Vaccines and vaccine trials

The development of new meningococcal vaccines

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Meningococcal meningitis represents a worldwide problem. It is caused by *Neisseria meningitidis* and occurs both in endemic and epidemic forms. The endemic disease, caused by different serogroups (A, B, C, Y, W135) occurs with an incidence of 1-5/100,000 and appears mainly in infants 6 months to 2 years old. Each serogroup of *N. meningitidis* produces a distinct capsular polysaccharide. Antibodies directed against the capsules can provide protection from meningococcal disease (1). The current antimeningococcal vaccine, composed of group A, C, Y and W135, is highly immunogenic, with no significant adverse reactions, in subjects older than 2 years. In very young children it does not induce a good antibody response (2). Poor immunogenicity in children is a limitation shared by many polysaccharides as a consequence of their T-independent character (3). This drawback has usually been overcome by coupling immunogenic proteins to polysaccharides or oligosaccharides to convert these antigens to a T-dependent form. In our laboratory we have developed a conjugate vaccine against Meningococcal A and C using CRM197 as the carrier protein. This vaccine has been successfully tested in adult volunteers in a pilot study. The vaccine proved to be safe and immunogenic (4). A second preparation of the vaccine has been tested in a phase I study involving volunteers in the US by NIAID, and also in this case the vaccine resulted safe and immunogenic, and comparable to the commercially available polysaccharide vaccine. The vaccine was tested in The Gambia in 2 month old infants. In addition, this lot of investigational Meningococcal A/C vaccine has been tested in toddlers in the USA and has recently received approval for testing in infants in the UK. To date, a total of about 335 humans have received our vaccine preparations and the total number of injections administered have been 46 in adults, about 50 in toddlers and about 540 in infants.

Although a strong immunogenic response was measured in all subjects, these results were tainted by the observation that in the Gambian study a rapid decrease in antibody titres was recorded 3 months after the third dose. These results have spurred us to re-examine the characteristics of our Meningococcal A/C vaccine, in particular to the size of the oligosaccharides which were conjugated to the carrier protein. Starting with acid hydrolysed Meningococcal C polysaccharide we have developed a chromatography method that exquisitely permits the removal of low molecular weight oligomers which in competition ELISA assay proved to be poorly inhibitory when compared to the intact polysaccharides and to oligomers having a higher

molecular weight.

The Oligomers of meningococcal C polysaccharides are polyanions, thus by choosing a buffer of adequate ionic strength it is possible to adsorb on an anion exchange column those species having a greater overall charge and elimination during the loading of the column unwanted species. The bound oligomers can then be recovered using an isocratic buffer at higher salt concentrations. A development study has been completed in which we were able to eliminate better than 95% of all those species having less than 6 sialic acid residues. We have successfully fractionated two large lots, 16 g each, and these oligosaccharides have been conjugated to CRM197, yielding 50,000-80,000 doses of vaccine. Clinical trials with these two lots are planned for this coming fall.

The development of a vaccine against group B *N. meningitidis* is much more problematic for two important reasons. One, the capsular polysaccharide is not immunogenic in humans (5) and two, its structure appears identical to polysialic acid present in many mammalian tissues (6), and it can be considered a self antigen. To increase its immunogenicity, in animal model systems, we have synthesized several conjugates of meningococcal B polysaccharide linked to carrier proteins (tetanus toxoid or CRM197), through an adipic acid dihydrazine (ADH) spacer. All conjugates induced a strong immune response in mice. However, most of the antibodies were not directed against the polysaccharides and could not be inhibited by the polysaccharide alone. Further studies established that many of antibodies recognized a new epitope involving the junction between the polysaccharide and the spacer generated during the coupling reaction. While the majority of the measured immune response was directed against the above new epitope, a small but significant response was also measured against the meningococcal B polysaccharide. The anti-meningococcal B antibodies elicited were of the IgM and IgG class, inhibited by the polysaccharides and showed good bactericidal activity. We will also present data that different adjuvants elicited a graded immune response.

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Immunization of juvenile rhesus monkeys with group B *Neisseria meningitidis* capsular polysaccharide-protein conjugate vaccines

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Group B *Neisseria meningitidis* continues to be a serious public health problem in many countries. Although several efficacy trials have been carried out with outer membrane protein (OMP) vaccines with an efficacy of between 50-80% (4), these vaccines as used have not protected young children. We are therefore evaluating better immunization schedules for protein vaccines and as reported here, polysaccharide-protein conjugate vaccines.

Group B meningococcal capsular polysaccharide (GBPS) is implicated in virulence and protection. Its poor immunogenicity (6) is attributed mainly to the presence of "(268) polysialic acid in fetal mammalian tissues (3). In recent years, GBPS is rendered immunogenic by covalently coupling with various protein carriers (2, 5). However, these conjugates have not been used in humans, because of concern for autoimmune consequences.

We obtained 7 different GBPS vaccines from different sources and evaluated their immunogenicity in juvenile rhesus monkeys. GBPS non-covalently complexed with OMV was obtained from Dr. W. Zollinger, GBPS-CRM_{197} conjugate from Dr. Rino Rappouli of Biocine-Sclavo, and N-propionylated GBPS- OMP3 conjugate mixed with aluminium hydroxide or stearyl tyrosine from Dr. Joseph Tai of NAVA Inc., GBPS-OMP conjugate with and without MPL+TDM and *E. coli* K92-TT conjugate were synthesized and characterized at the FDA (2).

One year-old rhesus monkeys were immunized intramuscularly three times at weeks 0, 6 and 14. Blood samples were subjected to chemistries at 0, 2, 8 and 16 weeks. Sera were collected at weeks 0, 2, 6, 8, 14, 16, 22 and 30 and analysed for capsular- and protein-specific antibodies by ELISA. GBPS complexed with methylated human serum albumin (HSA) (1) or GBPS-HSA conjugate were used to study the binding specificities of capsular antibodies.

There was variation in antibody response among individual monkeys. All GBPS vaccines except *E. coli* K92-TT elicited a two-fold or greater increase in GBPS

antibodies after the first immunization. All vaccines including the K92-TT elicited a rise in GBPS antibody level of five-fold or more after the third immunization. Most of the antibodies elicited by N-propionylated GBPS-OMP3 vaccines were directed to the N-propionylated GBPS-HSA antigen rather than to native GBPS. The K92-TT conjugate elicited antibodies to both groups B and C polysaccharides.

Rhesus antibody response to GBPS-OMP conjugate administered with MPL+TDM adjuvant was increased 4-fold compared to the conjugate given in saline after the first immunization. The GBPS response of monkeys immunized with N-propionylated GBPS-OMP3 vaccine in aluminium hydroxide was better than that elicited by the same vaccine in stearyl tyrosine. Both combinations showed an IgM to IgG class switch following the second immunization, thus showing a T cell-dependent memory response.

All conjugate vaccines induced booster antibody responses to the corresponding carrier proteins. GBPS-OMP conjugates elicited an increased antibody response to OMP compared to the GBPS non-covalently complexed with OMV. The bactericidal activity of vaccinated rhesus sera was examined using complement from different sources (7). Antibodies elicited by GBPS-OMP conjugate administered with and without MPL+TDM were most bactericidal in presence of human and rabbit complements. The conjugate-induced antibodies did not appear to cause any visible safety related symptoms in the rhesus.

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Development of conjugate vaccines against *Neisseria meningitidis*

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Neisseria meningitidis is the major cause of bacterial meningitis worldwide. Although the current polysaccharide vaccine is efficacious, its principle drawback is its inability to elicit protective and long-lasting immunity in young children and in some older individuals. Polysaccharide-protein conjugate vaccines have been demonstrated to overcome these deficiencies.

Groups A and C meningococcal polysaccharide (PS) conjugates were prepared by reductive amination to tetanus toxoid and tested for immunogenicity in mice. These preclinical studies have focused on four areas: (1) vaccine lot consistency, (2) antigen dose response, (3) time course of antibody response throughout the immunization schedule, and (4) immunoglobin subclasses. Results from these studies have clearly demonstrated that monovalent group A and group C, as well as divalent group A/C conjugate vaccines, elicited high levels of IgG, thereby converting these T-independent polysaccharide antigens into T-dependent antigens. In addition, both monovalent and divalent vaccines generated high bactericidal activities.

Vaccine consistency studies of various group A and C lots indicated that vaccines can be prepared in a consistent manner, both chemically and immunologically. Antigen dose response of the vaccines, measured by ELISA IgG and bactericidal titres, conforms to a plateau over a dose range of 0.5 to 5 mg PS concentrations. Time course of antibody response was evaluated with monovalent and divalent vaccines in mice injected 3 times at 2-week (short schedule) and 4-week (long schedule) intervals. ELISA and bactericidal titres showed a T-dependent response, with high and prolonged IgG titres that correlated well with the total antibody response. Booster effects were most evident when the conjugates were administered without adjuvant. It was also found that two injections by the long schedule gave the same responses as three injections by the short schedule. Antibody isotype profiles in mice are being determined and quantitated for all conjugates.

Chemically modified group B meningococcal polysaccharide (N-Pr GBMP) has also been conjugated to tetanus toxoid and group B meningococcal outer membrane protein. Both conjugate vaccines elicited high-titre, polysaccharide-specific, antibody responses in mice; however, only polysaccharide-specific antibodies raised with the outer membrane conjugate were bactericidal against group B meningococci. The antibodies showed essentially no cross reaction to the native polysaccharide and provided a broad spectrum of protection against various serotypes.

Development of a multivalent class 1 OMP containing meningococcal vaccine

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Vaccine strains were constructed expressing three different class 1 OMP. The expression of the class 3 OMP, some class 5 OMP, the B polysaccharide and the lacto-N-neotetraose was eliminated in these strains. Two strains were constructed having the following serogroup-serosubtyping characteristics:

Strain I : - : - : P.1.7,16; P1.5,2; P1.p,15: L-(PL16215)
Strain II : - : - : P1.(7),4; P1.12,13; P1.5,10: L-(PL10412)

The L- indicates that the usual L1,L2,L3,L8 monoclonals which type all group B organisms do not reveal binding. The only LPS specific monoclonal which binds is MN8D6A, also known as D6A.

Outer membrane vesicles (OMV) were prepared from these strains and formulated into a hexavalent class 1 OMP vaccine. Class 1 OMP in this vaccine represented 60-80% of the total protein. A phase I safety study was carried out in adult volunteers with one dose, two dosages. The increase in bactericidal antibody activity was measured against six strains differing by the *porA* gene only. These test strains were derived from strain H44/76 (B:15:P1.7,16:L3) by transformation with the respective *porA* genes.

A vaccine against group B meningococci composed of the outer membrane protein P1 produced in *Bacillus subtilis* and renatured *in vitro*

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The protective efficacy of single component vaccines has often been very good: diphtheria toxoid, tetanus toxoid, capsular polysaccharides (or their conjugates), hepatitis B. We therefore wanted to find such a component of *Neisseria meningitidis* group B (MenB) that could be developed into a vaccine. We were convinced that the MenB capsular polysaccharide could not be used for the purpose because of its similarity with polysaccharides in human proteins (4). We therefore decided to test, in collaboration with Jan Poolman (RIVM), a number of monoclonal antibodies binding to surface-exposed components of MenB (8). This analysis showed that antibodies to the class I outer membrane protein (P1) were the only ones showing consistently complete protection in the infant rat assay (8).

On this basis we proceeded to produce the P1 protein in *Bacillus subtilis*, an apathogenic microbe devoid of endotoxin (LPS) and other known toxic components, for which efficient expression systems had been developed in our laboratory (6,7). The protein was expressed from an efficient bacillar promoter linked to a truncated bacillar signal sequence; it was produced in large amounts as inclusion bodies (5). These could be isolated by simple centrifugation procedures, and the BacP1 protein (identical in primary sequence to the native P1 protein of MenB with the exception of an N-terminal extension of 11 amino acids deriving from the expression vector) purified to 70-95 % purity (5; poster by Muttilainen *et al.* at this conference).

The BacP1 protein could only be solubilized with SDS or a chaotropic agent (urea or guanidine hydrochloride) resulting in complete denaturation. Mice immunized with such preparations, administered together with a strong adjuvant (FCA), produced a high titre of antibodies to the denatured BacP1 but none or a marginal amount reacting with the meningococcal surface in either EIA or bactericidal assays. Because P1 in the meningococci is a typical outer membrane protein complexed with LPS, we asked whether it could be refolded into a more native-like conformation in the presence of LPS. As a test of the conformation we used immunization of mice: if the antibodies produced bound to native meningococcal surface in EIA or bactericidal assays, the preparation was concluded to have (at least some) native structure. If these reactions were specific to the P1 subtype of MenB, the formation of native-like VR1/VR2

epitopes could be surmised (9). These tests indicated indeed the presence of native subtype-specific epitopes in BacP1 refolded in the presence of a heterologous (*Salmonella*) LPS (5).

Since LPS is an inherently unwanted component in a vaccine, we then looked for other ways to refold BacP1 in a conformation resembling that of the native protein. In this we took advantage of recent knowledge of the tertiary structure of bacterial porins (1,10) and of studies of the refolding of outer membrane proteins of *E. coli* (2,3).

The purified BacP1 was first solubilized in SDS, which was then replaced by a milder detergent, e.g. octylglucoside (OG). Micelles of phosphatidyl choline in the same detergent were added, and the mixture submitted to detergent removal by dialysis or gel filtration. The process led to the formation of liposomes, which were then used to immunize mice at a dose of 2 µg BacP1/injection, two injections 6 weeks apart with or without FCA. The mice produced in both cases a high titre of antibodies reacting in EIA and bactericidal assays with group B meningococci in a subtype-specific manner. The sera were also protective in the infant rat assay.

These data show that it is possible to produce the P1 protein of MenB in the heterologous, nontoxic host *B. subtilis*, and to achieve its refolding *in vitro* into a structure displaying native-like epitopes. The liposomes consisting of BacP1 and phosphatidyl choline offer a basis for developing a single-component vaccine for MenB. To cater for the several subtypes of P1, the vaccine should contain a mixture of P1 proteins representing the most common P1 epitopes.

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Phase I and phase II clinical studies of group A serotype 4 protein vaccine in infants in China

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This study is one of the target projects in the National Vaccine & Serum Institute based on the following possibility and necessity: over 90% of meningitis is caused by group A strains, which last for about 30 years. Since 1980 the Institute has been able to produce group A polysaccharide vaccine of which the quality meets WHO requirements. Polysaccharide A vaccine has been proven to be less immunogenic in infants than in children and adults. The Institute had developed a simplified method for the cultivation and purification of meningococcal outer membrane protein antigen on a large scale. The serotype of serogroup A strains has been considered to have an extremely uniform outer membrane protein profile. For the above reasons, a lyophilized protein vaccine was prepared for this study. The vaccine contained 125: g protein/vial, $A1(OH)_3$ saline (2mg/ml) used for reconstitution of the vaccine. LPS content was less than 8%. The placebo consisted of the same $A1(OH)_3$ saline solution without the OMP.

The vaccine has tested consecutively for phase I and phase II studies in infants (6-24 months). In the Spring of 1993, 30 infants were enrolled for phase I study, 15 of them received 50: g of protein vaccine, and the others received 1mg of $A1(OH)_3$ saline as placebo by IM injection. Axillary temperature and local side reaction were examined at 6,12,24,48 and 72 hours of post vaccination. For phase II study, 742 infants were divided randomly into 4 groups, 1) 182 for 50: g protein vaccine; 2) 181 for 25: g protein vaccine; 3) 238 for polysaccharide A vaccine; 4) 141 for $A1(OH)_3$ as placebo. All infants received 2 injections of the same vaccine and same dose of phase I at 4 week intervals. After each injection, axillary temperature and side reactions were checked as in phase I. Blood samples were collected from some infants of each group at: 0, 4 and 26 weeks after second injection for bactericidal test. Vaccine serum antibody was detected by a microbactericidal test (BT), which was a modification of Ambroschs and Chen.

Results: Local and systemic reaction: erythema, induration and axillary temperature were followed. There is no difference in severity of local reaction among these groups, but 50: g dose gave more side effects and the moderate reacting rate was within 0.7%. However, most of the side effects were mild and all of these were short-

lived. There were no reports of sterile abscesses among 1-4 weeks after vaccination. Based on these results we do not consider the local side effects a problem in ordinary use of the vaccine. Systemic side effects: axillary temperature was recorded in the morning and evening for 72 hours after vaccination. The groups showed no difference in incidence of moderate fever rate (37°C-37.5°C). There was no statistically significant difference between these four groups (P>0.16). Serum antibody response: the GMTs of bactericidal tests (4 weeks sera) were 30.7 (50: g protein), 29.1 (25: g protein), 14.5 (30: g polysaccharide, 1.1 (placebo) and the GMTs of the 26 weeks sera were 15.7, 16.4, 6.4 and 1.4 respectively. Before vaccination the GMTs of the four groups were 1.9, 1.7, 1.4 and 1.1, no significant difference between them. Therefore, the GMTs of the two protein vaccines were significantly higher than that of the polysaccharide vaccine and the placebo groups. According to the above results, the group A serotype 4 protein vaccine will be a candidate vaccine in infants in China.

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Quality control of the Cuban and Norwegian serogroup B vaccines used in the Iceland study

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Two vaccines, one from the Finlay Institute (F), the other from the NIPH (N), were used in a WHO sponsored study in Iceland to evaluate the serum bactericidal assay and ELISA as potential immunological correlates of protection. Both vaccines have already been shown effective in separate clinical trials. Here we report on their characterization and quality control by the manufacturers and by an independent laboratory (NIBSC). Independent laboratory evaluation was considered to provide data complementary to that produced by the manufacturers, to improve vaccine characterization and to help identify difficult areas, specifications and a need for standard reagents.

The two vaccines consisted of outer membrane vesicles enriched in serotype and sub-type antigens treated to remove the bulk of the lipooligosaccharide. They also contained significant amounts of other membrane proteins. In addition, the F vaccine contained added polysaccharide C.

The vaccines were shown to be similar but not identical in protein composition, containing Class 1, 3, 4 and Opc proteins; class 5.5. protein was identified in the N vaccine only. Both preparations had low levels of LOS 3,7,9. The 70 KDa iron-regulated Frp B protein was identified amongst the higher molecular weight components of both vaccines, as was an 80 KDa protein. Neither preparation contained transferrin-binding proteins. More 70 KDa protein was present in the lot of N vaccine used in Iceland than in previous lots.

Both vaccines adsorbed well onto aluminium hydroxide although differences were detected in the ease of desorption. Differences were also noted between the stabilities of the two vaccines both prior to and after adsorption onto the carrier.

Interesting differences dependent on the vaccine and strain of mouse used, were noted in the immune responses detected by immunoblotting. This highlights a need for caution in interpreting murine immunogenicity data. Some minor proteins gave significant immune responses.

Immunogenicity of two outer membrane protein-based serogroup B meningococcal vaccines among young adults in Iceland

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Introduction: Two serogroup B meningococcal vaccines, one produced by the Finlay Institute (F) in Cuba & the other by the National Institute of Public Health (N) in Norway, have been shown to be effective using 2-dose regimens in large clinical trials with estimated protective efficacies of 83% and 57%, respectively. In this World Health Organization-sponsored study, we evaluated the serum bactericidal assay (SBA) & ELISA as potential immunologic correlates for protection conferred by these vaccines.

Methods: 408 persons ages 15-20 yrs were enrolled in a randomized, double-blind trial & given 2 (0, 6 weeks) or 3 doses (0, 6 weeks, 11 months) of a serogroup B vaccine or 2 doses (0, 6 weeks) of serogroup A/C vaccine. Blood samples were obtained before vaccinations and at 12 weeks, 12 months and 20 months. Sera were evaluated by SBA & ELISA against respective vaccine type-strains (CU385/83 [B:4:P1.15:L3,7,9] & 44/76-I [B:15:P1.7,16:L3,7,9], F & N, respectively) in F, N, & CDC laboratories. A "responder" was defined as a person with a > 4-fold rise in antibody titre compared to the pre-vaccination titre.

Results:

Time	% SBA Responders (Control)				% ELISA Responders (Control)			
after	F	F	N	N	F	F	N	N
enroll	2-dose	3-dose	2-dose	3-dose	2-dose	3-dose	2-dose	3-dose
12wks	25*(2)		71*(3)		54*(4)		74*(3)	
12mos	15 (13)	44*(13)	47*(10)	84*(10)	17 (9)	68*(9)	34*(1)	89*(1)
20mos	43*(20)	62*(20)	64*(13)	69*(13)				

* = p<0.05 compared to control. These results are from CDC. Similar trends were seen in all laboratories, and among sera collected at 6 wks and 11 months.

Conclusions: The relatively low proportions of SBA and ELISA responders among the Finlay vaccine recipients are difficult to interpret given the high clinical efficacy found in the Cuban trial. The proportions of SBA and ELISA responders among NIPH vaccine recipients are similar to the efficacy measured in the Norwegian trial. Three dose regimens with the Finlay and NIPH vaccines were associated with significantly higher proportions of SBA responders at 12 months, but not at 20 months, after enrolment. Efforts to understand mechanisms by which these vaccines confer protection should be intensified, and other tests should be evaluated as possible immunologic correlates for protection.

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Vaccines and vaccine trials, poster 160.

Antibody response of complement deficient patients to tetravalent meningococcal polysaccharide vaccine

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Complement deficiency predisposes to meningococcal disease, especially due to uncommon serogroups (Y, W135). Vaccination with tetravalent (ACYW135) meningococcal polysaccharide vaccine of healthy persons with an intact complement system confers immunity to meningococcal disease (2). Whether the antibody response to this vaccine is normal and confers protection in patients with different complement deficiencies still has to be established (3).

We studied the antibody response to tetravalent meningococcal polysaccharide vaccine (MencevaxACWY, SKB, Rixensart, Belgium) in 49 complement deficient persons with the following deficiencies: properdin deficiency (n=20); C3 deficiency syndrome (n=5): factor H (n=1), C3 deficiency (n=2) and C3NeF (n=2); 24 persons with a late component of complement deficiency: C5 (n=3); C6 (n=3); C7 (n=7) and C8 (n=11). In addition, 24 non-complement deficient relatives and an age and sex matched control group of 24 persons with a normal complement system were vaccinated. All patients were over the age of 9 years and healthy at the moment of vaccination. Serum samples were collected immediately before and 1, 2 and 6 months after vaccination. Antibodies (IgG, IgM and IgA) against the capsular polysaccharides A, C, Y, W135 were measured in ELISA according to the method described by Carlone (1). Serum bactericidal response was measured against the bacterial strains from which the purified polysaccharides of the vaccine were prepared, except for the highly serum sensitive NmY strain. Sera were tested with their own complement and with addition of exogenous human complement.

Results of the bactericidal activity and quantitative antibody response to the vaccine in the various groups will be compared and used to define guidelines for vaccination in complement deficient subjects.

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Bactericidal antibody responses of juvenile rhesus monkeys to *Neisseria meningitidis* conjugate B polysaccharide vaccines

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Six conjugate group B polysaccharide (GBPS) vaccines and one non-covalent complex of B polysaccharide with meningococcal outer membrane protein were obtained from different sources and tested for immunogenicity and safety in rhesus monkeys. The vaccines included: GBPS noncovalently complexed to outer membrane vesicles from strain 99M(B:2a:P1.2) [Dr. W. Zollinger], GBPS-CRM197 conjugate [Dr. Rino Rappouli, Biocine, Sclavo], N-propionylated GBPS-OMP3 conjugate combined with aluminum hydroxide or stearyl tyrosine [Dr. Joseph Tai, NAVA Inc.] (2), GBPS-OMP conjugate with OMP from strain M986(B:2a:P1.2:L3,7) with and without MPL+TDM [Dr. S.J.N. Devi and Dr. C. Frasch, FDA] (1), and E. coli K92 PS-TT conjugate [Dr. S. J. N. Devi and Dr. C. Frasch, FDA].

One year old rhesus monkeys were immunized intramuscularly three times at weeks 0, 6, and 14. Serum samples were collected at weeks 0, 2, 6, 8, 14, 16, 22, and 30 and analyzed for bactericidal antibodies against a group B case isolate, 8765(B:15:P1.3:L3,7). It has been shown that anti-GBPS antibodies induced by natural infections or by vaccination with noncovalent complexes of GBPS and OMPs are bactericidal with heterologous complement but not with homologous complement (4). We therefore did bactericidal assays using normal human serum as the complement source and repeated the assays using normal rabbit serum as the source of complement. In addition, some sera were tested using normal rhesus monkey serum as the complement source. The assays were done in 96 well microplates using a total volume of 0.1 ml. Viable colony forming units were enumerated after plating on solid medium by the tilt method and overnight incubation at 37/C. The dilution giving 50% killing was chosen as the endpoint.

When rabbit complement was used, nearly all the animals had relatively high pre-vaccination titres of bactericidal antibodies (range: 1:3 to 1:7290; GMT: 1:90). With one exception (*E.coli* K92 PS-TT conjugate), all the vaccines induced a 4-fold or greater increase in titre in most of the animals vaccinated. The increased titres, however, did not persist through the end of the study. By week 30 the serum bactericidal titres (determined with rabbit complement) of most monkeys had returned to prevaccination levels.

When human complement was used, most prevaccination sera showed no bactericidal activity and in most of the vaccine groups little or no increase in bactericidal activity was observed. Two vaccines, however, induced relatively high titres of bactericidal antibodies which persisted through the end of the study. These vaccines were the GBPS-OMP conjugate with and without the MPL + TDM prepared at the FDA. Since these vaccines contained meningococcal outer membrane proteins and some lipopolysaccharide, the specificity of the bactericidal antibodies was uncertain. A pool of the 8-week sera from the monkeys that received the GBPS-OMP conjugate with MPL + TDM was absorbed 4X with *E. coli* K1 cells to specifically remove antibodies to the GBPS. The absorption did not change the bactericidal titre with human complement. The titre observed with rabbit complement, however, was decreased by 90%. A significant increase in antibodies to lipopolysaccharide was observed in these animals by ELISA, and nearly all of the bactericidal activity with human complement could be inhibited with purified lipopolysaccharide. These results indicate that the crossreactive bactericidal antibodies induced by the GBPS-OMP conjugate vaccines and functional with homologous complement were directed against the lipopolysaccharide rather than the GBPS.

Some sera were also tested for bactericidal activity with rhesus monkey complement. These results closely paralleled the results obtained with human complement and suggest that human complement is an adequate substitute for homologous monkey complement in the bactericidal assay system. The failure of the GBPS conjugates to induce antibodies that were bactericidal with homologous complement suggests that conjugation of the GBPS to protein does not change the basic quality of the antibodies it induces (3).

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Serological analyses from preclinical studies on meningococcal conjugate vaccines

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In preclinical studies on meningococcal polysaccharide-protein conjugate vaccines with mice, serological problems have been encountered in three areas: (1) immune variations in mice from different sources for testing conjugate vaccine lots, (2) lack of correlation between total IgG and bactericidal titres, and (3) individual mouse variation in the immune response. The use of different suppliers for the same strain of mice consistently resulted in a 3 to 4-fold difference in total IgG and bactericidal titres when testing the same meningococcal C conjugate vaccines. Some supplier-related immune variation has also been observed for meningococcal B conjugate vaccines, but the differences were not as great or as reproducible. Incorporation of a reference control lot may be useful to compensate for this supplier-based immunogenicity discrepancy if supplier changes cannot be avoided. Bactericidal antibodies may be elicited in mice against meningococcal B conjugate vaccines constructed from native or chemically modified polysaccharide (N-Pr GBMP) and class 3 outer membrane protein (OMP 3). Individual mice have shown large variations in antibody responses to these B conjugate vaccines. All respond to the polysaccharide with IgG as measured by ELISA, but not all respond with bactericidal antibody. Contrary to what has been observed for the A and C conjugate vaccines, however, there appears to be no correlation between specific IgG and bactericidal antibody titres. In addition, bactericidal titres for pooled sera from different mice do not necessarily correlate with the average of the individual sera, suggesting the possibility of high affinity and blocking antibodies with respect to bactericidal activity. IgG isotyping is being investigated.

Vaccination of mice with outer membrane protein detoxified LPS complexes mainly induces antibodies that do not recognize intact outer membrane vesicles

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There are several problems associated with the use of LPS in a vaccine. The most obvious is its toxicity, but the LPS can be detoxified by mild acid hydrolysis which cleaves off lipid A, or by mild alkaline hydrolysis which removes ester-linked fatty acids from the lipid A. Presented alone, these molecules have poor immunogenicity and must generally be linked to a carrier molecule to be immunogenic. Among other protective antigens, neisserial lipopolysaccharide and outer membrane proteins (OMP's) are possible vaccine candidates.

We prepared vaccine consisting of noncovalent complexes of OMP's from a B:NT:NT strain isolated from the CSF of a patient and alkaline detoxified LPS (L3,7). The vaccine was analyzed by SDS-PAGE and showed the presence of OMP's of classes 1, 3, 4 and 5 (about 70-90% of total protein). The detoxified LPS remained antigenically active as determined by specific reaction with monoclonal antibody. Balb/c mice were immunized with 2 doses of vaccine with or without adsorption to aluminium hydroxide. The mice were bled at appropriate intervals and some of the mice were sacrificed to produce monoclonal antibodies. Hybridoma clones were screened by ELISA using purified outer membrane proteins and native outer membrane complex from several different strains as antigens. A larger number of clones were obtained when the mice received vaccine with adjuvant as compared to vaccine alone. Most of the clones produced antibody that bound to purified OMPs better or much better than to native outer membrane complex. Many of the clones showed specificity for the homologous OMP's suggesting that these antibodies may be directed against serotype or subtype specific determinants. The results suggest the importance of polyvalent vaccine approaches and of preparing OMP vaccines from strains prevalent in the area targeted for vaccine use, and using methods that preserve the native conformation of the OMP's.

Safety and immunogenicity of meningococcal lipopolysaccharide incorporated into liposomes for monkeys

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The reactogenic properties and immunogenic potency of meningococcal vaccine containing native lipopolysaccharide (LPS) incorporated into liposomes was evaluated in experiments on rhesus macaques. Six monkeys aged 6-7 years received the vaccine in 2 injections at an interval of one month. One dose contained 50 or 200 mg of LPS incorporated into liposomes (3 monkeys per dose).

In all monkeys febrile reaction to the vaccine was in physiological norm. The contents of leukocytes in the blood and ESR remain unchanged after both immunizations. No local reactions in the form of hyperaemia or infiltration were noted. All monkeys remained healthy with no signs of disease till the period of observation was over. As revealed in ELISA, a high level of IgM antibodies to LPS was determined in the serum prior to immunization. In one monkey immunized with 50 mg of LPS the antibody level remained unchanged, but after the second injection an undulating of the level of IgM was observed. An increase in the level was noted in 2 monkeys receiving a dose of 200 mg. After both injections the peak of response was observed by the end of the second week. In none of the monkey the pronounced effect of memory to the second injection of the vaccine was noted. Thus, after the second injection the level of IgG antibodies remained on the same level or rose to the level observed after the first injection. Before the end of the observation term (6 months) the circulation of antibodies was established. The total level of IgG and IgM exceeded the initial level observed prior to immunization 2- to 10-fold. Thus, the results of animal experiments indicate that the vaccine is safe and moderately immunogenic.

The use of meningococcal detoxified lipooligosaccharide in experimental vaccines against group B meningococci

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Meningococcal disease remains a significant health problem in many different countries. A number of alternative cell surface antigens have been investigated as potential group B vaccines. These include lipooligosaccharide depleted outer membrane vesicles (OMV), iron regulated proteins associated with outer membrane vesicles (IRPs) and detoxified lipooligosaccharides (dLOS). In this study, antibody response of mice given experimental vaccines containing different concentrations of OMV associated with IRPs and C polysaccharide (PC) without and with different concentrations of dLOS have been analyzed.

Three vaccines with no added dLOS have the following composition: 2.0 mg of protein and 2.0 mg of PC per dose; 5.0 mg of protein and 5.0 mg of PC per dose and 10.0 mg of protein and 10.0 mg of PC per dose. Other 3 vaccines contained dLOS in the concentration of 2 mg, 5 mg and 10 mg added respectively to 2 mg, 5 mg and 10 mg of protein and PC preparation were also studied.

Meningococcal OMV from a B:4:P1.15 strain (N44/89) were extracted from bacteria grown in Tryptic Soy Broth with ethylenediamine D O-hydroxy-phenylacetic acid (EDDA). Meningococcal LOS (L1,3,7,8) was extracted from OMV of N44/89 strain using a cold phenol procedure, and purified in a Sephacryl column S-300 HR. LOS was detoxified by mild alkaline hydrolysis procedure.

Total IgG antibodies were quantified by ELISA and functional activity was studied by bactericidal assay using a homologous strain. The ELISA results showed higher antibody levels when 2 injections of 5.0 mg doses of OMV and PC were administered. However, a significant decrease in antibody levels was observed when dLOS was added to the preparations. Nevertheless, the bactericidal activity did not show a significant difference among the vaccines studied.

An analysis of the results suggested that OMV purification with DOC displaced the natural LOS, exposing buried proteins that do not induce bactericidal antibodies. When dLOS was added to the preparation it disguised these epitopes providing a more specific antibody response.

Role of IgM antibodies against C polysaccharide in the bactericidal antibody titres of Brazilian vaccinees

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The incidence of meningococcal disease due to *Neisseria meningitidis* C in the Greater Sao Paulo area of Brazil has until recently been low since the epidemic situation in 1971 and 1972. However, the percentage of serogroup C cases rose from 9.32% in 1988 to 14.75% in 1989 and reaching 32.36% in 1990. In 1989 serotype 2b:P1.3, which had not been previously isolated in Brazil, started to replace serotype 2a.

Between 1989 and 1991, several immunization campaigns were conducted in Brazil using the Cuban antimeningococcal BC vaccine (VA-MENGOC BC). In a previous study, bactericidal antibody activity against C meningococci (strain C11) were detected in 57% (n = 14) of children 24-83 mo old and in all (n = 23) persons 10-14 years old studied. Because of the low correlation between IgG levels detected by ELISA and bactericidal activity (r = 0.39), we directed this study to analyze the importance of IgM in the bactericidal activity detected in individuals with bactericdal antibody titres higher than 1:32.

Serum samples collected 4 weeks after the second dose of the vaccine were studied. IgM was removed from each serum by immunoadsorption with anti-IgM monoclonal antibody bound to the surface of polystyrene beads. Control reactions were done using unadsorbed polystyrene beads. Bactericidal titres were determined before and after adsorption. ELISA measurements of IgM were also determined. Our results suggest an important contribution of IgM in the bactericidal antibody response since a significant reduction of antibody titre, minimum of 2-fold, was detected in 58% of the persons studied.

Because of the short duration of IgM antibodies, the results also suggest that more emphasis should be given to covalently conjugated polysaccharide-protein vaccines in the development of effective vaccines against group C meningococci.

Immune response of mice to a Brazilian group B meningococci vaccine

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Since 1988 *N. meningitidis* B:4:P1.15, ET-5 complex, has been responsible for a epidemic of meningococcal disease in Greater São Paulo, Brazil. Despite current trials to develop an effective vaccine against B meningococci, children less than 2 years old have not been protected.

It has been suggested that iron-regulated proteins (IRPs) should be considered as potential antigens for meningococcal vaccines. The vaccine under study consisted of outer-membrane vesicles depleted of lipooligosaccharide from a B:4:P1.15 strain (N44/89) isolated in our region, IRPs, C polysaccharide and aluminium hydroxide. Groups of ten 5 to 6 weeks-old-female Swiss mice were immunized with 2 intramuscular injections of 4 different protein and C polysaccharide concentrations: 0.25, 0.5, 1.0 and 2.0 mg/dose.

The immune response of vaccinated mice was analyzed by ELISA, bactericidal assay and immunoblot. The ELISA and bactericidal results showed a higher antibody response when 2 injections of 2.0 mg doses were administered. The ELISA results also showed a significant higher IgG reactivity against antigen preparations containing IRPs. However, the bactericidal activity was not increased if the target strain was grown in iron-restricted medium. The immunoblot studies showed IgG reactivity primarily to class 1 OMP. Studies are in progress to evaluate the effect of a third dose and the role of LOS in the vaccine's immunogenicity.

Phase I study of two meningococcal outer membrane protein vaccines prepared from a class 4 outer membrane protein negative mutant and its isogenic parent

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Previous studies of a purified outer membrane protein vaccine from group B *Neisseria meningitidis* in Chile showed that in many instances the bactericidal antibody responses were relatively weak as compared to the overall antibody response measured by ELISA and by Western blotting (3). Possible explanations for the low percentage of functional antibody induced include the induction of class 4 OMP specific blocking antibodies (1,2) and vaccination with purified proteins that had epitopes exposed that are normally not exposed on the intact organism. These possibilities were investigated by preparing vaccines using deoxycholate extraction to yield lipopolysaccharide-depleted outer membrane vesicles which are thought to present the OMPs in a more native configuration. Two vaccines were prepared, one from a mutant strain that lacked the class 4 OMP (2), 8765(B:15:P1.3)RecA- CL4-, and one from its isogenic parent, 8765(B:15:P1.3)RecA-. Although the intent was to prepare vaccines that were identical except for the presence or absence of the class 4 OMP, differences were noted in the presence of the 5C OMP and the L8 LPS determinant. For serological analyses, strains and antigens were chosen that were 5C and L8 negative.

The vaccines consisted of sucrose stabilized vesicles adsorbed to aluminum hydroxide, and they contained per dose either 25 : g protein (low dose) or 50 : g protein (high dose). These vaccines were initially tested for safety and immunogenicity in mice, rabbits and rhesus monkeys. The vaccine prepared from the class 4 negative strain consistently induced slightly higher bactericidal titres in mice and rabbits than the vaccine containing the class 4 OMP, but no difference was seen in monkeys. Each vaccine was then tested for safety and immunogenicity in 40 volunteers (20 at the low dose and 20 at the high dose). Two doses were given intramuscularly at 0 and 6 weeks. About half of the volunteers received a third dose at 34 weeks.

The vaccines were generally well tolerated. The most common complaint was soreness at the site of vaccination. Pain or tenderness at the vaccination site was reported in about 75% of the volunteers. Mild fevers within 24 hours after vaccination

were observed in about 10% of volunteers.

Bactericidal assays were done using the parent strain 8765 which expressed the class 4 OMP but did not express either the 5C OMP or the L8 determinant. Over the limited dosage range tested no dose response was observed. Geometric mean increases in bactericidal titre between 0 and 9 weeks post vaccination varied from 3.5 to 4.3-fold for the four vaccine groups. Based on this limited analysis the presence or absence of the class 4 OMP in the vaccines had no effect on the titres of bactericidal antibody induced.

IgG antibody levels as measured by ELISA against the OMC from strain 8765 increased 3 to 4 fold between 0 and 9 weeks. Antibody levels decreased to about 2-fold above the prevaccination level after 1 year. No clear dose response was observed, and no difference between the two vaccines was evident.

More detailed analysis of the antibody response is planned. These studies will include measurement of the amount of antibody induced to each of the individual major outer membrane proteins including the class 4 protein. The relative amount of anti-class 4 antibody induced relative to the amount of antibody to the porins and LPS will be determined and correlated with the bactericidal titre of the sera.

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Immune response to outer membrane vesicle vaccines of LPS mutants of the vaccine strain 44/76 of *Neisseria meningitidis*

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The Norwegian outer membrane vesicles (OMV) vaccine against group B meningococcal disease contains LPS (7% relative to protein). The major LPS component is of immunotype L3,7,9 possessing lacto-N-neo-tetraose (LNnT; Gal1-4GlcN1-3Gal1-4Glc-) (2). This tetrasaccharide is also found on human cells (1,3) and could theoretically reduce the immunogenicity of the vaccine, or induce autoantibodies. Thus, four pyocin-resistant LPS mutants (Mu-1, -2, -3 and -4) of strain 44/76 were isolated. Since no galactose was detected by gas chromatography, the four mutants possessed LPS devoid of LNnT.

Mu-1 LPS contained Kdo and GlcN from lipid A with a ratio of 2:2; Mu-2 contained Hep, Kdo and GlcN (2:2:2); Mu-3 LPS contained Hep, Kdo, GlcN (2:2:3) and Mu-4 LPS contained Glc, Hep, Kdo and GlcN (1.6:2:2:3). A phosphorylated Hep was detected in Mu-2, Mu-3 and Mu-4 LPS as the molar value of Hep increased by close to one mol upon dephosphorylation. None of the four mutant LPSs expressed the immunotype determinants of L3,7,9 or L8, and only Mu-4 showed a weak reaction towards an immunotype L11 specific monoclonal antibodies. The class 1 (P1.7,16), class 3 (P15) and Opc outer membrane proteins of the mutants were found to be identical to the parent strain.

OMV vaccines were made from Mu-1, Mu-4 and the parent strain 44/76. $Al(OH)_3$ was used as adjuvant. Altogether 6 groups (n= 8 to 11) of F1 hybrid mice (BALB/cA x C57-B) were immunised subcutaneously at day 0 and day 21 with two doses of either 0.5 or 4 g protein of one of the vaccines. Sera from each group of were collected at day 35 and pooled.

The total antibody levels to whole cells and OMV from the parent strain 44/76 were measured by ELISA. A significant dose response was evident with all three vaccines. But they induced similar amount of antibodies to the parent vaccine strain. In contrast, antibodies against LPS from strain 44/76 were only detected by ELISA in serum from mice immunised with 4 g protein per dose of the Mu-4 OMV vaccine.

The serum pools were tested for antibodies to components of LPS from strains 44/76 and the four mutants by immunoblots after polyacrylamide gel electrophoresis. The

serum pool from mice immunised with Mu-4 OMV vaccine reacted to LPS from Mu-2 (weakly), Mu-3 and Mu-4, but not to LPS from 44/76. Serum pool from mice immunised with Mu-1 reacted only with its homologue LPS, whereas serum from mice immunised with OMV vaccine from strain 44/76 did not react at all with LPS components.

In conclusion, mutant OMV vaccines devoid of LNnT were not less immunogenic than the OMV vaccine of the native strain 44/76. On the contrary, LPS in the Mu-4 OMV vaccine seemed more immunogenic than LPS from the strain 44/76. The specificity of the antibodies against LPS, induced by the Mu-4 OMV vaccine, seemed to be directed against the conserved inner core of LPS, containing heptose.

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Vaccines and vaccine trials, poster 170.

Human antibody kinetics to meningococcal outer membrane antigens after vaccination with the Norwegian group B outer membrane vesicle vaccine: Results from a pilot three-dose trial among Norwegian adults

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Antibody kinetics after vaccination with three 25 : g doses of the Norwegian serogroup B meningococcal outer membrane vesicle vaccine (1,2), as used in a teenager protection trial (1) and in the Iceland immunogenicity study with group B meningococcal vaccines, is described. Sera from 27 adult Norwegian vaccinees have been studied.

The volunteers, who originally participated in the Phase II-1 and Phase II-3 immunogenicity studies in Norway (3), received the first two 25 : g doses at about six week's interval. The third 25 : g dose was given four to five years later. Sera drawn prior to the first and second vaccination, six weeks and six months after the second vaccination, as well as sera obtained just before and up to one year after the third dose were studied.

Serum antibody activities to the vaccine antigens (outer membrane proteins, OMP, and lipopolysaccharides, LPS), were measured by ELISA (4), immunoblotting (5) and by serum bactericidal assay (SBA) (6) against a selection of meningococcal strains.

Six weeks after the second injection more than 95% of the vaccinees had responded in OMP-ELISA, and in the SBA applying the vaccine strain (44/76; B:15:P1.7,16), when this strain expressed high amounts of Opc protein (7). When variants of the strain expressing low amounts of Opc was used in the SBA, a significant reduction in bactericidal titres were observed. After two doses, the geometric mean (GM) IgG level against OMP had increased about 7-fold. Five months after the second dose the GM IgG level against OMP had declined to about 3-fold higher than the prevaccination value. After about four years, the GM IgG anti-OMP activity in the vaccinees was not significantly different from that prior to vaccination.

The third dose induced a brisk memory IgG anti-OMP and bactericidal response in most of the vaccinees, although large variations between individual vaccinees were observed. Two weeks after the third dose, the GM IgG level against OMP was about 14-fold higher than the prevaccination value. One year later the GM IgG OMP level

had declined significantly compared to the peak level, but still remained 4.5-fold higher than before vaccination. Also in the SBA a significant reduction in titre was observed one year later, but the reduction was relatively less than observed in ELISA. One vaccinee did not respond with bactericidal antibodies to the vaccine strain after any of the three doses.

Most of the vaccinees also responded well with IgG antibodies against LPS from the vaccine strain after the two first doses, but in contrast to the anti OMP-response, there was no strong booster response against LPS after the third dose. As judged from immunoblots, the IgG binding to each of the major outer membrane proteins showed different profiles throughout the five-year period observed. The third dose induced a remarkably strong response in IgG against the class 3 protein in 23 (85%) of the vaccinees.

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A retrospective cohort study of the possible association between demyelinating diseases and immunization with the Norwegian MenB outer membrane vesicle vaccine

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In 1988-90, 93,000 students born in the years 1972-77 received at least one dose of the Norwegian vaccine against group B meningococcal disease in a randomised double-blind placebo-controlled efficacy trial (phase IIIB) (3). Three cases of demyelinating diseases were reported as possible adverse events following immunization: myelopathy (ICD-9 code 336.9), onset on day 10 after last dose; possible multiple sclerosis (341.9), day 26; and transverse myelitis (323), day 42 (2). In 1991-92, 54,000 students, all placebo recipients from phase IIIB, received the vaccine. Two cases of demyelinating diseases were reported as possible adverse events following immunization: Guillain-Barré syndrome (357.0) onset on day 21 after last dose; and postinfectious encephalitis (323.6), day 49 (1).

We are performing a retrospective cohort study to test the hypothesis that the risk of demyelinating disease is not increased in the first eight weeks following immunization. The cohort consists of all persons born in 1972-77 and attending compulsory secondary school in Norway in 1988-90, a total of 233,000 persons. Outcome information was obtained from a nationwide, anonymous health services research database of all persons discharged from a hospital ward. We assumed that these diseases would always lead to hospital admission. Each record represents a stay in a hospital ward, and contain up to three ICD-9 diagnoses, sex, age in years, date of admission and discharge, and number of admissions in the same hospital within a calendar year. The database has no information on date of onset of diseases.

We identified 291 records (representing up to 240 patients) that met the following criteria: 1. Admitted 1988-90; 2. age 10-19 years; 3. at least one of the following as main, secondary or tertiary diagnosis (codes): encephalitis, myelitis or encephalomyelitis (323.n); other diseases of the medulla (336.n); multiple sclerosis (340.n); other CNS demyelinating diseases (341.n); hemiplegia (342.n); other paralytic syndromes (344.n), other encephalopathy (348.3); Guillain-Barré syndrome (357.0); adverse effects of bacterial vaccines (E948); or adverse effects of other vaccines or biological substances (E949). We also included code 781.n (symptoms from the nervous or musculoskeletal system) as this diagnosis is often used for unclear clinical pictures that later turn out to be multiple sclerosis.

Hospitals were requested to de-anonymize the 291 records (based on dates of admission and diagnosis) and send the discharge report to us. We obtained information on 260 of these 291 records (89,3%), representing 193 patients. The following patients were excluded from further analysis: 47 not born in 1972-77; 71 with onset of disease before 1988; 50 with diagnosis 781.n but no later neurological disease; 29 with either congenital, traumatic or neoplastic etiology of their neurological diagnosis; one with a penicillin reaction (E949); and four with miscoded diagnosis. Several patients had more than one reason for exclusion. Thus 54 persons of the total cohort of 233,000 had onset of one the outcomes in the observation period 1988-90: 21 had encephalitis, myelitis or encephalomyelitis (323.n); two had other diseases of the medulla (336.n); two had multiple sclerosis (340.n); one had other CNS demyelinating diseases (341.n); one had hemiplegia (342.n); eight had other paralytic syndromes (344.n), six had other encephalopathy (348.3); and 13 had onset of Guillain-Barré syndrome (357.0) in the observation period 1988-90.

Exposure information on these persons will be obtained from the trial database of the entire cohort. In this database each person is categorized as vaccinee, placebo recipient or non-participant, and dates of all injections of vaccine or placebo are recorded. We will calculate the relative risk to the vaccinees of these diseases in various time periods following immunization or placebo injection. For this analysis, non-participants will be assigned random "dates of injection". We will also calculate the incidence of these diseases in this teenager population for use as baseline data in future vaccine trials.

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Comparison of the class 1 outer membrane protein from B: 15:P1.16 *Neisseria meningitidis* strains isolated from patients previously immunised with a serogroup B outer membrane protein vaccine in Norway

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Current attempts to develop effective vaccines against infection caused by group B meningococci have led to investigation of experimental outer membrane vaccines. Since 1974 there had been a high incidence of meningococcal disease in Norway, particularly among teenagers, with 75% of cases due to *N. meningitidis* group B serotype 15, subtype P1.16 (1). An outer membrane protein vesicle vaccine was prepared from organisms of strain 44/76, a B:15:P1.7,16 strain isolated from a fatal case which occurred during the epidemic. A large scale, double blind efficacy trial was carried out in Norwegian teenagers with this vaccine. Over the period of the trial, the point estimate of protection was 57% (95% confidence limits = 19-79 %) with the majority of cases in vacinees occurring during the final years of the trial. Vaccine failures were found not to be due to complement deficiencies (3), low levels of manan - binding protein (2) or predisposition through ABH non - secretor status of the victims/patients (4).The distribution of vaccine failures suggests that the duration of protective immunity was limited or that infection had occurred with strains that had evolved so as to escape the consequences of the immune response.

The outer membrane protein vaccines contain a number of different antigens and the identity of those which induce protective immunity is not clear. The class 1 protein is one of the two most abundant proteins in the outer membrane. Antibodies directed against this protein promote complement mediated bactericidal killing and protect in the infant rat model of meningococcal infection (8). In addition to the major structural differences in class 1 protein that generate sero-subtype specificity, recent studies have revealed the occurrence of minor variations in protein sequence which may have major effects on immune recognition of meningococci. One such variant of subtype P1.16 which was originally isolated from a local epidemic of meningococcal infection in the Gloucester area of the UK, and subsequently found in other areas of England, was termed P1.16b (5). A further distinct variant (P1.16c) has recently been isolated from a small cluster of cases in southern Norway (7). These strains showed increased resistance to bactericidal killing by sera from individuals who had been immunised with the B:15:P1.7,16 vaccine. Similar sequence variations have also been seen which modify the P1.7 epitope (6,9,10).

In order to investigate the possibility that vaccine failures during the Norwegian trial may have had been caused by the occurrence of similar variation in class 1 protein, the *porA* genes (encoding class 1 protein expression) from isolates obtained during the trial were sequenced and the deduced amino acid sequences of the proteins compared with those which have been previously determined. During the trial 10 meningococcal group B strains were isolated from vaccinees and subjected to typing and subtyping. Any isolates which could possibly have arisen from the prevalent B:15:P1.7,16 strain by mutation in the *porA* gene were chosen for further study. These were defined as B:15 strains which reacted with any of the monoclonal antibodies used to detect either P1.7 or P1.16 epitopes or which were non-typeable. By these criteria five strains which were B:15:P1.7,16 and one which was B:15:P1.16 were selected. The latter strain failed to react with the P1.7 monoclonal antibody in outer membrane ELISA but reacted weakly in dot and Western blots. For comparison five B:15:P1.7,16 strains from cases which occurred in the placebo group were also studied.

The sequence of the complete *porA* genes from the vaccine failures and placebo cases were compared with those of the 44/76 vaccine strain (P1.7,16a), strain MC58 from Gloucester, UK (P1.7,16b), 104/84 from West-Agder, Norway (P1.7,16c) and with the sequence of a Norwegian strain 67/87 with a 'masked' P1.7 epitope (10). In the vaccine failures and placebo controls, the *porA* gene sequences and the deduced amino acid sequences were all identical to the sequence of strain 44/76 except for that of one isolate whose sequence was identical to the strains described in Norway (10) and England (6) which had 'masked' P1.7 epitopes. Between 1987 - 1990, B:15:P1.16 was the most common infecting strain in Norway with 81% of these also reacting with the P1.7 antibody and the remaining 19% all having 'masked' P1.7 epitopes with amino acid sequences identical to that of this one isolate. Strains of similar sequence have been noted (9) and designated P1.7b,16. The occurrence of one P1.7b,16 out of the ten vaccine failures studied is in agreement with the general incidence of P1.7b,16 strains in Norway. Thus it would appear that the major reasons for vaccine failure in the trial were likely to be associated with the failure to develop an adequate immune response on vaccination rather than the generation of a selection pressure leading to a predominance of escape mutants.

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Antibody response against the 64 kDa protein from *Neisseria meningitidis* after systemic meningococcal disease, and after vaccination with two different serogroup B meningococcal vaccines among young adults in Iceland

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A 64 kDa molecular weight protein has been identified from a Cuban group B meningococcal strain (2). The gene encoding this protein has been cloned and expressed in *Escherichia coli* (2). The protein was purified to homogeneity and murine monoclonal antibodies (MAb) were prepared. The protein appears to be an important vaccine candidate, but the presence of 64 kDa in both the Norwegian and Cuban serogroup B meningococcal vaccines (1,3) is not known yet. Here we report a study of the expression of the 64 kDa protein in different meningococcal strains and the humoral anti-64kDa response in human vaccinees immunized with both vaccines, and in patients who have suffered from systemic meningococcal disease.

Expression of 64 kDa protein in different strains: Screening of various *Neisseria* strains in whole-cell ELISA and immunoblotting with MAbs showed that the 64 kDa protein is expressed in all strains of meningococci tested, but not in *Neisseria lactamica* or *N. sicca*. When outer membrane protein complexes from the Norwegian vaccine strain (44/76) was extracted with lithium chloride (pH=5.8), the 64 kDa protein was detected by antibodies in immunoblots. However, when this preparation was treated with 0.5% sodium deoxycholate (DOC) as in the vaccine preparation, the intensity of the signal detected with the MAbs was strongly reduced. This suggests that the epitope recognized by this MAb either is modified after treatment with DOC or that the 64 kDa protein is removed by DOC extraction.

Bactericidal antibodies: Murine polyclonal antiserum against the 64 kDa protein was bactericidal against 44/76 (B:15:P1.7,16) and B385 (B:4:P1.15) while no bactericidal activity was detected against strains 8069 (B:2b:P1.2) and G1963 (B:4:P1.4,(7),14). The tested murine MAbs against 64 kDa were not bactericidal. Affinity purified human IgG immunoglobulin against this protein, isolated from a Cuban vaccine, was bactericidal against all meningococcal strains tested, although giving only moderately high bactericidal titres. The bactericidal titres were higher when the bacteria were grown in broth than when grown on agar plates.

Antibodies to the 64 kDa protein in sera from convalescent patients and vaccinees: Immunoblots of acute phase (at hospital admission) and reconvalescent sera (drawn about two weeks later) from 15 Norwegian patients with systemic meningococcal disease showed that ten out of 15 convalescent patients developed antibodies reacting with the 64 kDa protein after infection with *N. meningitidis* strains, and 5/15 had weak response against the protein before the infection, but all had enhanced recognition of the protein in the reconvalescent sera, as shown by Western blot. This indicates that the natural protein is highly immunogenic in humans.

In a randomized, double-blind, controlled trial in Iceland, 408 15-20 yr-old volunteers were enrolled and given 2 (0,6 wks) or 3 doses (0,6 wks, 11 mos) of either the Norwegian (1) or the Cuban (2) produced group B meningococcal vaccines. A control group was given 2 doses (0 and 6 wks) of a serogroup A/C meningococcal vaccine. Blood was obtained prevaccination and 6 wks after each vaccine dose. Antibody response against a recombinant 64 kDa protein were studied by ELISA and in immunoblots with a representative subset of sera from 1/4 of the participants. Only 1-2 out of the 20 vaccinees in each group showed significant increases in specific IgG against the recombinant 64 kDa protein. No significant differences between the different vaccine groups were observed and no statistically significant correlation between bactericidal activity and IgG against 64 kDa protein was demonstrated.

Conclusion: We were able to demonstrate the presence of the 64 kDa protein in a wide collection of meningococcal strains. The protein was found in both carrier and systemic disease isolates, suggesting that this antigen is broadly crossreactive within meningococci. Antibodies specific for 64 kDa protein were detected in all reconvalescent sera from patients with meningococcal disease, implying the expression of this protein *in vivo*, during infection and its immunogenicity in humans. Finally, neither the Norwegian nor the Cuban group B meningococcal vaccines induced significant amounts of antibodies against the recombinant 64 kDa protein in Icelandic vaccines.

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Cross-reactive bactericidal components in sera from the serogroup B meningococcal vaccine trial in Iceland

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Two serogroup B meningococcal vaccines based on outer membrane proteins, one produced by the Finlay Institute (F) in Cuba (1) and the other by the National Institute of Public Health (N) in Norway (2), have been shown to be effective using 2-dose regimens in large clinical trials (1,3). The F-vaccine was produced using a B:4:P1.15:L3,7,9 strain (385/83) and the N-vaccine was from a B:15:P1.7,16:L3,7,9 strain (44/76). To study cross-reactivities in serum bactericidal assays (SBA) with heterologous meningococcal strains and the influence on bactericidal activity of antibodies against various components in the strains, sera from young adults immunized with the F and N-vaccines in a immunogenicity trial in Iceland, have been analysed with a panel of meningococcal strains.

Sera collected before the first dose and six weeks after the second and third doses from a stratified subset of sera from 1/4 of the vaccinees receiving three doses of either the F-vaccine (n=18), or the N-vaccine (n=22), were tested in SBA as described (4). The first two doses of vaccines were given at a six week interval, and the third dose ten months later. Five different strains were tested: three variants of strain 44/76, expressing either low (44/76-SL) or high (44/76-SL Opc++) amounts of the Opc protein, and a mutant of strain 44/76 (M14) which neither expressed the class 1 nor the Opc protein (4); a recently isolated systemic serogroup B strain from Iceland (B:21:P1.16); and a serogroup A strain from Mali (A:21:P1.9). All sera were also tested in ELISA for antibodies to lipopolysaccharide (LPS) (L3,7,9) from the Norwegian vaccine strain.

The N-vaccine induced higher bactericidal titres than the F-vaccine against the homologous strains 44/76-I and II. For both the N and F-vaccinees, the observed titres were also higher against strain 44/76-II than against 44/76-I. This indicates that both the N and the F vaccine induced bactericidal antibodies against Opc, or that strain 44/76-II was more sensitive in SBA than 44/76-I.

With 44/76-M14 as target strain, a reduction in bactericidal titres was observed for both groups of vaccinees, when compared with strain 44/76-I. The relative reduction in SBA was less for the sera from the F-vaccinees than for the N-vaccinees, showing

that the serosubtype (class 1) specific component is more important in this assay for the N-vaccinees than for the F-vaccinees. However, with this subset of the sera, 16/22 (73%) of the N-vaccinees and 11/18 (61%) of the F-vaccinees tested, responded significantly (more than 2-fold increase in titre) against 44/76-M14 in SBA, after three doses. This demonstrates the importance for bactericidal activity of antibodies against other components than the Opc and the class 1 proteins. SBA results with the heterologous strains from Iceland and Mali will be reported.

Both the N and F-vaccines induced antibodies against LPS (L3,7,9), which is believed to be a component shared by both vaccines. With the N-vaccine, after two doses the mean increase in IgG against LPS was about 4-fold. No strong booster response was seen after the third dose. With the F-vaccine the mean IgG response after the two first doses was 2.6-fold and increased to 4.3-fold after the third dose. A relatively low (but significantly positive) correlation coefficient (r=0.5) was observed between IgG antibodies to LPS and $\log_2$ bactericidal titres against 44/76-M14.

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Immunoblot analyses of vaccination sera from the serogroup B meningococcal vaccine trial in Iceland

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In 1992-93, a vaccine trial comparing the immunogenicity of the two group B meningococcal vaccines, produced by the Finlay Institute in Cuba (F) (3) and the National Institute of Public Health in Norway (N) (1), took place among Icelandic teenagers. The aim of the present study was to use the immunoblotting method to identify immunogenic antigens in the vaccines and to semiquantitate their antibody binding, to identify crossreacting antigens in the four strains used in the bactericidal assays, and to identify antigens which might correlate with bactericidal or phagocytic assays. For this purpose, a randomly selected subset of sera from ¼ of the volunteers receiving three doses of the F-vaccine (n=17) or N-vaccine (n=19) were immunoblotted (5). The sera were taken before vaccination and 6 weeks after the second and third dose, respectively. In addition, sera from 7 volunteers showing bactericidal activity (2) against all or one of the strains were also analysed.

All sera were incubated with nitrocellulose filters of outer membrane vesicle (OMV) preparations from the following four meningococcal strains: 1) The N-vaccine strain 44/76 (B:15:P1.7,16); 2) the F-vaccine strains 385/83 (B:4:P1.15); 3) G1963 (B:4:P1.4,14 with a masked P1.7 epitope; 6) isolated in Austria; and 4) 8069 (B:2b:P1.2,5) isolated in South Africa. The IgG binding to the high molecular weight proteins (HMWP), the class 1, 2 or 3, 4 and 5 proteins, the low molecular weight proteins (LMWP) and LPS in each OMV was analysed in the presence and absence of 0.15% Empigen BB to increase renaturation of boiled antigens (4). Antibody binding was rated visually on a scale from 0 to 4, where 0 represented no binding and 4 a very strong binding. Intensity of the immunoreactive bands was then grouped into distinct (3-4), medium (2-2.5) and weak/none (0-1.5) binding.

Before vaccination, 3-30% of the vaccinees showed distinct or medium IgG binding to either HMWP, LMWP or the class 1,3 or 4 proteins, whereas 25-50% had such bands for the class 5 proteins. Therefore, these persons probably had been or were carriers of meningococci. About 75% of those given the N-vaccine demonstrated distinct or medium IgG binding to the P1.7,16 protein after the second dose, while about 50% reacted with the serotype 15 protein. Nearly all sera bound to the latter

protein after the third dose. The sera showed varying degrees of crossreactivity with OMV components from the other strains. Sera that were only bactericidal against 44/76 displayed little binding to the other OMVs, whereas those that were bactericidal against all strains usually reacted with several antigens in all OMVs. Of those given the F-vaccine, about 40% demonstrated distinct or medium binding to the P1.15 subtype protein after the second dose, while about 10% reacted with the serotype 4 protein. After the third dose, the corresponding numbers were 50% and 70%, respectively. Thus, for both the N- and F-vaccine the number of vaccinees with antibody activity against the serotype proteins increased after the third dose. Generally, the serotype antibodies showed little crossreactivity.

After the third dose of N-vaccine, about 40-90% of the sera reacted with the HMWP of the four OMVs, while the corresponding numbers for the F-vaccine were 20-60%.

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Opsonic activity against meningococci in vaccination sera from the serogroup B meningococcal vaccine trial in Iceland

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A vaccine trial among Icelandic teenagers was carried out in 1992-93 in order to compare the immunogenicity of two outer membrane vesicle vaccines against group B meningococci. The vaccines were produced by the Finlay Institute in Cuba (F) (4) and the National Institute of Public Health in Norway (N) (2). The aim of the present study was to compare the bactericidal and opsonophagocytic activity of the sera against the two vaccine meningococcal strains and two heterologous group B strains, since it is not clear whether the main protective activity of antibodies formed during vaccination is due to bactericidal activity i.e. bacterial lysis or phagocytosis i.e. bacterial ingestion by polymorphonuclear leucocytes, PMN (granulocytes).

To perform the phagocytosis assay, *Neisseria meningitidis* strains 44/76 (N vaccine strain)(B:15:P1.7,16), 385/83 (F vaccine strain) (B:4:P1.15), SA 8069 (B:2b:P1.2,5) and AG 1963 (B:4:P1.4,7,14) were fixed in 70% ethanol over night at 20/C, washed in HBSS, the concentration was adjusted to 10 x 10^8 bacteria/ml and stored at -85/C. Venous blood from healthy adult volunteers, heterozygous for the Fc(RII^{LRHR} allotype was drawn into heparin vacutainers, and the red cells lysed. No additional purification of PMN was required because further analyses were done using flow cytometry that easily discriminate PMN from other cells in the suspension. We analyzed sera from 1/8 of the material including that from 10 volunteers receiving N and 9 receiving F vaccine in three doses, respectively. All sera to be tested were heated to 56/C for 30 min to inactivate endogenous complement. Normal human serum from one healthy donor with no detectable antibody against *N. meningitidis* strain 44/76 was used as complement source for opsonization of meningococci. The respiratory burst (RB) analysis reflected the OP activity and was performed mainly as described (1). As an indicator for RB we used the non-fluorescent probe dihydrorhodamine 123 (DHR) (Molecular Probes, Eugene, OR, USA) that is oxidized to the fluorescent rhodamine 123 by superoxide anions. Dihydrorhodamine 123 probed effector cells (5x10^6 /ml) was added to the opsonized bacteria and the incubation continued for 8 min at 37°C with agitation. The reactions were stopped by placing the microtiter plates on ice bath until RB was measured by flow cytometry.

The flow cytometry was performed as previously described (1) using an EPICS Profile II flow cytometer (Coulter Electronics, Luton, Beds, UK) with an 15 Mw argon laser. As negative control the test serum was omitted and replaced by HBSS-BSA. This was used to set the correct region on the fluorescent axis. About 3000 effector cells were counted in each sample, and all determinations were performed in duplicate. The results are presented as sum percent of RB positive PMN at each dilution (threefold starting at 1/6).

The test for serum bactericidal activity was performed as described previously (3). Results are presented as log_2 of the reciprocal serum dilution of the highest dilution giving more than 50 % killing, counted as colony forming units. To test for correlations between the assays we plotted single results on logarithmic scale for BA against RB to calculate the correlation coefficient, r.

By using strain 44/76 we got relatively high correlation (r=0.8) between bactericidal activity and respiratory burst. However, there were several sera with high BA and low OP and others with low BA and high OP indicating a difference between these two protective activities of antibodies emphasising the importance of performing both tests in a vaccine trial. Measurements of BA and RO activity against the four strains will be performed and compared to estimate vaccine respond and cross reactivity.

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Reactogenicity of two outer membrane protein-based serogroup B meningococcal vaccines among young adults in Reykjavík, Iceland

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Introduction: Two serogroup B outer membrane protein-based vaccines, one produced by the Finlay Institute (F) in Cuba and the other by the National Institute of Public Health (N) in Norway have been shown to be effective in clinical trials (1,2). A randomized, double-blind trial comparing the reactogenicity of these two vaccines to a polysaccharide serogroup A/C vaccine (C) was conducted during 1992-1993.

Subjects and methods: A total of 408 subjects (15 - 20 years of age) were enrolled of whom 407 were analyzed for reactogenicity. A total of 942 vaccine doses were given (372 doses to the F group, 370 doses to the N group and 200 doses to the control group). The F and N vaccines were given in 2-dose regimes at a 6-weeks interval and a 3-dose regime (0, 6 weeks and 11 months). The C vaccine was given in a 2-dose regime at 6-weeks interval. Each participant received a monitoring form to record symptoms and signs in the first three days following vaccination. Data on adverse events more than three days after vaccination were collected through personal interviews. The overall return rate of the monitoring forms was 91.9% for the F group, 90% for the N group and 89.5% for the control group.

Results: Late local adverse event (>3 days after vaccination) occurred only in F and N. Tenderness was reported in 23% and 19% of F and N, respectively, 1-2 weeks after the vaccinations. Swelling was reported in 12% and 7% 1-2 weeks after the vaccinations of F and N, respectively. Three persons receiving the F vaccine developed sterile abscesses that ruptured. Hardness persisted for at least 3 weeks in 3% of F vaccine recipients. A case of Henoch-Schonlein purpura occurred one week after vaccination with the control vaccine.

Sign/ Symptom	% Reporting Sign/Symptom within 3 days after vaccination							
	1st vaccination			2nd vaccination			3rd vaccination	
	F (n=149)	**N** (n=150)	**C** (n=96)	**F** (n=127)	**N** (n=120)	**C** (n=83)	F (n=66)	N n=(63)
Tender-ness	93	96	84	99	94	72	94	98
Redness	46*	62*	15	42*	42*	12	64	65
Swelling[1]	34*	31*	10	52*	36*	4	71	52
Hardness	26*	26*	1	24*	31*	6	23	40
Pain	77	79	72	81	76	60	77	84
Fever[2]	8	7	4	3	4	0	6	6
Fatigue/ irritable[3]	21	25	21	20	25	14	20	30

* = $p<0.01$ when compared to control. [1]Swelling increased significantly in F after each vaccine dose ($p<0.01$ for dose 1 vs. dose 2, $p<0.05$ for dose 2 vs. dose 3). [2]Fever was reported most frequently at 24 hours after vaccination. [3]Other Systemic complaints (anorexia, nausea, vomiting, headache or myalgia) were rare without any significant difference between the three different vaccines.

Conclusions: Local and systemic complaints were similar in all study groups. Local signs were more pronounced in F and N than the control group and swelling were more pronounced in F than N. Late local signs were only reported in F and N. Swelling increased significantly in F after each vaccine dose.

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Comparison of serum bactericidal results using vaccine type-strains and heterologous target strains to evaluate immunogenicity of two meningococcal serogroup B vaccines in Iceland

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The two meningococcal serogroup B vaccines evaluated in Iceland are produced with single meningococcal serogroup B strains (44/76-I and CU385, for the NIPH and Finlay Institute vaccines, respectively). This has led to concern that protection elicited by these vaccines might be serotype, subtype, or strain specific. To investigate this possibility we determined serum bactericidal activity (SBA) as a possible correlate of protection using two heterologous, or non-vaccine type, strains (8069 and G1963, from South Africa and Austria, respectively) as target strains and compared results with those of vaccine type-strains. In addition, the Finlay Institute strain CU385 was considered a heterologous target strain when testing the NIPH study group sera, and NIPH strain 44/76-I was considered a heterologous strain when testing the Finlay Institute study group sera. NIPH and Finlay Institute study group sera were tested using all three heterologous strains as SBA target strains. A total of 403 15-20-year-olds were enrolled and given 2 doses (0, 6 wks) or 3 doses (0, 6 wks, 11 mos) of a meningococcal serogroup B vaccine or 2 doses (0, 6 wks) of a serogroup A/C meningococcal polysaccharide vaccine. A responder was defined as an individual with a $\geq$4-fold rise in SBA titre.

Table 1. % Responders (control group% responders) for NIPH Study Group Sera.

Time after enroll	44/76-I#		CU385		8096		G1963	
	2-dose	3-dose	2-dose	3-dose	2-dose	3-dose	2-dose	3-dose
12 wks	71*(3)		28		24(6)		21(10)	
12 mos	47*(10)	84*(10)	34	31	22(13)	38(13)	31 (13)	27(13)
20 mos	64(13)	69*(13)	43	48	34(11)	38(11)	39(22)	31(22)

NIPH vaccine type-strain; the other strains are "heterologous" * = $p<0.05$ vs. control

Vaccination geometric mean titres (GMT) for the NIPH study group were 4.6, 2.2, 4.4 and 6.6 for target strains 44/76-I, CU385, 8069, and G1963, respectively. The prevaccination geometric mean titres (GMT) for the Finlay Institute study group were

2.5, 5.2, 6.2 and 10.9 for target strains CU385, 44/76-I, 8069, and G1963, respectively.

Table 2. Geometric Mean Titres (GMT) for NIPH Study Group Sera.

Time after enroll	44/76-I#		CU385		8096		G1963	
	2-dose	3-dose	2-dose	3-dose	2-dose	3-dose	2-dose	3-dose
12 wks	30.2		4.4		7.3		10.9	
12 mos	16.8	64.6	5.2	4.4	7.4	10.6	13.3	11.1
20 mos	14.8	29.8	6.7	6.8	9.2	11.5	16.3	12.2

NIPH vaccine type-strain; the other strains are "heterologous"

Table 3. % Responders (control group% responders) for Finlay Institute Study Group Sera.

Time after enroll	CU385#		44/76-I		8096		G1963	
	2-dose	3-dose	2-dose	3-dose	2-dose	3-dose	2-dose	3-dose
12 wks	25*(2)		34		18 (6)		16 (10)	
12 mos	15 (13)	44*(13)	27	48	8 (13)	30 (13)	12 (13)	36 (13)
20 mos	43*(20)	62*(20)	45	51	25 (11)	43 (11)	36 (22)	45 (22)

Finlay vaccine type-strain; the other strains are "heterologous" * = $p<0.05$ vs. control

Table 4. Geometric Mean Titres (GMT) for Finlay Institute Study Group Sera.

Time after enroll	CU385#		44/76-I		8096		G1963	
	2-dose	3-dose	2-dose	3-dose	2-dose	3-dose	2-dose	3-dose
12 wks	4.8		12.3		8.5		15.3	
12 mos	3.9	9.2	7.9	22.7	5.5	13.3	10.9	21.8
20 mos	9.0	16.2	13.8	33.9	8.7	20.0	14.4	29.3

Finlay vaccine type-strain; the other strains are "heterologous"

In summary, SBA results for the homologous strains are not consistent with protective efficacy data for the Finlay Institute vaccine, but are compatible with efficacy data for the NIPH produced vaccine. The percentage of responders when heterologous, or non-vaccine type strains were used was generally lower among the Finlay Institute vaccine study group than the Norwegian vaccine study group, but there is evidence

of boosting with the Finlay Institute vaccine against all 3 heterologous strains. The prevaccination GMTs were low for both the NIPH and Finlay Institute study groups. For 2- and 3-doses, the homologous strain GMTs were higher than those for the heterologous strains for the NIPH study group sera. However, for the Finlay study group, the heterologous GMTs were, in general, higher than the homologous GMTs. SBA is applicable to the evaluation of vaccine immunogenicity only if it is a reasonable correlate for protection.

Quality control of a serum bactericidal assay and other laboratory protocols used in a *Neisseria meningitidis* group B vaccine immunogenicity study in Iceland

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Quality control of a serum bactericidal assay (SBA) was undertaken prior to testing of study sera to identify differences in methodology and reagents which might affect the ability to compare results from three collaborating laboratories (Centers for Disease Control and Prevention [CDC], National Institute of Public Health [NIPH] and the Finlay Institute).

A SBA was used to determine functional antibody activity and immune response to immunization using either homologous (vaccine type-strain) or heterologous (non-vaccine type-strain) target strains. These strains were as follows: NIPH homologous, 44/76-SL (B:15:P1.7,16:L3,7,9); Finlay Institute homologous, CU385 (B:4:P1.15:L3,7,9), and heterologous, 8069 from South Africa (B:2b:P1.2,5:L3,7,9) and G1963 from Austria (B:4:P1.4,7,14:L3,7,9). All three laboratories did periodic monitoring of subtype and serosubtype epitope expression (colony blot or dot blot analysis) among target strains. They verified that complement sources had little or no anticomplementary or complement independent killing activity. The CDC did antimicrobial susceptibility testing of all target strains against 8 antimicrobial agents; all target strains were susceptible to these agents.

The SBA protocol differences identified between laboratories included: (1) the CDC and NIPH used 70-80 CFUs per well of cells in log phase growth from plates and the Finlay Institute used 50 CFUs per well (cells had been grown up to log phase in broth, frozen, diluted and then they used in the assay), (2) the CDC and NIPH used a 25% complement source, whereas the Finlay Institute used a 33% complement source, and (3) the CDC and NIPH used TSB agar for cell growth and the Finlay Institute used Mueller-Hinton Broth agar plus supplements.

Each testing laboratory identified and collected their own source of complement. The CDC evaluated the NIPH and Finlay Institute homologous target strains with the complement sources used by the Finlay Institute, NIPH and CDC. Quality control sera provided by NIPH (n=4) and the Finlay Institute (n=7) were used to compare

the complement sources. The three complement sources were comparable to one another in terms of the bactericidal titres they yielded with a given serum and target strain.

A comparison between human serum and plasma as a source of complement was made using the NIPH target strain (44/76-SL) and 13 sera from individuals vaccinated with the NIPH vaccine. Only two sera had bactericidal titres that differed by more than $\pm$1 tube dilution (the plasma titres were higher in both cases). A similar comparison was made using the Finlay Institute target strain (CU385) and 13 sera from individuals vaccinated with the Finlay Institute vaccine. Only one serum had a bactericidal titre that differed by more than $\pm$1 tube dilution (the serum titre was higher).

Quality control sera designated KME and CU36 were provided by the NIPH and Finlay Institute, respectively. These sera were assayed once each time a group (approximately 6 plates) of homologous test sera was assayed. When testing serum KME, the CDC had 40 assays (59%) with a titre of 128, 7 assays (10%) with a titre of 64 and 21 assays (31%) with a titre of 256. The NIPH had 79 assays (60%) with a titre of 128, 51 assays (38%) with a titre of 64 and 2 assays (2%) with a titre of 256.

When testing serum QC36, the CDC had 40 assays (53%) with a titre of 128, 11 assays (19%) with a titre of 64 and 16 assays (28%) with a titre of 256. The Finlay Institute had 81 assays (52%) with a titre of 128, 52 assays (34%) with a titre of 64, 18 assays (12%) with a titre of 256, 2 assays (1%) with a titre of 32 and 1 assay (1%) with a titre of 512.

In the initial comparisons using serum QC36, the median titres reported by the CDC and the Finlay Institute were as described above. However, when testing begun a shift in absolute titres was observed for QC36. The Finlay Institute titre for a given test serum was now 3 to 4 tubes higher than the titres reported by the CDC and NIPH for the same sera. Further analysis of the CDC and Finlay Institute test data revealed that, for comparison of titres, it was necessary to take the difference between the postvaccination and prevaccination titre. When the difference between titres were compared, the CDC and Finlay Institute data were in good agreement.

In summary, this study was the first direct blinded multilaboratory comparison of meningococcal SBA titres. The results suggest that multilaboratory comparisons of SBA can be done for the evaluation of vaccine immunogenicity. However, based on the comparison of the CDC and NIPH data to the Finlay Institute data, we still have more to learn about the bactericidal assay if we are going to continue to use it to evaluate vaccines.

Assessment of the vaccination with meningococcal polysaccharide vaccine in two localities of the Czech Republic

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Introduction: Meningococcal polysaccharide vaccine is not used in the routine immunization scheme for several reasons and its use depends on the epidemiological indication (1,2). In some countries good vaccination campaigns have stopped the epidemics of meningococcal invasive disease. In the Czech Republic where meningococcal disease occurred only sporadically for a very long period and *Neisseria meningitidis* B prevailed, the use of meningococcal polysaccharide vaccine was never indicated. This situation has dramatically changed recently.

Results and Discussion: Increase of invasive meningococcal disease caused by a new clone of *Neisseria meningitidis* C:2a:P1.2(P1.5), ET-37 occurred in two districts of the Czech Republic in spring 1993. This new meningococcal clone was never found before 1993 in the Czech Republic where meningococcal strains have been monitored since 1970. The disease showed unusual epidemiological and clinical characteristics: high age specific morbidity in the age group of 15-19 years, high fatality rate (20% compared to 10% reported for all meningococcal invasive diseases in 1993) and atypical clinical course with higher incidence of Waterhouse-Friderichsen syndrome and meningococcal sepsis. In May 1993, highest age-specific incidence in the locality "1" was in the age group of 15-19 years (26.1/100 000), while in locality "2" meningococcal invasive disease affected in that period two age groups: 1-4 years (24.9/100 000) and 15-19 years (18.7/100 000). The respective data of age specific incidence for the whole Czech Republic that time were 1.4/100 000 for 1-4 years and 1.8/100 000 for 15-19 years. The vaccination campaign focusing on the most affected age group started in the locality "1" at the beginning of June 1993 (using polysaccharide meningococcal vaccine A+C, Merieux). During two weeks 6191 students of the age group of 15-19 years were vaccinated (i.e. 96% of all students of this age group, 72% of all population of the age group of 15-19 years, 5.6% of the whole population of the locality "1"). In the locality "2" a different approach was used: vaccination of contacts (137 vaccinees) and "on request" (908 vaccinees). Thus 0.9% of the whole population of locality "2" was vaccinated, irrespective of age. It is likely that the focused vaccination in the locality "1" prevented the spread of meningococcal invasive disease caused by *Neisseria meningitidis* C

in the vaccinated age group. In locality "2", where vaccination was not age targeted and its coverage was very low, the incidence of invasive disease caused by *Neisseria meningitidis* C was not reduced. The spread of *Neisseria meningitidis* C:2a:P1.2 (P1.5) to other districts of the country was observed in the next winter/spring period (1994). In these individual districts the total incidence and age specific incidence did not reach high levels, so no vaccination campaigns have been indicated there to date. The statistical significance of the differences in the occurrence of meningococcal invasive disease caused by *Neisseria meningitidis* C between the locality where age targeted vaccination was implemented, the non vaccinated districts and the locality "2" vaccinating the contacts and "on request" is assessed.

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The *Neisseria meningitidis* outer membrane protein P1 produced in *Bacillus subtilis* and reconstituted into phospholipid vesicles elicits antibodies to native P1 epitopes

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Neisseria meningitidis serogroup B remains a major cause of bacterial meningitis around the world, for which no effective vaccine is available. Many different components of meningococcus are under research as possible vaccine candidates; they include modified capsular polysaccharide, lipopolysaccharide (LPS) and outer membrane proteins (1,4). Class 1 outer membrane protein (P1) is one of the most promising vaccine candidates as P1 subtype-specific antibodies have been shown to be protective in an animal model (3).

We have previously described the production of P1 in the Gram-positive *Bacillus subtilis* as intracellular inclusion bodies, from which the protein (BacP1) is easily purified (2). These inclusion bodies need strong denaturing agents (SDS, urea, GuHCl) for solubilization and are not immunogenic as such. Refolding of BacP1 into a conformation capable of eliciting bactericidal and protective antibodies in mice directed to native meningococcal epitopes was first obtained with complexing the protein with heterologous LPS (2).

We now show that the detergent-solubilized, completely denatured BacP1 can be refolded with the formation of the native immunodominant surface epitopes by reconstitution into phospholipid vesicles without LPS. This was achieved by fusing the BacP1-detergent micelles with phospholipid-detergent micelles by detergent removal (dialysis or gel filtration) to yield protein-lipid vesicles (liposomes). According to sucrose density gradient analysis, approx. 10 % of BacP1 protein was integrated in the lipid vesicles with both dialysis and gel filtration methods.

When mice were immunized with the BacP1-liposome preparations, they produced high titres of antibodies reacting in a P1 subtype-specific manner with native meningococcal antigen indicating the presence of conformation-dependent epitopes. These antibodies were also bactericidal and protective in the infant rat model (Idänpään-Heikkilä *et al.*, poster at this meeting). These results suggest that the BacP1-liposomes are a promising vaccine candidate for meningococcal disease.

Furthermore, they demonstrate the feasibility of refolding a denatured Gram-negative outer membrane protein that has never been exposed to LPS into a native-like conformation.

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The antibody response to a prototype liposome vaccine containing *Neisseria meningitidis* outer membrane protein P1 produced in *Bacillus subtilis*

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Several candidate vaccines for *Neisseria meningitidis* group B (MenB) have been prepared by methods that lead to enrichment of serotype-specific outer membrane proteins (OMP). These vaccines have been shown to induce bactericidal responses in man and protect from disease (1,2,5). However, in field trials the level of protection has been only 50-70 %. These OMP-based vaccines contain several bacterial components, some of which are not desirable and difficult to purify e.g. lipopolysaccharide (LPS), which is an endotoxin. A purified, single-component vaccine would have obvious advantages in terms of both safety and immunogenicity. Monoclonal antibodies to class 1 OMP have been shown to be bactericidal and protective in an infant rat meningitis model (4). This suggests class 1 protein as a vaccine candidate for serogroup B.

Our approach has been to produce the class 1 protein (P1) as a recombinant protein in *Bacillus subtilis*, which is a Gram-positive organism and thus devoid of LPS. We have previously shown that the P1 protein could be efficiently expressed as inclusion bodies in *B. subtilis* (3). The isolated P1 protein could only be solubilized by denaturing agents. Such BacP1-preparations did not induce bactericidal antibodies upon immunization, possibly because the antigenic epitopes normally exposed on the surface of meningococci were not present in the denatured form of BacP1. However refolding of the protein in the presence of LPS resulted in the appearance of native-like epitopes as demonstrated by the ability of the preparations to elicit the synthesis of bactericidal antibodies in the mouse. Since LPS is a nondesirable vaccine component we subsequently looked for refolding conditions not requiring LPS, and could show the presence of native-like P1 epitopes in protein-lipid vesicles (liposomes) formed of BacP1 and phosphatidyl choline (Muttilainen *et al*., poster at this meeting).

In order to assess the feasibility of developing a vaccine based on BacP1-liposomes we have now prepared several parallel batches of them and characterized their immunogenic properties in mice, guinea-pigs and rabbits.

BacP1-phosphatidylcholine liposomes were prepared with the gel-filtration method as

described by Muttilainen *et al*. (poster at this meeting). Immunization of mice with these liposomes resulted in the production of antibodies directed to P1 epitopes exposed on the surface of intact meningococci as determined by EIA. These antibodies were also bactericidal and protective in the infant rat meningitis model; these effects were specific to the subtype of the meningococcal P1. A good immune response was obtained with a low dose (3-10 : g) of BacP1-liposomes after two subcutaneous injections and no adjuvant was needed. The three consecutive batches of the antigen gave similar results in all the assays.

The interindividual variation in the antibody response was analyzed in outbred mice, guinea-pigs and rabbits as well as in three inbred lines of mice. All animals clearly responded producing antibodies binding to the surface of meningococci in the same subtype-specific manner.

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Use of a parenteral component priming - oral immunization regimen to elicit protection against *Neisseria gonorrhoeae in vivo*

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Introduction: The challenge of controlling gonorrhoea remains formidable due to an increase in the proportion and variety of antimicrobial resistant strains of *Neisseria gonorrhoeae*. Development of a successful vaccine against *N. gonorrhoeae* would assist in reducing the substantial burden of morbidity and cost due to this sexually transmitted disease. Toward this goal, we have developed a Parenteral Component Priming - Oral (PCP-O) immunization regimen which elicited protection against gonococcal challenge of subcutaneous chambers implanted in mice.

Study design: Studies were performed using Institute of Cancer Research (ICR) outbred mice and protection was evaluated by a graded dose challenge with live *N. gonorrhoeae* strain 340 using the subcutaneous chamber model of infection (1). Outbred mice were used since they more accurately represented a normal population than did inbred strains. Initial PCP-O immunization experiments were performed using either native or recombinant Fbp (Iron binding protein), or a synthetic peptide derived from gonococcal Protein I (PI,3) for parenteral priming. Gonococcal strain 340 (also designated as strain N7,2) or a Protein III-deficient mutant of strain 340 (4), which were administered via a gastric tube, were used as the oral component. Infectious dose 50% (ID50) was determined for each test group (approximately 10 mice/group) using a graded dose challenge protocol (2) and compared to that of unimmunized controls to give protection factors (ID50 test group/ ID50 unimmunized controls). Initial experiments involved groups of mice which received either 10 parenteral injections of the priming component (priming controls), 14 oral doses of the oral component (oral controls), 10 priming injections followed by 14 oral doses (PCP-O test groups) or no treatment (unimmunized controls). Variations of the experimental design included: changes in the number of priming injections, oral doses or both; use of 4X or 8X branched-chained PI peptides; different combinations of parenteral priming and oral component; different concentrations of the parenteral priming component; and live versus gamma-irradiated gonococci as the oral component.

Results: An initial PCP-O study using Fbp as the priming component and strain 340 as the oral component resulted in the following protection factors: Unimmunized controls, PF=1; Parenteral priming controls, PF=1; Oral controls, PF=12; PCP-O groups, PF

ranged between 7,180 to >31,250. The use of recombinant Fbp, instead of native Fbp, as priming component did not affect protection levels. The ID50 of strain 340 for unimmunized mice ranged between 50 and 200 CFU. A second PCP-O regimen used the synthetic peptide DDQTYSIPSLFV derived from gonococcal Protein I as priming component and a Protein III-deficient mutant of strain 340 as the oral component. The protection factors were: Unimmunized controls, PF=1; Parenteral priming controls, PF=1; Oral controls, PF=4; PCP-O group, PF=31,250.

Results of various immunization regimens demonstrated that: 1) the priming/oral combination of Fbp/strain 340 (PF=26,875) was superior to Fbp/340 PIII$^-$ (PF=8) and the combination of PI peptide/340 PIII$^-$ (PF=31,250) was superior to PI peptide/340 (PF=375); 2) a combination of 4X branch-chained PI peptide priming/ 340 PIII$^-$ oral elicited greater protection than other branched peptide priming/oral combinations; 3) the use of gamma-irradiated gonococci as the oral component resulted in higher levels of protection than were obtained using live gonococci; and
4) using 50mg of PI peptide for each priming dose followed by oral immunization resulted in higher levels of protection than using 5mg or 20mg of PI peptide for priming.

Conclusion: We have developed a Parenteral Component Priming - Oral immunization regimen which elicited high levels of protection against the infection of subcutaneous chambers implanted in mice with virulent gonococci. Studies are ongoing to further optimize and shorten the immunization regimen. Future plans involve evaluating this immunization regimen in primates and humans to determine its effectiveness as a gonococcal vaccine.

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A possible influence of vaccine induced Por, LOS, and Rmp antibodies on the outcome of intraurethral challenge with *Neisseria gonorrhoeae*

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In 1985, a placebo-controlled vaccine trial was conducted at the University of Washington that used a prototype vaccine prepared from a sodium deoxycholate extraction of outer membranes (1) of gonococcal strain NRL 5767. The vaccine preparation was enriched with porin protein (Por-approximately 85%), but also contained reduction-modifiable protein (Rmp-approximately 15%) and small amounts of lipooligosaccharide (LOS) (3). 62 adult male volunteers who had been randomized to receive either vaccine (n=35) or placebo injections (n=27) were challenged intraurethrally with live gonococci (NRL strain 5767) between 69 and 85 days after vaccination, with inocula ranging from 3.7 x 10^3 to 1.0 x 10^4 CFU/ml. Overall, the vaccine had no effect in preventing the onset of experimental infection. Following intraurethral challenge, 18 (51%) of vaccine and 19 (68%) of placebo recipients became infected (p=NS).

Despite the apparent failure of the vaccine, 26 (41%) of the volunteers resisted infection upon challenge including 17 (49%) of the vaccine recipients and 9 (32%) of the placebo recipients. We therefore evaluated: a) the possibility that pre-existing protective immunity to gonorrhoea exists by virtue of differing antibody levels occurring naturally or as a result of vaccination; and b) the influence of heterogeneity of immune responses to individual antigenic components of the vaccine. Serum antibody (Ab) levels were measured (6) in volunteers to the three major components (Por, LOS, Rmp) prepared from the challenge strain - before administration of vaccine (or placebo) and again just prior to intraurethral challenge. No differences in baseline antibody levels prior to administration of vaccine or placebo were measured in the two groups. No significant changes in antibody levels were measured in placebo recipients between the two dates that blood samples were taken, spanning 69 to 85 days; changes in geometric means of Por, LOS, and Rmp Ab levels were 2.0-fold (2.20 to 4.42 : g/ml), 3.2-fold (0.76 to 2.43 : g/ml), and 6.85-fold (0.88 to 6.0 : g/ml), respectively, in vaccinees. By logistic regression analysis, we tested several hypotheses regarding the influence of these Ab levels and vaccination status on the acquisition of infection after intraurethral challenge. Because Por (protein 1) and LOS antibodies are known to have complement dependent bactericidal activity (4,5) and may

also facilitate C5a-driven chemotaxis of polymorphonuclear leukocytes (2) to the site of gonococcal infection, we reasoned that these antibodies might be protective in the human model of infection used here. Alternatively, Rmp (protein 3) antibodies have been shown to block bactericidal antibody function (6) and we considered that these antibodies might facilitate the experimental infections. In statistical models that explored the hypothesis that, independently, Por-Ab and/or LOS-Ab may be protective and Rmp-Ab subversive, each of the Ab levels at the time of challenge was examined for association with infectious outcome both in conjunction with and independent of vaccination. These Ab levels demonstrated no influence on this outcome independently of or in conjunction with vaccination. In a model that assumed interdependence of the three Ab levels, we used the ratio [(Por-Ab + LOS-Ab)/Rmp-Ab] to characterize a beneficial effect of Por and LOS antibodies and a subversive effect of Rmp antibodies (3). The level of this ratio just prior to challenge appeared to protect against infection when vaccination status was included in the model (p=0.04), but not when vaccination status was excluded.

While these results suggest a possible beneficial effect of vaccination, this trial was not designed primarily to test the interrelatedness of Por, LOS, and Rmp Ab in influencing the outcome of intraurethral challenge. Therefore these results should be interpreted cautiously and used to test new hypotheses regarding the use of vaccination to protect against gonococcal infection.

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Protective effect elicited by vaccination with a live, attenuated strain of *Neisseria gonorrhoeae*

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Research into anti-gonococcal vaccine development has been impaired by the antigenic variation and host mimicry exhibited by *Neisseria gonorrhoeae* during infection. Important virulence factors such as pili and the Opa protein, which are involved in attachment, show intense antigenic variation and may be absent (1). Early work demonstrated that vaccination with whole dead gonococci conferred some protection against homologous challenge in chimpanzees (2) but failed to confer protection in a field trial in humans (3). A pili vaccine which elicited antibodies to common epitopes failed to protect in a field trial (4), however, other surface proteins appear to be conserved. The major protein, PI, is heterogeneous between strains, but has antigenic domains common to all types which may be relevant in immunity (5), and antibodies raised against synthetic peptides of PI have been shown to be bactericidal *in vitro* (6). LPS is a major component of the outer membrane and promotes the production of bactericidal antibodies. However, gonococcal LPS is not a good vaccine candidate due to toxicity, antigenic variation within a strain (7), and host mimicry by sialylation during infection (8, 9).

This study was undertaken to evaluate the ability of an *aroA* mutant of *Neisseria gonorrhoeae* strain MS11 to protect against a subsequent challenge with the wild type strain using the guinea pig SCM. In addition, the intensity and specificity of the antibody response to both the vaccination and challenge infections were examined in order to investigate more closely the difference between animals which were protected from subsequent challenge and those which were not.

Eighteen animals were vaccinated with the *aroA* mutant at a range of doses from $\log_{10}$ 3 to $\log_{10}$ 5. Two animals were inoculated with a placebo, which was buffer alone. Infection kinetics were examined at various times following inoculation by performing viable counts on samples of aspirated chamber fluid. In addition, chamber fluid was routinely aspirated from a contra lateral uninfected chamber, for assessment of the antibody response.

All chambers inoculated with the *aroA* mutant became infected and exhibited bacterial growth. The evolution of infection in each chamber was found to be unrelated to the initial dose given. In total, 8 out of the 18 chambers inoculated with the *aroA* mutant underwent spontaneous self-cure by day 26 post inoculation. Five more chambers exhibited a

substantial decrease in bacterial numbers (at least half a log in a 7 day interval) immediately prior to rejection, and 5 chambers were rejected with no decrease in bacterial numbers.

Fifteen of the vaccinated animals and the 2 placebos were challenged with the wild type strain 3 weeks after the end of the vaccination infections and, for technical reasons, the remaining 3 animals were challenged 11 weeks after the end of the vaccination infections. The challenge dose given was that known to elicit infection in 100% of naïve animals (10).

The 2 placebo animals became infected and their chambers were rejected before day 15 post challenge. In contrast, 12 of the 15 challenged in the first instance were partially or completely protected from subsequent challenge and 3 were not protected. Of the group of 3 chambers challenged later than the rest, 1 was completely protected and the other 2 were not.

In summary, of the 13 animals which cleared or controlled the vaccination infection, 85% demonstrated some or complete protection from the challenge infection. This protective effect had no discernible correlation with the vaccination dose given or the evolution of the vaccination infections.

In an attempt to explain this protective effect, anti-gonococcal antibodies were examined during the vaccination and challenge infections using ELISA. A steady increase in antibody production to whole gonococci was seen throughout vaccination and challenge, irrespective of the number of viable bacteria present in the chambers. However, a negligible antibody response to LPS was noted in the vaccination infections. This apparent unresponsiveness was not observed in the challenge infections.

The total amount of antibody present and the antibody levels obtained in chambers immediately prior to challenge could not be correlated with protection from subsequent challenge. In addition, none of the antibodies detected were found to be bactericidal *in vitro*. The antibodies elicited during vaccination were observed to recognise a wide variety of protein antigens in Western blots. No relationship between antibody specificity and protection could be detected except in the case of a protein of approximately 30 kDa, which was observed in 75% of chambers protected from subsequent challenge. This suggested strongly that the protein was of significance in the protection of chambers following vaccination. Further investigations failed to identify the 30 kDa protein as any of the following gonococcal antigens: PI, Opa, PIII, PAN III, pili.

In summary, protection was found to be irrespective of the fate of the vaccination infection or total antibody levels present immediately prior to challenge, and could not be correlated with the presence of antibodies specific for any of the known gonococcal antigens. However, antibodies recognising a protein of approximately 30 kDa were found in 75% of protected animals. The characterisation of this protein is the subject of further study.

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