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Full Length Research Paper

Outer membrane vesicle of *Neisseria meningitidis serogroup B* as an adjuvant in immunization of rabbit against *Neisseria meningitidis serogroup A*

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Neisseria meningitidis **serogroup B outer membrane vesicle (OMV) was revealed to be an efficient carrier for polysaccharide antigens such as capsular antigens. Also, OMV of *N. meningitidis* was shown to be able to induce high levels of antibodies when applied as an adjuvant for T-independent antigens. In this study OMV was used as an adjuvant with group A meningococcal capsular polysaccharide (GAMP) and tested in New zealand white rabbits to evaluate bactericidal antibody response and opsonophagocytosis activity against serogroup A meningococci. Non-covalent combination of OMV and GAMP and three controls including GAMP, OMV and normal saline were injected intramuscularly into groups of four female New Zealand rabbits with boosters on days 14 and 28 after primary immunization. The serum samples were collected on days 0, 14, 28 and 42 and tested by complement mediated bactericidal assay and opsonophagocytosis activity against serogroup A meningococci according to the world health organization protocol. The results indicate that the combination of OMV with GAMP, in noncovalent form, would be able to induce a high level of bactericidal antibody and opsonophagocytosis activity response in comparison with GAMP alone after 42 days (P< 0.05) . The OMV of *N. meningitidis* showed to be a potent carrier protein in the induction of immune system but in this article the role of OMV is studied as an adjuvant to promote immune system in non-covalent form and without any conjugation process in order to induce immune response against three prevalent serogroups of *N. meningitidis.***

Key words: OMV, Adjuvant, *Neisseria meningitidis* serogroup A, Bactericidal activity.

INTRODUCTION

Nowadays adjuvants are extensively used as immuno-stimulatory compounds to design subunit vaccines. The challenge for developing new and improved adjuvants stems from the need for more potent vaccines and reducing reactogenicity, side effects and also to gain features such as antigen-sparing ability, more rapid protection, stimulation of T-cell immunity, and long-lasting protective immunity. However, significant regulatory and other hurdles exist for developing new adjuvants, as evidenced by the complete absence of new FDA-approved adjuvants. Many candidates are recommended for modulating an immune response against an antigen. The outer membrane vesicle (OMV) of *Neisseria meningitidis* is among the newly studied components with

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Abbreviations: GAMP, Grou.p A meningococcal capsular polysaccharide; HBsAg, Hepatitis B surface antigen; OMV, Outer membrane vesicle; PBS, Phosphate buffered saline

microbial origin, which could be applied as an adjuvant. The potency of OMV as a carrier (conjugated to a hapten) to promote T-cell dependent responses has been proved (Sharifat et al., 2009; Nejati et al., 2008; Ada and Isaacs, 2003; Siadat et al., 2007a, 2007b).

Among the described properties of OMV is that the mucosal and systemic antibody responses to an influenza virus vaccine are greatly augmented when co-administered with OMV (Haneberg et al., 1998). In addition, the adjuvant properties of OMV-derived particles have been demonstrated for potential cancer vaccines (Estevez et al., 1997). Overall, the previous studies have shown that the predominant outer membrane proteins (OMPs) (PorA, PorB and RmpM) from *N. meningitidis* present in the Meningococci B Cuban vaccine had differing capacities to prime the immune system.

It has been demonstrated that application of OMV from *Neisseria lactamica* can be considered as an alternative approach for meningococcal vaccines. Immunization with *N. lactamica* OMV provided protection against the lethal challenges with a range of meningococcal strains in a mouse model of meningococcal disease (Oliver et al., 2002). Also, immunization of mice with *N. lactamica* OMV induced cross- reactive antibodies with *N. meningitidis* and primes for an enhanced response to *N. meningitidis* immunization (Sharifat et al., 2009; Oliver et al., 2002). In addition, OMV isolated from commensal *Neisseria* can be a delivery vehicle for heterologous vaccine antigens. Sardinas et al. (2006) have successfully employed a hepatitis B surface antigen (HBsAg) vaccine as a model antigen to assess the adjuvant properties of *N. lactamica* OMV which leads to increased titer of HBsAb (Sardinas et al., 2006). In the present study, we used *N. meningitidis* serogroup B OMV as a noncovalent complex with GAMP and then evaluated serum bactericidal activity of hyperimmunized rabbits against the *N. meningitidis* serogroup A.

MATERIALS AND METHODS

Preparation of OMVs and GAMP

OMVs were prepared as described by Siadat et al. (2006). In brief, *N. meningitidis* serogroup B strain (CSBPI, G-245) was grown in a fermentor, under controlled sub-merged cultural condition 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, 10 mM EDTA and 0.5%w/v deoxycholate. Purification of OMVs was done by sequential centrifugation at 20,000 g for 30 min followed by ultracentrifugation at 125,000 g for 2 h. The OMV pellet was homogenized in phosphate buffered saline (PBS) pH 7.2. Thiomersal (100 mg/l) was added as preservative Siadat et al., 2007b; Claassen et al., 1996; Siadat et al., 2006).

Group A *N. meningitidis* capsular polysaccharide (GAMP) was provided by the department of Bacterial Vaccine and Antigen Production, Pasteur Institute of Iran. *N. meningitidis* serogroup A (CPSA, G-243) was cultivated on a modified Frantz medium, and their GAMPs were purified according to World Health Organization, 1976 protocol (WHO, 1976; Jin et al., 2003).

Physico chemical analysis

Analytical methods

Protein content of OMVs was measured according to Peterson (Siadat et al., 2007b; Claassen et al., 1996). Hestrin method was used to estimate O-acetyl groups present in the GAMP (Kheirandish et al., 2009).

Bioassay

The toxicity of LPS content in the OMVs was assayed by the *limulus amebocyte lysate* test and expressed in endotoxin units according to the U.S. standard. Also, the pyrogenicity of the OMVs was assayed in rabbits (Siadat et al., 2007b; Jin et al., 2003).

Electron microscopy

Outer membrane vesicles integrity was checked by electron microscopy as described previously (Siadat et al., 2007ª, 2007b).

Immunization procedures

Hyperimmune sera were prepared by repeated injection of New-Zealand white rabbits (weighing 2-2.5 kg) as described by Jennings and Lugowski (Jennings and Lugowski, 1981). Four groups of animals each consisted of five rabbits, were immunized intramuscularly according to the following pattern:

1. 0.2 ml GAMP (containing 50 µg polysaccharide) without any adjuvant,
2. 0.2 ml GAMP (containing 50 µg polysaccharide) with 0.5 ml Freunds complete adjuvant
3. 0.2 ml GAMP (containing 50 µg polysaccharide) with 0.5 ml Freunds incomplete adjuvant
4. GAMP with 0.033 ml OMV (containing 40 µg protein) as an adjuvant.

Another group consisted of five rabbits, intramuscularly received normal saline and considered to be the control group.

The booster injections were carried out in 14th and 28th days following the first injection. The immunized animals were bled on the days 0, 14, 28 and 42. The immune sera were separated, pooled and kept in -20°C.

Complement – mediated bactericidal assay

Anti-meningococcal A bactericidal assay was carried out using the standardized meningococcal serogroup A strain CSBPI,G-243 in 96-well plates as previously described (Siadat et al., 2007ª; Siadat et al., 2007a; Rezaei et al., 2007). Briefly Muller Hinton broth was inoculated with 5 to 10 colonies from a fresh culture of *N. meningitidis* and incubated for 2 h at 37°C with shaking. Cells were removed, washed with sterile 0.5% BSA in PBS (Sigma-Aldrich, Germany) and diluted to 800 CFU per 10 µl using an OD equivalence of 0.1= 2 ×10 8 CFU/ml at wavelength of 650 nm. 20 µl of diluted heat-inactivated test serum was mixed with 10 µl of 3 to 4-week old baby rabbit complement and 10 µl of bacterial suspension. After 1 h of incubation at 37°C, the contents of each reaction well were serially diluted; 10 µl aliquots were placed onto

plates containing blood agar and incubated overnight at 37°C with 5% CO2 before the colonies were counted. The number of CFU in each reaction well was then calculated, and the percentage of reduction in CFU was determined by comparison with control wells, which received no serum. A second set of control wells that contained serum but no complement was included in each test. The bactericidal antibody titer was defined as the dilution of serum resulting in 50% reduction in CFU.

Opsonophagocytic assay

The opsonophagocytic activity of specific antibodies produced against *N. meningitidis* serogroup A was measured as the ability of synthesized antibodies to induce respiratory burst as previously described (Siadat et al., 2007d; Behzadiyannejad et al., 2008). Briefly, viable meningococci (CSBPI G-243), grown to log phase, were used as target cells and dihydrorhodamine 123, primed healthy rabbits PMNs as effector cells. Each serum sample was heated to 56°C for 30 min to inactivate endogenous complement, and was measured for RB activity. 50 µl 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 for 30 min at 37°C with continuous agitation. Then, 5 µl of normal rabbit sera was added as the complement source and the incubation continued for 8 min at 37°C with agitation. Each dilution was tested in duplicate. Non-fluorescent probe dihydrorhodamine 123 (DHR) was used as an indicator for RB that will be oxidized to fluorescent rhodamine 123 through RB process of oxidative burst. The DHR solution was added to the effector cells to reach a final concentration of 10 µg/ml just before mixing 50 µl of the effector cells with the opsonized bacteria, and the incubation continued for 8 min at 37°C with agitation. 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 samples were run on a flow cytometer (Coulter EPICS - XL-Profile USA) with a 15 mW Argon laser. Lymphocytes and non- lymphocytes (monocytes and polymorphonuclear leukocytes, that is, the potentially phagocytosing cells) can be discriminated and quantified by combined measurements of the forward scatter (FS) which is related to the size of the cells and the side scatter (SS) which is related to granularity of the cells. The excitation wavelength was 488 nm, and standard Coulter filters were used in all the measurements (The standard Coulter filters were used in all measurements with excitation wavelength of 488 nm). Phagocytic function and RB by rabbit PMN against *N. meningitidis* serogroups A were measured using flow cytometry with, dihydrorhodamine-123 as probes. The results were presented by summarizing the percentage of RB positive PMNs at each sample. Activities below 10% at any dilution were defined as zero (Siadat et al., 2007d; Behzadiyannejad et al., 2008; Aase et al., 1995; Aase et al., 1998; Martinez et al., 2002; Romero-Steiner et al., 2006).

Statistic methods

The data were analyzed using repeated ANOVA and P 0.05 was considered as significant (Siadat et al., 2007a).

RESULTS

Serum bactericidal activity

The rabbits that received three dose of the complex serogroup B meningococcal OMV-GAMP developed high levels of serum bactericidal activity against serogroup A meningococci after 42 days in comparison with the GAMP and OMV control group ($P<0.05$) . Bactericidal titer against serogroup B meningococci of the GAMP plus OMV complex showed no significant difference in comparison with the OMV containing control ($P> 0. 05$).

Performance of the RB assay

The immune responses against strain CSBPI G-243 serogroup A meningococci after vaccination were measured by PMN-mediated RB. In the present study, oxidative burst is mostly assayed by the percentage of PMN with R-123, and by the mean of non- fluorescent DHR-123 conversion to the fluorescent R-123 compound during the respiratory burst (major H_2O_2 release). The serum samples taken at different times including before vaccination, 2 weeks after the first dose, 2 weeks after the second dose and 2 weeks after the third dose were analyzed for the evaluation of opsonophagocytosis activity of specific induced antibodies. The RB results were calculated from the area under the histogram bars where each dilution is plotted on the abscissa and the percent of fluorescence- positive PMN_S is plotted on the ordinate. The percent of positive PMN at each test was summarized to give RB% indicating opsonophagocytic responses.

The combination of GAMP-OMV induced a highly significant increase in the opsonophagocytic responses (RB%) against the serogroup A strain 2 weeks after the first dose ($P<0.05$). The booster effects were clearly seen after the second dose on the day 28 and strongly after the third dose on the day 42 (Figure 1). The RB% against the serogroup A strain was relatively high in the group injected with GAMP alone, but comparison of GAMP-OMV complex showed insignificant differences on the days 14 and 28 (Figure 2) ($P>0.05$). Quantitative flow cytometric analysis of rabbit PMN function in the hyperimmune sera induced with GAMP-OMV complex revealed a highly significant increase in the opsonophagocytic responses against the serogroup A meningococci after 42 days in comparison with the GAMP and OMV control groups($P<0.05$). 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 has been caused by the conversion of non- fluorescent DHR-123 into strong green fluorescent R-123 during the respiratory burst (Figure 2).

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

DISCUSSION

In contrast to the serogroup C capsular polysaccharide, serogroup A capsular polysaccharide does not show the

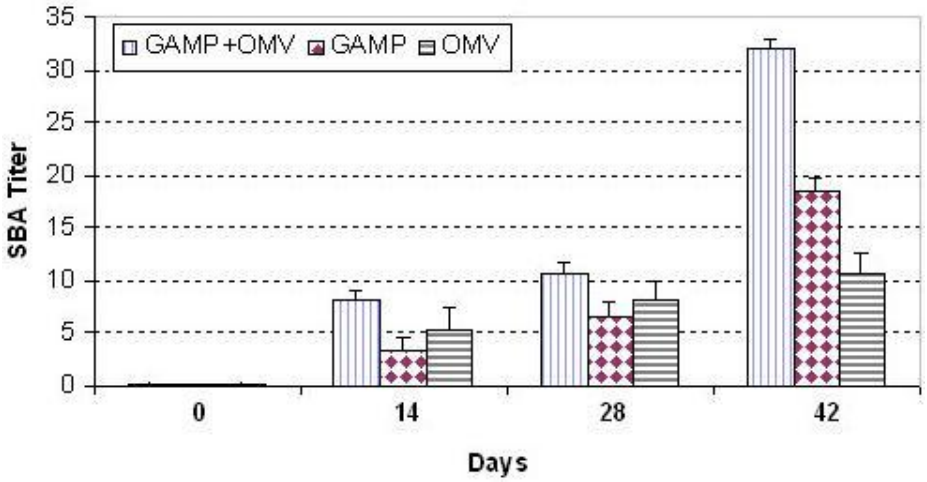

Figure 1. Bactericidal activity of rabbit sera against *Neisseria meningitidis* serogroup A strain CSBPI, G- 243 measured as serum bactericidal titres.

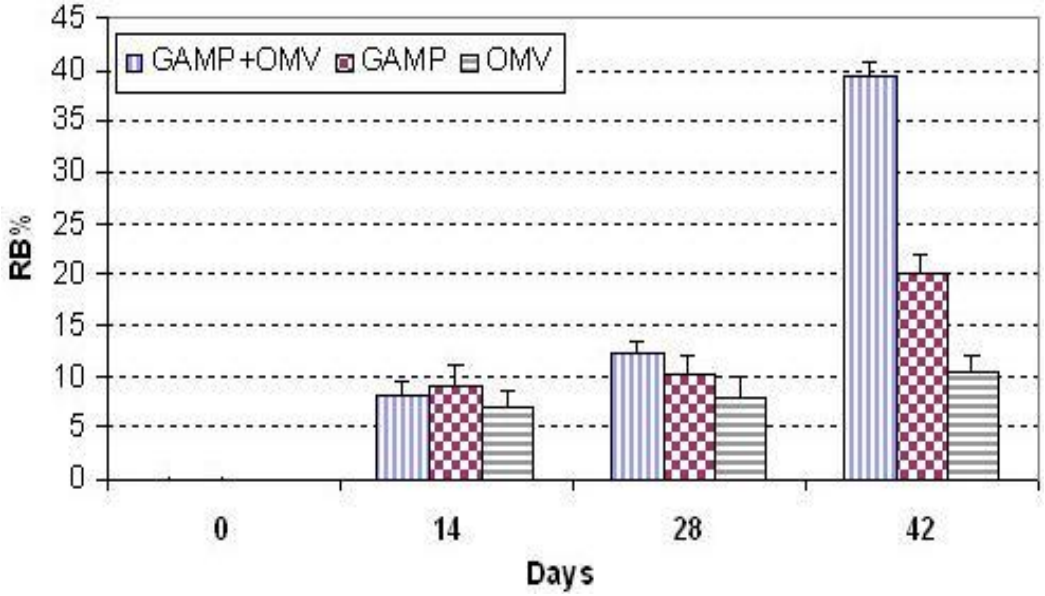

Figure 2. Opsonophagocytic activity of rabbit sera against *Neisseria meningitidis* serogroup A strain CSBPI, G- 243 measured as respiratory bursts (RB) in PMNs.

properties of a classic T-cell-independent antigen, therefore Serogroup A unconjugated vaccine has been shown to be immunogenic in children under 2 years of age and appears to stimulate affinity maturation following the vaccination. However it dose appear that there are differences in the quality of the responses induced if the serogroup A polysaccharide is conjugated or monovalent (Balmer and Bprrow, 2004). We previously synthesized a GAMP-OMV conjugate which was immunogenic in rabbit and the respective antiserum showed bactericidal and opsonophagocytosis activity against *N. meningitidis* serogroup A (Siadat et al., 2007a, Siadat et al., 2007b; Kheirandish et al., 2009), but the methods of conjugation is very complex and should be controlled by multi parameters such as the risk of conformational changes of dominant and essential epitopes as a result of the covalent reactions between GAMP and OMV. Therefore, protective antibodies against native polysaccharide or OMV proteins would not be induced following this conformational change. The covalent attachment of the GAMP to the OMV probably induced some modifications in the OMV protein epitopes through a directly covalent linkage or steric hindrance. Furthermore, a reactive reagent is used for coupling of polysaccharide to the protein, the control of conjugates as vaccines for human use may be rather complicated, to ensure the absence of

the coupling reagent and its reaction products. An alternative to these conjugates could be polysaccharide-OMV complexes that OMV can be act as a microbial adjuvant (Sharifat et al., 2009).

Most of the classic and introduced adjuvants cause local and systemic hypersensitivity reactions and are not licensed for human use; According to these drawbacks of currently applied adjuvants, OMV would be a safe adjuvant with a high potency to induce a typical secondary response, since the OMVs used in our vaccine formulation has been used previously in human trials and was found to be safe. Several reports have described that polysaccharide antigens, normally T - independent in animal model, stimulate immunologic memory when combined to *N. meningitidis* serogroup B OMP because it has been shown to have T helper cell mitogenic activity (Lillian et al., 1996; Bhattacharjee et al., 2006). The current explanation is related to PorA, a major protein component of OMV, acting as an adjuvant or mitogens inducing the expression of costimulatory molecules like CD 80, CD 86 or CD 40, which are essential for cognate T-cell- dependent antibody production. Meningococci group B OMP is also known to increase expression of costimulatory molecules on murine B cells and to enhance antibody responses to polysaccharide antigens by a CD40-CD40L- mediated mechanisim (Bhattacharjee et al., 2006).

In this study, we have specifically studied the modern vaccine adjuvants injected intramuscularly by complexing the GAMP with OMV. Although, the rabbits immunized with GAMP alone developed bactericidal antibodies titers and opsonophagocytosis activity against N. meningitidis serogroup A, two weeks after all two booster injections, our results suggest that usage of OMVs in complex with GAMP could stimulate significantly higher levels of bactericidal antibodies titers and opsonophagocytosis activity in rabbits than immunization with GAMP alone. The vesicular nature of the complex, demonstrated by electron microscopy, probably increases the uptake and processing of the GAMP by antigen presenting cells.

An unexpected result shown in this paper is the observation that the serum from rabbits immunized with OMV alone induced a low but efficient level of bactericidal antibodies against serogroup A meningococci. The probable explanation would be a cross-reactivity achieved due to the presence of protein components in the OMV.

Conclusion

This study reveals our purified OMV may be applied as a safe and potent intramuscular adjuvant to induce high titres of bactericidal antibodies and opsonophagocytosis activity and would be used in combination with group A meningococcal polysaccharide vaccines for human.

A highly significant increase in opsonophagocytic responses was elicited in the present research and the memory response appeared to be long-lasting.

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