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The immune response to group B streptococcus type III capsular polysaccharide is directed to the -Glc-GlcNAc-Gal- backbone epitope

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Abstract The structures of the branched capsular polysaccharides of group B streptococcus type III (GBSIIIPS) and *Streptococcus pneumoniae* type 14 (Pn14PS) are identical apart from the (α2→3)-linked sialic acid in the side chains of GBSIIIPS. The present study tries to determine the minimal epitope in GBSIIIPS, using both a panel of anti-Pn14PS mouse sera and sera of humans vaccinated with either Pn14PS or GBSIIIPS. Type-specific Pn14PS antibodies that recognize the branched structure of Pn14PS have a low affinity for the native GBSIIIPS. Desialylation of GBSIIIPS results in dramatically higher affinity of anti-Pn14PS antibodies. Epitope specific anti-Pn14PS mouse antibodies and human sera of PCV7 vaccinees only recognized structures with the branching element -Glc-(Gal-)GlcNAc-, in particular -Gal-Glc-(Gal-)GlcNAc- in Pn14PS. On the other hand anti-GBSIIIPS human antibodies recognize predominantly the linear structure in the backbone of Pn14PS or GBSIIIPS, *i.e.*, -Glc-GlcNAc-Gal-. This difference in antigenicity of Pn14PS and GBSIIIPS is in agreement with the difference in flexibility of the two polysaccharides caused by the presence or absence of sialic acid.

Keywords Group B streptococcus type III · *Streptococcus pneumoniae* type 14 · Polysaccharide · Epitope location

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Introduction

The relation between group B streptococcus type III (GBSIII) and *Streptococcus pneumoniae* (pneumococcus) type 14 (Pn14) bacteria, in particular the similarity of their capsular polysaccharides, has long been investigated.[1–4]. The structures of the branched capsular polysaccharides of Pn14 (Pn14PS) and GBSIII (GBSIIIPS) differ only in the absence (in Pn14PS) or presence (in GBSIIIPS) of the (α2→3)-linked sialic acid in their side chains (Fig. 1) [4]. In general, sialic acid on bacteria mimics mammalian cell surface sialic acid residues and thus can subvert immune clearance mechanisms [5]. The majority of healthy adults responding to GBSIII vaccines with a fourfold or greater increase in GBSIII-specific IgG antibodies developed antibodies cross-reacting with Pn14PS (*i.e.* desialylated GBSIIIPS) [3].

In previous reports, we have shown in a mouse model that the branched tetrasaccharide structure Gal-Glc-(Gal-)GlcNAc, which represents one repeating unit of Pn14PS, is essential and sufficient for inducing Pn14PS-specific anti-

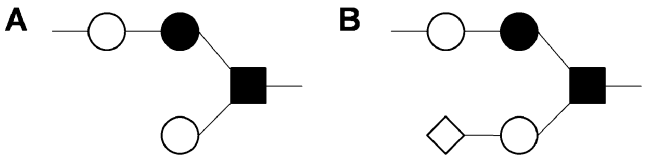


Fig. 1 Schematic polysaccharide structures of Pn14 and GBSIII. The primary structures of the branched capsular polysaccharides of Pneumococcus type 14, Pn14PS (**a**), and group B streptococcus type III, GBSIIIPS (**b**), differ only in that their side chains do not contain (Pn14PS) or do contain (GBSIIIPS) (α2→3)-linked sialic acid, *N*-acetylneuraminic acid (Neu5Ac): {→3)-β-D-Galp-(1→4)-β-D-Glcp-(1→6)-[±α-Neu5Ac-(2→3)-β-D-Galp-(1→4)-]β-D-GlcpNAc-(1→} n. *Open circle* = galactose (Gal); *filled circle* = glucose (Glc); *filled square* = *N*-acetylglucosamine (GlcNAc); and *open diamond* = sialic acid, *N*-acetylneuraminic acid (Neu5Ac)

bodies that promote opsonophagocytosis of pneumococcus [6, 7]. In fact, the branching element Glc-(Gal-)GlcNAc was fundamental for induction of an antibody response. The present study was aimed to elucidate the antigenic determinant in GBSIIIPS. Two approaches were followed. First, heat-inactivated GBSIII bacteria, which were treated or not treated with sialidase (desialylated GBSIII), were tested for their capacity to be recognized by specific anti-Pn14PS antibodies. The type-specific Pn14PS antibodies were prepared in mice using Pn14PS and a series of synthetic overlapping oligosaccharide fragments of Pn14PS, all conjugated to the cross-reactive material of diphtheria toxin, CRM_{197} (CRM-neoglycoconjugates) [7]. Second, sera of adult humans vaccinated with a pneumococcal conjugate vaccine containing a.o. the Pn14PS-CRM_{197} conjugate (PCV7, Prevnar, Wyeth) and with an experimental GBSIIIPS-tetanus toxoid conjugate vaccine were tested for their binding capacity to the immobilized synthetic Pn14PS oligosaccharide fragments, mentioned above.

Materials and methods

Group B streptococcus

Group B streptococcus (GBS) of different serotypes were obtained from the Reference Laboratory for Bacterial Meningitis, Department of Medical Microbiology, Academic Medical Center, Amsterdam, Netherlands. The GBS strains were plated on blood agar and incubated at 37°C overnight, after which they were inoculated into yeast extract broth containing 0.2% glucose and incubated at 37°C overnight. The culture was then centrifuged and washed with phosphate buffered saline (PBS) and the pellet was resuspended in PBS to an optical density (OD) value of 1.0 at 660 nm. The bacteria were inactivated by heating at 56°C for 1 h and stored at −20°C.

Mouse and human sera

A panel of sera was obtained from mice immunized with 15 different CRM-neoglycoconjugates, synthetic overlapping oligosaccharide fragments corresponding to *Streptococcus pneumoniae* type 14 polysaccharide (Pn14PS) (Code: JJ42; JJ141; JJ118; DM65; JJ153; JJ5; JJ1; JJ9; JJ6; DM35; DM66; JJ10; DM36; JJ4; ML2) which had a 6-aminohexyl/3-aminopropyl spacer and the natural polysaccharide (Code: Pn14PS) conjugated to the cross-reactive material of diptheria toxoid protein (CRM_{197}) (Table 1), as reported previously [7].

Seven human sera (Code: AH-1 to AH-7) were available from healthy individuals vaccinated with the 7-valent pneumococcal conjugate vaccine (PCV7; Prevnar, Wyeth, Madison, NJ, USA) containing Pn14PS-CRM_{197} as one of the constituents. The paired sera (pre- and post-vaccination) were kindly provided by the Department of Medical Microbiology and Immunology, St Antonius Hospital, Nieuwegein, Netherlands. Two batches Standard Human Reference Serum III (Code: SHRSIII-1 and SHRSIII-2), which each are a pool of five sera of healthy volunteers vaccinated with an experimental GBSIII-tetanus toxoid conjugate vaccine, were kindly provided by Dr. Dennis Kasper [8].

Direct-binding assay

Heat inactivated GBS bacteria (1×10^8 cells/ml) were treated without or with Arthrobacter ureafaciens sialidase (0.2 U/ml; Roche Diagnostic Corp., Mannheim, Germany) at 37°C for 1 h with shaking at 600 rpm. After washing with PBS, 50 μl of a series of diluted mouse sera (1:50; Table 1) were added, and the mixture was incubated at 4°C for 30 min with shaking at 600 rpm, then washed with PBS. Goat-anti-mouse IgG labelled with fluorescein isothiocyanate (FITC) (Dako, Glostrup, Denmark; diluted 1:100) was added and the mixture incubated again under the same conditions. After washing, the bacterial pellets were resuspended in PBS and analyzed by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA).

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was performed as previously described [7]. Briefly, a series of bovine serum albumin (BSA)-neoglycoconjugates of Pn14PS (1 μg/ml) was coated on flat-bottom 96-well plates (Corning, Inc., Corning, NY, USA) and incubated at 37°C overnight. The carbohydrate parts of the BSA-neoglycoconjugates comprise the synthetic oligosaccharide fragments JJ118, JJ141, JJ42, JJ9, JJ153, JJ5, DM65, DM35, JJ6, DM66, and JJ10, related to Pn14PS (Table 1),

Table 1 Summary of CRM-neoglycoconjugates and anti-Pn14PS antibody levels in mice, immunized with these conjugates

Code	Oligosaccharide fragment	Log_{10} IgG titer[1]
JJ42	Gal-Glc-GlcNAc-spacer	ND[2]
JJ141	Glc-GlcNAc-Gal-spacer	ND
JJ118	GlcNAc-Gal-Glc-spacer	ND
DM65	Gal-Glc-GlcNAc-Gal-spacer	ND
JJ153	Glc-GlcNAc-Gal-Glc-spacer	ND
JJ5	Glc-(Gal-)GlcNAc-Gal-spacer	1.1±0.2
JJ1	Gal-Glc-(Gal-)GlcNAc-spacer	3.0±0.3
JJ9	Gal-GlcNAc-Gal-Glc-spacer	ND
JJ6	Glc-(Gal-)GlcNAc-Gal-Glc-spacer	ND
DM35	Gal-Glc-GlcNAc-Gal-Glc-spacer	ND
DM66	Gal-Glc-(Gal-)GlcNAc-Gal-spacer	2.8±0.5
JJ10	Gal-GlcNAc-Gal-Glc-(Gal-)GlcNAc-spacer	0.5±0.4
DM36	Gal-Glc-(Gal-)GlcNAc-Gal-Glc-spacer	2.4±0.7
JJ4	Gal-Glc-(Gal-)GlcNAc-Gal-Glc-(Gal-)GlcNAc-spacer	2.3±0.4
ML2	Gal-Glc-(Gal-)GlcNAc-Gal-Glc-(Gal-)GlcNAc-Gal-Glc-(Gal-)GlcNAc-spacer	2.6±0.4
Pn14PS	{-(Gal-)GlcNAc-Gal-Glc-}$_n$	2.6±0.5

[1] Levels of serum antibodies against native *Streptococcus pneumoniae* type 14 polysaccharide (Pn14PS) obtained from mice immunized with 15 different CRM-neoglycoconjugates, synthetic overlapping oligosaccharide fragments corresponding to Pn14PS, which had a 6-aminohexyl/3-aminopropyl spacer and the natural polysaccharide type 14 (Code: Pn14PS) conjugated to the cross-reactive material of diptheria toxoid protein (CRM_{197}) by ELISA assay (Data from Safari *et al.* [7])

[2] *ND* not detectable (The limits of detection level is <0.1 (log10 IgG titer))

and mannose (BSA-mannose). After washing, the coated plates were treated with 3% (w/v) gelatin to block non-specific binding, then diluted human sera (1:100) were added and the plates incubated at 37°C for 1 h. This step was followed by incubation with horseradish-peroxidase-conjugated goat-anti-human IgG (Dako, Glostrup, Denmark) for 1 h at 37°C. Color development was initiated by incubation with a mixture of 3,3',5,5'-tetramethylbenzidine (Sigma Chemical Co, St. Louis, MO, USA) and H_2O_2 (Sigma Chemical Co), and the reaction stopped by addition of 0.5 M of H_2SO_4. Absorbance was measured at 450 nm.

Results

Recognition of native and desialylated GBSIII bacteria by mouse type-specific Pn14PS sera

Flow cytometry was used to detect interaction between antibodies induced in mice with a series of CRM-neoglycoconjugates of Pn14PS (Table 1) and heat-inactivated GBSIII in native or desialylated form. The sera, which were positive in a Pn14PS ELISA, obtained from mice immunized with the branched tetrasaccharide fragment of Pn14PS: JJ1 (one repeating unit), DM36 (one repeating unit with two extra monosaccharides), DM66 (one repeating unit with one extra monosaccharide), JJ4 (two repeating), and ML2 (three repeating), as well as polysaccharide conjugate (Pn14PS) itself displayed significant binding (mean fluorescence intensity) to the desialylated GBSIII (Fig. 2). Sera with low titers of anti-Pn14PS antibodies (Table 1; JJ5 and JJ10) showed also low binding to desialylated GBSIII. Sera in which anti-Pn14PS IgG antibodies were not detectable (Table 1) did not show any binding to desialylated GBSIII (Fig. 2). Native GBSIII bacteria were only bound by antibodies from mice immunized with neoglycoconjugates of JJ1, JJ4, and DM36, and even then to a much lower degree than desialylated GBSIII bacteria. Sera from mice immunized with the other CRM-neoglycoconjugates, showed virtually no binding to native GBSIII. In a subsequent series of experiments, the reactivity of type-specific Pn14PS mouse sera with the six GBS serotypes Ib, II, IV, V, VI, and VII were investigated. None of these sera showed binding to the different native GBS serotypes nor to their desialylated forms (Data not shown).

Recognition of oligosaccharide fragments related to Pn14PS by human sera

In subsequent studies the epitope specificity of human antibodies induced by vaccination with Pn14PS or GBSIII conjugate vaccines was investigated. To that end, BSA-neoglycoconjugates of linear JJ42, JJ141, JJ118, DM65,

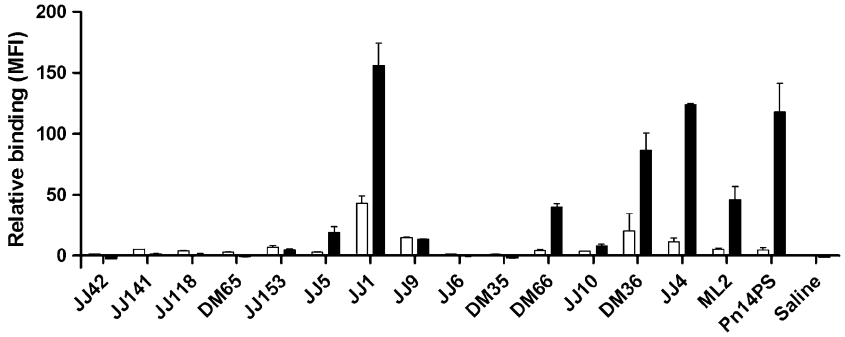


Fig. 2 Interaction between mouse antibodies against Pn14PS and GBSIII bacteria. Heat inactivated GBSIII bacteria were treated without (native form; *open bars*) or with (desialylated form; *filled bars*) sialidase at 37°C for 1 h with shaking. After washing, series of diluted mouse sera (Table 1) were added, and the mixture was incubated at 4°C for 30 min with shaking. Goat-anti-mouse IgG labelled with FITC was added and the mixture incubated again under the same conditions. Sera from mice injected with saline buffer were used as a negative control. The relative binding between mice sera and GBS III bacteria was measured by flow cytometry and was expressed as mean fluorescence intensity (MFI)

JJ153, JJ9, and DM35 (which do not induce detectable anti-Pn14PS titers when used as CRM-neoglycoconjugates), BSA-neoglyconjugates of branched JJ5, JJ10, and JJ6 (which induce low anti-Pn14PS IgG titers) and a BSA-neoglycoconjugate of branched DM66 (inducing a high anti-Pn14PS IgG titer) (Table 1) were used on coated plates. Vaccination with PCV7 resulted in the induction of antibodies, which bind to the branched tetrasaccharide DM66 (Gal-Glc-(Gal-)GlcNAc-Gal) by sera of the individuals AH-1, AH-2 and AH-3 (Fig. 3). Individuals AH-4, AH-5, and AH-6, while showing a comparable anti-Pn14PS antibody response after conjugate vaccination, did not recognize any of the linear or branched oligosaccharides tested. The human serum anti-GBSIIIPS interacts strongly with the BSA-neoglycoconjugates JJ141 (Glc-GlcNAc-Gal), JJ42 (Gal-Glc-GlcNAc), JJ153 (Glc-GlcNAc-Gal-Glc) but displayed lower binding to the other linear or the branched oligosaccharides, including DM66 (Fig. 3). It was noted that the pre- and post-PCV7 human sera of individual AH-3 bound equally well to JJ141, JJ42, and JJ153 in a pattern similar to that observed for SHRSIII (the human anti-GBSIII serum). Human sera AH-1 and AH-3 also interacted with BSA-JJ5 (Glc-(Gal-)GlcNAc-Gal) and BSA-JJ6 (Glc-(Gal-)GlcNAc-Gal-Glc); in fact JJ5 and JJ6 miss the galactosylated Glc residue at the non-reducing site, present in DM-66 (Gal-Glc-(Gal-)GlcNAc-Gal) (Fig. 3; Table 1).

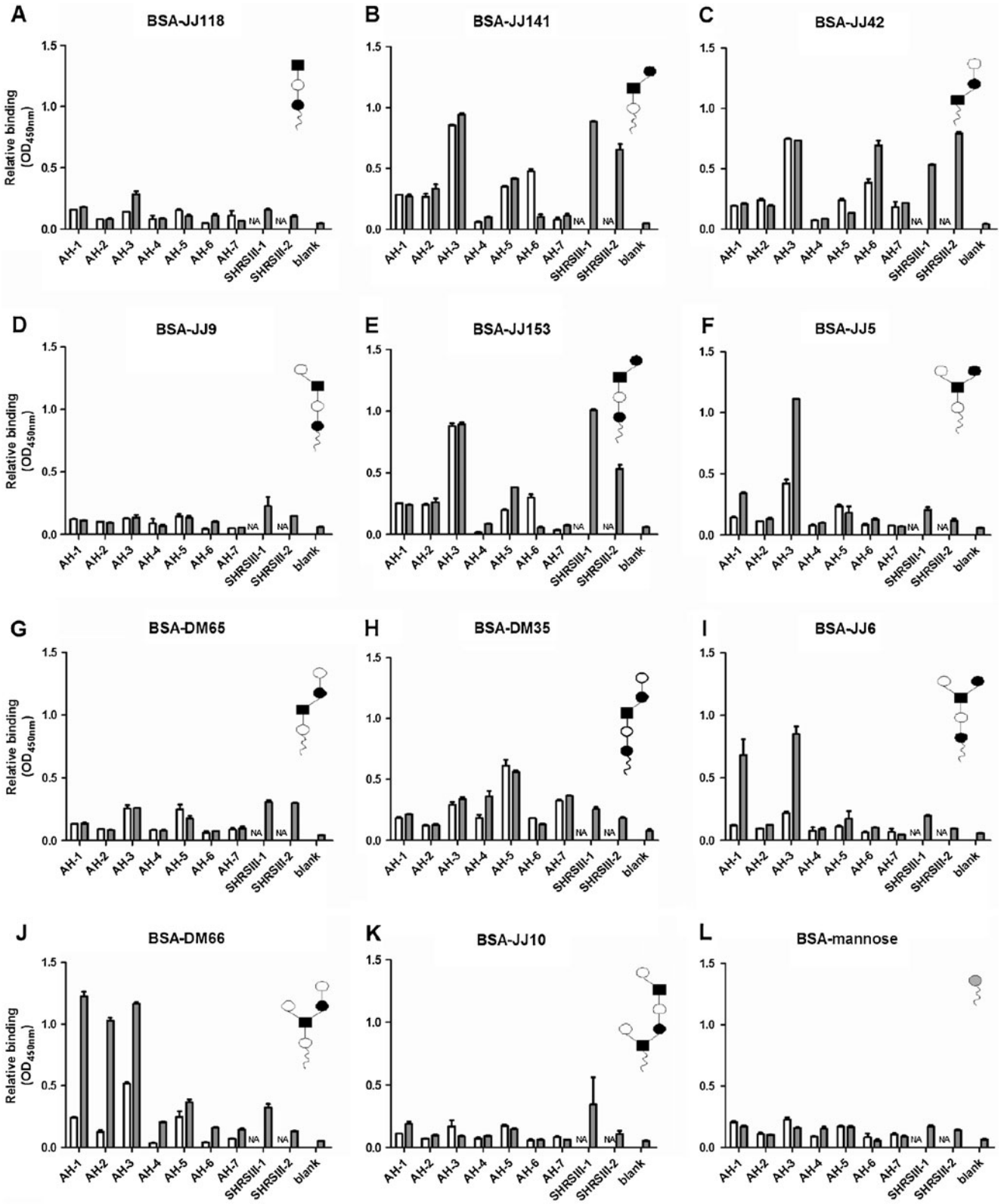
Discussion

The present study investigated the presence of common and unique epitopes in GBSIIIPS and Pn14PS by using type-specific mouse and human sera. The capsular polysaccharide in both GBSIII and Pn14 share a common polysaccharide backbone and differ only in their side chains: Pn14PS has β-D-Gal*p*-(1→4)- attached to the GlcNAc residue and GBSIIIPS α-Neu5Ac-(2→3)-β-D-Gal*p*-(1→4)-. The structure of Pn14PS (which is thus equivalent to desialylated GBSIIIPS) has been reported to be more flexible and disordered than the structure of GBSIIIPS. The rigidity of GBSIIIPS could have an impact on its immunogenicity [2, 4], and based on the GBSIIIPS model it has been suggested that anti-GBSIIII antibodies recognize the core and not the surface of the helix [2]. The conformation of the backbone is stabilized by interactions (hydrogen bonding) with the side chains, in particular with sialic acid.

Earlier, one structural repeating unit of Pn14PS, *i.e.* Gal-Glc-(Gal-)GlcNAc, has been reported to induce a specific antibody response to Pn14PS [6]. Recently, we confirmed that the branched trisaccharide element Glc-(Gal-)GlcNAc is essential in inducing Pn14PS-specific antibodies and that the extra galactose unit at the glucose residue contributes

Fig. 3 Direct binding between human serum and BSA-neoglycoconjugates. The paired sera (Code: AH-1 to AH-7) obtained from healthy individuals before (*open bar*) and after (*filled bar*) immunization with the 7-valent pneumococcal conjugate vaccine (PCV7; Prevnar, Wyeth, Madison, NJ, USA) and two human anti-GBSIII sera (Code: SHRSIII-1 and SHRSIII-2) obtained from healthy volunteers vaccinated with an experimental GBSIII-tetanus toxoid conjugate vaccine were tested in ELISA for their capacity to bind to a series of synthetic oligosaccharide fragments. The following BSA-neoglycoconjugates were used as coating material, *i.e.* JJ118 (A; GlcNAc-Gal-Glc), JJ141 (B; Glc-GlcNAc-Gal), JJ42 (C; Gal-Glc-GlcNAc), JJ9 (D; Gal-GlcNAc-Gal-Glc), JJ153 (E; Glc-GlcNAc-Gal-Glc), JJ5 (F; Glc-(Gal-)GlcNAc-Gal), DM65 (G; Gal-Glc-GlcNAc-Gal), DM35 (H; Gal-Glc-GlcNAc-Gal-Glc), JJ6 (I; Glc-(Gal-)GlcNAc-Gal-Glc), DM66 (J; Gal-Glc-(Gal-)GlcNAc-Gal), JJ10 (K; Gal-GlcNAc-Gal-Glc-(Gal-)GlcNAc), and mannose (L; man). Schematic structure of oligosaccharide fragments: *filled circle* = glucose; *open circle* = galactose, and *filled square* = *N*-acetylglucosamine. The data are expressed as the optical density (OD) value at 450 nm. NA = Not available

clearly to the immunogenicity of the epitope [7]. It has been shown in direct binding studies of GBSIIIPS with a panel of GBSIII specific monoclonal antibodies that 2 repeating backbone units form the minimum binding epitope [9]. The present study has shown that type-specific Pn14PS antibodies that recognize the branched structure of Pn14PS



have a low affinity for the native GBSIIIPS. Desialylation of GBSIIIPS, however, resulted in dramatically higher affinity of anti-Pn14PS antibodies in mice. These results demonstrate that GBSIII bacteria are protected from binding of antibodies against Pn14PS by a residue of (α2→3)-linked sialic acid, as described previously [10, 11].

The linear Gal-Glc fragment in the backbone of the rigid GBSIIIPS has been reported to be part of the immunodominant epitope of GBSIII [12], and cross-reactions between some anti-GBSIII antibodies and Pn14PS [3] likely arise from recognition of a small linear epitope that is associated with the shared immunodominant region [4]. In rabbits, immunized with GBSIII it was reported that two distinct populations of antibodies were induced: the major population of antibodies is dependent on the presence of sialic acid residues (the branched structure) and the other population of antibodies is not sialic acid dependent (located in the backbone of GBSIIIPS) [10]. In the present study, we showed that human anti-Pn14PS antibodies only recognized the branched structure of Pn14PS with -Glc-(Gal-) GlcNAc-Gal- as the minimum epitope while human anti-GBSIIIPS antibodies recognized the linear structure of the backbone of Pn14PS and GBSIIIPS: -Glc-GlcNAc-Gal-.

Mice immunized with CRM-neoglycoconjugates of linear oligosaccharide fragments of Pn14PS and GBSIIIPS, *e.g.* Gal-Glc-GlcNAc, Glc-GlcNAc-Gal, and GlcNAc-Gal-Glc, do evoke specific oligosaccharide antibodies [7] but these antibodies neither bind native nor desialylated GBSIII. These linear structures are either too small or too flexible to evoke antibodies to the linear structure of the backbone of Pn14PS and GBSIIIPS. The difference in antigenicity of Pn14PS and GBSIIIPS might be due to a difference in flexibility of the two polysaccharides caused to the presence or absence of sialic acid [4]. The structure of Pn14PS (which is thus equivalent to desialylated GBSIIIPS) has been reported to be more flexible and disordered than the structure of GBSIIIPS. The rigidity of GBSIIIPS could have an impact on its immunogenicity[2, 4] and based on the GBSIIIPS model it has been suggested that anti-GBSIIII antibodies recognize the core and not the surface of the helix.[2] The conformation of the backbone is stabilized by interactions (hydrogen bonding) with the side chains, in particular with sialic acid.

In conclusion, the sialic acid of GBSIIIPS effectively shields the inner core of the polysaccharide and prevents recognition by type-specific Pn14PS antibodies. Pn14PS immunization elicits antibodies that only recognize the branched structure of Pn14PS while GBSIIIPS immunization elicits antibodies to the backbone structure of GBSIIIPS and Pn14PS. The antigenic determinant for GBSIII is the -Glc-GlcNAc-Gal- epitope located at the linear structure of the backbone.

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