Available online at www.sciencedirect.com
SCIENCE DIRECT®
Vaccine 22 (2004) 2617–2625
Vaccine
www.elsevier.com/locate/vaccine

Outer membrane vesicles (OMVs) and detoxified lipooligosaccharide (dLOS) obtained from Brazilian prevalent *N. meningitidis* serogroup B strains protect mice against homologous and heterologous meningococcal infection and septic shock

Ellen Jessouroun[a,*], Ivna F.B. da Silveira[a], Andréa P. Larangeira[a,b], Solange Pereira[a], Solange A. Fernandes[a], Leon Rabinovitch[c], Carl E. Frasch[d], Hugo C. Castro-Faria-Neto[b], Patricia T. Bozza[b]

[a] *Laboratório de Tecnologias Bacterianas, Departamento de Desenvolvimento Tecnológico—Bio-Manguinhos, FIOCRUZ, Rio de Janeiro, RJ, Brazil*
[b] *Laboratório Imunofarmacologia, Departamento de Fisiologia e Farmacodinâmica, FIOCRUZ, Rio de Janeiro, RJ, Brazil*
[c] *Departamento de Bacteriologia, Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, RJ, Brazil*
[d] *Center for Biologics Evaluation and Research, FDA, Bethesda, MD, USA*

Received 6 September 2003; received in revised form 11 December 2003; accepted 11 December 2003
Available online 20 January 2004

Abstract

Neisseria meningitidis (*N. meningitidis*) is a serious bacterial pathogen that causes life-threatening invasive bacterial infections especially in children below 2 years of age, teenagers and young adults. We have investigated the protective potential of outer membrane vesicles (OMVs) and detoxified lipooligosaccharide (dLOS) obtained from Brazilian prevalent *N. meningitidis* serogroup B strains. Swiss mice were immunized with different combinations of OMV and dLOS from *N. meningitidis* serogroup B strains compared to a reference vaccine (VA-MENGOC-BC®, Cuba). The OMVs + dLOS from Brazilian prevalent strains induced higher bactericidal antibody titers against homologous and heterologous target strains and stronger inhibition of thrombocytopenia as compared to the reference vaccine. When the challenge was performed with the B strain, all immunogens tested showed similar survival rates (80%) significantly higher than the control group. Bacterial clearance against the group B strain was comparable for animals immunized with the tested immunogen and the reference vaccine. Inclusion of dLOS from the B strain with the OMV, induced a similar clearance of C strain bacteria as compared to VA-MENGOC-BC®. The immunogens, as well as the reference vaccine drastically inhibited increases in TNF-α and IL-6 plasma levels after challenge. In conclusion, the OMV/dLOS formulation obtained from Brazilian prevalent strains of *N. meningitidis* has a remarkable performance protecting mice against the lethal effects of meningococcal challenge showing a good potential as a vaccine and should be considered for clinical evaluation.

Keywords: *N. meningitidis* vaccines; Outer membrane vesicles; Cytokines

1. Introduction

Neisseria meningitidis (*N. meningitidis*) is a serious bacterial pathogen that infects only humans. It causes life-threatening invasive infections especially in children below 2 years of age, teenagers and young adults. Systemic meningococcal disease (MD) presents various clinical syndromes ranging from meningitis, fulminant septicemia with septic shock, multiple organ failure and death to benign meningococcemia. When characterized by widespread purpuric rash, sepsis and shock, the disease is associated with mortality rates up to 40% [1,2].

The MD remains a world wide health problem. In developing countries infections caused by *N. meningitidis* serogroup B has resulted in 35,000 deaths per year. MD in Brazil has been a public health problem since serogroup A and C epidemics occurred in the 1970s. From 1990 to 2000, the annual MD incidence in Brazil ranged from 1 to 3 per 100,000 inhabitants. Serogroup B presently accounts for 60% of clinical isolates from meningococ-

* Corresponding author. Tel.: +55-21-38829342; fax: +55-21-2260-4727.
E-mail address: ellen@bio.fiocruz.br (E. Jessouroun).

0264-410X/$ – see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2003.12.009

cal infections, followed by 40% due to serogroup C [3,4].

During logarithmic growth, *N. meningitidis* liberates high amounts of lipooligosaccharide (LOS) in outer membrane vesicles (OMVs) known as "blebs". The pathogenic role of OMVs, in plasma and cerebrospinal fluid has become increasingly evident. Active LOS in the bloodstream is believed to be the main pro-inflammatory factor produced by *N. meningitidis*. In severe MD, activation of complex cell and plasma factors occur with the release of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-8) from macrophages and other inflammatory cells [2,5].

The most important mechanism that confers protection against serogroup A and C is complement-mediated lysis induced by bactericidal antibody chiefly against the capsular polysaccharide [6,7]. By contrast, the serogroup B polysaccharide is poorly immunogenic and fails to generate bactericidal antibodies. The serogroup B polysaccharide is an alpha 2–8 linked sialic acid, structurally the same as sialic acid polymers found on human embryonal and new-born brain tissue [5]. Due to the poor immunogenicity of serogroup B polysaccharide, most efforts to develop effective vaccines have focused on the major outer membrane proteins and LOS as alternative immunogens. Proteins induced by iron limitation are also studied as vaccine candidates, despite the fact that they show type specificity in relation to the induction of bactericidal antibodies [8–11]. However, the responses to OMP vaccines, in young children, may be strain specific. It has been reported that the dose schedule may be important in both vaccine response and induction of cross-protective antibodies. The OMV based vaccines must contain some small amount of native membrane bound LOS in relation to protein to maintain its membrane structure. The use of detoxified lipooligosaccharide (dLOS) in a vaccine in combination with detergent treated OMVs was suggested to induce increased bactericidal antibodies and vaccine specificity [12].

Vaccines being developed against *N. meningitidis* serogroup B based on such surface components, have the problem of endotoxicity, low immunogenicity, serosubtype specificity and lack of good animal models for vaccine protection studies. However, OMVs treated with sodium deoxycholate (DOC) have about 300 times reduced LOS endotoxicity compared to purified LOS, making them safe as vaccine candidates [5].

In a hyperferremic mouse model of infection, DOC treated OMV and dLOS obtained from Brazilian prevalent *N. meningitidis* serogroup B strains, were compared to a reference vaccine (VA-MENGOC-BC®). The protective potential of the immunogens was evaluated by the induction of bactericidal antibodies and by mortality rates following meningococcal challenge, the ability to clear bacteria in immunized animals and quantification of cytokines that are important in the pathogenesis of sepsis, including TNF-α, IL-6, KC (IL-8) and IL-10, were studied in immunized mice after challenge as additional parameters of protection. Platelet counts were also determined as an important parameter in the evolution of MD.

2. Material and methods

Bacterial strains: *N. meningitidis*, strains N44/89 (B:4, 7:P1.19,15:P5.5,7:L1,3,7,8), N603/95 (B:4:P1,7,1:P5.5,7: L3,7), N1002/90 (C:2b:P1.3,6:P5,8: L2,3,7,8) and Cu385 (B:4:P1,7,1:P5.5,5c) were provided by Adolfo Lutz Institute, São Paulo, SP, Brazil.

2.1. Growth conditions

To obtain OMVs, the strains were grown over night on Agar Müeller Hinton (AMH) + 1% bovine serum albumin (BSA), 36.5 °C/16 h/CO_2 and transferred to incomplete Catlin medium in a series of two 4 h subcultures (36.5 °C, 120 rpm). The second 4 h culture was used to inoculate a 20 l fermenter (Bio Flow IV, New Brunswick Scientific Co. Inc., Edison, NJ) containing 15 l of complete Catlin medium (20 μM Fe + 42 μM ethylenediamino di-*o*-hydroxyphenylacetic acid (EDDHA) (Sigma), 36.5 °C/16 h [2]. The cells were concentrated from the fermenter by tangential microfiltration (Minisette System, Pall BioPharmaceuticals, Membrane Type OMEGA 0.3 μm open channel). The N44/89 and N1002/90 strains used in the challenge test, were grown overnight on AMH + 1% BSA 36.5 °C/16 h/CO_2 and transferred to Trypitic Soy Broth + 30 μM EDDHA in a 5 h culture. The cells were pelleted by centrifugation (10,000 × *g*, 10 min) [13,14].

2.2. Preparation of OMV

The OMVs from meningoococcal strains N44/89 and N603/95 were DOC-extracted. In addition to the major OMPs of classes 1–5, the OMVs contain iron-regulated proteins (60–90 kDa), small amounts of less characterized membrane proteins and LOS (20–50 EU/μg protein). The residual endotoxin was determined by Gel-clot assay with labeled sensitivity of 0.125 from Endosafe® (SINGLE-TEST vial for endotoxin detection). The LOS depleted OMVs were obtained from the cells by ultrasonic treatment, using 2% (w/v) sodium DOC and purified by ultracentrifugation on to a 60% sucrose bed. The OMVs were kept at −70 °C until formulation [15,16].

2.3. Isolation and detoxification of LOS

LOS was obtained from N44/89 OMVs after DOC treatment as described by Tsai et al. [7]. In brief, LOS from strain N44/89 was isolated by two successive extractions using 40 mM Tris–HCl, pH 8.5, containing 1% DOC and 4 mM EDTA. The extracted LOS was further purified on Sephacryl HR S-300 with 0.5% DOC and precipitated at

−20 °C with 4 vol. ethanol and 0.25 M NaCl. The purified LOS was detoxified (dLOS) with 0.25N NaOH at a final concentration of 75 μg/ml and incubated in a water bath at 60 °C/60 min. After detoxification, the samples were immediately neutralized with 0.25N HCl and their concentration adjusted to 25 μg/ml with pyrogen-free water [7].

Protein profile of OMVs by SDS-PAGE eletrophoresis: OMV proteins were analyzed on 12% polyacrylamide gels, electrophoresed in the presence of 2% (w/v) SDS. After electrophoresis, proteins were stained by 0.1% (w/v) Coomasie Brilliant Blue staining [17,18].

2.4. Immunogen preparation

The LOS depleted OMVs from *N. menigitidis* serogroup B prevalent strains, N44/89 and N603/95 were mixed in a ratio 1:1 in relation to protein content and 1:0.5 with dLOS (N44/89) + 650 μg of Al $(OH)_3$ and VA-MENGOC-BC® vaccine was diluted 10 times to immunize the mice. The Cuban vaccine is composed of protein antigens from Cu385 B strain (100 ± 20 μg/ml), polysaccharide from C strain (100 ± 20 μg/ml), aluminum hydroxide (4 mg/ml) and thimerosal (1 g/10,000 ml). The formulations tested were prepared as follows. Group 1: 5 μg of total protein (2.5 μg N44/89 + 2.5 μg N603/95) + 2.5 μg dLOS (N44/89) + 650 μg $Al(OH)_3$/dose; Group 2: 5 μg of total protein (2.5 μg N44/89 + 2.5 μg N603/95) + 650 μg $Al(OH)_3$/dose; Group 3: reference vaccine (VA-MENGOC-BC®) diluted 1/10 (human dose is 25 μg protein); Group 4: 2.5 μg dLOS (N44/89) + 650 μg $Al(OH)_3$/dose and Group 5: PBS + 650 μg $Al(OH)_3$/dose.

2.5. Animals and immunization

Swiss female mice weighing 12–20 g were obtained from Oswaldo Cruz Foundation breeding unit (Rio de Janeiro, Brazil) and caged with free access to food and fresh water in a room with temperature ranging from 22 to 24 °C and a 12 h light/dark cycle in the Bio-Manguinhos experimental animal facility until used. The experimental protocol was performed according to Colégio Brasileiro Experimentação Animal (COBEA) and approved by the Fundação Oswaldo Cruz ethical committee for animal experimentation. Five groups of 12 mice were immunized intraperitoneally (i.p.) with three doses at 0, 14 and 28 days. The animals were bled before the first, second and third dose and 1 month after the last immunization.

2.6. N. menigitidis group B infection

The hyperferremic mouse model for *N. menigitidis* group B infection for immunogen protection evaluation and bacteremia assay was performed as previously described [3]. In brief, 1 month after the last immunization mice from each group were challenged with *N. menigitidis* group B most prevalent (N44/89, (5–10) × 10^6 cfu/0.5 ml) or group C (N1002/90, (3–5) × 10^6 cfu/0.5 ml) strains. For each suspension, mice were infected in the presence of iron–dextran (9 mg/mouse, i.p.). Control group was injected with PBS. 12 h after challenge, the animals were bled and the blood was collected into a 3.4% sodium citrate solution for bacteremia evaluation. A 10-fold dilution was made and the bacterial counts were performed on AMH agar containing 1% BSA and Vancomicin, Nistatin and Colistin (VCN/Oxoid). For immunogen protection evaluation, mice were bled before (T0), and 3 and 24 h (T3 and T24) after bacterial challenge. Blood samples were used for platelet counts, evaluation of plasma cytokines and determination of bactericidal antibody titers. To establish the survival rate, groups of mice were followed for 72 h.

2.7. Bactericidal assay

Two-fold dilutions of sera were tested with an inoculum of 50–70 cfu per well of log phase meningococci grown on Triptic Soy Agar (TSA) [19]. Serum obtained from male guinea pigs was used as the source of complement (free of bactericidal antibodies to *N. meningitidis* serogroup B). The assay was carried out at 37 °C for 30 min, and 150 μl of TSA with 2% BSA was added to each well. Quantitative cultures were performed at time 0 and 30 min later by the tilt method in duplicate. The bactericidal antibody titers were expressed as $\log_2$ of the final dilution that gives at least 50% killing of the inoculum [19].

2.8. LOS ELISA

Determination of antibodies to meningococcal LOS was done by ELISA using purified LOS from N44/89 strain as a coating antigen. The assay was performed as described by Rosenqvist et al. [20].

2.9. Platelet counts

Analysis were performed at different time points after *N. meningitidis* administration. Samples were collected from blood and diluted (600×) in formalin solution (125 μl of 37% formaldehyde + 20 ml of PBS solution) and platelet counts were performed in Neubauer chamber under optical microscopy [21,22].

2.10. Cytokine analysis

Plasma samples were obtained by cardiac puncture from anesthetized animals (Ketalar-Parke Davis, i.p.), and maintained at −70 °C, until the cytokine serum levels were evaluated by sandwich ELISA. The levels of TNF-α were detected using the DuoSet ELISA Development Kit from R&D Systems (San Diego, CA, USA) according to instructions provided by the manufacturer. For IL-6, IL-10 and

KC measurements, antibody pairs (capture and detection antibody) and recombinant mouse cytokine standards were obtained from BD-Pharmingen (San Diego, CA, USA) and the ELISA was performed according the manufacturer's instructions.

2.11. Statistical analysis

Data were evaluated by Multifactorial Analysis of Variance (LSD test), with 95% of confidence level.

3. Results

As shown in Fig. 1, the OMVs from Brazilian strains fully expressed IRPs and Classes 1, 3, 4 and 5 proteins. Although, the OMVs from Cuban vaccine was obtained from Cu385 strain in the same growth conditions, utilized to obtain the experimental Brazilian immunogens, the protein profile of the Cu385 OMVs showed less IRPs and Class 5 proteins expression than either of the two other strains.

The co-administration of *N. meningitidis* and iron–dextran was able to induce lethal sepsis in mice (Fig. 2). Animals vaccinated using OMVs + dLOS or the reference vaccine (VA-MENGOC-BC®) and challenged with *N. meningitidis* serogroup B, showed statistically significant increase in the survival rate (80% survival rate) when compared to the non-vaccinated control group. Animals that received OMVs or dLOS alone failed to show significant increase in the survival rates. In non-protected animals, the majority of deaths occurred within 24–48 h and the animals became very ill, exhibiting extreme lethargy, tachypnea, piloerection and closed eyes.

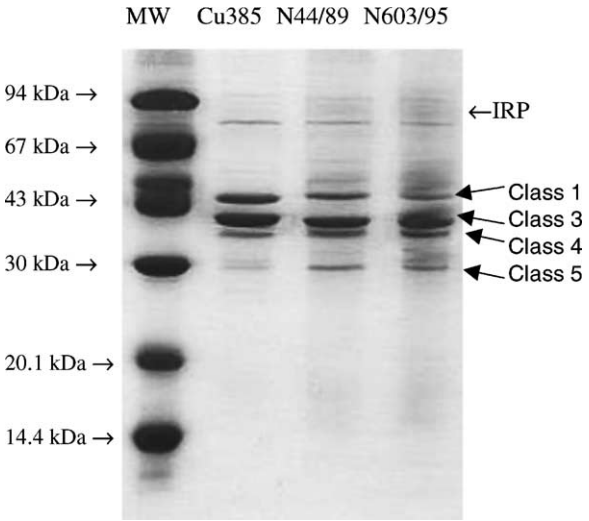

Fig. 1. Protein profile (10 μg/slot) of OMVs obtained from fermenter after 16 h cultures of Cuban vaccinal strain Cu385; Brazilian vaccinal strains N44/89 and N603/95. SDS-PAGE electrophoresis in a 12% gel stained by Coomassie Brilliant Blue.

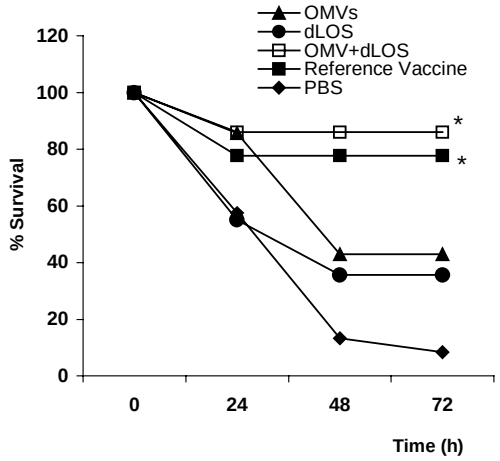

Fig. 2. Effect of immunogens on survival rates of mice challenged with *N. meningitidis* serogroup B (N44/89). The results are presented as average survival rates of 12 animals per data point. Significant differences ($P < 0.05$) from non-immunized PBS control mice are indicated by an asterisk. Reference vaccine (VA-MENGOC-BC®).

Mice immunized with OMVs + dLOS or PBS were bled 12 h after challenge with *N. meningitidis* serogroup B strain (N44/89). As shown in Fig. 3, leukocytes (mostly neutrophils) from non-immunized mice (A) were not able to control infection and cells dying full of bacteria in their cytoplasm as well as free bacteria in the peritoneal fluid were observed. In contrast, cells from OMVs + dLOS vaccinated animals with (B) were capable of efficiently kill the *N. meningitidis* and only sparse bacteria inside intact leukocytes were seen. Accordingly, the colony forming units count in the blood showed that non-vaccinated animals as well as those that received only dLOS as immunogen, were not capable of controlling bacteremia progression. Mice immunized with OMVs, OMVs + dLOS or with VA-MENGOC-BC® presented similar bacterial clearance efficiency 12 h after challenge with the *N. meningitidis* group B strain. Considering bacteremia evolution and protection rates against the B strain, the Brazilian complete preparation and the reference vaccine performed equally. In addition, using *N. meningitidis* group C strain (N1002/90) as challenge, the preparation formulated with antigens from a Brazilian prevalent group B strain (OMV + dLOS) induced total bacteria clearance within 12 h of challenge. The reference vaccine also showed efficacy on C strain clearance since it contains the group C meningococcal polysaccharide from C strain combined with OMVs from B strain Cu385 (Table 1).

The co-administration of *N. meningitidis* + iron–dextran was able to induce an acute increase in plasmatic levels of TNF-α, IL-6 and KC that were maximal within 3–24 h after infection (Table 2). Animals vaccinated with either OMVs + dLOS or the reference preparation drastically inhibited the increase in plasma levels of TNF-α, IL-6 and KC during the course of infection (Table 2). The values obtained for IL-10 in plasma of vaccinated animals, during

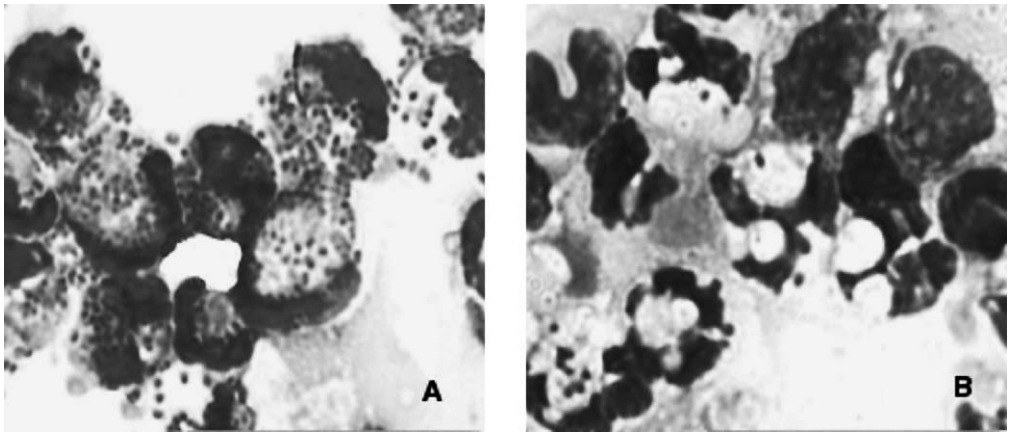

Fig. 3. Peritoneal cytosmear of non-immunized (A) or immunized (OMV + dLos) mice (B) 12 h after challenge with *N. menigitidis* B strain (N44/89). Peritoneal cavities were washed with 5 ml PBS. Leukocytes were stained by May Grunwald Giensa method.

Table 1
Bacteremia evaluation with *N. meningitidis* homologous and heterologous strains

Vaccines	N44/89 (group B) (challenge population (5–10) $\times$ 10^6 cfu/0.5 ml)	N1002/90 (group C) (challenge population (3–5) $\times$ 10^6 cfu/0.5 ml)
OMVs	1×10^2 to 1.5×10^4 cfu/0.5 ml	(2.5–3.5) $\times$ 10^5 cfu/0.5 ml
dLOS	$>1 \times 10^6$ cfu/0.5 ml	(1.0–4.0) $\times$ 10^5 cfu/0.5 ml
OMV + dLOS	1×10^3 to 2.4×10^4 cfu/0.5 ml	No growth
Vamengoc BC	1×10^2 to 4.0×10^4 cfu/0.5 ml	No growth
PBS	2.5×10^6 cfu/0.5 ml	1.6×10^6 cfu/0.5 ml

The bacteria counts were performed 12 h after challenge.

Table 2
Effect of vaccination on mice plasma cytokine levels (ng/ml) after challenge with *N. meningitidis* serogroup B strain

Plasma cytokines[a] (ng/ml)	Immunogen		
	Non-immunized mice	Immunized mice with Brazilian antigens (OMVs + dLOS)	Immunized mice with reference vaccine (VA-MENGOC-BC®)
TNF-α			
0 h	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01
3 h	0.92 ± 0.15	0.26 ± 0.17*	0.35 ± 0.11*
24 h	1.41 ± 0.58	0.16 ± 0.14*	0.03 ± 0.03*
IL-6			
0 h	0.10 ± 0.10	1.46 ± 0.89	0.00 ± 0.00
3 h	270.75 ± 26.09	3.75 ± 1.44*	12.80 ± 2.37*
24 h	228.25 ± 3.76	10.80 ± 1.31*	31.78 ± 17.67*
KC			
0 h	0.01 ± 0.01	0.06 ± 0.03	0.14 ± 0.10
3 h	29.95 ± 12.07	0.47 ± 0.25*	3.93 ± 3.40*
24 h	52.76 ± 14.05	0.07 ± 0.07*	0.01 ± 0.00*
IL-10			
0 h	0.03 ± 0.03	0.05 ± 0.05	0.00 ± 0.00
3 h	0.05 ± 0.03	0.01 ± 0.01	0.00 ± 0.00
24 h	1.61 ± 1.41	0.07 ± 0.04	0.05 ± 0.03

OMVs + dLOS − 5 μg (2.5 μg N44/89 + 2.5 μg N603/95) + 2.5 μg dLOS (N44/89) + 650 μg/dose. VA-MENGOC-BC® − diluted 1/10.

[a] Values are means ± standard error from at least five animals per group.

* Significant differences ($P < 0.05$) from non-immunized mice.

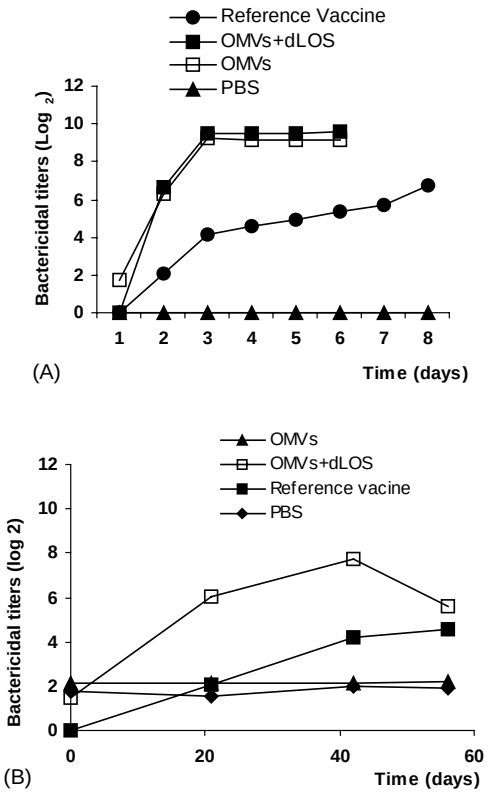

Fig. 4. Bactericidal antibodies induced by different antigen preparations with homologous N44/89 (A) and heterologous N1002/90 (B) as target strains.

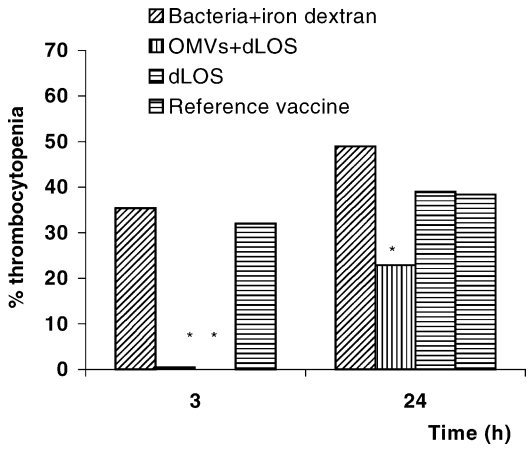

Fig. 5. Effect of immunogens on thrombocytopenia induced by *N. meningitidis*. The results presented were calculated based on normal platelets counts of non-infected mice (mean of at least 10 animals). Significant differences ($P < 0.05$) from non-immunized mice are indicated by an asterisk.

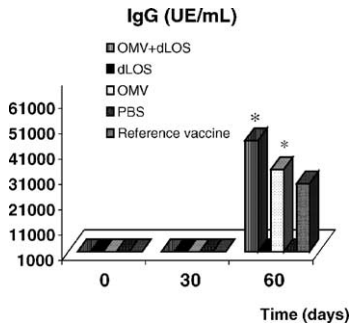

Fig. 6. dLOS antibody levels (ELISA) of mice immunized with different immunogens. Significant differences ($P < 0.05$) from non-immunized mice are indicated by an asterisk.

the period of evaluations, were not statistically different from the non-vaccinated and challenged animals.

In order to evaluate the functional antibodies induced by the experimental antigens, five pooled sera were evaluated in bactericidal assay using N44/89 as homologous and N1002/90 as heterologous target strains. As seen in Fig. 4A, the titers induced by OMVs alone and OMVs + dLOS, were the same against N44/89. However, both had statistically higher bactericidal titers than that induced by the reference vaccine. The bactericidal activity, of mice antibodies, observed against N1002/90 strain was higher in animals that received OMVs + dLOS (Fig. 4B), than in animals immunized with OMVs alone or VA-MENGOC-BC®. These results are in accordance to the enhanced C strain clearance capacity observed in the blood of these animals 12 h after challenge suggesting that dLOS may increase cross reactivity within different serogroups of *N. meningitidis.* Both groups B and C strains have the same L3,7 LOS immunotype. The animals that received only OMVs or the control group did not show bactericidal activity against N1002/90 strain.

As shown in Fig. 5, vaccination with OMVs + dLOS significantly inhibited *N. meningitidis*-induced thrombocytopenia within 3 and 24 h. Interestingly, VA-MENGOC-BC® failed to inhibit the observed thrombocytopenia. Animals immunized only with dLOS also showed a trend towards thrombocytopenia inhibition that was not significant compared to non-immunized animals.

Anti-LOS specific antibodies were measured by ELISA (Fig. 6). The animals that received the formulation containing OMVs from Brazilian prevalent strains with and without dLOS, 60 days after the last immunization, showed the highest antibody levels. The values obtained were statistically higher compared to the reference vaccine, dLOS alone and PBS.

4. Discussion

To design a Brazilian vaccine, the protein antigens were selected from two most prevalent B strains considering that the different IRPs, Classes 1 and 5 proteins should have increased the immunogen protective potential. The N44/89 B strain was chosen as the source of dLOS because its immunotypes (L1,3,7,8) included the immunotypes from N603/95 (L3,7).

According to the results presented in this study, noncapsular antigens from *N. meningitidis* serogroup B Brazilian prevalent strains induce protective antibodies against both groups B and C strains. Endotoxin release from the cell wall is thought to play an important role in the pathophysiology of *N. meningitidis* infection [23,24]. The lethality of MD is partially dependent of bacterial viability, but is also due to the over production of host cytokines after macrophage interaction with LOS and other cell envelop components. Though no animal model has been entirely adequate to reproduce MD as observed in humans, the noncapsular immunogens strongly suppressed cytokine induction following bacterial challenge [25].

Overproduction of host inflammatory mediators induced by infectious organisms and their products cause the peripheral cellular alterations that occur in sepsis and septic shock. The production of TNF-α and other endogenous mediators leads to several pathophysiological events including fever, leukopenia, thrombocitopenia, multiple organs failure and death. TNF-α together with IL-1 have been considered major endogenous mediators of sepsis and their interactions with target cells including macrophages, increase their cytotoxic capacity and induce the release of other pro- and anti-inflammatory cytokines [26–28]. Among the cytokines released during the development of sepsis, IL-6 has a central role as a marker for severity of the disease. Similarly, increased levels of other cytokine such as IL-8 have been observed in serum and cerebrospinal fluid from patients with meningococcal disease. The IL-8 serum levels were correlated with IL-6 and TNF-α levels and highest values were detected in patients with septic shock [23,28,29].

The experimental murine sepsis model that we used resembles the septic form of infectious disease in humans, where serum concentrations of TNF-α, IL-1β, IL-6 and IL-8 sharply increase as septic shock develops [15]. Our results showed that, during the course of infection, the circulating levels of pro-inflammatory cytokines were increased similar to that observed in MD. Moreover, in protected animals we observed a drastic reduction in both serum levels of TNF-α, IL-6 and KC (the murine homologue of human IL-8). It has been previously demonstrated that TNF-α neutralization decreases IL-6, KC and IL-10 production suggesting that TNF-α plays a key role in the positive loop of cytokine production [20,27,30]. The decrease of TNF-α, IL-6 and KC production observed in the immunized mice might have contributed to the increase in LOS resistance we found. The increased animal survival after challenge and the enhanced bacterial clearance capacity observed in protected mice, are likely due to the good vaccine potential of experimental immunogens. In addition, a synergic effect with the decreased responsiveness of host macrophages caused by the neutralization of LOS by specific antibodies and the down regulation of inflammatory cytokines production during the immunization procedures may also have contributed to the protective effect of these immunogens.

The bactericidal activity of antibodies induced by outer-membrane proteins of *N. meningitidis* serogroup B, was shown to be directed mainly against Por A, Class 5 and iron-regulated proteins (IRPs). These proteins have been described as principal targets for antibodies induced by meningococcal infection or vaccination [31–33]. Use of dLOS in combination with DOC treated OMVs appear to induce bactericidal antibodies and broaden vaccine specificity. These effects may be enhanced with the adjuvant effect of LOS stimulating Th1 and consequently the production of IgG 2 in mice [34–36]. Expressed proteins from two Brazilian strains may have induced higher bactericidal antibodies against homologous B strain, in animals that were immunized by OMVs or OMVs + dLOS. The common OMV residual LOS (L1,3,7,8) may also have contributed to the increase of bactericidal titers in mice that had received the experimental vaccines. It is in accordance to Steeghs et al., who studied the role of lipopolisaccharide (LPS) in immune response of outer membrane complexes from *N. meningitidis* [37]. They showed that LPS could direct the subclass of IgG towards to IgG 2a, IgG 2b and IgG 3, which have strong bactericidal activity.

Despite the fact that Brazilian most prevalent strain (N44/89) and Cuban vaccine strain (Cu385) belong to the same serotype, different OMV protein profile used in experimental vaccine may have induced different bactericidal antibody response. The OMV characteristic protein profile is related not only to the bacteria strain, but also to methods of cultivation and antigen purification [38,39]. The possibility of lower expression of Class 5 protein in OMVs from Cu385 may have explained the lower bactericidal titers against homologous strain. However, the titers decrease observed in animals immunized with Cuban vaccine may not have been enough to decrease its protection ability. The higher amount of aluminum hydroxide, in experimental vaccines may have increased Th2 response inducing IgG1 antibodies which do not have strong bactericidal activity [40]. Our results suggest that the Brazilian antigen combination may have increased the immune response specificity.

Previous studies in Swiss mice, showed that VAMENGOC-BC® induced lower functional antibody levels against vaccine homologous strain compared to experimental Brazilian OMV vaccines [19]. The cross-reactivity observed between serogroups B and C observed in mice that received OMVs + dLOS, is likely related to the common LOS within strains and dLOS inclusion as a vaccine component. It is clearly defined that the toxicity and adjuvant activity of LPS is determined by Lipid A but the intensity of its biological activity seems to be related to the integrity of the chemical structure. Alkali-hydrolyzed meningococcal LPS, which might have incomplete Lipid A structure retained adjuvant potential although shown reduced toxicity [24,37,41].

Controlling C strain bacteremia evolution after challenge, the Brazilian antigens from B strain performed as good as Cuban vaccine, which has C polysaccharide as vaccinal component. The cross-protection induced by experimental B

vaccine was confirmed by higher bactericidal titers against C heterologous strain, compared to Cuban vaccine.

Disseminated intravascular coagulation (DIC), is a frequent and serious complication in meningococcal sepsis. Coagulation activation, as well as, platelet activation and aggregation all contribute to the pathogenesis of DIC [42]. We observed an intense thrombocytopenia in non-vaccinated animals challenged by B strain of *N. meningitidis*. Interestingly, animals vaccinated with the Brazilian prevalent *N. meningitidis* strain based OMVs + dLOS formulation showed a significant decrease of *N. meningitidis*-induced thrombocytopenia, under conditions where the reference vaccine (VA-MENGOC-BC®) failed to have an effect. At the challenge, which occurred 30 days after the last dose, the presence of dLOS together with OMVs in Brazilian vaccine may have played an important role in the induction of enough LOS specific antibodies to decrease the platelet aggregation and thrombocytopenia. This could have caused beneficial effect on survival rates in mice immunized with this preparation. Due to the relevance of DIC for the MD outcome, the protection induced by OMVs + dLOS vaccine formulation could provide additional clinical benefit. These results suggest that the combination of antigens is necessary to obtain efficient protection.

In conclusion, the experimental murine meningococcal sepsis model used here resembles the disease in humans, and inclusion of measurement of sepsis markers to evaluate immunogens protective effects, provided valuable additional information. Our results demonstrated that the OMVs/dLOS formulation obtained from Brazilian prevalent strains of *N. meningitidis* serogroup B had a good performance in protecting mice against the lethal effects of bacterial challenge and could be a promising vaccine in the prevention of MD in Brazil.

Acknowledgements

We thank Dra. Diana Homma (FIOCRUZ, Rio de Janeiro, Brazil) for helpful discussions. This work was supported by Bio-Manguinhos, FIOCRUZ, Rio de Janeiro, Brazil.

References

[1] Brandtzaeg P, Kierulf P, Gaustad P, Skulberg A, Bruun JN, Halvorsen S, et al. Plasma endotoxin as a predictor of multiple organ failure and death in systemic meningococcal disease. J Infect Dis 1989; 159(2):195–204.

[2] Mirlashari MR, Hagberg IA, Lyberg T. Platelet-platelet and platelet-leukocyte interactions induced by outer membrane vesicles from *N. meningitidis*. Platelets 2002;13:91–9.

[3] Sifontes S, Infante JF, Perez P, Caro E, Sierra G, Campa C. The hyperferremic mouse model for the evaluation of the effectiveness of VA-MENGOC-BC against *Neisseria meningitidis* B clinical isolates. Arch Med Res 1997;28(1):41–5.

[4] Verheul AF, Snippe H, Poolman JT. Meningococcal lipopolysaccharides: virulence factor and potential vaccine component. Microbiol Ver 1993;57(1):34–49.

[5] Quakyi EK, Carter PH, Tsai CM, Marti GE. Immunization with meningococcal membrane-bound lipoolygosaccharide accelerates granulocyte recovery and enhances lymphocyte proliferation in myelosuppressed mice. Pathobiol 1997;65:26–38.

[6] Poolman JT. Development of a meningococcal vaccine. Infect Agents Dis 1995;4:13–28.

[7] Tsai CM, Frasch CE, Rivera E, Hochstein HD. Measurements of lipopolysaccharide (endotoxin) in meningococcal protein and polysaccharide preparations for vaccine usage. J Biol Stand 1989;17(3): 249–58.

[8] Saukkonen K, Leinonen M, Abdillahi H, Poolman JT. Comparative evaluation of potential components for group B meningococcal vaccine by passive protection in the infant rat and in vitro bactericidal assay. Vaccine 1989;7(4):325–8.

[9] Danve B, Lissolo L, Mignon M, Dumas P, Colombani S, Schryvers AB, et al. Transferrin-binding proteins isolated from *Neisseria meningitidis* elicit protective and bactericidal antibodies in laboratory animals. Vaccine 1993;11(12):1214–20.

[10] Banerjee-Bhatnagar N, Frasch CE. Expression of *Neisseria meningitidis* iron-regulated outer membrane proteins, including 70 kilodalton transferrin receptor, and their potential for use as vaccines. Infect Immun 1990;58(9):2875–81.

[11] Ala'Aldeen DA, Borrielo SP. The meningococcal transferrin-binding proteins 1 and 2 are both surface exposed and generate bactericidal antibodies capable of killing homologous and heterologous strains. Vaccine 1996;14:49–53.

[12] Pollard AJ, Galassini R, van der Voort EM, Booy R, Langford P, Nadel S, et al. Humoral immune response to *Neisseria meningitidis* in children. Infect Immun 1999;67(5):2441–51.

[13] Catlin WB. Nutritional profiles of *Neisseria gonorrhorae*, *Neisseria meningitidis*, and *Neisseria lactamica* in chemically defined media and the use of growth requirement for gonococcal typing. J Infec Dis 1973;128:178–94.

[14] Fu J, Bailey FJ, King JJ, Parker CB, Robinett RS, Kolodin DG, et al. Recent advances in the large scale fermentation of *Neisseria meningitidis* group B for the production of an outer membrane protein complex. Biotechnology 1995;13:170–4.

[15] Brandileone MCC, Zanella RC, Vieira VSD, Sacchi CT, Milagres LG, Frasch CE. Induction of iron regulated proteins during normal growth of *N. meningitidis* in a chemically defined medium. Rev Inst Med Trop (São Paulo) 1994;36(4):301–10.

[16] Milagres LG, Brandileone MCC, Sacchi CT, Vieira VSD, Zanella RC, Frasch CE. Immune response of mice to a Brazilian group B vaccine. FEEMS Immunol Med Microb 1995;561:1–9.

[17] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.

[18] Markwell MAK, Haas SM, Bieber LL, Tolbert NE. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochnol 1978;87:206–10.

[19] Milagres LG, Ramos SR, Sacchi CT. Immune response of Brazilian children to a *Neisseria meningitidis* serogroup B outer membrane protein vaccine: comparison with efficacy. Infect Immun 1994;62(10):4419–24.

[20] Rosenqvist E, Hoiby EA, Wedge E. Human antibody response to meningococcal outer membrane antigens after three doses of Norwegian group B meningococcal vaccine. Infect Immun 1995; 63(12):4642–52.

[21] Lima AO, Soares JB, Greco JB, Galizzi J, Cançado JR. Métodos de laboratório aplicados à clínica. 5ª edição. Guanabara Koogan; 1977.

[22] Castro-Faria-Neto HC, Bozza PT, Martins MA, Dias PM, Silva PM, Cordeiro RS. Platelet mobilization induced by PAF and its role in the thrombocytosis triggered by adrenaline in rats. Thromb Haemost 1989;62(4):1107–11.

[23] Quakyi EK, Hochstein HD, Tsai CM. Modulation of the biological activities of meningococcal endotoxins by association with outer membrane proteins is not inevitably linked to toxicity. Infect Immun 1997;65(5):972–9.

[24] Bjerre A, Brusletto B, Rosenqvist E, Namork E, Kierulf P, Ovstebo R, et al. Cellular activating properties and morphology of membrane-bound and purified meningococcal lipopolysaccharide. J Endotoxin Res 2000;6(6):437–45.
[25] Martin D, Cadieux N, Hamel J, Brodeur BR. Highly conserved *Neisseria meningitidis* surface protein confers protection against experimental infection. J Exp Med 1997;185(7):1173–83.
[26] Brandtzaeg P, Ovstebo R, Kierulf P. Compartmentalization of lipopolysaccharide production correlates with clinical presentation in meningococcal disease. J Infect Dis 1992;166:650–2.
[27] Hack CE, Arden LA, Thus LG. Role of cytokines in sepsis. Adv Immunol 1997;66:101–95.
[28] Prins JM, Lauw FN, Derkx BH, Speelman P, Kuijper EJ, Dankert J, et al. Endotoxin release and cytokine production in acute and chronic meningococcaemia. Clin Exp Immunol 1998;114(2):215–9.
[29] Halstensen A, Ceska M, Brandtzaeg P, Redl H, Naess A, Waage A. Interleukin-8 in serum and cerebrospinal fluid from patients with meningococcal disease. J Infect Dis 1993;167:471–5.
[30] Ziegler-Heitbrock HWL. Molecular mechanism in tolerance to lipopolysaccharide. J Inflamm 1995;45:13–26.
[31] Rosenqvist E, Harthug S, Froholm LO, Hoiby EA, Bovre K, Zollinger WD. Antibody responses to serogroup B meningococcal outer membrane antigens after vaccination and infection. J Clin Microbiol 1988;26(8):1543–8.
[32] Mandrell RE, Zollinger WD. Human immune response to meningococcal outer membrane protein epitopes after natural infection or vaccination. Infect Immun 1989;57(5):1590–8.
[33] Sacchi CT, Lemos AP, Popovic T, De Morais JC, Whitney AM, Melles CE, et al. Serosubtypes and PorA types of Neisseria meningitides serogroup B isolated in Brazil during 1997–1998: overview and implications for vaccine development. J Clin Microbiol 2001;39(8):2897–903.
[34] Gupta RK, Relyveld EH, Lindblad EB, Bizzini B, Ben-Efraim S, Gupta CK. Adjuvants—a balance between toxicity and adjuvanticity. Vaccine 1993;11(3):293–306.
[35] Gupta RK, Siber GR. Adjuvants for human vaccines—current status, problems and future prospects. Vaccine 1995;13(14):1263–76.
[36] Quakyi EK, Frasch CE, Buller N, Tsai CM. Immunization with meningococcal outer membrane protein vesicles containing lipooligosaccharide protects mice against lethal experimental group B *Neisseria meningitidis* infection and septic shock. J Infect Dis 1999;180:747–54.
[37] Steeghs L, Kuipers B, Hamstra HJ, Kersten G, van Alphen L, van der Ley P. Immunogenicity of outer membrane proteins in a lipopolysaccharide-deficient mutant of *Neisseria meningitidis*: influence of adjuvants on the immune response. Infect Immun 1999;67(10):4988–93.
[38] Jessouroun E, Danelli MGM, Almeida AL. Expression of iron-regulated outer membrane protein in *Neisseria meningitidis*: a comparison of three culture media. Biomed Lett 1995;51:85–92.
[39] Centro Nacional de Biopreparados. Vaccine against group B Neisseria meningitidis, gamaglobulin and tranfer factor. European Patent Specification, 0301992B1, Bulletin 1989.
[40] Gupta RK, Siber GR. Adjuvants for human vaccines-current status, problems and future prospects. Vaccine 1995;13(14):1263–76.
[41] Heumann D, Roger T. Initial responses to endotoxin and Gram-negative bacteria. Clin Chim Acta 2002;59–72.
[42] Pajkrt D, van Deventer SJ. The cellular response in sepsis. Curr Top Microbiol Immunol 1996;216:119–32.