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# Comparative Studies of Conjugated Capsular Polysaccharide of *Neisseria meningitidis* Serogroup A with Outer Membrane Vesicle of *Neisseria meningitidis* Serogroup B

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**Abstract:** In this study, polysaccharide obtained from *Neisseria meningitidis* serogroup A was purified according to published protocol of World Health Organization. The outer Membrane Vesicle (OMV) of meningococcal serogroup B was also extracted by ultracentrifuge employing deoxycholate reagent. The derivatives of polysaccharide of meningococcal serogroup A (GAMP) was conjugated to OMV of meningococcal serogroup B which was treated by 1-ethyl-3- (3 dimethylaminopropyl) carbodiimide (EDAC).The obtained polysaccharide was activated by cyanogen bromide (CNBr) and 1-cyano-4-dimethy laminopyridinium tetrafluoroborate (CDAP) separately. First Adipic acid dihydrazide (ADH) with EDAC was bound to GAMP activated with CNBr to form $GAMP_{CNBr}$ AH. Second ADH with EDAC was bound to GAMP activated with CDAP to form $GAMP_{CDAP}$ AH. Then these derivatives were conjugated to OMV by EDAC to form $GAMP_{CNBr}$ AH-OMV and $GAMP_{CDAP}$ AH-OMV. The yields of $GAMP_{CDAP}$AH-OMV was 45 to 48% which was higher than that of the other prepared conjugate (15-17%). The glycoconjugates were shown to induce hyper immunity in rabbits and formed antibodies against the above mentioned conjugates were detected by immunodiffusion technique.

**Key words:** Polysaccharide-protein conjugated vaccine, *Neisseria meningitidis* serogroup A and B, outer membrane vesicle

## INTRODUCTION

Many strategies are being explored to manipulate the immune system of humans/animals to fight better against a pathogenic organism. One of the most efficient and cost-effective methods is a prime/boost strategy with vaccines. Several types of vaccines, which comprise either an attenuated strain of the infectious organism, or a weakened form of a toxin that it produces, have been developed and employed. However, the possibility of infection with these agents has led to their replacement by subunit vaccines, that contain only the antigens triggering the immune system (Hossany *et al*., 2004; Ravvenscraft *et al*., 1999; Costantino *et al*., 1999). Carbohydrates in the form of capsules surround

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many pathogenic bacteria are important in bacterial survival and virulence within the host, are also one of the main bio-molecules that are recognized by the immune system. Therefore, targeting carbohydrate antigens is promising avenues in order to develop efficient vaccines. Some polysaccharides-based vaccines have been successfully developed, but generally have shown weak immunogenic effects, poor responses in infants, the elderly and in immunodeficient persons (Hossany *et al*., 2004; Rosenstin *et al*., 2001). The incidence of endemic meningitis and the frequency of epidemic meningitis caused by group A *Neisseria meningitidis* (GAM) are increasing in Africa and Asia. Although GAMP 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 GAMP 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, an age group which has significant disease burden, due to nature of their immune response. To overcome this limitation, glycoconjugate vaccines are being developed against A,Y and W 135 and some have been commercially exploited to vaccinate against serogroup C (Schmidt *et al*., 2001; Bethell and Pollard, 2001). Covalent linkage of the polysaccharide, or fractions thereof, to immunogenic carriers i.e., proteins create glycoconjugates which are t-dependent antigens and prime for boosting either with the glycoconjugate or the capsular polysaccharide. On the other hand, polysaccharide-protein conjugate has been proven to be effective in several cases and well-defined glycoconjugate vaccines have also been explored with a view to elicit discriminating immune responses (Lesinski and Westerlin, 2001; Ada, 2001; Lindberg, 1999; Perez-Melgosa *et al*., 2001). However, the development of a capsular vaccine against serogroup B is an obstacle due to weak immunogenicity believed to be associated with structural similarity between the capsular polysaccharide and human neural antigen. As a result, the other surface molecules, such as outer membrane proteins are being evaluated as potential candidate for vaccines against *N. meningitidis* serogroup B (Peeters *et al*., 1996, 1999; Ruggeberg and Polard, 2004; Arigita *et al*., 2004). We prepared conjugates with GAMP from *N. meningitidis* serogroup A and OMV from *N. meningitidis* serogroup B as a bivalent meningococcal vaccine candidate. We activated GAMP by two schemes, one by CNBr and the other by CDAP. Both $GAMP_{CNBr}$ and $GAMP_{CDAP}$ were treated with ADH to form an adipic hydrazide derivative (AH). EDAC forms an amide linkage between the hydrazide of the GAMP and the carboxyl of protein. This synthesis is accompanied by side reactions that include the formation of amide bonds between the ε-amino groups of lysines adjacent to carboxyl residues of the protein (intramolecular cross-linking) and other adjacent proteins (intermolecular cross-linking). Succinic anhydride (SA) or dihydro-2, 5-furandione reacts rapidly with ε-amino groups of lysines and ε-amino groups of N termini of protein at pH 7 to 8, forming an amide bond by replacing the amino with carboxyl groups. Succinic anhydride also reacts, to lesser extent, with tyrosyl, histidyl, cysteinyl and threonyl side chains of proteins that are hydrolyzed rapidly at alkaline pH. Theoretically, the conversion of ε-amino groups of lysines following succinylation of the protein should reduce EDAC to induce intra- and intermolecular amide formation. The additional carboxyls should also facilitate binding of AH-GAMP derivatives to protein (Gupta *et al*., 1995; Paviakova *et al*., 1999). In this study $GAMP_{CNBr}$ and $GAMP_{CDAP}$ were conjugated to OMV as a carrier protein and then, the level of obtained conjugates were compared in order to study the immunogenicity of the conjugates.

## MATERIALS AND METHODS

Adipic acid dihydrazide (ADH), 1-ethyl-3- (3 dimethylaminopropyl) carbodiimide (EDAC), 1-cyano-4-dimethy laminopyridinium tetrafluoroborate (CDAP), trinitrobenzen sulfonic acid (TNBS), agarose, bovin serum albumin (BSA), cyanogens bromide (CNBr), EDTA, L-cysteine, L-glutamic acid, dextran were of Sigma Chemical Co., USA., Sepharose CL-4B and Sephadex G-50 were purchased from Pharmecia., Triethylamine (TEA) was from Pierce, Rockford., Acetonitrile (T.J. Barker, Inc.), dialysis membrane (cutoff, 6000 to 8000) were procured from Spectra. Pro, Calif., Respectively. Pyrogen-Free Water (PFW) and Pyrogen-Free Saline (PFS) were used in all experiments.

### Preparation of OMVs and GAMP

OMVs were prepared as described by Claassen *et al*. (1996). 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 (upto early stationary phase). Outer membrane vesicles (OMVs) were extracted in 0.1 M Tris-HCl buffer, pH 8.6 containing 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 (Claassen *et al*., 1996; Norheim *et al*., 2005). *N. meningitidis* serogroup A (CSBPI, G-243) was cultivated on a modified Frantz medium and their GAMPs were purified according to the World Health Organization (WHO), (1976) protocol.

### Activation and Derivatization of GAMP

A-GAMP at a concentration of 10 mg $ml^{-1}$ in pyrogen free water was treated with CNBr (1 mg $mg^{-1}$ of polysaccharide) at pH 10.5 for 6 min at room temperature in a pH stat. The reaction mixture was brought to pH 8.5 by the addition of an equal volume of 0.5 M ADH and 0.1 M EDAC prepared in 0.5 M $NaHCO_3$ solution. The mixture was tumbled for 20 h at 4°C and the mixture was passed through a column of Sephadex G-50 (2.5×90 cm) using Pyrogen Free Water (PFW) as an eluant. The fractions of (each) 2.5 ml were collected and the peak was pooled, dialyzed against 6 liters of distilled water at 4°C with two changes of d/w for 3 days and freeze-dried (Jin *et al*., 2003).

B-CDAP was made at a concentration of 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 PS (10 mg $ml^{-1}$) and 30 seconds later, a volume of 0.2 M TEA equal to that of CDAP concentration was added. After 150 seconds an equal volume of 0.5 M ADH and 0.1 M EDAC in 0.5 M NaHCO3 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 (2.5×90 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.

### GAMPAH Conjugates of OMV

OMV at a concentration of 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°C and then centrifuged at 16,000 g, 4°C for 20 min, the supernatant was passed through a CL-4B Sepharose column (1.5×90 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°C and passed through a 0.45 μm membrane and stored at 3-8°C (Fukasawa *et al*., 1999; Schneerson *et al*., 1980; Gupta *et al*., 1995).

### Analytical Methods

Protein content of OMVs was measured according to Peterson (1977). The method of Hestrin (1949) was used to estimate O-acetyl groups present in conjugates of GAMP. 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 of the conjugates was assayed by the *Limulus amebocyte 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; Pyrogen test, 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 [0.01 M PBS supplemented with 0.5% BSA and 0.1% gelatin (PBG-Sigma)] and vesicles on the grids were fixed by 1% glutaraldehyde in PBS and negatively stained by potassium phosphotungstate at pH 6.0. The grids were examined in a Zeiss CEA902A electron microscope at 80 Kv (Claassen *et al*., 1996).

**Hyperimmune Antisera**

Hyperimmune sera were prepared by repeated injection of New Zealand white rabbit (weighing 2-2.5 kg) as described by Jennings and Lugowski (1981).

**Immunodiffusion**

Double immunodiffusion test was performed in 0.8% agarose prepared in PBS containing 2.0% polyethylene glycol 4000 (Jennings and Lugowski, 1981; Ouctherlony, 1962).

## RESULTS AND DISCUSSION

The O-acetyl contents of GAMP and $GAMP_{CDAP}$ AH were 2.7 mmol $mg^{-1}$ of protein and 1.14 mmol $mg^{-1}$ of protein and $GAMP_{CNBr}$ H 1.05 mmol $mg^{-1}$ of protein. Employing TNBS method, the contents of CNBr-activated GAMP AH was 1.00% (mean value) AH and CDAP- activated GAMP AH had 1.05% (mean value) AH. Figure 1 indicates elution profile of conjugated OMV-GAMP on Sepharose CL-4B column chromatography showing two peaks of conjugate and protein. The first peak is emerging in void volume with a protein/GAMP ratio of approximately 1:2.5. The yield of conjugate was found to be 45-48% in terms of the GAMP recovered from the conjugate. Likewise, $GAMP_{CNBr}$ AH-OMV and $GAMP_{CDAP}$ AH-OMV, showed two peaks of conjugate and OMV. But the yield of conjugate was 15-17% in terms of the GAMP recovered from the conjugates. The immunogenicity of polysaccharide component could be due to I) their molecular weight, ii) the density of carbohydrate on the carrier, iii) and the intactness of carrier protein (Costantino *et al*., 1999; Ada and Isaacs, 2003). Exclusively, the immunogenicity of GAMP, a linear homopolymer of (1, 6)-α-D-Manp NAc-1-PO4 O-acetylated at C-3, is related to its molecular size and O-acetyl content (Liu *et al*., 1971). GAMP is comparatively unstable as phosphodiester bond is labile for hydrolysis in both acidic/alkaline conditions and elevated temperature (Gudlavalleti *et al*., 2004). In order to avoid hydrolysis and maintain the immunogenicity, GAMP vaccine is stored at 4°C in freeze-dried state with a carbohydrate that competes for the residual moisture. CDAP-mediated activation at pH 8.0 is preferred over activator such as cyanogens bromide (CNBr), which requires pH of ≥ 10.5, as shown by the lesser effect on the molecular size of GAMP. CDAP activation at close to neutral pH makes it useful for pH-sensitive GAMP. Berry *et al*. (2002) showed the essential role of

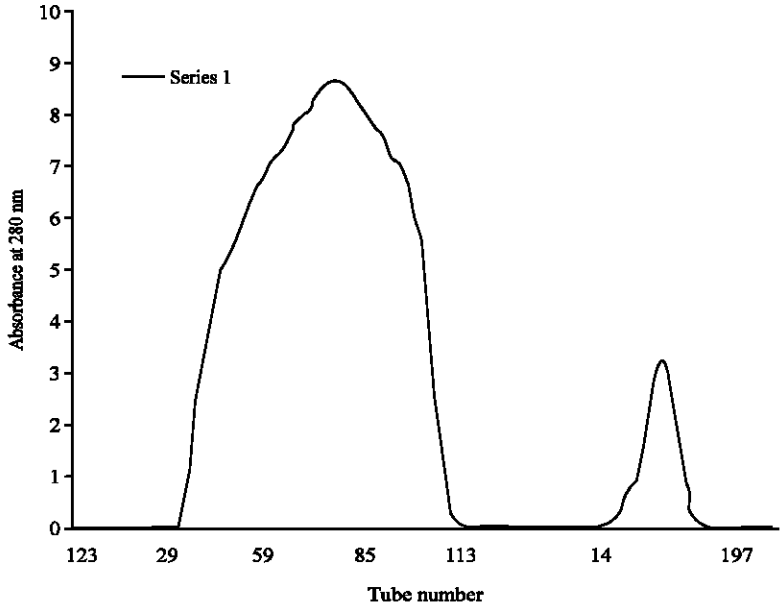


Fig. 1: Elution profile of conjugate of OMV-GAMP on Sepharose CL-4B column

O-acetyl in eliciting maximal levels of GAMP antibody production. Activation of GAMP by CDAP retained most of its O-acetyl content. Probably due to the ability of CDPA to activate GAMP at a pH closer to neutrality, the O-acetyl level was higher after GAMP activation than after CNBr activation (Jin *et al*., 2003). For this reason the average yield of $GAMP_{CDAP}$ AH-OMV in this process was high. Electron microscopy was used 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. 2A). Intactness of the vesicles in these preparations ranged from 50 to 80% of the vesicles. Figure 2B shows that the OMV maintained its original conformation even when coupled to GAMP. 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). 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, as compared to *E. coli* standard endotoxin. The endotoxin activity of the conjugates is in the range of DPT/polio vaccines and could be regarded as safe. The endotoxin activity of LPS in vesicles was found to be 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 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). This type of vaccine could be further exploited for human use as the LPS content is 10% w/w ratio as compared to OMP. Knowingly OMV structure has a shielding effect on the endotoxin properties of LPS (Claassen *et al*., 1996; Anderson *et al*., 1994; Cartwright *et al*., 1999).

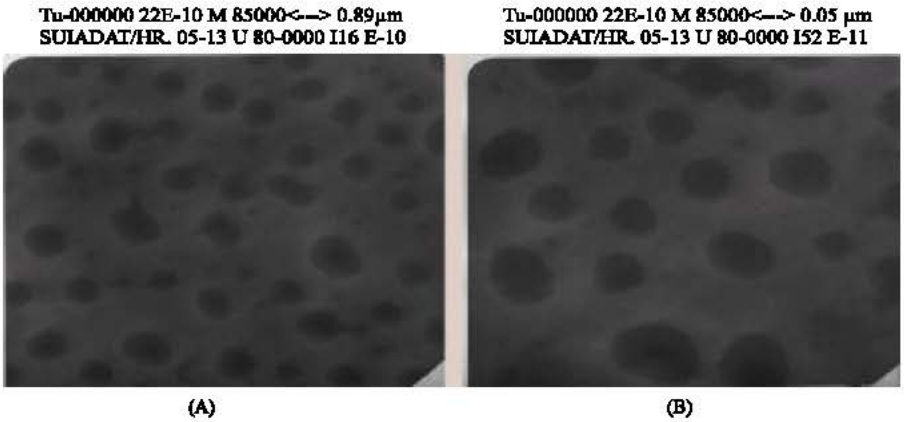


Fig. 2: Electron microscopy of Outer Membrane Vesicle (OMV) stained with 1% potassium phosphotungstate. (A) Control vesicles. (B) GAMP conjugated vesicles

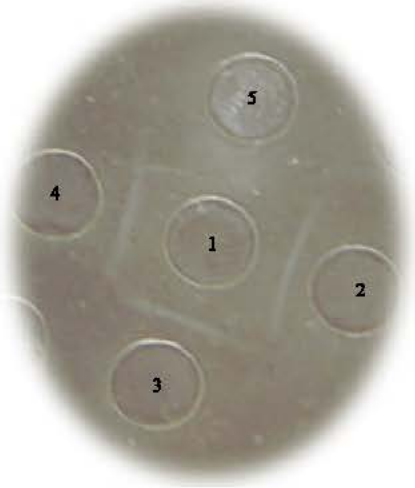


Fig. 3: Precipitation lines in Double Immunodiffusion test: 1: Hyperimmun sera rabbits with OMV-GAMP Conjugates (56 days) 2: Conjugate of OMV with GAMP 3: OMV 4: GAMP 5: Normal Saline

The $GAMP_{CNBr}$ AH-OMV conjugate, $GAMP_{CDAP}$ AH-OMV conjugate, GAMP and OMV were used as immunogens in rabbit and the antisera were evaluated by quantitative precipitin and immunodiffusion analysis. The precipitin curves obtained from each of the above antisera, when treated with its homologous antigen, indicated that a good antibody response was obtained using all the four immunogens. Three precipitin reactions were observed with the hyperimmune rabbit antisera, who were immunized by $GAMP_{CDAP}$ AH-OMV, as well as $GAMP_{CNBr}$ AH-OMV conjugate, raised against each of the $GAMP_{CDAP}$ AH-OMV conjugate, GAMP and OMV (Fig. 3). In a group A/B meningococcal vaccine, OMV could possibly be used as a group B antigen and as a polysaccharide carrier. For this purpose, OMV from the serogroup B strain CSBPI, G-245 was chosen as a carrier of group A capsular polysaccharide (Vella *et al*., 1990) and this conjugate was evaluated for the effect of two polysaccharide activator. It has been well demonstrated that the average yield of $GAMP_{CDAP}$ AH-OMV in this process was higher than that of $GAMP_{CNBr}$ AH-OMV.

In summary, the average yield of conjugation should be improved through a useful activating reagent. Thus, GAMP-OMV with immunogenicities improved over that of GAMP have been prepared and standardized. CDPA seemed to be a more useful activating reagent, because the treated GAMP had a higher molecular weight and content of O-acetyl than other activator (CNBr). Therefore, the development of a A/B bivalent anti-meningococcal vaccine could be a good candidate.

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