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Evaluation of Serum Bactericidal Activity Specific for *Neisseria meningitidis* Serogroup A and B: Effect of Immunization with *Neisseria meningitidis* Serogroup A Polysaccharide and Serogroup B Outer Membrane Vesicle Conjugate as a Bivalent Meningococcus Vaccine Candidate

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Abstract: Bacterial meningitis caused by different groups of *Neisseria meningitidis* is still one of the serious health problems world wide. The Serum Bactericidal Assay (SBA) to meningococci is the most important test in immunological evaluation of meningococcal infection after vaccination. The SBA has been adapted as the gold standard for immunity against different serogroups of *Neisseria meningitidis* after immunization. Capsular polysaccharide of serogroup A meningococci (CPSA) was conjugated to Outer Membrane Vesicle (OMV) from a serogroup B in order to test the possibility of obtaining a bivalent serogroup A and B meningococcus immunogen. The conjugate was prepared with adipic acid hydrazide (ADH) and 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDAC). The conjugate and control were injected intramuscularly into groups of five rabbit with boosters on days 14, 28 and 42 after the primary immunization. The following groups were used as control: 1-CPSA plus OMV; 2-CPSA; 3-OMV and 4-normal saline. The serum collected on days 0, 14, 28, 42 and 56 were tested by complement mediated bactericidal assay according to the world health organization protocol. The results of SBA in the glycoprotein conjugate group revealed a significant increase in serum bactericidal titer against serogroup A meningococci after 56 day in comparison with the CPSA and OMV control group. Bactericidal titer against serogroup B meningococci of the conjugate showed no significant difference in comparison with the OMV containing control. The results indicate that when polysaccharide A and OMV are in a covalent conjugate form, the complex is able to induce a high level of bactericidal antibody response. Therefore, this study shows that the CPSA--OMV conjugate could be a candidate for bivalent vaccine toward serogroup A and B meningococci.

Key words: *Neisseria meningitidis* serogroup A and B, conjugate vaccine, serum bactericidal activity, OMV

INTRODUCTION

Meningococcal disease remains as a significant global public health problem with approximately 1.2 million cases per year causing on estimated 135,000 deaths. Serogroups A, B and C account for most cases of meningococcal diseases throughout the world, with serogroups B and C responsible for the majority of cases in Europe and the Americas and serogroups A and C predominating throughout

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Asia and Africa (Rosenstin *et al.*, 2001). In addition, the meningitis belt is widening and spreading to cities within Africa and to other continents (Jodar *et al.*, 2002). However, hyperendemic disease caused by serogroup A was observed in Finland and Russia as recently as 30 years ago and in New Zealand in the 1980s and several pandemics have been described that originated in China. A recent study warns of the possible emergence of serogroup A in Greece (Ruggeberg and Pollard, 2004; Cartwright, 2001). Although CPSA vaccine confers immunity at all ages, the improved immunogenicity of a conjugate and its compatibility with the world health organizations extended program on immunization offers advantages over CPSA alone (Lesinski and Westerink, 2001; Jin *et al.*, 2003). Henceforth, disease caused by serogroup B strains remains an unsolved health problem in many part of the world and the lack of a serogroup B meningococcal vaccine is a serious public health limitation since these strains account for approximately one-third of meningococcal disease in North America and up to 80% in North Europe (Milagres *et al.*, 1998; Cartwright, 2001). During the 1960s, polysaccharide vaccines were developed against serogroups A, C, W135 and Y and these have been shown to be immunogenic in human being. Yet the immune response to these polysaccharide vaccines provides only limited protection for children under the age of 4 years of age, an age group which has significant disease burden, due to nature of their immune response (Lindberg, 1999). To overcome this limitation, monovalent group C conjugates are developed and widely used from 1999 in most European countries, Australia, Canada. Tetravalent conjugate vaccines are licensed in US and Canada (Schmidt *et al.*, 2001; Bethell and Pollard, 2001). Serogroup A and C polysaccharide vaccines were first shown to be immunogenic and protective in humans by Jodar *et al.* (2000). Since then antibody responses to these capsular polysaccharide vaccines have been measured by various serological methods. Serum Bactericidal Antibody (SBA) activity has been shown to correlate well with both natural and vaccine-induced immunity to meningococcal disease. The induction of complement-dependent bactericidal antibodies after vaccination with meningococcal polysaccharide or conjugate vaccines has, therefore, been widely accepted as evidence of the potential efficacy of these vaccines. Thus, a functional serological surrogate of vaccine efficacy exists that can be used to inform decisions about the licensure and implementation of meningococcal polysaccharide and conjugate vaccines. The original assays that associated bactericidal antibody with protection used human complement sources from individuals after natural infection, left permanently impaired by deafness or mental retardation. Serum bactericidal activity continues to be considered the most important response mechanism against this infection and so it is considered the most important parameter to be analysed when testing antigens to be used in vaccines (Jensen *et al.*, 2000; Jodar *et al.*, 2000; Holst *et al.*, 2003).

Present aim with this study have shown that *N. meningitidis* serogroup A capsular polysaccharide-serogroup B outer membrane vesicle conjugate induced complement dependent bactericidal antibodies against serogroup A and serogroup B meningococci.

MATERIALS AND METHODS

Reagent

1-ethyl-3-(3 dimethylaminopropyl) carbodiimide (EDAC), adipic acid dihydrazide (ADH), 1-cyano-4(dimethylamino) pridium tetrafloroborate (CDAP), trinitrobenzen sulfonic acid (TNBS), agarose, Bovine Serum Albumin (BSA), EDTA, L-cystein, L-glutamic acid, dextran were of Sigma chemical Co., St. Louis, Mo., Sepharose CL-4B and Sephadex G-50 were purchased from Pharmacia, triethylamine (TEA) was from Pierce, Rockford (T.J. Barker, Inc.), acetonitrile dialysis membrane (cutoff, 6000 to 8000) were purchased from Spectra.Pro, Calif, respectively Pyrogen-Free Water (PFW) and Pyrogen-Free Saline (PFS) were used in all experiments.

Preparation of OMVs and CPSA

OMVs were prepared as described previously (Siadat *et al.*, 2006a). In brief, *N. meningitidis* serogroup B strain (CSBPI,G-245) was grown under controlled-submerge cultural condition in

fermentor containing modified Frantz medium at 36°C for 24 h up to early stationary phase. Outer Membrane Vesicles (OMVs) were extracted in 0.1 M Tris-HCl, pH 8.6, 10mM EDTA and 0.5%w/v deoxycholate. Purification of OMVs was done by sequential centrifugation at 20,000 g 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 (100 mg L^{-1}) was added as preservative (Claassen *et al.*, 1996; Norheim *et al.*, 2005; Siadat *et al.*, 2006b). Capsular Polysaccharide of Serogroup A meningococci (CPSA) obtained from the department of bacterial vaccine and antigen production, Pasteur Institute of Iran. *N. meningitidis* serogroup A (CSBPI,G-243) was cultivated on a modified Frantz medium and their CPSAs were purified according to the World Health Organization (WHO) protocol (1976).

Synthesis of CPSA-OMV conjugates

Activation and Derivatization of CPSA

CDAP was made at 100 mg mL^{-1} in anhydrous acetonitrile and stored at -20°C for one month. CDAP (1 mg mg^{-1} of polysaccharide) was slowly pipetted into a vortexed solution of polysaccharide (PS) (10 mg mL^{-1}) and 30 sec later, a volume of 0.2 M TEA equal to that of CDAP was added. After 150 sec an equal volume of 0.5 M ADH and 0.1 M EDAC in 0.5 M NaHCO_3 were added and the mixture was tumbled for 20 h at 4°C . The reaction mixture was passed through a column of Sephadex G-50 ($2.5 \times 90 \text{ cm}$) in PFW and fractions of (each) 2.5 mL were collected, the peak was pooled, dialyzed against PFW as above (Jin *et al.*, 2003). All the reactions were performed in triplicate.

CPSA AH Conjugates of OMV

Ten mg of OMV (10 mg mL^{-1}) and EDAC at a final concentration of 0.1 M were added. The reaction mixture was tumbled gently overnight at $3-8^\circ\text{C}$ and then centrifuged at $16,000 \text{ g}$, 4°C for 20 min, the supernatant was passed through a CL-4B sepharose column ($1.5 \times 90 \text{ cm}$) that was equilibrated with 0.2 M ammonium acetate. The fractions of (each) 0.5 mL were collected and the peak was pooled, dialyzed against PBS, pH 7.0, at $3-8^\circ\text{C}$ and passed through a 0.45 nm membrane and stored at $3-8^\circ\text{C}$ (Fukasawa *et al.*, 1999; Schneerson *et al.*, 1980; Gupta *et al.*, 1995).

Physicochemical Analysis of Conjugates

Analytical Methods

Protein content of OMVs was measured according to Peterson (1977). Hestrin (1949) method was used to estimate O-acetyl groups present in conjugates of CPSA. The hydrazide content of the derivatized polysaccharide was estimated by trinitrobenzen sulfonic acid (TNBS) method and is expressed as mol of adipic hydrazide (AH)/mol (Schneerson *et al.*, 1980).

Bioassay

The toxicity of LPS content in the conjugates was assayed by the limulus amoebocyte lysate test and expressed in endotoxin units related to the U.S. standard. Also, the pyrogenicity of the conjugates was assayed in rabbits (Claassen *et al.*, 1996; Anonymous, 1995).

Electron Microscopy

Outer membrane vesicles integrity was checked by electron microscopy. OMVs were ultrasonically treated to disperse the vesicles and were attached to Formvar/carbon-coated nickel grids. Grids were washed with a 0.01M 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 at pH 6.0. The grids were examined in a Zeiss CEA902A electron microscope at 80 Kv (Claassen *et al.*, 1996; Siadat *et al.*, 2006b).

Immunization Procedures

New Zealand white rabbits weighing 2-2.5 kg each were used for the study (Jenning and Logowski, 1981). Five groups of 5 animals were injected intramuscularly with preparations in saline and Freund's complete adjuvant:

Group 1: 0.2 mL of conjugates, that containing 40 µg protein and 50 µg CPSA, with 0.5 mL Freund's complete adjuvant.

Group 2: 0.0333 mL OMVs (containing 40 µg protein) plus 0.2 mL CPSA (containing 50 µg polysaccharide) with 0.5 mL Freund's complete adjuvant.

Group 3: 0.2 mL CPSA (containing 50 µg polysaccharide) with 0.5 mL Freund's complete adjuvant.

Group 4: 0.0333 mL OMVs (containing 40 µg protein) with 0.5 mL Freund's complete adjuvant.

Group 5: Saline control.

Rabbit of all five groups were boosted with the same preparation 14, 28 and 42 days after the first immunization. Rabbits were bled at 0, 14, 28, 42 and 56 days after the first injection. Sera were pooled and stored at -20°C.

Complement-Mediated Bactericidal Assay

Anti-meningococcal B bactericidal assay was carried out using the standardized meningococcal serogroup B strain CSBPI, G-245 in 96-well plates, as previously described. Briefly Muller Hinton Broth (MHB) was inoculated with 5 to 10 colonies from a fresh culture of *N. meningitidis* and incubated for 2 h at 37°C with shaking. Approximately 100-200 mL of the stock bacterial cell suspension was added to 20 mL of sterile MHB pre-equilibrated at room temperature to yield an A₆₀₀ between 0.07 and 0.08. The culture flask was then incubated for approximately 2 h at 37°C with 160 rpm shaking until A₆₀₀ was between 0.23-0.24. This yielded approximately 1.10^9 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 (assay buffer) until a concentration of 1.10^5 CFU/mL was reached (bacterial working concentration). All rabbit sera to be tested were heat inactivated for 30 min at 56°C. Pooled sterile baby rabbit serum (3-4 weeks old;) without bactericidal activity against the strains to be tested was used as exogenous complement source in this assay. A sterile polystyrene U bottom 96 well microtiter plate was used for the micro serum bactericidal assay. The total volume of each well of the plate was 50 µL with 25 µL of serially diluted serum in assay buffer (final starting dilution 1:4), 12.5 µL of bacteria, 12.5 µL of complement (final concentration 20% (v/v) in assay buffer). Controls included samples with buffer, bacteria and complement and samples with serum, bacteria and buffer. A known positive serum sample (either monoclonal or polyclonal was included in each assay; the acceptable limit of variability was 1 well dilution). After all components were added to each well of the plate a 10 µL aliquot was taken of the control samples with buffer, bacteria and complement and pipetted onto a dry square formed GC agar plate containing 1% IsoVitaléX and allowed to run in lanes down the plate. The microtiter plate was then incubated for 1 h at 37°C. The GC agar plate with 1% IsoVitaléX was incubated 18 h at 37°C in 5% CO₂. After 1 h incubation a 7 µL aliquot was taken from each well of a lane using a multi-channel pipette and spotted onto a dry square formed GC agar plate containing 1% IsoVitaléX. After 18 h incubation at 37°C in 5% CO₂, the colonies on time zero and 60 min incubation plates were counted. The average number of CFU at time zero was used as 100%. The serum bactericidal titer was reported as the reciprocal of the serum dilution yielding ≥50% killing. Assays were made in duplicate. Anti-meningococcal serogroup A bactericidal assays were carried out using a meningococcal serogroup A strain CSBPI,G-245 and performed as described above (Peeters *et al.*, 1999).

Statistics

Groups of rabbit were compared by analysis of variance (ANOVA) according to the distribution of log titers for serum bactericidal assay against serogroup A and B. Statistical significance was defined as a P-value of <0.05 (Jin *et al.*, 2003).

RESULTS AND DISCUSSION

Characterization of Conjugate

Biochemical Assay

Ten mg of both protein and CPSA were used in the conjugation reaction.

The O-acetyl content of CPSA and CPSACDAP AH were 2.7 mmol mg⁻¹ of protein and 1.14 mmol mg⁻¹ of protein. Employing TNBS method, CDAP-activated CPSA AH had 1.05% (mean value) AH. The yield of conjugate, was 45-48% in terms of the recovery of CPSA in the conjugate. The immunogenicity of polysaccharide component has been related to their molecular weight, the density of carbohydrate on the carrier and the intactness of carrier protein (Costantino *et al.*, 1999; A da and Isaacs, 2003). Exclusively, the immunogenicity of CPSA, a linear homopolymer of (1-6)- α -D-ManpNAc-1-PO4 O-acetylated at C-3, is related to its molecular size and its O-acetyl content (Liu *et al.*, 1971). CPSA is comparatively unstable due to the ability of its phosphodiester bond to be hydrolyzed. Hydrolysis of this bond is favored by alkaline and acidic conditions and elevated temperature (Gudlavalleti *et al.*, 2004). For this reason, CPSA vaccine is stored at 4°C in (freeze-dried) state with a carbohydrate that competes for the residual moisture in order to prevent hydrolysis and preserve its immunogenicity. CDAP-mediated activation at pH 8.0 is preferred over activator, which requires pH levels of = 10.5, as shown by the lesser effect on the molecular size of CPSA. CDAP activation at close to neutral pH makes it useful for pH-sensitive CPSA (Jin *et al.*, 2003). Berry *et al.* (2002) showed the essential role of O-acetyl in eliciting maximal levels of CPSA antibody production. Activation of CPSA by CDAP retained most of its O-acetyl content. Probably due to the ability of CDPA to activate CPSA at a pH closer to neutrality, the O-acetyl level was higher after CPSA activation than other activator (Jin *et al.*, 2003; Siadat *et al.*, 2006b). For this reason the average yield of CPSACDAPAH-OMV in this process was high.

Electron Microscopy

Electron microscopy was used in order to verify the integrity of the OMV after preparation and conjugation. The size of OMV ranged from 70 to 120 nm in this process (Fig. 1A). Intactness of the vesicles in these preparations ranged from 50 to 80% of the vesicles. Figure 1B shows that the OMV maintained its original conformation even when coupled with CPSA. This evidence indicates that the carrier protein (OMV) with intactness form is an important factor for induction of immunogenicity where the polysaccharide component has to be protected (Hossany *et al.*, 2004; Constantino *et al.*, 1999; Lesinski and Westerink, 2001; Mond *et al.*, 1995).

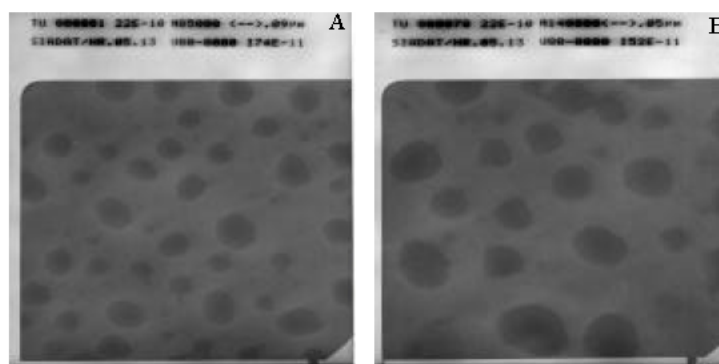


Fig. 1: Electron microscopy of Outer Membrane Vesicle (OMV) stained with 1% potassium phosphotungstate. (A) Control vesicles. (B) CPSA conjugated vesicles

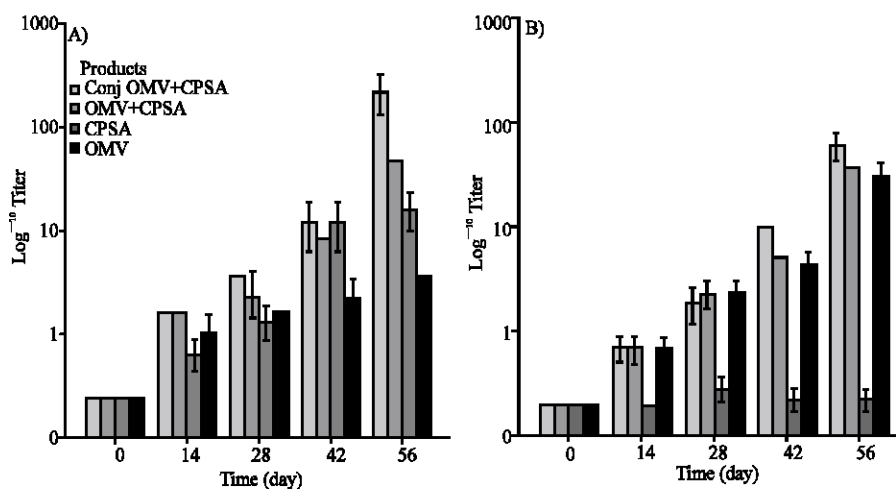


Fig. 2: Anti-meningococcal serogroup A (CSBPI,G-243) (A) and serogroup B (CSBPI,G-245) (B) bactericidal activity of rabbit sera after immunization with four doses of different vaccines

Biological Activity

The biological activity of the endotoxin was determined by limulus amoebocyte lysate (LAL) assay. Conjugate of final lot samples were tested in a five fold dilution containing 677 and 135 EU mL, respectively, when compared with *E. coli* standard endotoxin. 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 (Claassen *et al.*, 1996).

On the basis of the results obtained by pyrogenicity test, temperature rises of 1:300 and 1:1000 dilutions were comparable with those obtained with the placebo (data not shown). One: thousand dilution of the vaccine corresponds to those required for 23-valent pneumococcal polysaccharide vaccine and mono-, bi- and tetravalent meningococcal polysaccharide vaccines. The OMV was chosen instead of OMP, because of the poor immunogenicity of purified OMP, as compared to OMV and may be explained by the absence of LOS in these preparations, which has been reported to have immune response stimulating properties (In principal, OMV combines easy production procedures with potent delivery adjuvant characteristics) (Bethell and Pollard, 2002; Jensen *et al.*, 2000; Fukasawa *et al.*, 2004). The vaccine was also found to be safe for human use although LPS is present in a 10% w/w ratio as compared to OMP. It is known that the OMV structure has a shielding effect on the endotoxin properties of LPS (Claassen *et al.*, 1996; Anderson *et al.*, 1994; Cartwright *et al.*, 1999; Siadat *et al.*, 2006b).

Bactericidal Assay of CPSA-OMV Conjugate

The bactericidal assay was performed quantifying complement mediated bactericidal activity of rabbit sera against serogroup A CSBPI,G-243 (Fig. 2A) and serogroup B CSBPI,G-245 (Fig. 2B). CPSA-OMV conjugate induced high levels of bactericidal antibodies against the serogroup A strain after the second dose. The clear booster effects were seen after the third dose in 42 days and strongly after the fourth dose in 56 days (Fig. 2A). The bactericidal titer against the serogroup A strain was relatively high in the group of rabbit injected with CPSA or CPSA plus OMV, but in the comparison of two group showed insignificant (Fig. 2A). Regarding serogroup B, the CPSA-OMV conjugate as well as control, CPSA plus OMV and OMV induced bactericidal antibodies through in different amounts. As shown in Fig. 2 B, similar high titers were achieved with the CPSA-OMV conjugate, the

CPSA plus OMV and OMV control after two vaccine doses but in the CPSA-OMV conjugate group, the bactericidal titer was lower than that induced by the CPSA plus OMV and OMV control and it is significant. However, after three doses the CPSA-OMV conjugate and all the OMV containing control showed the high and significant titer. The bactericidal titer against the serogroup B strain was low and insignificant in the group of rabbit injected with CPSA. Among OMV proteins, class 1, named porin A, is the main protein involved in the bactericidal antibody induction, both in the animal model and in humans and it seems that class 1 protein induction was not by the conjugation (Arigita *et al.*, 2004; Claassen *et al.*, 1996; Jensen *et al.*, 2000). The bactericidal assay against serogroup A and B meningococcal strains demonstrate that it may be possible to produce a A/B conjugated vaccine. The integrity of OMV seems to be essential to achieve bactericidal activity against the serogroup B strain (Anderson *et al.*, 1994; Cartwright *et al.*, 1999; Claassen *et al.*, 1996; Vella *et al.*, 1990), since in a conjugate reaction in which the vesicles were damaged, the product failed to induce bactericidal activity against serogroup B, although this conjugate kept a similar bactericidal titer against serogroup A compared to undamaged OMV vaccine. An unexpected result shown in this paper is the observation that the serum from rabbit injected with the OMVs alone, derived from serogroup B strain, was able to induce a low but significant level of bactericidal antibodies against serogroup A strain. The probable explanation would be a cross-reactivity achieved from antigen similarity in the OMVs derived from serogroup B strain with serogroup A strain.

It has been well demonstrated that the polysaccharide must be conjugated with a carrier protein to induce antibodies in an efficient way (Ada and Isaacs, 2003; Perez-Melgosa *et al.*, 2001). This research shows that when polysaccharide and OMV are in covalent conjugate form, the complex is able to induce a high level bactericidal antibodies against serogroup A bacteria and a booster effect in this assay. We should remember that immunologic memory response to meningococcal serogroup A vaccination, both unconjugated and conjugate vaccine, have been evaluated using both the response to a polysaccharide challenged and avidity indices. It is interesting to note that the serogroup A capsular polysaccharide dose does not appear to be a traditional T-cell-independent antigen as is the serogroup C capsular polysaccharide. First, serogroup A unconjugated vaccine has been shown to be immunogenic in children under 2 years of age. Second, the serogroup A polysaccharide appears to stimulate affinity maturation, as indicated by an increase in avidity indices following vaccination. However, it does appear that there is a difference in the quality of the response induced if the serogroup A polysaccharide is conjugated or unconjugated. A great level of bactericidal antibody is produced following serogroup A polysaccharide conjugation and the memory response appears to be sustainable (Balmer and Borrow, 2004). The bactericidal assay against serogroup A and B meningococcal strains demonstrate that it may be possible to produce a A/B conjugated vaccine.

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