



Biological and Immunological Evaluation of *Neisseria meningitidis* Serogroup A Outer Membrane Vesicle as Vaccine Candidates

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ABSTRACT

Background: *Neisseria meningitidis* Serogroup A, is a major cause of bacterial meningitidis outbreaks in Africa and the Middle East. While polysaccharide vaccines have been available for many years, these vaccines have many disadvantages including the induction of T-cell independent responses which do not induce memory responses.

Objectives: Thus to overcome this problem, in this research outer membrane vesicle (OMV) containing PorA was extracted and evaluated by biological and immunological methods.

Materials and Methods: OMVs were extracted with deoxycholate and EDTA, and purification was performed by sequential ultracentrifugation. Physicochemical properties of extracted OMVs were analyzed by electron microscopy and SDS-PAGE. The toxicity of LPS content in its was assayed by LAL test. The Presence of PorA as a major component of OMV was confirmed by western blot. To study antibodies synthesis after immunization with OMV, ELISA method was used. Also serum bactericidal assay (SBA) was performed to determine the serum bactericidal activity against *N. meningitidis* serogroup A.

Results: The results revealed that the content of protein extracted was 0.1 mg/mL. The electron microscopy showed that intactness of the vesicle in these preparation ranged more than 70%. The SDS-PAGE showed that PorA as a major immunological part of outer membrane vesicle was located in 35-40kDa. LAL test showed that the endotoxin activity was around 126EU/mL which is safe for using. The ELISA test revealed that the IgG total titer was elevated after the first injection. SBA indicates that bactericidal antibodies rise after the second dose of booster.

Conclusions: The results showed that the extracted OMVs were conformationally stable, and there were no pyrogenic determinants in OMV. Also the results showed that the OMV elicited high level of specific antibodies against *N. meningitidis* serogroup A. These results indicate that the OMV obtained here, can be used as a meningococcal vaccine after further investigation.

Keywords: Outer membrane vesicle (OMV); *Neisseria meningitidis*, Serogroup A; Vaccines

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►Implication for health policy/practice/research/medical education:

The major focus of our research is to develop vaccines against meningococcal serogroup A infection, which is a global health problem worldwide, especially in Iran. In the present effort we tested the potential of OMVs as vaccine candidates to provide protection against *N.meningitidis* in mouse model. Our effort addresses a step towards the development of an effective vaccine for human use.

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1. Background

Neisseria meningitidis an encapsulated Gram-negative bacterium responsible for significant morbidity and mortality worldwide. *N. meningitidis* classified into 13 serogroups on the basis of differences in the structure and antigenicity of their capsular polysaccharides (1). *N. meningitidis* serogroup A strains are responsible for a large number of clinical bacterial meningitis in the Middle East and meningitis belt in Africa (2).

Immunization with capsular polysaccharide appears to be the only means to reduce the mortality and morbidity due to the meningococcal epidemics. Polysaccharide vaccines have a number of critical drawbacks which limit their utility in immunization programs (2). In general, the immunogenicity and efficacy of capsular polysaccharide vaccines are age-related, with the vaccine being poorly immunogenic in very young children, who are the most at risk of meningococcal infection.

This is a consequence of age-related differences in both the quantity and the quality of antibody responses to polysaccharide vaccines, which elicit a higher proportion of high-avidity, bactericidal antibodies in older children and adults than infants. With bactericidal antibody levels declining more rapidly in young children than adults, the duration of protective immunity induced by polysaccharide antigens is also age-related and tends not to be readily boosted by subsequent exposure to the antigen.

According to these drawbacks, many researches have focused on other parts of the organism to overcome these disadvantages (2-4). One of the alternative parts of the organism is outer membrane vesicle (OMV) (5). The OMV contains a restricted number of major proteins, phospholipids, lipooligosaccharide, and nucleic acid (6, 7). The porin proteins (PorA, PorB) are amongst the most abundant proteins produced by meningococci, and are the predominant proteins present on OMV (6, 7).

Specific antibodies and a functional complement system are of crucial importance in the host defense against systemic meningococcal infections. Serum bactericidal activity is the pivotal protective mechanism against invasive meningococcal infectious disease (1, 2). The bactericidal antibodies to PorA play an important role in the protection against meningitis and sepsis caused by meningococci.

Serogroup B meningococcal vaccines have been developed based on OMVs, and in clinical trials, these vaccines

have been shown to be safe and efficacious. Significant increases in bactericidal antibody response were observed in all age groups studied (6-9). We previously purified OMV of group B meningococci, which was immunogenic in rabbits, and the respective antisera showed bactericidal and opsonophagocytic activity against *N. meningitidis* serogroup B, and also has been of significant efficacy against *N. meningitidis* serogroup A, but in lower response (10, 11).

2. Objectives

The aim of this study was to extract the OMV from meningococci serogroup A and to evaluate bactericidal and total IgG antibody response induction in hyper immunized mice.

3. Materials and Methods

3.1. Bacterial Strains and Growth Conditions

Standard strain of *N. meningitidis* serogroup A (CSBPI, G243) was obtained from the standard Bacterial Collection of Pasteur Institute of Iran. Bacteria were grown in a defined Frantz medium, with main components as L-glutamate, glucose, L-cysteine and ammonium chloride, at 35 °C for 15h in a Contact-flow Bithoven unit system fermentor under pH control (10-12).

3.2. Preparation of OMVs

OMVs were prepared by Siadat et al. method. In brief, Outer membrane vesicles (OMVs) were extracted in 0.1M Tris-HCl, pH 8.6, 10mM EDTA, and 0.5%w/v deoxycholate. Purification of OMVs was performed by sequential centrifugation at 20,000g for 30 min, and finally followed by ultracentrifugation at 125,000g for 2h. The pelleted OMVs were homogenized in phosphate buffered saline (PBS) pH 7.2. Throughout the process, thiomersal (100mg/l) was added as preservative (10-13).

3.3. Composition of OMV

The protein content of OMVs was measured by spectrophotometer method using nano drop. The PorA content

(relative to total protein contentment) was determined by SDS-PAGE (see below), and the presence of LPS was analyzed by the Limulus Amebocyte Lysate (LAL) gelatin test. The lipid-A part of endotoxin molecule can activate the gelation of the limulus lysate. The OMVs were incubated in fivefold dilution with a fixed concentration of limulus lysate at 37 °C for 45 min. The endotoxin activity was visualized using Bromothymol blue. Unknown activity of endotoxin was compared to the *E.coli* standard endotoxin (FDA, Bethesda, USA) (11-15).

3.4. Electron Microscopy

To check the integrity and stability of OMV after extraction process, electron microscopy was performed as described previously (10-13). Briefly, OMVs were ultrasonically treated to disperse the vesicles and were attached to Formvar/carbon-coated nickel grids. Grids were washed with a 0.01 M PBS supplement with 0.5% bovine serum albumin (BSA-Sigma), and 0.1% gelatin (PBG-Sigma), and vesicles on the grids were fixed briefly with 1% glutaraldehyde in PBS and negatively stained with potassium phosphotungstate pH 6.0. The grids were examined in Zeiss CEA902A electron microscope at 80Kv (11-13).

3.5. SDS-PAGE and Western Blotting

The protein composition of OMV was analyzed by SDS-PAGE in 12% gel using a Mini-protean II electrophoresis apparatus (Bio-Rad). Gels were stained with Coomassie Brilliant Blue (CBB).

Western blotting was performed after SDS-PAGE under denaturing conditions. Briefly, Guide stripe from the blot, which was incubated with MAbs against PorA, to identify the position of the PorA. Binding of MAbs was detected with horseradish peroxidase (Dakopatts, Glostrup, Denmark) and further stained with 3-amino-9-ethylcarbazole (10-14). The value of ladder in Western blot was 3 µl.

3.6. Pyrogeny Test

To study pyrogenic determinants the pyrogeny test according to Pharmacopeia was performed (11). Briefly, four healthy adult New Zealand albino rabbits were used in the study. At the start of study, animals weigh ranged between 1.8 and 3.8 kg. Three test rabbits and one control rabbit were used in the study. The OMV was injected to the marginal ear vein of rabbits, at the dose based on the body weight of the animals. Digital thermometer was used to measure rectal temperature of the animals. If no rabbits showed an individual temperature rises of 0.6°C or more, or if the sum of the three individual maximum temperature rises did not exceed 1.4°C, the test article met the requirements for the absence of pyrogeny (12-15).

3.7. Immunization Program

To evaluate immunological responses to serogroup A OMV, one group of five BALB/c mice were immunized subcutaneously with 5µgr OMV of each of the vaccines on 0, 14, 28, and 42 days and bled on 0, 14, 28, and 42. Sera were stored at -20°C (12, 13).

3.8. Quantification of Total IgG by ELISA

To study specific total IgG antibodies response against meningococcal serogroup A OMV, uncompetitive ELISA was performed. Briefly, Maxisorp plates were coated with 100 µL/well of 5µg protein/mL of OMVs from strain CSBPI/G243 *N.meningitidis* serogroup A. Thus, in this study we used meningococcal serogroup A OMV as antigen and pooled sera as hyper immune sera, and antimouse IgG conjugated with HRP as the secondary antibody (16).

3.9. Complement Mediate Bactericidal Assay

Antimeningococcal A bactericidal assay was performed using the standardized meningococcal serogroup A strain CSBPI-G243 in 96-well plates, as previously described (13, 17, 18). Briefly, Muller Hinton Broth (MHB) was incubated with 5 to 10 colonies from a fresh culture of *N.meningitidis* and incubated for 2h at 37°C with shaking. Approximately 100-200mL of the stock bacterial cell suspension was added to 20 mL of sterile MHB pre-equilibrated at the room temperature to yield an A600 between 0.07 and 0.08.

The culture flask was then incubated for 2h at 37°C with 160 rpm shaking until A600 was between 0.23 and 0.24. This yielded approximately in t into o 1.109 CFU/mL. the bacterial cells were diluted in sterile 50 Mm Phosphate buffer ph 7.2 containing 10 mM MgCl₂, 10 mM CaCl₂, and 0.5% (w/v) BSA until a concentration of 1.105 CFU/mL was reached. All sera to be tested were heat inactivated for 30min at 56°C. Pooled sterile rabbit baby serum without bactericidal activity against the trains to be tested was used as the exogenous complement source in this assay.

A sterile polystyrene U bottom 96 well microtiter plate was used this assay. The total volume of each well of the plate was 50µl with 25 µl serially diluted serum in assay buffer, 12.5µl of bacteria, and 12.5 µl of complement. Controls included samples with buffer, bacteria and complement and complement and sample with serum, bacteria and buffer. After all components were added, 10µl pipetted on a (MHB) and incubated 18h at 37°C in 5% CO₂. The microtiter plate was then incubated for 1h at 37°C. After 18h incubation at 37°C in 5% CO₂, the colonies on time zero and 60min incubation plates were counted. The average number of the serum dilution yielding >50% killing (11, 13, 17, 18).

3.10. Statistical Methods

Analysis of variance was used for statistical evaluation of the data. Statistical significance was defined as a *P* value of < 0.05.

4. Results

4.1. OMVs Characterization and Identity Test

Total protein yield of the OMV production was determined by nano drops method based on spectrophotometry. Total protein yield was 0.1 mg/mL. Electron microscopy was used to verify the integrity of the OMV after preparation. The OMV size ranged from 50 to 150 nm in this process (Figure 1).

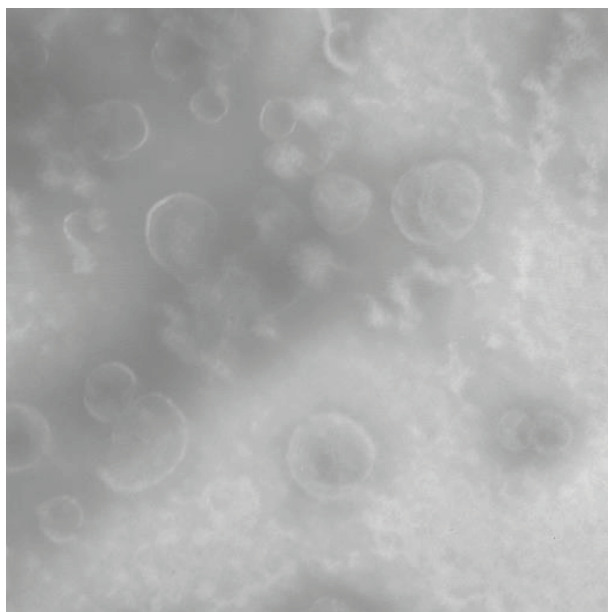


Figure 1. The Electron Microscopy of (OMV) Stained With 1% Potassium Phosphotungstate

The biological activity of the endotoxin was determined in the Limulus Amoebocyte Lysate (LAL) assay. OMV final lot samples were tested in a fivefold dilution and contained 126 EU ml⁻¹. The endotoxin activity is within the range of DPT/polio vaccines and is therefore regarded as safe. The endotoxin activity of LPS in vesicles is much lower than that of free purified LPS. On the basis of the results of the pyrogenicity test, no temperature raises were seen, thus, indicates the nonpyrogenic OMV extract.

According to the SDS-PAGE result, PorA as a major immunological protein of *N.meningitidis* serogroup A OMV was located on 35-40 kDa (Figure 2). The 35-40 kDa region

in the SDS-PAGE identified as PorA by reaction with monoclonal antibody against PorA (Figure 3).

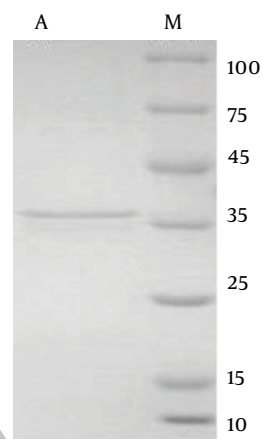


Figure 2. The SDS-PAGE Pattern of OMV Contains PorA. M: Marker, A:PorA

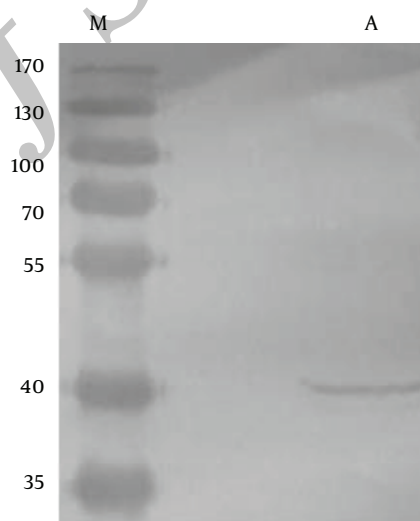


Figure 3. Western Blotting. M: Marker, A: PorA

4.2. Quantitation of OMV-Specific IgG Antibodies by ELISA

Quantitation of IgG responses towards OMVs from group A meningococci CSBPI, and G243 were performed for sera from BALB/c mice immunized with 5 µg of *N. meningitidis* serogroup A OMV. The result is shown in Figure 4.

4.3. Serum Bactericidal Assay

The bactericidal assay was performed quantifying complement mediated bactericidal activity of sera against *N. meningitidis* serogroup A CSBPI, G243. The result is shown in Figure 5.

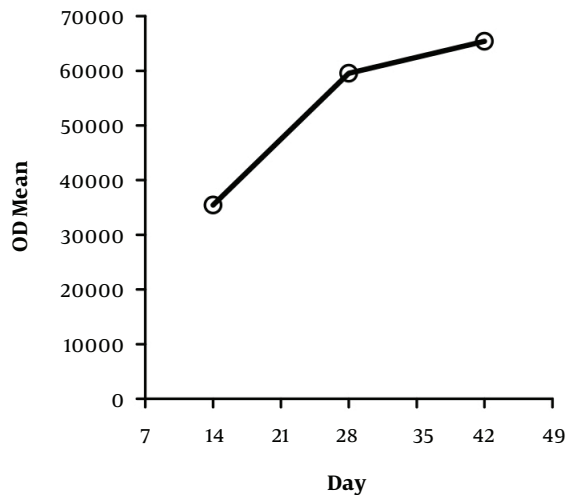


Figure 4. Total IgG Titers Elicited Against *N. meningitidis* Serogroup A OMV in Various Days.

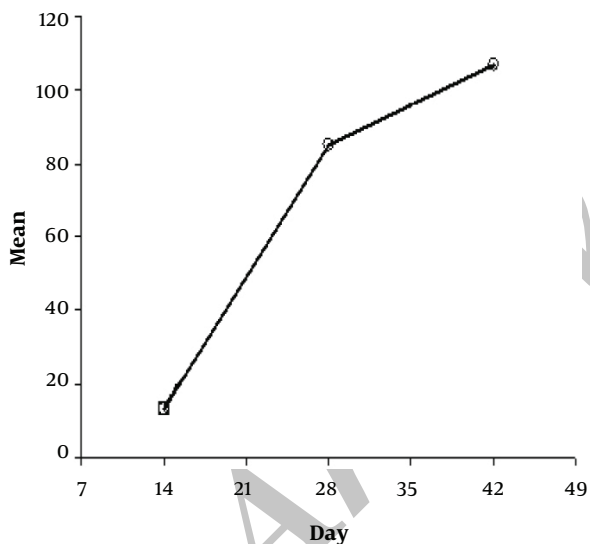


Figure 5. Serum Bactericidal Antibody Titers Against Meningococci Serogroup A

5. Discussion

N. meningitidis serogroup A causes most meningitis epidemic in Sub-Saharan countries in Africa and also in the Middle East (1, 2). The favorable intervention to prevent these epidemics would be an effective vaccine which induces long-term protection in all age groups. The group A polysaccharide vaccine is strongly protective in the first year after vaccination in adults and children above 5 years. It is recognized as effective for epidemic control purposes, but there is dispute about whether it should be

recommended in routine immunization programs. Thus, development of alternative protein-based vaccines has been encouraged.

One of the alternatives is OMV which can be prepared by detergent extraction from meningococci. The composition of OMV is complex. The main constituents are PorA, LOS, Residual DNA and residual detergent. The interaction of these compounds with each other through various noncovalent forces determines the physicochemical properties and stability of OMV (6, 7). The first OMV-based vaccines were developed by the Finlay Institute, Cuba and the National Institute of Public Health in response to the group B meningococcal outbreaks in these countries (2).

The OMV contains major class 1, 2, 3 proteins (porins). PorA and PorB are the major proteins in the *N. meningitidis* outer membrane. The OMV contains PorA which has the potency to elicit all the IgG subclasses. Classen *et al.* in 1996 introduced sequential Deoxycholate-Ultracentrifuge to prepare OMV from *N. meningitidis* serogroup B (12). In the same study, Siadat *et al.* prepared meningococci serogroup B OMV, and showed a significant serum bactericidal activity in hyper immunized rabbits after 3 times of injection against these bacteria (10, 11).

In this study, OMVs were prepared from *N. meningitidis* serogroup A (CSBPI, G243) grown in the chemically defined Frantz growth medium. After OMV extraction by sequential Deoxycholate-Ultracentrifuge method, 0.1mg/ml protein was prepared. In electron microscopy considerations more than 70% of OMVs were intact. The result was compatible with Classen results (12). The electrophoretic pattern of PorA (major immunological compound) on the SDS-PAGE was in the range of 35-40 kDa, while the Classen *et al.* the same as Siadat *et al.* results showed *N. meningitidis* serogroup B PorA locates on 40-45kDa (10, 12).

One of the advantages of this study was the lowest range of LOS in the OMV extract. In the extraction process Deoxycholate was substituted dramatically with LOS. It has been reported that the LOS in low range has immune response stimulating properties. The LAL results show that the OMV extract was safe to be used in biological products. The pyrogeny test results show lack of pyrogenic determinants in OMV extract.

Western blot confirms the presence of PorA in the range of 35-40 kDa. The ELISA results showed that antibody (IgG) titer increased (1.6 fold) after the second dose of *N. meningitidis* serogroup A OMV on day 28. Serum bactericidal assay showed that OMV induced high level of bactericidal antibodies against serogroup A after the second dose. The clear booster effects were seen after the third dose on day 42. The integrity of OMV seems to be essential to achieve bactericidal activity against meningococci serogroup A strain. This study confirms previous results that, OMVs based vaccine from *N. meningitidis* is highly immunogenic in mice, capable of inducing IgG and bactericidal antibodies, thus it can be a suitable candidate as

N. meningitidis serogroup A vaccine.

As we previously described the potential activity of OMV as a vaccine candidate, the OMV could be applied as an adjuvant with microbial origin (9). A number of scientific reports have demonstrated the adjuvant properties of *N. meningitidis* serogroup B OMVs. We have shown the adjuvant properties of *N. meningitidis* serogroup B OMV for inducing an immune response against the three prevalent serogroups of *N. meningitidis* (19). In a recent report, we further demonstrated the potency of noninfectious virus-like particles (mzNL4-3 VLPs) mixed with *N. meningitidis* serogroup B OMV to induce humoral and cellular responses against HIV-1 (20).

In conclusion, it is well known that meningococcal OMVs are effective and relatively safe vaccines, as demonstrated in several clinical trials. In addition, we have shown its capability to strongly induce an immune response when co-administered as a general adjuvant. Thus, the availability of such an OMV component would be of great importance for the development of subunit, combined and conjugate vaccines for a wide variety of meningococcal diseases.

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