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Preparation of Meningococcal Group A Polysaccharide-tetanus Toxoid Conjugate and their Immunogenicity in Mice

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SUMMARY. The development of polysaccharide-protein conjugate vaccines for *Haemophilus influenzae* type b, which have proven to be efficacious in infants and young children, has led to active development by a number of investigators of conjugate vaccines for other diseases. We describe here the obtention, by a new method, of a conjugate containing meningococcal group A polysaccharide (PsA) and tetanus toxoid (TT) as a carrier protein and the immune response evaluation in Balb/c mice. Amine groups generated by basic hydrolysis in PsA were successfully conjugated to carboxyl groups of tetanus toxoid (TT), using carbodiimide-mediated coupling. The conjugate induced anti-PsA IgG response and anti-PsA IgG subclasses (IgG1, IgG2a) in sera of immunized mice. In addition, the production of IFNγ by spleen cell of mice immunized with conjugate was observed. These results indicate that the conjugate changed the polysaccharide immune response from a thymus independent to a thymus dependent response.

RESUMEN. "Preparación de un Conjugado del Polisacárido del Meningococo Grupo A - Toxoide Tetánico y su Inmunogenicidad en Ratones". El desarrollo de las vacunas conjugadas para *Haemophilus influenzae* tipo b, ha demostrado ser eficaz en infantes y en niños pequeños y ha sentado las bases para el desarrollo de vacunas conjugadas dirigidas contra otras enfermedades. En este trabajo nosotros describimos la obtención, por un nuevo método, de un conjugado de polisacárido de meningococo grupo A (PsA) y toxoide tetánico (TT) como proteína portadora, así como la evaluación de la respuesta inmune de este conjugado en ratones Balb/c. Los grupos aminos generados por hidrólisis básica en el PsA fueron unidos a los grupos carboxilos del TT, por medio de la reacción con carbodiímida. El conjugado obtenido indujo una respuesta de IgG anti-PsA y de subclases de IgG (IgG1, IgG2a) anti-PsA en los sueros de ratones inmunizados. Además, se observó la producción de IFNγ por células de bazo de ratones inmunizados con conjugados. Estos resultados constituyen una evidencia del cambio de tipo de respuesta inmune, de timo-independiente a timo-dependiente, del polisacárido una vez conjugado.

INTRODUCTION

Neisseria meningitidis serogroup A causes epidemic meningococcal disease and is a major public health problem in many countries, particularly in the sub-Saharan "meningitis belt" region of Africa [1,2]. In 1996 one of the largest recorded epidemics of serogroup A meningococcal meningitis occurred in the meningitis belt, with more than 180,000 reported cases [3]. The occurrence of this and subsequent epidemics resulted in a renewed interest in vaccines for prevention of serogroup A meningococcal disease in Africa and elsewhere.

Polysaccharide (Ps) based vaccines against *N. meningitidis* serogroup A have been licenced since 1976. Immunization with meningococcal group A polysaccharide (PsA) elicits protective antibody responses. In some studies the PsA has been shown to elicit an anamnestic response that is not typically seen with others Ps vaccines [4,5]. This vaccine has been effective in controlling group A epidemics in Africa and other parts of the world [6]. However the Ps are classified as thymus-independent-2 (TI-2) antigens (Ags) because they do not require mature T cells to elicit a humoral response *in vivo* [7]. These Ags are immunogenic in adults but are only poorly or non-immunogenic in infants and young children who are highly susceptible to infection caused by encapsulated bacteria [8,9]. The response to capsular Ps is markedly different from the response to most protein Ags (thymus-dependent [TD] Ags) [10].

The immunogenicity of Ps can be improved

KEY WORDS: Conjugate vaccines, Group A meningococci, *Neisseria meningitidis*.
PALABRAS CLAVE: Meningococo grupo A, Vacunas conjugadas, *Neisseria meningitidis*.

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by conjugation to a carrier protein. Conjugations enhance the immune response, prime for immunological memory response, and are an effective approach for inducing the production of antibody to bacterial capsular Ps in young children. Four conjugate *Haemophilus influenzae* type b vaccines [11-13] and three conjugate *N. meningitidis* serogroup C vaccines have been developed [14-16]. There are many reports related to conjugates of serogroup A *N. meningitidis* that provide limited information on their syntheses and composition [17,18].

Therefore the aim of this study was to evaluate the anti PsA immune response (IgG, IgG subclasses, and cytokines) induced in mice by conjugates obtained with a new method and using tetanus toxoid as a carrier protein.

MATERIALS AND METHODS

Reagents

Ultrafiltration membranes (YM10) from Amicon, Inc.; 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide (EDAC) (Sigma); alkaline phosphatase-labeled goat anti-mouse immunoglobulin G (IgG) and poly-L-lisine were from Sigma Chemical Co., St. Lois, Mo.; avidin-biotinylated conjugate anti-IgG1 or anti-IgG2a, Primary and secondary biotinylated mAb, and recombinant IFN-γ and IL-5 were purchased from Pharmingen (San Diego, CA); Sepharose CL-4B from Pharmacia, Uppsala, Sweden; anti PsA rabbit serum (Murex Biotech Ltd); rabbit hyperimmune IgG anti-TT serum (800 U), PsA (Lot. 020/01 with Kav = 0.7) and TT (Lot. 011/01 with 1500 Lf) from Finlay Institute, Havana, Cuba.

Analytical methods

Proteins and phosphate were measured as described previously [19,20]. The amine groups were measured by OPA's methods using Glycine as standard [21], the free polysaccharide were measured separating the polysaccharide unconjugated in the supernatant by antigen-antibody reaction of conjugate with an anti-TT IgG and measuring phosphates in the supernatant. Double immunodiffusion was performed in 0.8 % agarose-0.15 M NaCl-0.01% sodium azide with an anti PsA rabbit serum.

Synthesis of PsA-protein conjugates

All reactions were performed at least five times to confirm their reproducibility, and the data for a representative lot is shown.

Derivatization of PsA

PsA was activated by a basic hydrolysis [22,23], in order to generate amines groups from acetamide groups present in their structure. Briefly, PsA (5 mg ml^{-1}) in 0.5 M NaOH was put in an oven at 70 °C for 3 h. Afterwards, the solution was cooled to room temperature and brought to pH 7.0 by the addition of 0.1 M HCl. The reaction mixture was passed through a 1.6 by 90 cm column of Sepharose CL-4B in 0.15 M NaCl. The fraction corresponding to 0.75 Kav was filtered by 0.2 µm and assayed for polysaccharide content and antigenicity by immunodiffusion.

PsA conjugates to proteins

In this study all conjugates were prepared binding the amine groups generated in PsA and the carboxylic groups present in the TT, by using carbodiimide-mediated coupling [22]. Briefly, the TT (5 $mg.mL^{-1}$) were treated with 0.1 M of EDAC by 30 min. at 4 °C and then PsA was added to a final concentration of 1 $mg.mL^{-1}$. The reaction mixture was carried out at 4 oC for 4 h with the pH maintained between 5 and 5.6. The reaction mixture was ultrafiltered against 3 L of 0.15 M NaCl. All conjugates were filtered by 0.2 µm and assayed for polysaccharide content, protein and antigenicity by double immunodiffusion.

Immunization

Groups of 8 to 10 BALB/c mice (CENPALAB, Cuba), 18-20 g each at the beginning of the experiments were injected intramuscularly with 0.1 ml of saline solution of 2 µg of PsA alone or conjugate on days 0 and 28. The mice were exsanguinated before each injection and 7 and 14 days after the second injection [24].

Enzyme-Linked Immunosorbent Assay (ELISA)

Antibody titres against PsA were determined by ELISA as described earlier [25]. Anti-PsA IgG was determined by using microplates coated with PsA/poly-L-lysine, and Peroxidase-conjugate anti-mouse IgG (Sigma) or avidin-biotinylated conjugate anti-IgG1 or anti-IgG2a was used as secondary antibody. 1,2- Phenylendiamin as substrate were used to detect antibodies bound to the antigen. The absorbance at 492 nm was read using an ELISA reader (Titertek Multiskan®). All results were expressed in OD of 1:800 dilution of the serum for IgG antibodies or 1:100 dilution of the serum for IgG subclasses because we do not have standard to quantify these anti-PsA immunoglobulins.

Biological assays

Complement mediated bactericidal antibodies were measured in serum of mice immunized by intramuscular route with unconjugated PsA or PsA-TT conjugated against *N. meningitidis*

serogroup A, using human serum as a source of complement. The bactericidal serum titre was defined as the reciprocal serum dilution yielding ≥50% killing compared to the number of target cells (CFU per well) present at To [26].

Cytokines

Cell culture and cytokine induction: single-cell suspensions of splenocytes from vaccinated Balb/c mice, were washed in Hanks' medium and treated for 4 min with lysine buffer (sodium chloride). The erythrocyte-free cells were then washed three times and resuspended in RPMI-1640 supplemented with gentamicin 50 µg x mL^{-1}, L-glutamine (2 mM), sodium-pyruvate (1 mM), Hepes (15 mM), and inactivated fetal calf serum (FCS) 10%. The viability of the isolated cells was ≥99% by trypan blue exclusion test. Finally, cell concentrations were adjusted at 4 x 10^6 cells per mL. A cell suspension was distributed (1 mL) in 24-well tissue culture plate (Costar). Non adsorbed polysaccharide were added at doses of 3 µg/ml 72 h at 37 °C in a humidified atmosphere containing 5% CO_2 (ASSAB, Sweden). The supernatants were subsequently harvested, centrifuged and stored at -70 °C until tested.

Cytokine ELISA: double MAb sandwich enzyme-linked immunoabsorbent assay were used to determine the presence of IFNγ or IL-5 as a secretion product in the supernatant of the same cultures.

Statistics

ELISA values are expressed as the OD values obtained at 492 nm. Standard deviations (SD) and repeated measures analysis of variance and chi-square analysis were used to compare values between groups of mice. Statistical significance was defined as a *P* value of < 0.05.

RESULTS

Composition of the conjugates

The activated PsA had an average content of 1.49 µmoles of amine groups.mg^{-1} of Ps. The O-acetyl content of PsA was 1,90 mmol.mg^{-1}. After activation the O-acetyl contents was reduced until 90% as a product of the basic hydrolysis. The Ratio of those conjugates obtained in all experiments (n=5) were similar, obtaining them with an average PsA / TT ratio of 0.77. Immunodiffusion analysis showed that the PsA after basic hydrolysis treatment was not recognized for the anti PsA rabbit serum which may suggest the importance of the eliminated group in the recognition of this polysaccharide. However after conjugation the recognition by the anti PsA antibody is restored (Fig. 1).

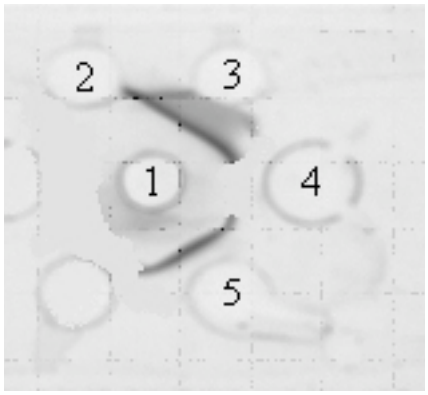

Figure 1. Double immunodiffusion to determine the identity of meningococcal group A Polysaccharide activated and after conjugation. **1**) anti PsA rabbit serum (Murex Biotech Ltd.); **2**) PBS; **3**) unconjugated PsA; **4**) activated PsA and **5**), PsA-TT.

Meningococcal group A Polysaccharide-specific IgG antibodies

The kinetic production of IgG antibodies was measured. It showed that the conjugates prepared elicited statistically significant rises ($P < 0.05$) in the levels of anti-PsA IgG antibodies after the second dose compared with unconjugated PsA. Lastly, after the second immunization there was an increased in the IgG response in animals immunized with the conjugate, which was not observed with unconjugated PsA (Fig. 2).

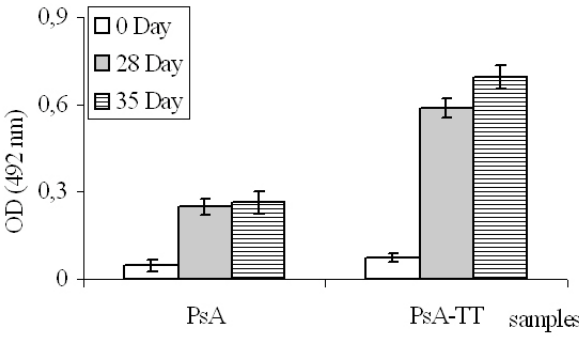

Figure 2. Anti-meningococcal group A polysaccharide IgG induced by conjugates. ELISA titre of pooled serum (n=5) obtained in 0, 28 and 35 days. The titre was expressed as OD 492 nm, of 1:800 dilution of the serum. PsA) meningococcal group A polysaccharide and PsA-TT) meningococcal A polysaccharide conjugated to tetanus toxoid.

Anti-meningococcal group A polysaccharide-specific IgG subclasses

Figure 3 shows that, after the second dose (35 days), the conjugate was able to induce both IgG subclasses (IgG1 and IgG2a), while very low levels of both subclasses were detect in sera following immunization with unconjugated PsA. No significant differences were ob-

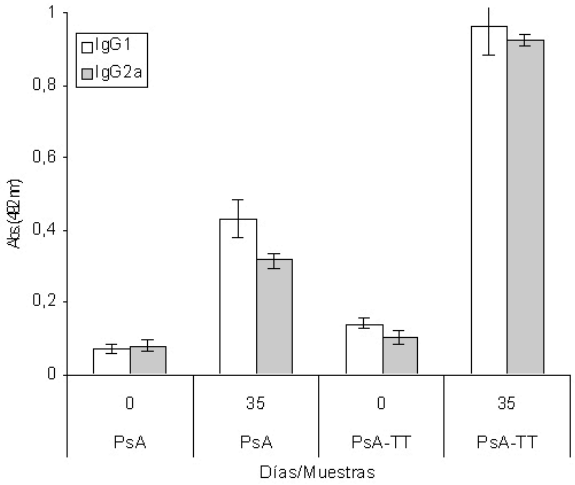

Figure 3. Anti-meningococcal group A polysaccharide IgG subclasses induced by conjugates. ELISA titre of pooled serum (n=5) obtained in 0 and 35 days. The titre was expressed as OD 492 nm, of 1:100 dilution of the serum. PsA) meningococcal group A polysaccharide and PsA-TT) meningococcal A polysaccharide conjugated to tetanus toxoid.

served in the levels of IgG1 antibodies compared with IgG2a antibodies.

Bactericidal antibodies

The level of functional antibody induced in each vaccinated group was measured in bactericidal assay. Mice injected two times with PsA-TT had bactericidal levels that correlated roughly with their antibody levels. The SBA titre in the group immunized with PsA- TT conjugate was more than fourfold higher than those in the group receiving native PsA (64 vs. 16) (Table 1).

Cytokines (IFNγ and IL5) anti Anti-meningococcal group A polysaccharide

The production of IFNγ and IL5 were measured in spleen cells from immunized Balb/c mice with PsA-TT conjugate or unconjugated PsA. The results (Table 2) show that PsA-TT conjugate drive the response to the production of IFNγ. On the contrary, the unconjugated PsA not induce IFNγ. Lastly, the production of IL5 was low and similar in all groups.

DISCUSSION

This study was carried out to obtain conjugates of PsA with TT and to evaluate, in balb/c mice, the immunogenicity of this. Reliable and efficient methods to activate the PsA and conjugation to TT by condensation with EDAC are described. The conjugate PsA maintained its antigenics properties being recognized by IgG antibodies present in the anti-PsA rabbit serum from Murex Biotech Ltd by Double immunodiffusion.

Sera	Title
Native PsA (T0)	<2
Native PsA (T35)	16
PsA-TT (T0)	<2
PsA-TT (T35)	64

Table 1. Bactericidal titre obtained in sera of mice immunized with PsA-TT conjugates and native PsA. The bactericidal serum titre was defined as the reciprocal serum dilution yielding 50% killing compared to the number of target cells (CFU per well) present at To. PsA) meningococcal polysaccharide group C; PsA) meningococcal polysaccharide group A; and PsA-TT) meningococcal polysaccharide group A conjugated to tetanus toxoid.

Cytokines	Anti-PsA	
	PsA	PsA-TT
IL5	1.381	5.364
IFNγ	43.240	156.279

Table 2. Determination of cytokines anti- meningococcal group A polysaccharide in spleen cells of Balb/c mice immunized (2 doses at 0 and 28 days) with PsA and PsA-TT conjugates. Concentration are expressed in pg.mL^{-1}; PsA) meningococcal A polysaccharide; PsA-TT) meningococcal A polysaccharide conjugated to tetanus toxoid.

The immunogenicity results agreed with the results mentioned previously, because the antibodies anti PsA obtained after conjugation showed an isotype change, the PsA-TT elicited IgG1 and IgG2a, while these results in mice immunized with PsA unconjugated were not observed. Furthermore the results show high concentration values of IFNγ anti PsA in spleen cell of mice inoculated with conjugated but not in the spleen cell of mice inoculated with unconjugated PsA. Lastly the results show high bactericidal titer in sera of animals immunized with conjugates and no for unconjugated Ps.

The antibodies response to TI-2 Ags develops late in ontogeny and in mice utilizes a particular late-developing subset of B cells that is defined by the expression of Lyb5 and other cell markers [27]. These Ags also generally fail to elicit a memory response or to show affinity maturation. In contrast, the ability to respond to a TD Ag is present at birth and results in the formation of memory cells, and the antibody response undergoes subsequent affinity maturation upon reimmunization [7]. For TI Ag, IgG3 and IgM are the major isotypes produced in mice, even after secondary immunization [28], whereas for TD Ag, the

ratio of IgG to IgM increases after the second dose, with IgG1 being the major subclass [29].

García-Ojeda *et al.* [10] used a conjugate PsC-TT and Balb/c mice for his work and concluded that the use of a TD form of PsC (conjugate), compared to the TI form (unconjugated), result in an isotype shift. The anti PsC antibodies are primarily of the IgG3 and IgM isotypes, whereas the PsC-TT elicited mainly IgG1.

Conjugates of PsC-TT, obtained by reductive amination [10,29,30] have been used in previous studies to immunize Balb/c mice. These studies showed that the conjugate, compared with unconjugated PsC, resulted in an isotype shift from predominantly IgM and IgG3 to predominantly IgG1.

All these results are an evidence of the change of immune response pattern for the PsA from TI to TD, after conjugation, in accordance with that outlined by other authors [10, 29, 30].

CONCLUSIONS

Our results showed that with the use of a basic hydrolysis for the activation of the PsA and the later use of the EDAC for the activation of the TT we were able to obtain a conjugate capable of eliciting high levels of IgG1 e INFγ in mice, unlike that response observed for PsA alone. Thus, a conjugate vaccine, obtained by this method, and composed by PsA and TT should protect against *N. meningitidis* serogroup A.

REFERENCES

1. Molesworth, A.M., M.C. Thomson, S.J. Connor, M.P. Cresswell, A.P. Mose, P. Shears, C.A. Hart & L.E. Cuevas (2002) *Trans. R. Soc. Trop. Med. Hyg.* **96**: 242-9.
2. World Health Organization. (2001) *Wly. Epi - demiol. Rec.* **76**: 282-8.
3. Robbins, J.B., R Schneerson & E.C. Gotschlich (2000) *Pediatr. Infect. Dis.* **19**: 945-53.
4. Peltola, H, P.H. Makela, H. Kayhty, H. Jousimies, E. Herva, K. Halstrom, A. Sivonen, V. Renkonen, O. Pettay, V. Karanko, P. Ahvonen & S. Sarna (1997) *N. Engl. J. Med.* **297**: 686-91.
5. Broker, M. (2003) *Arzneim.-Forsch./Drug Res.* **53**(2): 805-13.
6. Lennon, D., B. Gellin, D. Hood, L. Voss, H. Heffernan & S. Thakur (1992) *J. Infect. Dis.* **11**: 617-23.
7. Stein KE. (1992) *J. Infect Dis.* **165**: S49-S52.
8. Hutchins, W. A., G. M. Carlone & M.A.J. Westerink (1999) *J. Infect. Dis.* **179**: 1433-40.
9. Taunay, A.E., R.A. Feldman, C.O. Bastos, P.A.A. Galvao, J. S. Morais & I.O. Castro (1978) *Rev. Inst. Adolfo Lutz* **38**: 77-82.
10. García O'Jeda, P.A, M.E. Monser, L.J. Rubinstein, H.J. Jennings & K.E. Stein (2000) *Infect. Immun.* **68**: 239-246.
11. Eskola, J. & H. Kayhty (1996) *Rev. Med. Micro - biol.* **7**: 231-41.
12. Bergqvist, C., T. Lagergard & J. Holmgren (1997) *Infect. Immun.* **65** (5):1579-83.
13. Decker, M.D., K.M. Edwards, R. Bradley & P. Palmer (1992) *J. Pediatrics.* **120**: 184-9.
14. Snape, M.D. & A.J. Pollard (2005) *Lancet In - fect.* **15**: 21-30.
15. Borrow, R., P. Richmond, E.B. Kaczmarski, A. Iverson, S.L. Martin, J. Findlow, M. Acuna, E. Longwoth, R. O'Connor, J. Paul & E. Miller (2000) *FEMS-IM.* **28**: 79-85.
16. Ruggeberg, J. & P.T. Heath (2003) *Expert Opin Drug Saf.* **1**: 7-19.
17. Campbell, J.D., R. Edelman, J.C. King, T. Papa, R. Ryall & M.B. Rennels (2002) *J. Infect. Dis.* **186**: 1848-51.
18. Jin, Z., C. Chu, J.B. Robbins & R. Schneerson (2003) *Infect. and Immun.* **71**: 5115-20.
19. Lowry, O.H., N.J. Rosebrough, A.L. Farr & R. Randall (1951) *Biol. Chem.* **193**: 265-75.
20. Chen, P.S., T.Y. Toribara & H. Warner (1956) *Anal. Chem.* **28**: 1756-8.
21. Church, F.C., D.H. Porter, G.L. Calignani & H.E. Swaisgood (1986) *Anal. Biochem.* **146**: 343-8.
22. Cabrera, O., M. Cuello, V.G. Sierra, C.R. Soto & M.E. Martínez (2005) *European Patent Office* (http://v3.espacenet.com/textdoc?DB=EPODOC&IDX=EP1582217&F=0)
23. Cabrera, O, M. Cuello, C.R. Soto, M.E. Martínez, J.M. del Campo, O. Perez, J.F. Infante & V.G. Sierra (2006) *Vaccine* **24S2**: S76-8.
24. Fukasawa, L.O., M.C.O. Gorla, R.P.F. Schenkman, L.R. Garcia, S.M. Carneiro, I. Raw & M.M. Tanizaki (1999) *Vaccine* **17**: 2951-8.
25. Wessels, M.R., L.C. Paoletti, J. Pinel & D.L. Kasper (1995) *J. Infect. Dis.* **171**: 879-84.
26. Maslanka S.E., Gheesling L.L., Libutti D.E., Donaldson K.B., Haraek H.S., Dykes J.K. et al. (1997) *Clin. Diagn. Lab. Immunol.* **4**: 156-67.
27. Mosier, D.E., I.M. Zitron, J.J. Mond, A. Ahmed, I. Scher & W.E. Paul (1977) *Immunol. Rev.* **37**: 89-104.
28. Perlmutter, R.M., D. Hansburg, D.E. Briles, R.A. Nicolotti & J.M. Davie (1978) *J. Immunol.* **121**: 566-72.
29. Rubinstein, L.J., P.A. García-Ojeda, F. Michon, H.J. Jennings & K.E. Stein (1998) *Infect. Im - mun.* **66**: 5450-6.
30. Jennings, H.J. & C. Lugowski (1981) *J. Im - munol.* **127**: 1011-8.