# Prime-Boost Immunogenicity of Class 5C Protein from *Neisseria meningitidis* in Mice with Different Adjuvants

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**Abstract:** The nasal route for vaccination offers both mucosal and systemic immunity for the prophylaxis of respiratory diseases. In order to induce maximal protective mucosal immunity, mucosal adjuvant is essential. Current studies are searching vaccinal antigens that are derived from the outer membrane protein (OMP) of *Neisseria meningitidis*. This study investigated the immunogenicity of intranasally administered class 5C protein of *Neisseria meningitidis* B employing purified LPS of the immunotypes (L3, 7, 9 or L8), cholera toxin and whole cells of *Bordetella pertussis* as mucosal adjuvants for the development of new meningococcal vaccine. In conclusion, the nasal delivery of class 5C with mucosal adjuvants has considerable potential in the development of a mucosal vaccine against serogroup B meningococci in a prime boost immunization in the presence of the adjuvants used. A monoclonal antibody against class 5 C protein using popliteal lymph nodes was obtained.

**Key Words:** *Neisseria meningitidis* B, Opc protein, adjuvants, prime-boost immunization, monoclonal and popliteal lymph nodes.

## INTRODUCTION

*Neisseria meningitidis* is a bacterial pathogen that colonizes the mucosal surfaces of the upper respiratory tract in a significant portion of the population. Infrequently, meningococci penetrate the mucosal barrier and cause disseminated disease, which remains a serious health problem worldwide. During periods of endemic disease, about 10% of the general population harbor *N. meningitidis* in the nasopharynx [1].

The mouse nasal associated lymphoid tissue (NALT) is divided into the organized and diffuse NALTs (O-NALT and D-NALT), respectively [2].

O-NALT, which has been described as the equivalent of Waldeyers's ring in humans, is the only well organized mucosal associated lymphoid tissue in the upper respiratory tract [3]. Response in tissues other than the NALT, including lymph nodes, spleen and bone marrow, are also of importance upon intranasal (i.n.) immunization as they may contribute to the systemic response. Analysis of the immune responses after i.n. immunization or infections provoked in the upper respiratory tract generally show that the magnitude, kinetics, localization, and longevity of the response vary according to the antigens or microorganisms, immunization protocols and mouse strain used [4-7].

The major proteins of the outer membrane of *N. meningitidis* are designated class 1 to class 5 [8]. Studies investigating the ability of individual proteins to induce a protective immune response are facilitated by the availability of free purified proteins of the outer membrane components. Studies concerning the 5C (Opc) protein suggest that future vaccines employing proteins contained in the outer membrane vesicles (OMV) should be prepared from strains with high expression of Opc, although this protein may not provide broad protection if administered alone [9, 10]. In addition, each meningococcal strain can contain three or four Opa genes encoding distinct Opa proteins and one Opc gene encoding the Opc protein [11-15], which is known to be bactericidal [9], suggesting that the epitopes are exposed on the cell surface. Studies show that outer membrane proteins of *N. meningitidis* stimulate T-cell responses [16].

The Opc protein differs from Opa in genetic regulation [17] and in biochemical and immunological properties [13, 18]. While the variable regions of Opa proteins determine differences in size and immunological heterogeneity [11, 12], the Opc protein is of a constant size and antigenicity [11, 17].

The Opc is a protein that contributes to the protective effect of OMV vaccines [19] and it is believed to exhibit less sequence variability than class 1 proteins [20], although the levels of expression are hypervariable. Regulation mechanisms at the transcriptional level by variation in the length of a polycytidine stretch in the promoter region are responsible for protein expression at high levels (Opc++), lower levels (Opc+) or not at all (Opc-) [21]. The Opc protein has been shown to play an important role in the meningococcal adhesion and invasion of both epithelial and endothelial cells and perhaps, represents a common virulence factor [22, 23]. Although the protein is not a porin, it is believed to also adopt a β-sheet structure in the outer membrane, with six surface-exposed loops [24]. Studies made in Norway showed

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that the Opc is present in 17% of meningococci isolated from patients and 31% from healthy carriers in Norway [9].

In Brazil, the Opc protein is expressed in about 38% of the isolated strains from serogroup B [25], suggesting that a vaccine for *N. meningitidis* B for use in Brazil should contain the Opc protein. The highest incidence of meningococcal infection occurs in young children, which peaks between six months to two years old. Proteins contained in the outer membrane vesicles have been used as vaccinal antigens [26]. However, in contrast to older children and adults, infants produce antibodies that are not bactericidal after systemic infection with *N. meningitidis* [27-29].

A combination of pre-existing antibodies and a rapid secondary immune response is likely to be important in establishing protection against the meningococcal disease. Previous reports have shown that intranasal vaccines induce antibody production, but not at high levels [30-32]. This may reflect the priming for immunologic memory by these vaccines. The antibody avidity index has been used as a surrogate marker of immunological memory development upon vaccination against *N. meningitidis* and a high antibody index has been associated with a high bactericidal activity [33].

Cholera toxin (CT) and its subunit B (CTB) are considered very potent adjuvants [34] and a whole-cell pertussis vaccine induces antibodies in the respiratory secretions with a cross reactivity to meningococcal antigens [35].

Lipopolysaccharide (LPS) can be used as an adjuvant without causing adverse effects [36]. Additionally, it has been demonstrated that LPS loses its toxicity when present in intranasal vaccine preparations against *N. meningitidis* B [37-39].

Since the nasopharynx is the only natural habitat of meningococci, intranasal immunization with meningococcal antigens has been suggested to be an effective way of inducing both mucosal and systemic immunity. Recent studies of OMVs administered intranasally in rabbit and humans have provided support for this strategy [37-44].

In this study, we used BALB/c mice to investigate the effect of primary intranasal immunization with the purified Opc protein of *N. meningitidis* B, employing different adjuvant formulations, including LPS from *N. meningitidis* (L3, 7, 9 or L8), cholera toxin (CT) and whole cells of *B. pertussis* (BP). This was followed by an intramuscular (i.m.) antigen booster adsorbed onto $Al(OH)_3$, which is the standard adjuvant routinely licensed for human use. To our knowledge, this is the first report in which the Opc protein was investigated and shown to induce both mucosal (i.n.) and systemic immune responses.

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## MATERIAL AND METHODS

### Bacterial Strains and Adjuvants

The bacteria were grown overnight in a candle jar on Tryptic Soy Broth (TSB; Difco BRL products, Gaithersburg, MD) supplemented with 1% horse serum (Sigma, St.Louis, MO) in plates in a 5% $CO_2$ atmosphere at 37°C. The Opc protein was purified from *N. meningitidis* 44/76 (B:15:P1.7,16: P5.C), a human case isolate representative of the Norwegian group B epidemic (reference strain). The strain (IAL1929), a human case isolate representative from Brazil, was used in bactericidal assays [25]. The whole-cell pertussis consisted of a formalin-inactivated *B. pertussis* (18323 - IAL 0079), purified LPS (L8 and L3, 7, 9) from *N. meningitidis*, which was kindly provided by Dr. Wendell Zollinger (Walter Reed Army Institute, USA), and cholera toxin (Sigma) were used as adjuvants.

### Monoclonal Antibodies

The murine monoclonal antibodies (mAb) used for isolation and characterization of the meningococcal variant against Opc were produced in our laboratory as described previously [45] with some modifications [46]. BALB/c female mice (18-22 g - provided by the animal house of Adolfo Lutz Institute) were injected in the footpad with an emulsion of complete Freund's adjuvant containing 5μg of the purified Opc protein. Two weeks later, the animals received 10μg of the antigen in incomplete Freund's adjuvant by the same route and three days later, the popliteal lymph nodes were excised for fusion with a nonsecreting line, X63-Ag8.653 cell line, using 50% polyethylene glycol (MW 3500). The hybridomas were cultured in hypoxanthine-aminopterin-thymidine-containing medium (HAT) and cloned under limiting dilution conditions. Supernatants from growing hybrids were screened by ELISA using plates coated with the Opc protein [47, 48]. The cloned hybrid cells produced an IgG2 kappa mAb named 4A3Br6-mouse anti-Opc protein. The mAb class and subclass were determined by ELISA using plates coated with purified Opc protein. The specificity of the mAb anti-Opc protein was compared with the Opc protein MAb, 5F1F4-T3 [25].

### Colony Blots

For the detection and selection of the Opc variants, successive rounds of colony blotting with an Opc-specific MAb, 4A3Br6 were performed, to select Opc++. A suspension of meningococci was prepared using isolated colonies from overnight growth on solid medium. The suspension was diluted to give an optical density of 1.0 (OD 1.0) at 650nm and diluted again at 1:30, 000 in Hank's (Sigma). A 100μL sample of the suspension, which contained about 100 to 200 colony-forming units (CFU), was spread onto solid media (chocolate agar - Oxoid GCS agar base with growth supplement) and incubated overnight at 37°C. On the following day, the colonies were blotted directly onto nitrocellulose filter papers (90mm diameter, 0.22μm pore size). The mAb against Opc was used at a dilution of 1:4, 000. Bound mAb was visualized by incubating the nitrocellulose with 3-amino9-ethyl-carbazole (AEC; Pierce) and hydrogen peroxide according to the manufacturer. Colonies with the desired characteristics were identified, picked from the original plate and streaked onto agar. The purity, stability and desired characteristics of the isolated variant were verified after successive rounds of colony blotting [49].

### Purification of the Opc Protein

For the first phase of Opc protein purification, we used a method described by Blake and Gotschlich [50] with

some modifications. After purification, mAb against a 25kDa Opc protein were produced and characterized. A large amount of mAb was produced in pristane (2,6,10, 14-Tetramethylpentadecane; Sigma Chemicals Co., Mo.-USA) primed BALB/c mice. Antibodies were purified by ammonium sulfate precipitation and antibodies from ascites were collected and purified by affinity chromatography on a Sepharose-4B column (Amersham). Prior to the coupling procedure, the antibodies were dialyzed against 0.1M HEPES (Sigma, Buffer, pH 8.0) and later concentrated. In addition, for a better purification, target antigens were purified by immunoaffinity chromatography using a Sepharose-4B column as the matrix support. Briefly, purified mAb was coupled with the gel, according to the manufacturer and in the first phase, the antigen was passed through the column at 4°C. Then, the antigen was eluted with 0.1M glycine (pH 2.2) and the pH was neutralized by the addition of 0.1M Tris-HCl buffer (pH 9.0) and dialyzed against phosphate buffered saline 0.02M (PBS, pH 7.2). Then, the antigen was concentrated by ultra-filtration with a diaflomembrane of PM 10 (MW, cut off 10, 000; Millipore Corp., MA, USA). To collect a sufficient amount, eluted antigens from several purification runs were pooled and then concentrated as described above. Purity of the eluted antigen was demonstrated as a single band at the 25kDa position by (SDS-PAGE) with silver nitrate staining [51]. We decided to utilize this phase of purification so that we would avoid contaminates in our preparation. The protein concentration was determined as described elsewhere [52].

## Mice and Immunizations

Groups of BALB/c (H-$2^d$) adult female mice (18 to 22g, five mice in each group) were immunized in two schedules. The first was a four-dose intranasal immunization (i.n.) at weekly intervals with the Opc protein and one of the four different adjuvants, followed by an intramuscular (i.m.) dose with the Opc protein and $Al(OH)_3$. For i.n. immunization, 10μg of the Opc protein and whole-cells of *B. pertussis* ($2x10^4$cells), cholera toxin (Sigma-5μg) or LPS from *N. meningitidis* (L8 or L3, 7, 9-2μg) as the adjuvants (Fig. (**1**)). The second schedule used was a one-dose i.m. injection of Opc and $Al(OH)_3$. The standardization of the adjuvant quantity utilized was determined in studies carried out in our laboratory (data not shown). The study was conducted under an Institutional Animal Care and Use Committee Approved Protocol IAL/CCD/CEPIAL/CTC 01/2003.

### *ELISA and Avidity Index*

IgG, IgM and IgA antibodies raised against Opc were measured using high binding plates (Corning Costar). Anti-Opc antibodies were quantified using 100μl of Opc per well (2μg/mL) diluted in 50mM sodium carbonate-bicarbonate buffer (pH 9.6). To measure the anti-L3, 7, 9 or L8 response, microtiter plates were coated with 2μg/mL of LPS, 2μg/mL of cholera toxin or $1x10^2$/mL of BP (The concentrations were previously standardized (data not shown). For the IgG, IgM and IgA ELISA and Avidity Index determination, HRP-conjugated antibodies (Kirkegaard and Perry Laboratories,

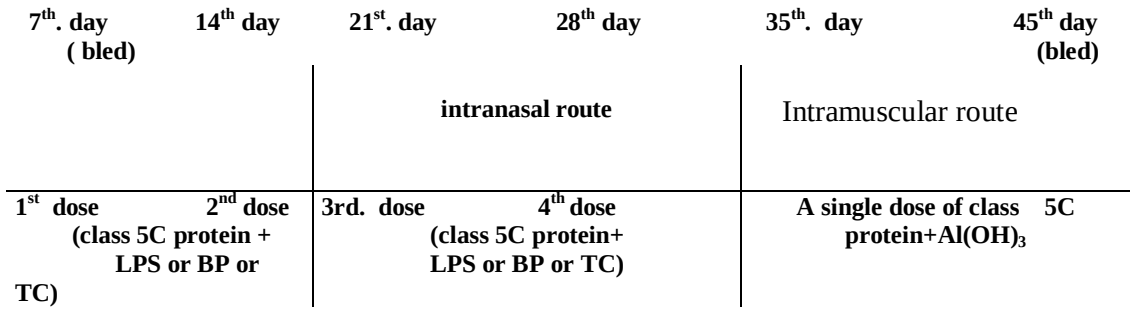


**Fig. (1).** BALB/c mice in a supine position immunized intranasally with *Neisseria meningitidis* antigen. Overview of the immunization and blood sampling schedules for the mice. Schedules of the immunization used**.** The mice were primed by intranasally immunization with 10μg of Opc protein and whole-cells of *B. pertussis* ($2x10^4$cells), cholera toxin (5μg) or LPS from *N. meningitidis* (L8 or L3, 7, 9-2μg) as an adjuvant.

Gaittersburg, MD) were used at 1:2000, 1:5000 and 1:1000, respectively for each isotype. The color reaction was developed with 3, 3', 5, 5'-tetramethylbenzidine (ZYMED, San Francisco, California, USA) and the end point titer was defined as the reciprocal dilution with the lower value presented in the reaction. To show the increase in the titers of IgG, IgM and IgA antibodies in the sera from immunized mice (i.n. and i.m.) compared to the normal mice sera, a ratio between the means of the optical densities of each antibody isotype obtained from the experimental group versus the control group (pre-immune) was determined. Similar determinations were carried out with antibodies against the adjuvants. The avidity index (AI) for IgG and IgM antibodies against the OpC protein was determined in ELISA plates according to the protocol described by Granoff *et al.* [53] with some modifications. Briefly, the immune sera diluted in phosphate buffered saline (PBS) were added and after three washings, 100 μl of 1.5M potassium thiocyanate (KSCN) was incorporated into each well and incubated for 15min. The titers obtained after treatment with KSCN were expressed as the ratio between the mean of three determinations of the absorbance in the presence of the KSCN/control.

### Bactericidal Activity

Briefly, the final reaction mixture (50μL) contained 25μL of serial two-fold dilutions of the test serum that had been heat inactivated at 56°C for 30 min, 12.5μL guinea pig serum as a complement source, which was screened for absence of anti-meningococcal activity, and 12.5μL of a suspension of exponential-phase meningococci (60cfu per well), grown in agar containing 1% (v/v) horse serum. The reaction mixture was incubated at 37°C for 30min, and 130 μL tryptic soy agar (TSA), cooled to approximately 45°C, was added to each well. The mixture was allowed to solidify and the plates were incubated for 18 h at 37°C in 5% $CO_2$. Viable bacteria counts were done at time 0 (*t*0), before incubation with complement to estimate the number of cfu for 100% survival, by plating 12.5μL of the bacterial suspension in TSA. The control wells included on each microtiter plate contained one of the following mixtures: (i) bacteria, complement and buffer (complement dependent control) or (ii) bacteria, heat-inactivated complement and buffer (complement-independent control). In addition, a positive control was included in each assay that consisted of serial dilutions of a known positive serum. Assays were carried out in duplicate. The bactericidal titer of the serum was defined as the reciprocal of the serum dilution that killed 50% of the bacteria at *t0*. The lower limit of the detection in this assay was 1:4.

### Dot-Blotting Analysis

The 369/88 strain (Opc++) was tested by blotting as described previously [47]. Briefly, whole-cell suspension (1μL of bacteria) was suspended in PBS, pH 7.4, with 0.02% sodium azide. Cells were heat-inactivated at 56°C for 30min and the absorbance of the suspension was adjusted to OD 0.1 at 650nm using a Coleman 6A Junior Spectrophotometer. The bacteria were dotted onto nitrocellulose membranes and the membranes were dried for 30min at room temperature (RT) and used immediately. Membranes were blocked for 30min with 1% Bovine Serum Albumin-PBS (BSA-PBS). Hybridoma culture supernatant was pipetted directly into blocking buffer at a final dilution of 1:100. After an overnight incubation at RT on a rotator, the membrane was washed four times with PBS and incubated for 2h with goat anti-mouse IgG conjugated to peroxidase (Sigma), diluted at 1:2, 500. The membrane was then washed four times with PBS and stained with 3-amino-9-ethylcarbazole (AEC).

### SDS-PAGE and Immunoblot Analysis

SDS-PAGE and immunoblot detection of IgG antibodies against the Opc protein purified from strain 44/76 were performed as described in [25].

## RESULTS

### Nasal Immunization of Mice

Fig. (**1**) shows BALB/c mice in the supine position immunized with 20μL of purified Opc of *N. meningitidis*, antigen preparation as shown in Fig. (**2**) (3) and the immunization schedule that was used.

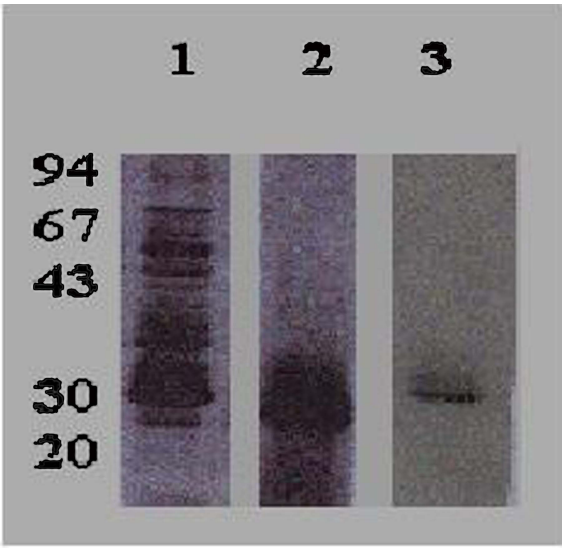


**Fig. (2).** Coomassie blue-stained SDS-PAGE analysis of (**1**) whole cells of the 44/76 strain, (**2**) Opc protein from the first phase of purification, and (**3**) Opc protein from the second phase of purification. The position of molecular mass marker (in kilodaltons (kDa) are shown on the left). The arrows indicate the position of the 25 kDa Opc protein.

### SDS-PAGE

Fig. (**2**) (3) shows the purity of the Opc protein isolated from the *N. meningitidis* 44/76 reference strain. The antigenic characterization of the *N. meningitidis* outer membrane was determined by 10% SDS-PAGE. In **(1)** we show the peptide profile of whole cells of the 44/76 strain. **(2)** Purified Opc protein of the *N. meningitidis* outer membrane (1st phase of purification) and in **(3)**, the Opc protein purified in a Sepharose 4B-column bound to an anti-Opc monoclonal antibody. The molecular weight is approximately 25 kDa.

### Dot-ELISA and Immunoblot

In Fig. (**3**), we can see the immunoreactivity of the new monoclonal antibody obtained using popliteal lymph node by Dot-ELISA. The monoclonal antibody of the isotype IgG recognizes the homologous strain 44/76 up to a dilution of

1/500 Fig. (**3A**). As seen in Fig. (**3B**), we observed the immunoreactivity of the monoclonal antibody against a 25 kDa Opc protein by means of the immunoblot. Analyzing Fig. (**3C**), we observe that there is immunoreactivity present in the IgG antibodies from the sera of mice after i.n. and i.m. immunization using different adjuvants. All lanes contain purified Opc protein and were probed with serum sample at a dilution of 1/50. Antibodies were detected with peroxidase-conjugated anti-mouse IgG. In Fig. (**3C**), the specificity of the IgG antibodies produced after i.n or i.m immunization is directed against the recognition a 25 kDa peptide. However, a strong reactivity against Opc can be observed when the mice received one dose of the purified Opc protein and aluminum hydroxide Fig. (**3C**), blot lane 10). This contrasts with the results obtained when using sera produced with Opc immunization, without adjuvant use Fig. (**3C**), blot lane 12. Furthermore, the band intensities

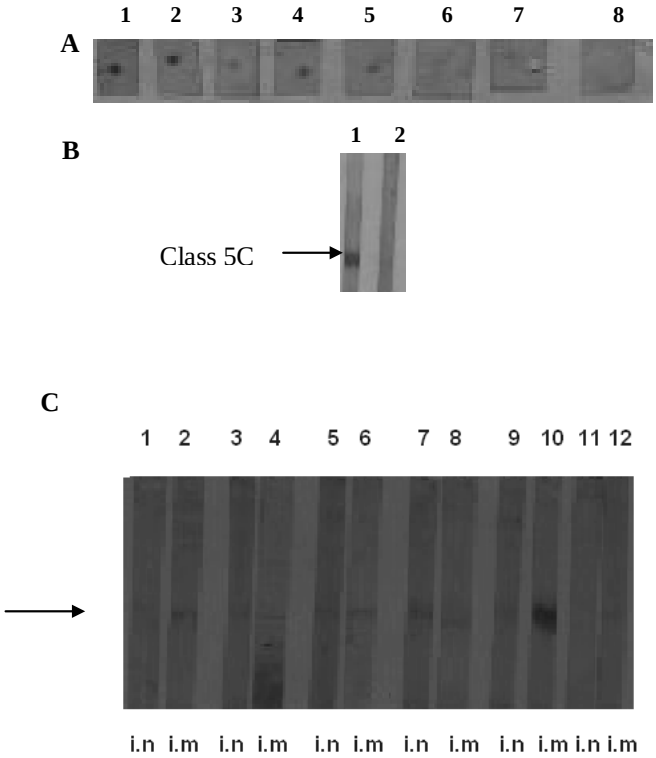


**Fig. (3).** Reactivity of the anti-Opc protein verified by Dot-ELISA using a monoclonal antibody and immunoblot. (**A**) Dot-blot assay with whole cells of *Neisseria* meningitidis 44/76. Culture monoclonal 1/500. (**B**) Immunoblot assay of Opc purified protein and monoclonal antibody. (**C**) All lanes contain purified Opc and were probed with homologous serum samples at a 1/50 dilution. Antibodies were detected with a peroxidase-conjugated anti-mouse IgG, IgM and IgA. (1) sera from mice immunized intranasally with Opc +L8, (2) sera from mice immunized intramuscularly with Opc +L8, (3) sera from mice immunized intranasally with Opc +L379, (4) sera from mice immunized intramuscularly with Opc +L379, (5) sera from mice immunized intranasally with Opc +BP, (6) sera from mice immunized intramuscularly with Opc +BP, (7) sera from mice immunized intranasally with Opc +CT, (8) sera from mice immunized intramuscularly with Opc +CT, (9) sera from mice immunized intranasally with Opc, (10) sera from mice immunized intramuscularly with Opc +$Al(OH)_3$, (11) sera from mice immunized intranasally with saline (control group), (12) sera from mice immunized intramuscularly with saline (control group).

were in agreement with the obtained ELISA results (Table **1**). Interestingly, Table **1** shows that a high level of IgM antibody was consistently produced, independent of the adjuvant used.

### Detection of IgG, IgM and IgA in the Sera from Mice by ELISA

We evaluated the immunogenicity of the Opc protein from *N. meningitidis* following intranasal delivery with CT, BP or LPS (L3, 7, 9 or L8) adjuvant by ELISA. We verified that the Opc antigen induced antibody titers in adult BALB/c mice in relation to the adjuvants used in the present investigation (Table **2**). To better interpret these results, we used the ratio between the values of the post immunization serum samples and the value of the pre-immune sera compared with the normal sera. Increases in the antibody titers suggest that four doses of purified Opc protein successfully primed the immune system of mice to generate IgG,IgM and IgA antibodies, using the prime/boost schedule described herein. We observed fast production of antibodies, within ten days after i.n. immunization. An exception to the general trend of priming by i.n. immunization was the low level of serum IgA recognizing the Opc after i.n. and i.m. immunizations.

### IgG and IgM Avidity

To investigate the type of antibody response produced, we determined the IgG and IgM avidity index (AI) after i.n. and i.m. immunizations in all of the studied groups (Tables **3** and **4**). We found that the AI of the IgM and IgG isotypes did not differ significantly between the different adjuvants used. Additionally, we observed a good correlation between AI and bactericidal activity after i.n. or i.m. immunization. Still, it is important to highlight that the avidity of the measured antibodies produced using the adjuvants was decreased when compared with the antibodies produced using the Opc alone. Interestingly, we also observed a good correlation between the bactericidal activity of post-immunization sera samples and the antibodies produced against Opc. Our data suggest that use of these adjuvants resulted in the production of low-avidity antibodies. In this experiment, the reactivity of the IgG, IgM and IgA antibodies was also determined for each adjuvant and expressed as fold-change Tables (**3** and **4**). The avidity index was considered during the analysis of the results (avidity index values less than 0.29 were designated as low-avidity; 0.30-0.49 were designated as intermediate-avidity and greater than 0.50 was high-avidity.

### Bactericidal Antibody Response

Table **5** shows the bactericidal titers of the antibodies. Sera collected after i.n. immunization showed lower antibody titers compared to the i.m. immunization. In spite of the fact that some bactericidal activity was present when we used LPS L8 and L3, 7, 9 as controls, we observed a significant difference between the bactericidal antibody response after the i.m. dose compared with i.n dose. These data suggest that high levels of bactericidal antibodies were produced that are specific for the Opc protein. The controls showed a lower bactericidal activity than that observed in the experimental groups.

**Table 1. Increase in the Levels of IgG, IgM and IgA Antibodies from BALB/c Mice Immunized with Opc Protein and Different Adjuvants - B. *Pertussis*, Cholera Toxin, and LPS (L3, 7, 9 and L8 from *N. meningitidis*) as Determined by ELISA to Class 5C Protein. Means of the Increase of the Antibodies in the Serum were Determined by (fold Change). We Pooled the Serum from Five Animals**

| Immunization Route | Immunoglobulin Isotypes | Sera from Immunized Mice | | | |
|---|---|---|---|---|---|
| | | *Class 5C Protein* + B. Pertussis | *Class 5C Protein* + Cholera Toxin | *Class 5C Protein* + LPS Immunotype L3, 7, 9 | *Class 5C Protein* + LPS Immunotype L8 |
| **Intranasal** | IgG | 3.7 | 4.4 | 3.8 | 2.4 |
| | IgM | 2.3 | 4.8 | 3.9 | 6.4 |
| | IgA | 0.6 | 2.8 | 0.6 | 1.0 |
| **Intramuscularly** (Class 5C protein + aluminum hidroxide) | IgG | 3.9 | 7.4 | 5.4 | 10.0 |
| | IgM | 10.9 | 13.2 | 12.0 | 23.8 |
| | IgA | 1.2 | 3.2 | 3.2 | 2.9 |

*Increment index*: The compare absorbance in ELISA for the sera obtained before with that after immunization that express the level of antibodies produced. The fold-increase will depend heavily on how much absorbance is found in the mouse sera pre-immunization. The results represents the mean of 10 mice per group in an experiment representative of two separate experiments.

**Table 2. Increase in the Levels of IgG, IgM and IgA Antibodies from BALB/c Mice Immunized with Opc Protein and Different Adjuvants - B. *pertussis*, Cholera Toxin, and LPS (L3, 7, 9 and L8 from *N. meningitidis*) as Determined by ELISA Immune Response to the Adjuvants. Means of the Increase of the Antibodies in the Serum were Determined by (Fold Change). We Pooled the Serum from Five Animals**

| Immunization Route | Immunoglobulin Isotypes | Sera from Immunized Mice | | | |
|---|---|---|---|---|---|
| | | *B. pertussis* | Cholera Toxin | LPS Immunotype L3, 7, 9 | LPS Immunotype L8 |
| **Intranasal** | IgG | 19, 0 | 33, 3 | 8, 9 | 3, 9 |
| | IgM | 32, 8 | 20, 6 | 5, 8 | 6, 6 |
| | IgA | 2, 9 | 3, 2 | 0, 8 | 2, 4 |
| **Intramuscularly** (Class 5C protein + aluminum hidroxide) | IgG | 25, 4 | 36, 5 | 19, 4 | 11, 4 |
| | IgM | 22, 4 | 25, 2 | 4, 1 | 1, 9 |
| | IgA | 2, 4 | 13, 4 | 2, 6 | 1, 2 |

*Increment index*: The compare absorbance in ELISA for the sera obtained before with that after immunization that express the level of antibodies produced. The fold-increase will depend heavily on how much absorbance is found in the mouse sera pre-immunization. The results represents the mean of 10 mice per group in an experiment representative of two separate experiments.

**Table 3. The Figure Shows the Avidity Index of the IgG and IgM Antibodies Present in Sera (Avidity Index Values Less than 0.29 was Designated as Low Avidity; 0.30-0.49 as Intermediate Avidity and Greater than 0.50 was High Avidity). Antibodies from Immunized Mice Immunized by Intranasal and Intramuscular Routes with Opc and the Adjuvants BP, L379, L8 or CT. We Pooled the Serum from Five Animals**

| | Immunoglobulin Isotypes | Avidity of Antibodies Against Class 5C Protein, According to the Adjuvant | | | |
|---|---|---|---|---|---|
| | | *B. pertussis* + Class 5C Protein | Cholera Toxin+ Class 5C Protein | LPS-L3, 7, 9 + Class 5C Protein | LPS-L8 +Class 5C Protein |
| **Intranasal** | IgG | 0.88 | 0.75 | 0.87 | 0.80 |
| | IgM | 1.00 | 0.86 | 0.75 | 0.71 |
| **Intramuscularly** (class 5C protein + aluminum hydroxide) | IgG | 0.84 | 0.75 | 0.87 | 0.83 |
| | IgM | 0.93 | 0.85 | 0.80 | 0.79 |

**Table 4. The Figure Shows the Avidity Index of the IgG and IgM Antibodies Present in Sera (Avidity Index Values Less than 0.29 was Designated as Low Avidity; 0.30-0.49 as Intermediate Avidity and Greater than 0.50 was High Avidity) to the Adjuvants Used. Antibodies from Immunized Mice Immunized by Intranasal and Intramuscular Routes with Opc and the Adjuvants BP, L379, L8 or CT. We Pooled the Serum from Five Animals**

| | Immunoglobulin Isotypes | Avidity of Antibodies Against Class 5C Protein, According to the Adjuvant | | | |
|---|---|---|---|---|---|
| | | ***B. pertussis* + Class 5C Protein** | **Cholera Toxin+ Class 5C Protein** | **LPS-L3, 7, 9 + Class 5C Protein** | **LPS-L8 +Class 5C Protein** |
| **Intranasal** | IgG | 0.05 | 0.35 | 0.61 | 0.73 |
| | IgM | 0.05 | 0.37 | 0.63 | 0.61 |
| **Intramuscularly** (class 5C protein + aluminum hydroxide) | IgG | 0.28 | 0.92 | 0.30 | 0.04 |
| | IgM | 0.27 | 1.00 | 0.16 | 0.02 |

**Table 5. The Figure Shows the Bactericidal Activity of the Sera from BALB/c Mice Immunized by i.n. and i.m. with 5C Purified Protein from the Outer Membrane of *N. meningitidis* B with Different Adjuvants**

| Adjuvants | Bactericidal Activity After i.n. Immunization* | Bactericidal Activity After i.m. Immunization |
|---|---|---|
| *B. pertussis* + OPC | 1/64 | 1/1024 |
| Cholera toxin + OPC | 1/1024 | 1/2048 |
| LPS (L3, 7, 9) + OPC | 1/512 | 1/5096 |
| LPS (L8) + OPC | 1/256 | 1/10192 |
| *B. pertussis* alone (control) | 1/4 | 1/32 |
| Cholera toxin alone (control) | 1/8 | 1/64 |
| LPS (L3, 7, 9) alone (control) | 1/4 | 1/64 |
| LPS (L8) alone (control) | 1/8 | 1/128 |

*OPC (class 5 protein).

## DISCUSSION

The body´s mucosal surfaces are constantly exposed to a myriad of benign foreign antigens. Relatively few molecules are highly immunogenic when they contact mucosal surfaces, in the sense that they generate strong humoral and secretory antibody responses. Meningococcal infection initiates from the adherence of the bacteria to human cells and results in the colonization of the organism on the nasopharyngeal mucosa [22]. An effective meningococcal vaccine should provide protection against group B organisms either at the level of initial colonization, or by inhibiting bacterial invasion of the bloodstream, or through a combination of both. Protection against serogroup B meningococci requires antibodies with high avidity and complement-binding bactericidal antibodies [26-28]. Using adequate adjuvants and new schemes of immunization, it is possible to develop these high-avidity antibodies and thus to improve the immune response to the OMV vaccine [54, 55].

Therefore, in the present study, we tested a prime/boost strategy with purified meningococcal Opc protein and the different adjuvants; LPS (L3, 7, 9 and L8), *B. pertussis* and cholera toxin, in addition to aluminum hydroxide, which is the adjuvant regularly used in OMV vaccines for *N. meningitidis*. This "prime boost" strategy is effective in generating high levels of T-cell memory. The strategy has been applied to vaccines developed against a variety of pathogens, such as HIV [56, 57] or other agents for which both mucosal and systemic immunity are important for protection [58].

Clearly, different prime-boost approaches are likely to generate distinct types of immunity and it is essential to ensure that only appropriate immune responses are targeted during immunization. The factors that regulate distinct types of immune responses are poorly understood and, depending on the route of delivery, it is difficult to predict in advance what type of immune response will be favored. We found that four i.n. priming immunizations followed by an i.m. boost significantly enhanced the serum Opc-specific antibody titers. Studies are underway in our laboratory that aim to determine the optimal number of doses and the quantity of Opc required for sensitizing the immune system of the mucosa and for stimulating the appearance of memory cells. We maintain that the quality of the antibody produced is another important factor to consider in choosing an adequate antigen.

Mucosal vaccines have a number of advantages, including avoiding the need for injections, which improves patient compliance and eliminate problems associated with the disposal of contaminated needles. Mucosal delivery also has the advantage of stimulating local as well as systemic immune responses, thus preventing the bacterial colonization

in naive individuals and the dissemination of bacteria that breach the mucosal barrier. However, most antigens are poorly immunogenic when delivered at mucosal sites and typically require the use of mucosal adjuvants.

Our study, which used different adjuvants with Opc, showed that levels of IgG against the Opc protein peaked after the fourth i.n. immunization. At this time, these IgG values were 3.8- and 2.8-fold increased and the IgM values were 3.9- and 6.4-fold increased, with Opc+LPS 3, 7, 9 and L8, respectively. In comparison, we saw a 3.7- and 2.3-fold increase in IgG and IgM, respectively, when we used *B. pertussis* as the adjuvant. At the 45$^{th}$ day, ten days after the i.m. booster dose, all antibody values were significantly enhanced (Table **1**). Results also demonstrated that antibodies to Opc could kill bacteria *in vitro* (Table **4**), showing a good correlation with the avidity index (Table **3**). In our study, Opc was poorly immunogenic when delivered intranasally without adjuvant (data not shown). Therefore, different adjuvants were used that substantially enhanced the immune response against the protein.

Our results clearly demonstrate that IgG and IgM antibodies contribute to the protection at the mucosal level. Possibly, IgG produced against Opc contributes more to the observed protection, due to its characteristics as already described [13, 18, 19]. However, in the literature, Rosenqvist *et al.* [9] observed that the Opc (5C)-dependent bactericidal activity was apparent only against bacteria expressing large amounts of the Opc protein. Besides, it was observed that most of the meningococci isolated from infected individuals are probably not susceptible to this mechanism of killing, because they express only small or undetectable amounts of Opc. In our study, we used a strain with a high level of Opc protein expression, which was identified by colony blot. More studies are necessary using other mouse strains, since this is the first analysis of the prime/boost system used to induce IgG and IgM antibodies by intranasal immunization using the purified Opc protein of *N. meningitides* B. Moreover, the bactericidal activity and avidity indexes of the induced antibodies varied from intermediary to high, depending on the adjuvant used, and correlated with the bactericidal activity. These antibodies may be effective for protection against serogroup B meningococci when this protein is administered i.n. with the appropriate adjuvant. Interestingly, since it is known that Opc is an adesin, we observed that even when the Opc protein was administered i.m. with aluminum hydroxide, IgA antibodies were produced. In this study, we observed a high avidity of the IgG antibodies produced.

The cholera toxin has long been established as a potent mucosal adjuvant [36]. However, in our study, it did not stimulate the production of anti-Opc IgA antibodies by nasal immunization. The lower levels of IgA could be explained by the high levels of IgG and IgM produced, which are likely to have blocked the sites for IgA attachment. Further assessment is required to verify if different concentrations of the Opc protein and cholera toxin can induce IgA antibodies intranasally or if this result can be attributed to the protein studied.

The use of purified LPS (L3, 7, 9 or L8) as a mucosal adjuvant in conjunction with Opc has not been previously reported. However, successful i.n. immunization with native outer membrane vesicles containing LPS has been described [38, 40]. Herein, the addition of purified LPS 3, 7, 9 or L8 with Opc induced highly significant anti-Opc IgG and IgM antibody responses with bactericidal activity and a high avidity index, compared to cholera toxin and *B. pertussis*. Recently, the use of LPS in a nasal vaccine formulation with satisfactory and safe results was reported [38, 40]. In our study, the additional i.m. booster with the Opc protein and aluminum hydroxide resulted in higher titers of IgG and IgM antibodies (Table **1**).

The immunogenicity of recombinant Opa B and Opa J proteins associated with liposomes administered in mice i.n has been described by de Jonge *et al.* [59]. We used a well-defined formulation based on an important and purified meningococcal antigen, but more studies are required to fully investigate its potential as a mucosal vaccine.

In a evaluation, the immunogenicity of three surface-exposed proteins of *N. meningitidis* from serogroup B (App, NhhA, and NadA), which were identified during whole-genome sequencing, was determined [34]. Mice were immunized intranasally with individual proteins in the presence of wild-type *Escherichia coli* heat-labile enterotoxin (LTwt), LTR72, a partially inactivated mutant, or LTK63, a completely nontoxic mutant, as the adjuvants. Each of the meningococcal proteins induced significant cellular responses; NhhA and NadA induced strong antibody responses, but only NadA induced bactericidal antibodies when administered intranasally with mucosal adjuvants. In addition, IgA and bactericidal antibodies were detected in the respiratory tract following intranasal delivery of NadA. Our data suggest that Opc is important for the induction of mucosal immunity to *N. meningitidis* B, and that the quality and magnitude of the immune responses generated by mucosal vaccines are influenced by the antigen as well as the adjuvant. Therefore, the nasal delivery of Opc with mucosal adjuvants seems to have considerable potential in the development of a mucosal vaccine against meningococci serogroup B, as suggested for other purified proteins of meningococci [59].

Whole-cell bacteria (WCB) vaccines containing endotoxin may cause adverse effects upon parenteral administration, but this risk is lower by mucosal route. Thus, inactivated WCB preparation delivered mucosally may confer effective, economical and simple immunization in humans. Additionally, nasal whole-cell pertussis vaccine induces specific systemic and cross-reactive mucosal antibody responses in human [60]. Our data using *B. pertussis* as an adjuvant to the Opc protein suggest that more investigations are necessary to determine which of the different doses of WCB would be the best.

In conclusion, anti-Opc antibodies were found in the sera of mice after intranasal immunization with purified Opc protein and different adjuvants. An important point was the utilization of *Bordetella pertussis* whole cells as an adjuvant. Studies are being carried out in our laboratory with neonatal mice utilizing *Bordetella* pertussis acellular antigens as mucosal adjuvant (data not shown). Although it is known that CT is an excellent adjuvant for mucosal immunization, there may be adverse effects when it is used in human vaccines. In

our studies, IgG antibodies with a low avidity were produced in response to some of the adjuvants used but some improvement was observed with CT, which is a powerful adjuvant at the mucosal level. It will be necessary to carry out a dose study and to investigate the quantity of the CT to be administered as well as the quality of the IgG antibody produced.

This study forms a promising approach to the design of novel meningococcal vaccines aimed at reducing mucosal colonization, using new adjuvant formulations. However, the contribution to protection can only be measured in an animal model and at the humoral level of the immune response. Studies regarding the cellular mechanisms are in development in our laboratory.

Serum bactericidal activity is widely regarded as the gold standard for the predictive correlate of protection against *N. meningitidis* infection [61]. In our study, bactericidal antibodies were produced after i.n. immunization with adjuvants (Table **5**). A significant increment of the bactericidal activity after i.m. immunization could be observed, indicating that the cells of the immune system were primed after i.n. immunization. Also, the bactericidal activity did not differ significantly between groups at any point analyzed.

In our results, we observed the bactericidal capacity of the antibodies produced against Opc in the destruction of the meningococci. In our opinion, more studies are necessary that utilize *N.meningitis* and LPS mutants. However, it should be stressed that when *B. pertussis* was used as a mucosal adjuvant, the produced antibodies were better able to neutralize the homologous strain.

Taking the data together, we demonstrated that the Opc protein was able to elicit high titers of IgG and IgM antibodies with good avidity indeces when administered intranasally, following the immunization schedule presented here.In addition the Opc protein seems an excellent candidate for inclusion in a vaccine against serogroup B meningococci.

We conclude that intranasal immunization is effective in generating high levels of antibodies that appear as early as 10 days following intramuscular immunization. This is in accordance with the previous observation that mucosal immunization is important for obtaining significant antibody responses [43, 44]. The antibody titers produced depended on the adjuvant used. Thus, it is evident that i.n. immunization with Opc protein induces an immune response rather than a tolerogenic effect on the systemic antibody response to the later i.m. immunization, using the dose and schedule presented herein.

Measuring the AI and IgG isotypes distribution of the antibodies after nasal immunization can be a supplementary method for predicting protective immunity and for evaluating the immune response against meningococcal serogroup B nasal vaccines.

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## ABBREVIATIONS

| | | |
|---|---|---|
| AI | = | Avidity Index |
| $Al(OH)_3$ | = | aluminum hydroxide |
| AEC | = | 3-Amino-9-ethylcarbazole |
| BP | = | Bordetella pertussis |
| CFU | = | Colonic Forming units |
| CT | = | cholera toxin |
| CTB | = | cholera toxin subunit B |
| i.m | = | intramuscular |
| i.n | = | intranasal |
| IgA | = | Immunoglobulin "heavy"α |
| IgG | = | Immunoglobulin "heavy" y |
| IgM | = | Immunoglobulin "heavy"μ |
| KSCN | = | potassium thiocyanate |
| KSCN | = | potassium thiocyanate |
| LPS L3, 7, 9 | = | *N. meningitidis* imunotye |
| LPS L8 | = | *N.meningitidis* imunotye |
| LPS | = | Lipopolysaccharide |
| MAb | = | Monoclonal Antibody |
| MW | = | Molecular Weight |
| N. meningitidis | = | Neisseria meningitidis |
| NALT | = | Nasal Associated Lymphoid Tissue |
| OMP | = | Outer Membrane Protein |
| OMVs | = | Outer Membrane Vesicles |
| PBS | = | Phosphate-Buffered Saline |
| Opa | = | Opacity associated Protein |
| Opc | = | Outer membrane protein class 5C |
| SDS-PAGE | = | Sodium Dodecyl Sulfate Polyacrylamide gel |
| TSB | = | Tryptic Soy Broth |
| WCB | = | Whole Cells Bacteria |

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