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Cross-reactivity with Brazilian strains of *Neisseria meningitidis* B after immunization with outer membrane vesicles

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Abstract

Background: Immunization against *Neisseria meningitidis* is important for public health. Vaccines composed of cross-reactivity antigens avoid strain-specific responses, ensuring more comprehensive protection.

Methods: The cross-reactivity between three strains from the last outbreak of *N. meningitidis* in Brazil was assessed in our studies, using enzyme-linked immunosorbent assay (ELISA) and immunoblotting assays.

Results: Both assays verifed a similar humoral response between the strains evaluated. Patterns of antigen recognition differed with each dose evaluated.

Conclusions: We observed that immunization with *N. meningitidis* B outer membrane vesicles (OMVs) led to the production of antibodies that recognized antigens of heterologous strains, indicating possible protection against these evaluated strains.

Keywords: cross-reactivity, immunization, *Neisseria meningitidis*, outer membrane vesicles, Swiss mice

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Introduction

Neisseria meningitidis is one of the main etiologic agents of bacterial meningitis, a disease with great impact on public health due to high rates of morbidity and mortality associated with it.[1,2] The main strategy for preventing infection by serogroups A, C, W, and Y is immunization with conjugate vaccines, composed of the polysaccharide capsule of the bacterium associated with a carrier protein.[3] The development of this type of vaccine is not viable for serogroup B, given the structural similarity between the polysialic acids $\alpha 2 \rightarrow 8$ present in the polysaccharide capsule and the neuronal cell adhesion molecule (N-CAM) present in human brain tissue, which results in low immunogenicity and the risk of developing autoimmunity.[4,5] Because of that, outer membrane vesicle (OMV) vaccines, composed of subcapsular antigens, which exhibit remarkable potential in immunomodulation of the immune response, have been developed. However, OMV vaccines exhibit some limitations, since most serum bactericidal activity was generated against PorA, which is highly variable between different strains, so these vaccines are indicated in epidemiological contexts where there is a specific circulating clone.[6] To overcome these limitations, two vaccines against meningococci B, based on recombinant proteins, were developed recently.[7,8] The vaccine 4CMenB/Bexsero® uses secondary and cross-reactivity outer membrane proteins (OMPs) to develop a more universal vaccine and to avoid the strain-specific response. This vaccine is composed of recombinant proteins: *Neisseria* adhesin A (NadA), factor H binding protein (fHbp), and Neisserial heparin binding antigen (NHBA), combined with OMVs from strain NZ98/254.[3] The other vaccine developed, rLP2086/Trumenba®, is composed solely of two fHbp variants, each one belonging to the two

different fHbp subfamilies described.[9] Continuing the studies of Trzewikoswki de Lima and De Gaspari,[10] the present study aims to evaluate the cross-reactivity generated by immunization of mice with *N. meningitidis* B OMVs, observing the humoral response and antigen recognition patterns after each immunization.

Material and methods

OMV isolation

OMVs from strains B:4:P1.9, B:8:P1.6 and B:4:nt used in this study were obtained as previously described,[11] and are representative strains of the last period of greater incidence of MenB, especially in Southeastern Brazil, at the end of the 1980s.[12]

Animals and immunization

Swiss mice were immunized as described in Trzewikoswki de Lima and De Gaspari.[10] In brief, when mice were approximately 21 weeks old, they were immunized intramuscularly with an antigenic preparation composed of 2 μg of *N. meningitidis* OMVs from strain B:4:P1.9 and 0.1 mM aluminum hydroxide. The animals were immunized again 20 and 30 days after the first dose. Blood was collected by the retro-orbital plexus puncture before immunization and after each dose. The Animal Ethics Committee of CEUA IAL/Pasteur (protocol number 2012/06) approved the procedures performed in this study.

SDS-PAGE

OMV proteins at a concentration of approximately 52 μg/mL were characterized by electrophoresis in a 10% polyacrylamide gel, in the presence of sodium dodecyl sulfate (SDS), following the protocol described by Laemmli.[13] After electrophoresis, the gel was stained with Coomassie Blue (PhastGel® Blue R, Pharmacia Biotech, Uppsala, Sweden).

Immunoblotting

A new 10% polyacrylamide gel was prepared. After electrophoresis, proteins were transferred to a 0.45 μm nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA) at 100 V for 18 h at 4°C. Strips, estimated to contain 14 μg of proteins from OMVs, were cut and blocked with skimmed milk (La Serenissima, Buenos Aires, Argentina) 5% for 2 h over agitation. Then, membranes were washed five times with PBS pH 7.2 and incubated overnight at 4°C with a pool of sera obtained before the immunization and after each dose, diluted 1:50 in 2.5% skimmed milk. Then, the membranes were washed and incubated for 2 h over agitation, with peroxidase-conjugated anti-mouse IgG (Fc specific) (Kirkegaard & Per Laboratories, KPL, Gaithersburg, MD, USA) 1:10,000, in 2.5% skimmed milk. After several washes, it was incubated with 4-chloro-1-naphthol (Sigma-Aldrich, St. Louis, MO, USA) for 20 min. The reaction was stopped by washing the membranes with distilled water.

ELISA

OMVs of *N. meningitidis* strains B:4:P1.9, B:8:P1.6 or B:4:nt were used as coating antigens. The plates were coated with 5 μg/mL of OMVs diluted in carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. After washing with PBS pH 7.2 containing 0.05% Tween 20, the plates were incubated with 5% skimmed milk at 37°C for 2 h. Then, the pre-immune sera and the sera obtained after the third immunization were added, which were evaluated individually, diluted 1:500 in 2.5% skimmed milk, and the plates were incubated overnight at 4°C. The next day, plates were washed and incubated with peroxidase-conjugated anti-mouse IgG whole molecule (Sigma-Aldrich) diluted 1:2000 at 2.5% skimmed milk for 2 h at 37°C. After washing, the enzymatic reaction was developed with the substrate tetramethylbenzidine (TMB) (Sigma-Aldrich) for 20 min at 37°C. The reaction was stopped with sulfuric acid 1 *N* and the plates were read at 450 nm in a microplate reader (Labsystem Multiskan, Thermo Fisher, Waltham, MA, USA).

Statistical analyses

The results obtained by the ELISA technique were analyzed using one-way ANOVA test followed by Tukey's post-test, using the software GraphPad Prism 5 version. Values of $p < 0.05$ were considered statistically significant.

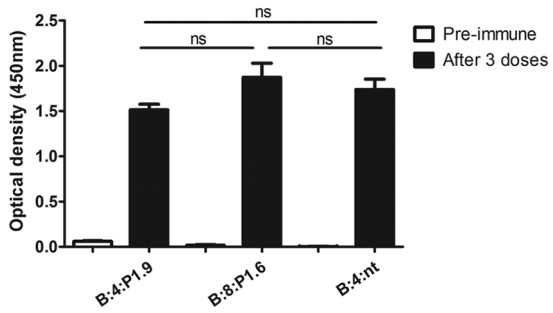

Figure 1. IgG antibodies levels, measured by ELISA, in pre-immune sera and sera obtained from mice immunized with three doses of antigenic preparation containing *Neisseria meningitidis* OMVs from strain B:4:P1.9 and aluminum hydroxide. The plates were coated with *N. meningitidis* OMVs from strains B:4:P1.9 (homologous strain), B:8:P1.6 or B:4:nt (heterologous strains) at a concentration of 5 µg/mL. The graph shows the mean of the optical densities obtained in sera diluted 1:500. Error bars represent the standard error of mean.
ELISA, enzyme-linked immunoabsorbent assay; IgG, immunoglobulin G; ns, not significant; OMV, outer membrane vesicle.

Results

Serum of mice immunized with three doses of an antigenic preparation containing *N. meningitidis* OMVs from strain B:4:P1.9 and aluminum hydroxide were evaluated by ELISA assay, in which wells were coated with OMVs from strains B:4:P1.9, B:8:P1.6 or B:4:nt. The evaluated serum presented high levels of IgG when tested with both homologous and heterologous strains, and there was no statistical difference between the IgG levels concerning the strain used to coat the plate (Figure 1). The pre-immune sera showed no reactivity, as expected.

The electrophoretic profile of the OMVs used in the present study was evaluated in a 10% polyacrylamide gel. The OMVs of the different strains presented similar electrophoretic profile; however, OMVs from strain B:4:P1.9 present more bands in the range of 35–40 kDa (Figure 2a).

In our previous study,[10] by dot-blot ELISA, the presence of cross-reactivity was noticed with most of the evaluated strains of *N. meningitidis* from serogroups B, W, and Y. In this study, an immunoblotting assay was used. Unlike dot-blot, which uses antigens in their native structure, immunoblotting denatures the proteins, altering the antigenic conformation structure, also separating them by molecular weight.

In sera collected after the first immunization, antigens were recognized in the 60–80 kDa range. After the second immunization, a variety of antigens were recognized, with molecular weights ranging from 200 to 24 kDa. The recognition pattern after the third immunization was similar to that observed after the second dose, except for OMVs from strain B:4:nt, in which antigen recognition was broader after the third immunization. The pattern of antigen recognition was similar among the OMVs of the evaluated strains (Figure 2).

Discussion

The strains included in this study were chosen because they were prevalent in the last epidemic of meningococcal B disease in Brazil, and, therefore, are believed to be virulent strains. Besides, these strains have been evaluated by our group in other studies and proved highly immunogenic.

The major criticism of OMV vaccines is that they possibly do not result in protection against strains of different subtypes. However, studies have shown that these vaccines are capable of leading to protection against heterologous MenB strains.[14–16] Besides, a retrospective study indicated that the MenZB® vaccine, consisting of MenB OMVs, may have generated a protective effect against *Neisseria gonorrhoeae*.[17]

The importance of cross-reactivity between *N. meningitidis* strains is described in the literature and should be considered when choosing vaccine strains; OMVs from the hypervirulent strain B:4:P1.19,15, for example, was chosen for the production of vaccine VA-MENGOC-BC®, which controlled epidemics in Cuba and São Paulo, considering OMPs that could also be expressed by heterologous strains.[18] The study by Williams and colleagues showed that this vaccine induced the production of bactericidal antibodies with cross-reactivity,[19] directed mainly against the antigens Rmp, Opa, PorB, ferric binding protein A (FbpA), exopolyphosphatase (NMB1467), γ-glutamyltranspeptidase (NMB1057), and a putative cell-binding factor protein (NMB0046).

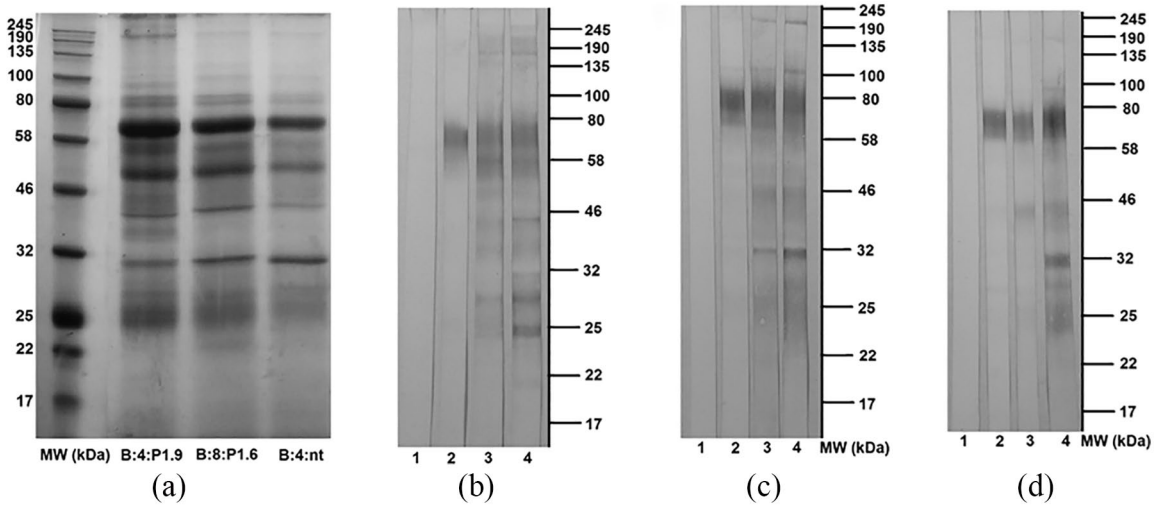

Figure 2. SDS-PAGE profile, after staining with Coomassie Blue, showing MW marker and *N. meningitidis* OMVs from strains B:4:P1.9, B:8:P1.6 and B:4:nt, respectively. (a) Immunoblotting for analysis of IgG antibodies reactivity with OMVs from strains B:4:P1.9; (b) B:8:P1.6; (c) and B:4:nt; and (d) Strip 1: pre-immune sera; strip 2: sera collected after one immunization; strip 3: sera collected after two immunizations; strip 4: sera collected after three immunizations.
IgG, immunoglobulin G; MW, molecular weight; OMV outer membrane vesicle; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

The results obtained by the ELISA technique confirmed the cross-reactivity between strains, as well as observed in the study of Harthug and colleagues, who found similar immune responses between patients infected by different serogroups of meningococcus when evaluated by ELISA assay using coating antigens of the strain B:15:P1.16.[20] Although there is no statistical difference, the optical density was higher when the heterologous strains (B:8:P1.6 and B:4:nt) were used as coating antigens than when homologous strain (B:4:P1.9) was used, which leads us to believe that these strains have greater expression of immunogenic antigens. The ELISA assay does not allow the verification of antibodies functionality generated by immunization, but Rosenqvist and colleagues demonstrated that IgG antibody levels, detected by ELISA, following immunization with a *N. meningitidis* OMV vaccine generate a good prediction of bactericidal activity against the vaccine strain, even if only a fraction of the antibodies are bactericidal.[21] Also, in our previous study,[10] it was verified that IgG antibodies produced after three immunizations with the same antigenic preparation used in the present study presented a high avidity index, which was correlated to bactericidal activity in some studies.[22,23]

In the present study, it was observed that a second dose of antigen preparation was beneficial, increasing the recognition of distinct antigens. A third immunization did not significantly alter the recognition profile concerning OMVs of strains B:4:P1.9 and B:8:P1.6, but resulted in greater antigen recognition in the 24–30 kDa range concerning OMVs from strain B:4:nt. Tunheim and colleagues also found increased antigen recognition after each immunization with meningococcal A and W OMVs compound vaccines.[24] Rosenqvist and colleagues and Gioia and colleagues found that three doses of the vaccine MenBVac®, in humans, generated higher levels of IgG antibodies and a decrease of the relative importance of specific antibodies directed to OMPs of Class 1, with the probable increase in cross-reactivity against heterologous meningococcal strains.[21,25]

Molecular biology techniques, mass spectrometry or the use of monoclonal antibodies would be necessary to identify which antigens are responsible for the cross-reactivity observed in this study. However, in many laboratories, it is not possible to implement these techniques, due to the required greater infrastructure or lack of monoclonal antibodies. The immunoblotting technique is relatively simple and can be employed in many

laboratories at a relatively low cost. Although it is not possible to determine precisely which antigens were recognized without the use of monoclonal antibodies, the technique allows the main antigens present in the sample to be estimated based on molecular weight.

Analyzing this data, and complementing our previous study, we observed that immunization with OMVs has the potential to protect against heterologous strains. In the present study, we observed that immunization with MenB OMVs led to the production of antibodies that recognized antigens of heterologous strains. Through the experiments performed, it is not possible to affirm that these antibodies are protective, for this purpose it would be necessary to perform the serum bactericidal activity assay (SBA), which is the correlate of protection against meningococcal disease. In a previous study, it was found that mice immunized with OMVs from the same strain used in the present study, complexed to aluminum hydroxide, presented serum bactericidal antibody titers above the levels considered as protective (manuscript in preparation). However, evaluation of OMV vaccines solely by inducing serum bactericidal activity may underestimate the protection offered by these vaccines, as other mechanisms may also contribute to protection against *N. meningitidis*.[26,27] The absence of SBA does not necessarily indicate an absence of protection.[27,28] As seen by several studies, opsonophagocytosis also appears to be important in protection against meningococcus.[26,29–31]

Finally, it is believed that the immunized mice produced antibodies directed to possible cross-reactivity antigens. It demonstrates the importance of monitoring the antigenic expression in *N. meningitidis* strains prevalent in the country, paying attention to those associated with the cross-reactivity, which contributes to assessing antigenic preparations that are representative of the epidemiology of meningococcal disease in Brazil, and that could help predict the effectiveness of a new vaccine in the population.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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