ORIGINAL RESEARCH

Immunization and chemical conjugation of Bm95 obtained from *Pichia pastoris* enhances the immune response against vaccinal protein and *Neisseria meningitidis* capsular polysaccharide

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Abstract: The ectoparasite *Rhipicephalus* (*Boophilus*) *microplus* causes severe economic losses to the cattle industry in tropical and subtropical regions, and transmits endoparasites, such as *Babesia bovis*. The glycoprotein Bm95 is homologous to Bm86, a surface membrane protein of gut epithelial cells in *R. microplus*, and has been shown to efficiently control this ectoparasite in regions of the Americas. The immunostimulant properties of Bm86 have already been demonstrated after its coinjection with hepatitis B surface antigen (HBsAg) and the infectious bovine rhinotracheitis virus. This study evaluated the carrier and immunostimulant properties of Bm95 using low immunogenic *Neisseria meningitidis* capsular C polysaccharide (Men CpS) and HBsAg. We produced two polysaccharide-Bm95 conjugates by carbodiimide (MenCpSBm-*c*) and reductive amination (MenCpSBm-*ra*) methods. These conjugates were characterized and evaluated in mice. Antibody titers against Men CpS were significantly higher in mice immunized with MenCpSBm-*ra* ($2{,}350 \pm 250$, $P < 0.01$) than in those immunized with MenCpSBm-*c* (250 ± 75) or Men CpS (570 ± 104). The study data indicate effective immunological memory after booster inoculation in mice immunized with MenCpSBm-*ra*. Additionally, significant humoral immunity against HBsAg was documented in mice coimmunized via the intranasal route with recombinant Bm95 ($11{,}400 \pm 345$) and HBsAg ($128{,}000 \pm 250$) compared with mice immunized only with HBsAg (400 ± 40) or Bm95 ($5{,}461 \pm 150$, $P < 0.01$). In conclusion, the immunostimulatory properties of recombinant Bm95 make it a useful element for developing safer conjugated vaccines against bacterial pathogens and for evaluation against ticks and tick-borne diseases in the context of a polyvalent veterinary vaccine.

Keywords: glycoconjugate, Bm86, capsular polysaccharides, carrier protein

Introduction

The Bm86 glycoprotein is localized on the membrane of gut epithelial cells in *Rhipicephalus* (*Boophilus*) *microplus*, and efficient control of this ectoparasite is possible in cattle vaccinated with this antigen.[1] The blood of actively immunized bovine contained Bm86 antibodies that inhibited the biological function of the Bm86 on the surface of the gut epithelial cells.[2,3] Additionally, immunological elements such as complement proteins work together with anti-Bm86 antibodies to lyse the intestinal epithelial cells of the tick.[4,5] Due to these biological alterations, a reduction in tick number, weight, and reproductive capacity has been documented in *R. microplus*.[6–8] The Bm86 antigen is expressed in the methylotrophic yeast *Pichia pastoris*, and the recombinant protein is obtained as a highly glycosylated and particulate antigen.[3,9] These characteristics of

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recombinant Bm86 confer strong immunogenic and protective properties in many populations.[10,11] Experimental reports confirm the adjuvant and immune-stimulating properties of Bm86.[11,12] Bm95 is the homologous gene of Bm86, with only 2.1% of divergence between them, corresponding to approximately 3.4% of divergence between their respective amino acid sequences.[13,14] The coding sequence of Bm95 was cloned and expressed in *P. pastoris* yeast; the recombinant protein was glycosylated, and the particulate antigen was similar to Bm86 and had similar immunogenic properties.[14] In this report, the features of Bm95 were harnessed to explore and design conjugated vaccines.

Numerous bacteria responsible for invasive diseases have a polysaccharide capsule enabling these organisms to survive through the transmission and colonization processes. This polysaccharide capsule enables persistence of bacteria in the blood during the pathogenesis of an invasive infectious disease.[15,16] The principal defense mechanism of the body against entry of these microorganisms is generation of an immune response against capsular polysaccharides. These antigens are thymus-independent and require the late development of a subset of B-cells.[16] The immune response generated is characterized by a brief period of latency followed by a rapid rise of antibodies in serum, predominantly the immunoglobulin (Ig)M subclass. This immune response is associated with the immunological unresponsiveness observed in infants and some animals, and with failure of stimulation of the immunological response at any age.

Conjugation of a bacterial polysaccharide with an immunogenic carrier protein could promote a protective anti-polysaccharide IgG and induction of immunological memory.[17–19] The effect of the carrier protein during the primary immunization consists of producing a change in IgG class and a population of B-cells for memory that can be stimulated by T-cell-independent type 2 or TD5 antigens.[16,20–23] The polysaccharide component of a conjugated vaccine can be obtained from important veterinary and human pathogens, including *Pasteurella hemolytica*, *Pasteurella multocida*, *Neisseria meningitidis*, *Clostridium* spp., and *Haemophilus* spp., that usually are not strong immunogens. Additionally, the most used carrier proteins in conjugated vaccines are the tetanus toxoid and diphtheria antigens. Consequently, there are a limited number of carrier proteins for humans and animals, which constitutes a risk for immunogenicity of the individual conjugates administered in multivalent preparations.[24,25] Previous studies have shown a reduced anti-polysaccharide antibody response attributable to the excess of carrier protein or carrier-mediated epitope suppression.[26–31] Clinical studies of coadministration of conjugate vaccines using the same carrier protein have yielded inconsistent data related to immune interference and vaccine efficacy.[32] The present study aims to take advantage of the immunogenic and particulate properties of Bm95 to develop glycoconjugated vaccines. To achieve this, recombinant Bm95 expressed in the yeast *P. pastoris* was conjugated by two chemical methods with the *N. meningitidis* capsular C polysaccharide. This bacterial polysaccharide and the hepatitis B surface antigen (HBsAg) were used to evaluate the carrier and immunostimulant properties of recombinant Bm95 in mice.

Mucosal surfaces such as in the gastrointestinal and respiratory tracts are the principal sites of contact and entry of a vast number of human and animal pathogens, hence stimulation of protective immunity at the mucosal level is very important for developing new vaccines. Also, mucosal vaccines have the ability to stimulate humoral-mediated and cell-mediated immune responses at the mucosal and systemic levels.[33] These elements, along with advances in needleless, noninvasive immunization are important and attractive features of mucosal vaccination. In the present study, we also investigated the immunostimulatory properties of recombinant Bm95 administered by the nasal route.

Materials and methods

Native meningococcal C polysaccharide was obtained from the serogroup C meningococcal strain (C11 American Type Culture Collection, Manassas, VA, USA). Recombinant Bm95 and HBsAg were both expressed in the yeast *P. pastoris* and homogeneously purified as described elsewhere.[14,34] The VA-MENGOC-BC® vaccine against *N. meningitidis* was produced at the Finlay Institute in Havana, Cuba.[35] The outer membrane protein of *N. meningitidis* was obtained as described by Perez et al.[36]

Conjugation of polysaccharide
Carbodiimide method

The *N. meningitidis* C polysaccharide (Men CpS) was modified according to the method described by Beuvery et al in 1983.[37,38] The polysaccharide (5 mg/mL in 0.2 M NaCl, pH 5.2) was reacted with 0.5 M adipic acid dihydrazide in the presence of 10 mM 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluenesulfonate of 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide (EDC). The reaction was maintained with 0.1 M HCl in the pH range of 5.2–5.5 for 4 hours at room temperature. The mixture was dialyzed extensively against a solution of 0.2 M NaCl at pH 7.2 and concentrated using an Amicon-10 filter (Merck Millipore,

Billerica, MA, USA). The residual adipic acid was separated by Sepharose-4BCL (Sigma-Aldrich, St Louis, MO, USA) chromatography from the modified Men CpS, which was concentrated using an Amicon-10 filter.

The conjugation was performed by slow addition of 0.5 mL EDC (100 mg/mL) into 20 mL of 0.2 M NaCl solution containing 50 mg of modified Men CpS and 50 mg of Bm95 to a final concentration of 2.5 mg/mL. The pH was maintained at 5.5 by addition of 0.1 M HCl. The reaction was carried out for 1 hour at room temperature. The conjugate was centrifuged for 30 minutes at $50,000 \times g$, and the unreacted protein and dihydrazide polysaccharide were removed by Sepharose-4BCL chromatography previously equilibrated with 0.2 M phosphate buffer (pH 7.2). The chromatography flow speed was 0.5 mL per minute, and the conjugate was sterilized by filtration for its conservation after separation. The conjugate was designated as MenCpSBm-*c*.

Reductive amination method
The Men CpS was oxidized by a modified method of reductive amination reported by Jennings and Lugowski in 1981.[39] A total of 200 mg of oxidized Men CpS was fragmented in 5% sodium acetate (10 mL, pH 8.1), and 200 mg of sodium metaperiodate was added at room temperature in the dark for 10 minutes. Next, 2 mL of ethylene glycol was added to expend the excess sodium metaperiodate, and the reaction mixture was left at room temperature for a further 60 minutes. The oxidized Men CpS with an average molecular weight of 40,000 Da was purified using a 1.5×90 cm Bio-Gel A 0.5 column (Bio-Rad, Hercules, CA, USA) by direct application of the reaction mixture treated with ethylene glycol. The column was previously calibrated with dextran (40,000 Da) and equilibrated with 250 mM sodium chloride and 25 mM disodium phosphate (pH 7). The oxidized Men CpS was desalted using a Sephadex G-25 column (Amersham Biosciences, Buckinghamshire, UK) and lyophilized.

The conjugation was performed by dissolving 5 mg of oxidized Men CpS, 5 mg of Bm95, and 5 mg of sodium cyanoborohydride in 1 mL of 0.1 M sodium hydrogen carbonate and 0.1 M NaCl solution (pH 8.3). The reaction mixture was maintained for 24 hours at 37°C with slow agitation. This mixture was applied to a Bio-Gel A 0.5 column (1.6× 100 cm) previously equilibrated with 25 mM of sodium phosphate and 250 mM of sodium chloride (pH 7.0) at a flow speed of 0.5 mL per minute. The conjugate eluted in the void volume of the column. The final product was designated as MenCpSBm-*ra*.

Determination of amino groups
The amino content was determined using the 2,4,6-trinitrobenzene sulfonic acid method[40] (Thermo Fisher Scientific, Waltham, MA, USA). The concentration of amino groups is expressed in µmol/mg of Men CpS. Alcohol dehydrogenase was used as the standard for the standard curve.

Conjugate characterization
The N-acetylneuraminic acid content was determined by the resorcinol method.[41] The polysaccharide content was expressed in mg/mL. Neuraminic acid was used as the standard in the standard curves, and the bicinchoninic acid assay was used to determine the protein concentration (Thermo Scientific Pierce Inc., Rockford, IL, USA).

Animals and immunization
Experiment 1
Female Balb/c mice aged 6–8 weeks of similar weight were used in five experimental groups. The mice were selected at random to form five groups with a total of five animals per group, and were maintained under controlled lighting conditions at 22°C, with access to water and laboratory chow ad libitum, as previously described.[42] The Bm95 conjugates and polysaccharides were mixed with incomplete Freund's adjuvant (Sigma-Aldrich, St Louis, MO, USA). The mice in group A were immunized with conjugated MenCpSBm-*ra*. Group B mice were immunized with MenCpSBm-*c*, group C mice were immunized with 2 µg of Men CpS, group D mice were immunized with 2 µg of unconjugated Bm95, and group E mice were vaccinated with VA-MENGOC-BC® as a positive control. Each dose of glycoconjugate was normalized to contain only 2 µg of Bm95 in a total volume of 100 µL administered subcutaneously. The second dose was administered 21 days after the initial dose. Blood was taken from the mice via the retroorbital vein, and the serum was collected and stored at −20°C until testing for antibodies against Bm95 and Men CpS by enzyme-linked immunosorbent assay (ELISA). Blood was taken before the first vaccination (pre-immune serum) and two additional blood extractions were performed 15 days after the first and last doses.

Experiment 2
To study the immunostimulant properties of Bm95, five groups of 10-week-old female Balb/c mice were immunized intranasally with the following: 10 µg of Bm95 (group 1), 5 µg of proteoliposome (group 2) or 2 µg of HBsAg (group 3), while group 4 was inoculated with 10 µg of Bm95 combined with 5 µg of the outer membrane protein of *N. meningitidis*

and group 5 was inoculated with 2 µg of HBsAg combined with 10 µg of Bm95. All immunogens were dissolved in 50 µL of phosphate-buffered saline, and a final volume of 100 µL was administered to each animal. Mice immunized in groups 4 and 5 received their respective immunogens mixed into the same preparation. The mice were immunized on days 0, 14, and 28. Samples of blood were obtained 12 days after the final immunization on day 40. Blood was collected from the retroorbital vein, and the serum was isolated and stored at −20°C until testing for antibodies.

Immunoassays

The antibody response against Men CpS was measured by enzyme-linked immunosorbent assay using a procedure described elsewhere.[42] Microtiter plates (Nunc Immunoplate I, Gibco Ltd., Paisley, UK) were activated using poly-L-lysine at a concentration of 2 µg/mL in carbonate/bicarbonate (0–0.5 M) coating buffer (pH 9.5). Three washes were done with Milli Q water (18.2 mΩ·cm; EMD Millipore, Bedford, MA, USA). The plates were coated with 1 µg/mL of Men CpS in phosphate-buffered saline at 4°C overnight. The plates were washed three times with T-PBS (0.05% [v/v] Tween-20 in phosphate-buffered saline) and blocked with 1% skim milk in T-PBS for 1 hour at room temperature. The specific antibodies against Men CpS were developed with goat anti-mouse IgG-horseradish peroxidase conjugate for 1 hour at room temperature. The plates were washed, and the chromogenic reaction was developed in substrate buffer (3% of H_2O_2, o-phenylenediamine [0.5 mg/mL] in 0.1 M citrate buffer, pH 5). The reaction was stopped after 15 minutes at 25°C with 50 µL of 2 M orthophosphoric acid per well. The optical density was read in a plate reader at 492 nm (Sensidentscan; Merck, Darmstadt, Germany). End-point titers were determined as two-fold optical density values of preimmune sera. An enzyme-linked immunosorbent assay for detecting the antibody response against Bm95 and HBsAg was conducted as described elsewhere.[12,13,43]

Statistical analysis

Log-transformed titer data were used. The Student's t-test ($P<0.01$) was used to test for statistically significant differences in antibody levels between the experimental groups.

Results
## Effect of conjugation method on immunogenicity of MenCpSBm

Synthesis of the glycoconjugates was done using the carbodiimide and reductive amination methods. The composition of both conjugates was determined by chemical assay. The results of these assays indicate that the composition of the conjugates obtained by amination (MenCpSBm-*ra*) and oxidation (MenCpSBm-*c*) methods have a molar ratio of Men CpS to protein of 2.019±0.137 and 0.878±0.044, respectively. Gel filtration chromatography (Bio-Gel A 0.5 column) was used to separate the protein conjugates from the polysaccharide and chemical residual. The crude conjugate (Men CpS-Bm95-EDC) contained a 280 nm absorbing material in the void volume fraction, indicating that the protein had been linked to the Men CpS (data not shown). The antibody titers against meningococcal C polysaccharide induced after the first dose of MenCpSBm-*ra* (group A), MenCpSBm-*c* (group B), Men CpS (group C) and VA-MENGOC-BC® were very similar between the experimental groups (550±175, 550±225, 700±203, and 250±100, respectively). However, after the second dose of the immunization scheme (day 36), groups B and C showed a plateau in the immune response, with titers of 250±75 and 570±104, respectively (Figure 1). The response in group A showed significantly higher IgG titers ($P<0.01$) of 2,350±250 compared with the control group E (1,100±100). The placebo group (group D) did not have IgG titers against Men CpS detectable by enzyme-linked immunosorbent assay. Consequently, we can conclude that MenCpSBm-*ra* induced a potent antibody response against Men CpS under our experimental conditions.

An enzyme-linked immunosorbent assay was used to measure the anti-carrier (Bm95) immune response, and showed only IgG titers against Bm95 in sera obtained from group A (MenCpSBm-*ra*), group B (MenCpSBm-*c*), and group D (Bm95) mice (Figure 2). The data obtained did not show interference by the anti-carrier IgG with the immune response against the meningococcal C polysaccharide fraction of the glycoconjugate.

Immunostimulant analysis by intranasal immunization

The immunostimulant capability of Bm95 was studied further by intranasal immunization using recombinant HBsAg. We observed that all animals immunized with Bm95 and HBsAg (group 5) seroconverted and reached mean titers of 11,400±345 and 128,000±250 against Bm95 and HBsAg, respectively. These titers were significantly ($P<0.01$) higher than the titers obtained in mice immunized only with Bm95 (5,461±150, group 1) or HBsAg (400±40, group 3). However, the anti-Bm95 titers (11,400±345) in group 5 were similar to the mean titers of group 4 immunized with Bm95 and the outer membrane protein of *N. meningitidis* (12,800±180, Figure 3).
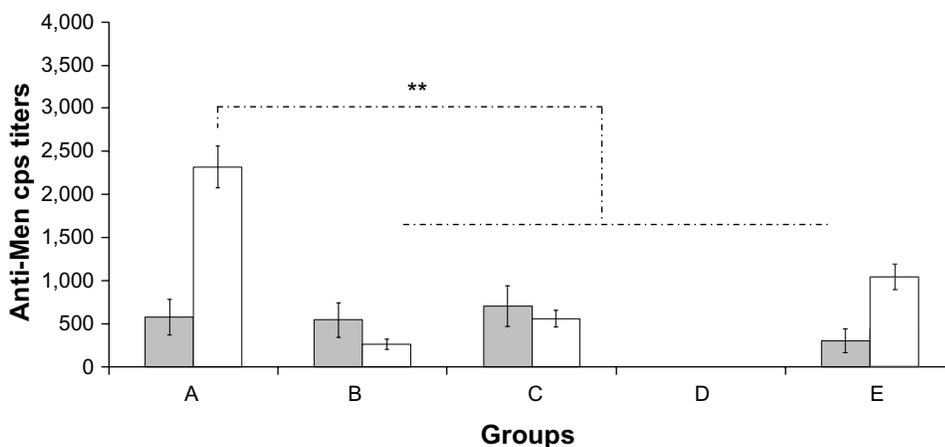
Figure 1 Immunogenicity of MenCpSBm95 conjugates obtained against Men CpS. The gray bars represent immunoglobulin G titers 15 days after the first dose. The white bars represent immunoglobulin G titers 15 days after the second dose. The experimental groups were immunized with the MenCpSBm-*ra* (**A**) and MenCpSBm-*c* (**B**) conjugates, *N. meningitidis* C polysaccharide (**C**), Bm95 (**D**), and VA-MENGOC-BC® (**E**). **P<0.01, statistically significant difference by Student's *t*-test.
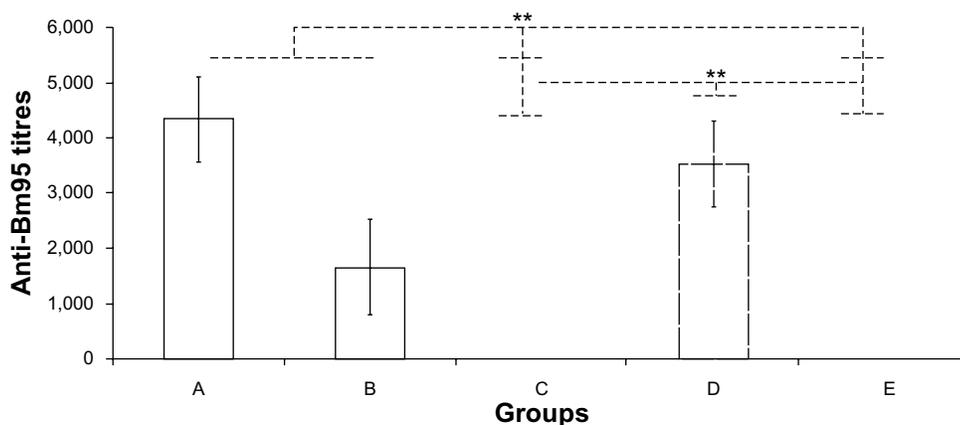**Notes:** VA-MENGOC-BC® is a commercial vaccine against *N. meningitidis* produced by Finlay Institute, Havana, Cuba. Anti-Men cps represents the level of immunoglobulin G against *N. meningitidis* capsular C polysaccharide.
Abbreviations: Men CpS, *Neisseria meningitidis* capsular C polysaccharide; MenCpSBm-*ra*, MenCpSBm95 conjugates produced by reductive amination; MenCpSBm-*c*, MenCpSBm95 conjugates produced by carbodiimide.

Discussion

The present study investigated the immunostimulatory properties of Bm95 used alone and as a carrier protein in a conjugated vaccinal preparation. Therefore, a rational approach for using Bm95 as a novel carrier molecule for preventing infections caused by polysaccharide encapsulated bacteria was conceived.[42] The results obtained in this study showed high immunogenicity of the Bm95-based conjugate (MenCpSBm-*ra*) compared with the meningococcal C polysaccharide. Additionally, an enhanced immune response typical of the T-cell-dependent immune response against Men CpS was observed. The Bm95 conjugates induced a more rapid and stronger immune response after the first dose against Men CpS, as high titers against Bm95 were maintained. However, MenCpSBm-*ra* induced a significantly higher antibody titer against Men CpS than MenCpSBm-*c* after the second dose, and similar results have been reported previously using the same conjugation reaction.[42] The effect of conjugation chemistry on the immunogenicity of polysaccharide-protein conjugates and the advantage of the reductive amination method to develop an effective immune response against polysaccharide antigens have already been reported.[42,44–48] The ratio of Bm95 to Men CpS had an important influence on the results. The conjugate

Figure 2 Immunogenicity of the MenCpSBm95 conjugates against Bm95. An enzyme-linked immunosorbent assay was used to determine immunoglobulin G titers against Bm95 as described in the methods. The experimental groups were immunized with MenCpSBm-*ra* (**A**), MenCpSBm-*c* (**B**) conjugates, Men CpS (**C**), Bm95 (**D**), and VA-MENGOC-BC (**E**). **P< 0.01, statistically significant difference by Student's *t*-test.
Notes: VA-MENGOC-BC® is a commercial vaccine against *N. meningitidis* produced by Finlay Institute, Havana, Cuba.
Abbreviations: Men CpS, *Neisseria meningitidis* capsular C polysaccharide; MenCpSBm-*ra*, MenCpSBm95 conjugates produced by reductive amination; MenCpSBm-*c*, MenCpSBm95 conjugates produced by carbodiimide
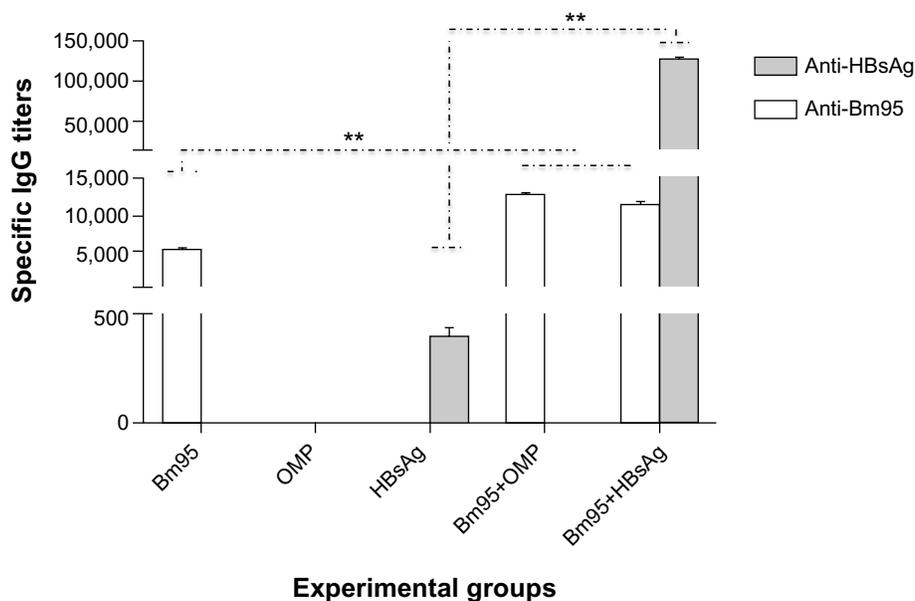
Figure 3 Immunostimulant properties of Bm95 in mice immunized by the intranasal route. The data are shown as the mean ± standard deviation. **$P<0.01$, statistically significant difference by Student's *t*-test.
Abbreviations: HBsAg, hepatitis B surface antigen; IgG, immunoglobulin G; OMP, outer membrane protein of *Neisseria meningitidis*.

obtained by the reductive amination method had a high ratio of Men CpS to Bm95 compared with the carbodiimide conjugate. Consequently, this ratio of carrier protein was observed to provide enough T-cell epitopes for the development of the most efficient T-cell-dependent response and improved the presentation of carbohydrate epitopes. This explains the robust immune response observed after administration of the second doses with MenCpSBm-*ra*.

Intranasal immunization is a distinct approach that offers advantages in terms of protection against infection at both local and distant sites.[49] The immune response after vaccination using the intranasal route is usually characterized by mucosal IgA, as well as systemic IgG. This response is mediated by type 1 and type 2 helper T cells lymphocytes and complements the protection against a number of pathogens affecting the upper and lower respiratory tracts of humans and animals.[50–54] Further, immunization via the mucosal surface is an effective approach for avoiding maternal antibody interference[53,55] and is a less stressful immunization procedure.

Consequently, the immunogenicity of Bm95 was further tested in this study by intranasal co-immunization with HBsAg. Enhancement of the humoral immune response against HBsAg following intranasal administration of both antigens was observed. However, the specific IgG titer obtained against Bm95 after coadministration of both antigens is similar to that of Bm95 mixed with outer membrane protein, which is an important immune potentiator.[56–60]

Previously, similar adjuvant properties were reported for Bm86 antigen obtained from *P. pastoris*, which is homologous with Bm95. A small number of proteins have adjuvant characteristics and act principally as B-cell mitogens capable of inducing high antibody levels. The particulate antigen obtained from *P. pastoris* could trigger important immunological signals to gather immunological factors that improve the immune response. Whereas the mechanism of interaction between Bm95 or Bm86 and the immune system is imprecise, there are important experimental data showing a better immune response to vaccinal proteins coadministered with these antigens.[12,56,61] However, this is the first study that similarly shows the adjuvant and immunostimulant properties of Bm95 administered intranasally in mice.

Conclusion
The present study demonstrates for the first time, to the authors' knowledge, that the conjugation procedure using recombinant Bm95 as a carrier protein improves antibody titers against bacterial polysaccharide antigens, which usually have poor immunogenicity. Hence, Bm95 seems to be an attractive candidate for use as a carrier protein or in combination with other vaccine antigens because of its availability in high purity, its yeast origin and its immunogenicity. This protein could be used with polysaccharide components obtained from different bacterial entities to develop multivalent vaccines conjugated against veterinary pathogens.

Acknowledgment

Disclosure

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