INFECTION AND IMMUNITY, Oct. 2008, p. 4615–4623 Vol. 76, No. 10
0019-9567/08/$08.00+0 doi:10.1128/IAI.00472-08

Identification of the Smallest Structure Capable of Evoking Opsonophagocytic Antibodies against *Streptococcus pneumoniae* Type 14[▽]

Dodi Safari,[1] Huberta A. T. Dekker,[1] John A. F. Joosten,[2] Dirk Michalik,[2] Adriana Carvalho de Souza,[2] Roberto Adamo,[2] Martina Lahmann,[3] Andreas Sundgren,[3] Stefan Oscarson,[3,4] Johannis P. Kamerling,[2] and Harm Snippe[1]*

Department of Medical Microbiology, University Medical Center Utrecht, Utrecht, The Netherlands[1]; Bijvoet Center, Department of Bio-Organic Chemistry, Utrecht University, Utrecht, The Netherlands[2]; Department of Organic Chemistry, Stockholm University, Stockholm, Sweden[3]; and Centre for Synthesis and Chemical Biology, University College Dublin, Dublin, Ireland[4]

Received 16 April 2008/Returned for modification 17 May 2008/Accepted 24 July 2008

Synthetic overlapping oligosaccharide fragments of *Streptococcus pneumoniae* serotype 14 capsular polysaccharide (Pn14PS), {6)-[β-D-Gal*p*-(1→4)-]β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→}$_n$, were conjugated to CRM_{197} protein and injected into mice to determine the smallest immunogenic structure. The resulting antibodies were then tested for Pn14PS specificity and for their capacity to promote the phagocytosis of *S. pneumoniae* type 14 bacteria. Earlier studies have reported that the oligosaccharide corresponding to one structural repeating unit of Pn14PS, i.e., Gal-Glc-(Gal-)GlcNAc, induces a specific antibody response to Pn14PS. The broader study described here, which evaluated 16 oligosaccharides, showed that the branched trisaccharide element Glc-(Gal-)GlcNAc is essential in inducing Pn14PS-specific antibodies and that the neighboring galactose unit at the nonreducing end contributes clearly to the immunogenicity of the epitope. Only the oligosaccharide conjugates that produce antibodies recognizing Pn14PS were capable of promoting the phagocytosis of *S. pneumoniae* type 14. In conclusion, the branched tetrasaccharide Gal-Glc-(Gal-)GlcNAc may be a serious candidate for a synthetic oligosaccharide conjugate vaccine against infections caused by *S. pneumoniae* type 14.

Synthetic carbohydrate-based vaccines are being investigated by many researchers for the prevention of diseases caused by *Streptococcus pneumoniae* (12), *Haemophilus influenzae* type b (7, 33), meningococcus group C (8), *Vibrio cholerae* (27), etc. Advantages of synthetic carbohydrate-based vaccines include their well-defined chemical structures (chain length, epitope conformation, and carbohydrate/protein ratio) and a lack of the impurities present in polysaccharides obtained from bacterial isolation (4). A breakthrough for this type of vaccines was made in 2004 by Verez-Bencomo et al. (33) when they reported the large-scale synthesis and the introduction of a synthetic oligosaccharide vaccine for *H. influenzae* type b for human in Cuba.

Pneumococcal disease is a major public health problem worldwide, and it is estimated that 1.6 million people die from this infection each year, 1 million of whom are children (36). Capsular polysaccharides (PS) are well known as the major virulence factors of *S. pneumoniae*. Numerous studies have demonstrated that antibodies to PS are essential for protection against pneumococcal disease (5, 24, 26). Based on the diversity of the chemical structures of PS (16, 23), more than 90 serotypes have been identified to date. This diversity determines the ability of the serotypes to survive in the bloodstream and possibly also the ability to cause invasive disease, especially in the respiratory tract (3, 16). In general, PS are poorly immunogenic: they mainly induce immunoglobulin M (IgM) antibodies and, as a vaccine, they are only moderately protective in adults and ineffective in young children (1).

S. pneumoniae type 14 PS (Pn14PS) consists of biosynthetic repeating units of the tetrasaccharide (19) {6)-[β-D-Gal*p*-(1→4)-]β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→}$_n$ (Fig. 1A). Pn14PS is structurally related to and has cross-reactivity with group B streptococcus type III (9). The immunogenicity of this polysaccharide and its depolymerized oligosaccharide fragments conjugated to a protein has been reported to produce specific anti-Pn14PS antibodies in mice (17, 34, 35). In 2002, a synthetic branched tetrasaccharide, corresponding to a single structural repeating unit of Pn14PS conjugated to the cross-reactive material of diphtheria toxoid (CRM_{197}), was found to induce anti-polysaccharide type 14 antibodies (20) (Fig. 1B). Based on this result, the present study was set up to investigate how small the minimal structure in Pn14PS can be and still produce specific antibodies to polysaccharide type 14. To do this, a series of oligosaccharide fragments of Pn14PS, varying from tri- to dodecasaccharides, were synthesized (14, 15, 21, 30). These oligosaccharide fragments were then conjugated to a protein carrier, i.e., either CRM_{197} or bovine serum albumin (BSA). The immunogenicity of the conjugates was subsequently studied in BALB/c mice.

MATERIALS AND METHODS

Synthetic oligosaccharide fragments. Sixteen different oligosaccharide fragments, varying from tri- to dodecasaccharide and representing fragments of the

* Corresponding author. Mailing address: Department of Medical Microbiology, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands. Phone: 31 (0) 887557628. Fax: 31 (0) 302541770. E-mail: h.snippe@umcutrecht.nl.

▽ Published ahead of print on 4 August 2008.

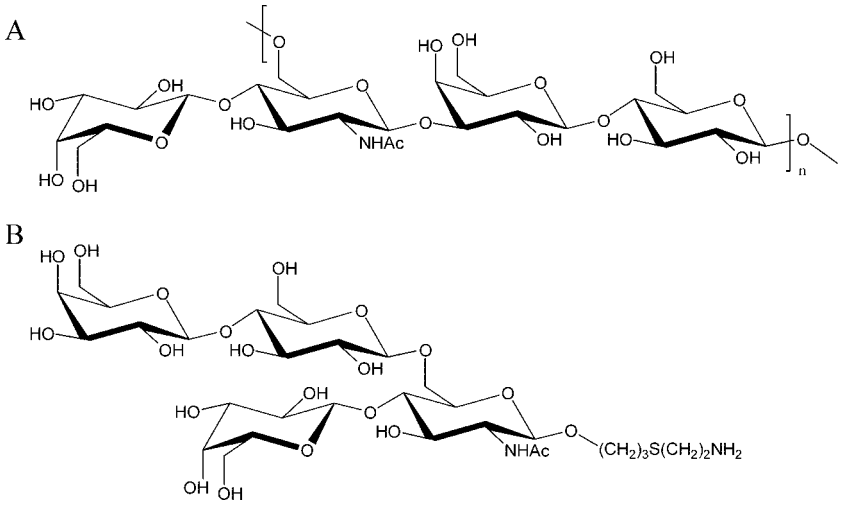


FIG. 1. Structures of the biosynthetic tetrasaccharide repeating unit of Pn14PS (19) (A) and the branched tetrasaccharide unit Gal-Glc-(Gal-)GlcNAc (B), synthesized and studied by Mawas et al. (20).

Pn14PS, were synthesized. Fourteen had a 6-aminohexyl spacer (14, 15, 21), and two had a 3-aminopropyl spacer (30) (Table 1).

Preparation of neoglycoconjugates. Diethyl squarate was used to link the oligosaccharide fragments to a protein carrier (either CRM_{197} or BSA) as described previously (4, 31). A total of 100 μl (4 μmol) of stock solution containing 74.7 μl of diethyl squarate in 12.8 ml of ethanol was added to a solution of spacered oligosaccharide fragments (1 μmol) in 0.1 M sodium phosphate buffer (75 μl, pH 7.0). After stirring for 16 h, the ethanol was evaporated, and the water layer was applied to a C18 cartridge (500 mg; Alltech, Deerfield, IL) that was preconditioned with methanol (5 ml), followed by the addition of H_2O (5 ml). The elution was effected with five 1-ml aliquots each of H_2O, ethyl acetate, and methanol. Fractions containing the pure desired compound were then concentrated to acquire the elongated oligosaccharide (80 to 99%), which was used for the conjugation reaction.

CRM_{197} conjugates. The elongated oligosaccharide fragments (1 μmol) were dissolved in 0.1 M sodium borate buffer (400 μl, pH 9.5), and 56 μl (0.05 μmol) stock solution containing 52.25 mg of CRM_{197}/ml (Novartis Vaccine & Diagnostics, Siena, Italy) was added. Incubations were carried out for 3 to 4 days, until the desired loading was reached as determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS; Voyager-DE PRO mass spectrometer; Applied Biosystems, Foster City, CA). The mixtures were then desalted in a 30-kDa Microsep microconcentrator (Filtron Technology Corp., Northborough, United Kingdom) and subsequently reconstituted with five 0.5-ml aliquots of sodium phosphate buffer (0.05 M; pH 7.2).

BSA conjugates. After the elongated oligosaccharide fragments (1 μmol) were dissolved in 0.1 M sodium borate buffer (1.5 ml), a solution containing BSA (0.05 μmol) in 0.1 M sodium borate buffer (pH 9.0; 0.6 ml) was added. The mixture was stirred for 2 to 3 days and then loaded into a 30-kDa Microsep microconcentrator and washed with five 2-ml aliquots of H_2O. The retained material was then freeze-dried.

Determination of carbohydrate/protein molar ratios. The average molar ratio of carbohydrate and protein was determined by MALDI-TOF MS analysis. Samples were prepared by mixing 1 μl of conjugate with 1 μl of sinapic acid (20 mg) in 70% acetonitrile containing 0.1% trifluoroacetic acid as a matrix solution. The carbohydrate/protein molar ratio of each of the conjugates is presented in Table 2.

Mouse immunization studies. The mouse vaccination study was approved by the Ethics Committee on Animal Experiments of University Medical Center Utrecht, Utrecht, The Netherlands. Inbred 6-week-old female BALB/c mice were maintained at the animal laboratory of Utrecht University. Five mice per group were immunized intracutaneously with 2.5 μg of carbohydrate at four different sites in the neighborhood of the lymph nodes of the axillae and the groins. Pn14PS conjugated to CRM_{197} (CRM_{197}-Pn14PS; Wyeth Research, Pearl River, NY) was injected into one group of mice, which then served as the antibody-positive control mice. The other control mice were injected, respectively, with either saline (0.9% [wt/vol] NaCl in water), unconjugated PnPS14, or CRM_{197} protein. When adjuvants were applied on the priming day, a fivefold-lower dose of carbohydrate (0.5 μg) was injected. In these cases, the conjugates were mixed with adjuvants (10 μg of monophosphoryl lipid-A [MPL derived from *S. minnesota* R595 lipopolysaccharide; Ribi ImmunoChem Research, Inc., Hamilton, MT] and 20 μg of Quil-A [Superfos Biosector, Vedbaek, Denmark] per animal) and injected intracutaneously at the four sites, as mentioned above. A booster of 2.5 μg of carbohydrate was given on day 35 without adjuvant. Using a retro-orbital puncture, blood samples were taken by from isofluran-anesthetized mice 1 week before the booster and 2 and 3 weeks after the booster.

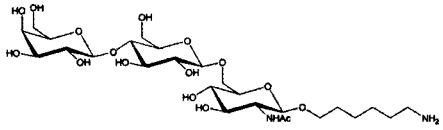
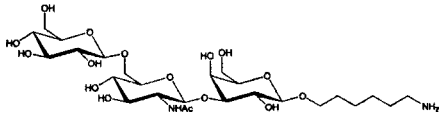
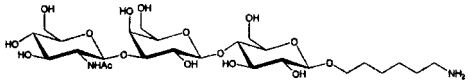
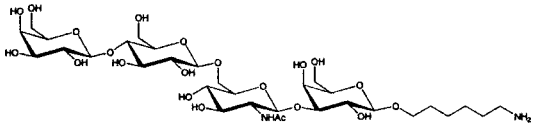
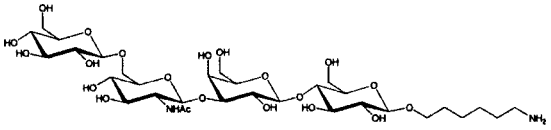
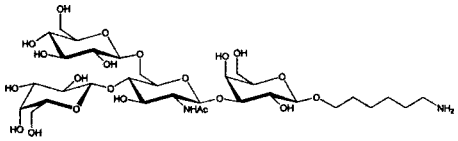
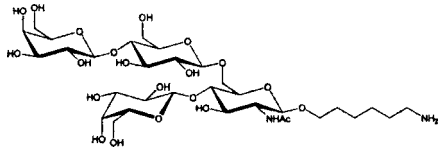
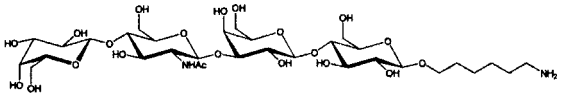
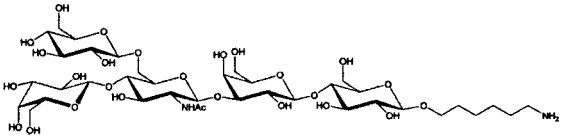
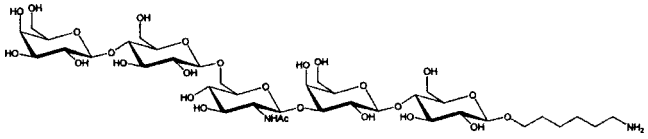
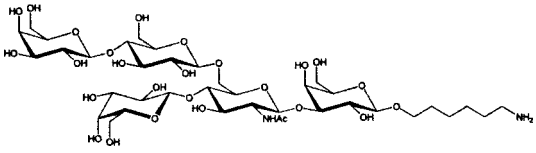
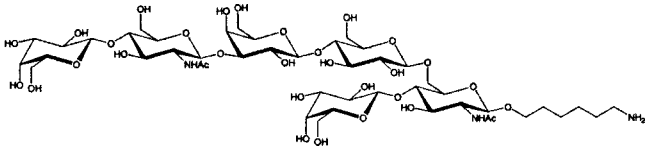
Measurement of Pn14PS-, protein carrier-, and spacer-specific antibodies by ELISA. Enzyme-linked immunosorbent assay (ELISA) was performed to measure the anti-Pn14PS antibodies, as described previously (4, 18). Briefly, diluted sera were incubated for 1 h at 37°C in flat-bottom plates (Corning, Inc., Corning, NY) which were coated with Pn14PS (0.3 μg/well) and blocked with 3% gelatin. After a washing step, horseradish peroxidase-conjugated goat anti-mouse IgM or IgG (Nordic Immunology Laboratories, Tilburg, The Netherlands) was incubated for 1 h at 37°C. A mixture of 3,3′,5,5′-tetramethylbenzidine (Sigma Chemical Co., St. Louis, MO), and H_2O_2 (Sigma Chemical Co.) was then added to visualize the amount of bound peroxidase. The reaction was stopped with the addition of 0.5 M H_2SO_4. Optical density (OD) values were obtained with a microtiter plate spectrophotometer at 450 nm (Bio-Rad, model 3550 UV; Bio-Rad Laboratories, Hercules, CA). Antibody titers were expressed as the $\log_{10}$ of the dilution giving twice the OD obtained for control mice (immunized with saline) with a cutoff value of 0.2.

CRM_{197}- and BSA-mannose-coated plates (0.1 μg/well) were also used to measure anti-protein carrier and anti-spacer titers, respectively. BSA-mannose was constructed by coupling 6-aminohexyl α-D-mannopyranoside, via diethyl squarate, to BSA.

Detection of oligosaccharide-specific antibodies. In order to investigate the immune response to the oligosaccharide fragments that did not induce anti-Pn14PS antibodies, ELISAs were performed after preincubating the sera with BSA-mannose to block the antibodies recognizing the spacer molecule (-C_6H_{12}-NH-C_4O_2-NH-). After the uncoated plates were blocked with 2% gelatin in phosphate-buffered saline (PBS) and washed with PBS–0.05% Tween 20 several times, diluted sera (1:100 in PBS supplemented with 0.05% Tween 20 and 3% Protifar) were incubated with BSA-mannose in a concentration ranging from 0 to 100 μg/ml for 1 h at 37°C and left overnight at 4°C. The mixtures were then transferred to three differently coated plates (0.1 μg/well): BSA-mannose, BSA-conjugates corresponding with CRM_{197} conjugates, and BSA-DM66 (for the structures, see Table 1). The amount of specific antibodies in these absorbed sera was detected by ELISA method as described above. The results were expressed as the OD changes of the sera incubated with BSA-mannose compared to sera alone.

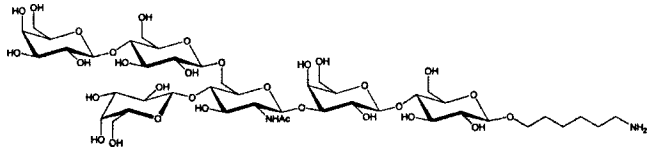
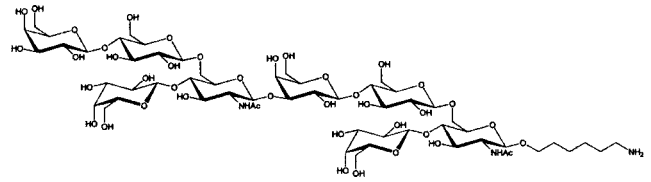
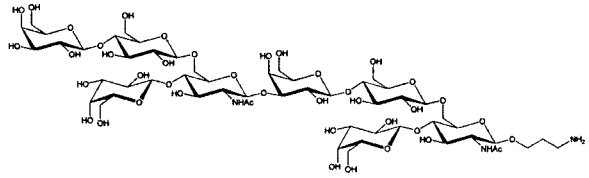
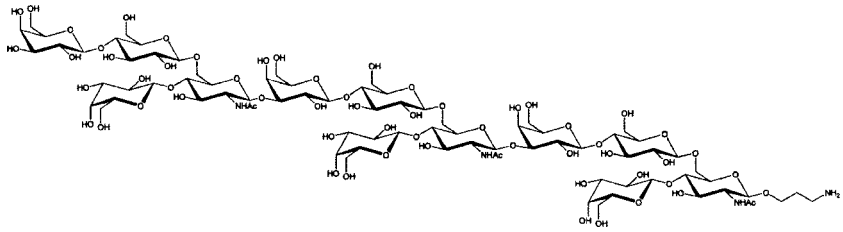
Measurement of avidity. The antibody avidity of mouse sera that recognized Pn14PS as the coating material was measured by ELISA using chaotropic sodium thiocyanate (NaSCN; Sigma Chemical Co.), as previously described (18, 25). Briefly, Pn14PS-coated plates were incubated with diluted sera (1:25 in PBS, 0.05% Tween 20 and 3% Protifar) and washed. Series of NaSCN concentrations

TABLE 1. Series of spacered oligosaccharide fragments of Pn14PS

Oligosaccharide fragment	Spacered structure	Code
Gal-Glc-GlcNAc		JJ42
Glc-GlcNAc-Gal		JJ141
GlcNAc-Gal-Glc		JJ118
Gal-Glc-GlcNAc-Gal		DM65
Glc-GlcNAc-Gal-Glc		JJ153
Glc-(Gal-)GlcNAc-Gal		JJ5
Gal-Glc-(Gal-)GlcNAc		JJ1
Gal-GlcNAc-Gal-Glc		JJ9
Glc-(Gal-)GlcNAc-Gal-Glc		JJ6
Gal-Glc-GlcNAc-Gal-Glc		DM35
Gal-Glc-(Gal-)GlcNAc-Gal		DM66
Gal-GlcNAc-Gal-Glc-(Gal-)GlcNAc		JJ10

Continued on following page

TABLE 1—*Continued*

Oligosaccharide fragment	Spacered structure	Code
Gal-Glc-(Gal-)GlcNAc-Gal-Glc		DM36
Gal-Glc-(Gal-)GlcNAc-Gal-Glc-(Gal-)GlcNAc		JJ4
Gal-Glc-(Gal-)GlcNAc-Gal-Glc-(Gal-)GlcNAc		ML1
Gal-Glc-(Gal-)GlcNAc-Gal-Glc-(Gal-)GlcNAc-Gal-Glc-(Gal-)GlcNAc		ML2

(0 to 3.0 M) were incubated on the plates at 37°C for 15 min. After five washes, horseradish peroxidase-conjugated goat anti-mouse IgG (1:10,000) in PBS was added, and the solution was incubated for 1 h at 37°C. The amount of antibodies left was detected by ELISA as described above. In the present study, the avidity index (AI) is expressed as the concentration of NaSCN needed to reduce the OD_{450} by 50%. The absorbance value of sera without NaSCN should be at least 0.5.

TABLE 2. Carbohydrate/protein molar ratios within CRM_{197} and BSA conjugates

Code	Carbohydrate/protein molar ratios[a]	
	CRM_{197} conjugate	BSA conjugate
JJ42	5.3	5.0
JJ118	6.3	6.0
JJ141	3.6	2.5
DM65	5.0	1.0
JJ153	6.2	3.0
JJ1	6.2	NT
JJ9	6.0	2.0
JJ5	7.0	4.0
JJ6	6.5	1.0
DM35	5.0	3.7
DM66	5.5	5.0
JJ10	5.6	0.5
DM36	6.6	NT
JJ4	3.0	NT
ML1	2.6	NT
ML2	1.0	NT

[a] Ratios were determined by MALDI-TOF MS analysis. NT, not tested.

Measurement of phagocytosis titer. The opsonic activity of mouse sera was determined as the uptake of *S. pneumoniae* type 14 by the mouse macrophage cell line J774A.1 (ATCC TIB67). The phagocytosis assay procedure was performed as described by Alonso De Velasco et al. (2) and Lefeber et al. (18). Twofold dilutions of heat-inactivated pooled sera in Hanks balanced salt solution–1% BSA were added with 2% complement (guinea-pig serum) in round-bottom plates (Greiner Bio-One, Frickenhausen, Germany). The assay was performed by mixing 20 μl of J774A.1 cells (3×10^6 cells/ml) and 20 μl of fluorescein isothiocyanate (FITC)-labeled *S. pneumoniae* type 14 (ATTC 634; 3×10^7 cells/ml) (11), followed by incubation at 37°C under vigorous shaking at 900 rpm for 50 min. The mixtures were then fixed with cold 2% paraformaldehyde and analyzed in a flow cytometer (FACScan; Becton Dickinson, Mountain View, CA). The percentage of FITC-positive J774A.1 cells was used as a measure of the phagocytic activity and corrected by using the values found in the mixtures without sera. The phagocytosis titers are expressed as the $\log_{10}$ of the serum dilution during phagocytosis that resulted in 25% of the J774A.1 cells being positive for FITC (13).

Statistical methods. An unpaired *t* test was used to determine the differences in antibody titers, and a *P* value of ≤ 0.05 was considered statistically significant. The $\log_{10}$ of the dilutions was used in all analyses.

RESULTS

Specific antibodies to Pn14PS in immunized mice without adjuvant. Groups of five mice were immunized intracutaneously with CRM_{197}-oligosaccharide conjugates (2.5 μg of carbohydrate per mouse) without adjuvant. All mice were boosted on day 35 with the same conjugate at the same dose used for the primary immunization. For the positive control, mice were immunized with CRM_{197}-Pn14PS; for the negative control, mice were mock immunized with saline solution. Small amounts of IgM antibodies that bound to Pn14PS were de-

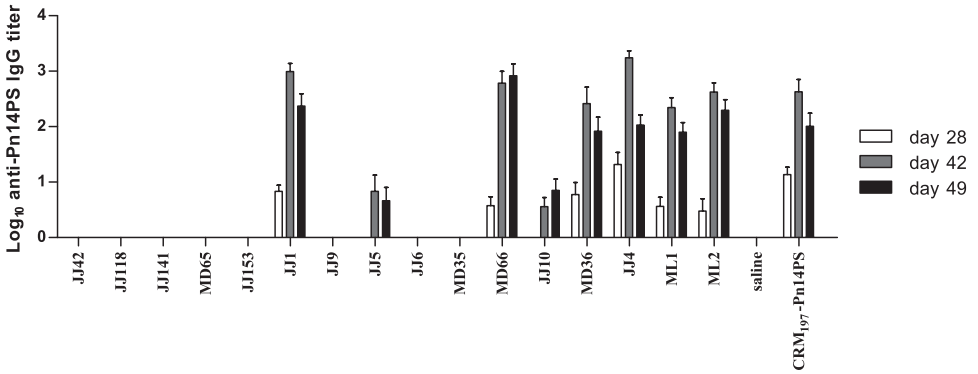


FIG. 2. Total IgG antibody titers recognizing Pn14PS as coating material. Groups of mice ($n = 5$) were immunized with the oligosaccharides conjugated to CRM_{197} and boosted on day 35 with the same preparation. Sera were collected on days 28, 42, and 49, and an ELISA was used to measure IgG antibody titers against Pn14PS. Antibody titers were expressed as the $\log_{10}$ of the dilution giving twice the OD obtained for control mice (immunized with saline).

tected in the sera of the mock-immunized mice. These IgM antibody levels did not rise or fall after primary and booster immunization with conjugate (data not presented). After primary immunization only, low IgG antibody titers against PnPS14 were detected in mice from the groups receiving JJ1, DM66, DM36, JJ4, ML1, and ML2 (Table 1 and Fig. 2). One week after the booster injection on day 35, these antibody titers had increased threefold (Fig. 2). Moreover, mice boosted with JJ5 and JJ10 also had a low IgG titer. The other groups of mice did not produce specific IgG antibodies against Pn14PS when it was used as the antigen-coating material. Additional experiments (data not shown) demonstrated that neither unconjugated Pn14PS nor the carrier protein CRM_{197} induced IgG specific antibodies against Pn14PS.

Structures of the branched tetrasaccharide Gal-Glc-(Gal-)-GlcNAc with one [Gal; DM66, Gal-Glc-(Gal-)GlcNAc-Gal] or two [Gal-Glc; DM36, Gal-Glc-(Gal-)GlcNAc-Gal-Glc] extra monosaccharides at the reducing end also elicited high titers of IgG antibodies against Pn14PS, similar to that elicited by the branched tetrasaccharide JJ1 [Gal-Glc-(Gal-)GlcNAc)] (Fig. 2). When two extra monosaccharides were added to the branched tetrasaccharide at the nonreducing end [Gal-GlcNAc; JJ10, Gal-GlcNAc-Gal-Glc-(Gal-)GlcNAc], specific antibody titers against Pn14PS were sharply reduced. Interestingly, a change in the position of the galactose unit of the glucose-linked galactose from the nonreducing [JJ1, Gal-Glc-(Gal-)GlcNAc] to the reducing [JJ5, Glc-(Gal-)GlcNAc-Gal] end resulted after immunization with a lower IgG titer against Pn14PS. The immunogenicity against Pn14PS was totally lost when one glucose residue was added to JJ5 at the reducing end, yielding JJ6 [Glc-(Gal-)GlcNAc-Gal-Glc] (Fig. 2). The linear tetrasaccharide (JJ9: Gal-GlcNAc-Gal-Glc), which lacked the branching Gal at the GlcNAc unit, did not elicit any antibodies against Pn14PS even after coadministration of the adjuvant (Table 3). In addition, the conjugates of smaller fragments (JJ42 [Gal-Glc-GlcNAc], JJ118 [GlcNAc-Gal-Glc], and JJ141 [Glc-GlcNAc-Gal]) were not able to produce specific antibodies against Pn14PS (Fig. 2). Finally, nonbranched conjugates, such as DM65 (Gal-Glc-GlcNAc-Gal), JJ153 (Glc-GlcNAc-Gal-Glc), and DM35 (Gal-Glc-GlcNAc-Gal-Glc), did not produce antibodies against Pn14PS (Fig. 2).

Effect of adjuvant administration on antibody levels against Pn14PS. To determine the effect of adjuvants on the immunogenicity and specificity of the conjugates, the adjuvants MPL and Quil-A were coadministered in combination with a lower conjugate dose (0.5 μg of carbohydrate per mouse), as reported previously (18). On day 35, the groups of mice were boosted with 2.5 μg of carbohydrate per mouse without adjuvant. The results are only presented for 10 groups of mice, and just the data from sera collected 1 week after booster are shown. In general, although anti-Pn14PS antibodies titers were higher than those in mice injected with the corresponding conjugates without adjuvant (1.6- to 2-fold), only mice immunized with JJ5 showed significantly increased anti-Pn14PS antibody titers (2-fold, P value of <0.05) (Table 3). In contrast, conjugates that were previously shown not to induce anti-Pn14PS antibodies (Fig. 2) were still unable to do so after coadministration of adjuvant.

Anti-carrier and anti-spacer antibodies within the conjugates. CRM_{197} protein was coated on microtiter plates in order to determine specific antibodies against the carrier protein.

TABLE 3. Effect of adjuvant administration on titers of specific IgG antibodies against Pn14PS[a]

Code	$\log_{10}$ IgG titer 2 wk after booster[b]:	
	Without adjuvant	With adjuvant
JJ42	0.0	0.0
JJ141	0.0	0.0
JJ5	1.24 ± 0.29	2.59 ± 0.25
JJ118	0.0	0.0
DM65	0.0	0.0
DM35	0.0	0.0
DM66	2.92 ± 0.48	3.67 ± 0.17
JJ1	2.10 ± 0.40	2.55 ± 0.37
JJ6	0.0	0.0
JJ10	1.03 ± 0.25	1.61 ± 0.67
CRM_{197}-Pn14PS	2.01 ± 0.53	2.85 ± 0.43

[a] Groups of mice ($n = 5$) were immunized with conjugates only or in the presence of adjuvants (10 μg of MPL and 20 μg of Quil-A per mouse). Antibody titers (mean ± SD) were expressed as the $\log_{10}$ of the dilution giving twice the OD obtained for control mice (immunized with saline).

[b] A booster without adjuvant was given at day 35. Sera collected 1 week before the booster showed no significant anti-Pn14PS IgG.

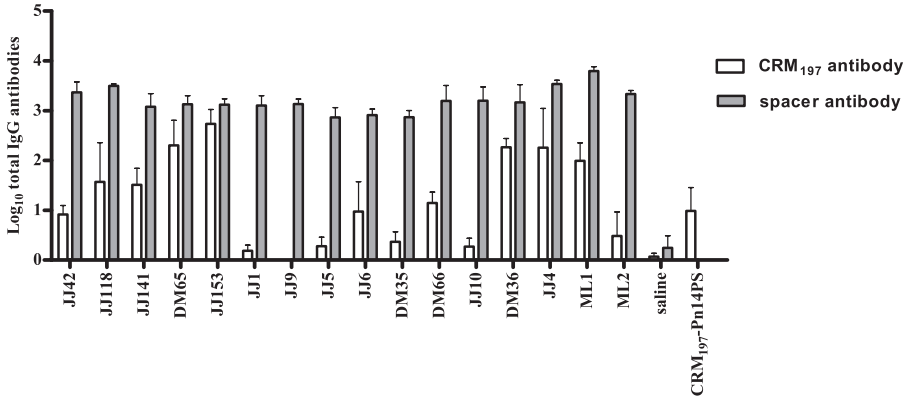


FIG. 3. Antibodies recognizing the carrier CRM_{197} (□) or the spacer present in BSA-mannose (▨) as coating material. The mouse sera (n = 5) were obtained from 2 weeks after the booster. Antibody titers were expressed as the $\log_{10}$ of the dilution giving twice the absorbance value corrected by buffer.

One week after the booster, IgG antibodies against CRM_{197} protein were detected in several sera (Fig. 3). Specific antibodies were not detected in the sera of group of mice immunized with JJ1, JJ9, JJ5, DM35, and JJ10.

In order to determine the antibodies that recognized the spacer present in the conjugates, BSA-mannose was constructed containing the same spacer (-C_6H_{12}-NH-C_4O_2-NH-) that was present in all but the ML1 and ML2 CRM_{197} conjugates. These two conjugates had a shorter spacer (-C_3H_6-NH-C_4O_2-NH-). BSA-mannose was used as the coating material on the microtiter plates. All immunized mice produced specific IgG antibodies against the spacer, including the one for ML1 and ML2 (Fig. 3). No anti-spacer antibodies were detected in the sera of mice immunized with saline or CRM_{197}-Pn14PS (Fig. 3), which was constructed without such a spacer.

Specific antibodies against the oligosaccharide fragment of conjugates. ELISA techniques were used to investigate whether the conjugates that were not able to produce Pn14PS antibodies (Fig. 2) were capable of producing oligosaccharide-specific antibodies. Pooled sera were preincubated with a serial concentration of BSA-mannose to absorb anti-spacer antibodies and then transferred to three different coated plates: BSA-oligosaccharide conjugates corresponding to CRM_{197} conjugates, BSA-mannose, and BSA-DM66 as a control.

The immune complexes formed by BSA-mannose and the anti-spacer antibodies were detected as an IgG precipitate on the uncoated plates, which also served as the preincubation plates (Fig. 4A). Absorption of sera with BSA-mannose showed a dose-dependent response to BSA-mannose-coated plates (Fig. 4B). When the BSA-mannose-absorbed sera were transferred to the BSA conjugate-coated plate (corresponding to CRM_{197} conjugate), just the sera immunized with JJ10 or DM65 showed a dose-dependent response (Fig. 4C). This suggests that, in most sera, oligosaccharide-specific antibodies are present that do not recognize Pn14PS. When the BSA-mannose absorbed sera were transferred to the BSA-DM66-coated plates [DM66, Gal-Glc-(Gal-)GlcNAc-Gal], there was response to BSA-DM66 as observed with BSA-mannose (Fig. 4B and D). This suggests the recognition of spacer-specific antibodies only. Sera obtained from JJ5-immunized mice were used throughout the absorption experiment as a control for the sera recognizing Pn14PS. After absorption with BSA-mannose, this serum displayed a dose-independent response when BSA-DM66 was used as coating material (Fig. 4D).

Antibody avidities of sera from mice receiving a booster injection. The thiocyanate elution ELISA was used to measure the anti-Pn14PS antibody avidity of the sera obtained 1 week after the booster (Fig. 5). All conjugates that elicited anti-Pn14PS antibody titers were evaluated with regard to the AI. Except for conjugates JJ5 and JJ10, the observed AI was not significantly different between conjugates, which could be a consequence of the lower anti-Pn14PS titer (Fig. 5).

Phagocytic capacity of sera from mice receiving a booster injection. To analyze the phagocytic capacities of groups receiving different conjugates, pooled mouse sera obtained 1 week after the booster were tested in a phagocytosis assay using *S. pneumoniae* type 14 and the mouse macrophage cell line J774A.1. Except for JJ10, all conjugates that elicited antibodies against Pn14PS (Fig. 2) were also capable of promoting the phagocytosis of *S. pneumoniae* type 14 (Fig. 6). All conjugates that did not induce Pn14PS-specific antibodies were not able to promote the phagocytosis of *S. pneumoniae* type 14.

DISCUSSION

The smallest immunogenic structure of Pn14PS was identified in this study by testing 16 overlapping synthetic oligosaccharide fragments of Pn14PS (Table 1). The oligosaccharides were conjugated to the protein carrier CRM_{197}. CRM_{197}-Pn14PS, which contains of the entire *S. pneumoniae* type 14 polysaccharide and was used as the positive control, produced a specific antibody response to Pn14PS, as reported previously (32, 34, 35). The conjugate of one defined structural repeating unit of Pn14PS, namely, the branched tetrasaccharide Gal-Glc-(Gal-)GlcNAc (JJ1), produced a specific antibody response to Pn14PS, similarly to that reported by Mawas et al. (20). The conjugates of two and three repeating units of this branched tetrasaccharide, as represented by JJ4/ML1 and ML2, respectively, also produced antibodies against Pn14PS (Fig. 2). No significant differences in immune response were observed between the conjugates JJ4 and ML1. These conjugates contain identical oligosaccharides but differ in the length of the spacer.

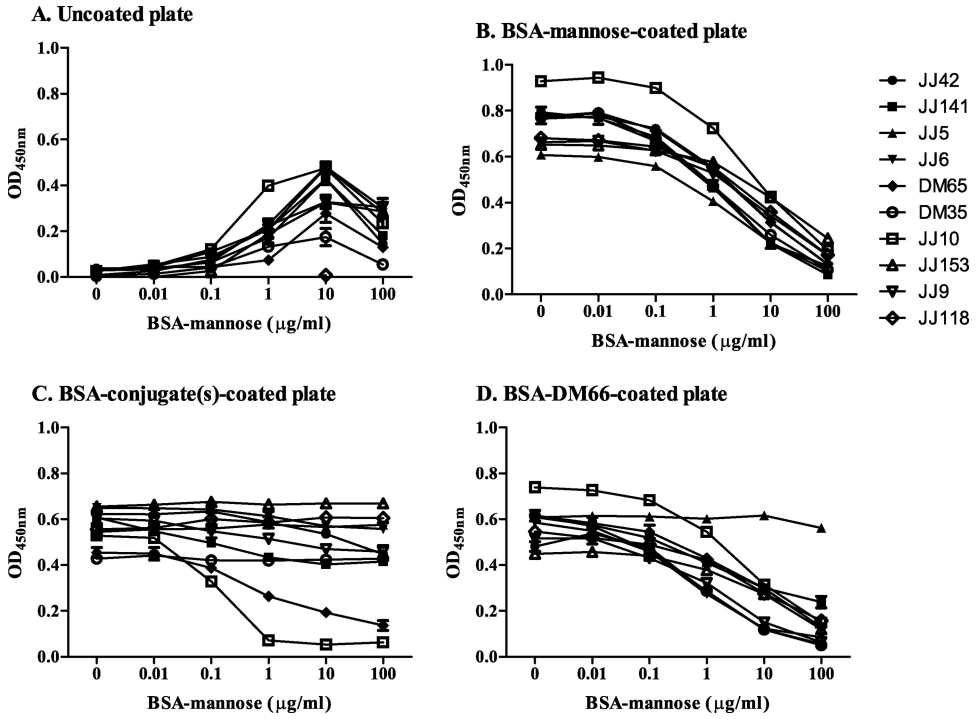


FIG. 4. Antibodies recognizing the specific oligosaccharide fragments within the conjugates. (A) Pooled sera (duplo) were combined with serial concentrations of BSA-mannose conjugate which could inhibit the binding of the antibodies to the spacer (in the conjugates) and incubated on an uncoated plate. After incubation, the mixture was transferred onto plates coated either with BSA-mannose (B) or BSA corresponding conjugates (C) and BSA-DM66 as a control (D). The results are expressed as the OD changes of the sera incubated with BSA-mannose and compared to results with serum alone.

Since several conjugates did not produce anti-Pn14PS antibodies, the question was raised whether oligosaccharide-specific antibodies could be demonstrated. BSA-mannose, which contains the spacer present in CRM_{197} and BSA conjugates, was used to block the anti-spacer antibodies. Its use was based on the results shown in Fig. 3, i.e., all conjugates produced specific antibodies to the spacer part of the conjugates. Almost all conjugates induced specific antibodies to their own oligosaccharide fragments (Fig. 4). It was noted that JJ10 did not induce oligosaccharide-specific antibodies, while low amounts of Pn14PS antibodies could be detected in the sera (Fig. 2, Table 3, and Fig. 4C); this is discussed further below. DM65 also did not evoke oligosaccharide-specific antibodies (Fig. 4C). Other studies have shown that anti-spacer antibodies can suppress the induction of antibodies against the carbohydrate antigen (6, 22). In the present study, we found that almost all conjugates produced antibodies against the specific oligosaccharide of the conjugate that was not suppressed by the simultaneous production of anti-spacer and anti-carrier antibodies (Fig. 3).

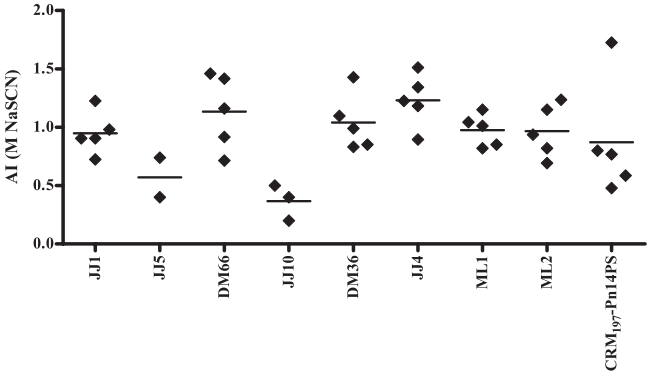


FIG. 5. AIs of sera that recognize Pn14PS as coating material. Sera were obtained from mice 1 week after booster injection (day 42). The AI is expressed as the concentration of NaSCN needed to reduce the OD_{450} by 50%. The absorbance value of sera without NaSCN was at least 0.5 (18).

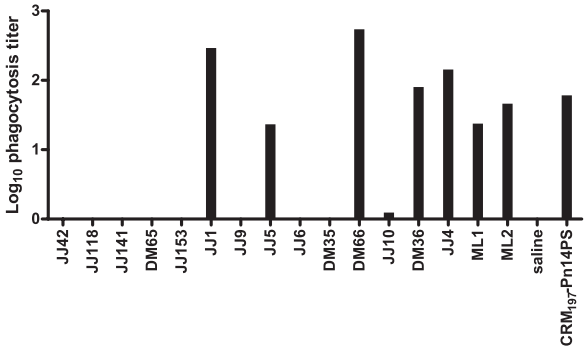


FIG. 6. Phagocytosis titer in sera obtained 1 week after the booster that were heat inactivated and supplemented with 2% complement. The assay was performed with heat-inactivated FITC-labeled *S. pneumoniae* type 14 and J774A.1 cells by flow cytometry. The titers are expressed as the $\log_{10}$ of the serum dilution during phagocytosis resulting in 25% of J774 cells being positive for FITC.

The biological relevance of the evoked antibodies was investigated by measuring the capacity of the sera to promote opsonophagocytosis of *S. pneumoniae* type 14. First, the antibody avidity of the sera was measured in order to evaluate the relative strength of the binding of antibodies to Pn14PS (25). Antibody avidity is an important characteristic of protection immune response (10) and strongly correlated with antibody titer and the ability to opsonize pneumococci in vitro and with the potency of an antibody to passively protect mice against pneumococcal infections (29). We found that the conjugates that elicited specific antibodies against Pn14PS have similar binding strengths, as indicated by the AI except for JJ5 and JJ10. Apparently, the antibodies (paratope) that were elicited by conjugates JJ5 and JJ10 might not fit precisely with the epitope of polysaccharide type 14 (Fig. 5). Opsonophagocytosis was performed using heat-inactivated FITC-labeled *S. pneumoniae* type 14 and the mouse macrophage cell line J774A.1. We observed that the sera containing antibodies against Pn14PS were also capable of promoting the phagocytosis of *S. pneumoniae* type 14. There was one exception: JJ10 (the conjugate that induced lower antibody titers against Pn14PS in the other tests) sera did not promote phagocytosis (Fig. 6). When whole-cell ELISA for *S. pneumoniae* type 14 was used, additional experiments with JJ10-specific antibodies did not detect any binding of these antibodies. This explains the inability of these antibodies to promote phagocytosis (data not shown). It has been hypothesized and proven by additional experiments (data not shown) that JJ10 does not evoke antibodies against the branched structure Gal-Glc-(Gal-)GlcNAc present in PnPS14 but does evoke antibodies against the nonreducing end of the oligosaccharide Gal-GlcNAc-Gal-Glc. We concluded that the JJ10-specific antibodies may recognize an epitope that is exposed in purified PnPS14 but hidden in the capsule of the bacterium. The other conjugates that did not evoke specific antibodies against polysaccharide type 14 also did not display phagocytic capacity. They did, however, elicit specific antibodies against their own oligosaccharide structure.

Our findings (28) and the work of others (7, 8, 27, 33) on synthetic oligosaccharide-protein conjugates should encourage more research on synthetic oligosaccharide-conjugate vaccines. Establishing whether animals and humans can produce antibodies against minimal synthetic oligosaccharides, however, is just the first step in the development of a synthetic pneumococcal vaccine. The next steps are to define the most immunogenic-protective epitopes on the PS and to optimize the presentation of these epitopes to the immune system.

In conclusion, the large number of *S. pneumoniae* serotypes should stimulate the generation of new synthetic saccharides. Then, natural PS can, when needed and when possible, be replaced sequentially by their (bio)synthetic oligosaccharide counterparts in future semisynthetic pneumococcal vaccines. The present study has shown that the branched trisaccharide Glc-(Gal-)GlcNAc is the core structure in inducing Pn14PS-specific antibodies and that the neighboring galactose at the nonreducing end significantly contributes to the induction of phagocytosis-promoting (functional) antibodies. Furthermore, the branching element within Pn14PS plays an important role in the recognition of the PS of *S. pneumoniae* type 14. Taking into account the various results from the present work, the structure, originally described by Mawas et al. (20), is a serious candidate for a synthetic oligosaccharide conjugate vaccine against infections caused by *S. pneumoniae* type 14.

ACKNOWLEDGMENTS

This study was supported by a grant from the European Commission under contract MRTN-CT-2004-005645.

We thank Novartis Siena, Italy for the generous gift of CRM_{197}.

REFERENCES

1. **Ada, G., and D. Isaacs.** 2003. Carbohydrate-protein conjugate vaccines. Clin. Microbiol. Infect. **9:**79–85.
2. **Alonso De Velasco, E., A. F. M. Verheul, A. M. P. van Steijn, H. A. T. Dekker, R. G. Feldman, I. M. Fernandez, J. P. Kamerling, J. F. G. Vliegenthart, J. Verhoef, and H. Snippe.** 1994. 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. **62:**799–808.
3. **Alonso De Velasco, E., A. F. M. Verheul, J. Verhoef, and H. Snippe.** 1995. *Streptococcus pneumoniae*: virulence factors, pathogenesis, and vaccines. Microbiol. Rev. **59:**591–603.
4. **Benaissa-Trouw, B., D. J. Lefeber, J. P. Kamerling, J. F. G. Vliegenthart, K. Kraaijeveld, and H. Snippe.** 2001. Synthetic polysaccharide type 3-related di-, tri-, and tetrasaccharide-CRM197 conjugates induce protection against *Streptococcus pneumoniae* type 3 in mice. Infect. Immun. **69:**4698–4701.
5. **Breukels, M. A., G. T. Rijkers, M. M. Voorhorst-Ogink, B. J. M. Zegers, and L. A. M. Sanders.** 1999. Pneumococcal conjugate vaccine primes for polysaccharide-inducible IgG2 antibody response in children with recurrent otitis media acuta. J. Infect. Dis. **179:**1152–1156.
6. **Buskas, T., Y. Li, and G.-J. Boons.** 2004. The immunogenicity of the tumor-associated antigen Lewis^y may be suppressed by a bifunctional cross-linker required for coupling to a carrier protein. Chem. Eur. J. **10:**3517–3524.
7. **Fernandez-Santana, V., F. Cardoso, A. Rodriguez, T. Cermenate, L. Pena, Y. Valdes, E. Hardy, F. Mawas, L. Heynngnezz, M. C. Rodriguez, I. Figueroa, J. Chang, M. E. Toledo, A. Musacchio, I. Hernandez, M. Izquierdo, K. Cosme, R. Roy, and V. Verez-Bencomo.** 2004. Antigenicity and immunogenicity of a synthetic oligosaccharide-protein conjugate vaccine against *Haemophilus influenzae* type b. Infect. Immun. **72:**7115–7123.
8. **Granoff, D. M., Y. E. McHugh, H. V. Raff, A. S. Mokatrin, and G. A. van Nest.** 1997. MF59 adjuvant enhances antibody responses of infant baboons immunized with *Haemophilus influenzae* type b and *Neisseria meningitidis* group C oligosaccharide-CRM197 conjugate vaccine. Infect. Immun. **65:** 1710–1715.
9. **Guttormsen, H.-K., C. J. Baker, M. H. Nahm, L. C. Paoletti, S. M. Zughaier, M. S. Edwards, and D. L. Kasper.** 2002. Type III group B streptococcal polysaccharide induces antibodies that cross-react with *Streptococcus pneumoniae* type 14. Infect. Immun. **70:**1724–1738.
10. **Harris, S. L., H. Tsao, L. Ashton, D. Goldblatt, and P. Fernsten.** 2007. Avidity of the immunoglobulin G response to a *Neisseria meningitidis* group C polysaccharide conjugate vaccine as measured by inhibition and chaotropic enzyme-linked immunosorbent assays. Clin. Vaccine Immunol. **14:** 397–403.
11. **Jansen, W. T. M., J. Gootjes, M. Zelle, D. V. Madore, J. Verhoef, H. Snippe, and A. F. M. Verheul.** 1998. Use of highly encapsulated *Streptococcus pneumoniae* strains in a flow-cytometric assay for assessment of the phagocytic capacity of serotype-specific antibodies. Clin. Diagn. Lab. Immunol. **5:**703–710.
12. **Jansen, W. T. M., and H. Snippe.** 2004. Short-chain oligosaccharide protein conjugates as experimental pneumococcal vaccines. Indian. J. Med. Res. **119:**7–12.
13. **Jansen, W. T. M., M. Väkeväinen-Anttila, H. Käyhty, M. H. Nahm, N. Bakker, J. Verhoef, H. Snippe, and A. F. M. Verheul.** 2001. Comparison of a classical phagocytosis assay and a flow cytometry assay for assessment of the phagocytic capacity of sera from adults vaccinated with a pneumococcal conjugate vaccine. Clin. Diagn. Lab. Immunol. **8:**245–250.
14. **Joosten, J. A. F., J. P. Kamerling, and J. F. G. Vliegenthart.** 2003. Chemoenzymatic synthesis of a tetra- and octasaccharide fragment of the capsular polysaccharide of *Streptococcus pneumoniae* type 14. Carbohydr. Res. **338:** 2611–2627.
15. **Joosten, J. A. F., B. J. Lazet, J. P. Kamerling, and J. F. G. Vliegenthart.** 2003. Chemo-enzymatic synthesis of tetra-, penta-, and hexasaccharide fragments of the capsular polysaccharide of *Streptococcus pneumoniae* type 14. Carbohydr. Res. **338:**2629–2651.
16. **Kamerling, J. P.** 1999. Pneumococcal polysaccharides: a chemical view, p. 81–114. *In* A. Tomasz (ed.), *Streptococcus pneumoniae*: molecular biology and mechanisms of disease. Mary Ann Liebert, New York, NY.
17. **Laferriere, C. A., R. K. Sood, J. M. de Muys, F. Michon, and H. J. Jennings.** 1998. *Streptococcus pneumoniae* type 14 polysaccharide-conjugate vaccines: length stabilization of opsonophagocytic conformational polysaccharide epitopes. Infect. Immun. **66:**2441–2446.

18. **Lefeber, D. J., B. Benaissa-Trouw, J. F. G. Vliegenthart, J. P. Kamerling, W. T. M. Jansen, K. Kraaijeveld, and H. Snippe.** 2003. Th1-directing adjuvants increase the immunogenicity of oligosaccharide-protein conjugate vaccines related to *Streptococcus pneumoniae* type 3. Infect. Immun. **71:**6915–6920.
19. **Lindberg, B., J. Lönngren, and D. A. Powel.** 1977. Structural studies on the specific type 14 pneumococcal polysaccharide. Carbohydr. Res. **58:**177–186.
20. **Mawas, F., J. Niggemann, C. Jones, M. J. Corbel, J. P. Kamerling, and J. F. Vliegenthart. G.** 2002. Immunogenicity in a mouse model of a conjugate vaccine made with a synthetic single repeating unit of type 14 pneumococcal polysaccharide coupled to CRM197. Infect. Immun. **70:**5107–5114.
21. **Michalik, D., J. F. G. Vliegenthart, and J. P. Kamerling.** 2002. Chemoenzymic synthesis of oligosaccharide fragments of the capsular polysaccharide of *Streptococcus pneumoniae* type 14. J. Chem. Soc., Perkin Trans. **1:**1973–1981.
22. **Ni, J., H. Song, Y. Wang, N. M. Stamatos, and L.-X. Wang.** 2006. Toward a carbohydrate-based HIV-1 vaccine: synthesis and immunological studies of oligomannose-containing glycoconjugates. Bioconjugate Chem. **17:**493–500.
23. **Park, I. H., D. G. Pritchard, R. Cartee, A. Brandao, M. C. C. Brandileone, and M. H. Nahm.** 2007. Discovery of a new capsular serotype (6C) within serogroup 6 of *Streptococcus pneumoniae*. J. Clin. Microbiol. **45:**1225–1233.
24. **Prymula, R., P. Peeters, V. Chrobok, P. Kriz, E. Novakova, E. Kaliskova, I. Kohl, P. Lammel, J. Poolman, J.-P. Prieels, and L. Schuerman.** 2006. Pneumococcal capsular polysaccharides conjugated to protein D for prevention of acute otitis media caused by both *Streptococcus pneumoniae* and non-typeable *Haemophilus influenzae*: a randomised double-blind efficacy study. Lancet **367:**740–748.
25. **Pullen, G. R., M. G. Fitzgerald, and C. S. Hosking.** 1986. Antibody avidity determination by ELISA using thiocyanate elution. J. Immunol. Methods **86:**83–87.
26. **Rennels, M. B., K. M. Edwards, H. L. Keyserling, K. S. Reisinger, D. A. Hogerman, D. V. Madore, I. Chang, P. R. Paradiso, F. J. Malinoski, and A. Kimura.** 1998. Safety and immunogenicity of heptavalent pneumococcal vaccine conjugated to CRM197 in United States infants. pediatrics. **101:**604–611.
27. **Saksena, R., X. Ma, T. K. Wade, P. Kovác, and W. F. Wade.** 2006. Length of the linker and the interval between immunizations influences the efficacy of *Vibrio cholerae* O1, Ogawa hexasaccharide neoglycoconjugates. FEMS Immunol. Med. Microbiol. **47:**116–128.
28. **Snippe, H., W. T. M. Jansen, and J. P. Kamerling.** 2008. Immunology of experimental synthetic carbohydrate-protein conjugate vaccines against *Streptococcus pneumoniae* serotypes, p. 85–104. *In* R. Roy (ed.), Carbohydrate-based vaccines. ACS Symposium Series 989, Washington, D.C.
29. **Sun, Y., Y.-L. Hwang, and M. H. Nahm.** 2001. Avidity, potency, and cross-reactivity of monoclonal antibodies to pneumococcal capsular polysaccharide serotype 6B. Infect. Immun. **69:**336–344.
30. **Sundgren, A., M. Lahmann, and S. Oscarson.** 2005. Block synthesis of *Streptococcus pneumoniae* type 14 capsular polysaccharide structures. J. Carbohydr. Chem. **24:**379–391.
31. **Tietze, L. F., M. Arlt, M. Beller, K. H. Glusenkamp, E. Jahde, and M. F. Rajewsky.** 1991. Squaric acid diethyl ester: a new coupling reagent for the formation of drug biopolymer conjugates, synthesis of squaric acid ester amides and diamides. Chem. Ber. **124:**1215–1221.
32. **Van Den Dobbelsteen, G. P. J. M., K. Brunekreef, T. Sminia, and E. P. Van Rees.** 1992. Effect of mucosal and systemic immunization with pneumococcal polysaccharide type 3, 4 and 14 in the rat. Scand. J. Immunol. **36:**661–669.
33. **Verez-Bencomo, V., V. Fernandez-Santana, E. Hardy, M. E. Toledo, A. Rodriguez, A. Baly, L. Harrera, M. Izquierdo, A. Villar, Y. Valdes, K. Cosme, M. L. Deler, M. Montane, E. Gracia, A. Ramos, A. Aguilar, E. Medina, G. Torano, I. Sosa, I. Hernandez, R. Martinez, A. Muzachio, A. Carmenates, L. Costa, F. Cardoso, C. Campa, V. Diaz, and R. Roy.** 2004. A synthetic conjugate polysaccharide vaccine against *Haemophilus influenzae* type b. Science **305:**522–525.
34. **Verheul, A. F. M., A. A. Versteeg, M. J. de Reuver, M. Jansze, and H. Snippe.** 1989. Modulation of the immune response to pneumococcal type 14 capsular polysaccharide-protein conjugates by the adjuvant Quil A depends on the properties of the conjugates. Infect. Immun. **57:**1078–1083.
35. **Verheul, A. F. M., A. A. Versteeg, N. A. C. Westerdaal, G. J. van Dam, M. Jansze, and H. Snippe.** 1990. Measurement of the humoral immune response against *Streptococcus pneumoniae* type 14-derived antigens by an ELISA and ELISPOT assay based on biotin-avidin technology. J. Immunol. Methods **126:**79–87.
36. **World Health Organization.** 2007. Pneumococcal conjugate vaccine for childhood immunization: WHO position paper. Wkly. Epidemiol. Rec. **82:**93–104.

Editor: A. Camilli