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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

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Streptococcus pneumoniae **type 23F capsular polysaccharide (PS23F) consists of a repeating glycerol-phosphorylated branched tetrasaccharide. The immunogenicities of the following related antigens were investigated: (i) a synthetic trisaccharide comprising the backbone of one repeating unit, (ii) a synthetic tetrasaccharide comprising the complete repeating unit, and (iii) native PS23F (all three conjugated to keyhole limpet hemocyanin [KLH]) and (iv) formalin-killed *S. pneumoniae* 23F. All antigens except the trisaccharide-KLH conjugate induced relatively high anti-PS23F antibody levels in rabbits. The epitope specificity of such antibodies was then studied by means of an inhibition immunoassay. The α(1→2)-linked L-rhamnose branch was shown to be immunodominant for immunoglobulin G (IgG) induced by tetrasaccharide-KLH, PS23F-KLH, and killed *S. pneumoniae* 23F: in most sera L-rhamnose totally inhibited the binding of IgG to PS23F. Thus, there appears to be no major difference in epitope specificity between IgG induced by tetrasaccharide-KLH and that induced by antigens containing the polymeric form of PS23F. Human anti-PS23F IgG (either vaccine induced or naturally acquired) had a different epitope specificity: none of the inhibitors used, including L-rhamnose and tetrasaccharide-KLH, exhibited substantial inhibition. These observations suggest that the epitope recognized by human IgG on PS23F is larger than the epitope recognized by rabbit IgG. Both human and rabbit antisera efficiently opsonized type 23F pneumococci, as measured in a phagocytosis assay using human polymorphonuclear leukocytes.**

Anti-capsular polysaccharide (anti-PS) antibodies (Ab), which are able to protect against infections by pneumococci (26), can be elicited not only by PS-containing antigens but also by disaccharide or trisaccharide fragments of PS coupled to a carrier protein (3, 5, 11). Thus, Ab induced by pneumococcal oligosaccharide (OS)-protein conjugates can induce protective immunity against the pneumococcal type from which the OS was derived (3, 10, 27). Apart from the existence and relative avidity of the binding of such Ab to the corresponding PS, little is known about the epitope specificity of the Ab induced by pneumococcal OS conjugates versus the specificity of the Ab induced by their respective PSs. This might be important in the design of OS conjugate vaccines, since the results of recent studies indicate that the conformational epitopes of PSs may play an important role in their immunogenicities (17, 22, 37).

Streptococcus pneumoniae type 23F is a well-studied serotype that is isolated mainly from children (12, 16). PS23F is a polymer of a tetrasaccharide repeating unit containing a phosphoglycerol substituent (Fig. 1) (24). In earlier reports, the α(1→2)-linked L-rhamnose was thought to be a major epitope recognized by horse Ab (14). In this work, we compare the specificities of immunoglobulin G (IgG) induced in rabbits by three antigens: a complete repeating unit of PS23F which was prepared by organic synthesis and a native purified PS23F (both coupled to keyhole limpet hemocyanin [KLH]) and formalin-killed *S. pneumoniae* 23F.

In the past, rabbit and horse sera have been used to detect epitopes present in PSs and to evaluate the degree of cross-reactivity among different pneumococcal serotypes or between pneumococci and other bacteria (13, 14). Intra- and interspecies differences in the amounts and epitope specificities of Ab induced by saccharide antigens have been reported for mice, rabbits, horses, and humans (20, 21, 29). Thus, the relevance of data derived from animal studies to the human situation is uncertain. To address this question for the Ab response to pneumococcal type 23F capsular antigens, the immunogenicities of various type 23F antigens were studied in mice and rabbits, and we compared the specificity of rabbit IgG elicited with the antigens described above with that of IgG derived from humans who were vaccinated with the 23-valent pneumococcal vaccine or who had naturally acquired anti-PS23F Ab. The opsonic activity of such Ab was assessed by means of a phagocytosis assay using human polymorphonuclear leukocytes (PMN).

MATERIALS AND METHODS

Chemicals. KLH was purchased from Calbiochem Co. (La Jolla, Calif.). *N*-Succinimidyl *S*-acetylthioacetate, *N*-succinimidyl bromoacetate, and *N*-hydroxysulfosuccinimide were from Pierce Chemical Co. (Rockford, Ill.). Cyanogen bromide, adipic acid dihydrazide, β-mercaptoethylamine, 1-ethyl-3-dimethylaminopropyl carbodiimide, and fluorescein isothiocya-

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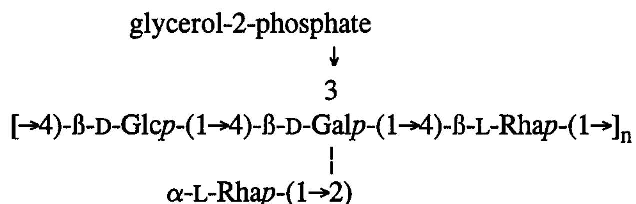

FIG. 1. Structure of the PS of *S. pneumoniae* serotype 23F (24).

nate isomer I were supplied by Sigma Chemical Co. (St. Louis, Mo.). Ficoll-Paque was from Pharmacia (Uppsala, Sweden).

OSs and inhibitors. The trisaccharide β-L-rhamnopyranoside-(1→4)-β-D-glucopyranoside-(1→4)-β-D-galactopyranoside-$O(CH_2)_3NH_2$ and the tetrasaccharide β-D-glucopyranoside-(1→4)-[glycerol-2-phosphate→3][α-L-rhamnopyranoside-(1→2)]-β-D-galactopyranoside-(1→4)-β-L-rhamnopyranoside-$O(CH_2)_3NH_2$, representing fragments of the PS23F structure (Fig. 1 and Table 1) were synthesized as described previously; 3-aminopropyl was used as a spacer at the reducing end to permit coupling to a carrier protein (32, 33). Two other trisaccharides, namely, (phosphate→3)-β-D-glucopyranoside-(1→4)-[α-L-rhamnopyranoside-(1→2)]-β-D-galactopyranoside-OMe and α-L-rhamnopyranoside-(1→2)-β-D-galactopyranoside-(1→4)-β-L-rhamnopyranoside-OMe, and one tetrasaccharide, (phosphate→3)-β-D-glucopyranoside-(1→4)-[α-L-rhamnopyranoside-(1→2)]-β-D-galactopyranoside-(1→4)-β-L-rhamnopyranoside-OMe, related to the PS23F structure (see Fig. 5), were synthesized without a spacer for use in inhibition studies (31).

Pneumococcal PS17F and PS6B were purified by fractional ethanol precipitation as described previously (3). C-polysaccharide (C-PS) was obtained from the Statens Seruminstitut (Copenhagen, Denmark). The other inhibitors were analytical grade L-rhamnose, D-galactose, D-glucose, and glycerol.

OS-protein conjugates. The trisaccharide and the tetrasaccharide containing a 3-aminopropyl spacer (see above) were each coupled to KLH in a stepwise reaction as described previously (3). The trisaccharide (16.5 μmol) and tetrasaccharide (6.9 μmol) were S acetylated with *N*-succinimidyl *S*-acetylthioacetate and precipitated by the addition of 90 μl of glacial acetic acid, 3 ml of acetone, and 2 ml of ethyl acetate. The carbohydrate content of the precipitate was more than 80% of the amount before the S acetylation (9). The efficiency of S acetylation was 80 and 99% for the trisaccharide and tetrasaccharide, respectively. Bromoacetylation of KLH with *N*-succinimidyl bromoacetate was performed according to the method of Bernatowicz and Matsueda (6). S-acetylated trisaccharide and tetrasaccharide were dissolved in a solution of bromoacetylated KLH (2.7 and 4.6 mg of KLH/ml, respectively, in 0.1 M phosphate buffer [pH 6.1] containing 5 mM EDTA and 0.01% NaN_3) to a final concentration of 3.0 mM (trisaccharide) and 3.9 mM (tetrasaccharide). The OS/KLH molar ratio during the coupling reaction was approximately 5,800 for the trisaccharide and 4,400 for the tetrasaccharide (assuming the molecular weight of KLH to be 5.25×10^6). After addition of 200 μl of 0.2 M NH_2OH, the solution was stirred for 48 h at room temperature under helium. To block the remaining bromoacetyl groups present on KLH, a molar excess of β-mercaptoethylamine was added, and the solution was stirred overnight at 4°C. The conjugates were purified over a Sepharose CL-6B column as described previously (3) and analyzed for protein (28) and sugar (9) contents (using KLH and PS23F, respectively, as standards) and for monosaccharide composition by gas chromatography (18); the linkage of both OSs to KLH was confirmed by the presence of rhamnose in the conjugates, since this monosaccharide, unlike galactose and glucose, is not present in KLH (Table 1).

PS purification and PS-KLH conjugates. PS23F was purified from strain ATCC 6323 (American Type Culture Collection, Rockville, Md.) by fractional ethanol precipitation. Crude PS23F was subjected to protein extraction by chloroform-butanol and to concanavalin A-Sepharose affinity chromatography to remove polymannan (34). Purified PS23F contained less than 2% protein (28) as determined with bovine serum albumin (BSA) as a standard. The A_{260} of PS23F (5 mg/ml) was 1.08, indicating a DNA concentration of about 1%. Free amino groups were introduced by cyanogen bromide activation and incorporation of adipic acid dihydrazide (34). Derivatized PS23F was analyzed for monosaccharide composition by gas chromatography (18); neither mannose nor *N*-acetylgalactosamine (a marker for C-PS) could be detected. Modified PS23F was coupled to KLH by means of 1-ethyl-3-dimethylaminopropyl carbodiimide and *N*-hydroxysulfosuccinimide (34). A C-PS–KLH conjugate was prepared by the same

TABLE 1. Properties of the synthetic oligosaccharide-KLH and native polysaccharide-KLH conjugates

Conjugate	Schematic structure	Sugar/protein ratio		Monosaccharide ratio[a]		
		wt/wt	mol/mol[b]	Rha	Gal	Glc
Trisaccharide-KLH	Rha-Glc-Gal-KLH	0.09	940	1.0	0.2	1.3
Tetrasaccharide-KLH	Gly-ol-2-PO_4^- | Glc-Gal-Rha-KLH | Rha	0.19	1,163	2.0	1.6	5.8
PS23F-KLH	Gly-ol-2-PO_4^- | [Glc-Gal-Rha]$_n$-KLH | Rha	0.60		2.0	1.0	1.1

[a] Monosaccharide amounts were determined by gas chromatography. Molar ratios were calculated on basis of the rhamnose residues after correcting for the monosaccharides present in KLH (Gal and Glc).

[b] The molar ratio between KLH and OS was calculated by assuming a molecular weight of 5.25×10^6 for KLH. The molar ratio of the PS23F-KLH conjugate was not calculated because of the variable length of the PS chains.

method as used for the PS23F-KLH conjugate. In both cases, a soluble fraction and an insoluble fraction were obtained during the coupling reaction; both were analyzed as described for the OS-KLH conjugates. In the present work, the insoluble fractions were used. Since C-PS does not react in the phenol-sulfuric acid assay (9), the amount of C-PS in the conjugate could not be determined directly. It is estimated to be 40% of the total weight of the conjugate, after subtracting the amount of KLH present.

Immunizations and sera. Inbred 10- to 12-week-old female BALB/c, C57BL/6, DBA/2, and CBA mice and female New Zealand White rabbits were supplied by Iffa-Credo (Someren, The Netherlands); they were maintained at the Central Laboratory of Experimental Animals (Utrecht University). Groups of five mice from each strain were immunized (day 0) subcutaneously or intracutaneously with PS23F-KLH conjugate (2.5 μg of sugar per mouse) alone or in combination with 20 μg of the adjuvant Quil A. Tetrasaccharide-KLH (administered subcutaneously with or without Quil A) was tested only in BALB/c mice. At day 21, each mouse was boosted with the same preparation used for primary immunization; the mice were bled at day 35. BALB/c mice were immunized intraperitoneally with formalin-killed *S. pneumoniae* 23F (10^8 bacteria in 0.5 ml of phosphate-buffered saline) at days 0 and 14 and were bled at day 21.

Rabbits weighing approximately 3 kg were immunized subcutaneously at four sites with trisaccharide-KLH conjugate, tetrasaccharide-KLH conjugate, or PS23F-KLH conjugate (two rabbits in each group) or C-PS–KLH conjugate (one rabbit). The dose used was 10 μg of sugar per rabbit (or 100 μg of conjugate per rabbit in the case of the rabbit given C-PS-KLH conjugate), emulsified with complete Freund's adjuvant, in a total volume of 0.8 ml. Booster immunizations were given intraperitoneally in incomplete Freund's adjuvant at days 21 and 49 after the first immunization. Rabbits were bled at days 0, 14, 35, and 57 after the first immunization. Rabbit sera used for inhibition studies were obtained at day 57 and are referred to as Ra-tetra-1 and -2 (rabbit anti-tetrasaccharide-KLH conjugate), Ra-PS-1 and -2 (rabbit anti-PS23F-KLH conjugate), and Ra-Spn (rabbit anti-*S. pneumoniae* type 23F). Ra-Spn was elicited by multiple intravenous injections of formalin-killed *S. pneumoniae* 23F in phosphate-buffered saline.

Human anti-pneumococcal PS plasma (referred to as Hu-PS-1) was provided by D. V. Madore (Praxis Biologics). After vaccination of adult volunteers with 23-valent pneumococcal vaccine, plasma samples from 18 donors with the highest levels of anti-PS Ab were pooled, defibrinated, and filtered. A serum pool from 10 nonvaccinated healthy medical students containing relatively high anti-PS23F IgG titers also was used in the inhibition studies. This serum pool is referred to as Hu-PS-2. Two other serum pools, obtained in the same way as Hu-PS-2, were also used. All sera used for inhibition studies are listed in Table 2.

IgG was purified from Ra-Spn and Hu-PS-2 by protein A/G affinity chromatography by the method described in the manufacturer's manual (Pierce Chemical Co.).

Anti-C-PS Ab were removed from Hu-PS-2 with C-PS-coated magnetic beads. Streptavidin-coated Dynabeads M280 (Dynal A.S., Oslo, Norway) were incubated with mixing for 60 min at room temperature with biotinylated C-PS (35) in phosphate-buffered saline (a fourfold molar excess of biotin, compared with the biotin-binding sites on the beads, was used). Beads were washed three times, and binding of C-PS to the beads was checked by flow cytometry after the beads were stained with rabbit anti-C-PS antiserum (see above) and fluorescein isothiocyanate-labeled goat anti-rabbit Ig. Hu-PS-2 (0.5 ml) was incubated for 1 h at 37°C with 8.3 mg of C-PS-coated beads. This incubation was repeated four times with new beads, and the last incubation was performed overnight. The decrease in anti-C-PS IgG titer (measured as described below) was more than 15-fold.

TABLE 2. Characteristics of the sera used in inhibition experiments

Serum	Host	Immunization	Anti-PS23F titer[a]		Anti-C-PS titer (IgG)
			IgG	IgM	
Ra-tetra-1	Rabbit	Tetrasaccharide-KLH	22,750	1,060	135
Ra-tetra-2	Rabbit	Tetrasaccharide-KLH	2,250	715	140
Ra-PS-1	Rabbit	PS23F-KLH	11,750	985	690
Ra-PS-2	Rabbit	PS23F-KLH	40,250	2,180	19,000
Ra-Spn	Rabbit	*S. pneumoniae* 23F	935,000	1,075	44,750
Hu-PS-1[b]	Human	Pneumococcal PS vaccine	1,800	1,400	4,100
Hu-PS-2	Human	None	1,450	1,700	11,000

[a] Titers were determined by ELISA using microtiter plates coated directly with either PS23F or C-PS.

[b] Defibrinated plasma. It should be noted that rabbit and human Ab titers may not be compared with each other, since different peroxidase conjugates were used for their determinations.

Measurement of anti-PS23F and anti-C-PS Ab. PS23F-specific Ab in rabbit and mouse sera were measured by an enzyme-linked immunosorbent assay (ELISA) using avidin-captured biotinylated PS23F (35). The horseradish peroxidase conjugates used were goat anti-rabbit IgM (Fc) (Nordic Immunological Laboratories, Tilburg, The Netherlands) and goat anti-rabbit IgG (heavy chain) (Southern Biotechnology Associates, Inc., Birmingham, Ala.). The specificity of the ELISA was checked by an inhibition ELISA (30) using PS17F, C-PS, and PS23F as inhibitors and various rabbit sera. PS23F, in contrast to the other inhibitors, displayed a concentration-dependent inhibition. Between 72 and 96% inhibition was observed for the different antisera when a 1.1 μM concentration of PS23F repeating units was used.

PS23F-specific Ab in human sera could not be measured in the avidin-biotin ELISA because of the presence of antiavidin Ab in these sera. Therefore, to measure human anti-PS23F Ab, polyvinylchloride microtiter plates (Titertek, 173-05 activated; Flow Laboratories, Amsterdam, The Netherlands) were coated directly with PS23F (10 μg/ml in saline) overnight at 37°C. The plates were washed four times with phosphate-buffered saline containing 0.05% Tween 20 and incubated for 2 h at 37°C with test serum dilutions (in saline containing 0.05% Tween 20, 1% BSA, and 5 μg of C-PS per ml). After being washed, the plates were incubated with a horseradish peroxidase-labeled conjugate [goat anti-IgG (Fc) (Pel Freez Biologicals, Rogers, Ark.) or sheep anti-IgM (ICN Biomedicals, Inc., Costa Mesa, Calif.)] diluted in saline containing 0.05% Tween 20 and 1% BSA, and after further washing a tetramethylbenzidine-H_2O_2 substrate solution was added. The reaction was terminated after 20 min by the addition of 1 M H_2SO_4, and the A_{450} was measured spectrophotometrically. The specificity of the ELISA was checked by inhibition with PS23F and PS6B. Detection of anti-C-PS Ab was blocked by the addition of 5 μg of C-PS per ml to the serum dilutions.

Anti-C-PS IgG in human and rabbit sera was measured by the same method as described for human anti-PS23F IgG, using C-PS (10 μg/ml in saline) for coating the plates and omitting the addition of C-PS to the serum dilutions. The amount of specific Ab in the serum is expressed in titers, calculated as the reciprocal dilution giving an absorbance of 0.5.

Inhibition ELISA. Plates were coated directly with PS23F (see above). Either rabbit or human sera or their purified IgG (all at a dilution giving an absorbance of about 0.5) were incubated in duplicate overnight at 4°C in the presence of different amounts of inhibitors. Fifty microliters of each serum-inhibitor mixture was then pipetted into the PS23F-coated ELISA plate and incubated for 90 min at 37°C with mixing. The ELISA was then performed as described above for human sera, using horseradish peroxidase-labeled goat anti-IgG. Percentages of inhibition were calculated as follows, after subtraction of the absorbance of control wells, to which no serum was added: percent inhibition = [1 − (absorbance of serum with inhibitor/absorbance of serum without inhibitor)] × 100%.

Phagocytosis assay. Human PMN were isolated from peripheral blood of healthy volunteers. A total of 30 ml of heparinized blood was mixed with 30 ml of phosphate-buffered saline (pH 7.4), layered on Ficoll-Paque, and centrifuged for 20 min at 400 × *g*. The precipitate containing PMN and erythrocytes was washed once in Hanks balanced salt solution containing 0.1% gelatin (HBSS), and the erythrocytes were lysed by two consecutive hypotonic shocks. The purity and viability of PMN were more than 95%. *S. pneumoniae* type 23 (strain ATCC 6323) was grown overnight at 37°C in a 5% CO_2 atmosphere in brain heart infusion medium containing 0.2% glucose. After being washed with HBSS without gelatin, bacteria were labeled with fluorescein isothiocyanate (0.5 mg/ml in HBSS without gelatin) for 1 h at 37°C, washed twice, and resuspended in HBSS. Samples of 5×10^6 bacteria were pipetted into round-bottomed microtiter plates, and dilutions of heat-inactivated (30 min at 56°C) rabbit or human sera or their purified IgG were made in HBSS and added. Each serum concentration was tested in duplicate. The opsonization, in a volume of 50 μl, was performed at 37°C with shaking on a microtiter plate agitator. Opsonization was performed in the absence of complement to favor the opsonic activity of IgG over that of IgM, which is a much stronger activator of complement (4, 25). After 30 min of opsonization, plates were placed on ice and 5×10^5 PMN (in 50 μl of HBSS) were added to each well. Phagocytosis was performed for 30 min at 37°C with shaking. After being washed twice with ice-cold HBSS, samples were resuspended in 200 μl of HBSS and transferred to tubes for analysis in a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.). Phosphate-buffered saline–2% paraformaldehyde (0.5 ml) was added to each tube. The mean fluorescence of the cells was used as a measure for both uptake and binding of bacteria by PMN (referred to as phagocytosis).

RESULTS

Immunogenicities of conjugates. A synthetic trisaccharide, a tetrasaccharide, and native PS23F were coupled to KLH. The molar ratios of trisaccharide and tetrasaccharide to KLH were similar (Table 1). The monosaccharide ratios in these conjugates do not correspond with the expected ratios because of the heterogeneous nature of the carrier. The total amounts of saccharide and protein in the conjugates were determined by the phenol-sulfuric acid assay and a protein assay, respectively. Since KLH contains substantial amounts of sugars, including galactose and glucose (detectable by the phenol-sulfuric acid assay), the determination of the weight/weight ratios is approximate, so that corrections for the monosaccharides found in KLH can give rise to errors.

The immunogenicities of pneumococcal type 23F antigens were initially investigated by immunizing mice with formalin-killed *S. pneumoniae* 23F, native PS23F-KLH conjugate, or

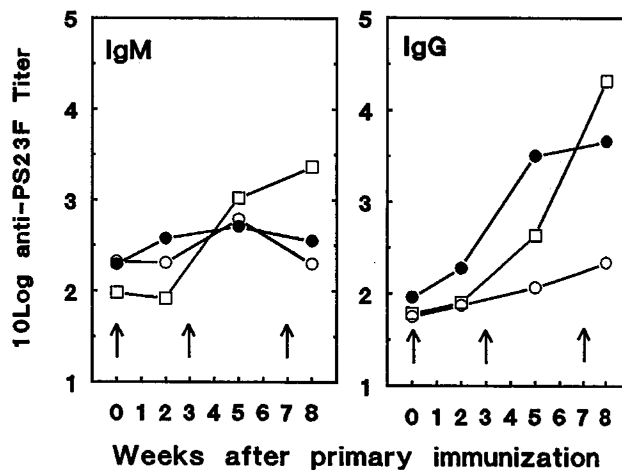

FIG. 2. Rabbit anti-PS23F Ab response to synthetic trisaccharide-KLH (○), tetrasaccharide-KLH (●), and native PS23F-KLH (□) conjugates. Rabbits (two in each group) were immunized subcutaneously with the conjugates emulsified in complete Freund's adjuvant and boosted intraperitoneally at days 21 and 49 (arrows) with the conjugates in incomplete Freund's adjuvant. IgM and IgG titers in the sera were determined by ELISA.

synthetic tetrasaccharide-KLH conjugate. The IgM and IgG responses induced by the first two antigens were weak, while tetrasaccharide-KLH did not induce any response (data not shown). In view of the low immunogenicities of these antigens in mice, rabbits were immunized with either trisaccharide-KLH, tetrasaccharide-KLH, or PS23F-KLH. A clear anti-PS23F IgM response was induced only by the PS23F-KLH conjugate, not by tetrasaccharide-KLH or trisaccharide-KLH (Fig. 2). The IgG response induced by tetrasaccharide-KLH had an earlier onset than the response to PS23F-KLH (Fig. 2). However, the magnitude of the response induced by the PS23F-KLH conjugate was greater. The trisaccharide-KLH conjugate induced a small increase in IgG titer.

Specificities of rabbit and human anti-PS23F IgGs. The synthetic glycerol-phosphorylated tetrasaccharide representing one repeating unit of PS23F, coupled to KLH, was able to induce anti-PS23F Ab in rabbits. We were interested in the specificity of these Ab compared with that of Ab elicited by antigens containing native PS23F. Sera from rabbits immunized with trisaccharide-KLH were not included in these experiments because of the low anti-PS23F IgG titer. The specificity of IgG was investigated by inhibition ELISA performed on plates coated directly with PS23F, in order to allow comparison of the inhibition patterns of rabbit and human IgGs.

The concentration of PS23F needed to reach 50% inhibition, which is a reciprocal estimation of the relative avidity of the Ab for PS23F (30), was similar for all rabbit antisera (Fig. 3). Rhamnose was a potent inhibitor of the rabbit IgG-PS23F interaction, except in Ra-PS-2, in which it was a relatively weak inhibitor. In some of the sera glycerol induced a slight inhibition. In Ra-tetra-2, glycerol, glucose, and especially galactose also exhibited substantial inhibition, but the concentrations needed to reach the same level of inhibition as with rhamnose were 10 to 200 times higher.

For human anti-PS23F IgG, PS23F exhibited a 50% inhibitory concentration similar to that for rabbit IgG, although it did not reach total inhibition (Fig. 4). However, rhamnose, like the other monosaccharides, did not exceed 20% inhibition,

FIG. 3. Inhibition ELISA of rabbit IgG, using as inhibitors PS23F, three monosaccharides, and glycerol. Rabbit sera (abbreviations listed in Table 2) were preadsorbed with different concentrations of inhibitors. Subsequently, anti-PS23F IgG was measured by ELISA on PS23F-coated microtiter plates. The concentration of PS23F is expressed in repeating units. Each value represents the mean of two to four experiments.

which is in contrast to the results obtained with the rabbit antisera. Glycerol inhibited the ELISA to approximately the same extent as in most rabbit antisera. The same results were also observed when inhibition experiments were performed with two other serum pools (anti-PS23F IgG titers, 1,100 and 900 [data not shown]). To exclude the possibility that inhibition by rhamnose might have been obscured by insufficient sensitivity of the assay, inhibition ELISA was performed with

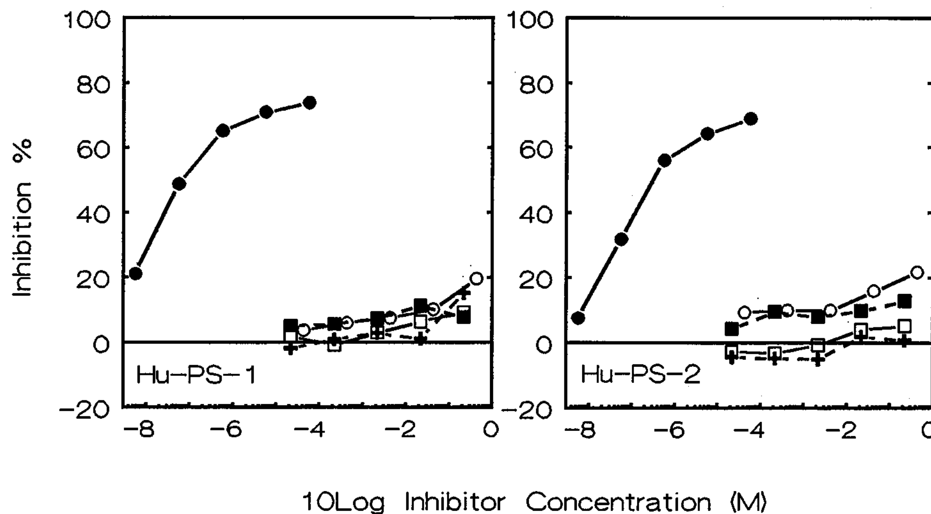

FIG. 4. Inhibition ELISA of human IgG, using as inhibitors PS23F, three monosaccharides, and glycerol. The abbreviations of IgG sources are shown in Table 2. The inhibition ELISA was performed as described in the legend to Fig. 3. Symbols are as in Fig. 3.

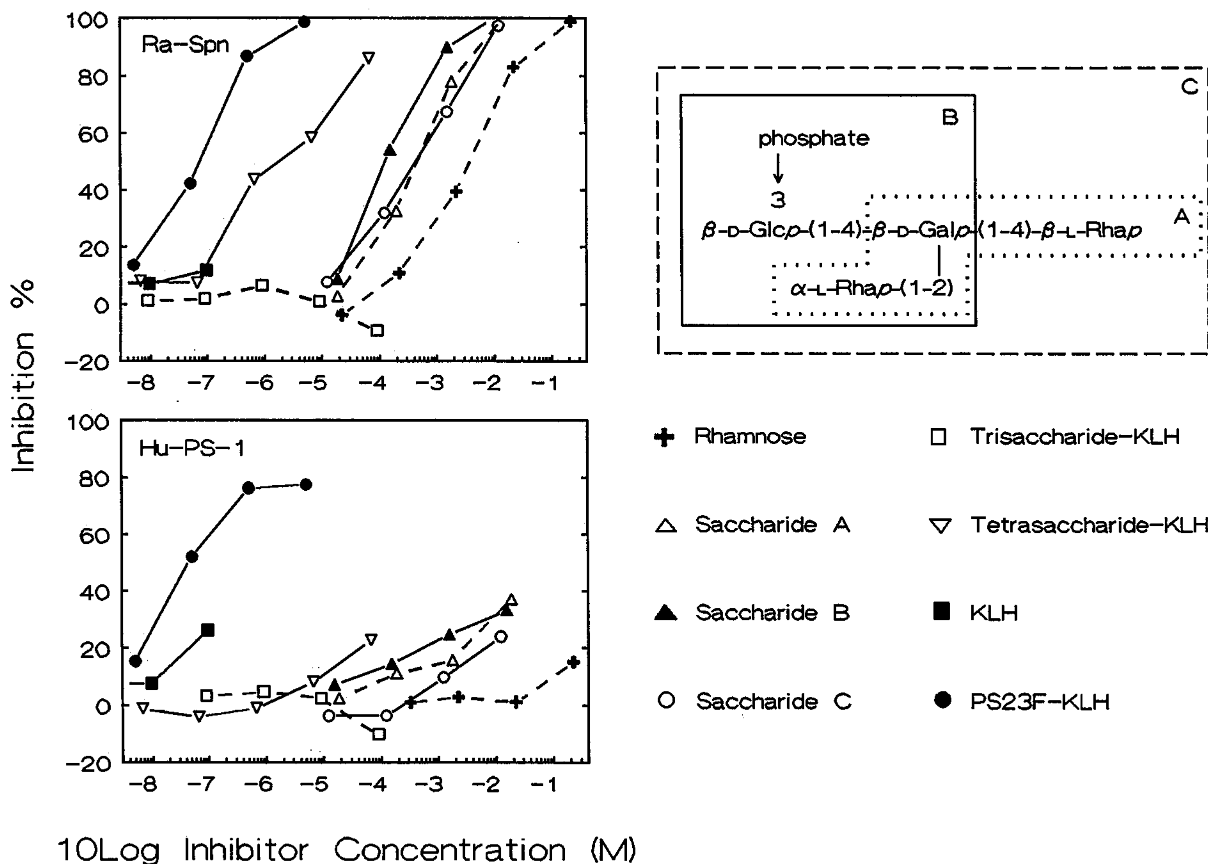

FIG. 5. Inhibition ELISA of rabbit and human IgGs, using as inhibitors two synthetic trisaccharides and one tetrasaccharide derived from an incorrect previously described PS23F structure (upper right panel) (15), synthetic trisaccharide-KLH, tetrasaccharide-KLH, and native PS23F-KLH conjugates (Table 1), and KLH. The inhibition curve of L-rhamnose is shown for comparison. Rabbit or human serum (abbreviations listed in Table 2) was preadsorbed with different concentrations of inhibitors. Subsequently, anti-PS23F IgG was measured by ELISA. The concentrations of OSs and their conjugates are expressed in antigenic determinants: tetrasaccharides, trisaccharides, or repeating units of PS23F. The highest concentrations of free KLH used exceeded the KLH concentrations present in the conjugates. Each value represents the mean of at least two determinations.

Hu-PS-1 and Hu-PS-2, both 10 times more diluted than in the experiments whose results are shown in Fig. 4, and an accordingly increased concentration of the peroxidase-labeled second Ab. Despite the increased sensitivity of the ELISA (the 50% inhibitory concentration of PS23F was three to four times lower), rhamnose still did not exceed 15% inhibition. When inhibition ELISA was carried out with Ra-Spn at a 10-fold-higher concentration than in the former experiments (thereby lowering the sensitivity), the 50% inhibitory concentration of both PS23F and rhamnose increased two- to threefold (data not shown).

In order to confirm the marked difference in the inhibition patterns of human and rabbit anti-PS23F IgGs and to exclude a possible (blocking) effect of IgM present in the sera, IgG was purified from Ra-Spn and from Hu-PS-2, and the ELISA was repeated with these fractions. The results were virtually the same: both PS23F and rhamnose inhibited by 100% the binding of Ra-Spn IgG, whereas the binding of Hu-PS-2 IgG was exclusively inhibited by PS23F (79%). Rhamnose did not exceed 3% inhibition. To exclude the possibility that differences among individuals might have been obscured by pooling the sera, 20 individual sera (of which 9 were from vaccinated donors) were tested for inhibition. The results were essentially the same: the mean inhibition ($\pm$ standard deviation) obtained at the highest rhamnose concentration was 4.7 $\pm$ 13%.

We wished to further define the binding site of rabbit anti-PS23F IgG compared with that of human IgG. Therefore, inhibition experiments were performed with Ra-Spn and Hu-PS-1, using as inhibitors various synthetic OSs and OS-KLH conjugates (Fig. 5). All structures containing the α(1→2)-linked L-rhamnose residue (synthetic saccharides A, B, and C as β-methyl glycosides and the tetrasaccharide-KLH and PS23F-KLH conjugates) were potent inhibitors of IgG in Ra-Spn. The tetrasaccharide-KLH conjugate was superior to the uncoupled OSs, and free OSs were in turn better inhibitors than rhamnose. Trisaccharide B, which bears a phosphate group at glucose instead of a glycerolphosphate group at galactose (as present in PS23F), was a slightly better inhibitor than the other OSs, including trisaccharide A, which is a nonglycerolphosphorylated fragment of PS23F. This was also observed in a precipitin inhibition system (data not shown). The trisaccharide-KLH conjugate, which lacks the α(1→2)-linked L-rhamnosyl residue, exhibited no inhibition. The inhibition pattern of Hu-PS-1 differed markedly from that of Ra-Spn, since none of the OSs or OS conjugates achieved inhibition exceeding 40%. Trisaccharides A and B induced between 35 and 40% inhibition, and the trisaccharide-KLH conjugate, like in the ELISA with Ra-Spn, showed no inhibition at all. Tetrasaccharide-KLH induced approximately the same level of inhibition as KLH.

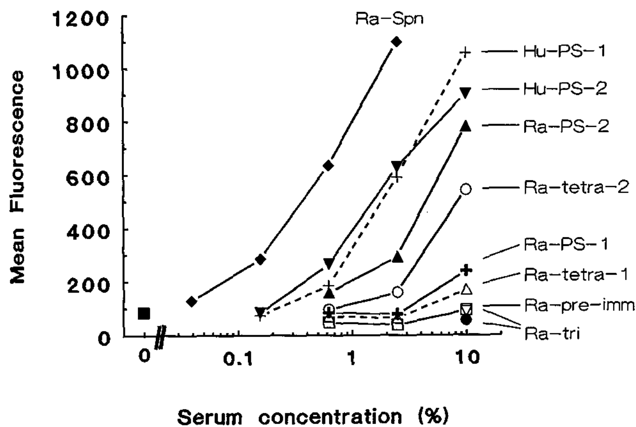

FIG. 6. Mean fluorescence of human PMN after phagocytosis of fluorescein isothyocyanate-labeled type 23F pneumococci previously opsonized with heat-inactivated rabbit or human sera at various concentrations. Most of the sera are listed in Table 2. Ra-pre-imm, pool of preimmune sera from the different rabbits; Ra-tri, rabbit sera obtained after three immunizations with synthetic trisaccharide-KLH conjugate. Each value represents the mean fluorescence of two determinations from a representative experiment.

Opsonic activities of human and rabbit anti-PS23F Ab. The effect of opsonization of *S. pneumoniae* 23F by various rabbit sera on phagocytosis by human PMN was investigated. Opsonization was performed in the absence of complement to favor IgG-dependent phagocytosis, since complement strongly increased the opsonic activity of all sera, including the preimmune sera (data not shown). Ra-Spn was the most potent serum in inducing phagocytosis of type 23F pneumococci (Fig. 6). Ra-PS-2 and Ra-tetra-2 were less efficient than Ra-Spn in inducing phagocytosis; about 15 and 25 times higher concentrations of Ra-PS-2 and Ra-tetra-2, respectively, were necessary to achieve a mean fluorescence of 400. Ra-PS-1 and Ra-tetra-1 induced a slight increase in phagocytosis only at the highest concentration tested. Sera from rabbits immunized with the synthetic trisaccharide-KLH conjugate had hardly any effect on the phagocytosis. The same result was obtained with individual preimmune sera from the different rabbits (only results for a pool of these sera are shown in Fig. 6). A rabbit antiserum obtained by immunization with a C-PS–KLH conjugate (anti-C-PS IgG titer, 100,000) strongly induced phagocytosis. This serum was only two times less efficient than Ra-Spn (not shown). Both human sera used for inhibition studies were also tested in the phagocytosis assay. Hu-PS-1 and -2 were equally opsonic, i.e., about five times less efficient than Ra-Spn. Human serum from a hypogammaglobulinemic patient, depleted of residual IgG by protein A affinity chromatography, had no effect on phagocytosis (mean fluorescence, <50). IgGs purified from Ra-Spn and from Hu-PS-2 induced similar levels of phagocytosis as did their respective sera; Ra-Spn IgG was about four times more efficient than Hu-PS-2 IgG. To study the possible opsonic activity of human anti-C-PS Ab, Hu-PS-2 was depleted of C-PS IgG by adsorption to C-PS-coated beads (about 93% of anti-C-PS IgG was removed). The opsonic activity of this depleted serum was tested in a phagocytosis experiment alongside the original Hu-PS-2. The depleted serum induced a mean ($\pm$ standard deviation) fluorescence of 258 $\pm$ 30, and the original serum induced a fluorescence of 204 $\pm$ 7 ($P < 0.05$ by Student's t test). This shows that removal of anti-C-PS IgG results in a slight increase in phagocytosis. The mean fluorescence of PMN after phagocytosis of nonopsonized bacteria in this experiment was 6 $\pm$ 0.5. In another experiment, performed with formalin-killed type 23F pneumococci, adsorption of anti-C-PS IgG also did not result in a decrease in phagocytosis (data not shown).

DISCUSSION

In this study we investigated the immunogenicities of pneumococcal type 23F antigens in mice and rabbits, we compared the epitope specificity of IgG elicited in rabbits with that of human anti-PS23F IgG, and we assessed the opsonic capacities of rabbit and human antisera in a phagocytosis assay. The immunogenicities of the pneumococcal type 23F antigens investigated differed between mice and rabbits. The low responses in mice to type 23F antigens might be due to the lack of appropriate V genes encoding PS23F-specific Ab, since mice display strong immune responses to similar antigens of pneumococcal serotypes 3, 4, 14, and 17F (2, 3, 23, 27, 34). Another possible explanation could be that type 23F antigens are rapidly cleared by a mechanism involving recognition of PS23F by lectin-like molecules (19), as seems to be the case for type 14 pneumococci (36). This is also suggested by the low virulence of type 23F pneumococci in mice (7).

In rabbits, most type 23F antigens induced moderate to strong responses (Fig. 2). Synthetic tetrasaccharide-KLH induced a clear anti-PS23F IgG response, whereas the trisaccharide-KLH conjugate evoked a very low IgG response. The low anti-PS23F response to trisaccharide-KLH might be due to the lack of immunogenicity of this conjugate. Alternatively, the Ab induced may not recognize the native PS23F. Since the trisaccharide and the tetrasaccharide conjugates have similar chemical characteristics (Table 1), the latter explanation seems more likely. The trisaccharide lacks the $\alpha(1\rightarrow2)$-linked L-rhamnose, which is part of an immunodominant epitope of PS23F in rabbits, and therefore the Ab induced by this conjugate probably do not recognize native PS23F.

Rabbit anti-PS23F IgG was inhibited by rhamnose regardless of the immunogen used (Fig. 3), as was observed in precipitin experiments with horse antisera (14). The inhibition pattern of rhamnose was similar in all rabbit antisera except Ra-PS-2, which suggests that the specificity of rabbit IgG is not dependent on the length of the type 23F saccharide used for immunization. Of the two rhamnose residues present in the PS23F repeating unit (Fig. 1), the $\alpha(1\rightarrow2)$-linked L-rhamnose (nonreducing end) is most likely involved in the interaction with IgG. All OSs (either free or conjugated) containing this residue were potent inhibitors of Ra-Spn, while trisaccharide-KLH, which lacks $\alpha(1\rightarrow2)$-linked L-rhamnose, displayed no inhibition (Fig. 5). Furthermore, the saccharide without $\beta(1\rightarrow4)$-linked L-rhamnose (saccharide B) was a somewhat stronger inhibitor than saccharides A and C, both of which contain $\beta(1\rightarrow4)$-linked L-rhamnose. These results demonstrate that the $\alpha(1\rightarrow2)$-linked L-rhamnose residue is a main part of the immunodominant epitope(s) of PS23F recognized by rabbit IgG, as was already suggested for horse antisera (14). Another structure which is presumably recognized by rabbit IgG is glycerol. Its contribution to the binding, however, is small. The specificity of IgG in Ra-tetra-2 differs slightly from those in the other rabbit antisera. It contains IgG which binds not only to rhamnose but also to galactose (as was observed in horse antisera by Heidelberger and colleagues [14]) and, to a lesser extent, to glucose and glycerol.

Although the inhibition ELISA of human IgG may not be fully comparable with inhibition of IgG in rabbit sera (because of the different peroxidase-labeled second Ab), the 50%

inhibitory concentration of PS23F is very similar in both species; therefore, one can compare the inhibition patterns generated by the different inhibitors in human and rabbit sera. The lack of complete inhibition of human IgG by PS23F and PS23F-KLH is probably due to greater nonspecific adherence of human IgG to ELISA plates compared with rabbit IgG. Both human sera, either induced by the 23-valent pneumococcal PS vaccine or from nonimmunized healthy adults, showed no inhibition by any monosaccharide. The inhibition by glycerol was similar to that in rabbit sera (Fig. 3 and 4). These results were shown to be representative, since two other serum pools and 20 individual sera displayed the same pattern of inhibition. Among the OSs and OS-KLH conjugates tested, none exceeded 40% inhibition in Hu-PS-1, although there was moderate inhibition by the three unconjugated synthetic OSs and especially the tetrasaccharide-KLH conjugate (Fig. 5). Furthermore, additional ELISAs were carried out under conditions which enhance the sensitivity of the assay. In order to detect any putative inhibition by rhamnose in human sera, we used serum concentrations at which the 50% inhibitory concentration of PS23F in Hu-PS-1 was 15-fold lower than that in Ra-Spn, but no inhibition of Hu-PS-1 by any monosaccharide was observed. Moreover, the results obtained with whole sera were confirmed by experiments performed with purified IgG, so that the possibility that other antibody classes (mainly IgM) might have interfered with binding of IgG to PS23F can be excluded. Since the specificity of human IgG appears to be similar in sera from vaccinated and nonvaccinated individuals, we believe that the observed specificity of human IgG is independent of how the immunogen is presented. The relatively high titer of Hu-PS-2 is probably caused by carriage of type 23F pneumococci by (some of) the donors, although the presence of cross-reacting Ab induced by related antigens can not be excluded. However, the latter possibility is unlikely because Hu-PS-1 and Hu-PS-2 seem to have the same specificity, and Hu-PS-1 IgG is a response to PS23F present in the pneumococcal vaccine. The different specificity of rabbit or horse versus human IgG is most likely due to differences in their V region repertoires for PS23F. Alternatively, it remains possible that humans immunized with a PS23F conjugate might produce IgG recognizing $\alpha(1\rightarrow2)$-linked L-rhamnose. In rabbits, however, the specificity of IgG is not influenced by the type of immunogen, and the unresponsiveness of mice was also not affected by using three different type 23F antigens.

The failure to find small structures with strong inhibitory capacities in human sera may imply that human IgG possesses a larger antigen-binding site than rabbit IgG. This seems in accordance with the weak inhibition by tetrasaccharide-KLH. It has been suggested that rabbits can produce two types of Ab against carbohydrate-containing antigens: Ab with a cavity-like antigen-binding site, which seems to recognize one or two monosaccharides, and Ab with a groove-like antigen binding site, which would recognize elongated sugar epitopes consisting of up to six or seven monosaccharides (38). A large portion of the IgG elicited by type 23F antigens in rabbits presumably has cavity-like binding sites and can be inhibited by monosaccharides. In contrast, human anti-PS23F IgG recognizes larger epitopes and therefore seems to have a groove-like binding site. Another explanation for the weak inhibition of human IgG by OSs or OS-KLH conjugates, which is compatible with the former one, is that the epitope recognized by human IgG on PS23F does not correspond with the arbitrary delimitation of the repeating unit, on which the synthesis of the tetrasaccharide is based. Hence, it is possible that human anti-PS23F IgG binds to an epitope which occurs in PS23F in its polymeric form but which has been split in the synthetic OS. Alternatively, human IgG may recognize conformational epitopes present only in the polymeric PS23F.

It is unlikely that small OSs express conformational epitopes, which might be present in the native form of PS23F. The lack of such epitopes in OSs may result in lower effectiveness of the Ab induced by OS-protein conjugates compared with that of Ab induced by PS-protein conjugates or whole pneumococci. It was recently shown that in group B streptococcal type III OS-protein conjugates, shortening the OS length from 14 to 6 repeating units resulted in impaired functionality of the Ab induced, in terms of passive protection and opsonizing activity (22).

We were not able to assess the passive protection mediated by these antisera, since *S. pneumoniae* 23F shows hardly any virulence in mice (7). However, we determined the opsonic activities of the different sera in a phagocytosis assay. Ra-Spn, followed by Ra-PS-2, were the most potent rabbit sera in inducing phagocytosis of type 23F pneumococci (Fig. 6). This seems in accordance with the high anti-PS23F titers of these antisera, although a role of anti-C-PS Ab in promoting phagocytosis cannot be excluded (Table 2). The relatively high opsonic capacity of Ra-tetra-2, in spite of its low anti-PS23F and anti-C-PS IgG titers, might be related to the slightly different specificity of IgG in this rabbit. Taking the anti-PS23F IgG titers of the different sera into account, none of the three type 23F capsular antigens is apparently superior in inducing highly opsonic Ab in rabbits. Rabbit antisera induced by trisaccharide-KLH were not opsonic, which is in accordance with their low anti-PS23F titers. It is noteworthy that rabbit anti-C-PS Ab were highly opsonic, in contrast to mouse anti-C-PS (1) and human antiphosphocholine Ab (8). This work also provides some evidence that human anti-C-PS Ab are not opsonic, since depletion of anti-C-PS Ab from Hu-PS-2 did not decrease (but rather slightly enhanced) the opsonic activity of Hu-PS-2. However, the opsonic activity of purified anti-C-PS Ab should be tested in order to definitely demonstrate that human anti-C-PS Ab are not opsonic in this phagocytosis assay.

The human sera Hu-PS-1 and -2 were equally potent in inducing phagocytosis, consistent with their similar anti-PS23F IgG titers. This may indicate that their IgG subclass distributions are similar, since IgG subclasses differ strongly in opsonic activity. On the basis of experiments with adsorbed serum, a substantial opsonic effect of human anti-C-PS Ab can be regarded as unlikely; hence, it seems that human anti-PS23F IgG is efficient in promoting phagocytosis of type 23F pneumococci, in spite of its different epitope specificity compared with rabbit Ab. Since the epitope specificity of human IgM was not determined in the present study, IgM with a specificity different from that of IgG might have interfered with the results of the phagocytosis assay. However, IgM may have only a blocking effect, because the opsonization of pneumococci was performed in the absence of complement, and the opsonic capacity of IgM is attributed to its highly efficient complement fixation and not to its direct interaction with phagocytes via Fc receptors (4, 25). However, even a blocking effect of IgM seems unlikely, because the opsonic activities of IgGs purified from Ra-Spn and Hu-PS-2 were similar to the opsonic activities of their respective sera. A reliable comparison between the opsonic activities of rabbit and human sera unfortunately cannot be made by using a phagocytosis assay as the read-out system, since the differences between the Ab of the two species may be due to their distinct interactions with Fc receptors on human PMN. It is likely that human IgG promotes phagocytosis more efficiently than rabbit IgG. Using the mouse macrophage cell line J774, we have developed a phagocytosis assay

similar to that described here which is suitable for testing the opsonic activity of mouse antisera specific for PS17F (1). Anti-C-PS Ab do not stimulate phagocytosis in that assay; hence, it is especially useful for the assessment of the opsonic capacity of mouse Ab induced by pneumococcal saccharide vaccines.

This study confirms that there exist differences among species in their responses to carbohydrate antigens and emphasizes the need for studies with humans before predictions about the putative efficacies of saccharide vaccines in humans are made.

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