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Characterization and immunogenicity of meningococcal group C conjugate vaccine prepared using hydrazide-activated tetanus toxoid

I.A.F.B. Silveira [a,*], R.C. Bastos [a], M.S. Neto [a], A.P. Laranjeira [a], E.F. Assis [a], S.A.R. Fernandes [a], M.L. Leal [a], W.C. Silva [a], C.-H. Lee [b], C.E. Frasch [b], J.M. Peralta [c], E. Jessouroun [a]

[a] *Laboratório de Tecnologia Bacteriana, Bio-Manguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil*
[b] *Center for Biologics Evaluation and Research (CBER), FDA, Bethesda, MD, USA*
[c] *Departamento de Imunologia, Instituto de Microbiologia Prof. Paulo de Góes, Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro, Brazil*

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Abstract

The steps to produce, purify and control an immunogenic Brazilian conjugate vaccine against group C meningococcus (MenCPS–TT) using hydrazide-activated tetanus toxoid were developed. The conjugation methodology reduced the reaction time easily allowing scale-up. One freeze-dried pilot vaccine lot purified by tangential filtration, showed satisfactory quality control results including safety and stability. The pilot vaccine was immunogenic in mice in a dose-dependent fashion generating a 10–20-fold rise in IgG response in mice. The vaccine also induced high bactericidal titers. Vaccine concentrations of 1 and 0.1 μg showed higher avidity indices, suggesting induction of immunologic memory. These results support initiation of Phase I clinical studies with the MenCPS–TT conjugate vaccine.

Keywords: Conjugate vaccine; Meningococcal group C; Reductive amination; High avidity IgG; Serum bactericidal activity

1. Introduction

Neisseria meningitidis (*the meningoccocus*) is one of the most important pathogens as causes of meningitis and other clinical manifestations. Meningococcal disease is primarily caused by only five meningococcal groups (A, B, C, Y and W-135) among 13 groups described, with a realistic prospect of disease elimination given the development of effective vaccines [1]. However, native polysaccharide (PS) meningococcal vaccines are poorly immunogenic in infants. In addition, some polysaccharides like group C may induce immunological tolerance after inoculation of repeated doses in adults and children [2–5]. These vaccines have been replaced by PS–protein conjugates in order to improve the immunogenicity in infants and young children inducing memory responses [2,6–8]. Several conjugate vaccines against groups A and C meningococci have been immunogenic and well tolerated in infants [9,10]. The impact of conjugate vaccine on disease and nasopharyngeal carriage of group C meningococci has been considerable, with efficacy over 90% and 67% reduction in carriage resulting in an important herd immunity effect (around 65%) [11]. Meningococcal C conjugate vaccines have been introduced in many national immunization programs in countries such as United Kingdom, Ireland, Spain, Netherlands, Belgium, Australia and Canada [12–20].

The incidence of meningococcal disease in Brazil is 1–3/100,000 inhabitants mainly due to group B (60%) followed by group C (40%) [21,22]. Despite the significant rate, Brazil has produced only polysaccharide vaccines against groups A and C since 1976. The country does not produce

* Corresponding author. Tel.: +55 21 38829342; fax: +55 21 22604727.
E-mail address: ivna@bio.fiocruz.br (I.A.F.B. Silveira).

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meningococcal C conjugate vaccines for routine immunization and control meningococcal disease caused by this group.

Several multi-step conjugation methods have been employed for covalently linking polysaccharides to proteins. One of them is reductive amination that is currently used to prepare licensed conjugate vaccines against *Haemophilus influenzae* type b, *N. meningitidis* and *Streptococcus pneumoniae* [23–26].

Here, we describe the development of a Brazilian conjugate vaccine against group C (MenCPS–TT). The MenCPS–TT vaccine was produced by reductive amination, using hydrazide-activated tetanus toxoid (TT) as a carrier protein. Current methodology was modified to reduce time of reaction easily allowing scale-up [27,28]. Conjugate immunogenicity in mice was demonstrated by the detection antibodies including avidity indice and bactericidal activity. The results support initiation of Phase I clinical studies with the MenCPS–TT conjugate vaccine.

2. Materials and methods

2.1. Meningococcus group C polysaccharide (MenCPS) and tetanus toxoid (TT)

Native MenCPS was produced by Bio-Manguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil. It was obtained from bacterial mass of *N. meningitidis* 2135 strain cultivated in Frantz medium and purified as described previously [29,30]. Sialic acid content of MenCPS was measured by resorcinol method [31]. MenCPS identity, structure and purity were evaluated by one-dimensional proton nuclear magnetic resonance spectroscopy (^{1}H NMR 1D) at 500 MHz at 37 °C using a Bruker Avance/500. The analyses were done without water-suppression. Dry samples (10 mg) were dissolved in deuterated water (D_2O 99.96% D, Cambridge Isotope Laboratories Inc.) [32–34]. TT was provided by Instituto Butantan, São Paulo, SP, Brazil. It was produced and purified according to the specifications of DTP vaccine [35]. The antigenic purity of the TT used in these studies was 1892 Lf/mg protein nitrogen, which is appropriate to be used in conjugation processes [33]. The protein content was evaluated by Bradford method [36].

2.2. MenCPS activation

Different batches of native MenCPS (10 mg/mL) in water were treated with sodium periodate (23.4 mM) overnight at 4 °C in the dark for generating aldehyde groups [23]. Glycerol was added to quench the excess sodium periodate. The activated PS was purified by diafiltration against water and concentrated by tangential flow ultrafiltration (Centramate System, Pall BioPharmaceuticals) [28]. The identity and presence of aldehyde groups in the polysaccharides were evaluated by ^{1}H NMR 1D spectroscopy using the same conditions described above [32,34]. The aldehyde group content of activated-MenCPS was measured by a formaldehyde assay using the Purpald reagent [37].

2.3. Protein activation

TT (3.5 mg/mL) was activated by introduction of hydrazine groups by carbodiimide (EDAC) methodology after treatment with hydrazine dihydrochloride in 50-fold excess at room temperature and under acidic conditions (pH 6.1) [27]. The hydrazide-activated tetanus toxoid (TTH) was purified by diafiltration against 0.02 M PBS pH 7.4 and concentrated by tangential flow ultrafiltration (Centramate System, Pall BioPharmaceuticals) [28].

2.4. Conjugation procedures

Activated-MenCPS (50 mg/mL) was covalently linked to TTH (60 mg/mL) in the presence of 1 M sodium cyanoborohydride (1 M; 10 mL) overnight (method derived from [23]). The reaction was stopped by addition of 0.5 M adipic acid dihydrazide (ADH; 50 mL) to block unreacted aldehyde groups. Conjugation was analyzed by size exclusion chromatography (SEC) using a TSK-G® 4,000 PWxl column (with ultraviolet detection at 280 and 206 nm), and by ^{1}H NMR 1D spectroscopy using the same conditions described above, however, the analysis were done with water-suppression [32,34]. The mixtures were diafiltrated against 0.02 M PBS pH 7.4, to remove unconjugated polysaccharides and concentrated by tangential flow ultrafiltration (Centramate System, Pall BioPharmaceuticals) [28]. Total sugar and protein contents in the intermediate products and the final conjugates were determined by resorcinol and Bradford methods, respectively [31,36]. The amount of free polysaccharide was evaluated by HPAEC-PAD after precipitation with DOC according to Lei et al. [38].

2.5. Pilot lot vaccine preparation

In order to evaluate the immunogenicity of MenCPS–TT conjugate in mice and also to determine its stability after storage, one lot corresponding to 27,000 human doses (10 μg/0.5 mL; Lot 40) was used to fill vials containing five human doses. The vials were freeze-dried and stored at 4 °C.

2.6. Quality control assays

The quality control assays required by WHO for meningococcal C conjugate vaccines were conducted. The final product was assayed for determination of saccharide content after dialysis against water to remove all sucrose residues and avoid any interference in the resorcinol assay. The residual moisture was done by Karl-Fisher methodology [39] and the amount of sucrose by size-exclusion chromatography (Shodex SC1011-sugar 8 column) with refractive index detection. The purified bulk conjugate was tested for bacterial and mycotic sterility, pyrogen “in vivo” and “in vitro”

("Limulus amebocyte lysate"; LAL; [40]), abnormal toxicity in mice and reactogenicity in rabbits were used for the characterization of the pilot vaccine lot [33].

2.7. Immunization procedures

Immunogenicity of the lyophilized pilot vaccine lot (Lot 40) was evaluated in dose–response studies. Swiss mice (15–22 g, 15 animals per group) were immunized intramuscularly with 0.1, 1 and 10 μg/dose (0.2 mL) of vaccine (Lot 40) or plain polysaccharide (5 μg/dose) with $Al(OH)_3$ (1 mg/dose) as adjuvant, three times with 15-day interval. Blood was collected by retro-orbital vein puncture before each dose and 20 days after the third dose. Sera were isolated and stored at −20 °C until use.

2.8. ELISA assay

Serum samples were assayed by ELISA for IgG antibodies against MenCPS as previously described by Gheesling et al. [41]. Briefly, Immulon plates type II (Dynex) were coated overnight at 4 °C with 100 μL of 5 μg/mL MenCPS in PBS pH 7.4 co-mixed with methylated human serum albumin (5 μg/mL). After washing four times with 200 μL washing buffer (Tris Buffer Saline; TBS with 0.05% Tween 20), antiserum samples and in-house standard serum at a serial twofold dilution starting from 1/5000 were added to each well. After overnight incubation, the plates were washed four times and incubated with 100 μL goat anti-mouse IgG whole molecule conjugated with alkaline phosphatase (1/3000 dilution in PBS pH 7.4) for 2 h. After washing *p*-nitrophenyl phosphate (1 mg/mL) was added and plates were incubated for 30 min. Absorbance was read at 405 nm. ELISA titers were calculated using arbitrary unit of ELISA in reference to a standard serum (1000 U/mL) and expressed by Ln-transformed values (Ln U/mL).

2.9. Antibody avidity-measurement

The avidity of conjugate-specific IgG antibodies in sera, taken at different intervals after vaccination, was studied using the ammonium thiocyanate elution method as described [42]. In brief, MenCPS coated (5 μg/mL) microplates were incubated overnight at 4 °C with a dilution of each serum sample predetermined to give an absorbance between 0.8 and 1.0. To each well different dilutions of ammonium thiocyanate ranging 0–0.75 M were added. The plates were then incubated for 15 min at room temperature, followed by a washing cycle. The assay then continued as described above. The IgG avidity index (AI) corresponding to ammonium thiocyanate concentration needed to reduce the absorbance by 50% and it was calculated as described below according to Goldblatt [42]:

$$\mathrm{AI} = \frac{(\log 50 - \log A) \times (B - A)}{\log B - \log A} + A$$

where AI is the avidity index, log of 50 = 1.70, A the lowest concentration of ammonium thiocyanate that gives reduction of absorbance lower than 50%, and B is the highest concentration of ammonium thiocyanate that gives reduction of absorbance higher than 50%.

2.10. Serum bactericidal activity assay (SBA)

Twofold dilutions of sera were incubated with 50–70 colony forming units (cfu) per well of log phase meningococci grown on Tryptic Soy Agar (TSA). Each well contained complement from male guinea pigs, previously shown to lack detectable intrinsic bactericidal activity. The plates were incubated at 37 °C for 30 min and the number of cfu was counted. The bactericidal antibody activity (SBA) titers are expressed as the reciprocal serum dilution yielding ≥50% killing as compared to the number of target cells present before incubation with serum and complement [43].

2.11. Stability of MenCPS–TT conjugate vaccine

The lyophilized vaccine stability was evaluated in real time at 0, 11 and 22 months of storage at 4 °C, after filling and freeze-drying, by SEC profile. IgG titers were also detected by ELISA as a vaccine stability parameter after 6 and 15 months of storage before each dose and 1 month after the third dose and after 26 months of storage only 1 month after the last dose. ELISA was performed as described above to detect IgG antibodies against MenCPS in mouse sera obtained after immunization with the pilot vaccine lot (1 μg/dose).

2.12. Statistical analysis

For analysis, SBA titers were logarithmically transformed (Ln). The significance of differences in antibody levels among groups and their SBA titers were assessed by using a multifactor analysis of variance (ANOVA) followed by the least significant difference (LSD) procedure for comparison of groups versus control. The avidity indices were compared for each vaccine concentration by the non-parametric Kruskall–Wallis test. Pearson correlation coefficients were determined in the correlation analyses. Mann–Whitney *W*-test was used for comparison between the IgG antibodies induced at 6, 15 and 26 months of vaccine storage. Statgraphics® Plus Version 4.1 software (USA) was used in all statistical calculations. Limit for statistical significance was set at $p < 0.05$.

3. Results

3.1. Detection and structural characterization of intermediary compounds and MenCPS–TT conjugates

The identity of native MenCPS produced as described by WHO requirements was evaluated by NMR spectroscopy

(Fig. 1A). The peaks in the 5.0–5.2 ppm range correspond to the H-7 and H-8 residues of *O*-acetylated *N*-acetyl MenCPS. The chemical shifts observed in the range of 3.3–4.2 ppm are related to H-4, H-5, H-6, H-9, H-9′, H-7′ and H-8′ residues of deacetylated MenCPS (Fig. 1A). The peaks related to equatorial H-3 are observed in the range comprised between 2.5 and 2.8 ppm and the chemical shift observed in the 1.5–1.8 ppm range correspond to axial H-3. The peaks corresponding to *N*-acetyl and *O*-acetyl have chemical shift at 2.00 and 2.20 ppm, respectively. The peaks at 1.2 and in the 3.3–4.2 ppm range are relative to chemical shifts of the CH_3 and CH_2 groups (ethanol), respectively. It can be observed that the peak of HDO is at 4.6 ppm. The ^{1}H NMR spectra contains all the important species whose resonances have been assigned to individual atoms in the repeating unit and are consistent with the published structure [32,44–46]

The activation of native MenCPS with sodium periodate is illustrated in Fig. 2 [(1) MenCPS activation]. The ^{1}H NMR spectra of activated-MenCPS presents the same assignments described above for the native polysaccharide, showing that the polysaccharide structure remains unchanged following sodium periodate treatment (Fig. 1B). However, chemical shifts around 8 ppm are present, corresponding to the novel end groups formed during periodate oxidation. These chemical shifts can be observed as a signal at 8.4 ppm with low intensity, consistent with the aldehydic group. In addition, two small peaks relative to CH_3 and CH_2 groups (ethanol) are seen at 1.2 and 3.3–4.2 ppm range and a new peak with chemical shift at 5.0 ppm corresponding to hydrated aldehyde (Fig. 1B). The aldehyde groups present in the activated-MenCPS were measured with the Purpald reagent [37]. Three lots of activated-MenCPS (50 mg/mL) showed similar amounts of aldehyde groups, above 220 nM, which were more than 10-fold higher than the amount present in two lots of native MenCPS (less than 20 nM), showing again the successful introduction of functional groups to react with amino groups present in the TTH.

The reaction of TT activation introducing hydrazine groups in the protein is illustrated in Fig. 2 [(2) Protein activation]. The activated-TT reacted with activated-MenCPS generating a meningococcal group C conjugate vaccine using a new high efficiency conjugation methodology [Fig. 2; (3) Conjugation reaction]. Several MenCPS-TT conjugate batches were produced at different scales (from 200 to 20,000 human doses) by modified reductive amination in presence of sodium cyanoborohydride. The success of this procedure was apparent from the SEC profile of the conjugation reaction product, which displayed a high molecular weight product clearly differentiated from the starting protein and polysaccharide (Fig. 3). The formation of conjugate is also clearly demonstrated due to the disappearance of the assignments at 8.4 ppm present in the activated-MenCPS corresponding to aldehyde groups, after the conjugation step (Fig. 1C). The NMR spectra of MenCPS–TT also show

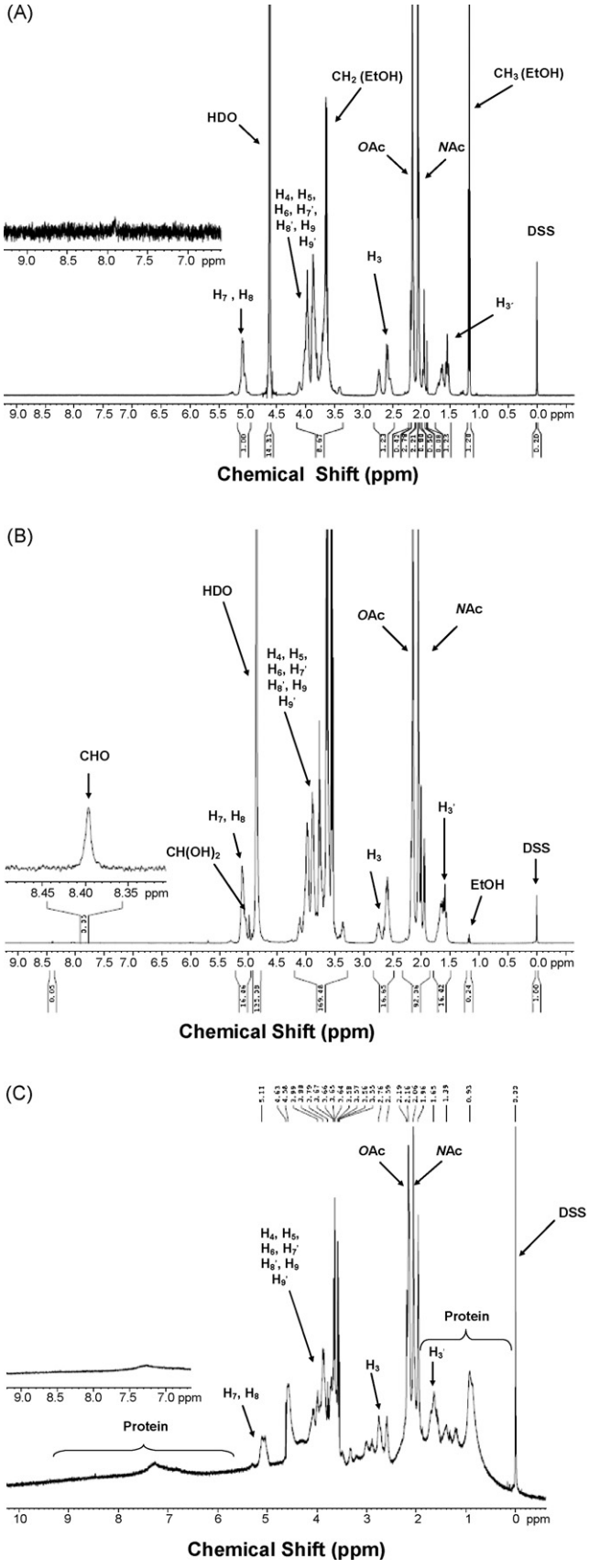

Fig. 1. Five-hundred megahertz ^{1}H NMR spectra of (A) native, (B) activated and (C) conjugated MenCPS.

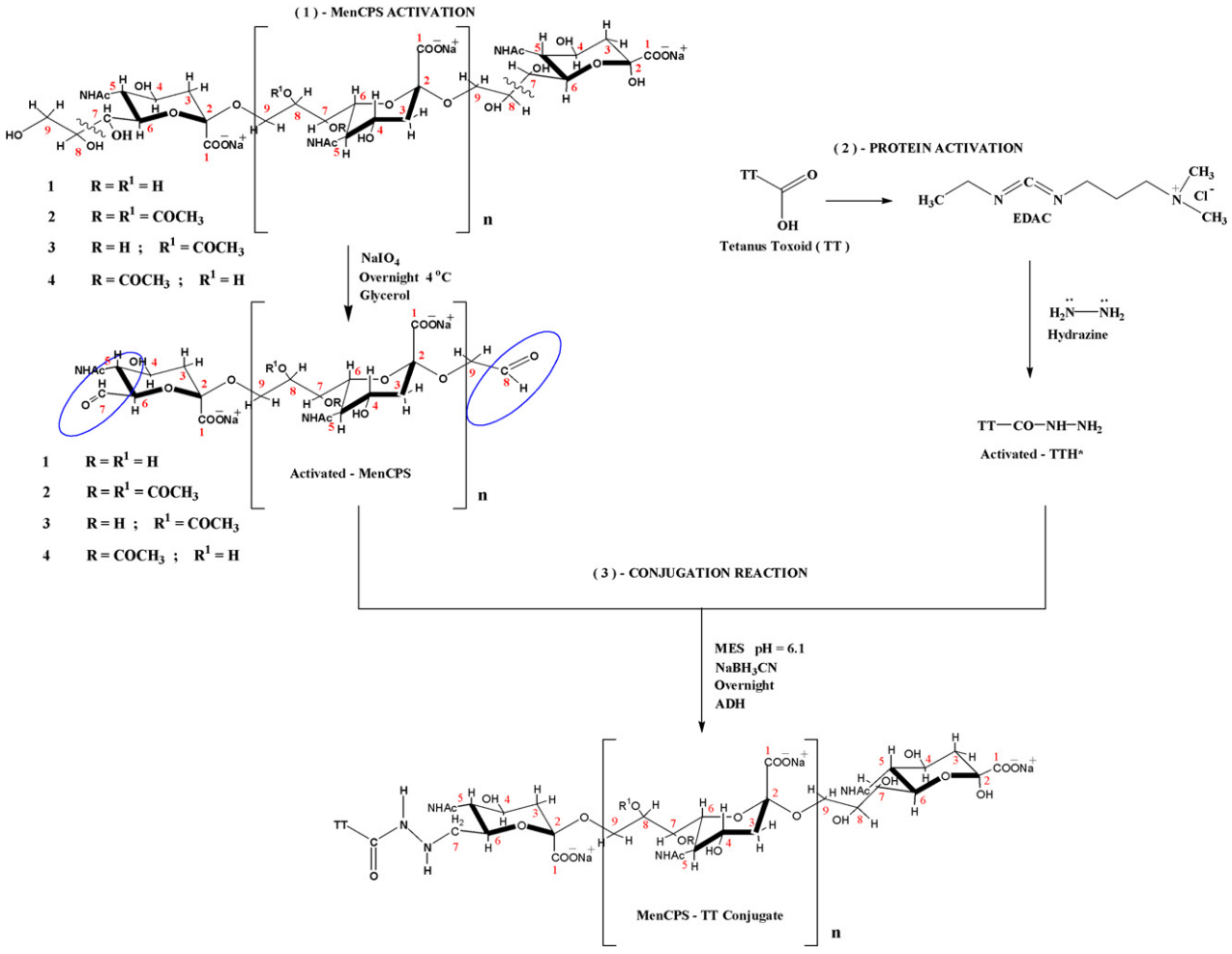

Fig. 2. Reaction schemes of native MenCPS and TT activation and conjugation by reductive amination.

the same assignments described for the native and activated molecule. In addition other assignments at 6.00–8.00 and 1.8–0.5 ppm corresponding to the protein aromatic and aliphatic amino acids were incorporated, respectively (Fig. 1C). The peak in the 4.4–4.7 ppm range corresponds to residual H_2O present in the molecule even after doing water-suppression.

The MenCPS–TT conjugate batches showed sugar:protein ratios from 0.3 to 0.5. The activated-MenCPS and TTH and MenC–TT conjugates were evaluated for all residual reagents present after the purification processes using several different methods, according to requirements [33]. All results were satisfactory, showing the efficiency of tangential flow ultrafiltration in the removal of reagents, by-products (data not shown) and unconjugated polysaccharide (average content of 11.3%).

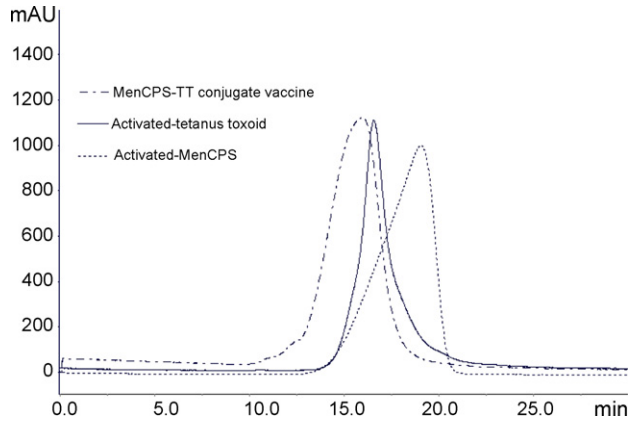

Fig. 3. Size-exclusion chromatography (SEC) profiles of MenCPS–TT conjugate vaccine, activated-TT and activated-MenCPS.

3.2. *Quality control assays of the MenCPS–TT conjugate vaccine*

The pilot vaccine lot (Lot 40) was evaluated using the quality control assays required by WHO for meningococcal C conjugate vaccines. All assay results were satisfactory including LAL test that showed less than 29.55 EU/μg, suggesting the conjugate vaccine has very low endotoxin and would be biologically safe (Table 1).

Table 1
Quality control assays used in analysis of lyophilized MenCPS–TT vaccine (Lot 40)

Final product controls	
Residual moisture	1.15%
Sucrose (HPLC)	32.45 mg/mL
Polysaccharide	19.11 μg/mL
Polysaccharide:protein ratio	0.3
Sterility	Approved
Pyrogen "in vivo"	Approved
LAL	<29.55 EU/μg
Abnormal toxicity	Approved
Reactogenicity (rabbits)	Approved

Tests required by WHO [33] for final product of meningococcal group C conjugate vaccines: Identity (serological test, NS); sterility; meningococcal polysaccharide content; residual moisture; pyrogen content; adjuvant content (NS); preservative content (NA); general safety test (abnormal toxicity); pH (NS). NS, not shown; NA, not applicable.

3.3. Immunogenicity of MenCPS–TT conjugate vaccine

Serum samples of mice immunized with three different concentrations of the pilot vaccine lot (Lot 40) were analyzed for specific IgG antibodies, avidity and serum bactericidal activity. Low levels of IgG antibodies were detected by ELISA in the pre-vaccination sera in all groups. The IgG titer of the serum samples obtained from mice immunized with plain MenCPS showed no increase with subsequent injections. In contrast, all concentrations of conjugate vaccine elicited significant antibody rise after the first injection. In addition, the second injection with 1 and 10 μg/dose elicited a boosting effect (above a four- to eightfold rise) as compared to plain MenCPS (Table 2; $p < 0.05$). The third dose of any vaccine leads to twofold increase of IgG titers without significant differences of the second dose (Table 2; $p > 0.05$).

The serum bactericidal activity of the elicited antibodies was determined using guinea pig complement (see Table 2). Serum samples after the first injection were negative. All animals immunized with the conjugate vaccine (Lot 40) showed detectable SBA values (above 1:8) after the second injection with significantly higher titers for the groups immunized with 1 and 10 μg/dose, correlated to protective titers ($p < 0.05$). As seen for ELISA titers, the third dose of vaccine elicits a 2–5.5-fold increase in the SBA titers in comparison to the second dose (Table 2; $p > 0.05$).

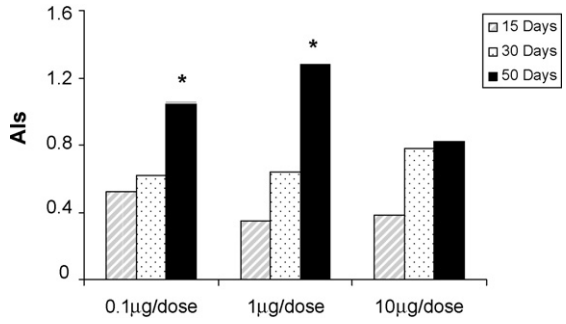

Fig. 4. Avidity of antibodies anti-MenCPS (IgG) after mice immunization. Avidity Index (AI) calculated by the ammonium thiocyanate concentration needed to reduce the absorbance by 50%. *Kruskall–Wallis test ($p < 0.05$).

In most pre-bleeds, antibody titers are below the detection limit. Therefore, avidity indices (AIs) cannot be calculated for those samples (Fig. 4). Groups immunized with the pilot vaccine (Lot 40) show a significant increase in the IgG avidity after the third dose in mice immunized with 0.1 and 1 μg (Fig. 4). Twenty days after the last injection, the median avidity indices of these two groups are 1 and 1.28, respectively ($p < 0.05$). Although the group immunized with 10 μg induced high IgG and SBA titers, the AI is lower than those of the other concentrations. These results demonstrate affinity maturation of IgG antibodies mainly after the third injection of conjugate vaccine at lower dosages.

To investigate the relationship between the IgG antibodies determined by ELISA and SBA titers and AI, Pearson coefficients were determined. We found high correlation between ELISA and SBA ($r = 0.91$) while the correlation between high-avidity ELISA and SBA was lower ($r = 0.70$).

3.4. Stability of MenCPS–TT conjugate vaccine

The SEC profiles of lyophilized vaccine stored at 4 °C for 11 and 22 months after filling and freeze-drying were similar to that analyzed at time 0 in regard of the depolymerization of the polysaccharide (Fig. 5). Likewise, titers of IgG in sera of mice immunized with vaccine stored at 4 °C for 6, 15 and 26

Table 2
Total IgG and serum activity bactericidal (SBA) of antibodies from mice immunized with different amounts of MenCPS–TT conjugate vaccine (dose–response studies)

	Dose							
	Pre		Post 1st dose		Post 2nd dose		Post 3rd dose	
	IgG	SBA	IgG	SBA	IgG	SBA	IgG	SBA
Conjugate (0.1 μg/dose)	1.05	2.00	4.15*	2.00	6.57*	21.86	7.44	48.50
Conjugate (1 μg/dose)	0.65	2.00	4.18*	2.00	7.89*	82.14*	9.04	451.94
Conjugate (10 μg/dose)	0.10	2.00	3.48*	2.00	7.45*	49.52*	8.69	103.97
Plain MenCPS	0.55	2.00	1.66	2.00	1.99	4.00	2.00	2.00

The IgG titers are expressed as Ln U/mL values. SBA titers are expressed as the reciprocal serum dilution yielding ≥50% killing and log-transformed for the calculations.
ANOVA (LSD test; *$p < 0.05$).

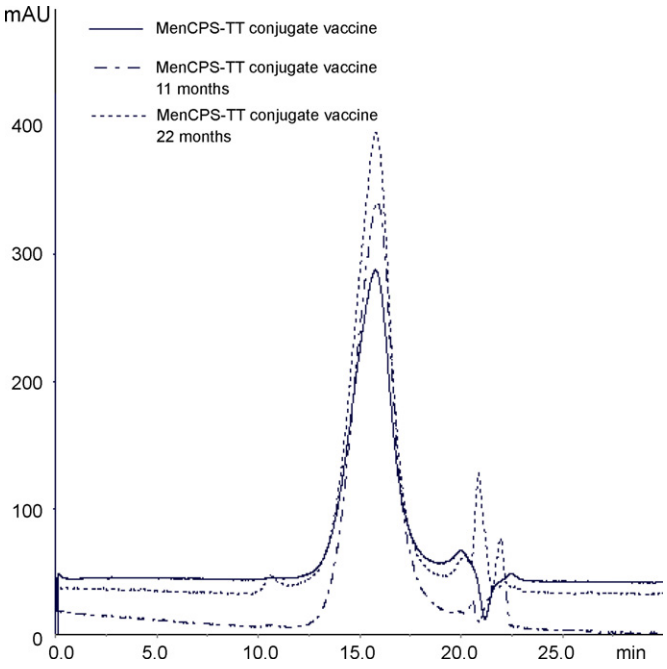

Fig. 5. Stability of lyophilized MenCPS–TT vaccine (Lot 40) storage at 0, 11 and 22 months at 4 °C after filling and freeze-drying by size-exclusion chromatography (SEC) profiles (206 nm).

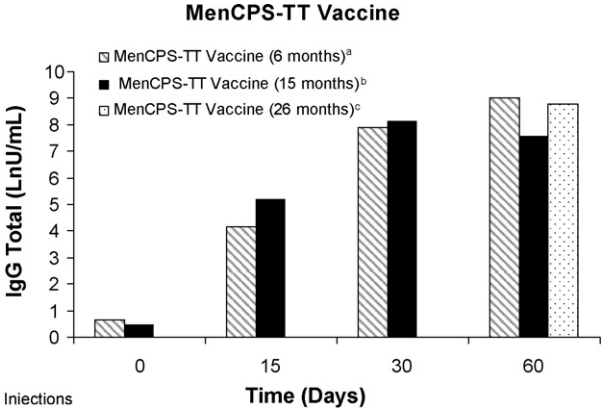

Fig. 6. Stability of lyophilized MenCPS–TT vaccine storage at 6, 15 and 26 months at 4 °C after filling and freeze-drying as measured by ELISA (total IgG) of serum from mice immunized. (a and b) IgG titers measured before each dose and 1 month after the third dose; (c) IgG titers measured only 1 month after the third dose. Mann–Whitney *W*-test; $*p > 0.05$.

months after filling and freeze-drying were not significantly different (Mann–Whitney *W*-test; $p = 0.69$) (Fig. 6). These results suggest that the vaccine is stable for more than 2 years.

4. Discussion

Chemical conjugation of bacterial polysaccharides to carrier proteins has proven to be an effective approach to improve the immunological response against capsular polysaccharides and in prevention of diseases caused by encapsulated bacteria like *N. meningitidis*, *H. influenzae* type b and *S. pneumoniae*. In this study we described the development, characterization and the immunogenicity of a Brazilian meningococcal C conjugate vaccine using hydrazide-activated tetanus toxoid as a carrier.

Reductive amination has been used to yield effective protein–polysaccharide conjugates whose structure is called "neoglycoproteins" in which there is only one reactive site on the carbohydrate polymer and no cross-linking of the protein [26]. Jennings and Lugowski [23] used for the first time the methodology to produce meningococcal conjugates against groups A, B and C. However, in our approach the treatment of polysaccharide with periodate overnight resulted in controlled depolymerization to generate saccharides with reactive aldehyde groups at both terminals [28]. Many effective conjugate vaccines prepared using this methodology are currently licensed [26]. However, reductive amination has a serious disadvantage related to the coupling reaction time. It has shown inefficiency, taking from 2 to 3 days to complete conjugation [23–26]. We obtained MenCPS conjugates after overnight reaction of an aldehyde-activated group C polysaccharide with a hydrazide-activated TT. This rapid rate of reaction is mainly due the introduction of highly reactive hydrazide groups to the carboxyl groups in the protein carrier [27,28]. The introduced hydrazide groups react more favorably with the aldehyde groups on the activated polysaccharide than the epsilon amino groups of proteins due to the low pK_a of the hydrazide groups [27]. Also, the amino groups in TT are compromised and de-populated with the detoxification procedure of tetanus toxin to toxoid after treatment with formaldehyde. In addition, due to defined structure of the molecules, the steps of MenCPS activation and conjugation could be monitored by NMR spectroscopy, which has proven a structurally sensitive and reproducible technique to control the identity of bacterial polysaccharides used in vaccine manufacture [46]. A meningococcus group A conjugate vaccine was developed using the same conjugation methodology provided by expertise from CBER/FDA as result of a partnership between WHO and PATH to promote vaccination campaign in Africa [27,47].

Accordingly, methods for the manufacture of polysaccharide–protein conjugate vaccines wherein the reaction proceeds at a rapid rate, with reduced production of undesired by-products, and with high yields of conjugated polysaccharide, are desirable. In this way, a reproducible procedure for the production of commercial volumes was developed including purification optimization. The downstream processing proposed here led to lower levels of reaction by-products and unconjugated polysaccharides. The average amount of free polysaccharide was 11.3%. The WHO requirements have not established a maximal value allowed for these vaccines [33]. But this rate would be acceptable considering the established limits for Hib conjugate vaccines (maximal of 20% free PRP, [48]). Beuvery et al. [49] produced a PSC–TT conjugate using carbodiimide methodology with 10% of free polysaccharide. In other studies Cuello et al. found an average content of

free polysaccharide of 21% for meningococcal group C conjugates [50]. This is an important aspect since tolerance has been shown after repeated injections of polysaccharide vaccines against meningococcal group C in adults and children [3,5].

In order to evaluate the efficiency of the conjugation the sugar:protein ratios were calculated. The conjugate batches produced showed low levels of carbohydrate loading as described for other studies using reductive amination [51–53]. In the near future the acceptable limits of the sugar:protein ratio could be established along the production process with larger batches and also be used to evaluate consistency of production [34].

The pilot freeze-dried vaccine (Lot 40) was evaluated by the quality control assays required by WHO for meningococcal C conjugate vaccines. All assays gave satisfactory results suggesting that the conjugate vaccine composition is safe. The stability of this vaccine stored for different times at 4 °C was evaluated by SEC profile and through mice immunization by measurement of IgG titers. The vaccine has remained stable for more than 2 years after filling and freeze-drying. The real time stability assessment of MenCPS–TT conjugate vaccine is in progress.

The assessment of immune responses to these conjugate vaccines has included a fourfold rise in antibody titer between pre- and post-immunizations sera with increased avidity of group C specific antibody [47,54,55]. It has been widely accepted since the studies of Gotschlich et al. [29] that serum bactericidal antibody levels correlate with immune protection against group C meningococcal disease. Based on efficacy estimates and the proportion of responders in various clinical trials of meningococcal C conjugate vaccines, it has been demonstrated that a SBA titer of 1:8 is the minimal titer required for short-term protective immunity [54,55]. The MenCPS–TT conjugate vaccine produced with our approach was immunogenic in mice in a dose-dependent fashion. All concentrations of MenCPS–TT conjugate were able to generate a 10–20-fold rise in IgG response higher than plain polysaccharide with a significant boosting effect after the second injection. This IgG response clearly showed T-cell dependent immune response after coupling the plain polysaccharide to an immunogenic protein carrier. It also showed a proper functional activity with bactericidal titers much higher than 1:8 for all vaccinated animal groups (0.1, 1 and 10 μg/dose), while the plain MenCPS induced no significant titers. A 695-fold increase of SBA titers after three doses (1 μg/dose) was observed in comparison to pre-immune sera. Despite this fact, in both assays the third dose of vaccine did not elicit a statistically significant increase in the ELISA and SBA titers in comparison to the second dose ($p > 0.05$). Cuello et al. [56] studied a MenCPS conjugate inoculating 2 μg/dose in mice and found a 256-fold increase of SBA titers after two doses. Ritcher et al. [57] observed an increase of the SBA titers higher than 256-fold using a mice immunization schedule with two doses of a MenCPS conjugate (2.5 μg/dose). In other study it was observed that two doses of a MenCPS conjugate (2.5 μg/dose) induced a threefold higher bactericidal response as compared with sera of the animals immunized with the plain polysaccharide [58].

Avidity maturation is a marker of induction of immunologic memory and it has been shown that higher avidity anticapsular antibodies are more active than lower avidity antibodies in eliciting complement-mediated bacteriolysis [42,54,55,59]. It is important to compare high-affinity antigen–antibody binding in presence of chaotropic agent as ammonium thiocyanate after determination of the appropriate serum dilution to give fixed absorbance. We use the approach described by Goldblatt [42] and cited by others [60,61]. The animal group immunized with 1 μg/dose of conjugate vaccine showed higher AI after repeated injections of conjugate vaccine followed by that one which received 0.1 μg/dose. Although the group immunized with 10 μg/dose has shown high IgG and SBA titers the AI was lower than the others, suggesting that the excessive amount of antigen located on the follicular dendritic cells may reduce the competition and permit production of antibodies with lower affinity maturation [62].

The correlation coefficient (r) between the standard ELISA, SBA and the high-avidity ELISA were calculated including pre- and post-vaccination serum samples. The standard ELISA showed a marginally higher correlation with SBA than the avidity ELISA. It is possible that conjugate vaccines in our animal model induce predominantly high-avidity antibodies as already published using human antibodies [63,64]. However, other studies found better correlation between SBA and the high avidity ELISA suggesting that not all the specific human or mice IgG antibodies induced by group C conjugate vaccines mediate SBA [41,42,52,60].

Altogether, these results support the production of a MenCPS–TT conjugate vaccine batches under Good Manufacturing Practices for Phase I clinical trial.

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