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Measurement of opsonophagocytic activity of antibodies specific to *Neisseria meningitidis* serogroup A capsular polysaccharide-serogroup B outer membrane vesicle conjugate in animal model

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Abstract - *Neisseria meningitidis* is efficiently phagocytosed by polymorphonuclear leukocytes (PMN_S) following opsonization with opsonic antibodies; opsonophagocytosis is the primary mechanism for clearance of meningococci from the host. Thus, in testing meningococcal vaccines, the level of opsonophagocytic antibodies appears to correlate with vaccine-induced protection. Our previous studies demonstrated that the conjugation of *N. meningitidis* serogroup A capsular polysaccharide (CPSA) to serogroup B outer membrane vesicle (OMV) could induce a high level of bactericidal antibody response against serogroup A meningococci in animals. The purpose of this study was to evaluate opsonophagocytic activity of the conjugate of CPSA to OMV (CPSA-OMV). In order to evaluate the potential efficacy of CPSA-OMV a flow cytometric opsonophagocytic assay was used. The conjugate and controls were injected intramuscularly into four groups of rabbits with boosters on days 14, 28 and 42 following primary immunization. The rabbits were bled prior to injection and two weeks after each injection. Opsonophagocytic activity of antibodies in hyperimmune sera through rabbit PMN_S were measured with flow cytometer, using dihydrorhodamine-123 as a probe. The results indicated that our conjugate could induce a highly significant level of opsonophagocytic activity against serogroup A meningococci after 56 days compared to the control groups ($P<0.05$). We conclude that this conjugate represents a vaccine candidate against serogroups A and B meningococci after further investigation.

Key words: *Neisseria meningitidis* serogroups A and B; glycoconjugate; opsonophagocytosis; flow cytometric assay; outer membrane vesicle.

INTRODUCTION

Meningococcal disease continues to be a significant worldwide cause of morbidity and mortality (Rosenstein *et al*., 2001; Siadat and Norouzian, 2007). Specific antibodies and a functional complement system play a crucial role in the host defense against systemic meningococcal infections (Siadat and Norouzian, 2007, Balmer and Borrow, 2004). *Neisseria meningitidis* is efficiently phagocytosed by polymorphonuclear cells (PMN_S), and antibodies are bactericidal in the presence of peripheral blood polymorphonuclear leukocytes and complement (Aase *et al*., 1998; Martinez *et al*., 2002). Consequently, patients harbouring defects in terminal complement pathway are highly susceptible to meningococcal disease, especially to meningococci of uncommon serogroups (Martinez *et al*., 2002; Rezaei *et al*., 2007). However, the presence of serum opsonins to facilitating phagocytic killing seems to be of great importance in the *in vivo* defence against their organisms. Previous studies have demonstrated that serogroup B meningococci is more resistant to bactericidal killing in comparison to serogroup A and C meningococci, though are highly susceptible to killing by PMN_S following opsonization (Aase *et al*., 1998). To engage in deposition of C3b, iC3b and C4b and ligation to the corresponding receptors on the phagocytes complement-mediated opsonization requires activation through C3. A synergistic opsonic effect is achieved once phagocytes are triggered through both complement receptors and Fcγ receptors (FcγRs). The potential for an antibody to bind FcR_S or activate the complement cascade is strongly dependent on the antibody isotype. IgM antibodies are powerful activators of the complement cascade, nonetheless, they probably cannot be induced through respiratory burst (RB) by FcR binding, as there are few FcμRs on neutrophils. Also, different IgG subclasses exhibit individual patterns regarding these effector functions. IgG antibodies

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bound to bacteria are also excellent opsonins, and a synergistic opsonic effect is achieved once the target is covered with both IgG and complement split products. PMN_S and macrophages constitutively express FcγRs and complement receptors (Aase *et al*., 1995, 1998). An immune response in which the effector function is based on phagocytosis rather than bacteriolysis may be beneficial to the host, since intracellular destruction could minimize intravascular release of bacterial endotoxin and thus reduce the risk for septic shock (Aase *et al*., 1998). Therefore, *in vitro* opsonophagocytic activities (OPA) of antibodies against meningococcal components are believed to reflect their in vivo functional activity (Vakevainen *et al*., 2001). Thus, to determine the serological correlates or surrogates of protection raised by the samples applied in ongoing efficacy trials, quantitative and qualitative characteristics of antibodies both have to be measured by reliable means (Romero-Steiner *et al*., 2006).

The present study is an attempt to measure the induction potency of opsonic antibody response by a bivalent conjugates of *N. meningitidis* serogroup A capsular polysaccharide (CPSA) and serogroup B outer membrane vesicle (OMV) using flow cytometric assay. Using complement-inactivated test sera we added a constant amount of an external complement source to each sample to quantitate the opsonic activity in the immunized serum samples.

MATERIALS AND METHODS

Preparation of OMVs and CPSA. OMVs were prepared as previously described (Claassen *et al*., 1996; Siadat *et al*., 2006). In brief, *N. meningitidis* serogroup B strain CSBPI, G-245 cells were grown in 40 L modified Frantz medium in a fermenter at 36 °C for 24 h. The OMV was extracted from the cells by the method of Claassen *et al.* (1996). The CPSA were obtained from the Department of Bacterial Vaccines and Antigen Production, Pasteur Institute of Iran (Tehran, Iran). *Neisseria meningitidis* serogroup A strain CSBPI, G-243 was cultivated on a modified Frantz medium, and its CPSAs were purified according to the World Health Organization protocol (WHO, 1976).

Synthesis of CPSA-OMV conjugates. As previously described, the polysaccharide derivatives of meningococcal serogroup A were conjugated with *N. meningitidis* serogroup B OMV using adipic acid dihydrazide (ADH) as linker and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) as coupling reagent (*Siadat et al*., 2007c). Briefly, the obtained polysaccharide (10 mg/ml) was activated by 1-cyano-4(dimethylamino) pyridium tetrafluoroborate (CDAP) (1 mg/mg of polysaccharide). Then, the activated polysaccharide was treated with 0.5 M adipic acid dihydrazide (ADH) and 0.1 M EDAC to produce an adipic hydrazide derivative (AH). The reaction mixture was passed through a column of Sephadex G-50 (2.5 x 90 cm) and fractions of 2.5 ml were collected. The peak was pooled and dialyzed against PFW as above (Jin *et al*., 2003; Siadat *et al*., 2007a). OMV (10 mg/ml) and EDAC at a final concentration of 0.1 M were added. The reaction mixture was tumbled gently overnight at 3-8 °C and then centrifuged at 16000 *x g*, 4 °C for 20 min; the supernatant was passed through a CL-4B Sepharose column (1.5 x 90 cm) equilibrated with 0.2 M ammonium acetate. Fractions of 0.5 ml were collected and the peak was pooled, dialyzed against PBS, pH 7.0, at 3-8 °C, and passed through a 0.45 µm membrane and stored at 3-8 °C (Schneerson *et al*., 1980, Siadat *et al*., 2007c).

Immunization procedure. Four groups of New Zealand white rabbits (each group consisted of five rabbits) were injected intramuscularly four times at 2-week intervals with CPSA-OMV (40 µg of protein and 50 µg of polysaccharide), CPSA (50 µg of polysaccharide) and OMV (40 µg of protein) in 0.2 ml of 0.9% NaCl with Freund's complete adjuvant. The rabbits were bled prior to injection and 2 weeks after each injection (Siadat *et al*., 2007a).

PMNs. Venous blood samples from healthy rabbits were drawn into heparinized vacuum tubes and the erythrocytes were lysed with a solution containing 8.3 mg/ml of NH_4Cl, 1 mg/ml of $NaHCO_3$, and 0.08 mg/ml of EDTA (pH 6.8) per ml. The leukocytes were washed twice with Hanks balanced salt solution (HBSS) using 0.2% bovine serum albumin (BSA), and the cell concentration was adjusted to 50 x 10^3/ml. No additional purification of PMN was required, as to discriminate PMN_S from other cells within the suspension, further analyses were done by flow cytometry (Aase *et al*., 1998).

Complement source for Respiratory Burst (RB) assay. Normal rabbit sera with no detectable antibody against *N. meningitidis* serogroup A CSBPI, G 243 and serogroup B CSBPI, G245 were used as the source of complement for opsonization of meningococci. We found no antibodies against the meningococcal antigens measured at the serum dilutions starting at 1:20 in an ELISA with whole-cell *N. meningitidis* as the antigen and developed with an alkaline phosphatase-conjugated anti-IgG. The complement source also did not give any measurable RB to the CSBPI, G 243 and CSBPI, G 245 strains. The serum was aliquoted in small volumes, stored at -85°C and thawed immediately before use (Aase *et al*., 1998, Siadat *et al*., 2007b).

Bacteria to be used in Respiratory Burst assay. *Neisseria meningitidis* serogroup A CSBPI, G-243 and serogroup B CSBPI, G-245 were grown as described previously for SBA, fixed in 70% ethanol overnight at 20 °C, and washed in HBSS. The concentration of bacteria was adjusted to 10^3 CFU/ml, aliquoted in 1 ml ampoules and stored at –85 °C (Aase *et al*., 1998).

Respiratory Burst assay. The RB analysis was performed mainly as described by *Aase et al*. (1998) with some modification (Fleck *et al*., 2003; Li *et al*., 2003; Siadat *et al*., 2007b). All the sera to be tested were heated to 56 °C for 30 min to inactivate the endogenous complement. Fifty microlitres of a threefold dilution of sera was mixed with 5 ml of serogroup A meningococci (1.10^3 CFU ml) in U-bottomed microtiter plates and incubated at 37 °C for 30 min with continuous agitation. Then, as the complement source, 5 µl of serum was added and incubation continued at 37 °C for 8 min with agitation. Each dilution was tested in duplicate. As an indicator for RB, we used the nonfluorescent probe dihydrorhodamine 123 (DHR) that will be oxidized to fluorescent rhodamine 123 through RB process. The stock solution was prepared by dissolving 10 mg of DHR in 1 ml of dimethyl sulfoxide. The obtained solution was aliquoted and stored at –85 °C until use. The DHR solution was added to the effector cells to give a final concentration of 10 µg/ml just before mixing 50 µl of the effector cells with the opsonized bacteria, and the incubation continued at 37 °C for 8 min with agitation (Siadat *et al*., 2007b). Each sample was tested in duplicate. The reactions were stopped by placing the microtiter plates in an ice bath until RB was measured by flow cytometry. The assay was performed with *N. meningitidis* serogroup B as described above.

The PMA (Phorbol 12-Myristate 13-Acetate) working solutions were freshly prepared before each assay of RB. This solution (100 ng/ml) was prepared by 1:10 dilution of PMA stock (1 mg/ml) in PBS. The following protocol was used for measuring the natural function of PMA (as positive control).

The reaction was performed in 100 µl of whole-blood specimen. First, the DHR solution (50 mM) was added to each tube and incubated at 37 °C for 5 min. Then, the PMA solution (100 ng/ml) was added to each sample and the tube was further incubated at 37 °C for 10 min in water bath. Finally, the reaction was stopped by washing the cells once with 4% PBS (3 ml).The cells incubated with DHR only served as negative controls. For the evaluation of RB all the control samples were tested according to the above protocol (Li *et al*., 2003; Siadat *et al*., 2007b).

Flow cytometry. The samples were run on a flow cytometer (Coulter EPICS - XL-Profile, USA) with a 15 mW Argon laser. The excitation wavelength was 488 nm, and standard coulter filters were used in all the measurements. Neutrophils, monocytes and lymphocytes could be clearly defined and separated on the basis of forward scatter and sideward scatter characteristics. On the scatter histogram, a gate was set on the PMN, and RB was measured as the percent of positive cells within the gate with three-decade logarithmic amplification on the fluorescence detector. Thus, PMN could be easily discriminated from monocytes and lymphocytes (as well as bacteria) without any further purification of the leukocytes fraction. As a negative control, the test serum was omitted and replaced by HBSS containing BSA. This mixture was used to set the correct point on the fluorescent axis. About 2500 effector cells were counted in each sample, and all determinations were performed in duplicate. Phagocytic function and RB by rabbit PMN against *N. meningitidis* serogroups A and B were measured with flow cytometer, using dihydrorhodamine-123 as probe. In these experiments, the viable meninigococci, CSBPI G-243 serogrup A and CSBPI G-245 serogroup B, grown to log phase, were employed as the target cells, and DHR-123 primed PMNs taken from a healthy donor rabbit were used as the effector cells. The results were presented by summarizing the percent of RB positive PMNs at each sample. Activities bellow 10% for the evaluation of RB at any dilution was defined as zero (Lehmann *et al*., 2000; Fleck *et al*., 2003; Siadat *et al*., 2007b).

Statistics. To determine whether the differences were statistically significant, the data sets were subjected to analysis using repeated ANOVA and at $P \leq 0.05$.

RESULTS

Ten mg of both protein and CPSA were used in the conjugation reaction. The yield of conjugate was 45-48% in terms of the recovery of CPSA in the conjugate.

Performance of the Respiratory Burst assay

The immune responses of strain CSBPI G-243 serogroup A and CSBPI G-245 serogroup B meningococci, following vaccination, were measured by PMN- mediated RB. In the present study, RB results were calculated from the area under the histogram bars where each dilution is plotted on the absence or presence of fluorescence-positive PMN_S. The percent of positive PMN at each test was summarized to give ΣRB% indicating opsonophagocytic responses.

CPSA-OMV conjugate induced a highly significant increase in the opsonophagocytic responses (ΣRB %) against the serogroup A strain, 2 weeks after the first dose ($P < 0.05$). The clear booster effects were seen after the exposure of the second dose on the day 28 and strongly after the fourth dose on day 56. The ΣRB% against the serogroup A strain was relatively high in the group of rabbits injected with CPSA, but comparison of CPSA-OMV conjugate showed insignificant differences on the days 14, 28 and 42 ($P > 0.05$) (Fig. 1). Regarding the serogroup B, the CPSA-OMV conjugate as well as the OMV induced the different amounts of ΣRB%. As shown in Fig. 2, two weeks after the first dose, similar high ΣRB_S% were achieved with the CPSA-OMV conjugate and OMV controls, Nevertheless, in the CPSA-OMV conjugate group, the ΣRB% was lower than that induced by the OMV controls, and the difference showed to be significant. However, 2 weeks after the second dose, the CPSA-OMV conjugate and all the OMV_s containing controls displayed higher and significant titer ($P < 0.05$). The ΣRB% against the serogroup B strain was low in the group of rabbits injected with CPSA. Altogether, the results of quantitative flow cytometric analysis of rabbit PMN function in the hyperimmune sera harbouring glycoprotein conjugate revealed a highly

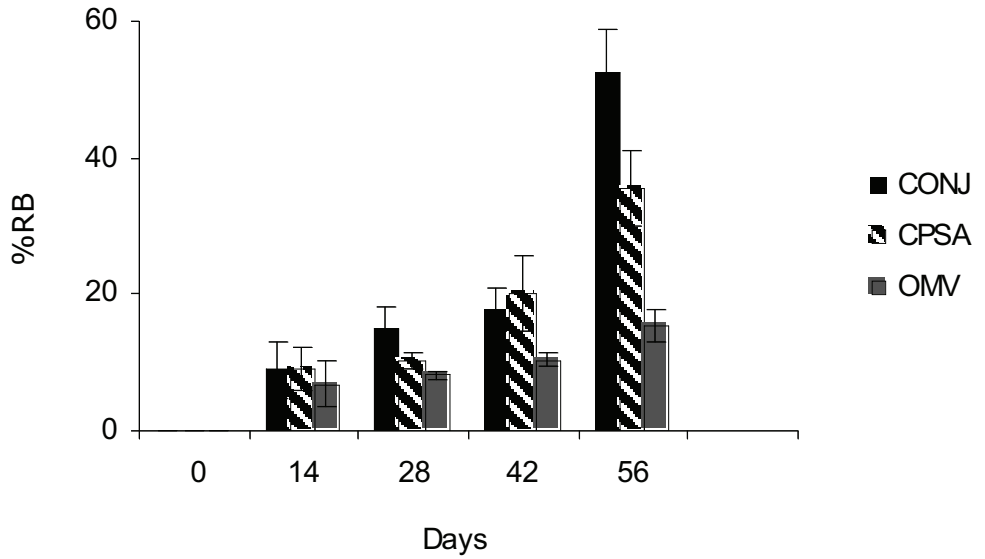

FIG. 1 - Opsonophagocytic activity of rabbit sera against *Neisseria meningitidis* serogroup A strain CSBPI, G-243 measured as respiratory bursts (RB) in PMNs.

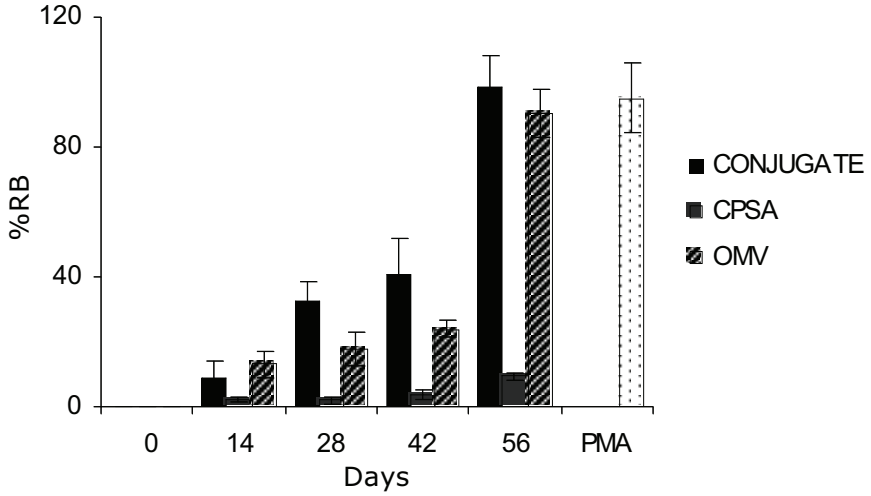

FIG. 2 - Opsonophagocytic activity of rabbit sera against *Neisseria meningitidis* serogroup A strain CSBPI, G-245 and positive control (PMA), measured as respiratory bursts (RB) in PMNs.

significant increase in opsonophagocytic responses against the serogroup A meningococci after 56 days compared to that of with the CPSA and OMV control groups ($P < 0.05$). Opsonophagocytic responses against the serogroup B meningococci of the conjugate showed no significant difference in comparison with that of the OMVs containing controls ($P > 0.05$).

A significant shift in the fluorescence histogram from a low fluorescence to a high fluorescence after PMA treatment was seen. This rise in the cell fluorescence is caused by the fact that nonfluorescent DHR-123 is converted during the respiratory burst into a strong green fluorescent compound R-123 (Fig. 2).

All the opsonophagocytic experiments were repeated at least twice. The difference between respective duplicates was regularly less than 5%.

DISCUSSION

It has been documented that polysaccharide (from bacterial capsule or LPS)-protein conjugates are usually immunogens in mice and rabbits as well as in humans. The immunogenicity of polysaccharide components has been attributed to many factors likely could generate differences of antibody response *in vivo*. These include quality and composition of the conjugates, conjugated methods, protein carriers, immunization routs, animal species, detecting methods, etc. (Costantino *et al*., 1999; Ada *et al*., 2003). Many studies have shown that in humans conjugated vaccines elicit antibodies to many pathogens, including *N. meningitidis, Vibrio cholera, Haemophilus influenzae, Shigella sonnei,* etc. the role of bactericidal and opsonic antibodies in conferring protective immunity against *N. meningitidis* infection have been elucidated.

We previously synthesized a CPSA-OMV conjugate which was immunogenic in rabbit and the respective antiserum showed bactericidal activity against *N. meningitidis* serogroup A (Siadat *et al*., 2007a, 2007c).

To investigate the additional protective capacity of antisera elicited by the conjugates, using normal rabbit sera as the source of complement, an opsonophagocytosis assay was performed by opsonizing meningococci and thus facilitating phagocytosis.

The ability of a serum sample to opsonize bacteria can be measured by various opsonophagocytosis assays (OPAs) *in vitro*. Thus, the evaluation of the protective capacity would be strongly dependent on demonstrating that the new vaccines can also induce sufficient opsonic titres for protection. Accordingly, various forms of opsonization assays have been developed, and there have been significant technical improvements in meningococcal antibody OPAs. Flow cytometry has long been the workhorse of immunology (Valdivia and Falkow, 1998; Li and Chung, 2003; Romero-Steiner *et al.*, 2006). In such applications, intracellular oxidation of compounds such as dihydrorhodamine-123 to fluorescent derivative, rhodamine-123 was taken as an indication of oxygen radical production. Therefore, in flow cytometric analysis, quantitative measurements of bacterial association and oxidative burst can be done on a per cell basis (Lun *et al*., 2000; Vakevainen *et al*., 2001; Romero-Steiner *et al*., 2006; Siadat *et al*., 2007b; Behzadiyannejad *et al*., 2008). Several reports have described chemiluminescence methods to measure the production of reactive oxygen intermediates generated during phagocytosis (Aase *et al*., 1998).

We preferred to measure RB rather than phagocytosis. This late event of phagocytosis is probably more related to bacterial killing rather than ingestion itself and is accordingly more relevant to glycoconjugate potency measurements in vaccine response studies. Phagocytosis (ingestion) might also be measured by a similar flow cytometric technique, though the bacteria have to be labelled with a fluorochrome such as FITC. Such surface labelling could modify or disguise the relevant antigens, and thus additional techniques is required to discriminate between internalized and adherent bacteria (Lun *et al*., 2000; Jansen *et al.,* 2001). We measured the phagocytosis and respiratory burst induction of PMN by the CPSA-OMV conjugate in a rapid, simple and reliable one-step flow cytometry method. Some characterizations of the flow cytometric method to evaluate the rabbits, PMNs were elucidated in this study. Important findings in this application was the elucidation of the number of bacteria (1.10^3 CFU/ml) used for opsonophagocytic assay by rabbit PMN_S and the fact that PMN_S demonstrated the most powerful capacity for respiratory burst after PMA stimulation (Siadat *et al*., 2007b).

Our experimental data revealed that the conjugated CPSA-induced rabbit sera showed high level opsonophagocytic activity against serogroup A meningococci and seems to have booster effect in this assay. It should be noted that immunologic memory responses to meningococcal serogroup A vaccination, either

unconjugated or conjugated vaccines, have been evaluated considering both the response to the polysaccharide component used and avidity indices. It is interesting to note the serogroup A capsular polysaccharide does not appear to be a traditional T-cell-independent antigen as shown for serogroup C capsular polysaccharide. Since serogroup A unconjugated vaccine has been shown to be immunogenic in children under 2 years old, the serogroup A polysaccharide appears to stimulate affinity maturation, as indicated by increase in avidity indices following vaccination. However, it does appear if there is a difference in the quality of the response induced regarding the conjugation state (unconjugated v.s. conjugated) of serogroup A polysaccharide (Balmer and Borrow, 2004). CPSA was rendered immunogenic when covalently bound to carrier protein. The resulting conjugates induced a highly significant increase in opsonophagocytic responses against *N. meningitidis* serogroup A. These data shows many correlates considering our previous studies indicating CPSA-OMV conjugate induced high level antibody responses with bactericidal and opsonophagocytic activities (Siadat *et al*., 2007a).

CONCLUSIONS

In conclusion, a high level of opsonophagocytic responses was induced by the CPSA–OMV conjugate. To study this aspect, a flow cytometric opsonophagocytic assay of glycol-conjugates against the serogroup A and B meningococcal strains was introduced; the results demonstrated that the CPSA–OMV conjugate might introduce an A/B conjugated vaccine (Siadat *et al*., 2007c).

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