Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 3 (3): 105-113, 2008
ISSN 1816-4935

Comparison Among Opsonic Activity and Serum Bactericidal Activity Against Meningococci in Rabbit Sera from Vaccines After Immunization with Outer Membrane Vesicle of *Neisseria meningitidis* Serogroup B

[1]Q. Behzadiyannejad, [2]S.D. Siadat, [3]M. Kheirandish, [2]B. Tabaraiee, [2]H. Ahmadi,
[2]D. Norouzian, [1]S. Najar Peerayeh, [2]M. Nejati and [2]M.H. Hedayati
[1]Faculty of Medicine, Tarbiat Modarres University, Tehran, Iran
[2]Department of Bacterial Vaccines and Antigen Production,
Pasteur Institute of Iran, Tehran, Iran
[3]Research Center of Iranian Blood Transfusion Organization, Tehran, Iran

Abstract: Production of effective vaccine formulations is dependent on the availability of assays for the measurement of protective immune responses. Antibody- and complement-mediated phagocytosis is the main defense mechanism against *Neisseria meningitidis*. Therefore, a newly developed phagocytosis assay based on flow cytometry (flow assay) and the Serum Bactericidal Activity (SBA) assay were using sera obtained from rabbit postvaccination with the Outer Membrane Vesicles (OMVs) of *Neisseria meningitidis* serogroup B was done in order to evaluation of the potential efficacy of (experimental) meningococcal vaccines. The OMVs were injected intramuscularly into of rabbits with boosters on days 14, 28 and 42 after the primary immunization. Phagocytic function of and intracellular oxidative burst generation by rabbit PMN, against *Neisseria meningitidis* serogroup B, was measured with flow cytometer (Coulter Epics-XL-Profile USA), using dihydrorhodamine-123 as probes, respectively. SBA titers are given as reciprocal Log 2 values of the dilution giving at least 50% killing of the inoculum measured as colony forming units. The results of SBA titers and quantitative flow cytometric analysis of rabbit PMN function in hyperimmun sera with the OMVs revealed a highly significant increase in opsonophagocytic responses and bactericidal antibody against serogroup B meningococci after 56 day ($p<0.05$). Both SBA and opsonic activity are crucial for the protection against meningococcal disease. In conclusion, we have shown a very high correlation between opsonic activity and SBA ($r = 0.91$). Present results indicated that the OMVs could be as a candidate for vaccine toward serogroup B meningococci.

Key words: *Neisseria meningitidis*, antibody and complement-mediated phagocytosis, flow cytometric analysis, outer membrane vesicle

INTRODUCTION

Meningococcal disease continues to be a significant cause of morbidity and mortality in the worldwide (Rosenstin *et al*., 2001). Specific antibodies and a functional complement system are of crucial importance in the host defense against systemic meningococcal infections (Balmer and Borrow, 2004). Specific antibodies and a functional complement system are of crucial importance in the host defense against systemic meningococcal infections. Serum Bactericidal Activity (SBA) correlates with protection as shown in several studies (Jodar *et al*., 2000, 2002).

Corresponding Author: Q. Behzadiyannejad, Department of Medical Microbiology, Faculty of Medicine, Tarbiat Modarres University, Tehran, Iran
Tel: 0098-21-88011001 Fax: 0098-21-88850053

Neisseria meningitidis is efficiently phagocytosed by polymorphonuclear (PMN) cells and antibodies are bactericidal in the presence of peripheral blood polymorphonuclear leukocytes and complement. Consequently, patients with defects in the terminal complement pathway are highly susceptible to meningococcal disease, especially from meningococci of uncommon serogroups (Aase *et al*., 1998; Martinez *et al*., 2002). However, the presence of serum opsonins to facilitate phagocytic killing is also likely to be of great importance in the *in vivo* defense against this organism. The previous studies have shown that serogroup B meningococci are more resistant to bactericidal killing than serogroup A and C meningococci but are highly susceptible to killing by polymorphonuclear leukocytes (PMN) after opsonization. Opsonophagocytic assays (OPA) offer several advantages over the standard SBA. OPA for other pathogens such as *Streptococcus pneumoniae* have been developed which are semi-automated, use noninfectious targets, provide useful laboratory correlates of protection and can be multiplexed (Aase *et al*., 1994).

During complement activation, C3 and C4 split products will be deposited on the bacterial surface and these fragments may be effective opsonins. IgG antibodies bound to bacteria are also excellent opsonins and a synergistic opsonic effect is achieved when the target is covered with both IgG and complement split products. PMN and macrophages constitutively express Fcγ receptors (FcγRs) and complement receptors. An immune response in which the effector function is biased against phagocytosis rather than bacteriolysis may be beneficial to the host, since intracellular destruction will minimize intravascular release of bacterial endotoxin and thus reduce the risk for septic shock. Therefore, the *in vitro* opsonophagocytic activity (OPA) of antibodies to meningococcal components are believed to be a measure of their functional activity *in vivo*. Thus, to determine the serological correlates or surrogates of protection from the samples of ongoing efficacy trials, both quantitative and qualitative characteristics of antibodies have to be measured reliably (Jack *et al*., 1994; Aase *et al*., 1994; Väkeväinen *et al*., 2001). This report compared a flow cytometric assay to measure Respiratory Burst (RB) in PMN and a bactericidal antibody assay, with B meningococci as the target cells.

MATERIALS AND METHODS

Preparation of OMVs

OMVs were prepared as described previously (Siadat *et al*., 2006). 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, 10 mM 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 125,000 g for 2 h, 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 (Arigita *et al*., 2004; Claassen *et al*., 1996; Norheim *et al*., 2005; Siadat *et al*., 2006).

Physicochemical Analysis of OMVs

Protein Determination

Protein content of OMVs was measured according to Peterson (1977).

Electron Microscopy

OMV 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 0.01 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 a Zeiss CEA902A electron microscope at 80 kV (Siadat *et al*., 2006).

Biological Activity

The biological activity of the endotoxin content of OMVs were estimated by the limulus amebocyte lysate (LAL) gelatin test. The lipid-A part of the endotoxin molecule can activate the gelation of the limulus lysate. The OMVs were incubated in a fivefold dilution with a fixed concentration of limulus lysate at 37°C for 45 min. The endotoxin activity was visualized using Bromthymol Blue. Unknown activity of endotoxin was compared with *Escherichia coli* standard endotoxin (FDA, Bethesda, USA).

To determine the pyrogenicity of the OMVs a study in rabbits was performed. Samples of the OMVs final lot were injected IV in three healthy rabbits (1 mL kg^{-1}), that have not been injected previously, at 1:100, 1:300 and 1:1000 dilution and the rise of body temperature was monitored. Furthermore a placebo which contains only Freunds complete adjuvant and thiomersal, was injected at 1:100 dilution. For the 1:100 vaccine dilution a repeat of the test was necessary (WHO, 1976).

Immunization Procedures

For immunization of rabbit the OMVs were adsorbed to the adjuvant Al $(OH)_3$ with a protein: Al $(OH)_3$ ratio of 1:66 (w/v) as previously described and diluted in saline. New Zealand white rabbit weighing 2-2.5 kg (n = 5 rabbit per group) were immunized intramuscularly with 40 μg protein of each of the vaccines on days 0, 14, 28 and 42 and bled on days 0, 14, 28, 42 and 56. Sera were stored at -20°C (Siadal *et al*., 2007).

PMN

Venous blood from healthy rabbit was drawn into heparinized vacuum tubes and the erythrocytes were lysed with a solution containing 8.3 mg mL^{-1} of NH_4Cl, 1 mg mL^{-1} of $NaHCO_3$ and 0.08 mg mL^{-1} of EDTA (pH 6.8) per mL. The leukocytes were washed twice with Hanks balanced salt solution (HBSS) with 0.2% Bovine Serum Albumin (BSA) and the cell concentration was adjusted to 5×10^3 mL^{-1}. No additional purification of PMN was required, because further analyses were done by flow cytometry that easily discriminates PMN from other cells within the suspension (Aase *et al*., 1994).

Complement Source for RB Assay

Normal rabbit serum with no detectable antibody against *N. meningitidis* serogroup A CSBPI, G-243 and serogroup B CSBPI, G-245 was used as the source of complement for opsonization of meningococci. We found no antibodies against meningococcal antigens measured at 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*., 1994).

Bacteria to Be Used in RB Assay

N. meningitidis serogroup B CSBPI, G-245 was grown as described previously for SBA, fixed in 70% ethanol overnight at 20°C and washed in HBSS. The concentration was adjusted to 1.10^3 CFU mL^{-1} bacteria, aliquoted in 1 mL ampoules and stored at -85°C (Siadat *et al*., 2007a).

RB

The RB analysis was performed mainly as described by Aase and Michaelsen (1998) with some modification. All sera to be tested were heated to 56°C for 30 min to inactivate endogenous complement. Fifty microliters of a threefold dilution of sera was mixed with 5 mL of

serogroup B meningococci (1.10^3 CFU mL^{-1}) in U-bottomed microtiter plates and incubated for 30 min at 37°C with continuous agitation. Then, 5 μL of serum as the complement source was added and the incubation continued for 8 min at 37°C 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 during RB. A stock solution of DHR was prepared by dissolving 10 mg in 1 mL of dimethyl sulfoxide. This solution was aliquoted and stored at -85°C until use. DHR was added to the effector cells to give a final concentration of 10 μg mL^{-1} 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 Phorbol Myristate Acetate (PMA) working solutions were freshly prepared before each assay of RB. The PMA working solution (100 ng mL^{-1}) was prepared by 1:10 dilution of PMA stock (1 mg mL^{-1}) in PBS. The following protocol was used for measuring naturally function of PMA (as positive control): the reaction was performed in 100 mL of whole-blood specimen. First, the DHR solution (50 mM) was added to each tube and incubated for 5 min at 37°C. Then, the PMA solution (100 ng mL^{-1}) was added to each sample and the tube was further incubated for 10 min in the 37°C water bath. Finally, the reaction was stopped by washing the cells once with 4°C PBS (3 mL). The RBCs were lysed using the following ammonium chloride lysing method. Cells incubated with DHR only served as negative controls. All control samples were tested according to the above protocol for the evaluation of RB (Chung and Li, 2003).

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 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 percent positive cells within the gate with three-decade logarithmic amplification on the fluorescence detector. By this method PMN could easily be discriminated from monocytes and lymphocytes (and bacteria) without any further purification of the leukocyte fraction. As a negative control, the test serum was omitted and replaced by HBSS containing BSA. This mixture was used to set the correct region on the fluorescent axis. About 10,000 effector cells were counted in each sample and all determinations were performed in duplicate. Phagocytic function and intracellular oxidative burst generation by rabbit PMN, against *Neisseria meningitidis* serogroup A and B, were measured with flow cytometer, using dihydrorhodamine-123 as probes, respectively. In these experiments, viable meninigococci, CSBPI G-243 serogrup A and CSBPI G-245 serogroup B, grown to log phase, were used as target cells and DHR-123 primed PMNs from a healthy donor rabbit were used as effector cells. The results are presented by summarizing the percent RB positive PMN at each sample. Activities below 10% at any dilution are defined as zero (Chung and Li, 2003; Lehmann *et al*., 2000).

SBA

The SBA assay was carried out as described earlier (Siadat *et al*., 2007b) SBA titers are given as reciprocal log 2 values of the dilution giving at least 50% killing of the inoculum measured as colony-forming units (Siadat *et al*., 2007).

RESULTS

OMVs Characterization and Identity Test

Total protein yield of the OMV production was determined after extraction and purification. Total protein yield ranged from 350 to 950 mg in the final product.

Electron microscopy was used order to verify the integrity of the OMV after preparation. OMV size ranged from 70 to 120 nm in this process. Intactness of the vesicles in this preparation ranged from 50 to 80% of the vesicles.

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 contain 677 and 135 EU mL^{-1}, 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.

On the basis of the results of the pyrogenicity test, temperature rises of 1:300 and 1:1000 dilutions were comparable with those obtained with the placebo (data not shown). 1:1000 dilution of the vaccine corresponds with the requirements for 23-valent pneumococcal polysaccharide vaccine and mono-, bi- and tetravalent meningococcal polysaccharide vaccines.

Performance of the RB Assay

The immune response of strain CSBPI G-245 serogroup B meningococci after vaccination was measured by PMN-mediated RB. We used a Coulter Epics XL-Profile USA with an argon laser operating at 488 nm. Lymphocytes and non-lymphocytes (monocytes and polymorphonuclear leukocytes, i.e., the potentially phagocytosing cells) can be discriminated and quantified by combined measurements of Forward Scatter (FS), which is related to the size of cells and Side Scatter (SS), which is related to granularity of cells. In our experiments, oxidative burst has mostly been assayed by the percentage of PMN with R-123 and by the mean that nonfluorescent DHR-123 is converted during the respiratory burst (major H_2O_2 release) into a fluorescent compound R-123. Sera taken from fifth different time points (prevaccination, 2 weeks after the first dose, 2 weeks after the second dose, 2 weeks after the third dose and 2 weeks after the fourth dose,) were analyzed. Each serum sample was heated to 56°C for 30 min to inactivate endogenous complement and each of sera was measured for RB activity. RB results were calculated from the area under the histogram bars where each sample is plotted on the abscissa and the percent fluorescence-positive PMN is plotted on the ordinate. The percent positive PMN at each test is summarized to give ΣRB% and have indicated opsonophagocytic responses.

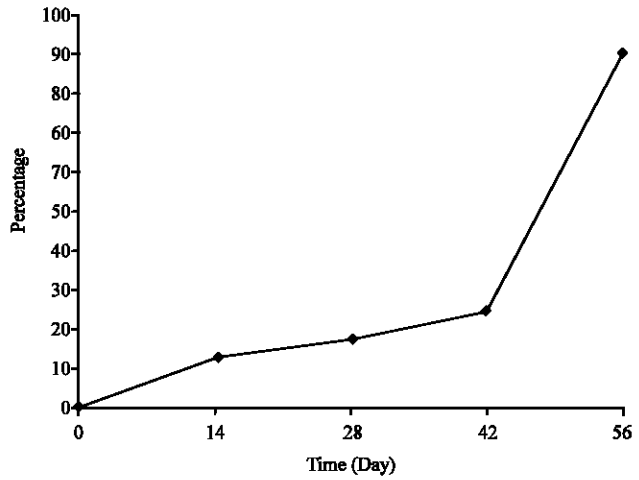

Fig. 1: Opsonophagocytosis titer of rabbit sera in *Neisseria meningitidis* serogroup B (CSBPI, G-245) after immunization with four doses of OMVs

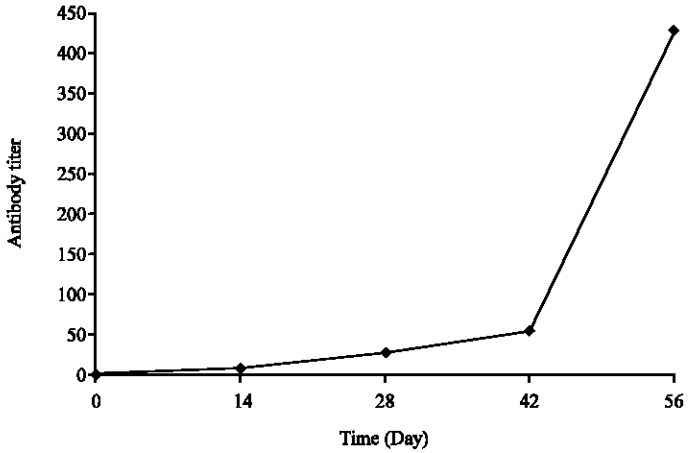

Fig. 2: Anti-meningococcal serogroup B (CSBPI, G-245) bactericidal activity of rabbit sera after immunization with four doses of OMVs

OMVs induced a highly significant increase in opsonophagocytic responses (ΣRB%) against the serogroup B strain 2 weeks after the first dose ($p<0.05$). The clear booster effects were seen after the second dose in 28 days and strongly after the fourth dose in 56 days (Fig. 1) ($p<0.05$).

A significant shift in the fluorescence histogram from a low fluorescence to a high fluorescence after PMA treatment was shown. This increase in cell fluorescence results from the fact that nonfluorescent DHR-123 is converted during the respiratory burst into a strong green fluorescent compound R-123 (data not shown).

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

Bactericidal Assay of CPSA-OMV Conjugate

The bactericidal assay was performed quantifying complement mediated bactericidal activity of rabbit sera against serogroup B CSBPI, G-245 (Fig. 2). OMVs induced high levels of bactericidal antibodies against the serogroup B strain after the second dose. The clear booster effects were seen after the third dose in 42 days and strongly after the forth dose in 56 days (Fig. 2). However, after three doses the OMVs showed the high and significant titer bactericidal antibody against serogroup B meningococci.

The results of SBA titers and quantitative flow cytometric analysis of rabbit PMN function in hyperimmun sera with the OMVs revealed a highly significant increase in opsonophagocytic responses and bactericidal antibody against serogroup B meningococci after 56 day.

DISCUSSION

In order to develop effective vaccines against serogroup B meningococci, the relative contributions of antibodies against serogroup B lipopolysaccharide and the various meningococcal outer membrane components in protection against meningococcal disease need to be established (Ruggeberg and Pollard, 2004; Plested *et al*., 2001). Among these components, 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 lipooligosaccharide (LOS) in these preparations, which has been reported to have immune response stimulating properties. In principal, OMV has potent delivery adjuvant characteristics (Bethell and Pollard, 2002; Jensen *et al*., 2000; Fukasawa *et al*., 2004).

The integrity of OMV seems to be essential to achieve bactericidal activity against the serogroup B strain, since in which the vesicles were damaged, the product failed to induce bactericidal activity against serogroup B meningococci. Therefore, the OMV maintained its original conformation in this preparation (Cartwright *et al*., 1999; Claassen *et al*., 1996; Vella *et al*., 1990).

The opsonophagocytic activity of antibodies have been documented by others using bacteria that generate high levels of antibodies. Lortan *et al*. (1993) showed a significant correlation between phagocytosis and the serum levels of anti-pneumococcal polysaccharide antibodies of the IgG, although they did not test each subclass as purified preparations. Bredius *et al*. (1993) studied opsonophagocytic activity of purified IgG1 and IGg2 antibodies against *Staphylococcus aureus* and *Haemophilus influenzae* type b. Similar data were also supplied by Amir *et al*. (1990).

Flow cytometry has been increasingly used to evaluate the functional capabilities of PMN in host defense. Investigators interested in studying the function of immune system cells have utilized bacteria to elicit cellular responses (Falkow and Valdivia, 1998; Lehmann *et al*., 2000).

RB and SBA are related effector functions, as both are induced by antibodies and complement. However, SBA depends on activation of the entire complement cascade through C9 for the generation of the membrane attack complex that subsequently causes membrane leakage. Complement-mediated opsonization needs only activation through C3 to cause deposits of C3b, iC3b and C4b that then ligate to the corresponding receptors on phagocytes. A synergistic opsonic effect is achieved when the phagocytes are triggered through both complement receptors and FcgRs. The potential for an antibody to bind FcR or activate the complement cascade is strongly dependent of the antibody isotype. IgM antibodies are powerful activators of the complement cascade, but they probably can not induce RB by FcR binding, as there are few FcμRs on neutrophils. Also, the different IgG subclasses reveal individual patterns for these effector functions (Aase *et al*.,1995, 1998).

Several protocols to measure opsonophagocytic activity against meningococci have been described and several reports describe chemiluminescence methods to measure the production of reactive oxygen intermediates generated during phagocytosis (Aase *et al*., 1995, 1998; Lun *et al*., 2000; Romero-Steiner *et al*., 2006; Väkeväinen, 2001; Falkow and Valdivia, 1998). All these reports use the test serum itself as the complement source, which may be inconvenient for several reasons: all sera must be collected by standardized procedures to preserve complement activity, sera must be tested at high concentrations to avoid diluting the complement components that will consequently make it difficult to differentiate between stronger responses (as some responses may be more or less off scale) and there is high consumption of serum. Also, the complement activity and other serum factors may differ from one rabbit to another and the responses measured may thus not reflect specific antibody responses (Aase *et al*., 1998). In RB assay we use an external complement source, ensuring identical complement activity among the different serum samples and the dilution effect is omitted, since a constant amount of complement source is added to all test serum dilutions. We prefer to measure RB rather than phagocytosis. This late event of phagocytosis is probably more related to bacterial killing than mere ingestion and is therefore more relevant to measure in vaccine response studies. Phagocytosis (ingestion) might also be measured in a similar flow cytometric technique, but then the bacteria have to be labelled with a fluorochrome such as FITC. Such surface labelling might modify or disguise relevant antigens and additional techniques must be introduced to discriminate between internalized and adherent bacteria (Lun *et al*., 2000; Jensen *et al*., 2001).

Present results showed that anti-meningococcal opsonic antibodies may all effectively contribute to protection against disease caused by this microorganism. It is not clear which of these effector functions is the most important, but we may anticipate that both are involved: followed by antibody binding the complement cascade is immediately activated and the complement- mediated destruction

will begin. Concurrently, PMNs and other phagocytic cells may start engulfing opsonized meningococci (viable, complement-killing and deberis). This may direct much of the microbial destruction to intracellular compartment and perhaps limit some of the damages induced by LPS. Whether one of these effector functions alone will be protective against group B meningococcal disease is highly uncertain.

The results of SBA titers and quantitative flow cytometric analysis of rabbit PMN function in hyperimmun sera with the OMVs revealed a highly significant increase in opsonophagocytic responses and bactericidal antibody against serogroup B meningococci after 56 day.

This study confirms previous results that a OMV vaccine from a serogroup B meningococcal strain is highly immunogenic, capable of inducing opsonophagocytic and bactericidal antibodies. Present study observed a very high correlation between bactericidal antibodies and RB ($r = 0.91$).

Both SBA and opsonic activity are crucial for the protection against meningococcal disease. In conclusion, we have shown that the OMVs could be as a candidate for effective vaccine toward B meningococci.

REFERENCES

Aase, A. and T.E. Michaelsen, 1994. Opsonophagocytic activity induced by chimeric antibodies of the four human IgG subclasses with or without help from complement. Scan. J. Immunol., 39: 581-587.

Aase, H.A. and T.E. Michaelsen, 1998. Opsonophagocytic and bactericidal activity mediated by purified IgG subclass antibodies after vaccination with the Norwegian group B meningococcal vaccine. Scand. J. Immunol., 47: 388-396.

Amir, J., M.G. Scott, M.H. Nahm and D.M. Granoff, 1990. Bactricidal and opsonic activity of IgG1 and IgG2 anticapsular antibodies to *Haemaphilus influenza* type b. J. Infect. Dis., 162: 163-171.

Arigita, C., W. Jiskoot and J. Westdijk *et al*., 2004. Stability of mono and trivalent meningococcal outer membrane vesicle vaccines. Vaccine, 22: 629-642.

Balmer, P. and R. Borrow, 2004. Serologic correlates of protection for evaluating the response to meningococcal vaccine. Expert. Rev. Vaccines, 3: 77-87.

Bethell, D. and A.J. Pollard, 2002. Meningococcal vaccines. Expert. Rev. Vaccines, 1: 75-83.

Bredius, R.G., C.E. Vrise and A. Torelstra, 1993. Phagocytosis of *Staphylococcus aureus* and *Haemophilus influenzae* type b opsonized with polyclonal IgG1 and IgG2 antibodies. J. Immunol., 151: 1463-1472.

Cartwright, K., 2001. Meningococcal disease in Europe: Epidemiology, mortality and prevention with conjugate vaccines. Report of a European Advisory Board Meeting Vienna, Austria, 6-8 October, 2000. Vaccine, 19: 4347-4356.

Chung, S.C.S. and W. Li, 2003. Flow cytometric evaluation of leukocyte function in rat whole blood. *In vitro* Cell Dev. Biol. Anim., 39: 413-419.

Claassen, I., J. Meylis, P. Ley, C. Peeters, H. Brons, J. Robert, D. Borsboom, A. Ark, I. Straaten, P. Roholl, B. Kuipers and J. Poolman, 1996. Production, characterization and control of *Neisseria meningitidis* hexavalent class 1 outer membrane protein containing vesicles vaccine. Vaccine, 14: 1001-1008.

Falkow, S. and R.H. Valdivia, 1998. Flow cytometry and bacterial pathogenesis. Curr. Opin. Microbiol., 1: 359-363.

Jack, R.M., B.A. Lowenstein and A. Nicholson-Waller, 1994. Regulation of C1q receptor expression on human polymorphonuclear leukocytes. J. Immunol., 153: 262-269.

Jensen, C., B. Kuipers, J. Van Der Bienzen, H. Cock, P. Van Der Ley and J. Tommassen, 2000. Immunogenicity of *in vitro* folded outer membrane protein PorA of *Neisseria meningitidis*. FEMS Immun. Med. Microbiol., 27: 227-233.

Jensen, W.T.M., M. Wäkeväinen and H. Kayhty *et al*., 2001. Comparison of a classical phagocytosis assay and a flow cytometry assay for assessment of the phagocytic capacity of sera from adults vaccinated with pneumococcal conjugate vaccine. Clin. Lab. Immun., 8: 245-250.

Jodar, L., K. Cartwright and I.M. Feavers, 2000. Standardisation and vialidation of serological assay for the evaluation of immune responses to *Neisseria meninngitidis* serogroup A and C vaccines. Biologicals, 28: 193-197.

Jodar, L., I.M. Feavers, D. Salisbury and D.M. Granof, 2002. Development of vaccines against meningococcal disease. Lancet, 359: 1499-1508.

Lehmann, A.K., S. Sqrnes and A. Halstensen, 2000. Phagocytosis: Measurement by flow cytometry. J. Immunol. Methods, 243: 229-242.

Lortan, J.E., A.S. Kaniuk and M.A. Monteil, 1993. Relationship of *in vitro* phagocytosis of serotype 14 Streptococcus pneumoniae to specific class and IgG subclass antibody levels in healthy adults. Clin. Exp. Immunol., 91: 54-57.

Lun, A., M. Schmitt and H. Renz, 2000. Phagocytosis and oxidative burst: Reference values for flow cytometric assay independent of age. Clin. Chem., 46: 1836-1839.

Martinez, J., T. Pilishvili and S. Barnard *et al*., 2002. Opsonophagocytosis of fluorescent polystyrene beads coupled to *Neisseria meningitidis* serogroup A, C, Y, or W135 polysaccharide correlates with serum bactericidial activity. Clin. Diag. Lab. Immun., 9: 485-488.

Norheim, G., A. Aase, D.A. Caugant, E.A. Hoiby, E. Eritzsonn, T. Tangen, P. Kristiansen, U. Heggelund and E. Rosenqvist, 2005. Development and characterization of outer membrane vesicle vaccines against serogroup A *Neisseria meningitids*. Vaccine, 23: 3762-3774.

Peterson, G.L., 1977. A simpilification of the protein assay of Lowry *et al*. Which is more generally applicable. Anal. Biochem., 83: 346-356.

Plested, J., B.L. Ferry, P.A. Coull, K. Makepeace, A.K. Lehmann, F.G. Mackinnon, H.G. Griffiths, M.A. Herbert, J.C. Richards and E.R. Moxon, 2001. Functional opsonic activity of human serum antibodies to inner core Lipopolysaccharide (gale) of serogroup B meningococci measured by flow cytometry. Infect. Immun., 69: 3203-3213.

Romero-Steiner, S., D. Libutti and L.B. Pais *et al*., 1997. Standardization of an opsonophagocytic assay for the measurement of functional antibody activity against streptococcus pneumoniae using different HL-60 cells. Clin. Diag. Lab. Immun., 4: 415-422.

Rosenstin, N.E., B.A. Perkins, D.S. Stephens, T. Popovic and J.M. Hughes, 2001. Meningococcal disease. N. Engl. J. Med., 344: 1378-1388.

Ruggeberg, J.U. and A.J. Pollard, 2004. Meningococcal vaccines. Pediat. Drugs, 6: 251-266.

Siadat, S.D., Q. Behzadiyannejad, B. Tabaraie, H. Ahmadi and M. Nejati, 2006. Extraction and molecular evaluation of *Neisseria meningitidis* serogroup b outer membrane vesicle containing porA. Danshvar, 65: 23-32.

Siadat, S.D., D. Norozian, B. Tabaraie, Q. Behzadian-Nejad, H. Ahmadi, S. Najar-Peerayeh, M. Nejati and B. Adibi Motlagh, 2007a. Comparative studies of conjugated capsular polysaccharide of neisseria meningitidis serogroup a with outer membrane vesicle of neisseria meningitidis serogroup B. Res. J. Microbiol., 2: 337-345.

Siadat, S.D., Q. Behzadian-Nejad, B. Tabaraie, H. Ahmadi, D. Norouziuan, S. Najar-Peerayeh, M. Nejati, M.H. Hedayati and M. Zangeneh, 2007b. Evaluation of serum bactericidal activity specific for neisseria meningitidis serogroup A and B: Effect of immunization with *Neisseria meningitidis* SerogroupA polysaccharide and serogroup B outer membrane vesicle conjugate as a bivalent meningococcus vaccine candidate. Res. J. Microbiol., 2: 436-444.

Väkeväinen, M., W. Jansen and E. Sadand *et al*., 2001. Are the opsonophagocytic activities of antibodies in infant sera measured by different pneumococcal phagocytosis assay comparable? Clin. Diag. Lab. Immun., 8: 363-369.

WHO (World Health Organization), 1976. Requirements for meningococcal polysaccharide vaccine. WHO Tech. Rep. Ser., 594: 50-75.