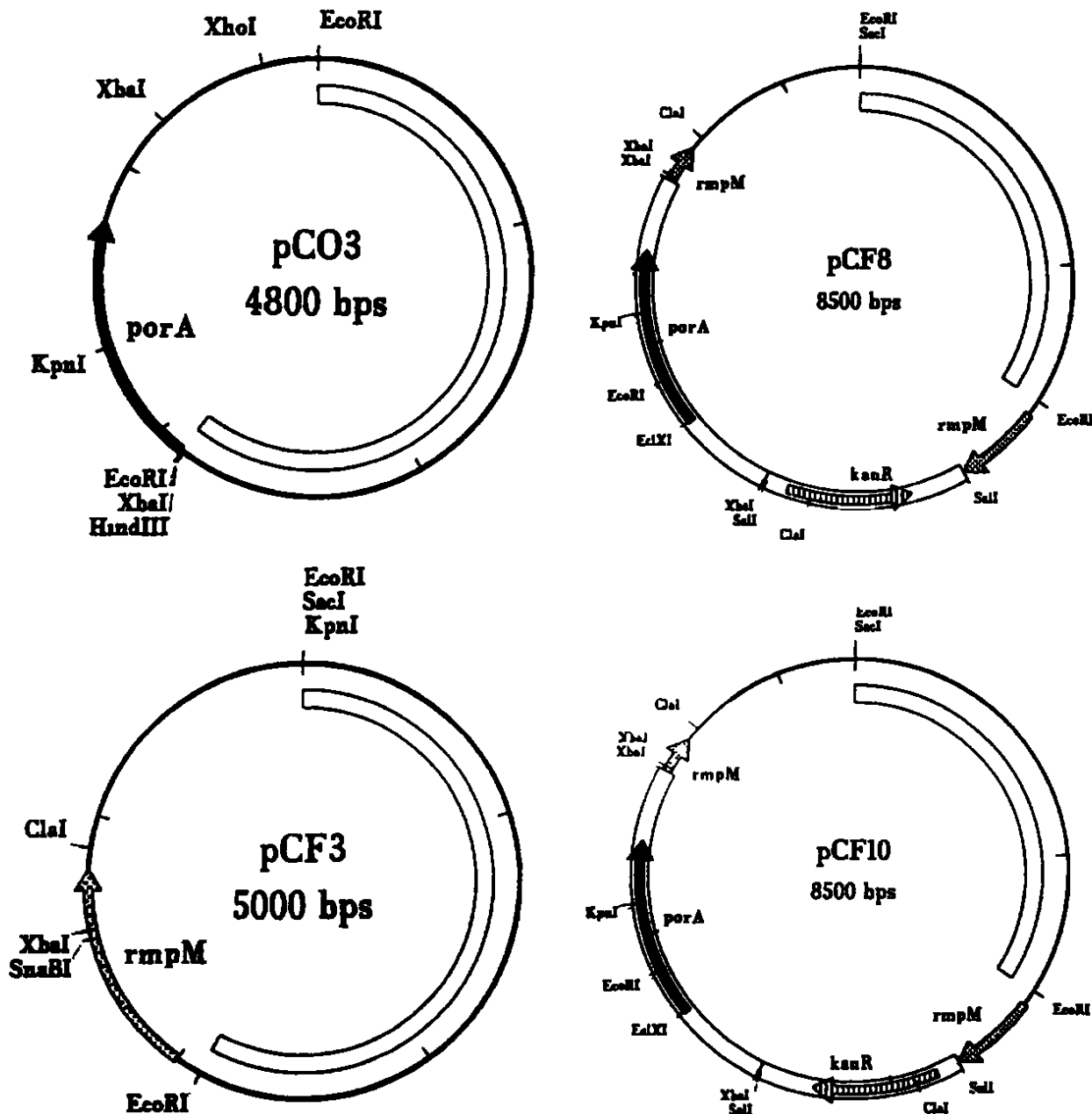


Figure 1 Restriction maps of plasmids used in this study. The part that consists of the vector pTZ19R is indicated by the open bar within the circles. Arrowheads indicate the direction of transcription. pCO3 carries the C-terminal part of *porA*. pCF8 was made by inserting a 3.5 kb fragment carrying *porA* and *kanR* into the *SnaBI* site within *rmpM* on pCF3. pCF10 was derived from pCF8 by inverting the 1.3 kb *Sall* fragment carrying *kanR*.

the *opa* genes⁶. The resulting strain, termed 1-2, expresses equal amounts of class 1 proteins of subtypes P1-7-16 and P1-5-2. This strain was used as the starting point for the further constructions described in this paper. First, transformation with plasmid pMF121 to erythromycin resistance was done in order to introduce an 18.5 kb deletion into the *cps* locus²⁰. This resulted in the inability to synthesize group B capsular polysaccharide and was done for the following reasons: (1) to make sure that no B polysaccharide is present in any vaccine preparation made from this strain, and (2) to render the strain avirulent and thus make it safer to handle in large-scale cultures. In addition, it was recently found that this deletion also removes the active copy of *galE*, thus leading to the synthesis of galactose deficient LPS²³. Since the lacto *N* neotetraose structure which is present on

Neisserial LPS is also found on human glycolipids, its lack of expression in a *galE* vaccine strain is also desirable. The deletion of the *cps* locus in the resulting strain 1-2B⁻ was verified by Southern blot (not shown). Absence of the immunotype L3 epitope from its LPS was demonstrated in a whole-cell ELISA with mAb MN4A8B2. Absence of the B polysaccharide was verified in a double-diffusion immunoprecipitation assay with specific antiserum.

Second strain 1-2B was transformed to kanamycin resistance with undigested plasmid pCF8 (Figure 1 and Materials and methods). In this plasmid, a 3.5 kb *Bam*HI-*Bgl*II fragment carrying a *kanR* marker and the *porA* gene from strain MC51 (subtype P1-15) has been inserted into the *Sna*BI site located within the *rmpM* gene. All kanamycin resistant transformants thus obtained

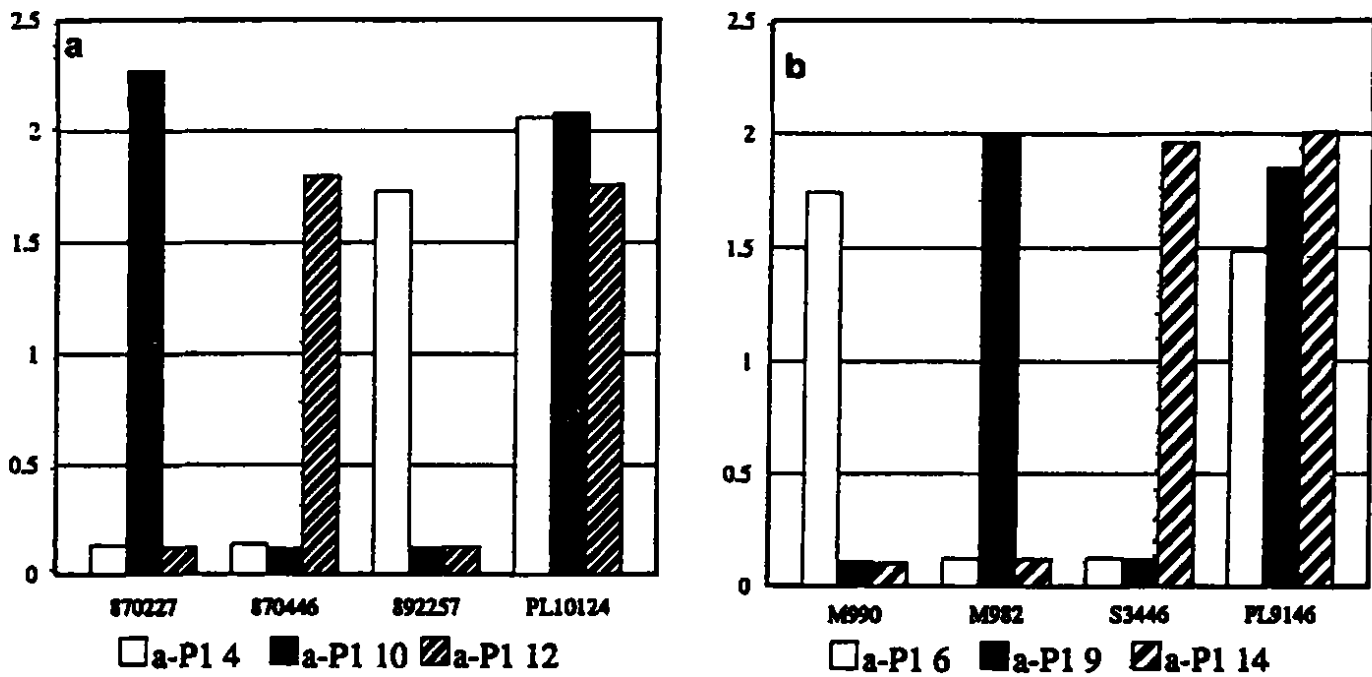


Figure 5 Whole-cell ELISA, with binding of subtype-specific mAbs shown as the extinction at 450 nm (a) Trivalent strain PL10124 and the three strains from which its *porA* genes were originally obtained (b) Same for PL9146

trivalent strains were thus constructed in the following way. First PCR fragments were prepared from the *porA* genes of strains 870227 (P1 5⁺ 10) 870446 (P1 12 13) and 892257 (P1 7^h 4), using primers 8 + 7 (see Materials and methods). *Hind*III-*Kpn*I digested PCR fragments from the P1 5⁺ 10 and P1 12 13 genes were cloned into similarly digested plasmid pCO3 which carries a 1.9 kb *Eco*RI fragment containing the C terminal part of the *porA* gene from strain 2996 (Figure 1). This resulted in two plasmids which both contain a complete *porA* gene, with the two variable regions derived from the P1 5⁺ 10 and P1 12 13 genes and the C-terminal part derived from strain 2996. The PCR fragment from strain 892257 (P1 7^h 4) was digested with *Fci*XI-*Kpn*I and used to replace the corresponding part of plasmid pCF8 resulting in plasmid pCF82. Transformation and allelic replacement with the constructed plasmids was subsequently used to change strain 1-2B⁻ in two steps from 7 16/5,2 into 5⁺ 10/12 13. Selection of transformants was done with a colony blot using mAbs specific for the incoming class 1 subtypes. Transformation of the resulting strain with pCF82 to kanamycin resistance and expression of P1 7^h 4 resulted in the trivalent strain PL10124.

In a similar fashion PCR fragments from the *porA* genes of strains M982 (P1 9), S3446 (P1 14) and M990 (P1 6) were used in the construction of a third trivalent strain PL9146. Again strain 1-2B⁻ was first used for allelic replacement of its two copies of *porA* from 7 16/5,2 into 9/14. Transformation with pCF83 to kanamycin resistance introduced the third *porA* gene (subtype P1 6).

Expression of the expected epitopes in strains PL10124 and PL9146 was checked in a whole-cell ELISA (Figure 5). Isolation of OMCs and analysis by SDS-PAGE demonstrated class 1 protein as the major component in contrast to strain PL16215. Resolution of the different subtypes was not possible for PL10124 and PL9146. Western immunoblotting with mAb 2D6D showed that class 4 OMP was still present in these strains as well

(Figure 6). Southern blotting with the *rmpM* gene as probe on *Clal*-digested chromosomal DNA also gave the same result as found previously with strain PL16215 i.e. integration of the complete plasmid into the chromosomal *rmpM* gene.

Construction of trivalent strains lacking class 4 protein

Our aim with respect to improved outer membrane vaccines focuses on the removal of class 2/3, 4 and 5 OMPs and the expression of multiple class 1 OMPs in order to obtain specific vaccines. As mentioned above transformation with plasmid pCF8 to kanamycin resistance never resulted in allelic replacement and insertional inactivation of the *rmpM* gene. Use of lower concentrations of kanamycin or other strains (for instance class 3 OMP proficient) did not make a difference. By accident it was discovered that when the *kanR* marker is used to inactivate a gene in meningococci transformants are only obtained when it has the same orientation as the gene into which it is inserted. Therefore, a derivative of pCF8 was constructed in which the *kanR* gene is reversed, now giving the same orientation as *rmpM* and *porA* genes (Figure 1). Transformation of strain 1-2B⁻ to kanamycin resistance with the resulting plasmid pCF10 was much more efficient than with pCF8 and all transformants tested were found to express the inserted P1 15 *porA* gene and lack class 4 OMP (Figure 6). Southern blotting of *Clal*-digested chromosomal DNA from the resulting strain JB16215 with the *rmpM* probe confirmed the occurrence of allelic replacement since two fragments of 3.9 and 3.5 kb were now found to hybridize (Figure 4b).

By using the 5⁺ 10/12 13 and 9/14 bivalent strains for transformation with plasmids pCF12 (carrying the *kanR* gene and the P1 7^h 4 *porA* gene in the same orientation as the *rmpM* gene) and pCF11 (carrying the *kanR* gene and the P1 6 *porA* gene in the same orientation as the

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Immunogenicity and reactogenicity in UK infants of a novel meningococcal vesicle vaccine containing multiple class 1 (PorA) outer membrane proteins

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Abstract

The development of effective vaccines against serogroup B meningococci is of great public health importance. We assessed a novel genetically engineered vaccine containing six meningococcal class 1 (PorA) outer membrane proteins representing 80% of prevalent strains in the UK. 103 infants were given the meningococcal vaccine at ages 2, 3 and 4 months with routine infant immunisations with a fourth dose at 12-18 months. The vaccine was well tolerated. Three doses evoked good immune responses to two of six meningococcal strains expressing PorA proteins contained in the vaccine. Following a fourth dose, larger bactericidal responses to all six strains were observed suggesting that the initial course had primed memory lymphocytes and revaccination stimulated a booster response. This hexavalent PorA meningococcal vaccine was safe and evoked encouraging immune responses in infants. Vaccines of this type warrant further development and evaluation. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Meningococcal vaccine; PorA; Infants

1 Introduction

Meningococcal disease remains a major problem worldwide. In 1995 in England and Wales, there were over 1800 laboratory-confirmed cases of meningococcal disease [1], an underestimate due to undernotification

[2] and changes in clinical and diagnostic practices [3]. Serogroup B strains, predominant in most temperate countries, have caused more than two-thirds of culture-confirmed cases in England and Wales since 1985.

Immunity to meningococcal disease is correlated with the presence of serum bactericidal antibodies (SBAs) to pathogenic meningococci at titres of 1:4 or more [4]. Recently, conjugated protein-polysaccharide vaccines for serogroup A and C meningococci have been shown to be safe and immunogenic when administered to UK infants [5].

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Though the serogroup B capsular polysaccharide is poorly immunogenic [6,7], the declining age incidence of serogroup B meningococcal disease and its rarity in the elderly indicate that natural protection is possible and indeed the norm. The immune response to colonisation and systemic infection with serogroup B meningococci is directed mainly against sub-capsular antigens [8–10], including outer membrane proteins (OMPs) and lipooligosaccharides.

Several different OMP vaccines have been subjected to efficacy studies in Norway, Cuba, Brazil and Chile [11–14], but none has been shown to protect infants. Though variable immune responses were observed [15], a link between PorA-specific antibodies and bactericidal activity was noted in all studies [15–17].

The *porA* gene encodes a transmembrane protein with eight surface-exposed loops [18] and hypervariable regions (VR1 and VR2) at the apices of loops 1 and 4 [19]. Monoclonal antibodies against these loops are particularly effective in serum bactericidal assays and in animal protection studies [20]. PorA OMPs have long been perceived as potential B vaccine components [21] despite variability in structure and level of expression [22,23].

Poolman and colleagues at the RIVM constructed a hexavalent PorA vaccine composed of two isogenic meningococcal strains in a vesicle formulation [24–26]. Promising SBA titres were obtained in adult volunteers [27]. The six PorA proteins present in the vaccine were represented in over 80% of UK meningococcal case isolates in 1996 (regardless of serogroup), with similar percentages in the preceding three years. A study was undertaken to evaluate the acceptability and immunogenicity of this vaccine in UK infants.

2. Methods

2.1 The hexavalent PorA-OMV vaccine

The construction of the vaccine strains, the production and contents of the vaccine, and the results of a phase 1 trial have been described elsewhere [24,27]. Briefly, two isogenic vaccine strains were constructed such that each expressed three PorA OMPs (P1 5.2, P1 7.16, P1 19.15, and P1 7^h.4, P1 5^e.10, P1 12.13, respectively). Purified vesicles from these strains were formulated into low dose (lot No. E9282) and high dose (lot No. E9281) vaccine preparations containing 45 or 90 µg PorA OMP respectively. PorA OMP comprised 90% of vaccine protein equivalent to 7.5 or 15 µg of each specific PorA OMP per low or high dose respectively. The vaccines were devoid of capsular polysaccharide, lacto-*N*-neotetraose and PorB OMP through gene deletion and expressed only small

amounts (7% and 3% respectively) of class 4 and class 5 OMP [26].

2.2 The patient population and the clinical study

Approval to carry out the study was granted by the local Research Ethics Committee. The study design was open, control infants were not recruited for the primary immunisation course. The study population consisted of west Gloucestershire infants aged 8–12 weeks who were eligible for routine primary immunisation. Following parental informed consent, infants were recruited between December 1994 and July 1995. Infants were excluded if their gestational age at birth was less than 26 weeks, they were immunocompromised sufficient to contraindicate live oral polio vaccine [28], or there were language difficulties in communicating with their parents.

At ages 2, 3 and 4 months infants received Wellcome (Manchester UK) Trivax-AD DTP vaccine (0.5 ml with AIOH₃ adjuvant) combined in the same syringe with Wyeth Lederle (Gosport Hants UK) HibTiter Hib vaccine (0.5 ml) by intramuscular injection into the left anterior mid thigh and 0.5 ml meningococcal vaccine, either low or high dose, in the right anterior mid thigh. Infants also received oral polio vaccine.

Between the ages of 12 and 18 months all children in the study received a dose of combined measles, mumps and rubella vaccine (MMR, Pasteur Mérieux, Maidenhead, Berks UK) by intramuscular injection into the left thigh, and a fourth dose of meningococcal vaccine of the same strength as that used previously, in the right thigh. Most received the two vaccines concurrently.

Thirteen control children recruited from the same general practitioner lists as the trial children were given a single dose of RIVM meningococcal vaccine at age 12–18 months, at the same time as their MMR immunisation.

Blood samples were obtained prior to each immunisation and one month after the third and fourth doses. Controls gave a blood sample prior to, and one month after the administration of MMR and meningococcal vaccine.

Study nurses visited or contacted the households of vaccinated infants 24 h and 7 days after each immunisation to complete a standard symptom questionnaire [5]. Specific systemic symptoms, the size of local reactions at the sites of immunisation, and twice-daily axillary temperatures were recorded by parents in a 7 day health diary. Parents were asked to note any other signs or symptoms of ill health and whether they had taken their immunised child to see their family doctor. Serious adverse events were as defined by the UK Departments of Health [29].

Table 1
Numbers and percentages of children who had local and systemic reactions following each dose of vaccine

	Meningococcal B low dose	Meningococcal B high dose	p value	DTwP&Hib	p value ^a	MMR	p value ^b
<i>Erythema ≥2.5 cm</i>							
Dose 1	3/52 (6)	2/50 (4)	0.68	17/103 (17)	0.007	-	-
Dose 2	1/52 (2)	0/51 (0)	0.32	7/103 (7)	0.03	-	-
Dose 3	4/52 (8)	3/51 (6)	0.72	16/103 (16)	0.046	-	-
Dose 4	9/51 (18)	5/47 (11)	0.32	-	-	4/79 (5)	0.044
Controls	2/9 (22)	0/4 (0)	0.31	-	-	1/12 (8)	0.59
<i>Swelling ≥2.5 cm</i>							
Dose 1	4/52 (8)	2/50 (4)	0.41	18/103 (17)	0.01	-	-
Dose 2	3/52 (6)	1/51 (2)	0.32	7/103 (7)	0.35	-	-
Dose 3	2/52 (4)	2/51 (4)	0.98	14/103 (14)	0.01	-	-
Dose 4	7/51 (14)	5/47 (11)	0.64	-	-	3/79 (4)	0.045
Controls	2/9 (22)	0/4 (0)	0.35	-	-	1/12 (8)	0.59
<i>Fever ≥38°C (up to 3 days)</i>							
Dose 1	6/52 (12)	4/51 (8)	0.53	-	-	-	-
Dose 2	5/52 (10)	7/51 (14)	0.52	-	-	-	-
Dose 3	5/52 (10)	10/51 (20)	0.15	-	-	-	-
Dose 4	8/51 (16)	8/47 (17)	0.86	-	-	-	-
<i>Systemic symptoms (≥3) in 1st 24 h</i>							
Dose 1	26/52 (50)	22/51 (43)	0.49	-	-	-	-
Dose 2	16/52 (31)	11/51 (22)	0.29	-	-	-	-
Dose 3	14/52 (27)	13/51 (25)	0.87	-	-	-	-
Dose 4	8/52 (16)	7/47 (15)	0.91	-	-	-	-
<i>Crying > 1 h in 1st 24 h</i>							
Doses 1-3	3/156 (2)	2/153 (1)	0.66	-	-	-	-
Dose 4	0/51 (0)	1/47 (2)	0.33	-	-	-	-
<i>Medication required within 7 days^c</i>							
Doses 1-3	111/156 (71)	110/153 (72)	0.95	-	-	-	-
Dose 4	36/51 (71)	25/47 (43)	0.08	-	-	-	-

^a p value calculated for comparison of low and high dose meningococcal B vaccines combined versus Hib&DTwP vaccines.
^b p value calculated for comparison of low and high dose meningococcal B vaccines combined versus MMR vaccine.
^c Medications required were mainly antipyretics.

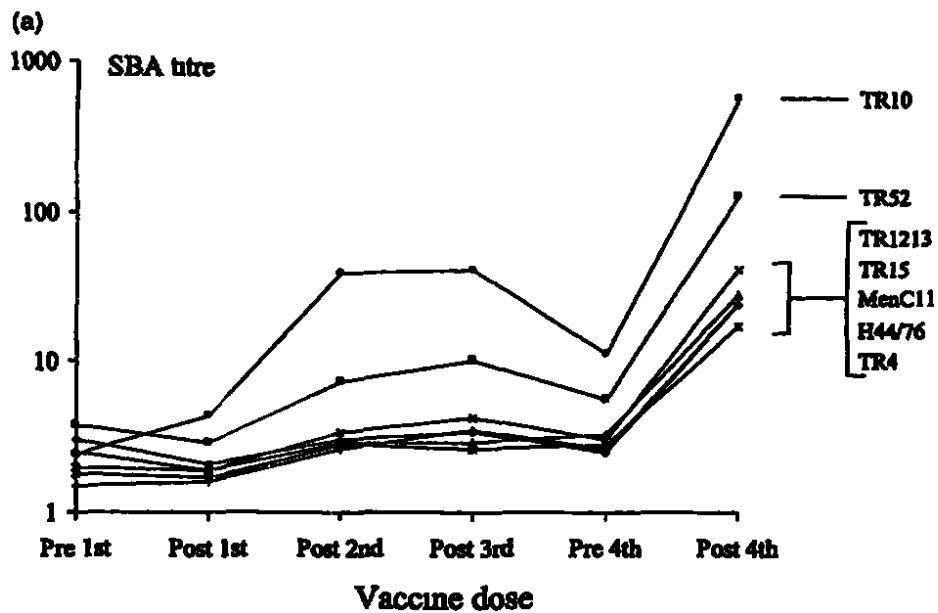
2.3 Serological studies

Sera were transported frozen to the Meningococcal Reference Unit and tested for the presence of SBAs against a panel of six meningococcal strains expressing the same PorA OMPs as those used to construct the vaccine strains. Strains H44/76 (B 15 P1 7,16 L3) and its isogenic *porA* derivatives TR52 (P1 5,2), TR15 (P1 19,15), TR1213 (P1 12,13), TR4 (P1 7^b,4), TR10 (P1 5^c 10) were used [24,27], together with a serogroup C strain MENC11 (C 16 P1 7^a,1). SBA assays were carried out according to standardised methodology

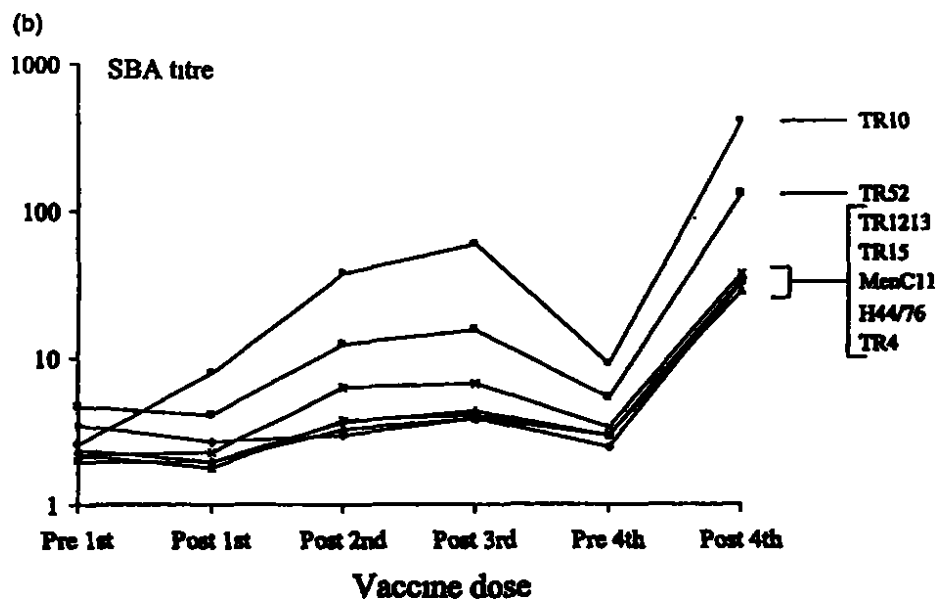
[30], with titres expressed as the reciprocal of the final dilution giving ≥50% bacterial killing at 60 min [27].

2.4 Statistical evaluation

Postvaccination GMTs with 95% confidence intervals (CIs) were calculated. The Mann-Whitney test was used to determine if differences in SBAs between doses and meningococcal vaccine strengths were significant. χ^2 tests were used to evaluate the significance of differences between reactions to high and low doses of meningococcal vaccines. McNemar's test was used



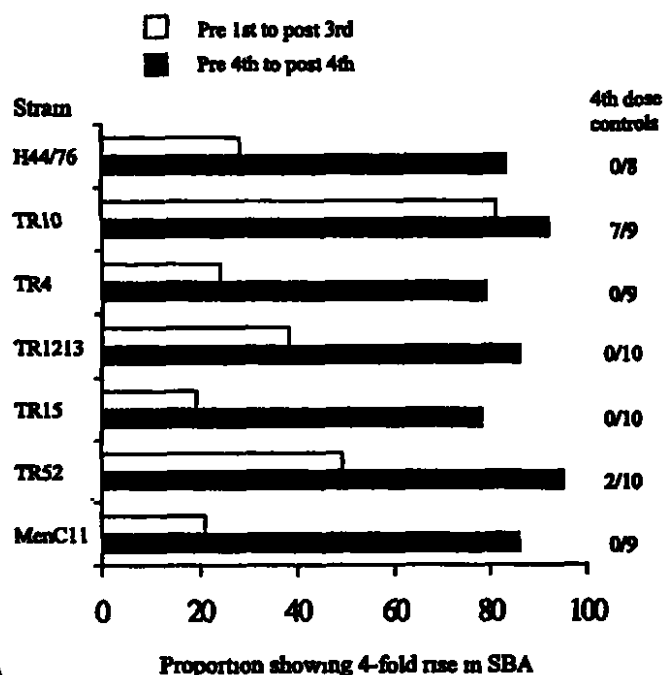
	Pre 1st	Post 1st	Post 2nd	Post 3rd	Pre 4th	Post 4th
TR10	2.4	4.4	38.7	40.7	11.1	562.8
TR52	3.1	2.9	7.3	10	5.6	126
TR1213	2	1.9	3.4	4.2	3	41.2
TR15	2.5	1.9	2.9	2.9	3.3	27.4
MenC11	3	2.1	3	3.4	2.5	23.8
H44/76	1.5	1.6	2.6	3.5	2.6	24.4
TR4	1.8	1.7	2.1	2.6	2.8	17



	Pre 1st	Post 1st	Post 2nd	Post 3rd	Pre 4th	Post 4th
TR10	2.6	7.8	37.1	59.4	9	402
TR52	4.7	4.1	12.3	15.3	5.3	130
TR1213	2.2	2.3	6.2	6.7	3.4	36.9
TR15	2.4	2	3.3	3.9	3	28.1
MenC11	3.5	2.7	3	3.9	2.5	32
H44/76	2	2	3.7	4.4	3	33.6
TR4	2.2	1.8	3.8	4.1	3	33

Fig. 1 Geometric mean titres of serum bactericidal antibodies for each episode of venepuncture: low dose (a) and high dose (b) Data for figures: (a) and (b)

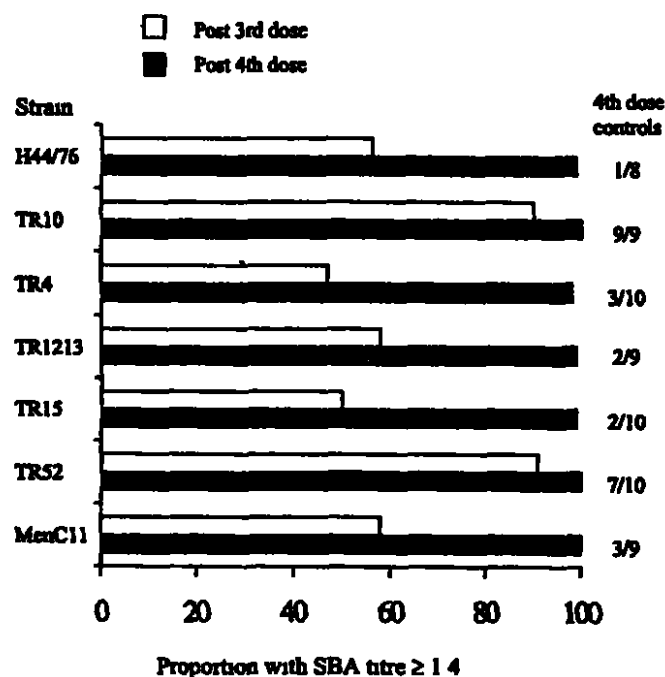
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* data from low and high dose vaccine cohorts merged

	Pre 1 st to post 3 rd	Pre 4 th to post 4 th
TR10	81	92
TR52	49	95
TR1213	38	86
TR15	19	78
MenC11	21	86
H44/76	28	83
TR4	24	79

Fig. 2. Proportions of children with fourfold or greater rises in SBA titres pre 1st and post 3rd, and pre and post 4th dose (including 4th dose controls).



* data from low and high dose vaccine cohorts merged

	Post 3 rd dose	Post 4 th dose
TR10	90	100
TR52	91	100
TR1213	58	99
TR15	50	99
MenC11	58	100
H44/76	56	99
TR4	47	98

Fig. 3. Proportions of children with SBA titres ≥ 14 post 3rd and post 4th dose (incl 4th dose controls).

to evaluate the significance of differences between local reactions due to meningococcal and DTP-Hib and MMR vaccines

3. Results

3.1 Patients and clinical procedures

One hundred and three infants (53 male) received a full primary course of three injections of meningococcal vaccine (52 low dose and 51 high dose) and 98 children received a fourth dose (51 low dose and 47 high dose). Of the 13 control children nine received a single low dose, and four a single high dose of meningococcal vaccine.

3.2 Adverse events

Rates of adverse events up to 7 days post-immunisation are shown in Table 1. No serious adverse events occurred. There were no significant differences in rates of local or systemic reactions between low dose and high dose cohorts. Local reactions were generally mild and shortlived. One child in the high dose cohort had a fever $\geq 39^{\circ}\text{C}$ after a fourth dose of the meningococcal vaccine.

3.3 Functional antibody

In some children, the volume of blood obtained was too small to permit completion of all serological tests, resulting in discrepancies in the totals of serological results.

SBA GMTs against the panel of seven meningococcal strains are summarised in Fig 1a and b. Significantly higher SBA GMTs between pre-vaccination and post third dose samples were observed against strains TR52 and TR10 than against strains H44/76, MENC11, TR4, TR1213 and TR15 ($p < 0.001$). SBA GMTs to all strains tested rose significantly following administration of a fourth dose of meningococcal vaccine ($p < 0.001$). Following a single dose of meningococcal vaccine given at 12-15 months, control children did not show significant rises in SBA titres to the seven meningococcal strains tested, with the exception of the rise in titre against strain TR10.

Greater proportions of infants showed fourfold or larger rises in SBA titres to the TR52 and TR10 meningococcal strains after three doses of meningococcal vaccine (84% and 49%, respectively) than those showing fourfold rises in SBA GMT to the remaining four strains (range 19% to 38%, $p < 0.001$, Fig. 2). Data from low and high dose cohorts showed no significant differences and were merged. After a fourth dose at age 14 months, more than 77% of all infants who had received the initial three doses showed fourfold or greater rises in SBA titres to all meningococcal strains tested when compared with pre-fourth dose samples. No child in the control group showed fourfold or greater rises to strains H44/76, TR4, TR1213, TR15 or MENC11 although 78% and 20%, respectively, showed fourfold or greater rises in SBA titres to strains TR10 and TR52.

The proportions of infants showing $\geq 1:4$ SBA titres after three and four vaccine doses respectively are shown in Fig. 3. Following three doses of vaccine, significantly higher proportions of infants had $\geq 1:4$ levels of SBA against TR10 and TR52 than against the other four isogenic strains ($p < 0.001$). Proportions of infants with SBA titres $\geq 1:4$ fell slightly by 12-15 months but following a fourth dose of the same strength of vaccine, almost all achieved this putative protective level of SBA. Control children responded better to TR10 than to the other isogenic variants.

4. Discussion

Infants recruited into this study were chosen to be as nearly representative as possible of UK children eligible to participate fully in the national childhood immunisation programme, with the same exclusion criteria being used. A small group of age-matched control children was recruited to aid the serological evaluation of the effect of a fourth dose of meningococcal vaccine given at age 12-18 months.

Both low and high doses of meningococcal vaccine

were associated with very low incidences of local and systemic symptoms. Local reactions to the meningococcal vaccine were less than those observed in the contralateral limb in which combined DTP and Hib vaccines had been administered. The incidences of local and systemic reactions following the fourth dose of meningococcal vaccine given at age 12-18 months were similar to those documented in children receiving a fourth dose of Hib vaccine who were enrolled in an earlier Gloucestershire study [31].

Individual titres of SBAs have remained the best immunological correlate of protection against meningococcal infection since their elucidation by Goldschneider and his colleagues in 1969 [4,32]. However, the lowest titre of SBA consistent with protection against invasive meningococcal disease is not known with certainty, though titres of less than 1:4 were associated with susceptibility to disease in early studies [4]. An age-associated correlation between SBA titre and efficacy was also observed in the Brazilian study of the Cuban (Finlay Institute) OMP vaccine [13].

Immunological evaluation of the RIVM vaccine was complex because it contained six different PorA OMPs and 12 different VR1 and VR2 epitopes. The serogroup B meningococcal strains used in the SBA assays were chosen because they expressed genotypically identical PorA VR1 and VR2 epitopes to those in the vaccine.

Consistent with results obtained in younger children in Brazil, in whom two doses of Cuban OMP vaccine were used [17], one dose of the RIVM hexavalent vaccine could not compensate for the natural decline in passively acquired SBA titres, with the exception of those against the TR10 strain.

Following three doses, SBAs were induced preferentially to one of the three PorA proteins expressed by each trivalent vesicle in the vaccine. In one vesicle TR10 induced higher SBA titres than the remaining PorA proteins. In the other, TR52 was the most immunogenic, suggesting that particular epitopes are immunodominant within the vesicles. Approximately 90% of infants achieved protective levels of antibody against these strains. These two strains express PorA proteins detected in 10% of UK meningococcal serogroup B case isolates in 1996. Approximately half the infants developed protective SBA titres against the other isogenic meningococcal strains after three doses of vaccine.

SBA titres had declined when re-measured at 12-14 months, a matter of concern since the peak incidence of serogroup B meningococcal disease in England and Wales occurs in infants aged 6-7 months [33]. However, after a fourth dose, SBA titres rose significantly to levels regarded as protective, against all seven test strains and only 6% of infants did not

achieve protective SBA titres against all isogenic strains tested, suggesting that immunological memory was built effectively during primary immunisation, even to non-dominant PorA epitopes, despite the observation that not all children demonstrated an SBA response

To ensure that this priming was not due to natural antigenic exposure or to age, sera from control children were studied who received a single injection of the meningococcal vaccine at age 12-18 months. With the exception of a small elevation in the SBA response to strain TR10, their titres did not rise.

SBA GMTs against strain MENC11 strain rose by an amount similar to that observed against the four non-immunodominant isogenic strains represented in the vaccine. Strain MENC11 has a VR1 serosubtype of 7^a, closely related antigenically to the VR1 7 and 7^b epitopes that are vaccine constituents. The functional immune response to MENC11 engendered by the vaccine could be due to cross-reaction with a closely related PorA epitope, or to an as yet uncharacterised component of the vaccine. It was found previously that the P1 7 epitope was critical for the induction of SBAs against strain H44/76, which contains PorA P1 7 16 [25].

Results of serological testing of the Norwegian OMV vaccine in 28 Chilean infants were promulgated recently (9th International Pathogenic Neisseria Meeting, Baltimore, USA, 1996). After two doses of this vaccine administered at two and four months of age, SBA GMTs of 1 64 were obtained against a strain expressing PorA P1 7,16 (i.e. a homologous strain), which comprised about 20% of vaccine protein. These titres are higher than those obtained with the Cuban vaccine tested in Brazil [17], the Walter Reed Army Institute vaccine tested in Chile [14] and the RIVM vaccine data reported in this study. Infants are probably primed by OMP immunisation but may respond differently to different vaccine formulations. All these vaccines induce SBAs in adults and older children who are likely to have been primed by earlier asymptomatic colonisation, and who may therefore respond to OMP vaccines with similar booster-type responses.

The development of safe and effective meningococcal serogroup B vaccines is a major goal in meningococcal research. Here, we present the first evaluation of a multivalent PorA OMP vesicle vaccine in infants. Side effects were infrequent and mild. A course of three doses induced modest but encouraging rises in SBA titres against representative meningococcal strains. The nature of the immune responses suggested that the immunogenicity of this vaccine might be improved. The size of the immune response after a fourth dose of vaccine was consistent with priming of the immune system and generation of immunological memory.

Meningococcal vaccines of this type warrant further development and evaluation.

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Field trial of inactivated oral cholera vaccines in Bangladesh: results from 5 years of follow-up

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To determine the protective efficacy (PE) of three doses of oral B subunit-killed whole cell (BS-WC) or killed whole cell-only (WC) vaccines against cholera a clinical trial was conducted among 62285 children over 2 years and adult women in rural Bangladesh. During 5 years of follow-up there were 144 cases of cholera in the BS-WC group (PE=49% P<0.001), 150 in the WC group (PE=47% P<0.001) and 283 in the K12 group. Protection by each vaccine was evident only during the first three years of follow-up. Long-term protection of young children was observed only against classical but not El Tor cholera. 3 year protection against both cholera biotypes occurred among older persons but at a higher level against classical cholera.

Keywords: Cholera, oral vaccination, Bangladesh

Recent efforts to develop new vaccines against cholera have focused upon vaccines administered orally since intestinal mucosal immunity is thought to be most relevant to protection against cholera and since peroral presentation of vaccine antigens is most efficient in eliciting mucosal immune responses¹. The first oral cholera vaccines to be evaluated for efficacy in preventing cholera in an endemic setting were killed preparations consisting of heat or formalin inactivated whole cells either with (BS-WC) or without (WC) the addition of cholera toxin B subunit². When tested in rural Bangladesh three doses of each vaccine were well tolerated and conferred about 50% protection over a 3-year period of follow-up³. In this paper, we present the results for the final 2 years of follow-up and examine the manner in which age at vaccination and the biotype of the infecting vibrios modified the magnitude and longitudinal pattern of vaccine efficacy.

MATERIALS AND METHODS

Methods for the trial which was conducted in the Matlab field studies area of the International Centre for

Diarrhoeal Disease Research Bangladesh (ICDDR B) have been presented in detail elsewhere³. The trial evaluated two vaccines. The BS-WC vaccine consisted of 1 mg cholera toxin B subunit together with 10¹¹ killed whole cells representing the El Tor and classical biotypes and the Inaba and Ogawa serotypes of *Vibrio cholerae* 01. The WC vaccine had the same cellular constituents, but lacked B subunit. In early 1985 persons 2–15 years of age and older females were randomized to BS-WC, WC or an *Escherichia coli* K12 strain placebo (K12). The regimen for each agent consisted of three doses at 6 week intervals. At least one dose was taken by 89 596 persons after giving verbal informed consent. Of these 62 285 ingested three complete doses.

For the first 3 years after vaccination surveillance for diarrhoea was conducted at all three diarrhoea treatment centres serving the Matlab population of nearly 200 000 persons. During the final 2 years of the cholera vaccine trial surveillance was maintained as rigorously as before, with two exceptions.

no team was delegated to verify the patient's identity by visiting the patient's stated home address, since during the previous 3 years only 12 of 2904 cases (0.4%) of misidentification had occurred.

one of the original three Matlab diarrhoea treatment centres was closed because during the first 3 years of the trial it had contributed only 189 cases (1.6%) to the trial population.

At each centre clinical data and faecal specimens were systematically obtained from all Matlab residents who presented for care of diarrhoea and microbiological evaluations of faeces were performed to detect

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V. cholerae O1 and to characterize each isolate by biotype and serotype

Cholera endpoints were analysed as episodes as previously described³. Vaccine protective efficacy (PE) was calculated as $[(1 - \text{Relative risk of cholera in vaccinees vs placebo recipients}) \times 100\%]$. Efficacy was evaluated for recipients of three complete doses of an assigned agent, during the period 14 days to 5 years after the third dose. Only initial cholera episodes occurring during this period were analysed. Denominators for calculation of cholera risk were persons who were still under follow up at the beginning of each year of follow-up and who had not previously had a detected episode of cholera.

Comparisons of the risk of cholera in vaccinees vs placebo recipients were statistically appraised with χ^2 tests or with Fisher's exact tests when mandated by sparse strata. Confidence intervals (95%) for protective efficacy were estimated with test based methods for relative risks. For reasons cited elsewhere⁴, all statistical appraisals of protective efficacy employed one-tailed tests to estimate *P* values and 95% confidence intervals (95% CI) for the latter only lower boundaries are cited. Overall comparisons of vaccinees and placebo recipients employed $P < 0.025$ as the threshold of statistical significance, whereas subgroup analyses used $P < 0.05$.

RESULTS

During the final years of the cholera vaccine trial the annual admission rate for cholera patients seen at the Matlab treatment centres had declined considerably, from 1258 in 1986 to 76 in 1989, this trend was noticed nationwide including at our Centre's diarrhoea treatment centre in the capital Dhaka.

As presented elsewhere⁴, at baseline the three groups were equivalent in size (each with ca 21 000 persons) and in the distributions of factors known to be associated with the risk of cholera in Matlab⁵. Table 1 shows the occurrence of cholera during the entire follow up period. A total of 580 episodes of cholera were detected three (two in the BS-WC group and one in the K12 group) were excluded since they represented recurrent episodes during the follow up interval. After these exclusions there were 144 cases of cholera in the BS-WC group (PE 49%, 95% CI lower boundary (LB) 38%, $P < 0.001$), 150 cases in the WC group (PE 47%, 95% CI LB 36%, $P < 0.001$) and 283 cases in the K12 group.

This pattern reflected relatively sustained and equivalent protection for each vaccine during the initial 2 years (Table 1) followed by a substantial drop in efficacy during the third year. During the fourth year 56 cases were noted in the three groups (15 in BS-WC, 23 in WC and 18 in K12), and no significant protection was noted for either vaccine. During the fifth year cholera declined to very low levels in Matlab, and no cases were detected among three dose recipients.

As shown in Table 1 there was a rather complex age-related longitudinal pattern of efficacy. Protection conferred by both vaccines was sustained at substantial levels in persons vaccinated at ages of 5 years or greater for the initial 3 years of follow up, but were not significant in the fourth year. Children vaccinated at ages below 5 years exhibited moderate protection (67%, $P < 0.05$ for all vaccinees) during the initial pre-epidemic period of the first year corresponding to the first 4–6

months of follow up. More modest protection of this age group was observed for the remainder of the first 2 years after which protection dropped to nil.

Because age at vaccination and the biotype of the infecting vibrios were earlier noted to be critical determinants of vaccine protection³, it was of interest to examine the trends of vaccine protection separately for classical and El Tor cholera, by age during each year of follow-up (Table 2). In this analysis the two vaccine groups were combined, in view of the similar constituents of the vaccines and the nearly identical performance of these vaccines in the field particularly after the pre-epidemic period of the first year.

Among children vaccinated before the age of 5 years protection against El Tor cholera was evanescent being observed only in the first 4–6 months of follow up. In contrast for classical cholera, significant protection (40–56%) of young children was sustained for the first 2 years of follow up.

For persons vaccinated at older ages vaccine protection against El Tor cholera declined gradually from 64% to 48% during the initial 3 years, after which protection disappeared. Vaccine protection against classical cholera was somewhat higher, but also exhibited a year-by-year decline from 76% to 58%. No classical cases were detected in the fourth year.

DISCUSSION

In this trial each vaccine demonstrated moderate (ca 60%) protective efficacy for 2 years of follow up. Thereafter protection declined but was still evident during the third year. In the fourth year neither vaccine conferred significant protection. No noteworthy differences in the protection conferred by the two vaccines were detected apart from the initial pre-epidemic period when the BS-WC provided superior protection⁴.

Analyses of all vaccinees in aggregate revealed moderate (PE=40–56%) vaccine protection against classical cholera during each of the first 2 years of follow-up of vaccinees aged 2–5 years but only evanescent protection against El Tor cholera in this age group. Among older vaccinees protection against classical cholera was more substantial and was sustained (PE=58–76%) during each of the initial 3 years, somewhat lower protection was maintained during this period against El Tor cholera (PE=48%–64%). It is a matter of speculation whether protection would have been more sustained against classical cholera after 3 years of follow up of the older group, since no classical cases were detected during the fourth year of follow up.

These results demonstrate that the killed oral vaccines were able to confer sustained protection against classical cholera for 2 years in both young children and older persons and for at least 3 years in the older group. In contrast, sustained protection against El Tor cholera was seen only in the older group in whom protection was evident for only 3 years.

The possible explanations for these biotype and age-related distinctions in vaccine efficacy are multiple. It has been speculated that the better vaccine performance in older than in younger subjects might reflect the fact that older subjects had a greater likelihood of being immunologically primed at the time of vaccination⁶. This explanation does not however account for the

Table 1 , Overall occurrence of cholera episodes during the 5 years of surveillance^a by age at vaccination^b

	Group BS WC	WC	Vaccinees ^c	K12 ^d
Year 1				
<i>Pre-epidemic</i>				
2-5 years	0 (100%) (80%)	6 (35%) (-55%)	6 (67%) (24%)	9
>5 years	4 (76%) (44%)	5 (71%) (37%)	9 (73%) (50%)	17
<i>Epidemic</i>				
2-5 years	26 (21%) (-20%)	24 (31%) (-7%)	50 (26%) (-6%)	34
>5 years	11 (78%) (64%)	17 (66%) (47%)	28 (72%) (60%)	50
<i>Entire year</i>				
2-5 years	26 (38%) (7%)	30 (31%) (-1%)	56 (35%) (9%)	43
>5 years	15 (78%) (66%)	22 (67%) (51%)	37 (72%) (62%)	67
Total	41 (62%) (50%)	52 (53%) (38%)	93 (57%) (47%)	110
Year 2				
2-5 years	16 (47%) (13%)	24 (24%) (-18%)	40 (35%) (5%)	31
>5 years	25 (63%) (45%)	18 (73%) (59%)	43 (67%) (56%)	67
Total	41 (58%) (44%)	42 (57%) (42%)	83 (58%) (46%)	98
Year 3				
2-5 years	24 (-36%) (-126%)	18 (2%) (-70%)	42 (-17%) (-85%)	18
>5 years	23 (41%) (10%)	15 (61%) (38%)	38 (51%) (30%)	39
Total	47 (18%) (-14%)	33 (42%) (18%)	80 (30%) (7%)	57
Year 4				
2-5 years	7 (-44%) (-276%)	6 (-19%) (-220%)	13 (-31%) (-211%)	5
>5 years	8 (38%) (-28%)	17 (-31%) (-140%)	25 (4%) (-69%)	13
Total	15 (16%) (-49%)	23 (-28%) (-114%)	38 (-6%) (-69%)	18
All years				
2-5 years	73 (23%) (1%)	78 (21%) (-1%)	151 (22%) (3%)	97
>5 years	71 (62%) (52%)	72 (61%) (52%)	143 (61%) (54%)	186
Total	144 (49%) (36%)	150 (47%) (36%)	294 (48%) (39%)	283

^aNo cases were detected during the fifth year of surveillance. Three recurrent cholera episodes were excluded: two in the BS WC group (one with episodes in the first and second years, the other with two episodes in the second year) and one in the K12 group (with episodes in the second and third years). ^bThe numbers in the table denote: Number of cases (protective efficacy in percentage) (lower boundary of the 95% confidence interval). ^cVaccinees refer to the aggregate of the BS WC and WC groups. ^dDenominators for (BS WC K12) were: Year 1—(3728 3900 3630) for 2-5 years (16 977 16 843 17 007) for 5 years Year 2—(3599 3745 3674) for 2-5 years (16 403 16 260 16 338) for 5 years Year 3—(3532 3669 3600) for 2-5 years (15 838 15 755 15 780) for 5 years Year 4—(3447 3600 3557) for 2-5 years (15 356 15 305 15 348) for 5 years $P<0.05$ $P<0.01$ $P<0.001$ (one tailed) for protective efficacy.

relatively sustained protection of young children against classical cholera, but the very transient protection of this age group against El Tor cholera.

The better protection against classical cholera might have resulted from the preponderance of classical whole cells in the vaccines: each vaccine contained classical and El Tor cells in a ratio of 3:1. It is also possible that the immunogenicity of the El Tor cells in the vaccines might have been deficient because the inactivation procedures for the vaccines failed to preserve mannose sensitive hemagglutinin, a potentially important immunogen for El Tor cholera.⁷ Finally, it may be relevant that after being absent for many

years in Matlab, classical cholera reappeared during the years immediately prior to the trial and nearly displaced El Tor cholera.⁸ As a result, it is conceivable that the field trial population was more effectively "primed" against classical than against El Tor cholera at the time of vaccination.

Whatever the explanation, the credible performance of the vaccines against classical cholera in young children lends optimism to the possibility of protecting young children with killed oral vaccines if reasons for the selectively poor performance against El Tor disease can be unravelled and overcome by suitable vaccine modifications.

Table 2 Occurrence of cholera episodes among vaccinees during the 5 years of surveillance by age and biotype*

	El Tor 2-5 years	>5 years	Classical 2-5 years	>5 years
Year 1				
Pre-epidemic ^b	6 (57%) (-5%)	8 (71%) (43%)	0 (100%) (38%)	1 (83%) (11%)
Epidemic	18 (-13%) (-127%)	7 (50%) (-19%)	32 (36%) (1%)	21 (75%) (63%)
Entire year	24 (20%) (-38%)	15 (64%) (39%)	32 (40%) (9%)	22 (76%) (64%)
Year 2	25 (11%) (-55%)	24 (57%) (32%)	15 (56%) (22%)	19 (75%) (62%)
Year 3	20 (-11%) (-115%)	22 (48%) (14%)	22 (-22%) (-134%)	16 (58%) (28%)
Year 4	13 (-31%) (-211%)	25 (4%) (-69%)	0 (-)	0 (-)
All years	82 (4%) (-31%)	86 (48%) (33%)	69 (35%) (12%)	57 (72%) (65%)

*The numbers in the table denote number of cases (protective efficacy in percentage) (lower boundary of the 95% confidence interval)
^bPre epidemic period corresponds to the initial 4-6 months of follow-up Table excludes one episode detected in a K12 patient during the first year in which *V. cholerae* 01 vibrios of both biotypes were simultaneously excreted No cases were detected during the fifth year of surveillance Three recurrent episodes were excluded as noted in Table 1 Denominators are given in Table 1 P<0.05 P<0.01 P<0.001 P<0.0001 for protective efficacy

Two earlier trials of parenteral cholera vaccines observed that protection of young children was followed by a 'rebound' higher incidence of cholera in vaccinees than of controls^{6,9,10} A similar rebound with long-term follow-up was also reported during long-term follow up of persons immunized with parenteral vaccines against typhoid¹¹ In this trial there was a suggestion of such a rebound in young vaccinated children during the third and fourth years of surveillance (Table 1) However the higher incidence among vaccinated children was not statistically significant The possibility of such a rebound and the corresponding need for long-term surveillance should be considered in the design of future trials of cholera vaccines

During the 5 years of follow-up the epidemiological pattern of cholera shifted from balanced coexistence of both biotypes to El Tor alone Moreover the incidence of cholera among placebo recipients began at an unexpectedly high rate of ca 5 cases per 1000 placebo recipients, and progressively declined, disappearing completely during the fifth year of surveillance These data highlight the unpredictability of cholera incidence, even in Matlab where cholera is hyperendemic⁵, and underscore the conservatism required for adequate planning of such evaluations¹²

During the years since initiation of this trial several important developments have occurred in the epidemiology of cholera worldwide El Tor cholera appeared in a massive outbreak in Peru in 1991, and now has spread to affect much of the remainder of South and Central America¹³ The Latin American epidemic elicited a renewed interest in vaccination against cholera as a public health measure As a result, two field trials of a newer version of the oral BS-WC containing the same cellular constituents with BS produced by recombinant genetic technology (rBS) currently in progress in Peru, are testing whether the use of booster doses of vaccine can extend the duration of protection against El Tor cholera Whether this different regimen will extend the rather transient protection observed against El Tor

cholera in the Bangladesh trial particularly in young children will be of great interest

The second major development occurred in late 1992 with the appearance of *V. cholerae* 0139 in Asia¹⁴ This organism is capable of causing life threatening cholera on epidemic scale and appears to have pandemic potential However because epidemiological and laboratory studies suggest that natural immunity to *V. cholerae* 01 is not protective against *V. cholerae* 0139¹² there is little likelihood that the killed oral vaccines tested in this trial will prove to be useful against this new form of cholera Thus in addition to improving long term vaccine protection against El Tor cholera especially in young children, future development of killed oral vaccines in a manner that will make them useful public health tools will require inclusion of additional *V. cholerae* 0139 antigens

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Killed whole bacterial cells, a mucosal delivery system for the induction of immunity in the respiratory tract and middle ear an overview

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Abstract

Infectious diseases remain a leading cause of morbidity and mortality worldwide with mucosal membranes being the most frequent portals of entry of pathogenic micro-organisms. This has prompted studies aimed at the development of vaccination protocols that would lead to an increased protection of mucosae through an understanding of the common mucosal immune system as an immune communication network between mucosal sites. Recent studies have suggested that preferential sub-networks exist within the system and these studies have exploited the gut associated lymphoid tissue (GALT)-lung sub-network in the development of oral vaccine strategies for infections of the respiratory tract and middle ear. Mucosal immunization with whole formalin killed *Pseudomonas aeruginosa* (Pa), *Branhamella catarrhalis* nontypable *Haemophilus influenzae* (NTHi) or *Streptococcus pneumoniae* (Spn) results in enhanced homologous bacterial clearance from the lung of immune animals challenged with live bacteria. These studies have been extended to the middle ear where similar results have been observed for NTHi and Spn. Mechanisms responsible for inducing enhanced bacterial clearance from the airways include opsonising antibody antigen specific CD4+ T helper cells cytokine responses and recruitment of activated polymorphonuclear neutrophils. The mechanisms induced by immunization which stimulates the immune system to rapidly mobilise both innate and specific immune responses during infection are the subject of ongoing research. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Mucosal Immunization Bacteria

1 Introduction

The possibility of achieving protection at mucosal surfaces is now feasible using new vaccine strategies. Preparations comprising non living bacterial components have been used for both human and animal mucosal immunization and been shown to provide humoral and cellular responses. The development of immunization approaches that are comprised of immunostimulatory components and maximise antigenicity make vaccination an effective alternative for prevention of bacterial infections.

2 Respiratory pathogens

The predominant pathogens of the respiratory tract are *Streptococcus pneumoniae* (Spn), *Haemophilus influ*

enzae (nontypeable [NTHi] and type b capsulated [Hib]), *Branhamella* (*Moraxella*) *catarrhalis* and *Pseudomonas aeruginosa* (Pa). Disease caused by these bacteria range from infections both acute and chronic primarily confined to mucosal surfaces of the respiratory tract to invasive disease. This paper will focus on studies demonstrating the potential of whole cell immunization delivered mucosally to protect against infection.

B. catarrhalis, NTHi, Hib and Pa are gram negative bacteria whereas Spn is gram positive. Each can exist as commensal microflora of the human respiratory tract and all are major causes of otitis media, lower respiratory tract infections and many other serious infections including invasive diseases [1–4].

Streptococcus pneumoniae is the most significant of these pathogens with respect to morbidity and mortality. It is a major cause of lobar pneumonia, otitis media, sepsis and meningitis [5]. The disease process commences with colonization of the nasopharynx followed by either aerosol aspiration of the Spn into the

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lower respiratory tract resulting in infection or by migration from the nasopharynx through the eustachian tube to the middle ear. Penetration of the mucosa at any of these sites enables the bacteria to enter the vascular compartment which can result in sepsis and meningitis. Many components from the bacteria assist in its virulence once the balance between the human host and the bacterium has shifted from symbiotic commensalism to infection. The presence of components such as pneumolysin facilitate damage to alveolar epithelial cells [6] and influence the levels of inflammatory cytokines particularly tumour necrosis factor alpha (TNF α) [7]. Inflammatory activation of human cells has been shown to shift the targeting of pneumococcus to a different receptor. Phosphorylcholine produced by the bacteria will bind to the G protein coupled platelet activating factor (PAF) receptor of activated cells facilitating enhanced adherence and invasion [8]. The presence of the polysaccharide capsule helps the organism avoid phagocytosis in the blood stream and other components also mediate attachment to host cells.

Until recently *B. catarrhalis* was not considered a significant respiratory pathogen. Studies have now shown the incidence of *B. catarrhalis* infections account for approximately 35% of all upper and lower respiratory tract infections [9], 30% of purulent exacerbations in adults with chronic obstructive pulmonary disease [10] and 15 to 20% of episodes of otitis media [11]. An infection by *B. catarrhalis* induces an immune response against outer membrane antigens of the bacterium [12, 13] and antibody to *B. catarrhalis* is present in secretions of the upper respiratory tract [12]. However many of the proteins on the surface of this bacterium are still not fully characterized nor has an immune response resulting in protection from infection by different *B. catarrhalis* strains been determined. This bacterium has very rapidly adopted significant levels of antibiotic resistance with greater than 80% of clinical isolates producing a β lactamase [14] hence the development of a vaccine strategy against *B. catarrhalis* should be a priority.

Pathogenicity of *H. influenzae* requires consideration of the strain type and as with each of these pathogens begins with colonization of a mucosal surface. Development of disease is dependent upon the ability of the bacteria to colonize the mucosal surface and induce a localized infection or in the case of invasive disease penetrate through the mucosa into the bloodstream [15]. An immune response following infection is strain specific and the diversity of strains that exist for NTHi assist its success in avoiding host defence mechanisms particularly in children, patients with damaged airways or those who are immunocompromised (reviewed in [16]). Several antigens have been proposed as vaccine candidates for this pathogen but

as yet the parameters of the immune responses in animal studies that correlate with protection from infection are still being determined.

P. aeruginosa is an opportunistic pathogen that causes significant morbidity and mortality. A variety of clinical conditions including intensive care support use of immunosuppressive agents, cystic fibrosis, bronchiectasis and many others place many individuals at risk of *Pa* infections. *Pa* is currently a leading causative agent for nosocomial pneumonia in the USA [3]. Once acquired infections with *Pa* are difficult to eradicate and individuals with cystic fibrosis [17] and bronchiectasis [18] remain colonized often with the same *Pa* strain despite therapy and recovery from clinical symptoms. This places them at risk of chronic lung infection and the subsequent deterioration of pulmonary function.

Each of these pathogens has evolved many different virulence characteristics that ensure its survival in the host. Many elegant studies over the last several years have identified bacterial factors that potentially contribute to colonization, virulence or pathogenesis. The importance however of any single virulence component or the interaction of several bacterial components that are essential to the mechanisms for colonization and pathogenesis are still not fully understood. There is substantial evidence to support the roles of the bacterial components in host colonization, tissue damage and disease state however more studies are still required. In addition to this important investigations are also determining the significance and suitability of many of these components whether singly or in combination as vaccine candidates. The development of preventative therapeutic approaches as an alternative to the continued extensive use of antibacterial drugs has several distinctive advantages. An effective vaccine should reduce the level of mucosal colonization thus reducing the incidence of disease, it should provide a cost effective approach to disease management, be more affordable to the world community for disease intervention and reduce the dependence of at risk patients on antibiotic treatment possibly slowing the emergence of antibiotic resistant bacteria.

3 Mucosal immune mechanisms

Mucosal membranes are the most frequent portals of entry of pathogenic micro organisms and as such studies aimed at the development of vaccination protocols that would lead to increased protection from infection are increasingly focusing on mucosal immunity. The common mucosal immune system encompasses areas of the respiratory, alimentary and genitourinary tracts which are guarded immunologi-

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cally by subepithelial accumulations of lymphoid tissue that are not constrained by a connective tissue capsule. The mucosal membranes on the luminal surfaces provide extensive specific and non specific defence mechanisms which interact to control the attachment of invading pathogens. Specific mechanisms of defence involve the action of secretory IgA (sIgA), antigen specific T cells and cytokines, while the mechanisms of non specific defence aim at creating an antagonistic environment for the pathogen. These include mucus secretion, bacterial antagonism and desquamation, recruitment of neutrophils, the release of lysozyme as well as other mechanisms, some peculiar to specific mucosal tissues such as the specialized function of alveolar macrophages.

Recovery from mucosal infection parallels the timing and magnitude of the secretory antibody response and there is reasonable evidence to indicate that specific IgA in a previously immunized individual could confer protection against a fresh challenge; however, there is a limited capacity for antibody to eliminate infection once a pathogen has overcome initial host defence mechanisms. It is under these conditions that if secretory antibody is to participate it would be in conjunction with factors involved in opsonization and phagocytosis. sIgA from bronchopulmonary secretions has been shown to block the bactericidal and opsonising activity of antibody against nontypeable *Haemophilus influenzae* [19]. sIgA can also prevent infection by an invasive enteric pathogen, probably as a result of immune exclusion at the mucosal site [20].

There is now sufficient evidence to show that intestinally-derived B and T-cells traffic to other mucosal tissues, including the lung [21–22] and that intestinal immunization followed by a lung boost provides protection against acute respiratory infection with NTHi in a rodent model [23–25]. The trafficking of effector B and T cells does not only occur with cells primed in the gut, but it has now been established that networks of immunological communication between a variety of mucosal sites exist [26–27].

A model of pulmonary defence mechanisms against bacterial infection induced by mucosal immunization has been developed based on data from animal models and human clinical trials [28]. In summary, bacteria which invade and colonise the airways release lipopolysaccharide which activates alveolar macrophages and up regulates the expression of endothelial cell adhesion molecules. Macrophages are the first line of defence against invading bacteria and in immune animals are able to phagocytose increased numbers of bacteria as well as secrete TNF α locally in the lung. In addition, INF γ is also locally produced, probably by CD4 $^{+}$ T cells. Both of these cytokines further up regulate the expression of endothelial cell adhesion molecules. TNF α also stimulates the release of IL 8 by lung fibro-

blasts and epithelial cells. IL 8 and possibly other chemotactants, including bacterial products, provide a chemotactic gradient from the endothelium to the alveolus. With the up regulation of endothelial cell adhesion molecules, neutrophils are rapidly recruited from blood to the lung tissues. The accumulation of neutrophils in the lung may be enhanced by polymeric IgA antibody which inhibits neutrophil locomotion. Neutrophil phagocytosis of bacteria is activated and maintained by the elevated local levels of TNF α , INF γ and possibly other cytokines such as TNF β and GM-CSF in the presence of opsonising antibody. In the immune state, the release of pro inflammatory cytokines are controlled. In studies comparing cytokine responses in immunized and non immunized rats to pulmonary infection with NTHi [29] or Pa, TNF α levels more rapidly increased to a higher level in the immunized rats, but also decreased very rapidly, indicating that the concentration of this cytokine was under the regulation of factors resulting from immunization. IL 1 α is down regulated in immunized animals [30]. In addition, an appropriate opsonising antibody response is generated with respect to antigen specificity and immunoglobulin isotype and subclass. Whilst the precise control mechanisms have not been identified, T cells are thought to play a major role in the regulatory process. The overall outcome in the immune state is that invading bacteria are cleared more rapidly from the respiratory tract and inflammatory damage in the lung is limited. Evidence to support this model has been derived from a number of studies, including mucosal immunization with whole killed cells in rodent models.

4 Vaccination against infection

The design of effective vaccines against infections by these respiratory pathogens would significantly reduce the incidence of disease, particularly in children, individuals with damaged airways and those who are immunosuppressed. Inactivated microorganisms offer certain advantages as a potential vaccine for mucosal immunization. They are naturally occurring microparticles, they possess multiple antigens and are relatively inexpensive to produce. There are certain limitations, however, that require consideration. Firstly, for Spn, NTHi and *B. catarrhalis*, recurrent infections occur because of strain heterogeneity, thus a single or even multiple strain vaccine with a killed whole cell formulation is possibly not the ideal vaccine. Secondly, the method of inactivation can affect the immunogenicity of essential antigens through denaturation. Thirdly, the potential toxicity of some of the bacterial components such as lipopolysaccharides and finally, if whole killed cell formulations are parenterally delivered.

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ered then inflammation at the site of injection and other side effects present problems with safety and compliance

Oral immunization with formalin killed Pa Spn and NTHi have all been used to protect rats against acute respiratory infection [23 31 32]. In human studies adult patients have been protected against acute bronchitis after receiving a series of immunization with 10^{11} killed *H influenzae* given in enteric-coated tablets [33]. An earlier study using this same vaccine reported a ten fold reduction in the incidence of infection [34] however in both these studies there was no clear correlation between clinical protection and either carriage of *H influenzae* or the level of anti bacterial antibody in saliva

Studies in our laboratory have shown that a regime where rodents received primary immunization targeting gut associated lymphoid tissue (GALT) via inoculation of intestinal Peyer's patches combined with a boost targeting the bronchus associated lymphoid tissue (BALT) was most effective in enhancing pulmonary clearance of *B catarrhalis* and NTHi than a regime of intestinal only or bronchus only immunization (Fig 1). This regime has not only been the most effective following immunization with whole killed cells but was also the most effective with protective antigens. In studies with proteins from NTHi immunization using this regime for P6 [24] P2 [35] and OMP26 [36] enhanced pulmonary clearance was observed. A study in our laboratory investigating immune responses to OMP26 following immunization with different regimes that involved mucosal systemic and combinations of both has found that the intestinal/lung combination was the most effective in protecting against pulmonary infection with NTHi (authors unpublished data). Interestingly however this regime was not the most effective for inducing the highest titres of serum IgG but was the most effective for high levels of serum IgA. Mucosal IgA levels were equivalent in all groups that included a lung immunization including those that had received a lung route only regime (although this regime did not protect). Similar results were also found in comparing mucosal and systemic immunizations for the *B catarrhalis* antigen CD [37]. Intestinal/lung combination mucosal immunization with CD was most effective for clearing pulmonary infection however systemic immunization induced much higher antibody titres both the serum and the lung. Investigation of the bactericidal activity of the antibody responses found that serum from mucosally immunized mice had significantly greater bactericidal activity despite the > 100 fold higher antibody level in the sera from systemically immunized mice.

Investigation of protection from middle ear infections by these pathogens has not been as well developed due to the difficulty in establishing suitable

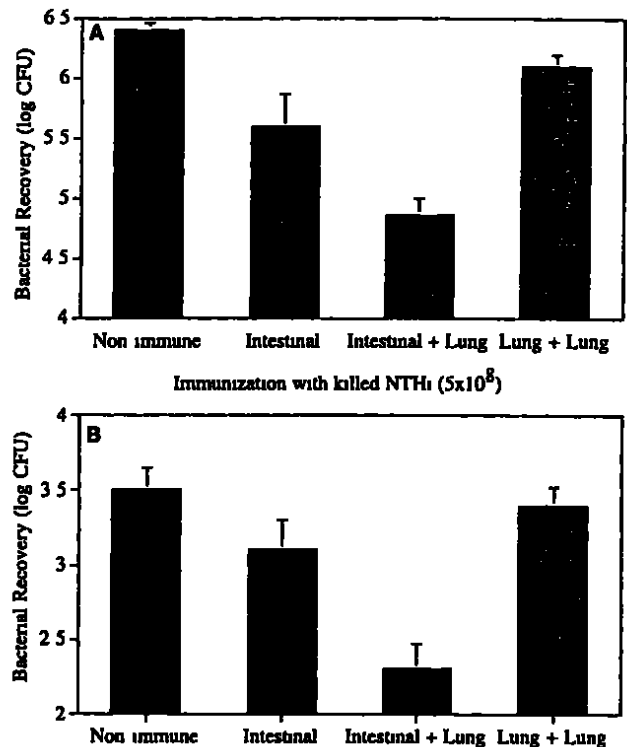


Fig 1 Bacterial recovery in the bronchoalveolar lavage of animals at 4 h post pulmonary challenge with live bacteria (A) On day 0 rats were immunized with 5×10^8 cfu formalin killed NTHi in incomplete Freund's adjuvant via Peyer's patch inoculation for intestinal protocol 5×10^8 cfu formalin killed NTHi in phosphate buffered saline (PBS) via intra tracheal cannulation for lung protocol. On day 14 a lung boost was administered the same as for day 0 protocol. Bacterial challenge was via intra tracheal cannulation with rats receiving 5×10^8 cfu live NTHi in PBS. Intestinal only groups were challenged on day 14 intestinal + lung and lung + lung were challenged on day 21 [23 24]. (B) Mice were immunized with formalin killed *B catarrhalis* by the same regimes as described for NTHi in the rat above except dosages were 10^8 cfu for immunization and 10^7 for bacterial challenge (manuscript in preparation)

animal models. Recently we have established a rat model for investigation of otitis media caused by either Spn NTHi or *B catarrhalis*. In establishing conditions for determining mechanisms involved in protection a whole killed cell formulation was used to immunize the rats. Our results have shown that of the different immunization regimes assessed intestinal immunization followed by a lung boost was the most effective (Table 1). A regime that had substituted an intra nasal boost for the lung boost was not as effective. Further work with the antigen OMP26 has also been found to enhance clearance of NTHi from the middle ear using this intestinal/lung regime (authors unpublished data).

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5 Improving whole killed cell vaccines

Technological advancements during the last decade have made it feasible to design more effective inactivated micro-organisms or their sub-components for mucosal immunization. In some instances success is as simple as ensuring that sufficient numbers of organisms are administered orally. This approach is feasible where disease is the result of infection by a homogenous organism or by bacteria whose strains have limited heterogeneity of essential antigenic components such as for *Vibrio cholera* [38]. For disease resulting from infection with heterogeneous strains for example respiratory infections with nontypeable *H. influenzae* major components of the organism exhibit significant strain heterogeneity hence prior exposure to one strain often does not prevent infection by a new strain [39]. Thus the design of effective oral vaccines to direct the immune response to highly conserved protective antigens in these bacteria presents a significant challenge.

New technologies in molecular biology combined with identification of protective antigens should enable manipulation of microorganisms such as to produce a whole cell vaccine that enhances the quantity and possibly the quality of immune responses to specific antigens. This could include the expression of antigens in the same organism in avirulent or attenuated organisms as well as the inclusion of adjuvants or other agents to manipulate the immune response. In the case of NTHi vectors have been identified for transformation of *Haemophilus influenzae* [40] and so have the potential to be used to design a better whole cell vaccine. Another alternative to the better development of a whole cell vaccine is the use of bacterial lysis technology which involves the incorporation of a temperature sensitive gene in the plasmid that induces bacterial lysis (bacterial ghosts) when growth conditions are

adjusted to a particular temperature [41]. The use of *Actinobacillus pleuropneumoniae* ghosts in an oral delivery was effective in protecting pigs against aerosol challenge [42]. The advantages of this technology include killing of the whole cell without antigen denaturation, removal of cytoplasmic contents which includes the plasmids and their antibiotic resistance genes used in the transformation and removal of the cytoplasm will result in a reduction of non relevant antigens.

Recombinant avirulent bacteria may offer an effective oral delivery formulation for some antigens. Of particular interest have been live attenuated typhoid and nontyphoid *Salmonella* species [43]. The genetic manipulation of these attenuated bacteria enables delivery of foreign viral, bacterial or parasitic antigens in an immunogenic form [44]. They have the potential to be multivalent vaccines and have been tested with a number of parasites, viruses and the bacterial antigen of enterotoxigenic *E. coli*, *Streptococci*, *Clostridium tetani* (tetanus) and *Shigella* spp. [44].

More recently *Lactobacillus* and *Lactococcus* have been investigated as potential non pathogenic recombinant vaccine delivery vehicles [45]. Mice immunized by subcutaneous and oral regimes with *Lactococcus lactis* expressing tetanus toxin fragment C developed significant protective titres of antibody to the tetanus toxin fragment C in their serum [46].

The concept of including cytokines as immunomodulatory agents in vaccine delivery has attracted attention. IL-1 has demonstrated adjuvant activity and peptide components of IL-1 are capable of acting as an adjuvant and provided they do not exhibit their natural proinflammatory action may be suitable for inclusion in human vaccines [47]. Recombinant IL-15 [48] and IL-12 [49] have both demonstrated potent adjuvant activity by enhancing the immune response to antigens. The use of recombinant bacteria as carrier vaccines and the inclusion of immune modulators for use in humans will depend upon whether an acceptable level of safety can be attained.

Table 1
Effect of mucosal immunization on bacterial clearance from the middle ear^a

Immunization group ^b	Bacterial recovery ^c (log ₁₀ cfu)
Non immune	3.74 ± 0.23
Killed NTHi	2.69 ± 0.50
Non immune	5.05 ± 0.60
Killed <i>Streptococcus pneumoniae</i> type 3	3.93 ± 0.50

Authors unpublished data. ^bSprague Dawley rats were immunized on day 0 intestinally via direct inoculation of Peyer's patches with $1-5 \times 10^8$ cfu killed bacteria and boosted in the lung on day 14 via intra tracheal cannulation. ^cBacterial recovery from middle ear lavage following challenge on day 21 with live bacteria inoculated into the middle ear through the tympanic membrane. Recovery post challenge was at 4 h for NTHi and 20 h for Spn. $P < 0.05$ as determined by *t* test.

6 Conclusion

The development of vaccines involving a whole cell formulation for any of the respiratory pathogens NTHi, Spn, Pa or *B. catarrhalis* utilizing some of the advances in molecular technologies has the potential to be an effective, cost efficient alternative to a parenterally administered component vaccine. Studies have shown that mucosal immunization with whole cell formulations are extremely effective at protecting against infections caused by these respiratory pathogens. However, optimization of this strategy will require the con-

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struction of new bio-engineered microbes as the delivery particle

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Extended recombinant bacterial ghost system

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Abstract

Controlled expression of cloned Φ X174 gene *E* in Gram negative bacteria results in lysis of the bacteria by formation of an E-specific transmembrane tunnel structure built through the cell envelope complex. Bacterial ghosts from a variety of bacteria are used as non living candidate vaccines. In the recombinant ghost system foreign proteins are attached on the inside of the inner membrane as fusions with specific anchor sequences. Ghosts have a sealed periplasmic space and the export of proteins into this space vastly extends the capacity of ghosts or recombinant ghosts to function as carriers of foreign antigens. In addition S layer proteins forming shell like self assembly structures can be expressed in candidate vaccine strains prior to E mediated lysis. Such recombinant S layer proteins carrying foreign epitopes further extend the possibilities of ghosts as carriers of foreign epitopes. As ghosts have inherent adjuvant properties they can be used as adjuvants in combination with subunit vaccines. Subunits or other ligands can also be coupled to matrixes like dextran which are used to fill the internal lumen of ghosts. Oral aerogenic or parenteral immunization of experimental animals with recombinant ghosts induced specific humoral and cellular immune responses against bacterial and target components including protective mucosal immunity. The most relevant advantage of recombinant bacterial ghosts as immunogens is that no inactivation procedures that denature relevant immunogenic determinants are employed in this production. This fact explains the superior quality of ghosts when compared to other inactivated vaccines. The endotoxic component of the outer membrane does not limit the use of ghosts as vaccine candidates but triggers the release of several potent immunoregulatory cytokines. As carriers there is no limitation in the size of foreign antigens that can be inserted in the membrane and the capacity of all spaces including the membranes periplasma and internal lumen of the ghosts can be fully utilized. This extended recombinant ghost system represents a new strategy for adjuvant free combination vaccines. © 1999 Elsevier Science B V. All rights reserved.

Keywords: Bacterial ghost, Combination vaccines, Adjuvants, Delivery system

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1 Introduction

In many countries all over the world unreliable sources of clean water inadequate food supply primitive sanitation overcrowding, and poor health conditions are factors which favour contagious infectious diseases. The perception that a multivalent vaccine given orally will provide the recipient with immunity against a variety of infectious diseases may be very ambitious. However it is now known that new generations of combination vaccines based on emerging new technologies may help to achieve this goal.

Vaccination with killed microorganisms enables the immune system to come into a risk free contact with an otherwise life threatening pathogen. The use of killed pathogens as substitutes for living infectious agents has been widely used as a principle for vaccine development. Non living vaccines can easily be produced by chemical or physical inactivation of pathogenic bacteria. Some of the new approaches to vaccine development have included the use of purified subunits synthetic peptides chemical conjugation of polysaccharides and proteins and the use of recombinant bacteria and viral vectors or nucleic acids as vaccines. While most vaccines are safe they often require additional adjustments to improve the quality of the vaccine such as adjuvants better methods of presenting antigens or improvement of vaccine stability.

In our view the extended recombinant ghost system represents new approaches in vaccine development and provides alternative platform technologies which can create better qualities of combination vaccines. In the end it will no longer be desirable mixing together existing vaccines and trying to overcome chemical and immunological obstacles for the combinations used. The extended recombinant bacterial ghost system is currently envisaged targeting and combining as many foreign antigens as possible into a multivalent vaccine. These candidate vaccines do not need any adjuvants should be stable without the requirement of a cold chain easy to administer and be effective early in life.

2 Protein E-mediated lysis of bacteria and production of bacterial ghosts

Bacterial ghosts are empty cells with intact envelopes. They have a natural outer surface make up which provide them with the original targeting functions of the pathogen they are derived from and are thus able to induce strong local immunity. Ghosts are produced by controlled expression of cloned bacteriophage Φ X174 lysis gene *E* (Witte et al. 1990a), which leads to the formation of a transmembrane tunnel structure through the cell envelope of pathogenic bacteria. The resulting bacterial ghosts share all functional and antigenic determinants of the envelope with their living counterparts.

Lysis gene *E* codes for a membrane protein of 91 aa which is able to fuse inner and outer membranes of Gram negative bacteria (Witte et al. 1990b, 1993). Through this tunnel, with border values fluctuating between 40 and 200 nm, the cytoplasmic content of the bacteria is expelled leaving an empty internal space devoid of nucleic acids, ribosomes or other constituents (Witte et al. 1992). The driving force for the release of cytoplasmic material is the osmotic pressure difference between the cytoplasm and the medium created by the opening of the tunnel structure. Measurements of membrane bound ATPase activities of ghosts showed higher specific activities when compared to membrane fragments produced by French press indicating that the inner membrane structure of ghosts is well preserved (Witte et al. 1992). Since tritiated water was taken up faster than labeled sucrose (which cannot diffuse through the inner membrane but can equilibrate through the tunnel) by the ghosts it was concluded that the water filling the interior of the ghosts is acquired during the lysis process from diffusion through the intact inner membrane. This fact also indicates that the portion of inner membrane not involved in tunnel formation remains intact during expulsion of cytoplasmic material (Witte et al., 1992). Electron micrographs clearly show a sealed periplasmic space at the border of the lysis tunnel (Witte et al. 1990a, 1992).

Except for the lysis hole the morphology of the bacteria including all cell surface structures and

appendices is not affected by the lysis event. Recently the hypothesis that a functional host cell autolytic system is required for E mediated lysis (Lubitz et al. 1984) was reinvestigated by comparing the murein composition of the bacteria after lysis with the induction of the autolytic system by EDTA. The analysis showed that the overall composition of murein is not changed by E mediated lysis. Nevertheless murein degradation seems to be stimulated by the action of protein E as shown by an increase in the total amount of murein turnover products by about 10%. However peptidoglycan sacculi prepared from E-lysed cells remain intact emphasizing the fact that the diameter of the E specific transmembrane tunnel structure is determined by the mesh size of the surrounding murein (Witte et al. 1998a). The changes in the host cell phospholipid composition mediated by protein E (Lubitz and Pugsley, 1985) most probably facilitate the membrane fusion of inner and outer membranes.

Protein E, a structural protein devoid of any enzymatic functions, can be divided into 4 domains (Witte et al., 1990b). The current working model of E mediated lysis divides the process into three phases (Fig. 1a–d). Phase 1 is characterised by integration of protein E into the inner membrane with its C terminal part facing the cytoplasmic side (Fig. 1a). Phase 2 (Fig. 1b, c) is initiated by a conformational change of protein E transferring its C terminus across the inner membrane and assembling into multimers at potential cell division sites (Fig. 1b, arrow). In phase 3 (Fig. 1d) a local fusion of the inner and outer membranes is achieved by a transfer of the C terminal domain of protein E towards the surface of the outer membrane of the bacterium (Schon et al. 1995). The initiation mechanism for such a conformational change is most probably a *cis-trans* isomerization of the proline 21 residue within the first membrane-embedded α helix of protein E. Exchange of P21 to other amino acids results in lysis negative E mutant proteins (Witte et al. 1997).

Proteinase K accessibility studies with a reporter group fused to the very C terminus of protein E are well in accordance with the proposed model. The C terminus of protein E is

moved through the intact envelope complex from the inner side of the cytoplasmic membrane to the outer side of the outer membrane. It should be emphasized that the very C terminal end of protein E domain 4 (aa 85–91), which is very hydrophilic, is not essential for this process but contributes to a better efficiency of the process (Schuller et al. 1985). The requirement of a two membrane system for E mediated lysis has been demonstrated. Protein E induces cell killing but not lysis in Gram positive cells (Halfmann et al., 1993).

Electron microscopic studies emphasize that the protein E specific transmembrane tunnel structure which permeabilizes the bacterium is not randomly distributed over the cell envelope but is restricted to areas of potential division sites predominantly in the middle of the cell or at polar sites (Fig. 1b triangle). Mutant strains with defects in cell division (*ftsZ84 ftsA12*) are tolerant to protein E mediated lysis whereas others like *ftsA3*, *ftsQ* and *ftsI* are lysed at conditions not permissive for cell division. These results suggest that initiation of cell division rather than specific functions of the septosome plays an essential role in protein E mediated lysis (Witte et al. 1998a).

3 Chimeric E-L lysis genes

By the construction of lysis gene E L (Harkness and Lubitz 1987) a chimeric lysis construct from phage Φ X174 and MS 2 lysis genes E and L respectively and the characterization of lysis events induced by protein E L and a family of internal truncated E L forms (Fig. 1e–h) it was shown that cell lysis and ghost formation can also be induced by an alternative mechanism (Witte et al. 1998b). Protein E L introduces a rather large hole in the cytoplasmic membrane of Gram negative bacteria and cell lysis occurs at the time point when the peptidoglycan is locally degraded at the rupture site of the inner membrane (Fig. 1f). As a consequence the outer membrane released from its rigid underlying layer is expanded until it can no longer withstand the osmotic pressure (Fig. 1g, h). The inner and

outer membranes are not sealed by this mechanism as detected by a great loss of periplasmic marker enzymes (Witte et al 1998c) Thus two different lysis mechanisms leading to

ghost formation can be distinguished depending on the nature of the membrane proteins penetrating either the inner membrane (E-L) or the inner and outer membranes (E)

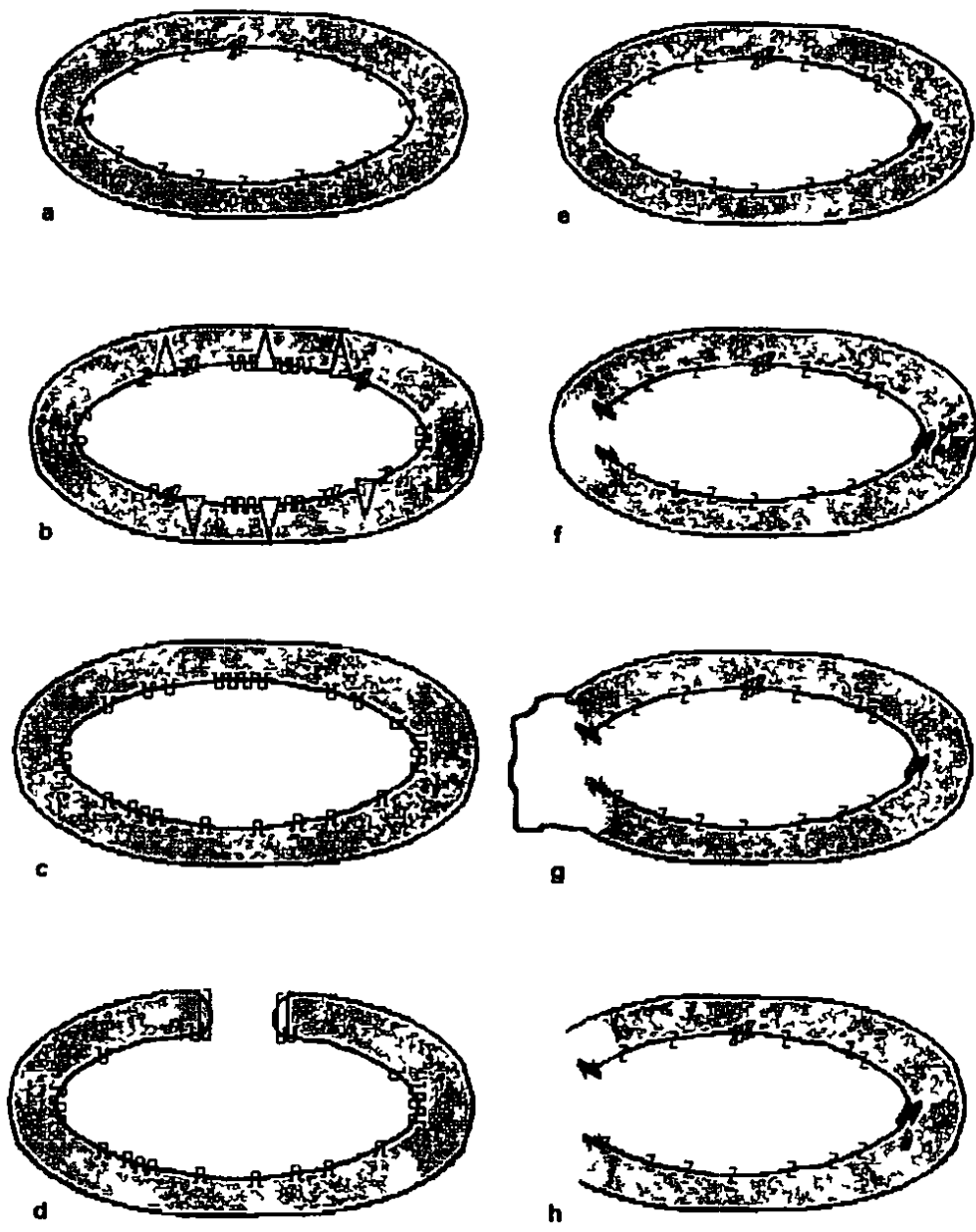


Fig 1 Schematic representation of ghost formation from Gram negative bacteria (a–d) E-mediated lysis (Δ) different conformations of protein E (▲) potential cell division zones (e–h) E-L mediated lysis (Z) protein E-L. The outer oval rings presents the envelope complex with inner membrane periplasmic space and outer membrane. The central area represents the cytoplasmic space

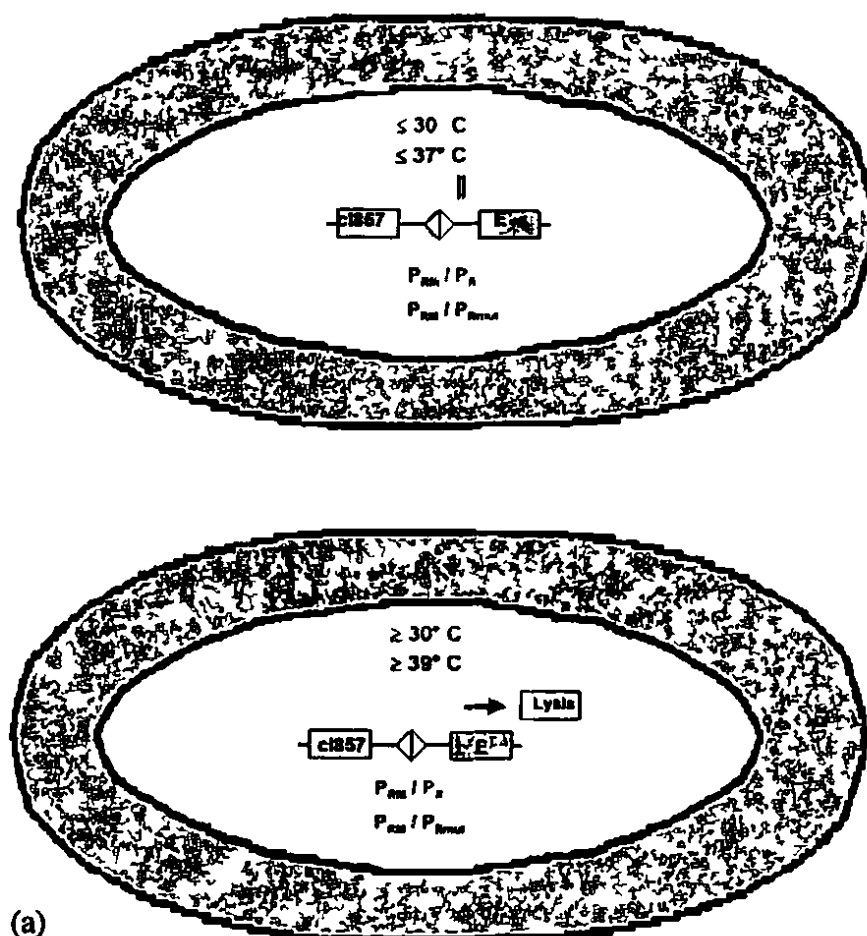


Fig 2 (a) E specific lysis cassettes The E specific lysis cassette consists of the lysis gene *E* of bacteriophage PhiX174 under transcriptional control of the rightward Lambda promoter and the corresponding temperature sensitive repressor *cI857* P_{RM}/P_R promoters of phage Lambda P_{RM} is analogous to P_R except a T $\rightarrow$ C mutation in O_R2 of the promoter (II) Repression of gene *E* expression ($\rightarrow$) gene *E* expression at temperatures given above promoter symbols (Φ) (b) Cold sensitive E specific lysis cassettes The *lacI* gene is under transcriptional control of the temperature sensitive (ts) *cI857* repressor and is expressed either from a wildtype (wt) Lambda P_R or from a mutated Lambda P_{RM} promoter Due to the inactivation of *cI857* repressor molecules at temperatures above 30 or 37 C the P_{RM} wt or P_{RM} mut driven gene *lacI* expression respectively results in high amounts of Lac repressor molecules which block the expression of gene *E* (top graph) At temperatures below 30 or 37°C *lacI* gene expression from P_R or P_{RM} mut respectively is prevented by functional *cI857* repressor molecules As a consequence the Lac repressor is no longer synthesized and after dilution of the Lac repressor below a critical value gene *E* expression from *lacPO* takes place (middle graph) leading to E mediated lysis of the cells and empty ghosts (bottom graph)

4 Control of gene E expression

Expression of lysis gene *E* can be placed under transcriptional control of either the thermosensitive $\lambda p_L/p_R$ -*cI857* or under λp_{PO} *lacI*^q promoter repressor systems Several E specific lysis plasmids with different resistance markers origins of replication and gene *E* expression control have been

published (Szostak et al 1996) Recently, a major breakthrough in temperature control of E mediated lysis has been achieved by mutating the Lambda p_R gene expression regulation circuit (Fig 2) For the rightward λp_R promoter and the temperature sensitive repressor *cI857* expression of gene *E* is stably repressed at temperatures up to 30°C, while gene *E* expression is induced at tem

peratures higher than 30°C with optimal induction at 42°C. Thus for the production of first generation ghosts bacteria were grown at 28°C to mid log phase before lysis was induced by temperature upshift to 42°C.

Because the antigenic determinants on the surface of bacteria can change under different environmental conditions and heat shock can be induced following temperature up shift to 42°C it seemed favourable to extend the heat stability of the λp_R promoter/ $cl857$ repressor system allowing pathogens to grow at 37°C before induction of lysis. The mutated λp_R promoter/operator region resulted in new expression systems (Fig. 2a) which stably repress gene *E* expression at temperatures up to 37°C but still allow induction of cell lysis at a temperature range of 39–42°C (Jechlinger et al

1998a). This small window of 2°C for temperature controlled induction of *E* mediated ghost formation of bacteria avoids induction of heat shock proteins. On the other hand it might also be of advantage to grow bacteria at 37°C and induce *E* mediated lysis at a lower temperature. Such a cold sensitive system for ghost formation could be obtained by combining the λp_R promoter/ cl repressor system with either the $lacI/lacPO$ or the phage 434 cl/pR system for control of gene *E* expression (Fig. 2b). In the latter system $cl857$ of λ and cl from phage 434 do not interfere with each other and recognize only their proper operator sites (Jechlinger et al 1998b).

E mediated lysis has been achieved in various Gram negative bacteria including *Escherichia coli*, *Salmonella typhimurium*, *Salmonella enteri*

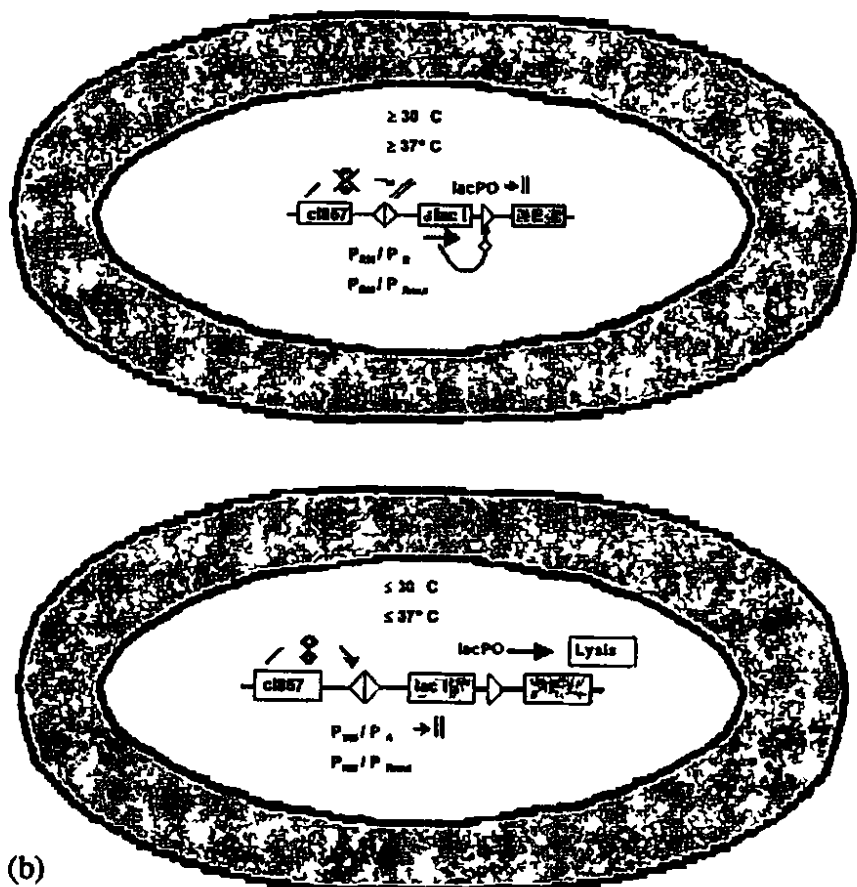


Fig. 2 (Continued)

tidis Klebsiella pneumoniae Bordetella bronchiseptica Helicobacter pylori Vibrio cholerae Actinobacillus pleuropneumoniae Haemophilus influenzae Pasteurella haemolytica Pasteurella multocida Pseudomonas aeruginosa Pseudomonas putida Ralstonia eutropha and Erwinia cyripedii. This broad spectrum of bacteria shows that E mediated lysis might work in every Gram negative bacterium provided that the E lysis cassette can be introduced into the new recipient by an appropriate vector allowing proper control of gene E expression.

5 Induction of immune responses to bacterial ghosts in experimental animals

In order to determine the immune response to bacterial ghosts different animals including mice rabbits foxes and pigs were used. They were immunized either by the intraperitoneal, subcutaneous or aerogenic route with or without booster immunizations. One of our most extensively studied model is the aerogenic immunization of animals. Inhalation and deposition within the airways are the initial steps preceding adherence and colonization of the respiratory tract by respiratory pathogens. Once the microorganisms are deposited in the lung lining fluids they do not remain at the location where they first came in contact with the mucous membranes. Lung clearance mechanisms translocate all deposited particles and most of them are swallowed and cleared via the gastrointestinal tract (Hensel and Lubitz, 1997).

Aerosol immunization is a safe way to induce complete protection against pleuropneumonia in pigs caused by the lung pathogenic bacterium *A. pleuropneumoniae*. For this purpose an aerosol infection model was developed for pigs with standardized inhalation conditions for the recipients. Methods have been established to determine deposition sites and to obtain specimens for analysis of local immune responses (Hensel et al., 1996). This infection model can induce several stages of the disease from acute pleuropneumoniae to chronic infections. Aerosol vaccinations of swine with *A. pleuropneumoniae* ghosts showed that pro-

TECTIVE immunity can be established by this route of application.

Detailed analysis of the immunological parameters for the protective immunity in swine against *A. pleuropneumoniae* will be given elsewhere (Brand, Hensel, Lubitz, in preparation). For the local immune response either ghosts or irradiated bacteria showed a net influx of plasma cells and lymphocytes into the lung lining fluid as well as significant increases in specific IgG. Concurrently the CD4⁺–CD8⁺ ratio was found to increase after aerosol immunization. The lymphocyte subsets of IgG⁺ and IgA⁺ cells were found to be significantly higher in the irradiated group than in the group that received bacterial ghosts. The latter group showed a significant increase in IgA and IgM and a net influx of lymphoid blasts and granulocytes in the bronchoalveolar lining fluid (Katinger et al., 1998).

Ghosts from *V. cholerae* (VCG) given intraperitoneally to mice evoke high levels of vibrio specific IgG responses and high titres of vibriocidal antibodies in sera (Eko et al., 1994a). Recently *V. cholerae* ghosts from the O1 and O139 serotypes expressing the toxin coregulated pilin protein (TCP) were shown to induce high vibriocidal antibody activity in rabbits. We have also shown that antibodies to VCG are sufficient to protect infant mice from experimental cholera when used to pre-treat the challenge inoculum. Interestingly sera from rabbits immunized with TCP positive O139 vibrios successfully protected infant mice against a TCP positive O1 bacterial challenge (Eko, Attridge, Lubitz, unpublished). In addition *V. cholerae* ghosts can be used as carriers of foreign antigens (Eko et al., 1994b).

Other experiments showed that single dose vaccination of one day old chicks with *E. coli* O78 K80 ghosts given either i.m. or in drinking water showed high survival ratios when compared to the control after challenge on day 20 (Brand, Hahn, Lubitz, unpublished). Dose dependent induction of protective immunity against *P. multocida* was induced in mice after two i.m. immunizations (on days 0 and 14) followed by challenge on day 24 (Marchart, Schlapp, Lubitz, unpublished).

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From the foregoing analysis of immune responses in different animal models indicate that ghosts induce humoral and cellular immune responses against cell envelope constituents including protective immunity against challenge infections (Hensel et al. 1996; Szostak et al., 1996). It should be pointed out that all ghost vaccination experiments were carried out with freeze dried ghosts (aerosol application) or freeze dried ghosts resuspended in saline or water for i.m. and oral route immunizations, without the addition of adjuvants, stabilizers or any other additives.

6 Bacterial ghosts as adjuvants

The ability of a vaccine to induce an immune response depends not only on the molecular properties of the antigen or on the immunogenic susceptibility of the host but also on the antigen formulation. Adjuvants like tapioca, Alum, Freund's adjuvants, Cholera toxin, *E. coli* heat labile toxin (LT), lectins, muramyl dipeptide, ISCOMS, lipid A and other cell wall constituents as well as microencapsulation with natural or synthetic polymers and liposomes have been used to potentiate non specifically the immune response to a target antigen. Although it is not fully understood how each of the adjuvants act to enhance an immune response it is believed that prolonged deposition of the antigen at the injection site and the induction of immune modulators are major properties of adjuvants. Other important roles include the activation of macrophages and antigen presenting cells. Bacterial ghosts contain some well known immune stimulating compounds such as LPS or lipid A and peptidoglycan. Thus ghosts per se or ghosts used as carriers of foreign proteins should enhance the immune response against target antigens.

In the simplest version of the use of ghosts as adjuvant a foreign antigen is mixed with a suspension of the bacterial ghosts. The resulting preparation combines soluble antigens together with antigens carried in the internal lumen of the ghosts (Fig. 3a). When the immunogenicity of HIV 1 reverse transcriptase (RT) formulated with

ghosts, Alum or CFA was compared following subcutaneous administration the immune response evoked by RT was comparable in all three formulations. With the intraperitoneal route RT mixed with ghosts induced a higher IgG response than when mixed with CFA or Alum (Szostak et al. 1993). Humoral and cellular immune responses to ghost structures have been determined and it was seen that both arms of the immune system are stimulated by the ghosts (Szostak et al., 1996) which is in accordance with the finding that bacterial constituents are presented by primary immune cells together with MHC I and MHC II (Svensson et al. 1997). The adjuvant component(s) of ghosts consist(s) mostly of cell specific receptor recognition abilities of the envelope contributing to effective uptake by macrophages, and other primary antigen presenting cells.

It has been shown that blood derived dendritic cells (DC) cultured in the presence of cytokines are more effective in endocytosis than any other cell types (Steinman and Swanson 1995). Granulocyte macrophage CSF stimulate dendritic cells and process live intact Gram negative bacteria for peptide presentation by MHC I and MHC II. These data suggest that dendritic cells may be important in priming both CD4+ and CD8+ T cells to bacterial antigens (Svensson et al. 1997). As we detect a strong humoral and cellular immune response against bacterial ghosts the capacity for uptake of ghosts by DC is of specific interest. This work is still in progress. The experiments performed showed that porcine DC from peripheral blood mononuclear cells can be enriched by adherence on plastic in combination with short term cultivation of the cells on collagen lattice. The enriched DC fraction failed so far to internalize ghosts whereas FITC conjugated dextran was taken up by pinocytosis.

The endotoxin content of the Gram negative cell wall has been suggested as a potential problem for non living vaccines. Bacterial ghosts prepared from *E. coli* and *S. typhimurium* which induced dose dependent antibody responses against bacterial cells or their corresponding LPS when administered intravenously to rabbits did not induce significant fever responses as deter-

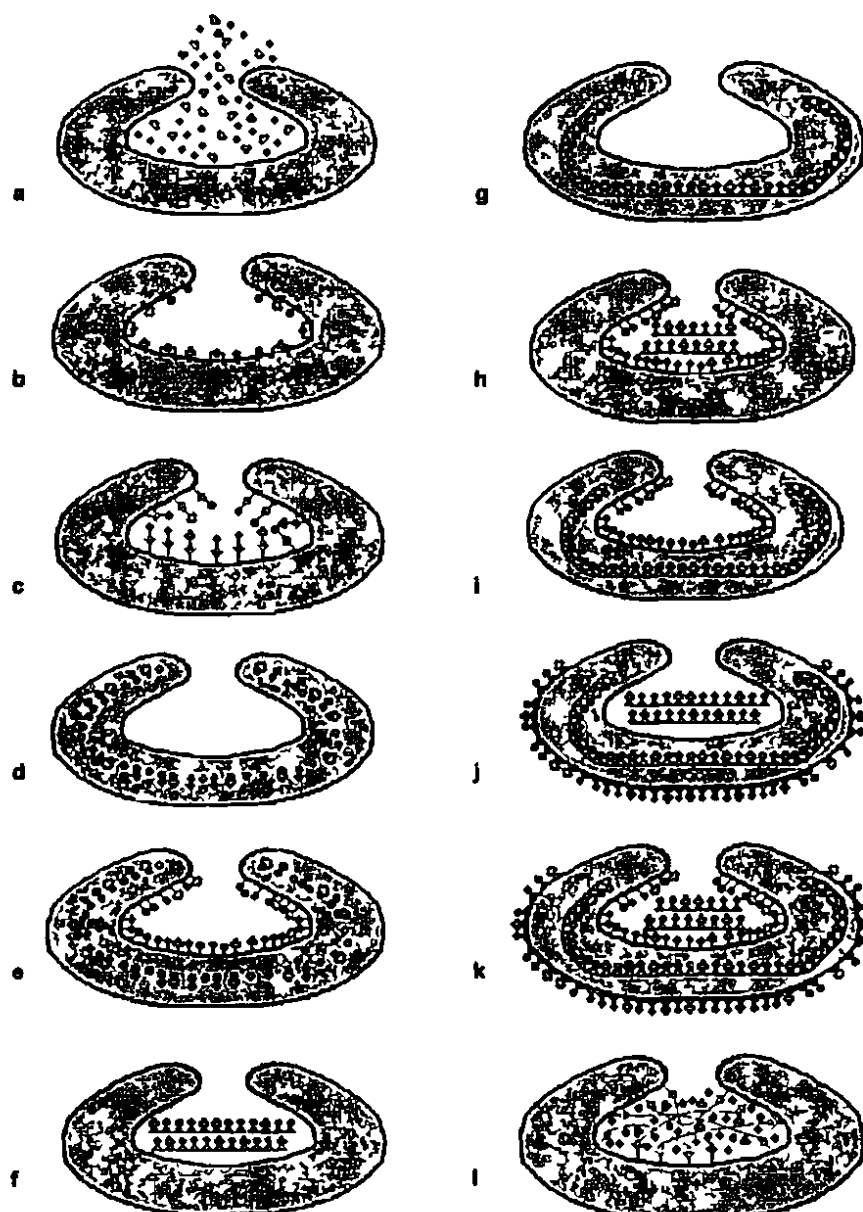


Fig. 3 Bacterial ghosts as adjuvant and/or carriers for foreign target antigens (a) Ghosts are mixed with different antigens () and act as carrier and adjuvant for the induction of the immune response (b) Antigens are attached to the inside of the cytoplasmic membrane via (J) N terminal (L) C terminal or (J L) N and C terminal anchor sequences (c) Membrane anchored streptavidin (Y) can bind any biotinylated (b) target antigen to the inner membrane (d) Target antigens can be exported to the periplasmic space (pp) either by their own signal sequence (●) or as fusion partner with polypeptides carrying a signal sequence (##) (e) Ghost with a combination of membrane anchored target antigens and antigens exported to the pp (f) Recombinant S layer proteins rSbsA ($\Delta \Delta \Delta$) or rSbsB ($\Delta \Delta \Delta$) as carriers of foreign antigens filling the cytoplasmic space of ghosts (g) rSbsA rSbsB proteins with foreign target antigens exported to the pp (h) Combination of membrane anchored antigens and rSbsA rSbsB target antigens in the inner lumen of the ghosts (i) Combination of membrane anchored target antigens and rSbsA and rSbsB antigens in the pp of ghosts (j) Combination of target antigens on rSbsA rSbsB carriers in the inner lumen the pp and on the outer surface of ghosts (k) Combination of target antigens immobilized by membrane anchors or on rSbsA rSbsB-carriers in the inner lumen pp and outer surface of ghosts (l) Ghost with membrane anchored streptavidin and biotinylated polymer filling the inner lumen of the ghosts together with soluble target antigens

mined by test methods recommended by the US pharmacopoeia. It is concluded that the LPS content of ghosts does not limit the use of bacterial ghosts as candidate vaccines (Mader et al. 1997).

7 Recombinant bacterial ghosts

For the production of combination vaccines against bacterial and viral pathogens or the use of bacterial ghosts as carrier systems for other antigens, a membrane targeting system was developed for the attachment of foreign protein entities to the inner side of the cytoplasmic membrane (Szostak and Lubitz 1991). By cloning the foreign DNA sequences into the membrane targeting vector pMTV5, any gene of interest can be expressed as a hybrid protein with N-C or N/C terminal membrane anchors directing and attaching the fusion protein to the envelope complex of the bacteria (Fig. 3b) prior to E-mediated lysis. The current list of membrane anchored target proteins comprises various viral core or envelope proteins and bacterial target antigens or enzymes. In the latter example, it has been shown that the enzymatic activities of β -galactosidase and PHB synthase were not impaired, indicating that the membrane anchors do not interfere with the proper folding of the target proteins and that clustering and self assembly is possible. The membrane anchored HIV1-RT and HIV1 gp41 target antigens carried by recombinant ghosts induced humoral as well as a cellular immune responses in animal models (Szostak et al. 1993, 1996). It should be noted that the system of membrane anchoring is not limited by the size of the foreign protein moieties attached to the inside of the inner membrane and that combinations of different antigens to be anchored are possible (Fig. 3b). If streptavidin is anchored to the inner membrane, biotinylated proteins can be attached to such sites by resuspending freeze-dried streptavidin ghosts in a solution of the biotinylated protein (Fig. 3c).

As the periplasmic space of ghosts is sealed, proteins exported to the periplasmic space prior to E-mediated lysis become part of the ghost (Fig. 3d) and thus potential immunogens. This finding

provides the possibility to combine membrane anchored target antigens with other antigens of either homologous or heterologous nature exported to the periplasmic space (Fig. 3e).

8 Recombinant S-layer proteins in combination with ghosts

Expression of the cloned surface layer (S layer) genes *sbsA* and *sbsB* of *Bacillus stearothermophilus* in *E. coli* (Kuen et al. 1994, 1995, 1997) led to accumulation of sheet-like self-assembling SbsA or SbsB proteins in the cytoplasm. The S layer self-assembly products consist of several hundred thousand monomers per cell and because of their ability to assemble into a superstructure do not form inclusion bodies. When expression of S layer sheets in *E. coli* is followed by E-mediated lysis, the S layer structures are not released to the external medium but are retained within the inner lumen of the cytoplasmic space (Fig. 3f).

Site-directed mutagenesis of *sbsA* and *sbsB* and structural-functional analysis of S layer domains essential for intra- and/or inter-molecular interactions revealed flexible surface loops in both proteins that accept foreign sequences coding of up to 600 amino acids (Truppe et al. 1997). In the latter case, the inserts tested were the enzymes PHB synthase and the Lux AB fusion protein which retained their enzymatic activities, indicating that the flanking S layer sequences do not interfere with the proper folding of the fused protein moieties. If antigens are used as inserts, S layers can act as carriers of target antigens (Fig. 3f–k). Most recently, it has been shown that *sbsA* S layer proteins equipped with a signal sequence for the periplasmic space can also assemble within this space (Fig. 3g, i–k).

It is not clear whether the immobilisation of the target antigen(s) within the S layer has any additional beneficial effect on the induction of the immune response with respect to adjuvanticity or whether the adjuvant property is dominated by the ghost envelope. It can be envisaged that multi-vaccine components can be derived by mixing together different recombinant S layer subunits, each carrying different immunodeterminants of

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pathogens which assemble into the S layer superstructure within a recombinant ghost carrying other membrane anchored target antigens in its envelope (Fig 3h–k). The nature of the bacterial envelope will determine to a large extent if a recombinant S layer carrying foreign target antigens can also be mounted on the surface of a ghost and be combined with recombinant S layers in the cytoplasmic and periplasmic spaces (Fig 3j, k).

9 Interaction of bacterial ghosts and S-layer proteins with human endothelial cells or macrophages and induction of immunomodulatory mediators

Endothelial cells strongly respond to bacterial cell wall components like LPS by IL 6 release and de novo expression of E-selectin. CD14, a membrane antigen expressed on the surface of monocytes and macrophages but not on endothelial cells, acts as a receptor for LPS and LPS complexed with LPS-binding protein (LBP). The soluble form of CD14 (sCD14) is present in the plasma of healthy individuals and several studies have revealed that LPS/LBP complex binds to serum derived sCD14 and activates endothelial cells. Human umbilical vein endothelial cells (HUVEC) responded to *E. coli* ghosts with IL 6 release and surface E-selectin expression, whereas the IL 6 release by *B. stearotheophilus* S layers was very weak. Anti-CD14 and anti-LBP antibodies both inhibited IL 6 release and E-selectin expression in a dose dependent manner after stimulation with ghosts but had no effect on S layer stimulated cells. These findings suggest that bacterial ghosts but not S layers activate HUVECs via sCD14 and LBP dependent mechanisms (Furst-Ladani et al 1998).

The synthesis of inflammatory mediators in human macrophages/monocytes seen after stimulation with LPS involves the binding of LPS complexed LBP to CD14. The effects of anti-mouse CD14 antibodies on the synthesis of TNF α and PGE₂ in mouse macrophages stimulated by *E. coli* and *S. typhimurium* ghosts, LPS, lipid A and S layer preparations has recently been

reported (Haselberger et al 1997). LPS ghosts and S layers all induced TNF α and PGE₂ synthesis as well as the accumulation of TNF α mRNA. Pre-incubation with anti-mouse CD14 antibodies resulted in a dose dependent inhibition of TNF α and PGE₂ synthesis after stimulation by LPS, lipid A and ghosts. The bacterial S-layer induced mediator synthesis remained unchanged following the addition of anti-mouse CD14 antibodies. Adding fetal calf serum (FCS) strongly enhanced the release of cell mediators stimulated by low doses of LPS and ghosts. These results show that CD14 is highly relevant for the activation of mouse macrophages by ghosts and that S layers might act through other activation pathways.

10 Bacterial ghosts as carriers and targeting vehicles

The fact that bacterial ghosts preserve the native cell wall components including broad adhesive structures like fimbriae of their living counterparts makes them suitable for attachment to specific target tissues. Envelopes made from pathogenic enterobacteria, for instance, could be used to target the gastrointestinal tract. Due to their antigenic surface, ghosts are readily taken up by macrophages (Haselberger et al 1997) and depending on the ghost should present an ideal tool for the targeting of M-cells in the gut with vaccines and drugs.

In a modified E-mediated lysis procedure for bacteria, high concentrations of MgSO₄ are used to inhibit transmembrane tunnel formation. This effect has been used to prime cells for lysis and allows the easy harvest of cells by centrifugation. Resuspension of the cell pellet either in water or low ionic strength buffers results in immediate lysis of the cells. Electron micrographs show that such a lysis procedure induces larger holes in the cell envelope located either in the middle of the cell or at the periseptal anuli (Resch et al 1998). Such ghosts with large holes can be used as empty bags in which various substances can be filled.

As the specific receptor recognition properties of ghosts are the same as their living counterparts, they attach to tissue surfaces specific for the

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pathogen which they are derived from. This quality makes the ghosts well adapted for targeting material entrapped within them or attached to them to specific surfaces of animal or human tissues. The intracellular space can be filled either with water soluble substances or emulsions such that the substance of interest or the matrix can be coupled to appropriate anchors on the inside of the cytoplasmic membrane of recombinant ghosts (Fig. 3).

A variety of different membrane anchored candidate molecules for binding different antigens and/or immunomodulators can be combined to produce multivalent vaccine components. Methods for preparing and filling ghosts with various materials are currently under development. For example, ghosts with streptavidin anchored on the inside of the cytoplasmic membrane can be filled by resuspending lyophilized ghosts in solutions carrying the biotinylated substances to be targeted. Biotinylated alkaline phosphatase has been coupled to the streptavidin anchor without loss of enzymatic activity. It has also been shown that nucleic acids either alone or attached to a matrix can be efficiently packaged into bacterial ghosts.

For various purposes it is advantageous to fill the internal space of the ghost with a substituted matrix which then binds the substance of interest. In model experiments, biotinylated fluorescent labeled dextran has been used to completely fill the internal space of streptavidin ghosts. As substituted dextrans have high capacity for binding peptides, drugs or other substances, therapy and prevention seems to be feasible with the extended recombinant ghost system.

11 Perspectives

The possibility of modulating the different arms of the immune response by regulating the growth and differentiation of T and B lymphocytes due to the context and type of presentation of the different antigens within a multivaccine ghost is currently under investigation. The capacity of all spaces of ghosts including the periplasmic membranes and the internal lumen to carry foreign

antigens seems to be surprisingly large and should be developed in the extended recombinant ghost system to design new combination vaccines.

Many questions remain to be answered, e.g. which formulation of the target antigens packed into the ghost envelope structures either as membrane anchored proteins or as paracrystalline recombinant S layer proteins is of advantage and whether loose packaging of antigens into the inner space of the envelopes or in the form attached to a matrix induces the best balanced immune response against the target antigens. It is obvious that different combinations of target antigens might need different components of the system for appropriate vaccine formulation and presentation to the immune system. The extended recombinant ghost system has great strategic potential in vaccine development and drug targeting. Thus this new platform technology warrants further investigation.

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201300

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT 800176089-2

RECIBO OFICIAL DE CAJA 05 - 59,183
FECHA AGOSTO 9 DE 2005

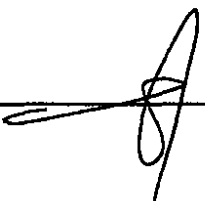
***** CONSIGNACION *****

DEPOSITANTE	TIPO PAGO	BANCO	CUENTA	No PAGO	FECHA PAGO	Vr PAGO
CASTILLO GRAU Y ASOCIADOS	CONSIGNACION	BANCO POPULAR	050-00110-6	33200965	08/08/2005	364 000 00

***** CONCEPTO *****

CANT	RENTISTICO	CONCEPTO	TOTAL CONCEPTO
1	50005-01-03	CAMBIO DE MODALIDAD Y MODIFICA	
		12 MARCAS Y LEMAS COMERCIALES	91,000 00
		TOTAL	\$ 91,000 00

SON NOVENTA Y UNO MIL PESOS

RESPONSABLE 

RECIBO DE CAJA APLICADO AL EXPEDIENTE No _____

37

**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISIÓN DE NUEVAS CREACIONES**

2020
Bogotá, D C ,

Doctor
GERMAN CASTILLO GRAU
Smithkline Beecham Biologicals
(Apoderado)

ASUNTO	Radicación	00-58242
	Trámite	002
	Evento	001
	Actuación	430
	Oficio	10296
	Folios	

Como resultado del examen de fondo, realizado con fundamento en el artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le comunica

Documentos estudiados

Los textos estudiados fueron la descripción (Folios 5 a 162) y las reivindicaciones 1 a 56 (folios 163 a 178)

La presente solicitud reivindica prioridad británica N° GB9918319 de agosto 3 de 1999 y cumple con lo establecido en los artículos 9, 10 y 11 de la Decisión 486 de la Comisión de la Comunidad Andina

La respuesta presentada el 25 de enero del 2001 no será tenida en cuenta, pues la redacción tan confusa y el alto grado de incoherencia como "Una ampolla o llamado farmacéuticamente ampolleta, es una vesícula con membrana exterior (ME) aislada a partir de cepas " no permiten bajo ningún concepto lógico comprender la idea que el solicitante desea expresar. Adicionalmente, dicho documento hace alusión a un nuevo capítulo reivindicatorio que no fue adjuntado

Objeto de invención

El objeto de la presente solicitud se refiere a preparaciones de vesículas para la elaboración de vacunas, obtenidas mediante la reducción de los antígenos inmuno dominantes o no-protectores. También se reclama la obtención de vesículas por otros procesos los que involucran la sobre regulación de la expresión de antígenos OMP dentro de la vesícula mediante la introducción de un promotor de alta eficiencia corriente arriba del gen que codifica dicho antígeno, la sobre regulación de la expresión de antígenos OMP protectores que son condicionalmente expresados eliminando los mecanismos de control represivo de estos, modificar la porción del lípido A de los LPS bacterianos dentro de la preparación de la vesícula reduciendo o apagando la expresión de un gen responsable de la toxicidad del lípido A, modificar la porción del lípido A del LPS bacteriano durante la preparación de la vesícula mediante la introducción de un promotor de alta eficiencia corriente arriba de un gen envuelto en la conversión de la porción del lípido A de LPS a menos tóxico, reducir la

toxicidad del lipido A e incrementar el nivel de los antígenos protectores incorporando un gen codificante para polimixina A, Crear antígenos OMP conservados deletando las regiones variables en el gen que codifica el antígeno, reducir la expresión de los antígenos que tienen alguna similitud con estructuras humanas, sobrerregular la expresión de antígenos OMP protectivos mediante la introducción de copias extras del gen codificante bajo la regulación de un promotor de alta eficiencia

El objeto de la solicitud definido anteriormente se clasificó en la IPC C12N 15/00, C12N 15/09, A61K 39/02

Claridad de la solicitud

El artículo 30 de la Decisión 486 de la Comisión de la Comunidad Andina establece que "Las reivindicaciones definirán la materia que se desea proteger mediante la patente. Deben ser claras y concisas y estar enteramente sustentadas por la descripción"

Las reivindicaciones podrán ser independientes o dependientes. Una reivindicación será independiente cuando defina la materia que se desea proteger sin referencia a otra reivindicación anterior. Una reivindicación será dependiente cuando defina la materia que se desea proteger refiriéndose a una reivindicación anterior. Una reivindicación que se refiera a dos o más reivindicaciones anteriores se considerará una reivindicación dependiente múltiple."

En general la invención adolece de concisión pues es muy difícil establecer el alcance real de las reivindicaciones dado su excesivo número y complejidad. En la presente solicitud se encuentra un número elevado de cláusulas independientes que podrían formularse de una manera dependiente dado su alcance. Adicionalmente, algunas reivindicaciones contienen un número demasiado alto de alternativas.

Por otra parte el término ampollas no corresponde a la traducción más apropiada para la palabra "bleb", se sugiere modificar dicho vocablo por "vesícula". De igual manera se sugiere modificar el título.

A lo largo del capítulo reivindicativo se reclama materia que no se encuentra bien definida y por lo tanto no es posible establecer el alcance de las reivindicaciones.

Ejemplo Reiv 41 "Un vector adecuado para realizar los sucesos descritos en las reivindicaciones 3-34" Un vector adecuado puede ser cualquiera de los existentes en el estado de la técnica. No se describen en ningún momento los componentes del vector, es decir secuencias regulatorias, promotores, secuencias codificantes, haciendo imposible la diferenciación de la materia reclamada, respecto de aquella existente en el estado de la técnica.

Reiv 42 Una cepa bacteriana gram-negativa modificada de la que se prepara la preparación de ampollas de las reivindicaciones 1 a 34. Las cepas reclamadas en la presente cláusula pueden ser cepas ya conocidas en el estado de la técnica, pues en la presente solicitud no se aporta ninguna información (Número de depósito) que permita diferenciarla de cualquier otra cepa modificada a partir de la cual se puedan generar vesículas para usarla en formulaciones de vacunas.

Reiv 43 Una vacuna de ampollas, fantasma o de células enteras muertas no tóxica adecuada para uso pediátrico. ¿Cuáles son ESPECIFICAMENTE las células muertas empleadas en la preparación? ¿Cuál es la formulación específica de la vacuna?

Reiv 43 Una secuencia polinucleotídica aislada que se hibrida en condiciones muy

estrictas con al menos una parte de 30 nucleótidos de las sec N° ID 2-23 etc
 Un poli nucleotido se define unicamente mediante la secuencia de nucleotidos que contiene, más no por las secuencias a las cuales se pueda unir, pues secuencias no complementarias pueden hibridizar entre sí. Adicionalmente, esta reivindicación es tan amplia que un numero muy alto de los poli nucleotidos existentes en el estado de la técnica podrian hibridizar con cualquiera de 30 nucleótidos de los 1000 que hacen parte de tan solo una de las secuencias descritas en la presente solicitud. Este tipo formulación en la reclamacion de poli nucleotidos es netamente especulativa y se recomienda retirarla de todo el pliego reivindicatono

Imprecisiones como las encontradas en las reivindicaciones 41 a 43 se encuentran a lo largo de todo el capitulo reivindicatono. Es necesario que el solicitante revise nuevamente todo el pliego de cláusulas y elimine todo elemento como los ejemplificados que le confieren a la solicitud un caracter vago y especulativo, y no delimitan el alcance de la protección que se pretende encontrar

Por otra parte las reivindicaciones 4, 7, 10-13 se refieren a secuencias X y Y, las cuales carecen de significado, no constituyen aspectos tecnicos de la invencion y no permiten establecer con clardad cual es la materia a proteger

De acuerdo con lo anterior, el capitulo reivindicatono en general no es conciso ni claro y por ende no cumple con los requerimientos del articulo 30 de la Decisión 486 de la Comisión de la Comunidad Andina

Evaluación de la Unidad de Invencion

El articulo 25 de la Decisión 486 de la Comisión de la Comunidad Andina establece "La solicitud de patente sólo podrá comprender una invención o un grupo de invenciones relacionadas entre sí de manera que conformen un unico concepto inventivo "

En la presente solicitud se identifican 9 objetos de invención diferentes

Invención 1 (Reiv 1-7, 12-15, 20-22, 26,27, 35-40, 42,54-56) Se refiere a preparaciones de vesículas para la elaboración de vacunas, obtenidas mediante la reducción de los antígenos inmuno dominantes o no-protectores

Invención 2 (Reiv 1-10, 13-15, 20-22, 26,27,35-40,42,54-56) Preparaciones de vesículas para la elaboración de vacunas que involucra la sobre regulación de la expresión de antígenos OMP dentro de la vesícula mediante la introducción de un promotor de alta eficiencia corriente arriba del gen que codifica dicho antígeno,

Invencion 3 (Reiv 1-7, 13-15, 20-22, 26,27,35-40,42,54-56) Preparaciones de vesículas para la elaboracion de vacunas que implica la sobre regulacion de la expresión de antígenos OMP protectores que son condicionalmente expresados eliminando los mecanismos de control represivo de estos,

Invención 4 (Reiv 1-7, 13-15, 20-22, 26,27,35-40,42,54-56) Preparaciones de vesículas para la elaboración de vacunas que comprende modificar la porción del lipido A de los LPS bacterianos dentro de la preparacion de la vesicula reduciendo o apagando la expresion de un gen responsable de la toxicidad del lipido A,

Invención 5 (Reiv 1-10, 13-15, 20-22, 26,27,35-40,42,54-56) Preparaciones de vesículas para la elaboracion de vacunas que implica modificar la porción del lipido A del LPS bacteriano durante la preparación de la vesicula mediante la introducción de un promotor de alta eficiencia corriente arriba de un gen envuelto en la conversion de la porcion del lípido A de LPS a menos toxico,

Invención 6 (Reiv 1-7, 13-15, 20-22, 26,27,35-40,42,54-56) Preparaciones de vesículas para la elaboracion de vacunas que involucra la reducción de la toxicidad del lipido A e incrementar el nivel de los antígenos protectores incorporando un gen

codificante para polimixina A,

Invencción 7 (Reiv 1-7, 13-15, 20-22, 26,27,35-40,42,54-56) Preparaciones de vesículas para la elaboración de vacunas que implica crear antígenos OMP conservados deletando las regiones variables en el gen que codifica el antígeno,

Invencción 8 (Reiv 1-7, 13-15, 20-22, 26,27 35-40,42,54-56) Preparaciones de vesículas para la elaboración de vacunas que implica reducir la expresión de los antígenos que tienen alguna similitud con estructuras humanas,

Invencción 9 (Reiv 1-10, 12-15, 20-22, 26,27,35-40,42,54-56) Preparaciones de vesículas para la elaboración de vacunas que envuelve sobreregular la expresión de antígenos OMP protectivos mediante la introducción de copias extras del gen codificante bajo la regulación de un promotor de alta eficiencia

Siendo que la preparación de vesículas preparadas mediante ingeniería genética a partir de bacterias gram-negativas es bien conocida (*Vaccine*, 13 no 4, 1995, pp 401-407 y *vaccine* vol 14, no 10 1 July 1996 pp1001-1008) y que el concepto de modificar la porción del lípido A de LPS para obtener vacunas adecuadas, son ampliamente conocidos en el estado de la técnica se conceptúa que no existe ningún concepto común inventivo entre las invenciones mencionadas. Por otro lado los métodos de obtención de vesículas de las invenciones 1 a 9 no guardan ninguna relación entre ellos

Según lo anterior la(s) reivindicación(es) de las anteriores invenciones no guardan unidad de invención entre ellas

Excepciones de Patentabilidad

El artículo 20 de la Decisión 486 se establece "No serán patentables a) las invenciones cuya explotación comercial en el territorio del País Miembro respectivo deba impedirse necesariamente para proteger el orden público o la moral. A estos efectos la explotación comercial de una invención no se considerará contraria al orden público o a la moral solo debido a la existencia de una disposición legal o administrativa que prohíba o que regule dicha explotación b) las invenciones cuya explotación comercial en el País Miembro respectivo deba impedirse necesariamente para proteger la salud o la vida de las personas o de los animales o para preservar los vegetales o el medio ambiente. A estos efectos la explotación comercial de una invención no se considerará contraria a la salud o la vida de las personas de los animales o para la preservación de los vegetales o del medio ambiente sólo por razón de existir una disposición legal o administrativa que prohíba o que regule dicha explotación c) las plantas los animales y los procedimientos esencialmente biológicos para la producción de plantas o animales que no sean procedimientos no biológicos o microbiológicos d) los métodos terapéuticos o quirúrgicos para el tratamiento humano o animal así como los métodos de diagnóstico aplicados a los seres humanos o a animales "

La reivindicación 55 reclama un procedimiento de inmunización de un huésped humano contra una enfermedad causada por la infección de una o más bacterias entre *Neisseria*, *Moraxella*, *Pseudomonas*, *Chlamydia*, tal procedimiento comprende la administración al huésped de una dosis inmunoprotectora de la preparación de ampolla de las reivindicaciones 1-34. Siendo que el literal d) del artículo 20 de la Decisión 486 exceptúa de patentabilidad los métodos terapéuticos de tratamiento humano o animal, se considera que la materia reclamada en la cláusula 55 no es patentable

Reivindicaciones de uso

El artículo 14 de la Decisión 486 de la Comisión de la Comunidad Andina establece "Los Países Miembros otorgarán patentes para las invenciones sean de producto o procedimiento en todos los campos de la tecnología siempre que sean nuevas tengan nivel inventivo y sean susceptibles de aplicación industrial"

La reivindicación 56 reclama "la utilización de la preparacion de ampolla de las reivindicaciones 1-34, en la preparacion de un medicamento para la inmunización de un huésped humano " Teniendo en cuenta que la Decision 486 unicamente contempla patentes para invenciones de productos o procedimientos, se considera que la utilizacion reclamada en la cláusula en mencion, se encuentra fuera del alcance del artículo 14 de la misma Decision

Determinación del estado de la técnica

El artículo 16 de la Decisión 486 de la Comisión de la Comunidad Andina establece que "El estado de la técnica comprenderá todo lo que haya sido accesible al publico por una descripción escrita u oral utilización comercialización o cualquier otro medio antes de la fecha de presentación de la solicitud de Patente o en su caso de la prioridad reconocida

Sólo para el efecto de la determinación de la novedad también se considerara dentro del estado de la técnica el contenido de una solicitud de patente en trámite ante la oficina nacional competente, cuya fecha de presentación o de prioridad fuese anterior a la fecha de presentación o de prioridad de la solicitud de patente que se estuviese examinando siempre que dicho contenido esté incluido en la solicitud de fecha anterior cuando ella se publique o hubiese transcurrido el plazo previsto en el artículo 40"

Considerando que la fecha de presentación de la primera solicitud de patente de la cual se reivindica prioridad es 3 de agosto de 1999 se buscaron documentos del estado de la tecnica relacionados, con fecha de publicación anterior a esta

Se hizo la busqueda de documentos relevantes en el estado de la técnica, entendiendo como estado de la técnica la definicion provista en el artículo 16 de la Decision 486 de la Comisión de la Comunidad Andina y las excepciones fijadas en el artículo 17 de la misma

Se encontró como antenondades (documentos cuya fecha de publicacion es anterior a la fecha de presentación de la primera solicitud de patente sobre la cual se reivindica prioridad o de la fecha de presentación de esta solicitud) que pueden afectar la patentabilidad del objeto de la presente solicitud

Nº	Documento	Titulo	Fecha de Publicación
1	Claasen I et al Vaccine vol 14 no 10 pp 1001-1008	Production Characterization and control of a Neisseria meningitidis hexavalent class"	1996-07-01
2	Van der Ley et al Vaccine vol 13 no 4 pp 401-407	"Construction of a Neissenameningitidis strain carryng multiple chromosomal copies of the porA gene for use in the production of a multivalent outer membrane vesicle vaccine	1995
3	Helminen et al Infection and immunity vol 61 no 5 pp 2003-2010	"A major outer membrane protein of moraxella catarrhalis is a target for antibodies that enhance pulmonary clearance of the pathogen in an animal model"	1993-05-01
4	US 5098997 Aniglonis Algis et al	24 de marzo de 1992	1992-03-24

Evaluación de los requisitos de Patentabilidad

El Articulo 14 de la Decision 486 de la Comisión de la Comunidad Andina el cual establece "Los Países Miembros otorgarán Patentes para las invenciones sean de producto o de procedimiento en

todos los campos de la tecnología siempre que sean nuevas tengan nivel inventivo y sean susceptibles de aplicación industrial"

Se procedió a evaluar los requisitos de novedad, nivel inventivo y aplicación industrial con el fin de establecer la patentabilidad de la invención reivindicada en la solicitud en estudio

Evaluación de Novedad

El artículo 16 de la Decisión 486 de la Comisión de la Comunidad Andina establece que "Una invención se considerará nueva cuando no esté comprendida en el estado de la técnica" "El estado de la técnica comprenderá todo lo que haya sido accesible al público por una descripción escrita u oral utilización comercialización o cualquier otro medio antes de la fecha de presentación de la solicitud de patente o en su caso de la prioridad reconocida

Sólo para el efecto de la determinación de la novedad también se considerará dentro del estado de la técnica el contenido de una solicitud de patente en trámite ante la oficina nacional competente cuya fecha de presentación o de prioridad fuese anterior a la fecha de presentación o de prioridad de la solicitud de patente que se estuviese examinando siempre que dicho contenido esté incluido en la solicitud de fecha anterior cuando ella se publique o hubiese transcurrido el plazo previsto en el artículo 40"

Esta se evaluó por comparación entre lo solicitado y los documentos encontrados en el estado de la técnica y que se relacionan con el objeto reivindicado y de acuerdo con la definición de novedad del Artículo 16 de la Decisión 486 de la Comisión de la Comunidad Andina

Las reivindicaciones 1-3, 6, 12-16, 21, 23, 26 y dependientes se refieren a una preparación de vesículas aisladas de *Neisseria meningitidis*, *Moraxella catarrhalis* o *Haemophilus influenzae* obtenidas mediante la reducción de antígenos variables inmunodominantes o no protectores durante la preparación de la vesícula

El Documento D1 revela vesículas de membrana externa (ME) las cuales son preparadas de cepas modificadas genéticamente de *Neisseria meningitidis*, las cuales expresan proteínas de membrana (Por A) Adicionalmente, para la construcción de las cepas apropiadas para vacunas se eliminó la expresión de los OMP de clase 3 porque esta proteína inducía muy pobremente la generación de anticuerpos bactericidas Por otra parte en el mismo documento también se menciona que la expresión del B-polisacárido y de lacto *N-tetraosa* son eliminados, pues dichas estructuras son comunes al huésped y generan respuestas inmunes muy pobres contra ellas mismas

El Documento D2 revela cepas de *Neisseria meningitidis* construidas para ser usadas en la elaboración de vacunas de vesículas de membrana externa El objetivo era remover los componentes de membrana externa no deseados y al mismo tiempo mejorar el rango de protección En este documento se emplean métodos similares a los reclamados en la presente solicitud en los que se describe la transformación con constructos de plásmidos y su recombinación homóloga en el genoma meningocócico

De acuerdo con lo anterior las reivindicaciones 1-3, 12-16, 31, 48, 53 a 54 no son nuevas en virtud de las anterioridades D1 y D2

Evaluación de Nivel Inventivo

El artículo 18 de la Decisión 486 de la Comisión de la Comunidad Andina establece que "Se

considerará que una invención tiene nivel inventivo si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica"

El Nivel Inventivo se evaluo por comparación entre lo solicitado y encontrado en el estado de la tecnica y que se relaciona con el objeto reivindicado y de acuerdo con la definicion de nivel inventivo del Artículo 18 de la Decisión 486 de la Comisión de la Comunidad Andina

Las reivindicaciones 3 a 13 que tratan aspectos relativos al evento de recombinación carecerian de nivel inventivo dado que las características específicas del proceso de recombinación y las diferentes variantes de este hacen parte de los conocimientos de una persona medianamente versada en la materia Así pues que contemplarlos en el desarrollo de la presente invención resulta mas que obvio

Las reivindicaciones 2, 3, 21, 26 , 53 y 54 reclaman preparaciones de vesículas obtenidas de *Moraxella catarrhalis* o *Haemophilus influenzae*, como tambien reclaman los procedimientos y las cepas a través de los cuales se elaboran

El Documento D3 describe vesículas de membrana externa de *Moraxella catarrhalis* y su importancia en el proceso de vacunación El documento D4 por su parte revela vacunas que comprenden proteínas de membrana externa de *Haemophilus influenzae*, PBOMP-1 y 2, obtenidas por tecnologia de ADN recombinante

A la luz de los documentos D1 o D2, resulta obvio para un técnico en la materia que las proteínas de membrana externa pueden ser formuladas efectivamente en vesículas como las descritas en D1 y D2 independientemente de la cepa bacteriana que las origine

Como se observa, el objeto de las reivindicaciones 2 a 13, 21, 26, 53 y 54 podria resultar obvio para una persona del oficio normalmente versada en la materia y derivarse de manera evidente del estado de la técnica

En conclusión los requisitos de patentabilidad de la presente solicitud se encuentran afectados así

Documento	Reivindicaciones afectadas	Requisito que afecta
1	1-3 12 16 31 48 53 a 54 2, 3, 21, 53 y 54	Novedad Nivel Inventivo
2	1 3 12-16 31 48 53 a 54 2, 3, 26 53 y 54	Novedad Nivel Inventivo
3	2 3 21 53 y 54	Nivel Inventivo
4	2 3 26 53 y 54	Nivel Inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del termino de sesenta dias contados a partir de la fecha de la notificación de la presente comunicacion En caso de no dar respuesta al presente requerimiento o si a pesar de la respuesta subsistieran los impedimentos para la concesion, se denegara la patente

Notifiquese el presente oficio conforme a lo dispuesto en el numeral 5 2, literal e), del capitulo quinto del titulo primero de la Circular Unica N° 10 de 2001

315308


ALIX CARMENZA CÉSPEDES DE VERGEL
Jefe de la División de Nuevas Creaciones

El anterior requerimiento fue notificado por fijación en lista, de
fecha 03 NOV 2005

CONCEPTO TECNICO N° 1725	EXAMINADOR TECNICO Código N° 202063	2005-10-28°
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EXAMEN TÉCNICO DE FONDO			
N° Radicación		Fecha de presentación	
N° Prioridad		Fecha de Prioridad	