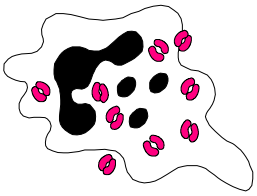

Noncapsular Vaccines

Clinical trials with outer membrane protein vaccines and PorA recombinant vaccines

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General childhood immunization against systemic infectious diseases such as meningitis caused by encapsulated bacteria has come within reach. The general introduction of a capsular polysaccharide protein conjugate vaccine has eliminated infectious diseases caused by Hib (Haemophilus influenzae type b).A major effort is now needed to ensure a maximum global health benefit through the Expanded Program on Immunization (EPI). The development of conjugate vaccines against infectious diseases caused by pneumococci and group A and C meningococci is in an advanced stage of development, including the performance of efficacy studies. An efficacious group B meningococcal vaccine is still lacking, because the group B capsular polysaccharide is poorly immunogenic. During the last decade a number of efficacy trials were undertaken with meningococcal outer membrane protein vaccines in Cuba, Brazil, Chile and Norway. The outcome of these studies indicated efficacies in the range of 50-80%, revealing no protection in the very young, showing a rapid decline in the antibody response over time. The results are summarized in Table 1.

The studies reveal a remarkable similarity in outcome. The differences in efficacies in the placebo controlled, double-blinded studies appear to be related to the study period, indicating waning immunity.
Two questions are important:

i) The specificity of protective antibodies
ii) The lack of protective immunity in the very young with the Finlay and WRAIR vaccines

In relation to question i) it will be extremely hard to correlate volunteers immune responses with protection while working with a multicomponent vaccine. The best we can achieve relates to the analysis of the specificity of bactericidal antibodies by *in vitro* methods.

The age-related differences with respect to protection as found in the Chilean and Brazilian studies appeared to be reflected by the bactericidal titers as measured in the laboratory (4,5). These results show that the Finlay and WRAIR vaccines do not induce bactericidal antibodies in the very young.

With respect to the specificity of the vaccine induced bactericidal antibodies the following has been found/published so far: the Chilean and Brazilian studies revealed evidence for the importance of antibodies against PorA, the class 1 OMP (5,6). The Norwegian study demonstrated the importance of Opc and PorA with respect to the induction of bactericidal antibodies (7). This study also demonstrated the beneficial effect of a third immunization, 4-5 yrs after the initial series of two. In addition to Opc and PorA, other antigens were found to

induce bactericidal antibodies after the third immunization, albeit in a minority of the vaccinees, but with a tendency of cross-reactivity towards strains with varying serosubtype composition (7).

Table 1. Efficacy trials with meningococcal OMP vaccines

Vaccine	Study	Efficacy
Purified total OMP in proteoliposome with added high mol. wt OMP (Finlay) 50 µg (B:4:P1.19,15)	Cuba, teenagers, randomized at school level, double-blinded placebo control, two immunizations, 16 mo study period	83% (Ref. 1)
Same vaccine	Brazil, case-control, two immunizations, 12 mo follow-up	-37% (3-23 mo) 47% (24-47 mo) 74% (48-83 mo) (Ref. 2)
Total OMP in vesicle (OMV) formulation (SIFF) 25 µg (B:15:P1.7,16)	Norway, teenager, randomized at school level, double-blinded, placebo control, two immunizations, near to three year study period	57.4% (Ref. 3)
Purified class 1, 3, 4 containing OMP (WRAIR) 100 µg (B:15:P1.3)	Chile, 1-21 yrs, randomized, double-blinded, placebo control, two immunizations, 20 mo follow-up	-39% (1-4 yrs) 70% (5-21 yrs) (Ref. 4)

Quo vadis? The results obtained with the first generation OMP vaccines are very promising. Improvement of these vaccines can be achieved in two ways:

I) to focus on the antigens with demonstrated ability to induce bactericidal antibodies

ii) to further investigate other vaccine candidates with special emphasis onto cross-reactive immunogens.

With respect to antigens with demonstrated ability to induce bactericidal antibodies, the class 1 OMP deserves further attention. The Opc protein can be considered as well, however, most case isolates do not seem to express appreciable amounts of this protein. Vaccines preferably will have to induce a consistent immune response in the vast majority of vaccinees. In order to achieve this, vaccines were constructed to contain PorA for the greater part (8). Since the serosubtype-specificity is a critical factor, a hexavalent PorA vaccine in vesicle formulation was prepared and immunization studies in adults, infant monkeys and infants were carried out.

The induced bactericidal antibodies were dependent upon PorA as proven by the use of various target strains having defined deletions/mutations within porA (9).
During the conference, I will also give data about infant and infant monkey studies with the multivalent PorA vaccine as well as the Norwegian OMP vaccine. Results obtained with the Norwegian OMV vaccine after immunization of Icelandic teenage volunteers will be discussed. A set of strains with defined deletions/mutations in Opc/PorA/PorB were used to characterize the bactericidal antibodies. One important outcome of these studies is the observation that the OMV vaccine formulation allows for the induction of bactericidal antibodies in the very young. The bactericidal antibodies are totally dependent upon PorA in case of the Dutch multivalent PorA vesicle vaccine. Results obtained with sera from vaccinees having received the Norwegian vaccine, indicate the critical importance of PorA although other antigens play a role.

The patterns of bactericidal antibody activity found with the multivalent PorA vaccine suggest some antigenic competition amongst PorA's within a vesicle (three PorA's are expressed simultaneously into one vesicle). The bactericidal antibodies were further analyzed by using target strains with deletions and point-mutations within the relevant PorA epitopes.

Other antigens/protection assays. Research is ongoing towards other possible vaccine antigens. In addition to the in vitro bactericidal assay, other protection assays can be of help in the finding of potential protective antigens. Such assays relate to the mechanism of phagocytosis or the combination of phagocytosis and direct bactericidal mechanism. In addition to the established value of the in vitro bactericidal assay, the following methods deserve further development:

i) an in vitro opsonophagocytosis assay
ii) an in vitro whole-blood assay, combining the bactericidal and opsonophagocytosis mechanisms
iii) passive immunization with vaccinees antibodies and challenge in an appropriate animal model.

A number of antigens in addition to PorA were identified to be able to induce bactericidal antibodies:

<u>LPS</u>**.**

Conflicting results have been obtained with respect to bactericidal activity of human antibodies against meningococcal LPS. Because of the abundance of expression and the inherent stability of saccharide epitopes, LPS still deserves further attention. The availability of well-defined mutants (10) allows a detailed analysis of human antibodies. A critical factor will be the potential cross-reactivity of LPS-specific antibodies with human tissue because of the structural similarities.
Studies with a collection of monoclonal antibodies in relation to bactericidal activity, tissue-crossreactivity and specificity as defined with mutant strains will be described in my presentation.

The definition of the LPS composition and selection of appropriate variants by way of these well-defined monoclonal antibodies, appears to be critically important in relation to the bactericidal assay.

Opa and Opc.

As mentioned, the Opc protein is associated with the induction of bactericidal antibodies. Since Opc is expressed by many carrier isolates, the question comes to mind if the induction of bactericidal antibodies against Opc will be able to kill the bacterium before Opc-non expressing bacteria enter the bloodstream.

In this context, the issue of parenteral versus mucosal immunization also appears relevant. As far as we understand the epidemiology and pathogenesis of meningococcal disease, carriage seems to be a self-limiting event by way of inducing bactericidal antibodies. The description of a highly conserved 22 kDa OMP that induces cross-reactive bactericidal antibodies (11) may relate to Opc. Until now, the feeling is predominant that the variability of Opa will be too high to be of vaccine relevance. However, the extent of this variability has not been delineated.

PorB.

PorB is the major OMP of the meningococcus and because of that a likely vaccine candidate. However, PorB appears not to be able to induce a consistent, relevant bactericidal antibody response in volunteers being immunized with vaccines containing appreciable amounts of this protein. The growth of meningococci under glucose limitation with an effect on LPS composition (sialylation most likely) completely eliminated the bactericidal effect of PorB-specific monoclonal antibodies (12). Monoclonal antibodies against PorB given passively to infant rats before challenge, were poorly protective (13).

Fe-limitation inducible OMPs/exotoxins.

Particularly Tbp2 and FrpB are interesting vaccine candidates (14,15). However, Tbp2 and FrpB are highly variable and it needs to be investigated how many types will be needed to ensure broadly reacting bactericidal antibody activities (16,17). Interestingly, meningococci appear to be able to express a LbpB with homology to Tbp2, indicating that the binding of lactoferrin resembles the binding of transferrin, involving two lactoferrin-binding proteins (18). Further studies are needed to investigate the vaccine potential of LbpB. Iron-limitation also appears to induce the expression of two proteins, FrpA and FrpC, which reveal homology to the RTX family of bacterial exotoxins (19). The role in pathogenesis as well as the potential vaccine implications still have to be established.

Still other antigens.

An overview is given in reference 20. Cross-reactive antigens such as Rmp, H8 have revealed disappointing results as vaccine candidates; Pili were found to be extremely variable. In addition to the porin functions (PorA, PorB); adhesion/invasion mechanisms (pili; Opa; Opc); Fe-uptake mechanisms (LbpA,B; Tbp1,2) there is some lack in our knowledge with respect to the OMPs

involved in protein secretion. On the basis of homology to enterobacterial proteins, the Omc protein seems important in this respect (21).

In conclusion. We have reached a critical stage in menB vaccine development.
Outer membrane proteins do induce protective immunity as found with the first generation OMP vaccines. These vaccines however contain many nonprotective components (i.e. not inducing bactericidal antibodies) and vaccinees respond in a scattered manner because of the complexity of the vaccine. PorA was found to be the most relevant antigen within first generation OMP vaccines. Developments with respect to homogeneous PorA containing vesicle vaccines are promising since a PorA dependent bactericidal antibody response can be induced, even in infants. Further studies, including aiming at efficacy, are needed with such vaccines. Laboratory research indicates that further human immunization studies are indicated with a few other vaccine candidates such as Tbp2, FrpB, LbpB, Opc. Because of the inherent stability of saccharide structures, research towards the B polysaccharide as well as LPS needs to continue.

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Recombinant and synthetic antigens from meningococcal class 1 protein

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Meningococcal class 1 outer membrane is an important candidate antigen for incorporation into vaccines designed to prevent infection by serogroup B meningococci. Monoclonal antibodies directed against the class 1 protein are highly bactericidal for meningococci and also passively protect infant rats in vivo against meningococcal infection (1). Most importantly, recent data from the Norwegian trial has shown that the presence of bactericidal activity in vaccinees' sera correlates with the presence of antibodies directed against the class 1 protein (2).

The cloning and sequencing of the *porA* genes which encode the class 1 protein (3) have permitted structural and antigenic studies which have led to a model of the organization of the protein within the OM (4). This predicts a structure composed of 16 amphipathic β-strands which traverse the outer membrane and generate eight surface exposed hydrophilic loops. Sequence variation is largely confined to two discrete variable regions designated VR1 and VR2, which are located in the longest surface-exposed loops 1 and 4 respectively. This restricted antigenic diversity is the basis for the sero-subtyping classification of meningococci. In addition, epitope mapping with synthetic peptides has localized the epitopes recognized by the bactericidal and protective subtype-specific mAbs to the apices of these loops (5).

Further studies on the protective effect of class 1 protein are hampered by the presence of additional components in the vesicle preparations so that the immune response to the different components varies between individuals. As an alternative, in this study a series of recombinant and synthetic antigens have been used to investigate optimal presentation of class 1 protein antigens for immunization strategies designed to induce biologically functional antibodies.

The *porA* gene encoding class 1 protein has been cloned in *E. coli* into the high level expression vector p-GEMEX^{TM}-1 in which the class 1 protein was expressed as a fusion with the bacteriophage T7 gene 10 capsid protein. The class 1 protein has also been expressed without the gene 10 leader peptide and with a poly (His) tag to facilitate purification by metal ion affinity chromatography. The recombinant proteins have been used in experiments designed to investigate refolding of the protein for immunization, including the use of liposomes incorporating the additional adjuvants monophosphoryl lipid A and muramyl dipeptide.

As an alternative strategy synthetic peptides have been used to focus the immune response to the protective epitopes. Previous studies utilized a multiple antigen peptide (MAP) containing a protective class 1 protein B-cell epitope together and promiscuous Th-cell epitope (6). Immunization using Freund's adjuvant produced a bactericidal immune response. In the current study this MAP has been used to investigate the use of adjuvants both acceptable for human immunization and with ability to induce relevant bactericidal IgG subclasses

With each of the antigens a good immune response to the immunizing agent was obtained but the antibodies produced differed markedly in antigenic specificity and their ability to activate complement mediated killing of the meningococci. The magnitude of the bactericidal effect could be correlated with production of a sero-subtype specific immune response, demonstrating that the presentation of the antigen in a conformation resembling the native protein produced the most functionally effective immune response. These data emphasize the importance of designing vaccination strategies to produce the most biologically effective immune response by optimizing epitope specificity, antibody subclass and avidity.

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Infant rat meningitis passive protection assay and protection evoked by human group B OMV vaccine induced antibodies.

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Infant rat meningitis model has previously been found useful for studying the passive protection afforded by mouse antibodies to meningococcal surface antigens (1). It has been used for comparison of the protective activity of monoclonal antibodies directed to capsular polysaccharide, lipopolysaccharide, PorB and PorA (2). Furthermore, the infant rat passive protection model has been used as a guide in our developmental work of PorA-based recombinant vaccine (3).

In this model 4 to 6 day-old pups are injected intraperitoneally with bacteria two hours after the passive administration of antibodies. The development of bacteremia and meningitis are followed by culturing blood and CSF sample 6 hours later.

We have now optimized this model for use with human sera. In these studies the i.p. challenge strain was rat passaged B:15:P1.7,16 (IH5341) (3). Doses of 10^5 and 10^6 cfu/pup proved most useful. The larger dose produced bacteremia and meningitis in all pups while the lower dose induced bacteremia in all animals and meningitis in a majority of them. Four human sera (bactericidal titers, SBA, <1:2, 1:4, 1:32 and 1:64, respectively) obtained after immunization with a group B meningococcal outer membrane vesicle vaccine (4) (OMV) were used for protection experiments. The sera were inactivated and diluted 1/10, 1/30 and 1/100; 100 µl were injected i.p. Saline was used as a negative and monoclonal antibody to group B capsular polysaccharide (2 µg/pup) as a positive control. There were 6 pups/group and to test the reproducibility of the assay, most of the experiments were repeated once or twice.

The reproducibility of the assay was good and results of the two to three repeat experiments were combined. The non-bactericidal serum (SBA <1:2) did not protect for either bacteremia or meningitis. Serum with weak SBA (1:4) showed protection against the lower dose, but not against the higher dose. Serum with the SBA titer 1:32 protected at the 1/10 dilution against both doses. The 1/30 serum dilution did not protect against higher challenge dose. However, with the lower dose of bacteria the majority of the animals were bacteremic but with low numbers of cfu (4 per cent of the negative control) recovered from the blood; correspondingly only 18 % had bacteria in the CSF. Serum with the highest SBA (1:64) protected at the 1/10 and 1/30 dilutions against both doses. No sera gave protection at the dilution 1/100. The development of meningitis clearly correlated with the number of bacteria in the blood; when the cfu in the blood exceeded 10^5/ml, the majority of the pups had bacteria also in CSF, whereas no bacteria were found in the CSF of animals with low grade or no bacteremia.

We conclude that the assay is entirely feasible with human sera, and its reproducibility is good. Two studies using this model with human sera are in progress: an evaluation of the model as a

surrogate of protection in humans by using the sera from the "Icelandic study" (5), and a study of the ability of human antibodies evoked by group B outer membrane vesicle vaccines (4,6) to protect against B:15:P1.7,16 variants with point mutations in the loop 4 of PorA (6).

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Immunization with a low molecular weight meningococcal outer membrane protein protects against lethal experimental infection

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Even though meningitis caused by *Neisseria meningitidis* is currently an important problem in both developed and developing countries, there is presently no effective vaccine available that can stimulate a long-lasting protective immunity in young children (1). Efforts are being made to improve the current polysaccharide vaccines or to find alternative meningococcal surface antigens that could serve as effective vaccinogens. In this respect, the great interstrain antigenic variability of the major meningococcal outer membrane proteins (OMP) could be restricting their protective efficiency to a limited number of antigenically related strains. Here, we report that the immunization of mice with a newly identified highly conserved OMP with an apparent molecular mass of 22 kDa called NspA, protects against lethal experimental infection.

Monoclonal antibodies (MAbs) specific for the NspA protein were generated (2). These MAbs were used to demonstrate that the NspA protein is exposed at the surface of all the meningococcal strains tested. These strains represented all major serological groups. In the presence of complement, two of the MAbs, Me-1 and Me-7, exhibited *in vitro* bactericidal activity against the four meningococcal strains tested: two strains of serogroup B and one strain from each serogroup A and C. These two bactericidal MAbs also protected mice against experimental *N. meningitidis* infection (3). In fact, the injection of ascitic fluid containing the NspA-specific MAbs 18 hours before the bacterial challenge increased the rate of survival of Balb/c mice from 8% observed in the control groups to 70%. This data clearly indicates the protective potential of antibodies directed against the meningococcal NspA protein.

The gene coding for the meningococcal NspA protein was identified and cloned into the expression plasmid vector pWKS30 (4). Similarity searches using the nucleotide and the deduced amino acid sequences of established databases confirmed that this protein has never been described previously. The affinity-purified recombinant NspA protein was then used to immunize Balb/c mice in order to evaluate its ability to confer protection against a bacterial challenge with a lethal dose of *N. meningitidis* strain of serogroup B. The mice were injected subcutaneously three times at three weeks intervals with 10 or 20 µg of affinity-purified recombinant NspA protein or control antigen preparations. Serum samples were obtained after each injection and the titers of these sera were determined by ELISA using meningococcal outer membrane preparations as the coating antigen. The results clearly showed that the purified protein is immunogenic when administered with an adjuvant such as QuilA. The serum titers varied from 1/2,000 to 1/51,000. The serum titers obtained in the control groups were below 1/200. Western immunoblotting experiments showed that the antibodies present in the sera obtained from the immunized mice recognized the recombinant NspA protein, but more importantly reacted strongly on the nitrocellulose membrane with the native meningococcal NspA protein. In both groups of mice injected with the purified recombinant NspA protein,

80% of the mice survived the bacterial challenge compared to 0 to 40% in the control groups. Analysis of the sera indicated that the NspA immunized mice who died following the bacterial challenge had the lowest serum titers suggesting that there is a correlation between the specific antibodies titers and the observed protection.

In conclusion, the newly identified meningococcal NspA protein can induce an immune response that can protect mice against a lethal challenge. This protein is antigenically highly conserved among meningococcal isolates and is exposed at the surface of intact meningococcal cells, where it is accessible to the antibodies. For all these reasons we believe that this protein possess all the important characteristics to be considered a potential candidate for the development of a new broad-range vaccine against meningococcal disease.

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Noncapsular Vaccines

Anti-endotoxin activity of monoclonal antibodies against meningococcal LOS and the memory response to LOS incorporated into liposomes

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In our previous study we showed that incorporation of the native lipopolysaccharide (LOS) from *Neisseria meningitidis* followed with decrease of toxicity and increase of immunogenicity (1). The bactericidal and endotoxin neutralizing activity of antibodies to meningococcal LOS is still subject of discussions. A potential disadvantage of using complete meningococcal LOS is the presence of structures which are also found in the human host (2). Moreover, LOS as a thymus-independent antigen is generally thought to be poor at inducing secondary immune response.

Murine monoclonal antibodies (MAbs) against meningococcal L3,7,9 *Neisseria meningitidis* LOS were tested for their endotoxin neutralization properties. The MAbs were characterized by using a set of LOSs from meningococcal strain H44/76 having defined stepwise truncations - mutants *lgtB, galE* and PB4 (3,4). Four groups of epitopes on the oligosaccharide part of LOS could be identified (terminal, inner and two middle). MAbs specific to terminal lacto-*N*-tetraose unit and deep structures of LOS were broadly cross-reactive with human tissues and mast cells respectively. Activity of outer membrane blebs and chemically extracted LOS was amplified with plasma to stimulate IL-6 production in human blood cells. Neutralization of purified LOS and blebs was demonstrated with MAbs specific to middle part of oligosaccharide and in both cases inhibition of IL-6 stimulation appeared to be up to 500 times less active in the presence of plasma derived factors. Besides endotoxin neutralization, one of these MAbs demonstrated bactericidal activity. Avidity of anti-LOS MAbs seems not to be related to neutralizing activity and interaction of plasma factors with both LOS and blebs in most cases improved the interaction with MAbs.

LOS preparations isolated from meningococcal wild strain H44/76 (L3,7,9) and mutant strains *lgtB* and *galE* were incorporated into liposomes and used for immunization. Primary immune response was similar in all tested strains of mice (BALB/c, C57BL/6, AKR and nude) and followed with high elevation of IgM antibodies. Compared to the primary response, the secondary anti-LOS response is characterized by the appearance of IgG antibodies (IgG3>IgG1>IgG2b>IgG2a). Booster effect was lower in AKR mice and significantly higher in C57BL/6. The study of liposomes prepared with different ratio of LOS/phospholipids showed that the secondary immune response corresponded with epitope density and did not require B-cell mitogenicity or cytokine stimulation (TNF-α, IL-6, IFN-γ). The priming effect of *lgtB* LOS was cross-reactive with wild H44/76 and *galE* LOSs and persisted for at least 8 weeks.

Thus antibodies to truncated L3,7,9 LOS can be expected to be more beneficial in induction of bactericidal and endotoxin neutralizing response against meningococcal infection. Our observations strongly support the hypothesis that memory response can also be induced with thymus-independent antigen.

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Noncapsular Vaccines

Design and production of meningococcal vaccine based on transferrin binding proteins

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Transferrin-binding protein 2 (Tbp2) from *Neisseria meningitidis* is involved in iron capture in the infected host and elicits bactericidal antibodies in animals (1); this is similar to what has been reported for recombinant TbpB from *Actinobacillus pleuropneumoniae* which was shown to induce protective immunity in pigs (2). When Tbp2 is purified to homogeneity from *Neisseria meningitidis* strain B16B6 (B:2a, P1.2) grown in 30l fermentors in an iron poor media (Mueller Hinton broth containing 30 µM EDDA) yields are low not exceeding 0.5mg/ml under our experimental conditions. The gene encoding Tbp2 has been cloned and sequenced and recombinant lipidated Tbp2 (67kDa) was produced in *E. coli* using the arabinose-inducible expression vector (3). A purification scheme was designed which involved the preparation of outer membranes, two ion exchange chromatographies and a polishing gel filtration step. The product obtained retained its ability to bind human transferrin (hTf) and was devoid of contaminants. Mice were immunized with different amounts of rTbp2 on days 0, 21 and 35 and bled on days 21, 35 and 42. The animals developed high IgG titers and bactericidal antibodies. The antisera were bactericidal versus strains expressing a 67kDa Tbp2 but did not induce the lysis of strains expressing a 85kDa Tbp2, these results were quite comparable to those reported earlier with Tbp1-Tbp2 purified from meningococcal cells (4) while others have described a broader cross reactivity (5). Because high molecular weight Tbp2s (>80kDa) are more divergent than low molecular weight (67kDa) Tbp2, we analyzed different truncated forms of Tbp2 before designing the vaccine antigen. Full length Tbp2 (85kDa) from strain M982 (B:9,P1.9) was produced in *E. coli* using the pMAL-c2 expression system. The fusion protein retained its ability to bind hTf and the system allowed to produce a series of truncated proteins including N-terminal domain (2-351) and C-terminal domain (352-691). The fusion proteins were purified by chromatography on amylose; the different Tbp2 molecules were analyzed for their ability to bind hTf and were inoculated to rabbits to produce antisera. We confirmed with this expression system that the N-terminal domain bound hTf as described earlier (6) but moreover identified a hTf binding site in the C-terminal domain of the protein. The analysis of the different antisera showed that the C-terminal domain induced more cross reactive antibodies than the N-terminal domain. These results allowed to design a rTbp2 from strain M982 which induced bactericidal antibodies broadly cross reactive against many strains of meningococci. Clinical grade Tbp2 will be tested in humans in the near future.

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Immunogenicity and safety of intranasal vaccination with meningococcal native outer membrane vesicles in mice and rabbits.

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Efficacy trials with meningococcal group B outer membrane protein (OMP) vaccines have demonstrated the potential of the OMPs to induce protective immunity (1,2,3,4). The OMP vaccines evaluated in efficacy trials, however, have been poorly protective in children under the age of 4 years (3,5). In these children, serum antibody responses as measured by ELISA were high, but bactericidal antibody titers were low. We have approached this problem by attempting to present the OMPs and the LOS in a lipid or native outer membrane environment. One of our strategies is to use native outer membrane vesicles (NOMV), never exposed to detergents, as an intranasal vaccine. The NOMV is an excellent antigen but has been considered too toxic to use as a parenteral vaccine in humans. We believe, however, that NOMV prepared from a genetically optimized vaccine strain can be a safe and effective vaccine if given intranasally (i.n.). This approach mimics the process of natural immunization and has the potential to induce a local antibody response against the OMPs that act as adhesins and invasins (6) as well as a serum bactericidal antibody response. NOMV were prepared under GMP from a mutant group B strain, 9162(-:15:Pl.3:P5.10,?:L3,7,9), deficient in sialic acid synthesis and therefore lacking capsule and sialylated LOS. This mutant was prepared using published sequence information (7) by deletion of a portion of the SynX gene and insertion of a Kanamycin resistance gene. For vaccine production the cells were grown on iron-deficient medium which resulted in the expression the iron regulated proteins. Two Opa proteins were expressed, but Opc was not expressed at significant levels. A vaccine strain expressing the L3,7,9 immunotype was chosen in spite of the presence of the lacto N-neotetraose group because of evidence we have obtained that bactericidal antibodies to this structure are often present in human sera following natural infections.

The NOMV vaccine was found to be non-pyrogenic in the rabbit when given intranasally (i.n.) at a dose of 400 µg protein (88 µg LOS). When given intravenously (i.v.) the maximum non-pyrogenic dose was about 0.05 µg/rabbit.

The mucosal and systemic immunogenicity of NOMV was determined in mice and rabbits. Mice were immunized intraperitoneally (i.p.) or i.n. with NOMV at day 0, and boosted i.n. at day 28. Mice developed serum bactericidal antibodies as well as high levels of specific serum IgA (4 µg/ml) and IgG (1-3 mg/ml) by day 42 as determined by ELISA. Mice immunized i.n. also responded with high levels of IgG and IgA antibody-secreting cells in the lungs as determined by ELISPOT. Rabbits immunized intranasally with three 100 µg doses of NOMV at days 0, 28, and 56 developed high serum bactericidal antibody titers (>512), and mean increases in serum IgG and IgA levels in excess of 450 and 370 µg/ml, respectively, at day 70. Western blot analysis of the rabbit sera showed IgG antibody responses to the Opa proteins, Tbp2, LOS, class1,3, and 4 OMPs, and several proteins in the 45-60 kDal range. ELISA vs purified LOS showed geometric mean levels of antibodies against L3,7,9 and L8 LOS increased from < 0.05 µg/ml to > 10

µg/ml. The results of these animal experiments demonstrate that NOMV given intranasally in animals is safe and induces both serum bactericidal antibodies and a mucosal immune response which may help prevent meningococcal infections by interfering with adhesion and/or invasion. We conclude that these results warrant further intranasal studies with NOMV in human volunteers.

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Opsonic activity induced by a monoclonal antibody against the Lip (H.8) antigen.

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Introduction: The Lip antigen (formerly called H.8) is an outer membrane protein expressed on all pathogenic *Neisseria* species. The gene has been cloned and sequenced and reveals an unusual protein built up of several pentapeptide repeats (1). However, the functional role of the Lip protein is unknown. Unlike many other outer membrane proteins on *Neisseria*, the Lip protein display no variability among strains and might thus be an interesting vaccine antigen. Also, patients with meningococcal or gonococcal infections readily make antibodies against the Lip antigen.

Several mAbs against Lip have been made, but none have revealed any bactericidal activity (2). Affinity-purified human polyclonal antibodies against Lip are also negative in bactericidal activity (3). After these observations were published, much of the interest concerning Lip protein as a vaccine antigen has declined. Phagocytosis is an other effector function that may confer protection against meningococcal disease. The aim of this study was to characterize the opsonophagocytic activity of a mAb specific for the Lip antigen.

Experiments: We have recently made a mouse mAb (denoted 215,C-1, of IgG2a isotype) against Lip, which reacted identically with mAb 2-1-CA2 (W. Zollinger) on Western blot. The 215,C-1 mAb did not induce any bactericidal activity, which is in accordance with the other reported anti-Lip antibodies. However, when tested against ethanol killed *N. meningitidis* strain 44/76 (B15: P1.7,16) for opsonophagocytic activity, 215,C-1 revealed a very high activity. The mAb was also opsonic against other meningococcal strains: G1963, 8069, B385, all previously alcohol killed and stored at -20 ◦C. However, when tested against live 44/76 or B385 the activity was almost negative and phagocytosis was induced in only about 20% of the neutrophils and few bacteria were ingested per cell. This low/negative opsonic activity remained also after ethanol fixation of the same inoculi.

The expression of the Lip antigen on the opsonic+ and opsonic− ethanol killed strains was further tested in ELISA, immuno electron microscopy (IEM) and by Western blot (WB).

In whole cell ELISA, using the 215,C-1 mAb, a much stronger binding against the opsonic+ preparation than against the opsonic− preparation was observed.

IEM of whole cells was carried out using an on-grid immunogold-labeling technique incubating with 215,C-1 as primary antibody and goat anti-mouse conjugated to 10 nm colloidal gold particles as secondary antibody. The opsonic+ preparation of strain 44/76 showed an even, dense labeling of all bacteria, as opposed to the opsonic− preparation of the same strain, which showed a strong variation in labeling density from moderately labeled cells to unlabeled cells. The results from ELISA and IEM might indicate that the Lip antigen is hidden within the membrane structure and scarcely exposed at the surface on live and recently ethanol killed bacteria, and that

prolonged storage might expose the 215,C-1 epitope of the Lip protein. However, this is unlikely since in WB the bacterial preparations that induced high opsonic activity showed strong staining of the Lip band, whereas the preparations that were negative in opsonic activity showed much fainter staining. This suggests that there is a difference in the total amount of the Lip protein between the opsonic+ and the opsonic– preparations, and that this difference is metabolically regulated, and not a result of live vs killed or stored bacteria. How the Lip antigen is expressed on the surface of in vivo growing meningococci remains to be elucidated.

	Phagocytosis	Bactericidal	ELISA	IEM	WB
			Mab against		
Batch	Lip P1.16	Lip P1.16	Lip P1.16	Lip P1.16	Lip P1.16
44/76-94	+++	n.t.	+++	+++	+++
	+++	+++	+++	+++	+++
44/76-95	+	–	+	–/++	±
	+++	+++	+++	+++	+++

Conclusion: These studies show that the Lip antigen is strongly opsonic *per se*, but that it may show a restricted and variable expression at least after in vitro cultivation.

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Expression and purification of meningococcal class 1 porin from *E. coli*: influence of adjuvants on specificity of the immune response to native protein.

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Currently licensed meningococcal vaccines based on group specific capsular polysaccharides offer no protection against serogroup B strains which currently cause the majority of European outbreaks, since the group B polysaccharide is non-immunogenic in humans, probably due to the structural similarity with human glycoproteins (1). Alternative sub-capsular antigens are being investigated as potential vaccine candidates. The class 1 porin protein is one of the two most abundant proteins in the outer membrane and the gene which encodes it, the *porA* gene has been cloned and sequenced (2).The class 1 protein is immunogenic (3) and antibodies raised against it are bactericidal to meningococci *in vitro* (4) and protect against infection *in vivo* (5).

The gene for the class 1 protein was isolated from a P1.16 strain (MC50) and ligated, in frame, into an appropriate 'XPRESS PRSET' vector (Invitrogen) overnight at 14°C. The Xpress system is designed for high level production and purification of recombinant proteins which are fused to a short leader peptide containing a polyhistidine sequence which has a high affinity for divalent cations enabling the protein to be purified by affinity chromatography on Ni^{+2} agarose. The plasmid gives high level expression of recombinant protein from a T7 promoter. T7 RNA polymerase can be introduced to the system via a M13 phage carrying the T7 polymerase gene, or by using *E. coli* JM109(DE3) which has its own T7 RNA polymerase. Thus, the ligated plasmid containing the *porA* gene was transformed into JM109 and expression was induced by the addition of IPTG. Crude cell lysates were analysed by SDS PAGE gel electrophoresis for the presence of class 1 protein. Large amounts of class 1 protein were present in cell lysate pellets, which was solubilized and purified by affinity chromatography on a Ni^{+2} agarose column. The effects of the protein on the immune response were studied using various adjuvants and liposomes.

Liposomes composed of L-α-phosphatidylcholine and cholesterol were prepared by a dialysis-sonication method (6). Recombinant class 1 protein was solubilized in 0.2% SDS and incorporated into liposomes both on its own and with the additional adjuvants muramyl tripeptide-phosphatidylethanolamine (MTP-PE) or monophosphoryl lipid A (MPLA) both at 1mg/ml concentrations. In each case immunogold electron microscopy with a P1.16 specific monoclonal antibody showed that the protective epitope was located on the surface of the liposomes. In addition solubilized class 1 protein was also adsorbed to alum, incorporated into an emulsions with (a) Ribi adjuvant containing MPLA, cell wall cytoskeleton and trehalose dimycolate, and (b) squalane and pluronic-block copolymer L121.

Groups of Balb-C mice and New Zealand half-lop eared rabbits were immunised at 0, 14, 28 and 42 days with 20 µg recombinant class 1 protein liposomes in a volume of 100µl (in PBS pH 7.2). In addition, purified class 1 protein in alum, Ribi and pluronic emulsions were used in

simultaneous immunisations. Animals were bled pre-immunisation, at regular intervals throughout the immunisation schedule and for a further three months after the last injection.

Sera from the animals given the emulsions and alum gave the greatest response in ELISA against native protein and homologous outer membrane preparations with pluronic giving the best titres. The addition of adjuvants to the liposomes produced significant increases in response to the purified class 1 preparation and against homologous outer membrane preparations. In Western blots against homologous outer membranes, all the sera reacted specifically with the class 1 protein.

The sera were analysed for their IgG subclass specificities. Sera from animals immunised with the Ribi and pluronic emulsions and liposome preparations containing MPLA gave antibodies of IgG2 subclass associated with bactericidal activity. Sera produced from all the other preparations only gave IgG1 subclass antibodies.

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Expression of the outer membrane protein complex of *Neisseria meningitidis* group B in different culture conditions.

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Neisseria meningitidis express outer membrane proteins complex (OMPsC) which are surface antigens of vaccine interest (1). The majority proteins can be present in numbers from 3 to 5 and with molecular weights between 25 and 46 KDa (2). Other outer membrane proteins of larger size molecules between 70 and 110 KDa were expressed when cultivated with iron limitation (3). The expression of OMPC is variable depending on the genetic features of the strain, medium and culture conditions.

The objective of this paper is to assess the potentiality of the expression of Neisseria meningitidis group B in fermentation with different culture conditions and concentrations of Fe^{+3} in the medium.

Cultures of strain B:4:P1.15 were carried out with successive passes in fermentors of up to 35L, in supplemented Frantz medium with dialyzed yeast extract (FM) under oxygen and pH controlled or uncontrolled conditions. The kinetics of expression of high molecular weight proteins was determined in FM medium and the character of the iron regulated were assessed by adding or depleting Fe^{+3} in the FM medium. Harvest took place at the end of the logarithmic growth phase. The OMPC was extracted with 0.2 M Lithium Chloride or 0.5 % Sodium Deoxycolate and was separated by ultra-centrifugation at 100,000 g. The proteins were analyzed through SDS-PAGE and quantified by laser densitometry.

The growth kinetics in the cultures conducted in FM medium with controlled pH showed larger growth speed. Harvest was accomplished in the maximum of feasible microorganisms. The yield of biomass and the composition of OMPsC were similar for each studied condition. The proteins present in the complex, class 1, 3, 4, 5, 70 kDa and 80 kDa, were expressed with a uniform composition. The kinetics of expression of the 70 kDa and 80 kDa proteins were related to the concentrations of Fe^{+3} present in the culture. In the iron depleted FM medium these proteins were expressed since the very first hours of culture, whilst the medium with enough Fe^{+3} were not expressed which shows their iron regulation.

The assessed culture conditions did not influence the OMPsC expression. Results were that high molecular weight proteins are iron regulated, therefore controlling this, OMPsC of well defined and uniform composition of these proteins can be obtained. This fact is of major importance for vaccines based on OMPsC (4).

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Consistency in the large scale production of the outer membrane protein complex of group B *Neisseria meningitidis*.

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GMP's establish controls for each and every stage of the vaccine production processes to assure quality, safety and efficacy of the final product and production consistency. Among all, the culture production processes is the main stage in the expression of target antigens.

In antimeningococcal vaccines that are based on outer membrane protein complex (OMPsC), it is very important that the compound contain proteins that will induce broad spectrum antibodies, because the antigenic diversity of the meningococcus, makes it dificult to know which are directly responsible for the control of the disease. The defined composition of the complex and their presence in outer membrane vesicle (OMVs) are crucial for them to confer immunity (1). Using detergents, OMVs should be extracted from the biomass obtained in rational harvest time and well defined fermentation processes. (2-3).

The objective of this paper is to show large scale consistency in the expression of OMPsC of group B Neisseria meningitidis and characterize the purified antigens regarding their composition, structure and contaminant levels.

Thirty fermentation processes of strain B:4:P1.15 were studied in Frantz medium supplemented with yeast extracts dialyzed at a scale of 300 litters. The extraction of OMVs was carried out with 0.5% sodium deoxycolate and was separated by ultra-centrifugation. After purification, the composition and structure of OMPsC were studied. Protein analyses were conducted using Lowry method, SDS-PAGE and laser densitometry. Electronic microscopy was used to determine vesicle composition. Lipopolysaccharides (LPS) were quantified by KDO method. Polysaccharide and nucleic acids contaminants were assessed by determining the sialic acid and 260 nm spectroscopy, respectively.

Uniform and vesicle structured proteins class 1, 3, 4, 5, 70 kDa, and 80 kDa forming part of the complex were shown during fermentation. The purification of the OMVs ensured stable concentrations of LPS. The level of contaminant polysaccharides and nucleic acids were kept below the acceptance limits.

The proteins present in the studied OMVs concurred with those reported in the batch of VA-MENGOC-BC studied in Iceland (4). Contrary to other papers (5 - 6) iron regulated proteins 70 kDa and 80 kDa were consistently produced and under control.

The large scale production of OMPsC met the established requirements for their use as vaccine antigens of VA-MENGOC-BC and had a well defined composition in the form of vesicles and having low contaminant levels.

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Recombinant Opc reconstituted into liposomes elicits opsonic antibodies.

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Opc is an antigenically conserved outer membrane protein of *Neisseria meningitidis* which is widely distributed among different serogroups. This protein is highly immunogenic in humans and induces bactericidal antibodies (1). The expression of Opc is regulated at the transcriptional level (2), with the amount of expressed protein ranging from very high in bacteria isolated from the nasopharynx to low in bacteria isolated from blood or cerebrospinal fluid. Opc seems to function as a bacterial adhesin, mediating some step of the meningococcal interaction with host epithelial cells (3).

The *opc* gene from the *N. meningitidis* strain B385 (B:4P1:15) has been cloned in *E. coli* fused to the first 44 amino acids of the P64k protein . The resultant 32 kDa protein was expressed as inclusion bodies, and after a washed pellet procedure it was obtained in soluble form with more than 80% of purity (4). In order to renature the recombinant polypeptide into a conformation resembling its native state, we have included it into synthetic liposomes by freeze-drying and analyzed the immunogenicity of the resulting preparation. Briefly, a mixture of empty vesicles and Opc was frozen and lyophilized three times, obtaining 30% incorporation of the protein into dried-rehydrated vesicles (DRV). Opc was inserted in the lipid bilayer rather than enclosed in the vesicles; and surface exposed as demonstrated by protease treatment of the liposome suspension. Furthermore, the intact vesicles bound the human monoclonal antibody LuNmO3, which recognizes a conformational epitope on natural 5C (5).

To further investigate the immunogenicity of this preparation, DRVs containing Opc mixed with the beta subunit of cholera toxin (CTB) and $Al(OH)_3$ as adjuvants were used to immunize Balb/c mice. The resulting sera showed high titers against pure recombinant 5C, as well as against OMPs of meningococci belonging to different serotypes. Moreover, it recognized $5C^{++}$ meningococci in colony blots, demonstrating that at least a portion of 5C in the immunizing liposomal preparation was in a native-like conformation. The antibodies, however, were not bactericidal, although they did show opsonic activity on a Fluorescence-Activated Cell Sorter (FACS) based assay. The reasons for their lack of bactericidal activity are currently under investigation.

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Influence of adjuvants on the humoral immune response towards a synthetic peptide containing a B-cell epitope from meningococcal class 1 protein.

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A major problem encountered in developing modern vaccines using chemical, recombinant or other technologies, is their weak immunogenicity. This has traditionally been overcome by the use of adjuvants, which are defined as compounds which can increase the humoral and/or cell-mediated immune responses to an antigen (1). A large number of structurally unrelated compounds, which can act via different pathways, are known to augment immune responses to weak antigens. Adjuvants commonly used or considered for human vaccines include mineral compounds, oil emulsions, natural structures and synthetic analogues derived from bacteria, saponins (of vegetal origin), surfactants, cytokines, and delivery vehicles such as liposomes, iscoms and microspheres.

Synthetic peptides as vaccines present a major problem because they are generally haptenic in nature and often require covalent linkage to carrier proteins to become effective immunogens. In addition, such highly purified peptide vaccines require very strong adjuvants to achieve early, high and long-lasting immune responses. In this study, a chimaeric synthetic peptide containing a meningococcal B-cell epitope in tandem with a defined tetanus toxin T-cell epitope, and assembled on a polylysine multiple antigen peptide core (BT-MAP) (2), was used as a model immunogen to assess the influence of various adjuvants on the humoral immune response. The adjuvants compared are either licensed preparations or have been used in human clinical trials.

Groups of mice were immunized with peptide and the following adjuvants:
i) adsorbed to aluminum hydroxide (Alhydrogel) and calcium phosphate gels, the only adjuvants licensed for clinical use in humans, and which have the longest history of proven use, ii) the saponin Quil A, iii) Ribi adjuvant (RAS) emulsion, containing monophosphoryl lipid A (MPLA), cell wall cytoskeleton and trehalose dimycolate, iv) Syntex adjuvant formulations (SAF). Syntex adjuvant contains muramyl dipeptide, the smallest unit of the mycobacterial cell wall which retains adjuvant activity, and a non-ionic block copolymer which is a simple linear polymer of hydrophobic polyoxypropylene (POP) and hydrophilic polyoxyethylene (POE), in a squalane-in-water emulsion (3). In addition, water-in-oil and oil-in-water Syntex emulsions were prepared with the addition of MPLA. For control immunizations, peptide was emulsified with Freund's complete and incomplete adjuvants, and with TitreMax, a squalane-in-oil emulsion containing a block copolymer which offers comparable adjuvanticity to Freund's emulsions but with greatly reduced toxicity. Also, peptide in saline alone and in a squalane-in-oil emulsion was used for immunization.

When administered in saline alone, the peptide induced a weak but significant humoral response against the B-cell epitope. This adjuvant effect is probably two-fold: a combination of increased epitope density on the peptide core with stimulation of Th-cells. In addition, immunization in a squalane-in-water emulsion alone also induced a similar response. However, when administered

with adjuvants, the immune response to the peptide was markedly increased. The most adjuvant-active formulations were the oil-in-water Syntex emulsion containing MPLA and the water-in-oil Syntex emulsions and RAS. The mean ELISA titres with these adjuvants were significantly greater than those observed with aluminum hydroxide and Freund's adjuvant. The least adjuvant-active formulations were the TitreMax emulsion, Quil A and calcium phosphate. In addition, the anti-peptide IgG titers elicited by peptide with adjuvant(s) declined only slowly with time, thus fulfilling the criterion of inducing a long-lived response. The RAS and water-in-oil SAF-MPLA emulsions were also able to elicit early IgG antibody titres after 1-2 doses only. No differences were observed in the epitope specificity of antisera, as determined by reactivity with overlapping peptides synthesized on pins. In addition, all antisera also reacted with homologous outer membranes.

The antibody isotype profile was markedly influenced by the adjuvant used. When peptide was administered in saline, with squalane, the saponin Quil A, adsorbed to $Al(OH)_3$ or $CaHPO_4$, or emulsified with TitreMax, only antibodies of the IgG1 isotype were elicited. Freund's adjuvant, RAS and the Syntex formulations, however, were able to induce antibodies of the IgG2a and IgG2b subclasses, which may be important for protection (4). Indeed, significant differences were seen in the bactericidal activities of antisera, suggesting an important role for adjuvant in modulating the immune response towards production of functional antibodies.

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IgG antibodies specific to the linear B-cell epitope on the class 3 outer membrane protein can promote opsonophagocytic killing of *Neisseria meningitidis*

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The class 3 outer membrane protein (OMP) of *Neisseria meningitidis* has been reported to be one of the potential targets for bactericidal and opsonic antibodies in humans, which recognize mostly surface-exposed epitopes [1,2]. The serotype 15 class 3 OMP constitutes one of the components of Norwegian group B meningococcal outer membrane vesicle (OMV) vaccine, which was used recently to immunize about 90 000 adolescents in Norway [3,4]. The aim of this study was to identify linear epitopes occurring on the serotype 15 class 3 protein and to study the antigen-binding and effector properties of the specific antibodies.

Synthetic peptides spanning the entire sequence of the class 3 OMP from the vaccine strain 44/76 (B:15:P1.7,16) were synthesized on pins and screened with paired sera from vaccinees from the Norwegian vaccination trial and from patients with systemic meningococcal disease (SMD). Basing on the differences in reactivity patterns in pre- versus post-vaccination sera, an immunodominant epitope consisting of 14 residues (17-30) was identified on the putative loop 1 (VR1) region close to the N terminus of the molecule. To study specific immune responses in more detail, a soluble 23mer peptide D63b2 covering the VR1 region was synthesised. Then quantitative peptide D63b2-specific IgG responses were measured in sera from 27 volunteers immunized with the Norwegian group B OMV vaccine, and from 132 SMD patients. The 17-30 epitope was found to be efficiently recognized by post-vaccination sera taken after three doses of the Norwegian group B OMV vaccine in 74% vaccinees, while no clear linear epitopes were recognized by four different murine monoclonal antibodies [5]. In contrast, both the class 3 OMP and the 17-30 epitope were low immunogenic in the course of SMD, as judged from both immunoblotting studies (responded 24/132; 18.2%) and reactivity with peptide D63b2 (responded 18/132; 13.6%), respectively. Peptide D63b2 significantly inhibited IgG binding to the denatured PorB protein on immunoblots, suggesting that this B-cell epitope was one of the main linear epitopes on the PorB protein recognised by sera from vaccinees and some SMD patients.

To study antigen-binding and effector properties of the specific antibodies, we purified the class 3 OMP from the PorA-deficient isogenic mutant M14 [6]. The purified class 3 OMP and peptide D63b2 were immobilised on the highly cross-linked (6%) spherical agarose beads with N-hydroxysuccinimide (NHS)-activated 6 atoms-long spacer arms. Peptide- and protein-specific IgG preparations were affinity purified from a post-vaccination serum with high levels of peptide-specific IgG antibodies. To study antigen-antibody interaction in more detail, we measured the affinity constants characterizing binding of the specific IgG preparation to bacteria, to the OMV from strain 44/76, or to peptide D63b2. Whatever the antigen studied, the peptide D63b2-specific antibodies possessed higher affinity compared to the class 3 OMP-specific antibodies, which supports the interpretation that the superior accessibility of the target epitope on peptide D63b2 selected for high-affinity antibodies during purification. The affinity constants characterizing IgG binding to bacteria were significantly higher than that for OMV complex or

peptide D63b2, which may suggest higher target epitope density on the bacterial cell surface. Both the class 3 OMP- and peptide D63b2-specific IgG preparations were found not bactericidal, but enabled complement-dependent opsonophagocytosis of *Neisseria meningitidis*, measured as respiratory burst of human neutrophils and/or internalization of opsonized meningococci by PMNL. Blocking experiments clearly suggested involvement of both FcγRII (CD32) and FcγRIII (CD16) into the observed antibody-dependent receptor-mediated phagocytosis. Taken together, the data presented here indicates that the PorB-specific antibodies may contribute to vaccine-induced protection against systemic meningococcal disease via the opsonophagocytic route of pathogen clearance.

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Immune response of mice to *Neisseria meningitidis* serogroup B protein antigens

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Neisseria meningitidis is one of the most common meningeal pathogens which causes systemic disease in humans. Depending on the clinical manifestations, the disease shows mortality rates of 10 to 70% (1). The identification of meningococcal antigens which induce functional protective antibodies constitutes an important approach to vaccine development (2). The polysaccharide antigens in general show age dependent protection, an immune response of short duration and no induction of immunological memory. In addition, the polysaccharide of the B serogroup shows poor immunogenicity relative to the other serogroups. This situation has stimulated interest in protein antigens and conjugated vaccines (3). In our laboratory, the immune response of Swiss mice to 0.1, 1.0 and 2.5 μg doses of outer membrane vesicles (OMVs) obtained by Sodium desoxycholate (DOC) treatment of the cells of *N. meningitidis* serogroup B was studied (4). The response was evaluated in terms of total antibodies and their bactericidal activity against prevalent Brazilian strains. The two higher doses induced high titers of total and functional antibodies with no statistical difference between them. These antibody levels were sustained during a four month period. The animals receiving the lowest dose received a booster dose after one month and showed an immune response equivalent to the high dose animals. These results suggest that OMVs protein antigens represent potentially protective components of a vaccine against *N. meningitidis* serogroup B.

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Vaccine potential of meningococcal transferrin binding proteins: mouse protection studies.

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There is much interest in the vaccine potential of *Neisseria meningitidis* transferrin binding proteins (Tbp1 and Tbp2) which are possessed by all meningococcal strains and are involved in the uptake of iron from transferrin (1). They have been shown to elicit protective and bactericidal antibodies against the homologous *N. meningitidis* strain in laboratory animals (2,3). There is, however, heterogeneity of molecular size and lack of a cross-reactive immune response in laboratory animals, particularly for Tbp2, which would appear to limit the usefulness of Tbps as vaccine antigens. In contrast, human antibodies to meningococcal Tbps have been shown to be highly cross-reactive (4), strengthening the case for their inclusion in a vaccine against meningococcal disease. We have used a mouse IP model of meningococcal infection to determine the protective potential of transferrin binding proteins (Tbp1 and Tbp2) against challenge by homologous and heterologous strains.

Tbp1+2 were purified from detergent extracts of iron-limited bacteria using transferrin-Sepharose affinity chromatography (5) and separate Tbp1 and Tbp2 were obtained by ion exchange chromatography on MonoS (Pharmacia). Both of the isolated proteins retained the ability to bind transferrin.

Mice (CAMR-NIH) were immunized with Tbps on days 1, 21 and 28 and challenged on day 35. Mice received 10mg human transferrin IP with the challenge and a further 10mg at 24h. Bacteria for the challenge were grown under iron-limited growth conditions. Animals immunized with Tbp1+2 isolated from serogroup B meningococci were protected against challenge with other serogroup B organisms, with greater protection seen with the homologous strain and strains expressing a Tbp2 with a similar molecular weight. Little or no protection was observed against challenge with meningococci possessing Tbp2 with a very different molecular weight. Mice immunized with Tbp1+2 from a serogroup B strain were also protected against infection with a serogroup C but not a serogroup A strain.

IgG1 was the predominant immunoglobulin type induced in response to Tbp1+2 immunization and antibodies cross-reacted with purified Tbps with a similar Tbp2 molecular weight. Mouse serum also strongly inhibited transferrin binding to homologous Tbps, determined by ELISA.

The protective potential of separate, functionally active, Tbp1 and Tbp2 was also determined. Previously, protection with Tbp1 was not investigated as it did not elicit bactericidal antibodies (3) but this may be because the purification methods used produced Tbp1 that no longer bound transferrin. It was found that Tbp2 provided protection against the homologous strain which was equivalent to that provided by Tbp1+2 or recombined Tbp1+Tbp2. Tbp1 provided a much reduced degree of protection compared with Tbp2. The cross-reactivity of sera raised against separate Tbp1 and Tbp2 (of both high and low molecular weight) will be presented. Preliminary

results indicate that Tbp1 in the separate form is less immunogenic compared with Tbp1 in the Tbp1+2 complex. This may account for the apparent lack of protective potency of Tbp1 alone. These results strengthen the case for inclusion of Tbps in a meningococcal vaccine but confirm that, in mice, Tbp2 is the predominant protective antigen.

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Prospects for a nasal vaccine against group B meningococcal disease

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Outer membrane vesicles (OMV) from *N. meningitidis*, the main constituent of the Norwegian vaccine against group B meningococcal disease (1), have proved to be strongly immunogenic when administered intranasally in mice (2). Based on these findings, similar studies were initiated in humans. We have searched for evidence that non-proliferating mucosal vaccines might be effective without the use of toxins as so-called mucosal adjuvants.

Twelve volunteers were immunized with OMV in the form of nose drops or nasal spray four times at weekly intervals, followed by a fifth dose six months later. Each dose consisted of 250 µg protein (i.e. 10x the intramuscular dose) in 0.5 ml PBS, with half the amount given on either side. Blood for serum and peripheral lymphocytes, nasal fluid and whole saliva were collected for up to seven months.

Five of the vaccinees responded to the first series of four immunizations with at least a two-fold rise in serum IgG antibodies to OMV as measured by ELISA. Five individuals also developed more than four-fold increases in serum bactericidal titers to the vaccine strain, but these results were concordant with the IgG ELISA results only for two of them. On the other hand, eight of the vaccinees showed increases in serum bactericidal activity against a variant of the vaccine strain expressing the Opc protein, which correlated well with the increases in serum IgG antibodies. The Opc protein may thus be important for induction of immunity via the mucous membranes.

Following the first four immunizations, all 12 vaccinees responded with at least two-fold increases in IgA antibodies specific to OMV in nasal fluid, and eight of them also developed such antibodies in saliva. Thereafter the antibody levels in the secretions decreased gradually although significantly increased levels persisted for five months, until the fifth dose were given. No significant IgA response in secretions was detected in a control group of 11 vaccinees given the OMV vaccine, with aluminum hydroxide, twice intramuscularly. Thus, the nasal vaccine induced mucosal antibodies most consistently at the site of stimulation.

On immunoblots, serum antibody responses to the nasal vaccine were mainly directed against the class 1 and 5 meningococcal protein and LPS, which are also strong immunogens by intramuscular vaccinations (3). The IgA antibodies in nasal fluid and saliva from some vaccinees were mainly directed against the class 1 protein.

After the fifth intranasal immunization dose it appeared to be signs of a booster effect on the antibody levels in serum, as well as in secretions. However, the changes in antibodies following this last dose were not statistically significant.

A transient proliferative response of peripheral lymphocytes from 10 of the 12 vaccinees could be detected against the class 1 protein two-to-four weeks after start of the nasal vaccinations. This indicates that an antigen specific T-lymphocyte response was also elicited.

Our results indicate that the administration to human volunteers of a meningococcal OMV vaccine directly onto the mucous surfaces, either as nose drops or nasal spray, can stimulate local mucosal as well as systemic immune responses. These effects which were not dependent on the addition of any other adjuvant, might eventually be shown to convey protection against invasive meningococcal disease.

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Analysis of the human Ig isotype response to individual Tbp1 and Tbp2 from *Neisseria meningitidis*

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There is as yet no vaccine available for prevention of serogroup B meningococcal disease. The poor immunogenicity and low affinity response to the group B capsule (1) has focused attention on meningococcal subcapsular antigens as vaccine candidates for group B disease (2). Meningococcal transferrin-binding proteins are currently being investigated for their vaccine potential (3). This study examined the human isotype antibody response to purified meningococcal Tbp1 and Tbp2 from two strains (SD and B16B6) expressing both high and low molecular weight Tbp2s (4).

The IgM, IgG and IgA responses to meningococcal Tbps in seventy well-defined sera from proven cases of meningococcal disease were examined by ELISA.

Tbp1 isolated from both strains was recognized more frequently and produced higher ELISA absorbance values than Tbp2s from either strain. This antibody response was independent of the serogroup or serotype of the infecting meningococcal strain. Tbp1 from strain B16B6 produced the most pronounced reactivity and was the most frequently recognized antigen. The reactivity of all four proteins was highly variable between individuals and differed significantly between all four antigens.

The reactivity of the majority of sera examined and the durability of the anti-Tbp1 response may indicate a role for these antigens in a vaccine. However, the variability of immune responses to each class of Tbp from the two strains suggested that a successful vaccine would need to include a combination of Tbps of varied specificities.

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Inducement and duration of cellular response to VA-MENGOC-BC® in babies and children.

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VA-MENGOC-BC® is the commercial name of a Cuban vaccine against *Neisseria meningitidis* of serogroups B and C. The vaccine consists of purified proteins from the outer membrane of group B meningococci enriched with proteins of "High Molecular Weight", which form a proteoliposome with controlled amount of phospholipid and lipopolysaccharide. The whole complex is mixed with C polysaccharide and then adsorbed to aluminum hydroxide gel (1,2).

The preclinical and clinical (phase I and II) studies showed the vaccine to be innocuous, safe and induce antibodies against different pathogenic serotypes (3). A double blind placebo-vaccine trial had been conducted in secondary students (11-16 years), with an efficacy of over 80 % (4). A second field trial with children from 5 months to 24 years of age had an efficacy of 83-90 %. This vaccine was included in the Cuban National Immunization Scheme at the age of 3.5 months and a mass vaccination campaign was organized in 1989-90 in babies under 6 months, with an efficacy of 92.5 % (2). This vaccine induces long-lasting specific IgG and bactericidal antibodies against some of the most frequent serotype B pathogens. After a 3rd dose these antibodies increased various times, showing an excellent anamnestic specific response, which allows a high efficacy (2).

VA-MENGOC-BC® has been applied to over 40 millions of persons in Cuba and other countries (Brazil, Colombia, Argentina, etc.). The Cuban strategy made it possible for the morbidity and mortality rate caused by *Neisseria meningitidis* B to drop since 1988, due to the vaccine application in provinces (5). Nevertheless, the cellular evaluation induced by VA-MENGOC-BC® has been less studied, so our group working in this field has shown the existence of Lymphoproliferative response *in vitro* in vaccinated mice and in humans (6). Also, and even more important, is the presence of delayed-type hypersensitivity (DTH) response that was determined in vaccinated humans (7) and this response was transferred by cells in mice (unpublished results).

Methods. Immunization**.** A population of nursing Infants (3.5 months old), children (from 2 to 6 years old) and teenagers (from 11 to 13 years old) were included to carry out the present work. The ages were selected regarding the time of vaccination (from 0 to 7 years). All were immunized or had been vaccinated with a two dose scheme of VA-MENGOC-BC®, spaced from 6 to 8 weeks apart, accordingly to producer indications. The teenagers were challenged with a third dose.

Prospective evaluation. To evaluate the induction of a cellular response, the Nursing Babies were studied in terms of the dermal test of DTH, before the vaccination program started, after the first dose (6 months of age) and 28 days after second dose.

Retrospective evaluation. To determine the duration of induced cellular response, children and teenager previously vaccinated between 2 and 7 years ago were evaluated. A dermal test was carried out in all, and in addition, in teenagers two blood extractions were carried out for the evaluation of Lymphoproliferative (LP) response and antibody forming cells (AFC), using ELISPOT techniques.

Dermal test of delayed-type hypersenitivity. This test was carried out by multi-puncture, using 14 µg of proteoliposome (protein component of VA-MENGOC-BC®) diluted in glycerol-PBS-phenol, taking previous aseptic and antiseptic measures. Determinations were carried out at 4, 12, 24 and 48 hours, with delimitation of hardness with a pen, transferring it to paper and measuring its diameters (mm).

ELISPOT The peripheral mononuclear cells (MNC) were obtained from the extraction of 10 ml of blood, before (t = 0) and after a third dose (t = 7, 14 and 21 days), by a Ficoll-Hypaque cushion. MNC were employed in the trial. Antigen (proteoliposome) was fixed at 10 µg/ml on a 96 nitrocellulose bottomed-well plate, incubated at 4 °C over-night and then cells were added at variable concentrations. Incubation took place during 4 hours at 37 °C in a 5 % CO_2 atmosphere. Finally, horseradish peroxidase labeled conjugated was added, being revealed with Diaminobenzidine. The reading was conducted in a stereo-microscopy and the results were expressed as AFC per million of MNC. The mean and standard deviations were calculated and the T test was applied to compare the results between experiments, considering that $p < 0.05$ indicates a significant difference.

Lymphoproliferation (LP). The MNC were cultured in complete medium RPMI-10 % FCS and were faced *in vitro* with proteoliposome at 2, 5 and 10 µg/ml for 5 days. The culture were pulsed with [^{3}H] Thymidine during the last 18 hours, harvested and counted their incorporation in a ß liquid scintillation counter. The mean count per minutes (CPM) of triplicates and Stimulation Index (SI) were determined. The SI > 2 were considered positive.

Ethical features. Because of the inclusion of children in this work, the authorization by National Group of Pediatrics and Health Municipality was necessary, once the characteristics of innocuity were known. Furthermore, the agreement by writing of each father or guardian was included.

Results. The cellular immune response is very important to be evaluated, since T cells are the principal orchestrator of any kind of immune response, intervening in and directing the specificity (by determination of which antigen and epitope will be in fact recognized); the effector mechanism selection; helping in the proliferation of effector cells selected; increasing phagocytic functions and activating other effector cells; allowing Ig isotope switching; participating in the long-lasting memory inducement and intervening in suppression. T Cell Response, according to the cytokines profile produced, can be split into T_H1 (IL-2, IFNγ) and T_H2 (IL-4, IL-5 and IL-10) subsets.

DTH is an *in vivo* test that translates an induced T_H1 cellular immune response. In previous projects the presence of DTH in Balb/c mice was demonstrated and proteoliposome concentrations were standardized for their use in human adults. Because of this, the evaluation in children was indispensable, even more when in Cuba, this vaccine is included in the National Program of Immunization. The first dose beginning in nursing babies 3.5 months after birth, with a coverage of over 98 %.

The absence of DTH in nursing babies before vaccination, being positive after the first dose and its subsequent increase after this, emphasizes that VA-MENGOC-BC® induces a strong cellular response, not only in adult, but also in nursing infants. Finally, no response was observed 4 and 12 hours after DTH test application, and the maximum hardness was after 48 hours. These results explain the good and previously observed antibody response in these ages and that the dermal response is mainly DTH.

The T cell response duration, evaluated by DTH, was maintained for 2 to 7 years, without meaningful differences ($p < 0.5$) between them. These results evidence a long-lasting memory induced by the vaccine or their natural booster or by the cross-reactive stimulation of other microorganisms.

T Cell response was also evaluated *in vitro* for Lymphoproliferation, and the B cell response by AFC with ELISPOT. For teenager vaccinated 5 years ago, both responses were determined in kinetic. Twenty six percent were LP positive (SI > 2) before the third dose and then was a decreased in the subsequent determinations. This decrease could be associated with the recruitment of specific T lymphocytes toward the site of immunization. The specific AFC were absent, and there was a positive conversion in 78% ($p > 0.05$) of those evaluated at 7 days. The rest of the determinations were negative. These activated cells, after proliferation and differentiation may be directed to the effector sites of the immune response.

Taking into account the results in the teenagers vaccinated 5 years ago, the *in vitro* immune response in the seven year group was only determined before and 7 days after challenge. The LP was positive before the challenge in a 34 percent, and B cell response was positive in a 12 percent before and 98 percent 7 days after the challenge. It should be emphasized that the number of AFC in the positive population was very low at the beginning (0.73×10^6 MNC); but showed a greater increases after challenge (67.68 vs 32.24×10^6 MNC). Furthermore, the number of AFC in teenagers vaccinated 7 years ago (73.9×10^6 cells) was significantly higher ($p<0.001$) compared to those vaccinated 5 years ago (3.5×10^6 cells).

The greater LP and AFC response of children after 7 years (Holguín), than those after 5 years (Havana City), could be due to a different traffic of *N. meningitidis* or other cross-reactive microorganisms circulating in both groups (provinces). This result was analogous to the epidemiological and microbiological studies developed previously to vaccination. We are currently working on the monitoring of this hypothesis.

The presence of a meaningful percentage of teenagers with positive Lymphoproliferation, all with positive dermal tests, and the absence or low percentage of responders at B cell level in

those vaccinated 5 and 7 years ago, allow us to suggest that the long-lasting response (memory) induced by VA-MENGOC-BC® is mainly T cell-mediated.

Conclusions.

1. VA-MENGOC-BC® induces a T-cell immune response, not only in adults, but also in children and nursing babies.
2. The response induced by VA-MENGOC-BC® is long-lasting, at least 7 years, and is fundamentally T cell-mediated.
3. The ELISPOT was shown to be a useful technique for humoral response evaluation at the cellular level.

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Anamnestic B cell response by ELISPOT after a challenge in humans vaccinated with VA-MENGOC-BC^{Ò} 5 years ago.

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Introduction. Meningococcal diseases is a health problem in many South American countries, not only because of its substantial incidence, but also because of its high lethality in children (1). Until recently, no vaccine had been available for the prevention of infection with *Neisseria meningitidis* B (2). The Finlay Institute developed and carefully tested a vaccine against *N. meningitidis* B and C serogroups, that mainly consists of outer membrane proteins from group B meningococci enriched with protein from the High Molecular Weight Complex, integrating a well defined and stable proteoliposome with a controlled amount of LPS (3). In 1989 and 1990, children under the age of 6 years were vaccinated in a national campaign, with a great decrease in the incidence of the disease.

Limited studies about duration of the immune response after vaccination have been carried out. The present study was conducted considering the existence of immunological memory and cellular migration that allow antigen recognition and rapid proliferation in the inflammatory sites due to the expression on the cell surface of accessories and adhesion molecules. All this was examined in order to assess the effects of the vaccination in the B cell compartment of the population immunized several years before, and the kinetics of occurrence of these cells in peripheral blood, using mononuclear cells (PBMC).

The objectives are to evaluate B cell immune response, at cellular level, in teenager immunized with VA-MENGOC-BC® 5 years before and after a third dose, and to determine the kinetics of occurrence of B cells recirculating in peripheral blood, after a third dose of VA-MENGOC-BC®.

Methods. Immunziation. A population of 80 teenagers, age ranging from 11 to 12 years, were included in this study. All were immunized with two doses of VA-MENGOC-BC® 5 years before, following manufacturers' instructions, and challenged with a third dose.

Elispot. Extraction of 10 ml of peripheral blood were carried out, before and after the third dose of VA-MENGOC-BC®. The kinetics for the study of the occurrence response of peripheral Antibody Forming Cells (AFC) were made with the evaluation of the response at 0, 7, 14, 21 and 28 days after challenge. The universe was separated in four groups (I-IV) of 20 individuals each, measuring all responses at time (t)=0 days and at t = 7 (Group I); t = 14 (Group II); at t = 21 (Group III) and t = 28 (Group IV). The PBMC were purified by a Ficoll-Hypaque gradient and were used at 5 x 10^5 per well, in Nitrocellulose 96 bottomed-wells plates. Antigen (proteoliposome 10 µg/ml) was fixed, incubated at 4 °C over-night and the cells were added and incubated during 4 hours at 37°C, in 5% CO_2 atmosphere. The peroxidase labeled conjugate was added and exposed to diaminobenzidine. The reading of the wells was made in a stereo-

microscope and the results were expressed as AFC per million of PBMC. The mean and standard deviations were calculated, and the T test was applied to compare the results between experiments, considering that $p < 0.05$ indicates a significant difference.

Results. The ELISPOT is a new method that uses the properties of B lymphocytes, carrying a surface immunoglobulin as antigen receptor for its recognition and specifically proliferates after stimulation, with the production of antibodies molecules that are measured by the formation of spots. In this study, we report the results after the evaluation of AFC response in teenagers' PBMC challenged with a third dose of VA-MENGOC-BC®.

The evaluation showed the absence of specific circulating B lymphocytes response in the whole group that was vaccinated 5 years ago (t = 0) and their conversion in 78 % of cases 7 days after the challenge in group I. This was the only group with a positive response after vaccination; group I (t = 14), group II (t = 21) and group III (t = 28) was negative when AFC in PBMC was measured. This lymphocytes behavior suggests that B cell memory is non-long lasting, at least 5 years after of primary vaccination (two doses of VA-MENGOC-BC®) Due to their complexity, proteoliposome induce the T cell compartment (4, 5) with a T cell-mediated long-lasting memory. Significant lymphoproliferation was observed 5 years after vaccination (6) that could explain the B cell response conversion after a third dose. T cells cooperate with B cells, inducing their activation and proliferation with a high antibody response, not only in animal but also in humans. The presence of some antigen-specific memory B cells in germinal center in lymph nodes (LN) is also possible, and may explain IgG-producer B cell as early as a week in peripheral blood. The cells proliferated specifically against the antigen taken and carried by APC toward the LN, with their differentiation in AFC after undergoing affinity maturation (7).

The expression of adhesion molecules or homing receptors on cell surface, allow cellular migration from LN to the inflammatory sites (8, 9). This cellular migration can be determined as optimal around 7 days after vaccine booster. After that, the determination of B cell in blood is almost impossible, maybe because cells leave the vascular stream toward the inflammatory sites.

Conclusions.

1. For the first time anti-proteoliposome AFCs of humans vaccinated with VA-MENGOC-BC® were evaluated.
2. The occurrence of recirculation of B cell response after a third dose of VA-MENGOC-BC®in teenager was determined, with an absence of circulating B lymphocytes before vaccination and with a great increase after vaccine booster.
3. ELISPOT was shown to be a useful method to measure B cell response in humans.

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IgG subclass response after systemic immunization with the Norwegian outer-membrane vesicle vaccine against group B meningococci.

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IgG is the main antibody class in serum and represents the secondary immune response with raise in antibody affinity and the generation of immunological memory. There is both overlapping and distinct protective effector functions attributed to each of the four human IgG subclasses (1). Thus, analysis of the IgG subclass response after vaccination will give valuable information concerning anticipated protective effect of the vaccine.

Two groups of ten volunteers were analyzed for IgG subclass response against OMV after vaccination. The first group was given a third vaccine dose after having received two doses 4-5 years before. The second group consisted of previously unimmunized volunteers and selected for low antibody level against group B meningococci. This group was given three doses of the vaccine by a schedule of 1 month between the first and the second dose and 9 months between the second and the third dose. We measured the antibody response in gravimetric units based on monoclonal chimeric, mouse V and human C, hapten antibodies of all four human IgG subclasses as calibrators (2).

There was a substantial individual variation among the vaccinees. The overall dominating subclass was IgG1.

Nine of ten in group one had measurable IgG1 level (range 1-14 mg/ml), while undetectable IgG3, 4-5 years after the second vaccine dose. All vaccinees in the first group showed at least two fold increase in the maximum IgG1 level achieved four weeks after the third dose (range 5-41 and median 15.5 mg/ml). Eight of ten in this group showed a measurable IgG3 response with maximum at two weeks after the third dose (range 2-25 and median 6.5 mg/ml). The IgG1 level prevailed for at least 14 weeks, while the IgG3 level quickly vanished and was almost not detectable at 14 weeks. Only two of ten in this group showed an IgG2 response (range 2-5-mg/ml), while none showed any IgG4 response.

For the second group of previously unvaccinated individuals, only two of them had detectable level of IgG1 (1 mg/ml). In this group, all responded at least two fold in IgG1 level after the first vaccine dose with a maximum after two weeks (range 1-24 and median 8.5 mg/ml). There was also a good booster effect on the IgG1 level of the second dose with maximum response after two weeks (range 4-50 and median 11.5 mg/ml). The highest median IgG1 level after the third dose for this group was fifteen weeks after the immunization (range 7-43 and median 14.5 mg/ml). In this group only three of ten gave an IgG3 response after the first dose, while eight of ten gave an IgG3 response after the second dose (median 3 mg/ml two weeks after the third dose), which was undetectable 39 weeks after the second dose. The maximum IgG3 response in this group was two weeks after the third dose (range 0-50 and median 7 mg/ml) with only one of

ten as non responder. Only one vaccinee in group two gave a strong IgG2 response and one vaccinee gave a measurable IgG4 response.

There was similarities but also differences in subclass response after the third dose for both groups, indicating that the immunological memory with regard to IgG subclass response lasted for at least 5 years. The IgG1 response seemed to last longer for group two than for group one indicating an other memory response in this group.

The IgG response after the first dose in group two could represent the beginning of a secondary respond as a result of prior exposure or as a result of prolonged immunological stimulation due to the adjuvant.

The kinetics of the antibody response was quite different among the subclasses. The IgG3 responded fastest but also gave the most rapid fall, while IgG1 responded slightly slower, but lasted longer. IgG2 had a very slow respond, but remained for a long time. IgG4 was only formed for a few vaccinees.

We also measured by a capture ELISA technique the total level of the IgG subclasses showing that the total IgG subclass levels did not change during the test period. Thus, the vaccination did not lead to a detectable polyclonal B-cell activation. There was neither any striking correlation between the total IgG subclass level and the corresponding OMV-vaccine response within each separate subclass.

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Epitope specificity and functional activities of human and murine antibodies against class 4 outer membrane protein from *Neisseria meningitidis*.

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The class 4 protein of *Neisseria meningitidis* is a highly conserved outer membrane protein (OMP), closely related to protein III (PIII) of *Neisseria gonorrhoeae*. Human IgG antibodies against PIII and some murine monoclonal antibodies (mAbs) against PIII and class 4 OMP have been reported to block bactericidal activity (SBA) of other antibodies (1, 2). Furthermore, in volunteers with previous gonococcal infection, who were immunized with gonococcal protein I vaccine, containing < 10% PIII, had a fall in bactericidal activity after vaccination. This fall in bactericidal activity was associated with the development of anti-PIII antibodies.

In order to investigate if antibodies against class 4 OMP elicited after vaccination with the Norwegian group B outer membrane meningococcal vaccine (3) block the bactericidal activity of other antibodies, sera from 8 vaccinees were selected. Six had antibodies, on immunoblots, against class 4 OMP before and after vaccination and 2 showed an increase in such antibodies after vaccination. In our experiments, we also used three new mAbs against class 4 OMP generated at National Institute of Public Health (185H-8, 155B-4, 173G-1), one mAb generated at Fundação Oswaldo Cruz, Rio de Janeiro, Brazil (AE3) and six mAbs against PIII, generated by J. Heckels *et al*. (SM50-SM55) (4).

Specific antibodies to class 4 OMP were purified by absorption with OMP from a strain lacking class 4 OMP (44/76 rmp-), after isolation the immunoglobulin (Ig) fraction from serum using protein G-Sepharose chromatography. After ultracentrifugation, the supernatant was precipitated with 50 % ammonium sulfate and the pellet resuspended and dialyzed against PBS.

Purified Igs reacted well in immunoblots against the OMP, recognizing only the class 4 protein, while no band was observed when OMP from 44/76 rmp- strain was used. However, when ELISA experiments were performed with OMP and whole cells from 44/76 strain, only weak reactions were observed, indicating that purified antibodies reacted with the parts of the molecule that were not exposed.

In order to detect linear epitopes and identify specificity of the mAbs, peptides of 14 residues were synthesized on polyethylene pins, using a commercially available kit (Cambridge Research Biochemicals). According to the predicted amino acid sequence of class 4 OMP, peptides spanning the entire molecule were synthesized with adjacent peptides overlapping by 7 residues. We found that the epitopes recognized by the mAbs raised against class 4 OMP from meningococci were different from those recognized by mAbs against PIII developed by Heckels *et al.* The amino acid sequences of peptides that these antibodies recognized were: SM50 and SM51: ^{43}**NYGECWKNAYFDKA**^{56}, AE3 ^{92}**DETISLSAKTLFGF**^{105}, 185H-8 and 155B-4:

^{197}**GAKVSKAKKREALI**^{210} and for 173G-1: ^{190}**EAEVAKLGAKVSKA**^{203}. MAbs SM52-SM55 and purified human anti-class 4 OMP Igs were also tested and showed binding to multiple peptides, indicating that these antibodies were reacting mainly with conformational epitopes.

Functional activities of the class 4 OMP specific antibodies, were assayed in bactericidal and opsonic tests. Neither of the human Igs were bactericidal or opsonic against strain 44/76. Only mAbs 155B-4 and 185H-8 were weakly opsonic, while no SBA were observed for any mAbs.

To investigate the blocking activity of purified Igs, SBA were performed in two ways. In the first one, the inoculum was incubated with dilutions of the purified anti-class 4 OMP Igs, after 15 min, the appropiate dilution of the 151F-9 P1.16 mAb (as a bactericidal antibody) and the human complement were added, followed by incubation for 30 min. In the second procedure, dilutions of the purified Igs were mixed with the P1.16 mAb before the inoculum and complement were added, followed by 30 min incubation at 37°C. After this time for both assays, agar was added and the plates were incubated overnight at 37°C, in 5% CO_2, and the colonies counted. In this experiment, one of eight Igs revealed a blocking effect in the highest concentration (1/5 dilution, 200 µg/ml total Ig concentration), while the other Igs did not block the bactericidal effect of P1.16 mAb. In the concentration used, neither of the mAbs inhibited the bactericidal activity of the effector P1.16 mAb.

The Norwegian group B vaccine contains about 10% class 4 OMP. Only a few vaccinees responded against the class 4 OMP after vaccination with this vaccine. In those who responded, no decrease in SBA titers were observed after vaccination and there was no negative correlation between anti-class 4 OMP IgG in immunoblots and bactericidal SBA titers (5). These studies give no evidence for induction of blocking antibodies against class 4 OMP after vaccination with the Norwegian group B outer membrane meningococcal vaccine.

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Recombinant Opc meningococcal protein, folded *in vitro*, elicits bactericidal antibodies after immunization.

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The meningococcal Opc protein, whose expression is restricted to a large subset of *N. meningitidis* strains(1), may mediate attachment to both endothelial and epithelial cells(2). Meningococci which lack capsular polysaccharide not only adhere to, but can also invade human cells when they express large amounts of Opc protein. Moreover, bacteria expressing large amounts of Opc protein are more commonly isolated from the nasopharynx than from the cerebrospinal fluid or bloodstream of patients.

The Opc protein is highly immunogenic in humans and stimulated bactericidal antibodies after vaccination with the Norwegian meningococcal group B outer membrane vesicle vaccine(3). Taking this fact into account, we wanted to evaluate this antigen as a vaccine candidate. For this purpose, the *opc* gene was cloned and expressed in *E. coli*. Then, the Opc protein was produced as a fusion protein with the N-terminal part of the high molecular weight meningococcal protein P64k as a stabilizer (4, 5).

The protein was found as inclusion bodies and after cell disruption and successive washing of the insoluble fraction of the cells, the proteins were solubilized in presence of the chaotropic agent Guanidium hydrochloride. The extract was applied on a Reverse Phase High Performance Liquid Chromatography (RP-HPLC) C4 column, for a further step of purification. The so obtained recombinant Opc protein was refolded *in vitro*, by adding several compounds to the solution where it was suspended. After several periods of time, the progress of the folding was tested by immunoblot with the human monoclonal antibody LuNm03 against the meningococcal Opc protein. LuNm03 recognizes a conformational epitope. The two unique conditions that favoured the folding were: (I) 1 M Urea, 400 µg/mL of PEG and 0.2 M L-Arginine and (II) 0.5 M GuHCl, 400 µg/ml of PEG and 0.4 M L-Arginine.

To evaluate the immunogenicity of the recombinant polypeptide, Balb/c mice were immunized subcutaneously with three doses of 0.5 mL injections, containing 20 µg of the refolded recombinant protein in Freund's adjuvant, at two weeks intervals. The immune serum was collected two weeks after the last injection. In this case other variants were used as controls: (III) Opc protein in 1 M Urea, (IV) Opc protein in 0.5 M GuHCl and (V) recombinant Opc protein after 4°C storag, in phosphate balanced salt solution (PBS), for 2 months at 100 µg/mL.

Sera collected during the immunization schedule were studied by western blotting, colony blotting and ELISA experiments. In colony blots, the natural protein was recognized only by the antibodies elicited against the two refolded variants, and the protein stored in PBS. In contrast, antibodies elicited against all variants recognized the Opc protein from strain H44/76 in western

blotting. Likewise, the recombinant protein was recognized in western blotting by all collected sera.

Antisera elicited against all variants recognized the recombinant Opc protein in ELISA, prior to folding. Antisera against the refolded proteins recognized the Opc protein in the OMP preparation, while preimmune sera and antisera elicited against conditions III and IV did not show any binding at all. A correlation between ELISA and colony blot experiments was observed. The natural protein was recognized by the sera of the variants where the Opc protein was refolded.

To study the functional activity of the antibodies elicited against the hybrid recombinant protein, sera were tested in a bactericidal assay, against strains having high, low and no level of expression of the Opc protein. Complement mediated bacterial killing was observed only when sera against refolded proteins were assayed. Strains with a higher amount of Opc protein were more complement-killed by the antibodies elicited, than strains having lower amounts of this antigen. A correlation was seen between the bactericidal titer of the antisera and the level of expression of this antigen on the surface of meningococci. This is in agreement with the results published by others (3), where bactericidal antibodies can only kill Opc^{++} bacteria and not Opc^{+} or Opc^{-} variants. Variable expression of Opc protein is due to phase variation at the transcriptional level (6).

These experiments described here, indicate that is feasible to fold properly the recombinant Opc protein produced in the *E. coli* system. It could be confirmed by the bactericidal activity of the murine antibodies elicited against it. Taking together these results with those reported by Rosenqvist *et al.,* attention should be paid to this meningococcal antigen, as a vaccine candidate.

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Induction of antigen specific human T cell responses after nasal immunization with the Norwegian group B meningococcal outer membrane vesicle vaccine.

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Systemic (intramuscular) vaccination with the Norwegian group B meningococcal outer membrane vesicle (OMV) vaccine has earlier been shown to induce protection against disease (1). We have studied the ability of this OMV vaccine to induce systemic human T cell responses when administered without adjuvant on the mucosal surface of the nose. A group of 12 vaccinees were given doses of 250 ug OMV once a week for 4 weeks (primary immunizations) and one single dose (250 µg) 6 months later. Peripheral blood mononuclear cells were assayed at several intervals for proliferative response (thymidine incorporation) against OMV, purified class 1 protein, class 3 protein as well as control antigens. In the majority of the vaccinees mucosal immunizations with OMV induced a significant increase in the specific T cell response to the meningococcal class 1 protein antigen, wheras only two vaccinees responded to whole OMV as antigen. Only one vaccinee showed a weak increase in the antigen specific T cell response to the class 3 protein antigen. The last dose given 6 months after the primary immunizations did not result in a proliferative response level higher than obtained within the first immunizations as far as all three antigens tested are concerned. In conclusion, we have shown that it is possible to induce vaccine antigen specific T cell responses after nasal administration of OMV in humans, but the condition required to obtain immunological memory remains to be eluciated.

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Cellular immune response after immunization with the Norwegian group B *Neisseria meningitidis*outer membrane vesicle vaccine.

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Vaccination with protein antigens will usually result in a both a cellular (T cell) and humoral (B cell) immune response. B-cells are dependent on help from T cells in order to produce antibodies efficiently (1). T cells are also necessary for the generation of immunological memory. For protection against extracellular bacterial infections like *Neisseria,* antibodies are of crucial importance while T cells will have a more indirect influence by regulating the antibody response. T cells can also indirectly induce killing of bacteria by activating phagocytes. Detailed mapping of the cellular immune response after immunization with bacterial vaccines is largely lacking, but such studies can lead to valuable information. We have therefore analyzed the cellular immune responses after vaccination with the Norwegian outer membrane vesicle (OMV) vaccine made from B:15:P1.7,16 meningococcal epidemic strain (2). The vaccine has previously been shown to induce protection against disease, after two doses given intramuscularly (3). In this study 10 volunteers, selected for low IgG antibody levels against group B meningococci, were given two doses of vaccine (25 μg outer membrane proteins) 6 weeks apart and a third dose after 9 months. Peripheral blood mononuclear cells (PBMC) were isolated before vaccination, 2 and 6 weeks after the 1. and 2. dose, and 1, 2 and 6 weeks after the 3. dose. The cellular immune response was analyzed by measuring T cell proliferation (thymidine incorporation) of freshly isolated PBMC from the vaccinees against OMV, purified class 1 and class 3 outer membrane proteins as well as the vaccine antigen BCG as control. Although individual variations occurred, we observed a significant primary and secondary T cell response. The response was usually highest against purified class 1 protein with stimulation indexes (SI) between 40 and 500 after the 1. dose (median SI = 155). The response against OMV gave SI values between 30 and 1100 after the 1.dose (median=80) and a further increase after the 2. dose with SI values up to 1300 (median=100). The response against the class 3 protein was highest after the 2. dose and varied from SI = 6 up to above 200 (median=38). The effect of the 3. dose is not yet evaluated. Furthermore, ELISPOT was used in parallel experiments to detect PBMC producing OMV-antibodies of IgM, IgA and IgG isotypes and IgG subclasses. This response was highest one week after vaccination. Analysis to correlate the corresponding serum antibody responses and cellular responses will be presented.

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Analysis of the continuous epitopes recognized by antibodies against a recombinant meningococcal high molecular weight antigen.

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Neisseria meningitidis is a pathogen responsible for a serious invasive disease throughout the world (1). The lack of an effective vaccine against all serogroups constitutes a problem in the control of meningococcal disease (2). Several outer membrane proteins (OMPs) have been examined as vaccine candidates (3). Besides the major OMP, other surface proteins are under investigation in several laboratories, in special highly conserved OMPs that could potentially confer protection against meningococcal disease in humans (4).

Our group has previously isolated , cloned and expressed in *E. coli* the gene coding for a high molecular weight protein (P64k, LpdA) which is common to many meningococcal isolates (5). To further characterize this meningococcal antigen at the molecular level, we have evaluated its immunogenicity in mice, rabbits and monkeys. The antigen was highly immunogenic in all three animal species (6), inducing antibodies that recognized the natural antigen in the membrane of intact meningococci. Besides, we have generated a group of monoclonal antibodies (Mabs) against the recombinant protein which recognize 4 non-overlapping epitopes , as shown using competition assays with biotinylated Mabs (7).

To characterize the epitopes on P64k recognized by these Mabs and by antisera obtained in the three species, multiple overlapping peptides were synthesized on pins and screened for binding by the antibodies (8). The complete P64k sequence was synthesized as 20 amino acid peptides overlapped by 10 a.a. stretches.

Also, the sequences involved in antibody binding were located in the previously determined 3-D structure of the protein (9). All 8 Mabs reacted with some of the overlapping peptides, indicating that they recognized linear epitopes.

A number of continuous epitopes were detected in mouse, rabbit and monkey sera, when immune and preimmune bleeds were compared. For mouse and monkey sera there appear to be four or five major antigenic regions, the response of the rabbit antisera being much more heterogeneous. In rabbits the epitopes seem to be less precisely defined. Despite variation in the exact location of continuous epitopes defined by different anti-P64k sera , we found an immunogenic core region within the molecule. Its sequence is AETGR. Consistently, these five residues are located in a β hairpin loop which is exposed to the solvent. This region is protruding, accessible to an sphere of 9 Å radius.

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Evaluation of the antigenic and molecular conservation of a new neisserial low molecular weight outer membrane protein

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An antigenically conserved protein that would confer protection against all *Neisseria meningitidis* isolates would be the antigen of choice for the development of a more efficient meningococcal vaccine. However, the conserved proteins already described, such as the class 4 or the Lip proteins do not elicit bactericidal or protective antibodies (1). Here, we present findings clearly indicating that a newly identified meningococcal protein, called NspA, is not only antigenically highly conserved among all *Neisseria meningitidis* strains, but is also present at the surface of certain *N. gonorrhoeae* and *N. lactamica* isolates. We also present results showing that the gene coding for that protein can be found among all *N. meningitidis, N. gonorrhoeae* and *N. lactamica* strains.

The antigenic conservation of the NspA protein was clearly established with monoclonal antibodies (MAbs). In a dot immunoassay done on intact bacterial cells, a series of six MAbs specific for this meningococcal protein recognized more than 94%, or even all, in the case of Me-7, of the 71 meningococcal strains chosen to represent the major serological groups (2). Five of these MAbs also reacted with 2 out of the 16 *N. gonorrhoeae* tested. The five MAbs reacted also with at least one out of the 5 *N. lactamica* strains. These results indicate that the protein is also present on closely related neisserial species. Further testing using Western immunoblotting experiments indicated that the protein is present on *N. gonorrhoeae* strains that were not reactive in the dot immunoassay. A radioimmunobinding assay which is used to evaluate the surface exposure of antigens confirmed that the protein is only accessible to the MAbs at the surface of intact bacterial cells on a limited number of *N. gonorrhoeae* strains. This protein was not detected by the MAbs on any other non pathogenic *Neisseria* species.

To corroborate these results, a DNA dot blot hybridization assay was used to evaluate the molecular conservation of the gene coding for the NspA protein among *Neisseria* isolates. First, the *nspA* gene was cloned from a meningococcal DNA library derived from strain 608B (B:2a:P1.2) and sequenced. The nucleotide sequence revealed an open reading frame of 525 nucleotides coding for a polypeptide of 174 amino acid residues, with a predicted molecular weight of 18,000 and a pI of 9.93. A comparison between the predicted amino acid sequence and the one obtained by N-terminal sequencing of the native meningococcal outer membrane protein showed the presence of an 19 amino acid residues leader peptide which is typical of outer membrane proteins. Similarity searches using the nucleotide and the deduced amino acid sequences of established databases confirmed that this protein has never been described previously.

The *nspA* gene was amplified by the polymerase chain reaction. After DNA dot blot hybridization using this probe, all the 71 *Neisseria meningitidis* strains tested were shown to

have the *nspA* gene in their genome. This latter result is in perfect agreement with the observed reactivity of MAb Me-7 which indicated that the NspA protein is produced by all the meningococcal strains and is accessible at the surface of intact cells. The DNA probe also hybridized with the chromosomal DNA of each of the 16 *N. gonorrhoeae* and 5 *N. lactamica* strains tested, but did not hybridize to the chromosomal DNA of any other neisserial species. Further studies are presently under way to determine whether the lack of reactivity of the NspA-specific MAbs with the gonococcal strains is due to the lack of expression of the *nspA* gene in most gonococcal strains, or to the absence of epitopes recognized by the NspA-specific MAbs on most gonococcal NspA proteins.

The *nspA* gene was cloned from two other meningococcal strains, one strain from each of serogroup B and A and from one *N. gonorrhoeae* isolate in order to further evaluate the level of molecular conservation. Alignment of the four *nspA* genes and the predicted amino acid residues sequences revealed very high degrees of identity of 90% and 95% respectively. Moreover, the observed variations were not clustered in a particular region of the sequences. These results clearly indicate that the *nspA* gene is highly conserved not only among *N. meningitidis* isolates, but also among *N. gonorrhoeae* strains.

The facts that the NspA protein is highly conserved, expressed at the surface of intact bacterial cells and that it can elicit the production of bactericidal and protective antibodies (2) greatly emphasize its potential use in a broad-range vaccine.

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Bactericidal effect of human neutrophils on meningococci incubated in pre- and post-vaccination serum of complement deficient patients.

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Of 45 Russian patients with late complement component deficiency (LCCD) who experienced one-to-five meningococcal infections, thirty-three were immunized with meningococcal polysaccharide vaccine (A+C+W135+Y) and followed for one to five years. The partial protective efficacy of vaccination was demonstrated (1). As far as bacteriolytic activity should be absent in LCCD plasma and serum, the potential bactericidal effect of human neutrophils on meningococci of groups A, W135 and B was studied in LCCD serum samples collected from vaccinees.

When meningococci were incubated at 37°C in 50% LCCD serum alone, exponential growth of meningococci occurred despite the presence of meningococcal antibodies. In LCCD serum the concentration of meningococci increased 1.5-3 times per hour, while meningococci were rapidly killed in all 20 control samples of 50% serum with normal complement activity. After the addition of human neutrophils ($5x10^6$ cells/ml) to meningococci ($5x10^3$ cells/ml) in LCCD serum, the growth was partly inhibited or even reversed to bacterial elimination. These experimental conditions were selected specially to simulate the conditions of initial bacteremia in meningococcal disease, to exclude some methodical errors, and to discriminate better the situations of high and low bactericidal effects. In all experiments there were used neutrophils of the same healthy donor, having heterozygous combination of Fcgammma-RIIa-receptors (Fc-RIIa-R131/Fc-RIIa-H131).

The rate of bactericidal effect of neutrophils depended on type of serum and group of meningococci. Group A, W135 and B meningococci were killed efficiently by neutrophils in 37% prevaccination LCCD serum samples. 84% of serum samples, collected one month to one year after vaccination, promoted the bactericidal effect of neutrophils against group A and W135 meningococci ($p < 0.05$). Three years after vaccination only 58% of LCCD serum samples were "bactericidal" in these conditions. 91% of serum samples, collected during one year after revaccination, promoted the bactericidal effect of neutrophils against group A and W135 meningococci. In contrast, no increase of bactericidal effect against group B meningococci was found in post-vaccination serum samples. Interestingly, bactericidal effect against both groups A, W135, and B was observed in 68% of serum samples collected from LCCD patients 3 months after systemic meningococcal disease. The latter effect was not polysaccharide-specific and possibly was caused by cross-reacting antibodies. These samples were excluded from the analysis below.

The rate of bactericidal effect of neutrophils correlated with the concentration of serogroup-specific IgM and, to some extent, IgG. For example, 88% of 54 LCCD serum samples, having

more than 1.5 mg/l anti-group A IgM and/or more than 5 mg/l anti-group A total Ig ("high" levels), supported the killing of group A meningococci. In contrast, only 31% of 35 samples, having specific antibodies under these limits ("low" levels), were "bactericidal" ($p < 0.05$). The same effect was observed also in experiments with group W135 meningococci. The cross-reacting antibody might function in synergy with polysaccharide-specific antibody. We measured the level of antibodies to inner core of lipopolysaccharide of gram-negative bacteria in the same LCCD serum samples. 92% of LCCD serum samples, having both high level of anti-polysaccharide antibody and high level of anti-core antibodies supported the killing of meningococci. Only 25% of samples, having low levels of both antibodies, were bactericidal. The samples, where one antibody was in high concentration and another in low one, exhibited the intermediate bactericidal effect. No significant bactericidal effect was demonstrated by neutrophils in heat-inactivated serum samples in spite of the concentration of specific antibodies, suggesting that the action of antibodies was complement-mediated. The data of Schlesinger et al. (2), who studied three C7-deficient vaccinees, agreed in general with our observations.

In conclusion, neutrophils could kill meningococci upon incubation in LCCD serum; this effect increased after vaccination and depended on specific antibody and complement content. Protection obtained from vaccination might be caused by an increased killing capacity of neutrophils.

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Neisseria meningitidis LOS micelle-based vaccine

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Endotoxin (LPS/LOS) is a glycolipid considered as one of the most significant antigens expressed by Gram negative bacteria and responsible for the toxic effects leading from endotoxicosis to septic shock. Toxicity is associated with the induction of endogenous cytokines like tumor necrosis factor (TNF) through the interaction of the conserved lipid A moiety of LPS with cell receptor proteins like CD14. We have recently elucidated the features needed by peptide structures to competitively bind and detoxify the conserved lipid A structure (1). These studies, using synthetic anti-endotoxin peptides (SAEP) to inhibit the toxicity of LPS systemically and in organ tissues, have revealed that a fundamental factor related to the full expression of the biological toxicity of LPS resides in its micellar architecture (2). Studies investigating the molecular architecture of LPS by Nuclear Magnetic Resonance (NMR) have come to similar conclusions (3). Lipid A is responsible for the micellar configuration of LPS and any approach which tends to chemically modify or eliminate the lipid A structure leads to the lack of the supramolecular architecture of LPS with the consequent reduction or elimination of toxicity. There is very limited but significant information about the importance that such supramolecular architecture may also have for optimal expression of antigenic and immunogenic activity (4,5). Since meningococcal group B LOS can be considered an important vaccine candidate, we have studied a vaccine formulation which is based on the use of purified LOS micelles detoxified by complex formation (1) with an appropriate amount of SAEP in order to reach the necessary level of safety, for investigation of the role of anti-LOS antibodies in conferring protection against bacteremia and endotoxemia. LOS purified from N. meningitidis A1, which is cross-reactive with Group B LOS but that does not contain the lacto-N-neotetraose structure similar to human glycolipids (5), has been therefore reacted with a synthetic cyclic peptide (SAEP-2). In selected experiments, after complex formation, the bound peptide has been "locked" into the lipid A binding site by covalent cross-linking with tailored bifunctional spacers in order to achieve its irreversible binding to the lipid A moiety. For either model of vaccine, the non-covalent or covalent complex, detoxification was ascertained in a variety of assays which included inhibition of LPS-induced LAL clotting, systemic and local TNF release by LPS challenge in mice, inhibition of LPS-induced local hemorragic dermonecrosis in rabbits. The vaccines and a control of purified LOS were then injected subcutaneously in SW mice at the dose of 5 ug/mouse in various schedules of treatment in order to follow the kinetic of the induced antibodies. Sera were obtained from the animals on weekly basis and the anti-LOS antibodies induced were quantitated by ELISA and characterized for isotype (IgG and IgM) and sub-isotype (IgG1, IgG2a, IgG2b, IgG3). The antibody response induced in all groups of animals contained essentially IgG antibodies which peaked after three injections with end-point titers in the range of dilution 10^{-4}-10^{-5}. The sub-isotype present within the IgG population of the animal groups were mainly IgG2 (44 %, equally distributed between 2a and 2b) followed by IgG1 (36 %) and IgG3 (20 %). Interestingly, the sub-isotype IgG distribution induced by either the vaccines or native LOS in mice was similar to that reported in febrile patients affected by typhoid to LPS of Salmonella typhi (6). The murine anti-LOS antibodies induced were biologically functional in fixing and activating guinea pig complement thus resulting in the lytic activity on N. meningitidis A1 LPS-coated sheep erythrocytes at serum dilution of 1:200. Analysis of immunochemical specificity of the induced antibodies for different antigenic regions of A1 LOS, performed by inhibition ELISA, revealed that all antibodies were directed against the carbohydrate region and none of them recognized the lipid A moiety. Accordingly, no cross-

reactivity of the anti-A1 LOS antibodies was detected against heterologous LPS purified from P. aeruginosa, S. typhosa, S. enteritidis, S. flexneri, H. influenzae and B. pertussis. In contrast, the induced antibodies were cross-reactive with purified N. meningitidis group A and B LOS as well as with three bacterial strains (Group A, strain A1; Group B, strain BB431; Group B, strain 44/76) sharing the immunotype L8 determinant (5).
These results show that peptide-detoxified LOS represents a novel new method for safely administering LOS/LPS in micellar configuration which induces an immunogenic response in mice comparable to that qualitatively and quantitatively induced by native (toxic) LOS, in contrast to lipid A-deprived LOS conjugated to carrier proteins that have shown a lower level of immunogenicity when compared to native LPS (5). Studies are in progress for investigating in appropriate animal models the efficacy of the anti-LOS antibodies for prevention of meningococcal bacteremia and endotoxemia.

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Heterogeneity of *tbp*2 genes among *Neisseria meningitidis* B strains belonging to the ET-5 complex

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Since 1974, serogroup B meningococci of the ET-5 complex have caused epidemics in Europe, Cuba and South America; these epidemics elevated disease rates for many years (1,2) and led to sustained efforts for vaccine development. The aim of this study was to assess the level of variability of *tbp2* genes encoding Transferrin binding protein 2 (Tbp2) among 23 strains of the ET-5 complex representing different serotypes and subtypes and nine different geographic origins. The size of *tbp2* genes of these strains determined after PCR amplification was 2.1 kb, indicating that they all belonged to the M982 family according to the classification previously described (3). We cloned and sequenced the *tbp2* gene of a strain isolated in Chile in 1987 (strain 8680) and the homology was only 65.5% with the nucleotide sequence of the reference strain M982. The multialignment of nucleotide sequences of five M982-like strains (4, 5), this ET-5 strain (8680) and another strain of the ET-5 complex, BZ83, previously described (5) allowed us to design two oligonucleotides in the N-terminal part of the gene giving a *tbp2*, the length of which was characteristic for three different types (type M982, type BZ83, type 8680). These primers were used to type the *tbp2* gene of each strain and this classification was confirmed with an analysis of the fragment length restriction pattern using specific enzymes. We found that ET-5 complex strains are heterogeneous for the *tbp2* gene as four different types were found. While types M982 and BZ83 were the most represented (10 and 9 strains out of the 21 studied respectively), we only found one other 8680-like strain. Among 7 Chilean strains isolated from 1986 and 1987, the four different types were represented. Overall, these data indicate that the *tbp2* gene is variable within the ET-5 complex.

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Human B- and T-cell responses after three doses of a hexavalent PorA meningococcal outer membrane vesicle vaccine

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Adult volunteers were immunized three times with 100 μg of a hexavalent PorA outer membrane vesicle (OMV) vaccine. This vaccine consists of OMV preparations derived from two trivalent meningococcal vaccine strains, each one of them expressing three different PorAs [1-2]. Previously, a phase I safety study was conducted in adult volunteers, who received one immunization of 50 or 100 μg of the vaccine [3]. This study investigates human B- and T-cell responses as a consequence of three consecutive immunizations. The vaccine was given with intervals of two and six months. Blood samples were taken weekly to study B- and T-cell responses. Antibody responses against the six PorAs as present in the vaccine were analyzed by ELISA using the trivalent vaccine OMVs as antigen. Six isogenic PorA target strains, each carrying a different PorA in an identical meningococcal background, were constructed to investigate the bactericidal immune response against individual PorAs [3]. Subsequently, PorA loop-deletion and point-mutation variant strains carrying PorA subtypes P1.7,16 or P1.5c,10 were constructed to investigate the epitope specificity and cross-reactivity of the induced antibodies by using such strains as targets in the bactericidal assay [4 and this study]. Lymphocyte proliferation assays were carried out to study the T-cell responses in both vaccinated and non-vaccinated donors. Monovalent OMV preparations of six wild type meningococcal strains, each one of them carrying one of the PorAs as present in the vaccine, and purified PorA P1.7,16 were used to study the antigen-specific stimulation of human peripheral blood cells. The first immunization induced a strong B-cell response resulting in high IgG levels in OMV ELISA and at least a four-fold increase in bactericidal activity as compared to pre-vaccination titers. Clonal specificity was observed in the bactericidal assay for one of the three PorAs as expressed by the two trivalent vaccine strains. The second and third immunizations did not induce booster responses. This was confirmed by the T-cell proliferation responses. A decline over time with respect to PorA specific antibodies was observed after each immunization. This was also reflected in the T-cell responses.

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Kinetics of bactericidal antibodies and specific IgG and IgA antibodies elicited by a meningococcal B:4:P1.15 outer membrane protein vaccine.

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Meningococcal B:4:P1.15 outer membrane proteins (OMP) vaccine produced in Cuba (VA-MENGOC-BC) was administered in two doses to 220 male recruits at an interval of 8 weeks. Thirteen serum samples were obtained in different times along one year. Specific IgG and IgA antibodies and serum bactericidal activity (SBA) elicited by the vaccine were measured by enzyme-linked immunosorbent assay (ELISA) and serum bactericidal assay, respectively. Arithmetic means and SD were calculated for all variables. Also a multivariate profile analysis was done. Nasopharyngeal carriage of meningococci was tested by a t-test for independent groups to compare meningococcal carriers with non-carriers with respect to specific IgA and IgG antibodies. After the second dose the higher increments in SBA and specific IgG were obtained. A significant decrease in these variables was observed from week 12 to week 52, although the negative slope showed by them after the second dose was smaller than that observed after the first one. In contrast to the kinetics showed by IgG, the increase of IgA was greater after the first dose. The kinetics of IgA and IgG stimulated by the vaccine reflect the presence and persistence of immunological memory at B cell level which could be protective since a similar behavior of SBA and specific IgG was observed. No significant effect of the carrier state on IgA and IgG was found.

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Cloning and expression of human immunoglobulin gene encoding anti-meningococcal P1.7 epitope antibody SS269

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Introduction. Human monoclonal antibody SS269 (γ3,λ) reacts with linear peptides containing NGGAS which is located in VR1 region, corresponding to serosubtype P1.7, of meningococcal PorA protein (1). This antibody has been shown to react with P1.7,13a and P1.7,16 PorA proteins but not with P1.7,10 protein, suggesting that SS269 recognizes a conformational epitope which is affected by the sequence of the VR2 region of PorA protein (1). As to function, SS269 stimulates inefficient bactericidal killing possibly due to its low avidity but mediates efficient opsonophagocytosis (1). In contrast, a murine monoclonal antibody targeting meningococcal P1.7 epitope reacts with all P1.7 epitope-containing PorA proteins, shows high avidity and mediates efficient bactericidal killing (1). It will be important to determine the structural basis for such differences and to determine whether these differences would have any implications in the design of meningococcal vaccine, because the PorA protein has been recommended as one of the components of a new meningococcal vaccine based on experiments with murine mAbs (2). Phage-display of immunoglobulin genes is a powerful technique which was originally designed for performing quick screening of phage-displayed antibody libraries (3). We now report on the use of Phage-display technology to clone and express human immunoglobulin genes of hybridoma SS269.

Materials and Methods. Total RNA was prepared from 10^6 hybridoma cells. 5 μg of total RNA was used for a first strand cDNA synthesis reaction. Each Hot-Start PCR was performed in a 50 μl volume containing 2.5 units of Extend™ Long Template DNA polymerase. PCR products were obtained by using primers gaggtgcagctcgaggagtctggg and tgtgtcactagttggggttttgagctc for heavy chain and acaggbtcybkskccgagctcrwrbtgacdca and gcattctagactattatgaacattctgtaggggc for light chain after denaturing at 94° C for 3 min., 40 cycles of 92°C for 40 sec., 55°C for 40 sec. and 72°C for 1.5 min., and a long extension time of 5 min.

The heavy chain and light chain PCR products were sequentially ligated into a phagemid vector pComb3 after double digestions by *Xho* I and *Spe* I and *Xba* I and *Sac* I, respectively. Both cloned inserts were then sequenced. Soluble Fab was prepared according to a standard protocol (4) and tested in ELISA as described before (5).

Results and discussion. DNA sequence analysis indicated that heavy and light chain variable regions of SS269 belong to immunoglobulin gene family VH3a and family Vλ1-b, respectively. Amino acid sequences of Fd and lambda chains of SS269 are shown below .

Fd fragment (1-98: VH, 99-116: J and 117-221: CH1):
VKLLEESGGGVVQPGRSLTLSCAASGFTFSSYGMYWVRQAPGKGLEWVAVIWYDGTK
KYYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCVRDQYYGSGWGQGTLVTV
SSASTKGPSVFPLAPCSRSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQ
SSGLYSLSSVVTVPSSSLGTQTYTCNVNHKPSNTKVDKRVELKTPTS

Lambda chain (1-98: Vλ, 99-111: J and 112-216: Cλ):
ELVVTQPPSASGTPGQRVTISCSGSISNIGINSVYWYQQLPGTAPKLLFYRNNQRPSGVPD
RFSVSKSGTSASLAISGLRSEDEADYYCAGWDDSLSGWVFGGGTKLTVLGQPKAAPSVT
LFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAAS
SYLSLTPEQWKSHKSYSCQVTHEGSTVEKTVAPTECS

Whole cell ELISA showed that both the soluble Fab_{SS269} and its parental mAb SS269 reacted with P1.7,16 meningococcal strain 44/76, but not with P1.7,10 strain 29019 and P1.2 strain 7379. This result indicates that Fab_{SS269} has the same binding specificity as its parental antibody.

The same procedure is being undertaken for three murine mAbs which are all directed against the P1.7 epitope. A comparison of DNA sequences and of variable region 3-D structures between the human and murine Fabs will help us in understanding how the differences in DNA sequences result in differences in their binding specificity and function.

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IgG antibody activity against meningococcal class 1 and 3 outer membrane proteins in patient sera: Comparison between immunoblot and ELISA analyses

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Previously, quantitative IgG antibody activities against purified class 1 and 3 outer membrane proteins from strain 44/76 (B:15:P1.7,16) in sera from patients, falling ill with meningococcal disease, have been determined in ELISA (1, 2). We wanted to compare these results with those obtained after immunoblotting of the same sera and density scanning of the blots. Outer membrane vesicles from 44/76 served as antigens, and all sera were incubated with and without Empigen BB to improve renaturation of boiled antigens (3). The sera were routinely used at 1:200 dilution, but those giving strong immunoreactive class 1 (P1.7,16) or class 3 (serotype 15) protein bands at this dilution, were diluted up to 1: 40 000. To control for variations between the blotting experiments, four strips with a reference serum were included on each blot. IgG binding was determined with peroxidase-conjugated rabbit anti-human IgG antibodies. Densities of immuno-reactive class 1 and 3 protein bands were measured with a video camera and a software program from Kem-En-Tec A/S, Denmark. In all, 56 acute and convalescent sera from 25 patients were studied; seven of the patients had been vaccinated with the Norwegian group B meningococcal OMV vaccine (4).

When 1:200 dilutions of the sera were used for immunoblotting, the Spearman rank correlation coefficient between IgG binding in ELISA (µg/ml IgG) (1, 2) and densities of the scanned class 1 protein band on blots was 0.38 ($p < 0.004$). The correlation coefficients for the vaccinees and non-vaccinees were 0.10 ($p = 0.72$) and 0.53 ($p < 0.001$), respectively. The low correlation coefficient for sera from vaccinees could possibly be related to a prozone effect , as the antibody binding was weaker at a 1:200 dilution compared to higher dilutions. Six patients had class 1 protein bands with scan values > 1 000, corresponding to medium or strongly stained bands. They were all infected with strains expressing subtype proteins different from that of the vaccine strain. Three were vaccinees, and their convalescent sera bound to protein P1.7,16 in the absence of Empigen BB, whereas the non-vaccinees' sera showed a strictly detergent-dependent binding. These results indicate that antibodies against more linear epitopes were preferentially raised after disease of the vaccinees.

When distinct class 3 protein bands were obtained on blots, they were generally much stronger than the corresponding class 1 protein bands. Eight patients had scanned class 3 protein bands > 1 000; seven of these were infected with serotype 15 strains and six were vaccinees. Anti-class 3 porin antibodies in the convalescent sera from these patient either showed a detergent-independent binding or a partial detergent-dependent binding, indicating that the antibodies were mainly directed against linear epitopes (5) or against conformational ones renatured during the incubation steps with a smaller contribution of antibodies against epitopes refolded by the

detergent. The correlation coefficient for IgG binding with all sera to class 3 porin with the two methods was 0.49 ($p < 0.001$). For vaccinees and non-vaccinees, the coefficients were 0.63 ($p = 0.007$) and 0.16 ($p = 0.32$), respectively. Distinct prozones were observed for sera with high anti-class 3 protein levels. When such sera were tested in 1:2 000 dilutions, the correlation coefficient was 0.89 ($p = 0.002$).

In conclusion, immunoblot analyses of IgG binding to the class 1 and 3 proteins of strain 44/76 with 1:200 dilutions of patient sera corresponded weakly to IgG levels measured by ELISA using purified class 1 and 3 proteins. The low correlation coefficients may be due to prozone effects observed at this serum dilution, as a high correlation was obtained at 1:2 000 dilution. Even though the use of Empigen BB allowed a differentiation of antibody activity directed mainly against conformational or linear epitopes, a possible loss of IgG antibodies against conformational epitopes using the blotting technique has to be taken into account. From a practical point of view, more work is involved in making serum dilution curves with the blotting method as compared to ELISA. These factors limits the use of scanned immunblots as a method to determine accurate antibody levels against class 1 and 3 proteins.

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Immunoblot analyses of antibody responses in sera, saliva and nasal fluids from volunteers immunized nasally with the Norwegian group B meningococcal vaccine.

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The immune responses in sera, saliva and nasal fluids from 12 volunteers, who received the Norwegian group B outer membrane vesicle (OMV) intranasally, were studied by the immunoblotting method. The immunizations were given as nose drops or nasal spray four times at weekly intervals followed by a fifth dose six months later. Samples for blotting analyses were taken before vaccination, two weeks after the fourth dose and two weeks after the fifth dose. OMV from the vaccine strain 44/76 served as antigen, and all samples were incubated with and without Empigen BB to renature boiled antigens (1). In sera, the IgG response was analyzed, whereas in saliva and nasal fluids the IgA responses were determined. All sera were also studied for crossreactive IgG antibodies against OMV from the Cuban vaccine strain 385/83.

Prevaccination sera from four volunteers showed distinct antibody activity against high molecular weight proteins (HMWP), class 1 porin and/or LPS of 44/76 OMV. In contrast, nine volunteers had similar bands against 385/83 OMV, in addition to class 5 proteins and lower molecular weight protein bands. Thus, preexisting antibodies crossreacting with antigens in the Cuban vaccine strain were more prevalent than those against the Norwegian vaccine strain.

Following nasal immunization, seven volunteers showed a distinct increase in antibody binding to one or more OMV antigens. Three vaccinees responded to class 1 porin, four to class 5 proteins (including Opc) and four to LPS of 44/76. Only one vaccinee had a weak response against the class 3 protein. None showed increases in IgG binding to HMWP as usually observed after intramuscular vaccination (2). The fifth immunization dose gave no distinct changes in antibody patterns. The vaccine-induced class 1 protein antibodies showed negligible crossreaction with 385/83, indicating a subtype-specific antibody response.

Serum bactericidal assays (SBA) were performed with strains 44/76-SL and 44/76-1, expressing low and high levels, respectively, of the Opc protein. Among five vaccinees, who developed more than 4-fold increases in serum bactericidal titers against 44/76-SL after immunization, three showed distinct class 1 porin bands, one a distinct LPS band, and the remaining a weak class 5 protein band. Postvaccination sera demonstrating more than 8-fold increases in bactericidal titers when SBA was performed with 44/76-1 compared to 44/76-SL, gave distinct bands in the class 5 protein region. Thus, for most post-vaccination sera there was a correlation between distinct class 1 or class 5 protein bands (including Opc) and titers in SBA.

After vaccination, a rise in IgA binding to class 1 and 5 proteins with nasal fluids from six volunteers was found. Compared to IgG binding patterns with sera, nasal fluids showed no distinct IgA antibodies against HMWP or LPS, and only three vaccinees demonstrated reactions matching those seen with serum IgG antibodies. IgA binding to class 4 protein was observed in

two volunteers both before and after vaccination; corresponding IgG activity was not found in their sera. In saliva, only three volunteers demonstrated induction of IgA antibodies against the class 1 protein, and similarly to nasal fluids, no HMWP or LPS responses were detected.

Generally, sera giving distinct immunoreactive bands on blots had high IgG levels. A similar correspondence was also observed IgA in nasal fluids, but not for saliva. A correlation between distinct LPS bands on blots and high levels as measured in ELISA was also noted.

In conclusion, nasal immunization induced serum IgG antibodies against class 1 and 5 proteins and LPS in 58% of the vaccinees. Compared to intramuscular vaccinations, little or no increase in antibodies against class 3 porin and HMWP were found. Fifty percent of the vaccinees showed a vaccine-induced IgA response against the class 1 and 5 proteins in nasal fluids, while 25% demonstrated class 1 protein IgA antibodies in saliva. The IgA binding patterns in secretion generally did not reflect those seen with serum IgG antibodies.

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Liposomes as a vaccine delivery system for neisserial transferrin binding proteins

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There is currently much interest in the use of transferrin binding proteins (Tbps) as antigens for incorporation into a meningococcal vaccine. It has previously been shown that antibodies elicited to Tbps are bactericidal (1) and anti-Tbp antibodies that prevent binding of transferrin (TF) to Tbp can inhibit growth of *N. meningitidis* when TF is the sole source of iron (2). Two proteins have been identified that form the TF binding complex; Tbp1 (M.W. 95 kDa) and Tbp2 (M.W. 68-85 kDa). They are associated with the outer membrane, and Tbp2 has been found to be a lipoprotein (3).

The use of liposomes as a delivery system for Tbps desirable because a) Tbp will be presented to the immune system in a potentially native state mimicking *in vivo* orientation and b) liposomes can better target protein antigens to antigen presenting cells (APC). This was shown by Gregoriadis (4) who demonstrated that liposomes injected subcutaneously are retained at the site of injection and are taken up by APCs.

In this study, liposomes consisting of synthetic phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in a 4:1 ratio were used. Lipids were rotary evaporated, shell dried and reconstituted in Tbp solubilized in 10% octylglucoside. Tbp was purified from detergent extract of *N. meningitidis* by affinity chromatography on TF-Sepharose 4B (2). Liposomes were formed by removing the detergent by dialysis against PBS. A homogeneous population of small unilamellar vesicles was obtained by extruding the Tbp/liposomes through 0.2 μm filter (5).

We found that intact Tbp/liposomes bound TF in a liquid-phase, detergent free binding assay, indicating that the Tbp complex was in a transmembrane location and in a functional conformation. The immunogenicity of the Tbp/liposomes was compared to Tbp alone, Tbp/Freunds and Tbp/alum in mice (n = 5). All preparations, containing 50 μg Tbp, were administered subcutaneously three times at two week intervals. Using purified Tbp as the coating antigen in an ELISA assay, we found that Tbp alone elicited equivalent amounts of IgG antibody to Tbp/Freunds and elicited more IgG antibody than Tbp/alum and Tbp/liposomes preparations. However, the Tbp/liposome antisera contained a higher percentage of whole cell-specific Tbp antibodies (16.4%) as compared to Tbp alone (5.2%), Tbp/Freunds (8.0%) or Tbp/alum (10.1%) antisera determined by whole cell ELISA. The only vaccine that elicited cross-reacting antibodies to *N. meningitidis* of different serosubtype was the Tbp/alum preparation.

In summary, liposomes proved to be an effective way to present TBP in a more native state since intact TBP liposomes bound TF and compared to the other TBP vaccine preparations produced the highest percentage of antibody to native TBP on whole cells. In

addition, TBP alone was immunogenic without additional adjuvant. Further studies regarding functional activity of antibodies elicited from the various TBP preparations are required.

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Antibody response of rabbits to intranasally administered meningococcal native outer membrane vesicles

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Delivery of vaccines via the intranasal route has gained much interest in recent years as a way to induce a mucosal antibody (IgA) response that may play a role in inhibiting the attachment or invasion of pathogenic microorganisms to mucosal surfaces. Many investigators have used the mouse as a model to examine effectiveness of intranasally delivered vaccines (1, 2). Investigators typically administer 20-50 µl of vaccine intranasally in mice. Much of the vaccine reaches the large mucosal surface area of the lungs and typically induces a good mucosal response. In humans, however, it may not be desirable to have lung involvement, especially with vaccines containing native endotoxin. We have used a larger animal model, the rabbit, as being more representative of intranasal immunization in humans with regards to vaccine disposition. We have found using dye experiments that intranasal delivery of 0.5 ml of vaccine in the rabbit provides good coverage of the nasopharyngeal region of the rabbit without getting to the lungs.

Immunizing with meningococcal native outer membrane vesicles (NOMV) would be an excellent way to present meningococcal antigens in their native conformation; however, NOMV does contain native endotoxin that would cause unwanted local and systemic reactions if given intramuscularly (i.m.) in humans. Endotoxin is highly pyrogenic, and we have found that in rabbits as little as 0.1 µg of NOMV caused a pyrogenic response when delivered intravenously. When administered intranasally, however, 400 µg of NOMV in rabbits caused no pyrogenic or other untoward responses.

In this study we investigated the immunogenicity of NOMV administered intranasally in rabbits. Rabbits were immunized 3 times at 28-day intervals. Vaccines that were used included: 1) NOMV from the parent strain 9162 (B:15:P1.3:P5.10,?:L3,7,9); 2) a capsule-deficient mutant of the parent 9162 strain; or 3) a vaccine containing detoxified meningococcal L12 LOS complexed with meningococcal (9162) outer-membrane protein (OMP-dLOS). We used two immunization schemes: 1) i.n. three times, or 2) i.m. once with OMP-dLOS followed by two i.n. boosters of parent or capsule-deficient NOMV. Vaccines were administered to unanesthetized New Zealand White rabbits i.m. at 25 µg protein or i.n. at 100 µg protein in a 0.5 ml volume.

Rabbits immunized i.n. three times with capsule-deficient NOMV had the highest serum bactericidal titers (mean $\log_2$ reciprocal endpoint titer having > 50% kill was > 9), highest serum IgG levels (500 µg/ml), and highest serum IgA levels (> 350 µg/ml). The NOMV from the encapsulated strain induced lower serum bactericidal activity (mean $\log_2$ reciprocal endpoint titer = 7.0) and lower serum IgG (400 µg/ml) and serum IgA (190 µg/ml) levels. Rabbits immunized i.n. three times with OMP-dLOS or i.m. once with OMP-dLOS and twice with two i.n. NOMV boosters had similar lower levels of serum bactericidal activity (mean $\log_2$ reciprocal endpoint titers of 5.7-6.2), and lesser amounts of serum IgG (110-230 µg/ml) and IgA (30-220 µg/ml).

Immunoblots showed that day 70 sera reacted with a wide variety of immunoreactive bands, including class 3 and 4 proteins, both class 5 proteins, as well as L3,7 LOS.

We have shown that intranasally administered NOMV are safe and induce good antibody responses against a variety of immunoreactive proteins in rabbits. Three i.n. doses of NOMV from a capsule-deficient mutant induced the best bactericidal and overall antibody response. It is not presently known if intranasal immunization in humans will induce high serum bactericidal activity as we observed in rabbits. We believe that the most effective strategy to immunize humans against group B meningococcal disease may involve a two-pronged approach using an intranasal vaccine to induce a local mucosal response and a parenteral vaccine to induce high titers of serum bactericidal antibodies.

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Immune response of mice to intranasally administered meningococcal native outer membrane vesicles

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The human nasopharyngeal region provides the sole natural habitat for *Neisseria meningitidis*. We believe that stimulating production of a local mucosal (IgA) antibody response may aid in preventing systemic meningococcal disease by interfering with meningococcal adhesion to and invasion of the mucosal surface. Secretory IgA has been shown to inhibit adherence of *Streptococcus* strains to human epithelial cells (1). Other investigators (2, 3) immunized humans intranasally with a purified M protein vaccine against group A *Streptococcus* and found reduced colonization and clinical illness after challenge with homologous streptococci. Furthermore, fewer vaccine side effects were observed in intranasally immunized subjects compared to parenterally immunized subjects (3). An added advantage of mucosal immunization is that vaccines containing lipopolysaccharide may be able to be delivered safely via the mucosal route without the adverse side effects seen with parenteral delivery (4).

We have conducted studies using an intranasal mouse model to examine the immunogenicity of meningococcal antigens delivered via the intranasal route. Meningococcal native outer-membrane vesicles (NOMV) were prepared from a mutant group B strain 9162 (--:15:P1.3:P5.10,?:L3,7,9) deficient in capsule and sialylated LOS. The NOMV were delivered intranasally (i.n.) to mice at a 20 μg dose of protein in a 25 μl volume using a micropipettor. For comparison, some groups of mice received one or both doses of NOMV intraperitoneally (i.p.) at a dose of 1 mg protein. Mice were immunized at days 0 and 28. Numbers of anti-meningococcal antibody-secreting cells (ASCs) were measured in lungs and spleens at day 33 using an ELISPOT assay. Antigens delivered intranasally in mice do reach the lungs; we therefore examined the histology of formalin-fixed H&E-stained mouse lungs collected at days 1, 2, 4, and 7 post i.n.-immunization.

Mice immunized and boosted i.p./i.n. or i.n./i.n. had 90 and 525 IgA ASCs per 10^6 lung lymphocytes, respectively, in lung tissue at day 33, whereas mice immunized i.p./i.p. had no detectable IgA ASCs in lung tissue. Mice immunized i.n./i.n. had 190 IgA ASCs per million spleen lymphocytes, compared to 30 and 43 IgA ASCs per 10^6 spleen lymphocytes, respectively, for mice immunized i.p./i.p. or i.p./i.n. Mice immunized i.p./i.p. or i.n./i.n. had similar high numbers of IgG ASCs in the spleen (105 and 91 per 10^6 spleen lymphocytes, respectively). In the lung, however, only 70 IgG ASCs per 10^6 lung lymphocytes was observed in the i.p./i.p group, compared to over 300 IgG ASCs per 10^6 lung lymphocytes in the i.n./i.n. group. Few IgM ASCs were observed in spleen and lung lymphocytes of any group. Serum bactericidal activity was highest in mice immunized i.p./i.n. (mean $\log_2$ reciprocal endpoint titer having > 50% kill = 8.8), followed by the i.p./i.p. group ($\log_2$ mean = 7.8) and the i.n./i.n. group ($\log_2$ mean = 7.0). Mice immunized intranasally had serum IgG antibodies directed against several immunoreactive proteins including class 1 and class 5 proteins as observed on immunoblotting. Serum IgA antibodies which bound to class 5 proteins were also evident.

Histological evaluation of mouse lungs on days 1 and 2 post-i.n. immunization revealed a predominantly granulocytic inflammatory response, which by day 7 had significantly diminished in severity and in general consisted of small aggregates of perivascular and peribronchiolar mononuclear cells. The inflammatory reaction we observed in the lungs was not unexpected since NOMV contains native endotoxin. Mice that received an intranasal dose of 20 μg of NOMV had an average weight loss of 1.8 g by day 3, probably due in part to the poor condition of the lungs; mice recovered to pre-immunization weights by day 7.

Our results show that intranasal administration of meningococcal NOMV can induce a mucosal IgA response in mice. Importantly, intranasal immunization also induced production of serum bactericidal antibodies in mice. However, NOMV does cause unwanted inflammatory responses in the lungs when administered intranasally to mice. We have therefore initiated further intranasal experiments in a larger animal model, the rabbit, in which intranasally administered antigens do not reach the lungs but do provide good coverage of the nasopharyngeal region. We believe that the rabbit model will provide a better physical model than the mouse with respect to disposition of intranasally delivered vaccines.

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(Two additional abstracts by AK Lehmann, et al., and J Tappero, et al. on the topic of *Noncapsular Vaccines* are found on pages 596 and 598)