RESEARCH

# *Polysaccharide of meningococcal group C conjugated to different quantities of outer membrane vesicle from* Neisseria meningitidis *serogroup B*

Maribel Cuello, ✉ Osmir Cabrera, Judith del Campo, Oliver Pérez

Department of Immunology, "Finlay" Institute
Ave 27 No. 19805, PO Box 16017, Ciudad de La Habana, Cuba
E-mail: ocabrera@finlay.edu.cu

***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 from the animals 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

*Biotecnología Aplicada 2005;22:122-126*

***RESUMEN***

**Polisacárido del meningococo grupo C conjugado con diferentes cantidades de vesícula de membrana externa de *Neisseria meningitidis* serogrupo B.** Se evaluó la inmunogenicidad a varios conjugados polisacáridos-proteínas en ratones. En el proceso de obtención de los conjugados del polisacárido de *Neisseria meningitidis* serogroup C (PsC) se utilizaron diferentes cantidades de vesículas de membrana externa (VME) de *Neisseria meningitidis* serogroup B (3, 6 y 9 mg) mediante la reacción con carbodiimida. Se inocularon tres dosis de 10 mg de PsC en ratones Balb/c, por vía intraperitoneal. Los animales fueron sangrados antes de cada inoculación y 7 y 14 días después de la última dosis. Por medio de un ELISA indirecto se evaluaron los niveles de IgM, IgG y las subclases IgG1 e IgG2a anti-PsC, en los sueros de estos animales. Los resultados mostraron títulos elevados de IgG anti-PsC con predominio de la subclase IgG1, y no se observaron diferencias significativas entre los conjugados. La utilización de las VME en los conjugados favoreció la presencia de IgG2a anti-PsC en los sueros de los animales inmunizados. Se concluye que 3 mg de VME son suficientes para obtener conjugados de PsC inmunogénicos.

*Palabras claves: Neisseria meningitidis*, inmunogenicidad, vacunas conjugadas

## *I*ntroduction

Polysaccharides (Ps) are the main factor of virulence of many pathogenic bacteria, such as *Neisseria meningitidis* that induce invasive diseases. The antibodies generated to against 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 mmunoglobulins, 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 of 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

1. Gotschlich EC, Luí TY, Artenstein MS. Human immunity to the meningococcus. I. The role of humoral antibodies. J Exp Med 1969;129:1307-26.

2. Goldschneider I, Gotschlich EC, Artenstein MS. Human immunity to the meningococcus. III. Preparations and immunochemical properties of group A, group B, and group C meningococcal polysaccharides. J Exp Med 1969;129: 1327-48.

3. Rosenstein NE, Perkins BA, Stephens DS, Popoviv T, Hughes JM. Meningococcal Disease. N Engl J Med 2001;344(18): 1378-85.

4. Ruggeberg JU, Pollard AJ. Meningococcal vaccines (Review). Paediatric Drugs 2004;6(4):251-66.

5. Poolman JT. Development of a meningococcal vaccine. Infect Agents Dis 1995;4:13-28.

6. Richmond P, Borrow R, Miller E, Clark S, Sadler F, Fox AJ, Begg NT *et al*. Meningococcal serogroup C conjugate vaccine is immunogenic in infancy and primes for memory. J Infect Dis 1999;179:569-72.

✉ Autor de correspondencia

those factors during the processes for the obtainment 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-Llysine (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 ophtalic dialdehyde (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 conjugates 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 ºC 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 exclusión 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.

### Immunization

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 ºC 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*-phenylendiamine (Merck) or *p*-nitrophenyl-phosphate was used as substrates, respectively, and the absorbances of the samples were measured in Titertek 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-streptadividine amplification on 96-wells polystyrene plates (Maxisorp, Nunc). After a treatment with 100 µL/well poly 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 ºC. 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 ºC. The next day, the plate was washed with the washing solution and the antiIgG1 or anti-IgG2a biotinilated conjugates (Sigma). After a step of washing similar to the former steps, streptavidine (Sigma) was added. As substrate, *o*-phenylendiamine (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

7. Jodar L, Griffiths E, Feavers I. Scientific challenges for the quality control and production of group C meningococcal conjugates vaccines. Vaccine 2004; 22: 1047-53.

8. Trotter CL, Ramsay ME, Kaczmarski EB. Meningococcal serogroup C vaccination in England and Wales: coverage and initial impact of the campaign. Common Dis Public Health 2002;5:220-5.

9. Bröker M. Development of new vaccines against meningococcal disease. Azneim. Forsch. Drug Res 2003;53(12): 805-13.

10. Madore DV, Johnson-Kraines CL, Rothstein EP, Smith DH. Kinetics of antibody response to *Haemophilus influenzae* type b vaccines. Pennridge Pediatric Associates. Curr Med Res Opin 1999;15(2):105-12.

11. Bramley JC, Hall T, Finn A, Buttery RB, Elliman D, Lockhart S *et al.* Safety and immunogenicity of tree lots of meningococcal serogroup C vaccine administered at 2, 3 and 4 months of age. Vaccine 2001;19:2924-31.

12. Finn A. Bacterial polysaccharide-protein conjugate vaccines. Br Med Bull 2004;70:1-14.

13. Cuello M, Cabrera O, Pérez O, Del Campo J, Balboa J, Soto CR *et al.* Influencia del tamaño del espaciador utilizado en la unión covalente de un polisacárido a una proteína sobre la respuesta inmune. Rev CENIC Ciencias Biológicas 2002; 33(2):71-5.

14. Lowry OH, Rosebrough NJ, Farr AL, Randall R. Protein measurement with the Folin phenol reagent. Biol Chem 1951; 193:265-75.

15. Svennerholm L. Quantitative estimation of Sialic II. A colorimetric resorcinol hidrochloric acid method. Biochem Biophy Acta 1957;24:609.

16. Church FC, Porter DH, Calignani GL, and Swaisgood HE. An o-phthalaldehyde spectrophotometric assay for proteinases. Anal Biochem 1986;146(2):343-8.

17. Ouchterlony, O. Diffusion in gel methods for immunological analysis. Prog Allergy 1958;5:1.

18. Fukasawa LO, Gorla MCO, Schenkman RPF, Garcia LR, Carneiro SM, Raw I, *et al. Neisseria meningitidis* serogroup C polysaccharide and serogroup B outer membrane vesicle conjugate as a bivalent meningococcus vaccine candidate. Vaccine 1999;17:2951-8.

19. Ruthus S, Driedijk PC, Weening RS, Out TA. ELISA procedures for the measurement of IgG subclass antibodies to bacterial antigens. J Immunol Methods 1991;140(1):67.

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 found 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 con-jugates 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.

Table 1. Characterization of the conjugates of polysaccharide of N meningitidis serogroup C to OMV of *N. meningitidis* serogroup B.

| Conjugate (n=5) | 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 |

PsC: Polysaccharide de *N. meningitidis* serogroup C
OMV: outer membrane vesicle of *N. meningitidis* serogroup B.

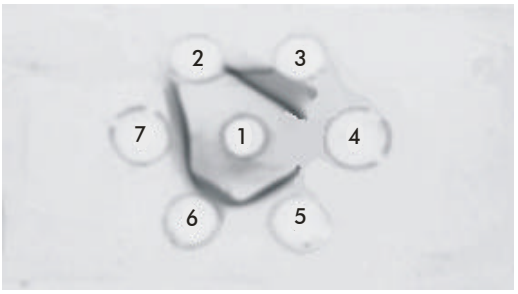


Figure 1. Double immunodiffusion of the polysaccharide of *N. meningitidis* C (PsC) to determine the identity of the polysaccharide in the conjugates. 1: anti-PsC rabbit serum (Murex Biotech Ltd); 2: PBS; 3: Non-conjugated PsC; 4: PsC activated by basic hydrolysis; 5: PsC-OMV (3 mg); 6: PsC-OMV (6 mg);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

## 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-

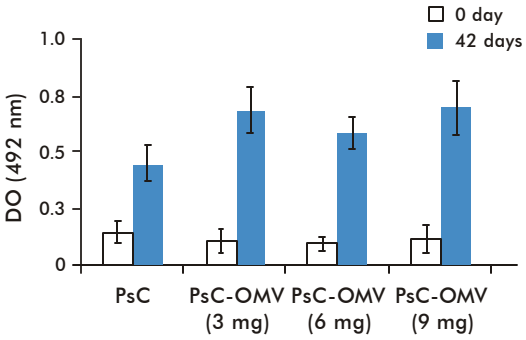


Figure 2. Determination of anti-PsC IgM antibodies in sera of mice inoculated with the tested conjugates and nonconjugated PsC with three doses on 0, 14 and 28 days, intraperitoneally and extraction on the days 0 and 42. The sera were diluted 1:200 for determination. PsC, polysaccharide of *N. meningitidis* serogroup C, PsC-OMV (3 mg), Conjugated of polysaccharide of *N. meningitidis* serogroup C to 3 mg OMV of *N. meningitidis* serogroup B; PsC-OMV (6 mg), conjugated of polysaccharide of *N. meningitidis* serogroup C to 6 mg OMV of *N. meningitidis* serogroup C to 9 mg of OMV of *N. meningitidis* serogroup B.

20. Pawlowski A, Svenson SB. A new simple method for producing antigenic fragments of bacterial polysaccharides for the preparation of conjugates vaccines. FEMS Microbiol Lett 1999;174:255-63.

21. Rüggeberg J, Heat P. Safety and efficacy of meningococcal group C conjugate vaccines. Expert Opin. Drug Saf 2003;2(1):7-19.

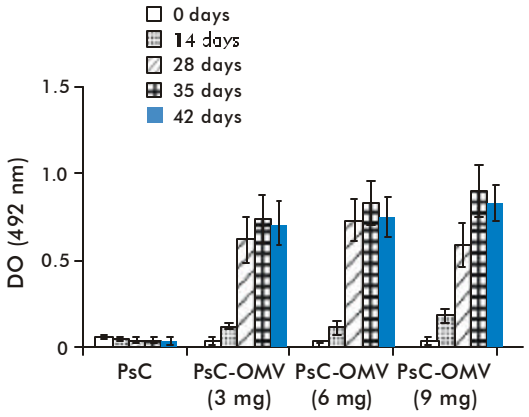


Figure 3. Determination of anti-PsC IgG antibodies in sera of mice inoculated (three doses on 0, 14 and 28 days, intraperitoneally and extractions on the days 0, 14, 28, 35 and 42) with the conjugated studied and the non-conjugated PsC. The sera were diluted 1:400 to determine. PsC, polysaccharide of *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 ofpolysaccharide 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 ME of *N. meningitidis* serogroup B.

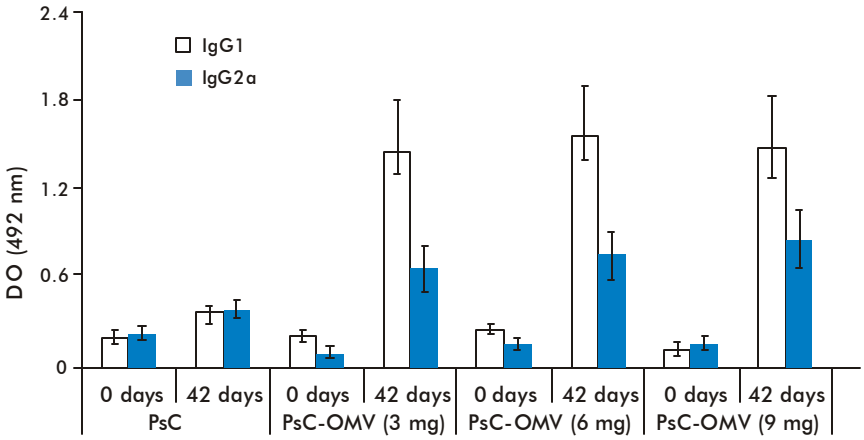


Figure 4. Determination of subclasses of anti-PsC IgG (IgG1 e IgG2a) antibodies in sera of Balb/c mice intraperitoneally inoculated with three doses on the 0, 14 and 28 days and extractions on the 0 and 42 days) with the studied conjugates and the nonconjugated PsC: PsC: polysaccharide of *N. meningitidis* serogroup C; PsC-OMV (3 mg): conjugate of polysaccharide of *N. meningiditis* serogroup C to 3 mg OMV of *N. meningiditis* serogroup B, PsC-OMV (9 mg): conjugate of polysaccharide of *N. meningiditis* serogroup C to 9 mg OMV of *N. meningiditis* serogroup B; PsC-OMV (9 mg): conjugate of polysaccharide of *N. meningitidis* serogroup C to 9 mg OMV of *N. meningiditis* serogroup B.

teins 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 VA-MENGOC-BC® (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 Balb/c 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 IgM 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.

22. Shaffer DE, Toll B, Schuman RF, Nelson BL, Mond JJ, Lees A. Activation of soluble polysaccharides with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) for use in protein-polysaccharide conjugate vaccines and immunological reagents. II Selective cros-slinking of proteins to CDAP-activated poly-saccharides.Vaccine 2000; 18(3):1273-81.

23. Sierra GVG, Campa HC, Valcarcel NM, Izquierdo PL, Sotolongo PF, Casa nueva GVC *et al*. Vaccine against group B *Neisseria meningitidis*: protection trial and mass vaccination. Results in Cuba. NIPH ANNALS 1991;14(2):195-207.

24. Frediksen JH, Rosenqvist E, Wedege E, Bryn K, Bjune G, Froholm LO *et al.* Production, characterization and control of MenB-vaccine Folkehelsa. An outer membrane vesicle vaccine against group B meningococcal disease. NIPH Ann 1991; 14:67-80.

25. Peeters CCAM, Rumke HC, Sundermann LC, Van der Voort EMR, Meulenbelt J, Schuller M *et al.* Phase I trial with a hexavalent PorA containing meningococcal outer membrane vesicle vaccine. Vaccine 1996;14(10):1009-15.

26. Peeters CCAM, Tenbergen-Meekes AM, Poolman JT, Beurret M, Zegers BJM Rijkers GT. A comparative study of the immunogenicity of pneumococcal type 4 polysaccharide and oligosaccharide Tetanus toxoid conjugates in adult mice. J Immunol 1991;146:4308-14.

27. Woodrow GL, Kaper JB, Cobon GS, editors. Klein D, Ellis RW. Conjugate vaccine against *Streptococcus pneumoniae*. In: New generation vaccines. New York: Marcel Dekker;1998.p.503-25.

28. García-Ojeda PA, Hardy S, Kozlowski S, Stein KE, Feavers IM. Surfase Plasmon Resonance Analysis of antipolysaccharide antibody specificity: Responses to meningococcal group C conjugate vaccines and bacteria. Infect Immu 2004;72(6): 3451-60.

29. Rubinstein LJ, García-Ojeda PA, Michon F, Jennings HJ, Stein KE. Murine Immune Responses to *Neisseria meningitidis* group C Capsular Polysaccharide and a Thymus-Dependent Toxoid Conjugate. Infect Immu 1998;66(11):5450-6.

30. Reddin KM, Crowley-Luke A, Clark SO, Vincent PJ, Gorringe AR, Hudson MJ, Robinson A. Bordetella pertusis fimbriae are effective carrier proteins in *Neisseria meningitides* serogroup C conjugate vaccines. FEMS Immun and Med Microbiol 2001;31:153-62.

31. García-Ojeda PA, Monser, ME, Rubinstein LJ, Jennings HJ, Stein KE. Murine Immune Response to *Neisseria meningitidis* Group C Capsular Polysaccharide: Analysis of Monoclonal Antibodies Generated in Response to a Thymus-Independent Antigen and a Thymus-Dependent Toxoid Conjugate Vaccine. Infection and Immunity 2000;68:239-46.

*Received in february, 2005. Accepted for publication in march, 2005.*