ABSTRACT

The immunogenicity to several meningococcal polysaccharide-protein conjugates was evaluated in mice. The purified polysaccharide from N. meningitidis serogroup C (PsC) was linked to different quantities of outer membrane vesicle from N. meningitidis serogroup B (OMV) (3, 6 and 9 mg) as carrier protein, via carbodiimide-mediated reaction. The conjugates were inoculated in Balb/c mice in three doses of 10 mg de PsC by intraperitoneal route and sera samples were collected before each dose and 7 and 14 days after the last immunization. The anti- PsC IgM, IgG and IgG subclasses antibodies (IgG1 and IgG2a) were evaluated in sera of mice by an indirect ELISA test. The results showed high titers of anti-PsC IgG with IgG1 as isotype predominant in all the mice that were inoculated with conjugates. No significant differences were observed between conjugates obtained with different quantities of OMV. The use of OMV as a carrier also induced IgG2a anti-PsC antibodies. We conclude that 3 mg of OMV are sufficient to obtain immunogenic conjugates of PsC.

Key words: Neisseria meningitidis, immunogenicity, conjugated vaccines

Introduction

Polysaccharides (Ps) are the main factor of virulence of many pathogenic bacteria, such as Neisseria meningitidis that induce invasive diseases. The antibodies generated to the capsular polysaccharide of Neisseria meningitidis serogroup C (PsC) are bactericidal and protective [1, 2]. The endemic type of meningococcal meningitis is induced by the serogroups A, B, C, Y and W135, while the epidemic type is induced by the serogroups A, B and C [3]. Some univalent, bivalent or tetravalent antimeningococcal vaccines against serogroups A, C, Y and W135 have been created to prevent that disease. They are immunogenic and safe for adults and children over 2 years old, but not for younger children, for whom those vaccines are usually poorly immunogenic. This is due to the thymo independence (TI) of Ps [4, 5].

Conjugation solves the thymo independence of Ps, by converting it in thymo dependent (TD) [6, 7]. This change in the immunological behavior occurs because the protein has epitopes that are recognized by T cells, what makes possible the cooperation with the B cells that recognize the polysaccharide portion to produce a change of isotypes of immunoglobulins, especially IgM to IgG or IgA.

As Ps conjugates behave as TD antigens, the induction of the immune response is enhanced in small children that are the population with the highest risk to contract those diseases [8, 9]. Some immunological studies in humans with conjugated vaccines demonstrate the above-mentioned statement; among them, with the vaccines of Haemophilus influenzae type b (Hib) currently commercialized, composed of the covalent union of the Ps of that microorganism with different proteins of bacterial origin, as well as, in more recent studies of conjugate vaccines against Neisseria meningitidis serogroup C [10-12].

For obtaining a conjugated vaccine, we should take into account the following factors: the molecular weight of Ps, the length of the molecules of the saccharide, the carrier, the union between those two molecules and the degree of substitution of the proteins with the saccharide. The relationship of the power of the conjugate with its molecular architecture becomes difficult because of the differences between

those factors during the processes for the obtaining of the conjugates [13].

The conjugation of the Ps with the proteins has been tested by different obtainment methods, different proteins and by the use of spacing or not spacing arms. However, the quantities of proteins that should be used in the obtainment process have not been studied enough. That is why we have studied how the immune response generated against the polysaccharide C in Balb/c mice changes when we use different quantities of proteins to obtain the conjugates, from the covalent union of the Ps of N. meningitidis serogroup C with Outer Membrane Vesicles (OMV) of N. meningitidis serogroup B.

**Materials and methods**

**Reagents**

Carbodiimide of 1-ethyl-3 (3-dimethylaminopropyl) (EDAC) (Sigma); ultrafiltering membranes (YM10) (Amicon Inc.); anti-mice IgG obtained in sheep and marked with alkaline phosphatase and poly-L-lysine (Sigma); anti-IgG1 or anti-IgG2a biotine-avidine conjugate (Pharmigen); Sepharose CL-4B (Amersham Biosciences); anti-PsC rabbit serum (Murex Biotech Ltd.) and (batch 2012C with Kay = 0.2) and PsC OMV (batch 2006B) (“Finlay” Institute, Cuba).

**Analytical methods**

The determinations of proteins and sialic acid were performed according to the papers by Lowry et al., and by Svennerholm, respectively [14, 15]. The concentration of the amino groups was determined by ophatic diodehyde (OPA), with glycine as a standard [16]. The double immunodiffusion was performed in agarose 0.8% - NaCl 0.15 mol/L - sodium azide 0.01% by using an anti-PsC rabbit serum [17].

**Synthesis of the Neisseria meningitidis serogroup C polysaccharide conjugate to outer membrane vesicles of Neisseria meningitidis serogroup B**

**PsC Activation**

The PsC to generate amine groups was activated through a basic hydrolysis, from the acetamide groups present in Carbon 5. The PsC (5 mg/mL) in NaOH 0.5 M was placed in an oven at 90 °C for 3.5 h. Subsequently, the solution was cooled at room temperature and the pH was adjusted to 7.0 by adding HCl 0.1 mol/L. The resulting solution was applied to a chromatography column 1.6 x 90 cm with Sepharose CL-4B as a matrix of molecular exclusion and NaCl 0.15 mol/L as buffer solution of elution. The fractions corresponding to 0.4 Kav were passed through 0.2 µm and samples were collected for the controls of PsC content and the antigenicity by double immunodiffusion [17].

**Preparing the polysaccharide conjugates serogroup C to outer membrane vesicles of Neisseria meningitidis serogroup B**

The conjugates were prepared by mixing the amine groups generated in PsC and the carboxylic groups present in the OMV. For activating the carboxylic groups, EDAC was used. The OMV (3, 6 y 9 mg) was treated with EDAC 0.1 mol/L. and later, 4 mg previously activated PsC were added. The reaction was kept at 4 oC and a pH 5 - 5.6 for 4 h. The mixture of the reaction was ultrafiltrated with a Phosphate Buffer Saline solution at pH 7.2. Samples were collected for the determinations of PsC, protein, antigenicity by double immunodiffusion and immunogenicity.

**Immunojugation**

Groups of 8 Balb/c (female) mice, whose starting weight were 18 - 22 g, were used. Three doses of the different conjugates prepared from OMV and PsC were intraperitoneally administered to the mice on 0, 14 and 28 days (10 µg PsC/ dose) [18]. Three control groups were also used as placebo: native PsC, OMV and PBS. The sera were obtained before each inoculation and on 35 and 42 days after the first dose; they were separately collected and stored at -20 °C until their utilization.

**Determination of IgG and IgM antibodies**

The immunogenicity of the conjugates was determined through the ELISA test, by using polystyrene plates (Costar). As a recovering antigen, the starting PsC was used. It was fixed after a previous treatment with poly L-lysine (3 µg/mL) (Sigma). The samples were diluted 1:400 for determining IgG antibodies, or 1:200 to determine the IgM antibodies in PBS-Tween 20. A phosphatase anti-IgG conjugate (Sigma) from mice and a peroxidase anti-IgM conjugate (Sigma) from mice were used. o-nitrophenyl-phosphate was used as substrates, respectively, and the absorbances of the samples were measured in Tintertek Multiskan equipment at an optical density (OD) of 405 nm for phosphatase and 492 nm for peroxidase.

**Determination of IgG subclasses anti-PsC of Neisseria meningitidis**

The determination of IgG subclasses was carried out through an ELISA test [19] of biotine-streptavidine amplification on 96-wells polystyrene plates (Maxisorp, Nunc). After a treatment with 100 µL/well poli L-lysine (3 µg/mL) at room temperature for 30 min in a humid chamber, and the first washing with PBS-Tween 20 (washing solution), they were covered with a PsC solution (5 µg/mL) and kept in incubation overnight in a humid chamber at 4 °C. Subsequently, the plate was washed and blocked with PBS-BSA-Tween 20. The samples were applied in a dilution 1:100 in PBS-BSA-Tween 20 and incubated over-night at 4 °C. The next day, the plate was washed with the washing solution and the antiIgG1 or anti-IgG2a biotinitated conjugates (Sigma). After a step of washing similar to the former steps, streptavidine (Sigma) was added. As substrate, o-phenylenediamine (Sigma) was used and the reaction was stopped with H2SO4 2.5 mol/L. Absorbance was measured at 492 nm in the ELISA reader.

**Statistical analysis**

The statistics of results was performed from a variance analysis (ANOVA) with a 5% significance level to compare the averages among the groups. When
differences were found, the multiple comparisons tests of lower significant difference (LSD) were used. The statistical package (Version 2.1) and the Microsoft Excel program were used to find the averages and the standard deviation of the values obtained through the ELISA test.

**Results**

**Composition of the conjugates**

The activated PsC had an average of 1.55 μmol of amino groups for each milligram of sialic acid and the yield of the conjugates in all the experiments (n = 5) was similar for each group. Its content of average free PsC was 26% (Table 1). The three conjugates and the unconjugated polysaccharide were recognized by the anti-PsC antibodies present in the commercial anti-PsC rabbit serum, what formed a precipitation halo in the double immunodiffusion (Figure 1). However, the activated polysaccharide was not recognized by those antibodies, due to the absence of the O-acetyl groups as a consequence of the process of its activation.

**Determination of anti-PsC IgM antibodies**

Figure 2 shows that the unconjugated polysaccharide induced an increase of the IgM titers after the third dose (42 day). Nevertheless, the conjugated obtained with the different quantities of the carrier reached higher values in the determination of that antibody. The statistical analysis of the results showed that there are no significant differences among the titers obtained for the sera of the animals inoculated with the 3 and 9 mg OMV conjugates. However, significant differences (p < 0.05) were observed when the values of the 3 mg conjugate were compared with the OMV 6 mg conjugate and the 6 mg conjugate were compared with the 9 mg OMV conjugate.

**Determination of anti-PsC antibodies**

Figure 3 shows the results of the determination of anti-PsC IgG antibodies in the sera of the tested mice. The sera of the animals inoculated with the conjugates showed high and significant (p < 0.05) anti-PsC IgG antibodies, while in the sera of the animals inoculated with the unconjugated PsC no titers of that kind of antibody were found. All the animals showed a secondary response after the immunization of a second and a third dose of the conjugates, and among the conjugates no significant differences in the IgG titers determined in the mice’s sera were observed.

**Determination of the IgG subclasses generated in the immunized mice**

Figure 4 shows how the three conjugates induced high and significant titers of both subclasses of IgG antibodies (IgG1, IgG2a) 14 days after the last dose (42 day); while the sera of mice inoculated with the unconjugated PsC did not induce those subclasses. In the sera of the mice inoculated with the conjugates no significant differences were observed in the determination of IgG1. However, in the determination of IgG2a, significant differences (p < 0.05) were found in the sera of the animals inoculated with the conjugates for which 3 mg and 9 mg of OMV were used, while no significant differences were found between 3 and 6 mg OMV or between 6 and 9 mg OMV.

**Discussion**

Many reports describe the contribution of the conjugated vaccines with different chemical couplings between polysaccharides and proteins [20], as well as the differences regarding the obtainment of pro-

| Table 1. Characterization of the conjugates of polysaccharide of N meningitidis serogroup C to OMV of N. meningitidis serogroup B. |
|---|---|---|---|---|
| Conjugate | PsC (mg/mL) | OMV (mg/mL) | PsC/OMV Ratio | Free PsC (%) |
| PsC-OMV (3 mg) | 1.76 | 1.3 | 1.35 | 32 |
| PsC-OMV (6 mg) | 1.82 | 2.9 | 0.63 | 26 |
| PsC-OMV (9 mg) | 1.85 | 4.3 | 0.43 | 20 |

Figure 1. Double immunodiffusion of the polysaccharide of N. meningitidis C (PsC) to determine the identity of the polysaccharide in the conjugates. 1: anti-PsC rabbit serum (Murex Biotech Ltd); 2: PBS; 3: Non-conjugated PsC; 4: PsC activated by basic hydrolysis; 5: PsC-OMV (3 mg); 6: PsC-OMV (6 mg); 7: PsC-OMV (9 mg).

Figure 2. Determination of anti-PsC IgM antibodies in sera of mice inoculated with the tested conjugates and nonconjugated PsC with three doses on 0, 14 and 28 days, intraperitoneally and extraction on the days 0 and 42. The sera were diluted 1:200 for


The sera were diluted 1:400 to determine. PsC, polysaccharide of N. meningitidis serogroup C, PsC-OMV (3 mg), Conjugate of polysaccharide of N. meningitidis serogroup C to 3 mg OMV of N. meningitidis serogroup B; PsC-OMV (6 mg), Conjugate of polysaccharide of N. meningitidis serogroup C to 6 mg OMV of N. meningitidis serogroup B; PsC-OMV (9 mg), Conjugate of polysaccharide of N. meningitidis serogroup C to 9 mg OMV of N. meningitidis serogroup B.

Other PsC conjugates, obtained by reductive amination [28-30], have been used in previous studies on proteins that vary from the carriers used in the conjugation [21], the concentrations of the antigens, the use of spacing arms [13], the molecular sizes [4] and the ways of activating the saccharides. This leads to different degrees of substitution [22]. It is difficult to perform any comparison among the vaccines because of those differences in the process to obtain them.

The most varied components of the conjugate vaccines are the carriers. The N. meningitidis serogroup B OMV has been used in this type of vaccines [18] and also in vaccines against N. meningitidis serogroup B that have been the most tested in humans. Among the OMV vaccines available in the market, there are the Cuban vaccine VAMENGO-C-BC6 (recommended for the vaccination against the serogroups B and C of N. meningitidis) [23], the vaccine NIPH N. meningitidis B, from Norway, the purified OMV of the 44/76 strain [24] and the PorA-OMV hexavalent, from Holland [25].

The OMV has been successfully used as a carrier in a vaccine against H. influenzae type b [26] and in conjugated vaccines against Pneumococcus [27], both commercialized by Merck & Co. Recently, Fukasawa et al. [18] conjugated PsC to OMV with carbodiimide coupled to adipic acid of hydrazide (ADH) as spacing arm. They studied the immune response in C3H/Hepas mice and found that those conjugates generated high titers of IgG with a great increase (21 times) 42 days after the first inoculation, by using three doses of conjugates the 0, 14 and 28 days.

Our results corroborate the results of Fukasawa et al. [18] that OMVs are good carriers for conjugated vaccines and also demonstrate that only 3 mg are required to obtain conjugates with high immunogenic results.

Other PsC conjugates, obtained by reductive amination [28-30], have been used in previous studies on Bablic mice. In those analyses it was observed that the conjugates, compared to the unconjugated PsC, showed a change of IgM and IgG3 isotypes in the native PsC to IgG and IgG1 in the conjugates.

García-Ojeda et al. [31] used PsC-TT conjugates that were inoculated to Balb/c mice. They concluded that the use of a PsC TD type that is obtained when conjugating PsC, compared to the TI type, induces a change of isotype. The anti-PsC antibodies are mainly of IgM and IgG3 isotypes, while the PsC-TT conjugates induced mainly IgG1.

This study demonstrated that PsC was conjugated to different quantities of OMV and that titers of IgG antibodies higher than those induced by the native PsC were induced in the three cases tested. It was also shown that when PsC was covalently joined to OMVs, there was an increase in the immune response and that effect was not observed in the unconjugated PsC.

As in papers by other authors [28-31], the results of this research showed that neither the IgG antibodies nor the subclasses evaluated were produced by PsC unconjugated and that IgG and IgG1 were the predominant isotypes in the sera obtained from mice inoculated with the different conjugates, with high titers in the three cases. Those results also showed the significant levels of IgG2a generated by those conjugates.

Considering these results and the results obtained by other authors, we concluded that in the three conjugates, a change in thymo dependence of PsC from TI to TD was obtained, since the IgG and IgG1 relationship increased for the TD antigens and the values of IgG1 antibodies were the predominant subclass, after the second immunization.

Conclusions

By the conjugation of different quantities of OMV to PsC, highly immunogenic conjugates were obtained in Balb/c mice. The sera from those animals showed a
prevalence of IgG and the anti-PsC IgG1 subclass as isotype, even though they also induced IgG2a. Moreover, a change in the thymo independence of PsC to its thymo dependence was observed. Thus, it was demonstrated that 3 mg of this protein were enough to obtain immunogenic PsC-OMV conjugates.


