Vaccine 30 (2012) 7090–7096

Contents lists available at SciVerse ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Relevance of O-acetyl and phosphoglycerol groups for the antigenicity of *Streptococcus pneumoniae* serotype 18C capsular polysaccharide

Janoi Chang*, Yohanna Serrano, Raine Garrido, Laura M. Rodríguez, Jessy Pedroso, Felix Cardoso, Yury Valdés, Dagmar García, Violeta Fernández-Santana[1], Vicente Verez-Bencomo

Center for Biomolecular Chemistry, Playa, Havana, Cuba

ARTICLE INFO

Article history:
Received 11 May 2012
Received in revised form 3 September 2012
Accepted 19 September 2012
Available online 2 October 2012

Keywords:
Streptococcus pneumoniae serotype 18C
O-acetyl group
Phosphate-glycerol
Antigenicity
Conjugate vaccine

ABSTRACT

Capsular polysaccharides are important virulence factors of *Streptococcus pneumoniae*. The polysaccharide has been used as a component of vaccines against pneumococcal diseases either as plain polysaccharide or better conjugated to a protein. The last one is the vaccine of choice to target child protection. The immune responses depend on several polysaccharide physicochemical properties that can be affected during either purification or modification in the case of conjugate vaccines. In serotype 18C, the repeating unit has a complex structure having a branched pentasaccharide with two apparently labile subtituents: glycerol-phosphate and O-acetyl group. The loss of these groups may potentially reduce the ability of the 18C polysaccharide to induce the desired immune response. Therefore, the relationship of both groups with the antigenicity and immunogenicity of 18C capsular polysaccharide is explored. It is shown that glycerol-phosphate must be preserved for conserving adequate antigenicity of the 18C capsular polysaccharide. At the same time, it was proved that O-acetyl groups do not play any role for the antigenicity and immunogenicity.

1. Introduction

Streptococcus pneumoniae (*S. pneumoniae*) is involved in several human infections like pneumonia, meningitis, otitis media, bacteraemia and sepsis representing an important morbidity and mortality worldwide [1]. It is well known that anti-capsular polysaccharide antibodies protect against pneumococcal infections, therefore, vaccine ability to induce such antibodies became an excellent tool to prevent pneumococcal infections [2,3]. Anti-capsular polysaccharide antibodies are induced by two types of vaccines composed of polysaccharides either in a pure form or conjugated to a carrier protein [4]. Purification of the capsular polysaccharides and, specially, modification and conjugation could alter their structure mainly by the loss of labile groups. However, this is not obvious as, in some cases; the lack of groups does not affect the ability of antibodies to recognize the bacteria [5,6].

In our approach for developing carbohydrate conjugate vaccines, we focus on the definition of the final products at the molecular level [7,8]. The capsular polysaccharide of *S. pneumoniae* serotype 18C (PS) is composed of a repeating unit having a tetrasaccharide backbone with D-galactose highly branched by D-glucose and glycerol phosphate (Fig. 1) [9,10]. The O-acetyl and glycerol-phosphate groups are potentially labile to the chemistry used for the modification-conjugation procedures. In the present paper we intend to clarify the importance of their respective conservation.

2. Materials and methods

2.1. Materials

Capsular polysaccharide serotype 18C and tetanus toxoid were provided by the Finlay Institute for Serum and Vaccines, Havana, Cuba.

2.2. Sera

Standard rabbit antiserum group 18 (16910) was purchased from the Statens Serum Institute, Copenhagen. Other sera from immunized mice and rabbits were collected and frozen at −20 °C until used. Besides we used Lot-89SF standard human pneumococcal serum and sera from human volunteers immunized with PNEUMO 23® (Sanofi-pasteur) after informed consent from donors.

* Corresponding author at: Center for Biomolecular Chemistry, Road 200 y Avenue 21, Atabey, Playa, PO Box 16042, cod 11600, Havana, Cuba. Tel.: +53 7 208 5104; fax: +53 273 6471.
E-mail address: janoi.chang@cqb.cu (J. Chang).
[1] Deceased, 20th November 2011.

0264-410X/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.vaccine.2012.09.047

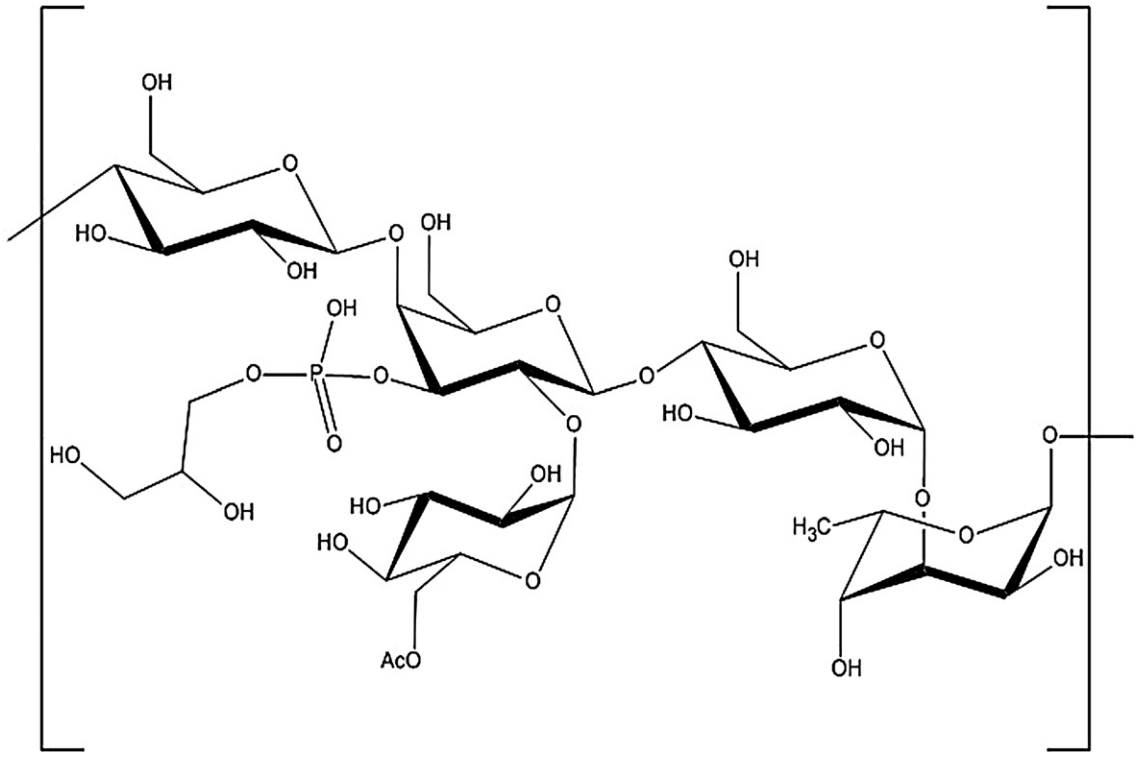

Fig. 1. Repeating unit of *Streptococcus pneumoniae* capsular polysaccharide 18C [10].

2.3. Preparation of capsular polysaccharide serotype 18C derivatives

Removal of glycerol-phosphate (P-Gro) and O-acetyl (O-Ac) groups was performed as described by Richards and Perry [11]. The capsular polysaccharide was treated with 1 M NaOH at 100 °C in presence of sodium borohydride. After incubation for 6 h, the reaction was neutralized with 0.1 M acetic acid. The fragment designated as dephosphorylated (de-P-Gro) was obtained by purification, using two consecutive molecular sievings against water (Amicon system) and selecting the fraction between 10 and 30 kDa.

Fragments of 18C PS with different degree of O-Ac, but maintaining the P-Gro group, were obtained by acid hydrolysis. The process was performed with trifluoroacetic acid (TFA) or acetic acid. Capsular polysaccharide was initially dissolved in water at 4 mg/mL. The acid was added to adjust the specific concentration, ranging from 2.5 to 250 mM. The mixture was heated at 70 °C or 100 °C during 1.5–6 h. The solution was then neutralized with 0.1 M NaOH and purified by ultrafiltration to obtain the fraction between 10 and 30 kDa, as described above.

2.4. NMR and colorimetric assay

The structural integrity of all PSs derivatives as well as the O-Ac content were evaluated by ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy. All samples were prepared dissolving 10 mg in 0.6 mL of deuterium oxide, lyophilized and redissolved in 0.6 mL of deuterium oxide with 3-(trimethylsilyl) 2,2,3,3-tetra-deuteropropionic acid sodium salt as internal reference.

NMR analysis was carried out on a Bruker/Avance DPX 250-MHz instrument at a probe temperature of 52 °C. The free induction decay (FID) for the q1HNMR experiments was acquired at 7 μs (90° pulse), 25 s of last delay, 512 transients with GARP (Globally Optimized Iternating-Phase Rectangular Pulses), decoupling method for ^{13}C. The total correlation spectroscopy (TOCSY) experiment was run with presaturation at 200 ms of mixing time. In addition, heteronuclear multiple quantum coherence (HMQC) experiment was performed with BIRD (Bilinear Rotation Decoupling) filter without decoupling during acquisition. Topspin 1.3 software was employed to process the resultant FID. The residual signal of 3-(trimethylsilyl) 2,2,3,3-tetra-deuteropropionic acid sodium salt was referenced at δ 0.00 ppm to calibrate all the spectra.

In order to ascertain the concentration of polysaccharides and derivatives, the carbohydrate concentration was quantified using the orcinol–sulfuric acid method [12]. The aldehyde content on the activated oligosaccharide was determined by the Park Johnson's colorimetric assay [13], and the protein content in the final conjugate was determined using the Lowry method [14].

2.5. Conjugation of totally de-O-acetylated (de-OAc) fragment to tetanus toxoid

A fragment obtained by acid hydrolysis without O-Ac group (18C de-OAc) was oxidized with 2 mM sodium periodate as previously described [15]. The reagents were removed by ultrafiltration through a 10 kDa membrane. The efficiency of reaction was ascertained by determination of aldehyde content. Oxidized 18C de-OAc and tetanus toxoid (TT) were mixed at a mass ratio of 1:1, in 0.1 M PBS pH 7.0 and lyophilized. The powder was resuspended with a solution of sodium cyanoborohydride 25 mM and kept at room temperature during four days. After incubation, the unreacted aldehyde groups were reduced with ten equivalents of sodium borohydride at room temperature for three hours. The conjugated was separated from unconjugated polysaccharide fragment and reagents by ultrafiltration using a 100 kDa membrane. Conjugate was characterized by carbohydrate:protein ratio. The distribution constant (K_{av}) and the content of unconjugated carrier protein were determined by high performance liquid size exclusion

chromatography (HP-SEC). Data were collected and analyzed using the ClarityChrom software.

2.6. Immunization methods

Animals: Six to eight-week-old female BALB/c mice and four-week-old female New Zealand rabbits were purchased from National Center for Laboratory Animal Production (CENPALAB, Havana, Cuba).

All experiments were carried out in accordance with the ethical guidelines for investigations with laboratory animals and were approved by the Ethical Committee for Animal Experimentation of the Center of Biomolecular Chemistry. All experimental protocols were approved by the Quality Department from the same institution.

Groups of ten mice and five rabbits were subcutaneously immunized with 2 μg and 5 μg of polysaccharide conjugated to TT respectively. The control group was immunized with 25 μg of 18C capsular polysaccharide and the placebo group received saline solution. Three immunizations were performed at 14 days intervals and serum samples were collected at 7 days post-immunization.

2.7. Evaluation of immunogenicity by Enzyme Linked Immunosorbent Assay (ELISA)

NUNC Immuno plates (Maxisorp; PGC Scientifics Corp., Gaithersburg, MD) were coated with native PS 18C (10 μg/mL), in a phosphate buffer solution (PBS) at 37 °C overnight. Plates were blocked with 2% BSA for 30 min at 37 °C. Two-fold serial dilutions of mouse or rabbit sera (starting 1:100 dilution) were added to the plates and incubated for 90 min at room temperature. The corresponding anti-immunoglobulin type G (IgG) whole molecule horseradish peroxidase-conjugate (1:10 000 dilution) was added and the plate was incubated for 90 min. After each incubation, plates were washed three times with PBS containing 0.05% Tween 20. Finally, a mixture of O-phenylendiamine and, H_2O_2 in citrate buffer pH 5 was added as enzyme substrate. After 20 min in darkness, the reaction was stopped with 3 M HCl, and read at λ = 492 nm using an ELISA Sumrise reader. The antibody titers were defined as the log10 of the highest dilution giving twice the absorbance value of pre-immune sera. Log 10 Titer lower than 1.69 (corresponding to 1:50 dilution) was considered negative.

2.8. Inhibition experiments in ELISAs

The antigenicity of different polysaccharide derivatives was tested by competition ELISA using the following sera: the rabbit standard antiserum group 18, the human reference serum 89SF against pneumococcus and the sera from volunteers vaccinated with the commercial vaccine PNEUMO 23®. Besides, the specificity of sera from mice and rabbits (immunized with the conjugate) was evaluated by incubating sera with native PS 18C. ELISAs were performed as previously described except that all sera were pre-incubated overnight at 4 °C with the inhibitors in concentrations ranging from 500 to 0.005 μg/mL.

For human serum, ELISA was performed using alkaline phosphatase-conjugated goat anti-human IgG at 1:10000 dilution and 1 mg/mL of p-nitrophenylphosphate in 1 M diethanolamine, 0.5 mM MgCl2, pH 9.8 as substrate. The reaction was stopped with 3 M NaOH and absorbance was detected at λ = 405 nm using an ELISA Sumrise reader. All incubating lasted 2 h for the entire ELISA protocol.

The percentages of inhibition were calculated as follows: percent inhibition = [1 − (absorbance of serum with inhibitor/absorbance of serum without inhibitor)] × 100.

2.9. Opsonophagocytosis assay (OPA)

The OPA was performed as described by Romero-Steiner et al. [16]. The OPA titter was calculated as the reciprocal of the serum dilution that caused a 50% reduction of the unit forming colony (CFU), compared to the CFU from the control wells containing all reagents except test serum.

For the immunogenicity study, statistical analyses were performed using Graph Pad Prism 4.03. The significant differences between the groups were determined by the Kruskal–Wallis non-parametric test and $P < 0.001$ was considered statistically significant.

3. Results

3.1. Fragments of PS 18C

Available fragmenting procedures for 18C PS include basic or acid hydrolysis. The basic hydrolysis NaOH 1 M provides fragments completely devoid of P-Gro and O-Ac groups. Fragments that conserved the repeating unit, except for the content of the O-Ac group, were obtained by hydrolysis with TFA or acetic acid (Table 1). Trifluoroacetic acid reduced the size very efficiently, with high recovery (>80%) on the fraction between 10 and 30 kDa obtained by ultrafiltration, but at the same time it removed most of the O-Ac. Fractions usually had less than 50% of O-Ac even with low TFA molarity. The acetic acid was less efficient in sizing the polysaccharide, with low recovery of the desired fragments but at the same time, it conserved more than 90% of O-acetyl groups. Therefore, this is the method of choice for the preparation of fragments with high degree of O-acetylation.

NMR spectroscopy has been used for the evaluation of PS identity [17] and its fragments. In the ^{1}H NMR spectra of 18C PS, we corroborate the presence of five anomeric protons at δ 5.40, δ 5.12, δ 4.87, δ 4.79 and δ 4.70 ppm and of the O-acetyl group and the L-rhamnose unit at δ 1.99 and δ 1.18 ppm respectively (Fig. 2). We

Table 1
Conditions and results obtained during the hydrolysis acid or basic of the capsular polysaccharide 18C.

Treatment	c (x) (mM)	Time (h)	Tem. (°C)	Yield (%)[a]	OAc (%)[b]	K_{av}[c]
TFA	250	1.5	70	27.6	0	n.d.
	100	1.5	70	>80	0	0.43
	5	3.5	70	66	36	n.d.
Acetic acid	200	3	100	17	50	0.43
	100	6	100	10	95	0.41
NaOH	100	6	100	n.d.	0	0.40

n.d.: not determined.
[a] Yield of carbohydrate was determined by orcinol–sulfuric methods.
[b] Content of O-Ac group measured by ^{1}H NMR.
[c] $K_{av} = \frac{(Ve - Vo)}{(Vt - Vo)}$.

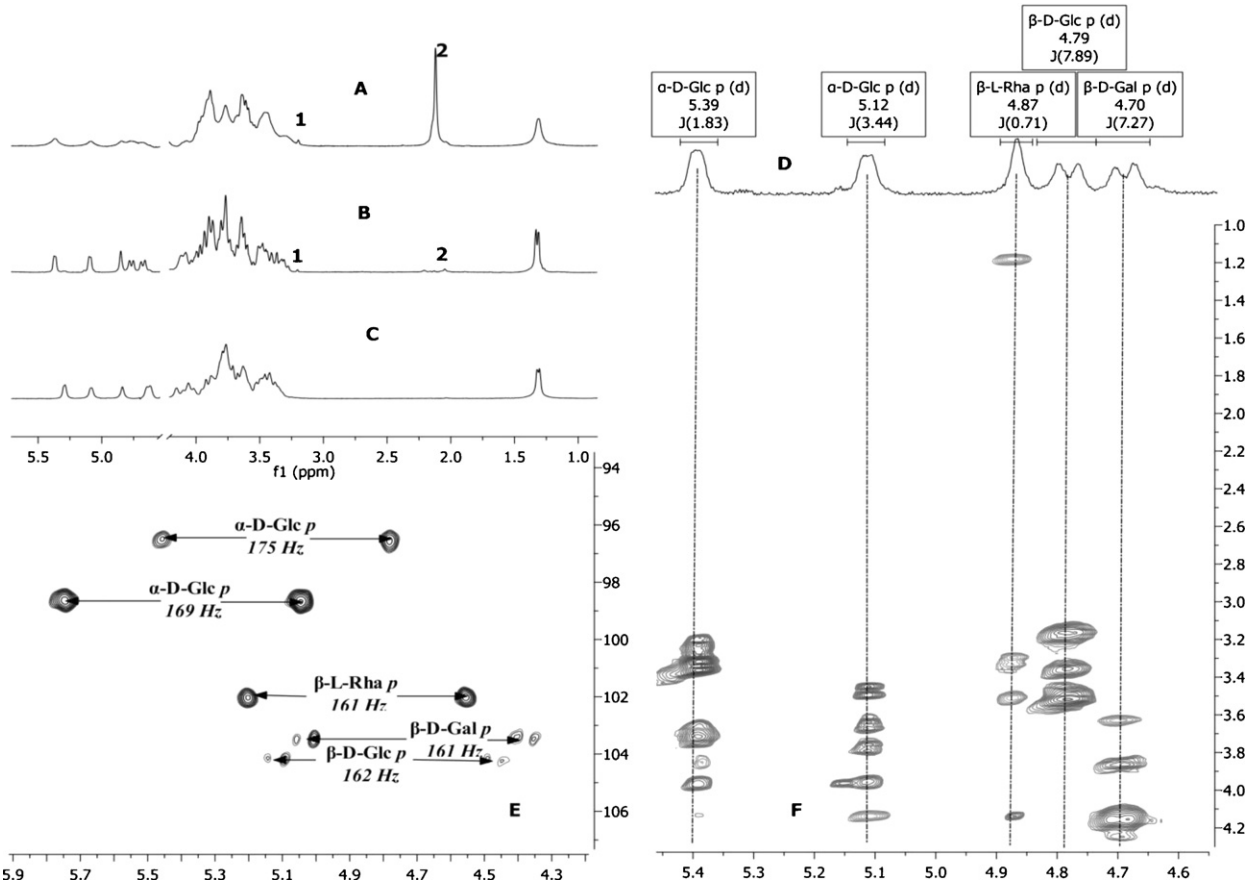

Fig. 2. NMR spectra. (A)–(C) Solvent Pre-saturated ^{1}H NMR spectra from Capsular polysaccharide, fragment 18C de-OAc and a fragment dephosphorylated (de-P-Gro) compound respectively. The spectra of capsular polysacharide and 18C de-OAc show the low signal corresponded to phosphocholine residue (1) from C-polysaccharide impurity. The lack of O-acetil residue (2) not implicated changed on anomeric region. Dephosphorylation causes shielding of anomeric protons. (D) Assignment ^{1}H NMR (anomeric region). (E) Assignment HMQC without decoupling during acquisition (anomeric region). (F) 150 ms TOCSY experiment (anomeric region). The spin systems of each monosaccharidic residue are marked by a dashed lines.

also corroborated the anomeric configuration of every unit by their scalar coupling constant value. In the particular case of L-rhamnose, the assigned β-configuration [10] was further confirmed by 3JH-H > 1.0 Hz and 1JC-H = 161 Hz.

De-O-acetylation did not influence on the signal of the corresponding monosaccharide unit. On the contrary, dephosphorylation caused the shielding of all anomeric protons and the overlapping of the signals corresponding to the anomeric of β-D-Glc at δ 4.79 ppm and β-D-Gal δ 4.70 ppm to the signal at δ 4.59 ppm (Fig. 2).

3.2. Antigenicity of fragments by inhibition ELISA

The influence of the O-Ac or the P-Gro groups on the recognition of 18C PS by antibodies was tested by inhibition ELISA. The previously described derivatives with different contents of acetylation and de-P-Gro were used as inhibitor for the rabbit antiserum group 18 from the Statens Serum Institute. Fig. 3 shows that the serum was inhibited to a similar extent (>95%) by the fragments with varying grade of acetylation (de-OAc, 50% O-Ac and full O-Ac), suggesting that the O-acetyl group is not implicated on the recognition by specific antibodies against 18C. On the contrary, the fragment de-P-Gro was not able to completely inhibit the recognition of serum against 18C PS, reaching only the 75% of inhibition at high concentration. Furthermore, only 50% inhibition was attained by a concentration 100 fold higher than any other fragment assayed.

In order to verify the aforementioned results, human sera from volunteers vaccinated with PNEUMO 23® vaccine and the international reference human serum against pneumococci 89SF were tested with the same kind of experiment. The human sera showed similar inhibition curves for fragment de-OAc, 50% or fully O-acetylated (Fig. 3). De-P-Gro did not inhibit tested sera and required approximately 100-fold higher concentration than any other deacetylated fragment to reach 50% of inhibition, similar to previous ELISA.

3.3. De-O-Ac-TT conjugates induce specific antibodies to 18C PS in mice and rabbits

Considering the very poor involvement of O-Ac groups in PS recognition and their high recovery rate, the TFA modification was further selected for the adjustment of the polysaccharides for the development of prototype conjugates. Fragments hydrolysed and deacetylated with 100 mM TFA (de-OAc) were oxidized with sodium periodate. The process proceed smoothly, yielding compounds with one aldehyde group per seven repeating unit. The conjugation was performed by reductive amination, using the standard procedure with some modification, consisting of co-lyophilize the activated polysaccharide and the carrier protein. The conjugate showed a ratio sugar: protein of 0.71. The HP-SEC patterns on the TSK 5000 PW column of the conjugate had a homogeneous peak, with a K_{av} = 0.36 and no unconjugated TT was detected (Fig. 4).

The immunogenicity of the de-OAc-TT conjugate was evaluated on mice and rabbits, proceeded the schedule described above. In both animal species the conjugate induced high IgG titers against

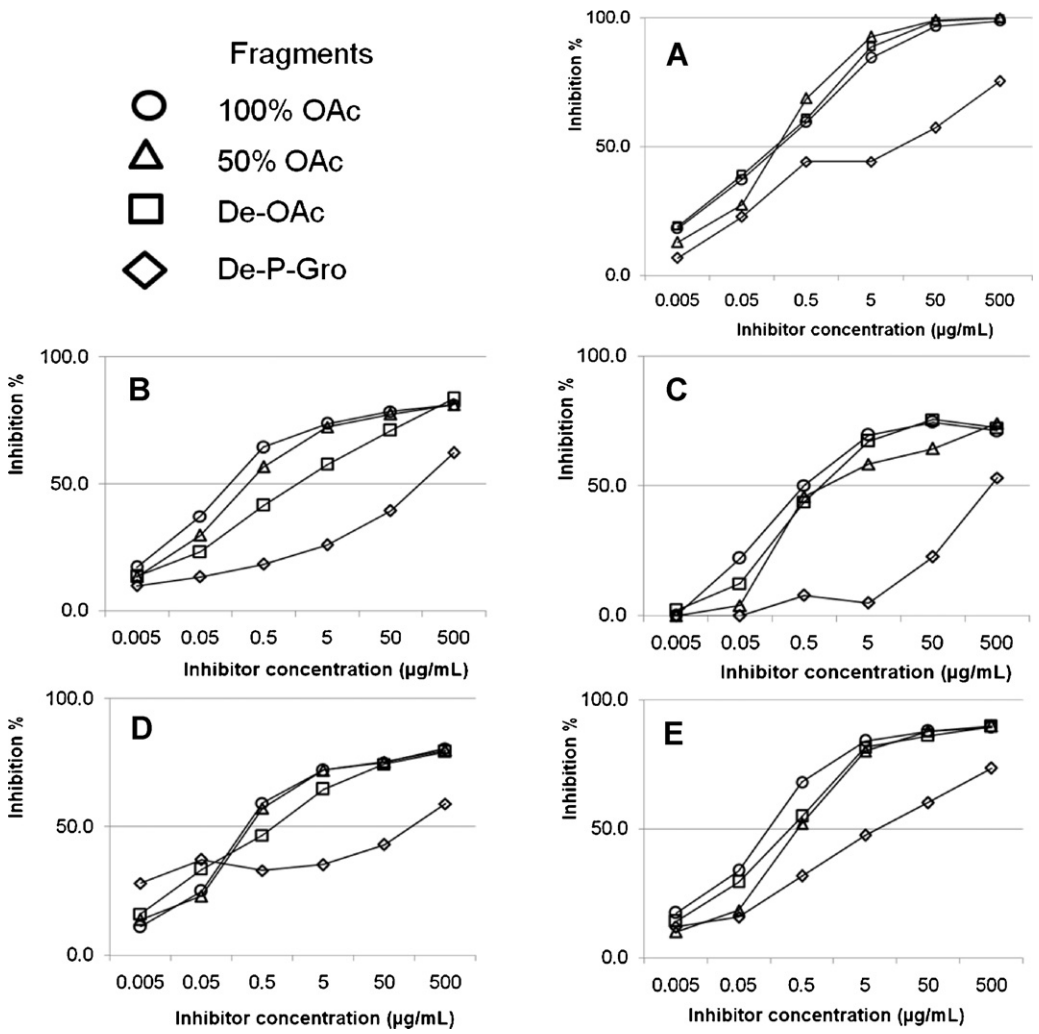

Fig. 3. Competitive ELISA, using as inhibitors fragments with 0% (de-OAc), 50%, 100% of O-acetylation and a fragment de-P-Gro. Sera were pre-adsorbed with different concentrations of inhibitor overnignt at 4 °C. ELISA was performed on 18C native PS-coated plate. Results are expressed as % of inhibition of IgG response respect to unabsorbed serum. (A) Standard rabbit antiserum group 18, (B) human reference serum 89SF. (C–E) sera from vaccinated volunteers with PNEUMO 23. Each value represents the mean of at least two determinations.

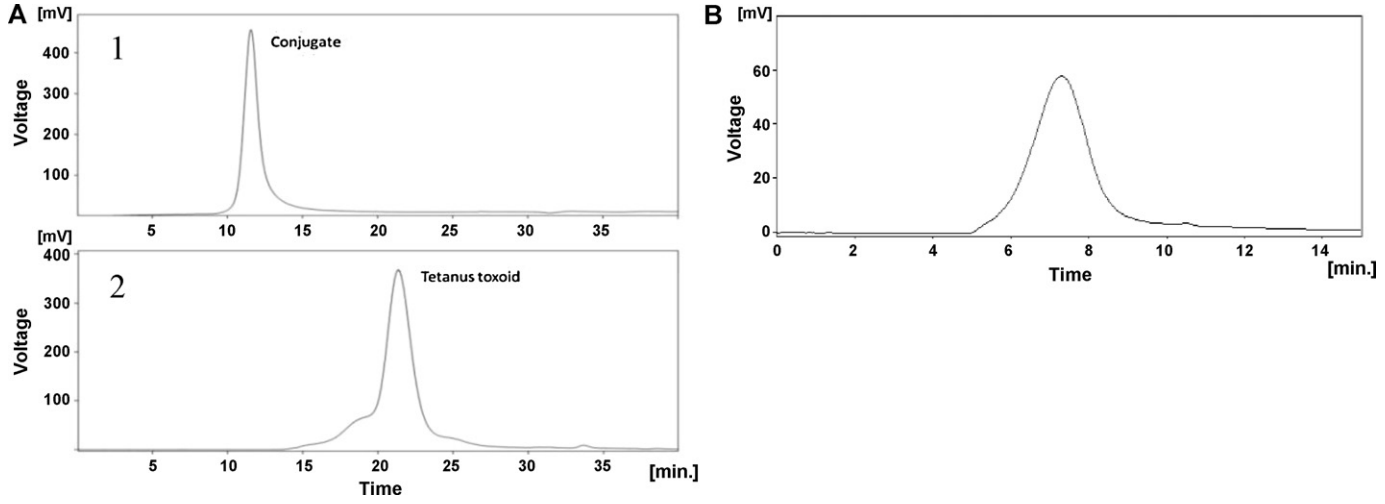

Fig. 4. (A) No unconjugated proteins were present in the conjugate (1) compared to TT, (2) on column Superose 12 (10 mm × 300/310 mm) with a flow rate of 0.5 mL/min with 0.05 M PBS (pH 7). (B) Profile of conjugate on column TSK 5000 PW (7.5 mm × 300 mm) running on 0.9% NaCl at a flow rate of 1 mL/min.

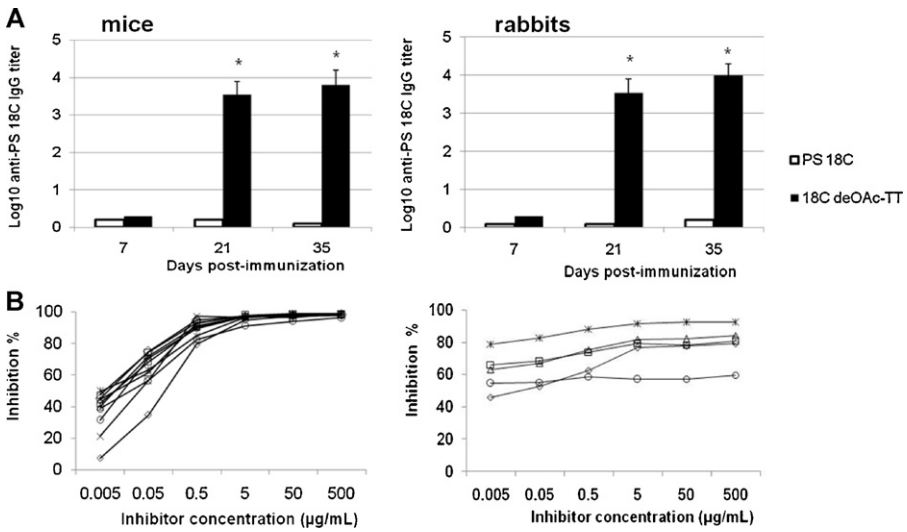

Fig. 5. Groups of ten mice and five rabbit were immunized (0, 14 and 21 day) with 18C de-OAc conjugated to TT (closed bars) and PS 18C (open bars) as a control. (A) Total IgG antibody titers against 18C PS coating material seven days post-immunization. The results are expressed as log 10 of the dilution giving twice the O.D. obtained for the pre-immune (*$p < 0.001$). (B) Sera from individual animals (day 35) were preabsorbed overnight at 4 °C with increasing concentration of native PS 18C. Results were expressed on percent of inhibition calculated as previously described. Inhibition ELISA were performed on PS 18C coated plate.

18C PS. After the second dose, a dramatic increment in IgG levels was observed, as animals immunized with the conjugate reached 1000-fold higher titers than those immunized with the native 18C PS (Fig. 5).

In order to confirm the specificity of the elicited antibodies against 18C PS, an inhibition ELISA was performed. The sera from mice and rabbits after the third dose with the de-OAc fragment conjugate to TT were inhibited with increasing concentrations of 18C PS, prior to the measurement of the anti-18C response on 18C-coated plate of ELISA (Fig. 5). In both cases, the animal sera were completely inhibited by the 18C PS, confirming the low involvement of the O-Ac group in the epitopes, because its presence is not necessary for the induction of anti-18C specific antibodies.

Additional proof of the usefulness of the de-O-Ac-TT conjugate was obtained by testing the functional capacity of the antibodies generated in rabbits. Rabbit sera showed high OPA titer (128) compared to a 4 baseline, confirming that the antibodies elicited by the de-O-Ac-TT not only recognized the 18C PS, but they also induced the opsonofagocitic killing of the bacteria by human peripheral blood leukocytes.

4. Discussion

The capsular polysaccharide of *S. pneumoniae* serotype 18C is incorporated in all the commercially available conjugate vaccines recently developed to prevent pneumococcal infections. However, the need of preservation of O-acetyl and glycerol-phosphate potentially labile to the chemistry used for conjugation was not systematically studied.

Other factors can also affect the immunogenicity, for example, the molecular size of the carbohydrate antigen [18,19]. We design our study in order to remove all affecting factors and to focus on the importance of O-acetyl and glycerol-phosphate. The information obtained here is valuable for the development of a suitable modification-conjugation procedure for 18C capsular polysaccharide.

There are several methods to reduce the size of PS, but acid hydrolysis is one of the most extensively used and inexpensive. We evaluated acetic and TFA acid to obtain fragments from 18C PS. The hydrolysis with TFA was very efficient, giving a product with the desired size and high recovery. But TFA removed almost completely the O-acetyl groups. Although TFA acid has been reported to hydrolyze the phosphodiester bond [20], in our case no signals were found on the spectra corresponding to the lost of P-Gro groups.

Hydrolysis with acetic acid was less efficient, providing lower recovery of the 10–30 kDa polysaccharide. But, at the same time, it affected less the O-Ac group than the TFA.

To evaluate the contribution of the groups P-Gro and O-Ac to the recognition of capsular polysaccharide, fragments which only differed on the content of O-Ac and de-P-Gro were used to inhibit sera with antibodies against 18C PS. Based on reported differences among the epitopic specificity of antibody elicited in different species [21], we included rabbit and human sera. No differences were found for any sera on inhibition curves between the fragments with different degree of O-Ac. Nevertheless, none of the sera were completely inhibited by de-P-Gro, suggesting that the P-Gro group is an important element for the recognition of 18C PS. Therefore, de-OAc 18C was conjugated to TT, using a reductive-amination procedure [22] and mice and rabbit were immunized to evaluate the immune response. As expected, the conjugates elicited high titers of IgG type antibodies compared to the group immunized with native PS. Also, the sera were specific against 18C PS measured by inhibition ELISA, confirming that the O-Ac was not required to obtain specific antibodies against 18C PS on mice and rabbits.

Rabbit sera against de-OAc 18C conjugate were also evaluated on OPA opsonophagocytic activity against the bacteria. A titer ($\geq$128) confirms further that the O-Ac group was not part of the protective epitope. Due to lability of the O-acetyl groups in the polysaccharide, this fact simplifies the process for developing a prototype conjugate vaccine.

The serogroup 18 includes four different serotypes, 18A, 18B, 18C and 18F. Types 18C, 18B [23] and 18F [24] are identical except for the presence of the O-Ac group. The cross-protection among the serogroup 18 [25] should be expected to be high and could be interpreted in the light of the finding from our studies. Particularly, 18C de-OAc, in fact 18B, elicited functional antibodies against the 18C as we proved in the present study and it should also be protective to 18B. The presence of O-acetylation in several capsular polysaccharides has been shown to be crucial, for example, in serotypes 1 and 15B from *S. pneumoniae* [26,27] and serogroup A from *Neisseria meningitidis*, while in others they are not involved as, for example, serotype 9 V [28] serogroup C and W135 from *Neisseria meningitidis* [29,30] and 18C (present study). Further systematic studies are needed to understand the reason of the

involvement or not of the O-acetyl group on antibody recognition of capsular PS.

5. Conclusions

The O-acetyl group in *S. pneumoniae* capsular polysaccharide serotype 18C is only marginally involved in recognition. The polysaccharide fragment devoid of the O-acetyl group could be used in a production of conjugate vaccine.

Acknowledgments

The authors are deeply grateful to Oralys Sanchez Ferras and Merlín Pérez Pérez whose dedication and work made possible this study and to Tania Carmenate for technical expertise. We thank to our coworkers from the Center of Bimolecular Chemistry due to their valuable collaboration. In addition, we are grateful to Dr. Alina Ruiz for writing assistance of the manuscript.

Conflict of interest

The author declares no financial or commercial conflicts of interest.

References

[1] O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, McCall N, Lee E, Mulholland K, Levine O, Cherian T, et al. Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. Lancet 2009;374:893–902.
[2] American Academy of Pediatrics Committee on Infectious Diseases. Policy statement: recommendation for the prevention of pneumococcal infections, including the use of pneumococcal conjugated vaccine (Prevnar), pneumococcal polysaccharide vaccine and antibiotic prophylaxis. Pediatrics 2000;106:362–6.
[3] Rodgersa GL, Klugman KP. The future of pneumococcal disease prevention. Vaccine 2011;29S:C43–8.
[4] Poolman J, Borrow R. Hyporesponsiveness and its clinical implications after vaccination with polysaccharide or glycoconjugate vaccines. Expert Rev Vaccines 2011;10:307–22.
[5] Berry DS, Lynn F, Lee CH, Frasch CE, Bash MC. Effect of O-acetylation of *Neisseria meningitides* serogroup A capsular polysaccharides in sera from vaccines and carriers. Infect Immun 2002;70:3707–13.
[6] Michon F, Huang CH, Farley EK, Hronowski L, Di J, Fusco PC. Structure activity studies on group C meningococcal polysaccharide-protein conjugate vaccines: effect of O-acetylation on the nature of the protective epitope. Dev Biol (Basel) 2000;103:151–60.
[7] Fernández-Santana V, Cardoso F, Rodriguez A, Carmenate T, Peña L, Valdés Y, Hardy E, Mawas F, Heynngnezz L, Rodríguez MC, Figueroa I, Chang J, Toledo ME, Musacchio A, Hernández I, Izquierdo M, Cosme K, Roy R, Verez-Bencomo V. Antigenicity and immunogenicity of a synthetic oligosaccharide-protein conjugate vaccine against Haemophilus influenzae type b. Infect Immun 2004;72:7115–23.
[8] Verez-Bencomo V, Fernandez-Santana V, Hardy E, Toledo ME, Rodriguez A, Baly A, Harrera L, Izquierdo M, Villar A, Valdes Y, Cosme K, Deler ML, Montane M, Gracia E, Ramos A, Aguilar A, Medina E, Torano G, Sosa I, Hernandez I, Martinez R, Muzachio A, Carmenates A, Costa L, Cardoso F, Campa C, Diaz V, Roy R. A synthetic conjugate polysaccharide vaccine against Haemophilus influenzae type b. Science 2004;305:522–5.
[9] Lugowski C, Jennings HJ. Structural determination of the capsular polysaccharide of *Streptococcus pneumoniae* type 18C (56). Carbohydr Res 1984;131:119–29.
[10] Karmerling JP, Tomasz A. Pneumococcal Polysaccharide: A Chemical View – *Streptococcus pneumoniae* – Molecualr Biology and Mechanisms of Disease. Larchmont, NY, USA: Mary Ann Lierbert, Inc.; 2000. p. 81–114.
[11] Richards JC, Perry MB. Structure of the specific capsular polysaccharide of Streptococcus pneurnoniae type 23F (American type 23). Biochem Cell Biol 1988;66:758–71.
[12] Bruckner J. Estimation of monosaccharides by the orcinol–sulfuric acid reaction. Biochem J 1995;60:200–5.
[13] Porro M, Vitti S, Antoni G, Neri P. Modifications of Park-Johnson ferricyanide submicromethod for the assay of reducing groups in carbohydrates. Anal Biochem 1981;118:301–6.
[14] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with Folin phenol reagent. J Biol Chem 1951;193:265–75.
[15] Che-Jen Lee. Quality control of polyvalent pneumococcal polysaccharide – protein conjugates vaccine by nephelometry. Biologicals 2002;30:97–103.
[16] Romero-Steiner S, Libutti D, Pais LB, Dykes J, Anderson P, Witin JC, Keyserling HL, Carlone GM. Standardization of an opsonophagocytic assay for the measurement of functional antibody activity against *Streptococcus pneumoniae* using differentiated HL-60 Cells. Clin Diagn Lab Immunol 1997;4:415–22.
[17] Jones C. NMR assays for carbohydrate-based vaccines. J Pharm Biomed Anal 2005;38:840–50.
[18] Daum RS, Hogerman D, Rennels MB, Bewley K, Malinoski F, Rothstein E, Reisinger K, Block S, Keyserling H, Steinhoff M. Infant immunization with pneumococcal CRM197 vaccines: effect of saccharide size on immunogenicity and interactions with simultaneously administered vaccines. J Infect Dis 1997;176:445–55.
[19] Laferriere CA, Sood RK, de Muys JM, Michon F, Jennings HJ. *Streptococcus pneumoniae* type 14 polysaccharide-conjugate vaccines: length stabilization of opsonophagocytic conformational polysaccharide epitopes. Infect Immun 1998;66:2441–6.
[20] Charlotte C, Yu Ip, Veda Manam, Robert Hepler, John Jr PHennessey. Carbohydrate composition analysis of bacterial polysaccharides: optimized acid hydrolysis conditions for HPAEC-PAD analysis. Anal Biochem 1992;201:343–9.
[21] Alonso De Velasco E, Verheul AFM, van Steijn AMP, Dekker HAT, Feldman RG, Fernandez IM, Kamerling JP, Vliegenthart JFG, Verhoef J, Snippe H. Epitope specificity of rabbit immunoglobulin G (IgG) elicited by pneumococcal type 23F synthetic oligosaccharide- and native polysaccharide-protein conjugate vaccines: comparison with human anti-polysaccharide 23F IgG. Infect Immun 1994;62:799–808.
[22] Jennings, HJ, Lugowski C. Immunogenic polysaccharide-protein conjugates. Patent 4356170; 1982.
[23] Karlsson C, Jansson P, Wildmalm G, Skov Sørensen UB. Structural elucidation of the capsular polysaccharide from *Streptococcus pneumoniae* type 18B. Carbohydr Res 1997;304:165–72.
[24] Per-Erik J, Lindberg B, Lindberg J, Henrichsen J. Structural studies of the capsular polysaccharide from *Streptococcus pneumoniae* type 18F. Carbohydr Res 1988;173:217–25.
[25] Butler JC, Breiman RF, Campbell JF, Lipman HB, Broome CV, Facklam RR. Pneumococcal polysaccharide vaccine efficacy an evaluation of current recommendations. JAMA 1993;270:1826–31.
[26] Corné, Stroop JM, Qiuwei Xu, Retzlaff M, Abeygunawardana Ch, Bush CA. Structural analysis and chemical depolymerization of the capsular polysaccharide of *Streptococcus pneumoniae* type 1. Carbohydr Res 2002;337:335–44.
[27] Rajam G, Carlone GM, Romero-Steiner S. Functional antibodies to the O-acetylated pneumococcal serotype 15B capsular polysaccharide have low cross-reactivities with serotype 15C. Clin Vaccine Immunol 2007;14:1223–7.
[28] Mcneely TB, Staub JM, Rusk CM, Blum MJ, Donnelly JJ. Antibody responses to capsular polysaccharide backbone and O-acetate side groups of *Streptococcus pneumoniae* type 9V in humans and rhesus macaques. Infect Immun 1998;66:3705–10.
[29] Fusco PC, Farley EK, Chun-Hsien Huang, Moore S, Michon F. Protective meningococcal capsular polysaccharide epitopes and the role of O acetylation. Clin Vaccine Immunol 2007;14:577–84.
[30] Gudlavalleti SK, Lee Ch, Norris SE, Paul-Satyaseela M, Vann WF, Frasch CE. Comparison of *Neisseria meningitidis* serogroup W135 polysaccharide-tetanus toxoid conjugate vaccines made by periodate activation of O-acetylated, non-O-cetylated and chemically de-O-acetylated polysaccharide. Vaccine 2007;25:7972–80.