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Immunogenicity of antigens from outer membrane vesicles of *Neisseria meningitidis* associated with bilayer fragment of dioctadecyldimethylammonium in Swiss adult mice

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Purpose: In the present study, meningococcal serogroup B outer membrane vesicles (OMVs) were associated with bilayer fragments of a cationic lipid, dioctadecyldimethylammonium (DDA-BF), used as adjuvant, in an antigenic preparation tested in adult female outbred mice. This adjuvant was compared to the traditional adjuvant aluminum hydroxide.

Materials and Methods: The potential in generating humoral response was evaluated by enzyme-linked immunosorbent assay (ELISA). Individual serum was collected and immunoglobulin G (IgG), IgG1, IgG2a, and IgG2b were quantified. Analyses were carried out 15 and 60 days after immunization. Antibodies avidity index were also analyzed by ELISA. Immunoblot and dot-ELISA were carried out to evaluate specific reaction for homologous strains and cross-reactive antigens present in other meningococcal strains isolated in 2011–2012 year, in Brazil. Delayed type hypersensitivity was used as indicative of cellular immunity and compared between two experimental groups, 24 hours after homologous strain challenge.

Results: The OMVs of *Neisseria meningitidis*, and *N. lactamica* (related species) were characterized by electrophoretic separation of proteins in 13% polyacrylamide gel. The strains presented antigens in the range of 8 to 130 kDa, showing a heterogeneous protein migration pattern. In the group immunized with OMVs/DDA-BF, we found no significant production of total IgG 15 days after the first immunization. On the other hand, 60 days after first immunization both adjuvants act benefiting total IgG production similarly. The antibodies of the IgG isotype produced by animals immunized after one or two doses after first immunization, showed intermediate and high avidity, independent on the adjuvant used. In both experimental groups the swelling of the footpads was significantly higher than those of the controls, suggesting that only one dose was enough to stimulate the generation of cellular immunity.

Conclusion: The use of this cationic adjuvant for *N. meningitidis* OMVs preparation revealed good potential for future new antigen preparation for *N. meningitidis* vaccine.

Keywords: Neisseria meningitidis, Dimethyldioctadecylammonium, Bacterial outer membrane, Immunity, Animals, Outbred strains

Introduction

Most licensed vaccines for protection against diseases caused by serogroup B are derived from outer membrane vesicle (OMV) and employ the aluminum hydroxide as adjuvant [1,2]. Although these vaccines are capable of generating protective antibod-

ies in sera from vaccinated people, the main limitation of its use is regarding the specificity of their strains of which. The basis for the immunogenicity of antigenic preparations is focused on PorA, an outer membrane protein, which has variable regions, responsible for the great variability in strains of serogroup B [3]. However, technological advances in the search for new vaccine antigens rely on reverse vaccinology, which in turn, relies on genomic in-silico studies [4].

The reverse vaccinology has also been used in the development of a vaccine against the meningococcal B. The vaccine covers five components identified vaccine 4MenB, whose name is based on its composition of four components [5].

Since the development of first-generation vaccines based on OMVs, which were able to contain epidemics of specific strain but were not suitable for universal use, huge advances in prevention of *Neisseria meningitidis* B were made. The first vaccine multicomponent, Bexsero, is authorized for use; other vaccines bivalent vaccines and rLP2086, OMVs of next generation, are under development [6]. The new vaccines may contribute substantially to reduce invasive bacterial infections, as they could cover most strains of *N. meningitidis* B. Moreover, other candidates potentially effective serogroup B vaccine are being studied in preclinical settings.

Adjuvants are molecules or macromolecular complexes that stimulate the immune response against different antigens [7]. A good adjuvant should not promote high toxicity; and at the same time, must increase, sustain, and direct the immunogenicity of antigens by modulating the immune response in an appropriate manner. It must also reduce the amount of antigen or the number of immunizations required for a good immune response, confer immunity for all age groups, and in immunosuppressed patients, which is essential to characterize a molecule as well adjuvant [8].

Dioctadecyldimethylammonium (DDA) can stimulate humoral and cellular immune responses against antigens of different natures and complexities. The complexity of antigens to the DDA has shown to increase immune response against microorganisms, viruses, proteins, hapten-conjugated proteins, synthetic antigens, and so forth [9-13].

The bilayer fragments of DDA (DDA-BF) displays good colloidal stability when complexed with antigens, with complete absence of toxicity in mice and a remarkable induction of T helper type 1 (Th1) type immune response observed in reduced doses of lipid [14]. DDA-BF was studied with different antigens preparations, such as, bovine albumin, purified 18 kDa/14 kDa *Taenia crassiceps* antigens [15,16] and also been investigated in pharmaceutical formulations [14,17].

The number of adjuvants able to induce delayed type hypersensitivity (DTH) reactions is limited [18]. DDA is effective to induce DTH surveyed in many animals in the laboratory, as seen in response to various antigens including *Brucella abortus* [18,19], bovine serum albumin [19], the forest of Semliki forest virus [20], and tetanus toxoid [21,22]. It worth mentioning that DDA-BF have also shown to be efficient in generating DTH, when associated to antigens (Gaspar, personal communication).

Recently our group showed that OMVs of *N. lactamica* were effective in generating cross-reactive immunoglobulin (Ig)G antibodies to *N. meningitidis* B in different studies. Interestingly, DDA-BF was superior to aluminum hydroxide as adjuvant for subcutaneous immunization with OMVs, both with measures of humoral and cellular immunity. In this study, we extend our research analyzing the cellular and humoral immune response promoted by immunization of female individual immune response in mice immunized with OMVs of *N. meningitidis* B associated with DDA-BF or aluminum hydroxide as adjuvant [16,23-25].

A similar approach has been studied previously with *N. lactamica* instead of *N. meningitidis* [16]. The investigation of *N. meningitidis* immunogenic proteins and its association with novel adjuvants to establish efficient vaccines against *N. meningitidis* are worthy of investigation. Thereby, the aim of this study was to evaluate immunological responses with a novel cationic lipid nano-adjuvant based on DDA-BF in Swiss adult mice.

Material and Methods

Strains of bacteria

The strains of *N. meningitidis* B:4:P.1.9, B:4.P1.15, and *Neisseria lactamica* were obtained by the core facility of Bacteriology Department of the Instituto Adolfo Lutz. OMVs of B:4:P.1.9, B:4.P1.15, and *N. lactamica* strains were used in experiments to test the cross reactivity of antibodies [16]. Whole cells of *N. meningitidis* used in dot-enzyme-linked immunosorbent assay (ELISA) were obtained [26].

Antigen preparation

N. meningitidis, OMVs were used as antigen and obtained under agitation at 45°C in 0.1 M sodium acetate buffer (pH 5.8), containing lithium chloride 0.2 M Sigma, in the presence of glass beads to stimulate OMVs release, as previously de-

scribed [27]. After extraction, we performed the necessary processes to reduce lipopolysaccharide (LPS) in the antigenic preparations, essential for inoculation in mice. The OMVs used to immunize animals were detoxified through polymyxin B column (Detoxi gel endotoxin removing; Pierce, Rockford, IL, USA) [16]. The limulus test was used as described by the manufacturer (Thermo Scientific Pierce LAL; Thermo Fisher Scientific, Waltham, MA, USA).

The dosage of proteins from the outer membrane vesicles of *N. meningitidis*

Protein concentration was determined by the Bio-Rad protein microassay (Bio-Rad, Hercules, CA, USA) based on the method of Bradford [28] in 1976.

Characterization of antigens of *N. meningitidis* by sodium dodecyl sulfate–polyacrylamide gel electrophoresis

The OMVs protein antigens were characterized by electrophoresis in 13% polyacrylamide gel in the presence of sodium dodecyl sulfate, following protocol established by Laemmli [29] in 1970 and as described in [30].

Preparation of lipid dispersions

A stock solution of DDA bilayer fragments (DDA-BF) was obtained by sonication with a titanium macrotip in 1 mM NaCl prepared with Milli-Q water at a final concentration of 2 mM DDA-BF [16,31].

Preparation of the complex DDA-BF/OMVs

To study the interaction between OMVs and DDA-BF the complexes were always prepared in 1 mM NaCl. The concentration of DDA-BF was set at 0.1 mM and the stock solution of OMVs (1,345 μg of protein/mL) was diluted to obtain OMVs/DDA-BF complexes with a range of protein concentrations: 5, 10, 25, 50, 100, and 200 μg/mL (Table 1). This procedure aims to choose the highest protein concentration, when adsorbed to the DDA-BF, produces a colloidal dispersion with positive final charge and average size of particles less than 500 nm. We used 1 μg in each immunization dose of OMVs with DDA-BF.

Table 1. Physical properties of DDA, OMVs of *Neisseria meningitidis* B, and DDA-BF/OMVs interactions

Sample	DDA (mM)	OMVs (μg/mL)	Average diameter (nm)	Polydispersity index	Zeta potential (ζ)
1	1	-	204.4±22.3	0.342±0.01	28.17±15.64
2	0.1	5	349.1±8.9	0.263±0.02	49.51±0.34
3	0.1	10	465.7±11.8	0.263±0.017	106.56±28.70
4	0.1	25	3,010.6±181.4	0.474±0.02	51.89±19.66
5	0.1	50	1,462.4±89.0	0.397±0.029	-24.6±15.01
6	0.1	100	259.5±3.1	0.267±0.017	-136.2±13.38
7	-	50	334.3±10.0	0.306±0.009	-29.53±0.5

Each size, zeta-potential, and polydispersity index value were the mean value obtained for at least 20 independent measurements for each sample and is presented as the mean value±standard deviation.
DDA, dioctadecyldimethylammonium; OMV, outer membrane vesicle; DDA-BF, bilayer fragments of dioctadecyldimethylammonium bromide.

Animals

Female Swiss mice (6 to 8 weeks old) were created and maintained in Adolfo Lutz Institute (IAL) facilities in São Paulo. All procedures with the animals were in accordance with the guidelines of the Brazilian Code for the Use of Laboratory Animals (CEUA-IAL/Pasteur no., 06/2012). The mice were separated by groups and received the antigenic preparations by subcutaneous injection, in the abdomen, in two sites. Ten mice were assessed in each experimental group. The animals (n=3) used for DTH experiments were removed from the experiment since the antigen injected in the footpad act as a booster dose.

Experimental design and delayed type hypersensitivity determination

The animals were immunized as described in Table 2. The DDA-BF and $Al(OH)_3$ were compared as adjuvant when *N. meningitidis* B:4:P.1.9 OMVs were used as antigen. The controls included OMVs, DDA-BF, and $Al(OH)_3$. The animals were immunized twice with 15-day interval. Fourteen days after the first immunization, two animals were challenged with 50 μL of 10 μg/mL of homologous *N. meningitidis* OMVs in the left hind footpad to measure the DTH. Footpad swelling was measured 24 hours with a caliper, and the percentage of swelling was calculated using the contralateral (not injected) footpad

Table 2. Immunization schedule

Group	No. of animals	OMV (μg/mL)	DDA-BF (mM)	$Al(OH)_3$ (mg/mL)
G1	15	10	0.1	-
G2	15	10	-	0.1
G3	5	10	-	-
G4	8	-	0.1	-
G5	9	-	-	0.1

OMVs, *Neisseria meningitidis* outer membrane vesicles; DDA-BF, bilayer fragments of dioctadecyldimethylammonium bromide; $Al(OH)_3$, aluminum hydroxide.

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Table 3. Analysis of *Neisseria meningitidis* strains isolated of different states in Brazil used by dot-enzyme-linked immunosorbent assay with sera of mice immunized with DDA-BF or alum as adjuvants (2011)

Strain no. (IAL)	Year	City	State	Strains	Serogroups	Serotypes	Subtypes
N.02	2011	SG	SP	*N. meningitidis*	C	23	P1.14-6
N.03	2011	SG	SP	*N. meningitidis*	C	23	P1.14-6
N.05	2011	LCR	SP	*N. meningitidis*	C	23	P1.14-6
N.06	2011	SG	SP	*N. meningitidis*	C	23	P1.14-6
N.08	2011	SG	SP	*N. meningitidis*	B	4, 7	P1.19,15
N.15	2011	LCR	SP	*N. meningitidis*	Y	19,10	P1.9
N.18	2011	LCR	BA	*N. meningitidis*	B	4, 7	P1.22-1,16
N.22	2011	LCR	SP	*N. meningitidis*	C	23	P1.19
N.26	2011	SR	DF	*N. meningitidis*	B	4, 7	P1.19,15
N.30	2011	LCR	BA	*N. meningitidis*	C	2a	P1.5
N.32	2011	LCR	BA	*N. meningitidis*	C	2a	P1.5
N.33	2011	LCR	PE	*N. meningitidis*	C	23	P1.14-6
N.35	2011	SR	PE	*N. meningitidis*	C	23	P1.14-6
N.36	2011	LCR	PE	*N. meningitidis*	C	2b	P1.14-6
N.37	2011	SR	BA	*N. meningitidis*	C	23	P1.14-6
N.39	2011	LCR	BA	*N. meningitidis*	C	23	P1.14-6
N.41	2011	LCR	SP	*N. meningitidis*	B	19,7	P1.14
N.56	2011	LCR	SP	*N. meningitidis*	B	4,7	P1.19
N.60	2011	LCR	RN	*N. meningitidis*	B	4,7	P1.7,1
N.61	2011	LCR	RN	*N. meningitidis*	B	4,7	P1.7,1
N.63	2011	LCR	SP	*N. meningitidis*	C	23	P1.5
N.67	2011	LCR	SP	*N. meningitidis*	C	21	NT
N.68	2011	LCR	AC	*N. meningitidis*	B	4,7	P1.19,15
N.70	2011	LCR	SP	*N. meningitidis*	B	4,7	P1.14-6
N.74	2011	LCR	RS	*N. meningitidis*	W135	2a	P1.5,2
N.75	2011	LCR	RS	*N. meningitidis*	B	15	P1.16
N.77	2011	LCR	SP	*N. meningitidis*	B	19,1	P1.5,2
N.82	2011	SG	SP	*N. meningitidis*	B	4,7	P1.19,15
N.85	2011	LCR	PR	*N. meningitidis*	B	4,7	P1.19,15
N.95	2011	LCR	MA	*N. meningitidis*	B	4,7	P1.19,15
N.101	2011	LCR	SP	*N. meningitidis*	W135	2b	P1.5,2
N.149	2011	LCR	SP	*N. meningitidis*	C	4,7	P1.19,15
N.155	2011	LCR	SP	*N. meningitidis*	C	2b	NT
N.180	2011	LCR	SP	*N. meningitidis*	Y	22	P1.3
N.184	2011	SG	PR	*N. meningitidis*	B	23	P1.14-6
N.186	2011	LCR	AM	*N. meningitidis*	B	4,7	P1.19,15
N.187	2011	LCR	SP	*N. meningitidis*	B	4,7	P1.9
N.188	2011	LCR	SC	*N. meningitidis*	B	15	P1.16
N.189	2011	LCR	SC	*N. meningitidis*	B	19	P1.22-1,14
N.193	2011	SG	SP	*N. meningitidis*	B	4,7	P1.19,15
N.205	2011	LCR	PE	*N. meningitidis*	Y	17,7	P1.5
N.210	2011	SG	BA	*N. meningitidis*	W135	2b	P1.5,2
N.219	2011	LCR	GO	*N. meningitidis*	C	2a	P1.5
N.220	2011	LCR	GO	*N. meningitidis*	C	4,7	P1.19,15
N.225	2011	LCR	GO	*N. meningitidis*	W135	2a	P1.5
N.267	2011	SG	ES	*N. meningitidis*	Y	22	P1.3

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Table 3. Continued

Strain no. (IAL)	Year	City	State	Strains	Serogroups	Serotypes	Subtypes
N.357	2011	LCR	SP	*N. meningitidis*	Y	19	P1.5
N.458	2011	LCR	BA	*N. meningitidis*	W135	2b	P1.2
N.472	2011	LCR	SP	*N. meningitidis*	C	2a	P1.5
N.552	2011	LCR	SP	*N. meningitidis*	C	2a	P1.5
N.553	2011	SG	SP	*N. meningitidis*	C	2a	P1.5
N.554	2011	LCR	SP	*N. meningitidis*	C	2b	P1.14-6
N.556	2011	LCR	SP	*N. meningitidis*	C	23	P1.14-6
N.601	2011	LCR	BA	*N. meningitidis*	C	23	P1.14-6
N.604	2011	SG	SP	*N. meningitidis*	C	23	P1.14-6
N.606	2011	LCR	PR	*N. meningitidis*	C	23	P1.3
N.609	2011	LCR	SP	*N. meningitidis*	C	23	P1.14-6
N.611	2011	LCR	SP	*N. meningitidis*	C	23	P1.14-6
N.617	2011	LCR	SP	*N. meningitidis*	C	23	P1.14-6
N.669	2011	LCR	SP	*N. meningitidis*	C	2a	P1.14-6

DDA-BF, bilayer fragments of dioctadecyldimethylammonium bromide; IAL, Instituto Adolfo Lutz; NT, not tested.

as control. The results were expressed as the mean±standard error (SE). Since this challenge can act as an antigen boost, after DTH measurement, the animals were removed from the experimental group. Therefore, only the remaining animals received the second immunization dose. The animals were bled 15 and 60 days after first immunization dose (45 days after second immunization dose) for humoral immunity evaluations [16].

Immunoblotting

The immunoblotting was performed as described by Towbin et al. [32] in 1979 with some modifications. This technique was used to demonstrate the reactivity of sera of animals immunized with *N. meningitidis* OMVs, comparing two different adjuvants, DDA-BF and $Al(OH)_3$, against homologous strain (B:4:P.1.9), to demonstrate specificity, against heterologous strain (B:4.P1.15) and against the correlated specie *N. lactamica*, to demonstrate cross reactivity.

For immunoblot, sera were incubated with the membranes at a dilution of 1/500 for 1 hour. Secondary antibodies used was IgG (anti-mouse IgG–peroxidase-conjugated; Sigma Chemical Company, St. Louis, MO, USA) at a concentration of 1/1,000 for 2 hours. The membranes were developed with 670 μL of 0.4% AEC (3-amino-9-ethylcarbazole) in 10 mL of sodium acetate pH 5.0 and 10 μL of hydrogen peroxide (H_2O_2). The enzyme reaction was interrupted with MiliQ water after 20 minutes. The strips were dried and digitalized for evaluation of proteins recognized by sera tested.

Enzyme linked immunosorbent assay

ELISA technique was performed to compare humoral immunity induced by immunization of mice with *N. Meningitidis* OMVs, using two different adjuvants, DDA-BF and $Al(OH)_3$. Serum from five animals per group were collected 15 and 60 days after first immunization for determination of total IgG levels and 60 days after first immunization for determination of IgG1, IgG2a, and IgG2b [16]. The serum was tested individually and the results were expressed as the mean±SE. Statistical analysis was performed only for dilution 1/100.

Avidity index determination

The determination of avidity index (AI) of IgG antibodies produced against the *N. meningitidis* OMVs/DDA-BF or OMVs/$Al(OH)_3$ was conducted with serum collected 60 days after the first immunization. The methodology used was described as phosphate-buffered saline of KSCN [33-35]. The results were interpreted as follows: AI above 0.5, high avidity; AI between 0.3 and 0.49, intermediate avidity; and AI below 0.29, low avidity.

Dot-ELISA

This technique was used to evaluate the cross-reactivity of antibodies produced against *N. meningitidis* B:4:P.1.9 OMVs, with meningococcal strains isolated from patients in 2011 and 2012. For realization of the dot-ELISA, 1 μL whole cell bacteria preparation of each prevalent *N. meningitidis* strain, isolated from patients, was applied in a nitrocellulose membrane of 0.2 μm (Bio-Rad) [26]. Three membranes were con-

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Table 4. Analysis of *Neisseria meningitidis* strains isolated of different states in Brazil used by dot-enzyme-linked immunosorbent assay with sera of mice immunized with DDA-BF or alum as adjuvants (2012)

Strain no. (IAL)	Year	City	State	Strains	Serogroups	Serotypes	Subtypes
N.01	2012	SG	SP	*N. meningitidis*	Y	22	P1.3
N.04	2012	LCR	SP	*N. meningitidis*	B	19,1	NT
N.08	2012	LCR	PR	*N. meningitidis*	C		P1.14-6
N.10	2012	LCR	SP	*N. meningitidis*	Y	19	NT
N.11	2012	SG	SP	*N. meningitidis*	C	23	P1.14-6
N.12	2012	SG	SP	*N. meningitidis*	C	23	P1.14-6
N.14	2012	SG	SP	*N. meningitidis*	C	23	P1.14-6
N.15	2012	LCR	RS	*N. meningitidis*	W135	NT	P1.5,2
N.16	2012	LCR	RS	*N. meningitidis*	B	19,7	NT
N.17	2012	SG	SP	*N. meningitidis*	B	19,1	NT
N.19	2012	SG	PR	*N. meningitidis*	B	4,7	P1.19,15
N.20	2012	SG	CE	*N. meningitidis*	C	23	P1.14-6
N.23	2012	SG	CE	*N. meningitidis*	C	23	P1.14-6
N.26	2012	LCR	CE	*N. meningitidis*	B	4,7	P1.19,15
N.29	2012	LCR	SP	*N. meningitidis*	B	19,1	NT
N.30	2012	LCR	SP	*N. meningitidis*	B	4,7	P1.19,15
N.33	2012	LCR	ES	*N. meningitidis*	B	4,1	P1.16
N.34	2012	SG	SP	*N. meningitidis*	B	4,7	P1.19,15
N.35	2012	LCR	SP	*N. meningitidis*	C	4,7	P1.19,15
N.37	2012	LCR	SP	*N. meningitidis*	C	23	P1.14-6
N.39	2012	SG	MG	*N. meningitidis*	C	23	P1.14-6
N.40	2012	SG	MG	*N. meningitidis*	C	23	P1.14-6
N.41	2012	LCR	MG	*N. meningitidis*	C	23	P1.14-6
N.42	2012	LCR	MG	*N. meningitidis*	C	23	P1.14-6
N.43	2012	LCR	MG	*N. meningitidis*	C	23	P1.14-6
N.44	2012	LCR	MG	*N. meningitidis*	C	23	P1.14-6
N.45	2012	LCR	MG	*N. meningitidis*	C	23	P1.14-6
N.47	2012	LCR	MG	*N. meningitidis*	B	4,7	P1.19,15
N.52	2012	LCR	SP	*N. meningitidis*	B	4,7	NT
N.54	2012	LCR	PE	*N. meningitidis*	B	4,7	P1.1
N.62	2012	LCR	DF	*N. meningitidis*	B	23	P1.14-6
N.63	2012	SG	DF	*N. meningitidis*	W135	NT	P1.2
N.64	2012	LCR	DF	*N. meningitidis*	W135	NT	P1.5,2
N.65	2012	LCR	DF	*N. meningitidis*	B	4,7	P1.19,15
N.72	2012	LCR	SP	*N. meningitidis*	C	NT	P1.5
N.76	2012	SG	BA	*N. meningitidis*	C	NT	P1.5
N.85	2012	LCR	SP	*N. meningitidis*	Y	22	P1.3
N.89	2012	LCR	BA	*N. meningitidis*	C	NT	P1.5
N.90	2012	LCR	SP	*N. meningitidis*	C	23	P1.14-6
N.99	2012	SG	BA	*N. meningitidis*	W135	NT	P1.2
N.101	2012	LCR	BA	*N. meningitidis*	B	4,1	P1.9
N.104	2012	SG	SP	*N. meningitidis*	C	NT	P1.5
N.105	2012	SG	SP	*N. meningitidis*	B	19,1	P1.19
N.106	2012	LCR	RN	*N. meningitidis*	B	19,1	NT
N.107	2012	SG	ES	*N. meningitidis*	B	1	NT
N.109	2012	LCR	SP	*N. meningitidis*	C	23	P1.14-6

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Table 4. Continued

Strain no. (IAL)	Year	City	State	Strains	Serogroups	Serotypes	Subtypes
N.110	2012	LCR	BA	*N. meningitidis*	C	23	P1.14-6
N.111	2012	LCR	BA	*N. meningitidis*	C	23	P1.14-6
N.112	2012	LCR	BA	*N. meningitidis*	C	23	P1.14-6
N.115	2012	LCR	SP	*N. meningitidis*	C	23	P1.5
N.116	2012	SG	SP	*N. meningitidis*	C	23	P1.14-6
N.117	2012	LCR	SP	*N. meningitidis*	C	23	P1.14-6
N.118	2012	LCR	SP	*N. meningitidis*	C	23	P1.14-6
N.119	2012	SG	SP	*N. meningitidis*	C	23	P1.14-6
N.120	2012	LCR	SP	*N. meningitidis*	C	23	P1.14-6
N.123	2012	LCR	SP	*N. meningitidis*	W135	NT	P1.5,2
N.125	2012	LCR	SP	*N. meningitidis*	Y	22	P1.3
N.126	2012	LCR	PE	*N. meningitidis*	B	4,7	P1.1
N.134	2012	LCR	RN	*N. meningitidis*	B	19	NT
N.220	2012	SG	SP	*N. meningitidis*	Y	NT	NT

DDA-BF, bilayer fragments of dioctadecyldimethylammonium bromide; IAL, Instituto Adolfo Lutz; NT, not tested.

structed with the same sequence of strains described in Tables 3 and 4. The membranes were incubated with anti-mouse IgG (anti-mouse IgG–peroxidase-conjugated; Sigma Chemical Company) and developed as in immunoblotting.

Ethics statement

The study protocol was approved by the institutional review board of Adolfo Lutz Institute (no., CTC 41/2011). The animal studies were performed after receiving approval of the Institutional Animal Care and Use Committee by CEUA/IAL (no., 03/2012).

Statistical analysis

The significance of the results was assessed by analysis of variance with post-test method of Tukey. Values of $p \leq 0.05$ were considered significant.

Results

Characterization of *N. meningitidis* OMVs antigens

The OMVs of *N. meningitidis* B:4:P1.9 (used in immunization, referred in this paper as homologous strain), B:4:1.15 (heterologous strain), and *N. lactamica* (related species) were characterized by electrophoretic separation of proteins in 13% polyacrylamide gel to characterization. The strains presented antigens in the range of 8 to 130 kDa, showing a heterogeneous protein migration pattern (Fig. 1).

To compare DDA-BF and OMV/$Al(OH)_3$, used as adjuvant with *N. meningitidis* OMVs antigen, the animals were immunized twice with 15-day interval. We performed evaluations 15 days (only one immunization dose) and/or 60 days (two immunization doses) after first immunization. Sera of each animal were tested in triplicate and results represent mean±SE.

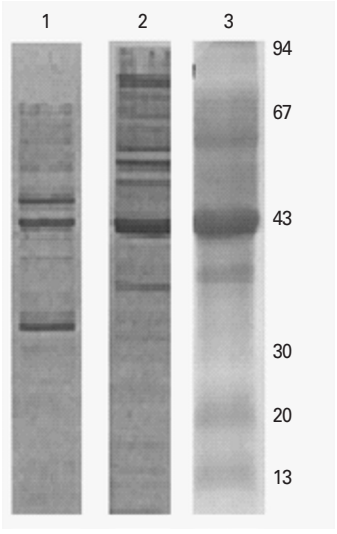

Fig. 1. Electrophoretic protein migration profile of the outer membrane vesicles of *Neisseria meningitidis* B:4:P1.9 (lane 1), *N. meningitidis* B:4:1.15 (lane 2), and *N. lactamica* (lane 3) by sodium dodecyl sulfate–polyacrylamide gel electrophoresis stained with Coomassie brilliant blue.

Determination of mean diameter, size distribution, polydispersity, and zeta potential (ζ) of DDA-BF and its association with OMVs of *N. meningitidis*

The distribution of sizes, medium diameter, polydispersity,

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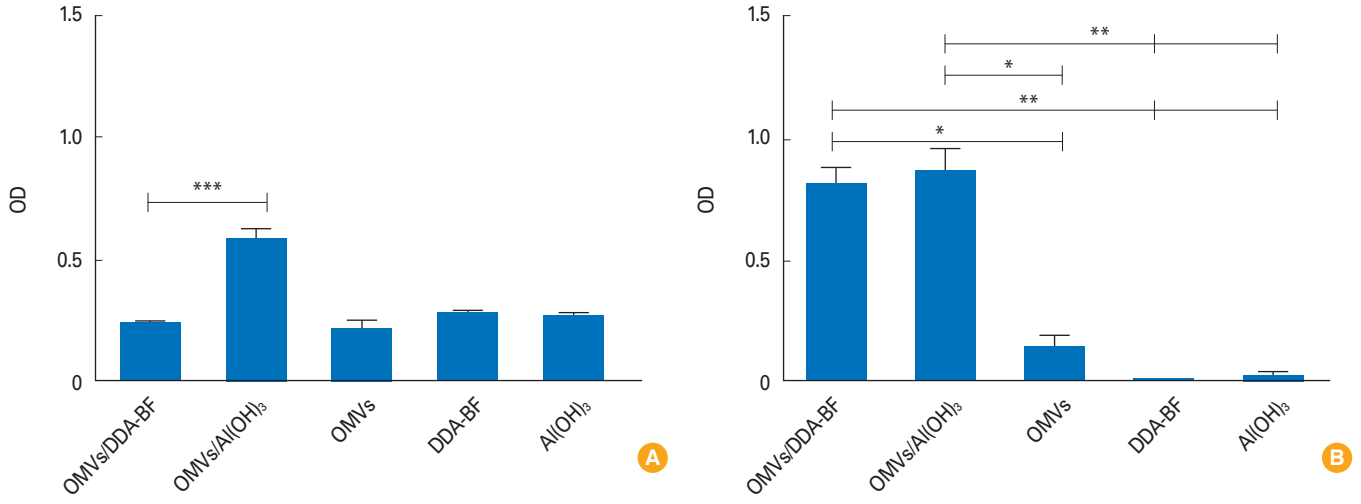

Fig. 2. (A) Total immunoglobulin G (IgG) levels in serum of mice immunized with one dose of antigen/adjuvant, 15 days after first immunization (A) or two doses of antigen/adjuvant, 60 days after the first immunization (B). Total IgG level was determined by enzyme-linked immunosorbent assay (ELISA). The plates were adsorbed with antigens of homologous strain (*Neisseria meningitidis* B:4:P1.9). The results represent the serum diluted 1:100. Bars represent the standard error. *p<0.05, **p<0.01, and ***p< 0.001 in relation to controls. Sera were analyzed individually, in triplicate. (B) IgG1, IgG2a, and IgG2b levels in serum of mice immunized with two doses of antigen/adjuvant, 60 days after the first immunization. Ig levels were determined by ELISA. The plates were adsorbed with antigens of homologous strain (*N. meningitidis* B:4:P1.9). The results represent the serum diluted 1:100. Bars represent the standard error. *p<0.05 and ***p<0.001 in relation to controls. Five mice in each group were analyzed. OMV, outer membrane vesicle; DDA-BF, bilayer fragments of dioctadecyldimethylammonium bromide; $Al(OH)_3$, aluminum hydroxide; OD, optical density.

and the zeta (ζ) potential of DDA-BF, OMVs, and their association were determined using the Zeta Potential Analyzer Zeta-Plus (Brookhaven Instruments Corp., Holtsville, NY, USA), equipped with a laser of 677 nm and dynamic light-scattering (photon correlation spectroscopy) at a 90° angle to the measurement of sizes for OMVs of *N. meningitidis* [16].

Humoral immunity induced by immunization of mice with *N. meningitidis* OMVs/DDA-BF in comparison with *N. meningitidis* OMVs/$Al(OH)_3$

Antibody production was evaluated by ELISA performed with homologous strain. IgG was measured 15 and 60 days after first immunization (Fig. 2). IgG1, IgG2a, and IgG2b were measured only 60 days after first immunization (Fig. 3). The results represent the serum diluted 1:100.

In the group immunized with OMVs/DDA-BF we found no significant production of total IgG 15 days after the first immunization. The total IgG level produced 15 days after immunization with OMVs/$Al(OH)_3$ was significantly higher than in animals immunized with OMVs/DDA-BF (Fig. 2A). On the other hand, 60 days after first immunization both adjuvants act benefiting total IgG production similarly (Fig. 2B).

We also analyzed antibody subtypes IgG1, IgG2a, and IgG2b production in the animals immunized twice 60 days after first immunization. When used as adjuvant of *N. meningitidis* OMVs, $Al(OH)_3$ was superior to DDA-BF in stimulating IgG1 production (Fig. 3A, B). The IgG2a and IgG2b production not influenced by the adjuvant used since there was no significant difference between IgG2a and IgG2b production in animals immunized with OMVs/DDA-BF and OMVs/$Al(OH)_3$ (Fig. 3B, C)

Avidity index

Since the quantification of antibody by ELISA can offers no information about the functionality of antibodies present in the serum, a modified ELISA assay, using the chaotropic substance, potassium thiocyanate (KSCN) was performed to determine IgG avidity [33,34]. Depending on the ratio between the optical density in presence/absence of KSCN, it is possible to classify the avidity in low (<0.29), intermediate (0.3 to 0.49), or high [35].

The antibodies of the IgG isotype produced by animals immunized after one or two doses after first immunization, showed intermediate and high avidity, independent on the adjuvant used in the antigenic preparations. The test was performed in both, pool of sera of each experimental group or serum from individual mouse immunized with *N. meningitidis* OMVs in association with the adjuvants DDA-BF or $Al(OH)_3$. The pool

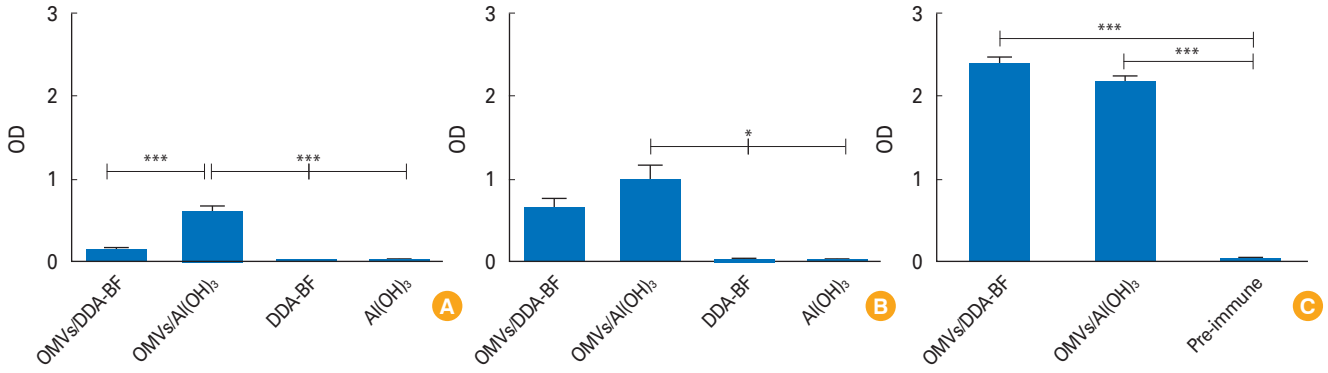

Fig. 3. IgG1 (A), IgG2a (B), and IgG2b (C) levels in serum of mice immunized with two doses of antigen/adjuvant, 60 days after the first immunization. IgG levels were determined by ELISA. The plates were adsorbed with outer membrane vesicles (OMVs) of homologous strain (*Neisseria meningitidis* B:4:P.1.9). The results represent the mean±standard deviation of optical density (OD492 nm) of serum diluted 1:100. Bars represent the standard error. IgG, immunoglobulin G; DDA-BF, bilayer fragments of dioctadecyldimethylammonium bromide; Al(OH)3, aluminum hydroxide. *p<0.05; ***p<0.001 in relation to controls. Sera were analyzed individually, in triplicate.

of sera of animals immunized with OMVs/DDA-BF presented intermediate avidity, while the pool of sera of animals immunized with OMVs/Al(OH)$_3$ presented high avidity. In individual mouse serum, both experimental groups presented AI intermediate to high.

Cellular immunity induced by immunization of mice with *N. meningitidis* OMVs/DDA-BF in comparison with *N. meningitidis* OMVs/Al(OH)$_3$

The DTH is an important parameter for assessing the cellular immunity induced by effective immunization [16]. Five animals from each experimental group as well as control animals were separated and challenged on the left hind footpad 14 days after immunization with *N. meningitidis* OMVs obtained from a homologous strain (B:4:P1.9). These animals received only one immunization dose. The footpad swelling, an indicative of cellular immunity, was measured 24 hours later with a caliper. In both experimental groups, the swelling of the footpads was significantly higher than those of the controls, suggesting that only one dose was enough to stimulate the generation of cellular immunity. In the control groups, the small swelling that occurred was probably a consequence of nonspecific inflammatory response caused by injection, which causes physical damage in local tissue (Fig. 4).

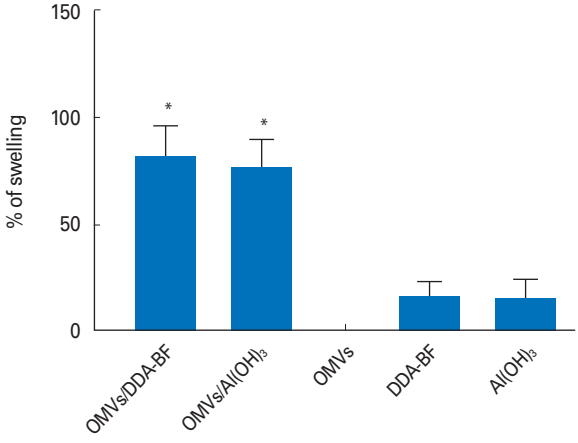

Fig. 4. Delayed type hypersensitivity reaction. Fourteen days after the first immunization, the animals were challenged, on the left hind footpad, with 0.5 μg outer membrane vesicles (OMVs) of *Neisseria meningitidis* strain B:4:P1.9. The diameter of the footpads was measured 24 hours after challenge and expressed as percentage of swelling over the contralateral footpad. The percentage of swelling of the footpads of the animals immunized, independent on adjuvant used, was significantly higher (*p<0.05) than the swelling of the footpads from controls animals. Five mice in each group were analyzed. DDA-BF, bilayer fragments of dioctadecyldimethylammonium bromide; Al(OH)$_3$, aluminum hydroxide.

Specificity and cross reactivity of IgG antibodies generated after immunization with *N. meningitidis* OMVs associated to the adjuvants DDA-BF or Al(OH)$_3$

The specificity of the total IgG antibodies produced after immunization with *N. meningitidis* OMVs associated to the adjuvants DDA-BF and Al(OH)$_3$ was tested by immunoblotting. The membranes were plotted with material from electrophoretic separation, of homologous strain OMVs, from *N. meningitidis* B:4:P.1.9. Serum was collected 15 or 60 days after first immunization (Fig. 5A, B). The serum collected 15 days after immunization represents animals immunized with a single dose, while the serum collected at day 60th represent animals

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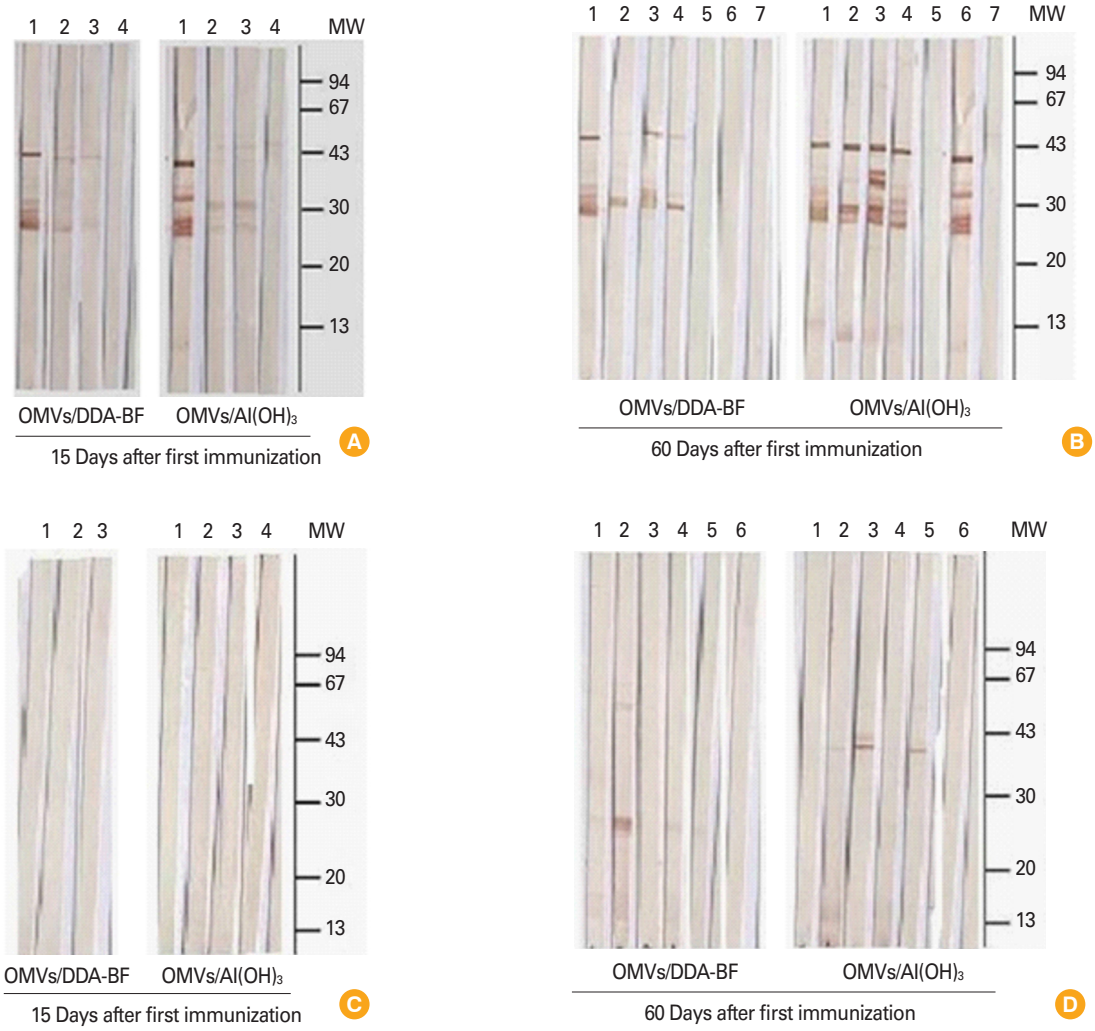

Fig. 5. Specificity and cross-reactivity of total immunoglobulin G (IgG) antibodies generated after immunization with *Neisseria meningitidis* by immunoblot. Outer membrane vesicles (OMVs) associated to the adjuvants DDA-BF or Al(OH)3. The serum was collected 15 (A, C) or 60 (B, D) days after first immunization. The serum collected on day 60th represent animals immunized twice, in an interval of 15 days. The nitrocellulose membrane was blotted with proteins from electrophoretic separation of homologous strain OMVs, from *N. meningitidis* B:4:P.1.9 (A, B) or heterologous strain from *N. meningitidis* B:4.P1.15 (C, D). (A, C) Lane 1: pooled serum; lanes 2 and 3: individual mouse serum; lane 4: serum from control animals that received only DDA-BF or Al(OH)3. (B) Lane 1: pooled serum; lanes 2 to 6: individual mouse serum; lane 7: serum from control animals that received only DDA-BF or Al(OH)3. (D) Lane 1: pooled serum; lanes 2 to 5: individual mouse serum; lane 6: serum from control animals that received only DDA-BF or Al(OH)3. Five mice in each group were analyzed. DDA-BF, bilayer fragments of dioctadecyldimethylammonium bromide; Al(OH)3, aluminum hydroxide; MW, molecular weight.

immunized twice with 15-day interval. Sera pooled (sera for all animals together; Fig. 5A, B line 1) or from individual mouse (Fig. 5A lines 2 and 3 and Fig. 5B lines 2–6) were tested. Sera from animals receiving only DDA-BF or $Al(OH)_3$ (Fig. 5A lines 4 and Fig. 5B lines 7) were used as controls.

The recognition of antigens was homogeneous when analyzing the pool of sera in animals immunized with OMVs associated to both adjuvants and independent on receiving one or two immunization dose (Fig. 5A, B), except for an additional band strongly marked by pooled serum from animals immunized once with OMVs/$Al(OH)_3$. In addition, the bands recognized by the sera pool also appear more strongly marked that the recognized by serum from individual animals. In antigens detected by pooled sera it is possible to notice the appearance of additional bands (Fig. 5A, B), in comparison to individual sera, except for sera from animals immunized with OMVs/$Al(OH)_3$, collected 60 days after first immunization.

Although it seems that the recognized antigens are similar, independent on the adjuvant used, the number of immunization and time after first immunization, in animals immu-

nized with OMVs/Al(OH)$_3$, the antigens were more heavily recognized by serum from 60 days after first immunization. Besides, serum from two animals immunized twice with OMVs/DDA-BF (Fig. 4B lines 5 and 6) and one animal immunized twice with OMVs/Al(OH)$_3$ (Fig. 4B lines 5) did not recognize any antigen.

The recognition of peptide by all serum was focused on proteins/peptides with molecular weight ranging from 20 to 67 kDa. High molecular weight antigens were not recognized. In animals immunized with OMVs/Al(OH)$_3$ appear some additional bands, both in individual sera and in pools, independent on receiving one or two immunization doses (15 or 60 days, respectively). The pool of sera recognizes antigens similarly to serum from individual animals, but those were more heavily marked, besides being possible the observation of additional bands. Controls sera did not recognize any peptide, as expected.

It is possible to note a heterogeneity of individual response of animals immunized twice, independent on adjuvant used (Fig. 4B). The antigens were more strongly recognized by antibodies produced by immunization with OMVs/Al(OH)$_3$.

In addition to specificity test, immunoblotting was also used to evaluate cross reactivity of the total IgG antibodies against the heterologous strain. The nitrocellulose membranes were plotted with material from electrophoretic separation of whole cell extract from *N. meningitidis* B:4.P1.15 and tested against serum from animals immunized with *N. meningitidis* OMVs associated to the adjuvants DDA-BF and Al(OH)$_3$. Serum was collected 15 or 60 days after first immunization (Fig. 5B, C). The serum collected 15 days after immunization represents animals immunized with a single dose, while the serum collected at day 60th represent animals immunized twice with 15-day interval.

It was not possible to note any IgG cross reactivity with *N. meningitidis* heterologous strain 15 days after immunization in animals immunized once with *N. meningitidis* OMVs independent on the adjuvant used (Fig. 5C).

On the other hand, 60 days after immunization, in animals that were immunized twice, we noted total IgG cross-reactive to the heterologous strain, *N. meningitidis* B:4.P1.15. However, that recognition did not occur for sera of all animals (data not shown). Although this cross-reactive recognition was heterogeneous and weak, it is possible to speculate that both adjuvants are able to benefit the production of cross-reactive IgG induced by the antigen.

Since cross-protection between non homologous strains is one of the most important limiting factor in production of vaccines against group B *N. meningitidis*, sera from animals immunized twice with OMVs/DDA-BF or OMVs/Al(OH)$_3$ where collected (day 60th), pooled, and tested against strains isolated from patients with diabetes mellitus in 2011–2012 to search total IgG cross reactivity by dot-ELISA (Fig. 6). Serum from animals injected twice with the adjuvants alone were also collected 60 days after first injection, pooled, and used as control (Fig. 6). Since each membrane was incubated with a different pool of serum, all strains were plotted in the membranes in the same order.

Although it is possible to note a small degree of unspecific reaction in membrane developed after control serum interaction, this reaction was weak and restricted to some strains (Fig. 6). On the other hand, in the experimental groups (Fig. 6) a greater number of antigens were recognized and the reactions were stronger. It worth mentioning that DDA-BF was superior to Al(OH)$_3$ to benefit the generation of cross reactive IgG promoted by the antigen, since it is possible to note stronger reactions, as well as, greater number of strains recognized

Fig. 6. Cross-reactivity of total immunoglobulin G antibodies generated after immunization with *Neisseria meningitidis* against clinical strains isolated in Brazil during the 2011–2012 period, by dot-blot. Sera were collected 60th days after the first immunization. Animals were immunized with two doses of OMV/DDA-BF (A), OMV/Al(OH)3 (B), or saline solution (C). OMV, outer membrane vesicle; DDA-BF, bilayer fragments of dioctadecyldimethylammonium bromide; Al(OH)3, aluminum hydroxide.

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by serum from animals immunized with OMVs/DDA-BF, in comparison with serum from animals immunized with OMVs/$Al(OH)_3$ (Fig. 6).

Discussion

This study is a comparison of DDA-BF and aluminum hydroxide used as adjuvants in two different meningococcal serogroup B OMV vaccine formulations. The formulations have been evaluated in our laboratory and tested in mice in different ages as well with *N. lactamica* [16,36,37]. The immunogenicity of the vaccine formulations was tested by ELISA, immunoblots, and dot blots for humoral responses and by DTH for cellular responses. Aluminum hydroxide was superior to DDA-BF in generating IgG antibodies and also high avidity IgG against the homologous vaccine strain. The adjuvants induced similar amounts of cellular responses as measured by the DTH reaction. DDA-BF was superior in inducing cross-reactive antibodies to heterologous meningococcal strains isolated from patients [38].

As we know, polysaccharide-based vaccines against *N. meningitidis* serogroups A, C, W135, and X are available for use; however, capsular polysaccharide of the serogroup B are similar to polysaccharide acid, present in human glycoproteins and cannot be used as vaccines because these antigens are little immunogenic and can cause autoimmunity [39].

Therefore, OMVs antigens against serogroup B of *N. meningitidis* have been used since the 1970s. Interventions on public health in Cuba, Norway, and New Zealand have suggested that vaccines consisting of outer membrane proteins present in the OMVs can prevent meningococcal disease caused by serogroup B. Because OMVs represent the components of the outer membrane of Gram-negative bacteria, these vesicles contain a variety of antigens that can be immunogenic and have been shown to be safe when used as vaccines [40]. Currently, the OMVs used in vaccines are extracted with detergent, to decrease the LPS of the native vesicles, and thus become safer for intramuscular use in humans [41].

The present study aimed to evaluate the immunogenicity of antigenic preparations based on OMVs of *N. meningitidis* B associated to cationic lipid DDA-BF used as adjuvant (OMVs/DDA-BF). In addition, DDA-BF effect on improvement of OMVs immunogenicity was compared to the widely used adjuvant, aluminum hydroxide ($Al(OH)_3$). For this purpose, the antigen was characterized, female, non-isogenic mice were immunized subcutaneously, and both humoral and cellular immunity were evaluated. Since one of the main obstacle in achieving a good *N. meningitidis* B vaccine concern the ability of antibodies generated against one strain recognized heterologous strains, the cross reactivity of antibodies generated against *N. meningitidis* B:4:P1.9 was evaluated against the heterologous strain *N. meningitidis* B:4:P1.15, the strains of *N. meningitidis* isolated from Brazilian patients in 2011 and 2012 in dot-ELISA assay.

An electrophoretic separation of proteins (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) was used to demonstrate differences in protein migration pattern in OMVs obtained from *N. meningitidis* B:4:P1.9 (vaccine strain), B:4:P1.15 (heterologous strain), and *N. lactamica* (correlate specie). As expected, the protein migration patter was different for each OMVs since *N. meningitidis* B presents genetic diversity and *N. lactamica* is another specie of Neisseria [12].

Since DDA-BF are small disks, positively charged, the complexation of DDA-BF to OMVs is evidenced by the change in load and in particle size, after association to OMVs, showed in results.

The colloidal stability of the complex is associated with the obtaining of small particles [15]. Since the concentration of DDA-BF was fixed in 0.1 mM, the final charge of the complex depends on both, the nature and the concentration of the adsorbed protein (proteins in OMVs). *N. meningitidis* OMVs were adsorbed to DDA-BF in a range of protein concentrations (5, 10, 25, 50, and 100 μg/mol) (Table 5).

This test allows determining the best concentration of protein to be used as antigen. This must be the largest concentration of protein adsorbed by the DDA-BF in which the final charge remains positive and that there is no particle aggregation in large complexes (above 500 nm), since large colloidal stability depends on obtaining small particles [15]; and it is expected that particles smaller than 500 nm have ideal size for being internalized by dendritic cells, promoting huge immune response [13,42]. For this study, the protein concentration of 10 μg/mL was chosen because allowed the ideal protein concentration (positive charge and small particles).

One of the biggest advantages to use cationic lipid-based adjuvants is the ability of this in adsorb negatively charged antigens, such as the OMVs *N. meningitidis*. The complex formed between the antigen and the DDA-BF occurs by electrostatic attraction due to the negative charge of OMVs, as well as, by hydrophobic effect as the OMVs have phospholipids and lipoproteins [27]. Antigen-adjuvant particles with positive final charge, as in the case of antigen preparation OMVs/

Table 5. Avidity index of total immunoglobulin G present in serum of animals immunized with *Neisseria meningitidis* OMVs associated to two different adjuvants, DDA-BF and $Al(OH)_3$, 60 days after first immunization

Immunization	Avidity index	Avidity classification
OMVs/DDA-BF pool of sera	0.47	Intermediate
OMVs/DDA-BF[a]	0.45	Intermediate
OMVs/DDA-BF[a]	0.4	Intermediate
OMVs/DDA-BF[a]	0.43	Intermediate
OMVs/DDA-BF[a]	0.65	High
OMVs/Al(OH) pool of sera	0.55	High
OMVs/$Al(OH)_3$[a]	0.54	High
OMVs/$Al(OH)_3$[a]	0.63	High
OMVs/$Al(OH)_3$[a]	0.58	High
OMVs/$Al(OH)_3$[a]	0.49	Intermediate

OMV, outer membrane vesicle; DDA-BF, bilayer fragments of dioctadecyldimethylammonium bromide; $Al(OH)_3$, aluminum hydroxide.
[a]Serum collected of individual mouse.

DDA-BF, facilitate the endocytosis by antigen-presenting cells by electrostatic interactions since cell membranes are negatively charged. When used as adjuvant DDA-BF promotes Th1-type response, generating long-lasting antibodies [10].

The animals used in this study are Swiss Webster. These animals were chosen because they represent, in the best way, the human population due to their non-isogenic characteristic. Besides, sera analyses were made individually, being a good parameter to evaluate how specific and heterogeneous can present the immune response against *N. meningitidis* B. The animals were immunized twice with an interval of 15 days and were bled 15 and 60 days after first immunization.

The production of total IgG was evaluated after one and two doses of immunization. After a dose, antibody production was significantly higher in the group of mice immunized with OMVs/$Al(OH)_3$. However, 60 days after the first immunization, when animals had already received two doses of antigen, there were no significant differences in the production of total IgG between groups immunized with OMVs/DDA-BF and OMVs/$Al(OH)_3$.

Since AI of the antibodies has a positive correlation with serum bactericidal activity (SBA), the measurement of the AI can be an additional method to predict a protective immunity during the evaluation of a vaccine against serogroup B [34]. This association is independent on the specific antibody levels and distribution of isotype. Thus, AI was evaluated 60 days after the first immunization, in animals that received two doses of the antigenic preparations. The avidity was tested in pool of sera and also in individual sera. Both adjuvants associated to *N. meningitidis* OMVs promoted the production of high to intermediate avidity antibodies suggesting that the two adjuvants, when complexed to OMVs, were able to induce production of IgG antibodies with good bonding strength to the antigen. As SBA is considered the gold standard for correlate of protection against meningococcal disease [41]. I saw avidity in the antibodies produced, which they point out has been shown to be correlated with SBA with DDA-BF as adjuvant as well as bactericidal activity but the reason for using avidity as an alternative to SBA is used or order to see the force of antibodies binding [35].

IgG subtypes were also evaluated 60 days after the first immunization. When used as adjuvant of *N. meningitidis* OMVs, $Al(OH)_3$ was superior to DDA-BF in stimulating IgG1 production. Although only animals immunized with OMVs/$Al(OH)_3$ presented levels of IgG2a significantly higher than controls, there was no statistical difference between the production of IgG2a among groups immunized with OMVs/$Al(OH)_3$ or OMVs/DDA-BF. The production of IgG2b was similar for both experimental group and significantly higher than control. The cytokines secreted by T cells may differentially promote changes of classes in antibodies produced by B-lymphocytes in mice [43]. Cytokines involved in type T helper type 2 (Th2) response, such as interleukin (IL)-4, promote the change of isotype of IgM and IgD to IgG1 and IgE, while the cytokines involved in Th1 response, such as IL-2 and interferon-γ promote change of class to IgG2a and IgG2b [43-45]. While the OMVs/$Al(OH)_3$ promoted the production of all IgG subtypes, the OMVs/DDA-BF only induced significant production of IgG2b, suggesting a driven of the immune response to a Th1 pattern. This subclass of IgG antibody is opsonizing and can also be related to cellular immunity, which has already been described in earlier work when using DDA-BF as adjuvant in our laboratory [16,36,37].

DTH is a good denotative of cellular immunity. The results suggest that both, DDA-BF and $Al(OH)_3$, when complexed to OMVs were able to generate cellular immunity after a dose of the antigenic preparations. The small swelling observed in control groups, however, can be due to local inflammation caused by injection of a relatively large volume of fluid. The result presented here diverged from our previous result, when we demonstrated that a second immunization dose with the antigen complexed with *N. lactamica* OMVs was necessary to achieve the same DTH generated after one dose of *N. lactamica* OMVs associated to DDA-BF [16].

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The analysis of the recognition of specific antigens with the homologous strain showed that sera from mice immunized with one dose (15 days after the first immunization) presented a relatively homogeneous IgG response independent on adjuvant used. In pool sera it is possible to note an additional band in animals immunized with OMVs/Al(OH)$_3$. The recognition was focused on peptides ranging 20–43 kDa, suggesting that the molecular weights may be related to the five classes of OMPs: class 5 (Opa and Opc, 26 to 30 kDa), class 4 (33–34 kDa); class 2/3 (PorB, 37–42 kDa); and class 1 (PorA, 43 kDa) [27].

After the second dose (totaling 60 days after first immunization), pool sera of animals immunized with OMVs/DDA-BF or OMVs/Al(OH)$_3$ recognized peptides in a similar way, while individual response, was quite heterogeneous. Despite this heterogeneity, the peptides were also in a range of 20–43 kDa, as for animals immunized with one dose. Minor weight antigens (below 13 kDa) were recognized in some individual serum from animals immunized with OMVs/Al(OH)$_3$.

The major proteins of *N. meningitidis* outer membranes and consequently OMVs are PorA and PorB. The PorA is often recognized by bactericidal antibodies present in the serum and have been widely used in studies for meningococcal vaccines. PorB, either, class 2 or 3 proteins, are the major porins constituent present in outer membranes. These proteins are able to insert in the membranes of target cells to facilitate attachment and subsequent invasion by *Neisseria*, and can also affect the phagosome maturation, once internalized. It has been shown that PorB may induce extracellular Ca^{++} influx and induce apoptosis in target cells [5].

Class 4 proteins may also be a target for blocking antibodies. Class 5, Opa and Opc are important in the pathogenesis. The Opa are variables regarding the number of expression, size, and antigenicity. These proteins facilitate adherence through human CD66 receptors. The Opc is a heparin-binding protein. It is also involved in the process of adherence and invasion of eukaryotic cells [46].

There was no cross-reaction of total IgG, collected 15 days after first immunization, with antigens of the heterologous strain B:4.P1.15 or *N. lactamica* antigens (data not shown), independent on adjuvant used. After the second immunization dose, in animals immunized with OMVs/DDA-BF it is possible to note a weak staining in a large number of antigens, but there was no uniformity in the individual response. We observed recognition of antigens of molecular weights of approximately 15–29 kDa, suggestive of the NspA antigens and class 5 (Opa and Opc); 43–67 kDa suggestive of class 1 (PorA), and 20–30 kDa (also suggestive of class 5). In the group immunized with OMVs/Al(OH)$_3$, a smaller number of antigens were recognized, and also there was no uniformity, and antigens between 30–43 kDa (compatible with class 2/3, PorB or class 4, Rmp) were weakly recognized by individual and pooled sera. In a single serum there was recognition of antigens in the range of 43–67 kDa (possible class 1) [3].

Protection promoted by OMVs-based vaccines of *N. meningitidis* is due to the production of serum bactericidal antibody [41]. which depends on the PorA present on the surface of the strain, which has the disadvantage of being variable in each meningococcal strain, leading to the need for periodic changes in vaccine formulation [41]. These vaccines are known as the most effective when used in epidemics in which the homologous strain, or a clone presenting the same PorA is used. When used in endemic disease or outbreaks, in which different strains are involved, the level of effectiveness generally tends to be lower. In addition, multiple doses of the same vaccine should be administered to promote primary protection and receiving a second dose is required to ensure that there will be long-term protection, especially when vaccine is applied in babies [41]. Then, to find a vaccine presenting good cross reactivity with heterologous strain is a goal. Here we show that antibodies generated after immunization with OMVs/DDA-BF were able to recognize antigens of non-homologous strain *N. meningitidis* B:4:P1.15, as well as 104/119 (88%) strains isolated from Brazilian patients, while serum from animals immunized with OMVs/Al(OH)$_3$ recognized only 23/119 (19%) in a more weak manner.

The licensed vaccine Bexsero is presented as described in the introduction important to be mentioned again not only by the presence of antigens that cause cross reactivity but also by its applicability in different countries. Bexsero 2017 presents cross binding results. We must not also forget the Trumemba is the serogroup B. DDA-BF is presented as an adjuvant that induces a Th1 response. However, it is known that bactericidal antibodies are very important in protecting against meningococcal disease (hence, the SBA being the correlate of protection). At least in humans, it is thought that a Th2 response to meningococcal is more beneficial than a Th1 response and aluminum hydroxide is known to induce a Th2 response [4].

Carmona-Ribeiro [46] in 2014 did an interesting review about the immunogenicity of DDA as adjuvant with different antigens preparation.

In conclusion, *N. meningitidis* OMVs in concentration of 10 μg/protein, both complexed the DDA-BF as well as $Al(OH)_3$, were able to generate humoral and cellular immune response in non-isogenic mice; $Al(OH)_3$ was superior to the DDA-BF in relation to the ability to assist the generation of humoral immunity since a dose of immunization with DDA-BF/$Al(OH)_3$ was enough to induce the production of total IgG, whereas to achieve this effect, two doses of DDA-BF/OMVs were required. Moreover only $Al(OH)_3$ induced the production of significant levels of IgG1 and IgG2a, in relation to control. In association to OMVs, $Al(OH)_3$ was also higher in promoting the generation of IgG avidity antibody, since the number of animals displaying. AI higher than 0.5 was bigger in animals immunized with OMVs/$Al(OH)_3$ in relation to animals immunize with OMVs/DDA-BF as well as bactericidal activity [23]. Both adjuvants did not differ in ability to assist the generation of cellular immunity, as evidenced by DTH results. In animals immunized with OMVs/$Al(OH)_3$, there was recognition of a greater number of antigens of the homologous strain. Furthermore, by analyzing the individual response, after the second dose of immunization, the recognition of these antigens were also more homogeneous in animals immunized with OMVs/$Al(OH)_3$. On the other hand, DDA-BF was superior in helping the generation of cross-reactive antibodies both, when the laboratorial heterologous strain *N. meningitidis* B:4:P1.15 or when strains isolated from patients were evaluated by dot-ELISA. Both adjuvants were able to help the generation of immunity against *N. meningitidis* OMVs and presented distinct advantages that should be better evaluated in future experiments.

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