INFECTION AND IMMUNITY, Aug. 1994, p. 3236–3243
0019-9567/94/$04.00+0

Vol. 62, No. 8

Neonatal Mouse Protection against Infection with Multiple Group B Streptococcal (GBS) Serotypes by Maternal Immunization with a Tetravalent GBS Polysaccharide-Tetanus Toxoid Conjugate Vaccine†

LAWRENCE C. PAOLETTI,[1]* MICHAEL R. WESSELS,[1,2] ARIANE K. RODEWALD,[1] AMY A. SHROFF,[1] HAROLD J. JENNINGS,[3] AND DENNIS L. KASPER[1,2]

Channing Laboratory, Brigham and Women's Hospital,[1] and Division of Infectious Diseases, Beth Israel Hospital,[2] Harvard Medical School, Boston, Massachusetts 02115, and Division of Biological Sciences, National Research Council of Canada, Ottawa, Ontario K1A 0R6, Canada[3]

Received 14 January 1994/Returned for modification 7 April 1994/Accepted 4 May 1994

Most cases of neonatal sepsis and meningitis caused by group B streptococci (GBS) are attributable to one of four major capsular serotypes: Ia, Ib, II, or III. Because resistance to infection with GBS has been correlated with the presence of serum antibodies to the type-specific capsular polysaccharides in both experimental animals and human neonates, efforts have been made to elicit protective immunity with GBS capsular polysaccharide vaccines. However, the GBS capsular polysaccharides alone are not highly immunogenic in either animals or human volunteers. Therefore, we and other investigators have attempted to enhance immunogenicity by coupling individual capsular polysaccharides to a carrier protein. Here we report the synthesis and immunogenicity in rabbits of a GBS type Ib polysaccharide-tetanus toxoid vaccine prepared by the direct, covalent attachment of tetanus toxoid to a selected number of sialic acid residues on the type-specific polysaccharide. In addition, the Ib polysaccharide-tetanus toxoid conjugate vaccine was combined with similar tetanus toxoid conjugates of GBS type Ia, II, and III polysaccharides to form a tetravalent GBS conjugate vaccine. Protective efficacy of the GBS tetravalent conjugate vaccine was demonstrated in a mouse maternal immunization-neonatal challenge model of GBS infection. The results support testing in human subjects of a multivalent GBS conjugate vaccine of this design, with the eventual goal of protecting newborns against GBS infection.

Streptococcus agalactiae, or Lancefield's group B *Streptococcus* (GBS), is a major cause of sepsis, meningitis, and pneumonia in the newborn infant (2). Early-onset disease (manifested within the first 7 days of age) has an incidence of 1.3 to 3.0 per 1,000 live births and is associated with a mortality rate of 20%, whereas late-onset infection (developing at >7 days of age) has an incidence of 1.0 to 1.7 per 1,000 live births and is associated with a mortality rate of 10% (2). Four of the seven GBS serotypes identified thus far (Ia, Ib, II, and III) are responsible for the majority of reported cases of infection in both neonates and adults in the United States (2, 12). In addition to these four recognized major serotypes, recent reports of invasive infections caused by type V GBS suggest that this newly characterized serotype also may account for a significant fraction of neonatal infections now or in the future (15, 17, 28).

Immunologic strategies proposed for the prevention of GBS disease include treatment of newborns with intravenous immunoglobulin preparations (13, 31) and active immunization of women with a vaccine against GBS (4, 6). The goal of both approaches is to supply the newborn with protective levels of immunoglobulin G (IgG) specific for the GBS capsular polysaccharide antigen, because antibodies to this cell surface component are protective (1, 3, 4, 7, 9, 11). Capsular polysaccharides of types Ia, II, and III have been tested as experimental vaccines in volunteers (5). The rate of immune response among adults with low preexisting antibody levels who were given purified GBS capsular polysaccharide ranged from 40% for type Ia to 88% for type II (5). To enhance their immunogenicity, type Ia, II, and III GBS polysaccharides have been coupled to immunogenic protein carriers to form polysaccharide-protein conjugate vaccines (20, 25, 26, 32, 33). Despite differences in coupling strategy and in the choice of protein carrier, all GBS glycoconjugate vaccines exhibited greater immunogenicity in laboratory animals than did uncoupled GBS polysaccharides. Lagergard et al. (20) coupled cyanogen bromide-activated GBS type III capsular polysaccharide to tetanus toxoid (TT) by carbodiimide reduction, with adipic acid dihydrazide as a spacer. When administered to mice, this conjugate vaccine elicited type III-specific IgG that opsonized GBS type III organisms (20). A coupling method similar to that used by Lagergard et al. (20) was used in the generation of a trivalent (types Ia, II, and III) GBS glycoconjugate vaccine, with detoxified *Pseudomonas aeruginosa* toxin A as the carrier protein (26). In rabbits, this trivalent vaccine elicited antibodies that were opsonically active in vitro against type Ia, II, and III GBS (26).

In contrast to the glycoconjugates described above, all saccharide-protein conjugates synthesized and tested in our laboratory have utilized reductive amination as the coupling method. We have reported the synthesis and immunogenicity testing of oligosaccharide-protein conjugate vaccines based on the GBS type III polysaccharide (23, 24) and of type Ia, II, and

* Corresponding author. Mailing address: Channing Laboratory, 180 Longwood Ave., Boston, MA 02115. Phone: (617) 432-2678. Fax: (617) 731-1541. Electronic mail address: lcpaolet@warren.med.harvard.edu.

† National Research Council of Canada paper 37390.

III polysaccharide-protein conjugates (25, 32, 33). While the type III oligosaccharide-TT conjugates were useful in relating particular structural features of glycoconjugate vaccines to the immune response, their synthesis is time-consuming and relatively inefficient. Conjugates such as those described in this report have been constructed by a much simpler method with the full-length polysaccharides of types Ia (33), Ib (this report), II (25), and III (32). The full-length polysaccharide-TT vaccines were synthesized by controlled periodate oxidation of a limited number of side chain sialic acid residues and covalent coupling of the modified residues to TT to produce glycoconjugates in which the polysaccharide was linked at multiple sites to the carrier protein. Coupling at multiple sites along the saccharide may help to optimize the physical relation between B-cell epitopes on the polysaccharide and T-cell epitopes of the carrier protein, accounting for the high-titer, polysaccharide-specific IgG response to these vaccines. Antiserum raised in rabbits to each of the GBS polysaccharide-protein conjugate vaccines was opsonic in vitro and protected mice against challenge with organisms of the vaccine serotype. The goal of the current studies was to formulate a multivalent vaccine capable of stimulating antibodies to at least 90% of GBS clinical isolates. This objective may be achieved by a tetravalent vaccine containing TT conjugates of the type-specific polysaccharides of the four major capsular types of GBS.

We now report the synthesis and immunogenicity testing in animals of a GBS type Ib polysaccharide-TT (Ib-TT) conjugate vaccine. Rabbit antiserum to the Ib-TT vaccine was opsonic for GBS type Ib organisms and was serotype specific. The Ib-TT vaccine was included as a component of a GBS tetravalent conjugate vaccine that also included Ia-TT, II-TT, and III-TT vaccines. When administered to female mice, the GBS tetravalent conjugate vaccine protected their neonatal pups against an ordinarily lethal challenge with organisms of the four homologous GBS serotypes. These results demonstrate the ability of a tetravalent GBS glycoconjugate vaccine to protect neonatal mice against multiple GBS serotypes by means of active maternal vaccination.

MATERIALS AND METHODS

Bacterial strains. GBS type Ia strain 515, type Ib strain H36B, type II strain 18RS21, and type III strain M781 were used to challenge neonatal mouse pups in a model of GBS infection. The 50% lethal doses for these GBS strains in neonatal mouse pups, as determined by the method of Reed and Muench (27), were as follows: 1.6×10^3 (type Ia strain 515), 4.0×10^5 (type Ib strain H36B), 1.6×10^4 (type II strain 18RS21), and 4.0×10^4 (type III strain M781). Strains 090 (type Ia), H36B (type Ib), 18RS21 (type II), and M781 (type III) were used to prepare type-specific polysaccharide by methods described previously for the purification of type III polysaccharide (32).

Protective efficacy of GBS trivalent conjugate vaccine. A maternal immunization-neonatal challenge model of GBS infection in mice was used to assess the protective efficacy of GBS vaccines (22, 29). Female CD-1 outbred adult mice (Charles River Laboratory, Wilmington, Mass.) were vaccinated with either a GBS trivalent conjugate vaccine (types Ia-TT, II-TT, and III-TT) or a mixture of GBS type Ia, II, and III polysaccharides and uncoupled TT. The GBS trivalent conjugate vaccine was composed of 2 μg each of Ia-TT, II-TT, and III-TT in a total volume of 0.3 ml of phosphate-buffered saline, pH 7.0 (PBS). The vaccine mixture was combined with an equal volume of complete Freund's adjuvant (CFA), and each of 10 mice received 0.6 ml by the intraperitoneal route. The trivalent mixture of unconjugated GBS polysaccharides and TT was prepared by combining 1 μg each of purified type Ia, II, and III polysaccharide with 3 μg of TT in a total volume of 0.4 ml of PBS. This vaccine was mixed with an equal volume of CFA, and each of four mice received 0.8 ml of this combination intraperitoneally. Booster doses of these vaccines were administered intraperitoneally with incomplete Freund's adjuvant on day 21. Mice were bred after receiving the second dose of vaccine. The first and second litters of pups were born to these dams approximately 46 and 79 days, respectively, after primary vaccination. Frozen GBS cultures prepared as challenge inocula were thawed, diluted to the appropriate concentration with Todd-Hewitt broth, and injected intraperitoneally (in a volume of 0.05 ml) into neonatal mouse pups of less than 36 h of age. In these experiments, the challenge doses per pup were as follows: 2×10^4 CFU for strain 515, 10^5 CFU for strain H36B, 2×10^5 CFU for strain 18RS21, and 4×10^4 CFU for strain M781. Newborn pups remained with their own dam for the duration of the 48-h observation period after challenge. Survival of pups was assessed 48 h after challenge.

GBS Ib-TT conjugate vaccine. Direct, covalent attachment of GBS type Ib capsular polysaccharide to TT was accomplished by reductive amination, as detailed previously for the conjugation of GBS type III polysaccharide (32). In brief, 31 mg of purified GBS type Ib capsular polysaccharide ($>10^6$ M_r) was oxidized with 310 μl of 0.01 M sodium metaperiodate in a total of 1.5 ml of deionized water for 90 min at room temperature in the dark. Ethylene glycol was used to consume residual periodate, and the oxidized polysaccharide was dialyzed overnight at 4°C (Spectropor no. 1, 6,000- to 8,000-molecular-weight cutoff; Spectrum Medical Industries, Inc.) against 17 liters of sterile deionized water. The degree of sialic acid oxidation was confirmed by gas-liquid chromatography-mass spectrometry of trimethylsilyl derivatives of a sample of the oxidized polysaccharide (32). Covalent attachment of TT to the free aldehyde residues on the sialic acid moieties was accomplished by reductive amination with sodium cyanoborohydride. Oxidized type Ib polysaccharide (29 mg) was combined with 29 mg of monomeric TT (150,000 M_r) in a total volume of 2.0 ml of 0.1 M sodium bicarbonate, pH 8.3. TT (kindly provided by Amvax Inc., Beltsville, Md.) was purified to its monomer form by gel filtration chromatography on a Sephacryl S-300 HR column (2.6 by 91.5 cm; Pharmacia LKB, Uppsala, Sweden). After addition of sodium cyanoborohydride (41 mg), the type Ib polysaccharide-TT mixture was incubated at 37°C for 37 days. The progress of the conjugation was monitored by analysis of aliquots, with use of a Superose 6 gel filtration column and a fast protein liquid chromatography system (Pharmacia LKB), as described previously (32). GBS Ib-TT vaccine was purified by gel filtration chromatography on the Sephacryl S-300 HR column. Fractions corresponding to the void-volume peak from this column were pooled, unreacted aldehydes were reduced by the addition of sodium borohydride, and the mixture was dialyzed against a total of 32 liters of sterile water at 4°C. The purified GBS Ib-TT vaccine was analyzed for carbohydrate content by the phenol-sulfuric acid assay (10), using galactose as the standard; for protein content by the method of Larson et al. (21), with bovine serum albumin as the standard; and for sialic acid content by the thiobarbituric acid assay (30), with sialic acid (Sigma Chemical Co., St. Louis, Mo.) as the standard.

Immunogenicity of Ib-TT vaccine in rabbits. Three female New Zealand White rabbits (Millbrook Farms, Amherst, Mass.) weighing ≈2 kg each were vaccinated subcutaneously at four sites on the back with 50 μg of Ib-TT conjugate vaccine emulsified with CFA in a total volume of 2 ml. One rabbit

TABLE 1. Composition of GBS polysaccharide-TT tetravalent conjugate vaccines

Vaccine	Polysaccharide size (M_r)	Sialic acid residues oxidized (%)	Protein (%, dry wt)	Carbohydrate (%, dry wt)	Carbohydrate/protein ratio (wt/wt)	Reference
Ia-TT	$>10^6$	25	29	71	2.4	This study
Ib-TT	$>10^6$	12	32	68	2.1	This study
II-TT	200,000	7	32	68	2.1	25
III-TT	200,000	29	53	47	0.9	This study

received a mixture of 25 μg of oxidized type Ib polysaccharide and 25 μg of TT (Ib_{ox}+TT) emulsified with CFA by the same route. Booster doses of vaccine, emulsified with incomplete Freund's adjuvant, were administered by the same route 20 and 41 days after primary immunization. Serum samples were obtained before immunization and 20, 34, 41, 55, and 69 days after the initial dose.

ELISA. Titers of polysaccharide- and TT-specific antibody were determined by an enzyme-linked immunosorbent assay (ELISA). Type Ib capsular polysaccharide was coupled to poly-L-lysine as described previously (14, 32) and was used to coat 96-well microtiter plates (Immunlon; Dynatech). Type Ib-specific antibody was detected with goat anti-rabbit IgG-conjugated alkaline phosphatase (γ and light chain specific; Tago, Inc., Burlingame, Calif.) diluted 1:3,000. The antibody titer was defined as the reciprocal serum dilution that resulted in an A_{405} of ≥0.3 when the reference serum (rabbit antiserum raised to whole GBS type Ib strain H36B cells and diluted 1:100,000) reached an A_{405} of 0.3.

Microtiter plates coated with monomeric TT were used to determine the amount of TT-specific antibody in maternal mouse sera. TT-specific IgG was detected with goat anti-mouse IgG-conjugated alkaline phosphatase (Cappel, Organon Teknika Corp., West Chester, Pa.) diluted 1:1,000. The antibody titer was defined as the reciprocal serum dilution that produced an A_{405} of ≥0.3 when the reference serum (mouse antiserum raised to a GBS oligosaccharide-TT conjugate and diluted 1:200 [23] reached an A_{405} of 0.3.

Opsonophagocytosis assay. The ability of Ib-TT vaccine- or Ib_{ox}+TT vaccine-induced rabbit antibody to opsonize GBS type Ib strain H36B cells for subsequent phagocytosis and killing by human peripheral blood leukocytes in the presence of active complement (human serum adsorbed with GBS type Ib strain H36B) was assessed in an in vitro opsonophagocytosis assay (8). Serum used in this study was collected 41 days after primary vaccination and was tested at a final concentration of 10% (vol/vol). The mean and standard deviation of triplicate determinations for each serum sample were calculated.

The opsonic capability of pooled sera (diluted 1:50) from mice given GBS tetravalent conjugate vaccine, GBS tetravalent polysaccharide vaccine, or TT against four GBS serotypes was also tested. Pooled sera were heated to 56°C for 30 min to destroy complement components. An exogenous complement source was prepared by adsorption (1 h, 4°C) of normal human serum with cells of the homologous GBS serotype and was used in the assay at 1% (vol/vol). The difference in GBS count (CFU) was determined after incubation for 1 h at 37°C.

Composition of GBS tetravalent conjugate vaccine. The GBS tetravalent conjugate vaccine was prepared by combining individually synthesized capsular polysaccharide-TT vaccines. The Ia-TT vaccine used in these studies was composed of 71% (wt/wt) carbohydrate and 29% (wt/wt) protein and was generated from polysaccharide ($>10^6$ M_r) that had 25% of the sialic acid residues oxidized as sites for protein coupling (Table 1). Synthesis of the GBS Ib-TT vaccine has been described above, and chemical composition is detailed in Table 1. The composition of II-TT vaccine (Table 1) has been reported previously (25). A III-TT vaccine was prepared for efficacy studies by the procedure detailed previously (32). The III-TT vaccine used in these studies was composed of 47% (wt/wt) carbohydrate and 53% (wt/wt) protein and was generated from polysaccharide (200,000 M_r) that had 29% of the sialic acid residues oxidized as sites for protein coupling. The ratio of carbohydrate to protein in these vaccines ranged from 0.9 for the III-TT vaccine to 2.4 for the Ia-TT vaccine (Table 1).

Protective efficacy of GBS tetravalent conjugate vaccine. GBS tetravalent conjugate vaccine was made by combining the same three individually prepared conjugates used in the trivalent GBS conjugate vaccine and the newly prepared Ib-TT vaccine. Stock solutions (1 mg/ml in PBS) of each conjugate (Ia-TT, Ib-TT, II-TT, and III-TT) were prepared, and equal volumes were combined and diluted with PBS to obtain a final concentration of 128 μg of GBS tetravalent conjugate vaccine per ml. This preparation was mixed with an equal volume of 3% alum (Alhydrogel; Superfos Biosector, Vedbaek, Denmark), and 0.25 ml was administered intraperitoneally to each of 12 adult CD-1 female mice (18 to 20 g; Charles River Laboratory). With the GBS tetravalent conjugate vaccine, each mouse received approximately 2 μg of each type of polysaccharide. The GBS tetravalent polysaccharide vaccine was prepared similarly. Stock solutions (1 mg/ml in PBS) of each polysaccharide (types Ia, Ib, II, and III) were prepared, and equal volumes were combined and diluted with PBS to obtain a final concentration of 64 μg of GBS tetravalent polysaccharide vaccine per ml. This preparation was mixed with an equal volume of 3% alum, and 0.25 ml was administered intraperitoneally to each of 12 adult CD-1 female mice. With the GBS tetravalent polysaccharide vaccine, each mouse received 2 μg of each type of polysaccharide. Eight adult female mice received a final concentration of 2 μg of uncoupled TT, combined with alum, in a total volume of 0.25 ml of PBS. All mice were bred after administration of the primary dose. Booster doses of vaccine, also mixed with alum, were administered by the same route 2 weeks after the primary dose. The first litters of pups were born to these dams 26 to 39 days and the second litters were born 54 to 66 days after primary vaccination. Within 36 h of birth, pups were challenged intraperitoneally with 0.05 ml of GBS. The GBS inoculum was prepared as follows: frozen stock cultures of GBS were thawed and streaked for isolation on a blood agar plate, and the plate was incubated overnight at 37°C. The following day, the culture was assessed for purity and was used to seed 5-ml tubes containing Todd-Hewitt broth. Cells incubated at 37°C with end-over-end mixing were grown to an A_{650} of 0.3. Serial 10-fold dilutions were made in Todd-Hewitt broth, and the appropriate dilution was used as the challenge inoculum. Cells were enumerated by the standard plate count method. Newborn pups remained with their own dam for the duration of the 48-h observation period after challenge. Survival of pups was assessed 48 h after challenge.

TABLE 2. Efficacy of GBS trivalent conjugate vaccine (Ia-TT, II-TT, and III-TT) or a mixture of GBS trivalent polysaccharide and TT vaccine (Ia, II, and III plus TT) in protection of neonatal mice against GBS challenge

Challenge (GBS serotype, strain)	Protection (no. of animals surviving/no. challenged) with indicated vaccine[a]					
	First litter			Second litter		
	Trivalent conjugate	Trivalent PS[b] + TT	P	Trivalent conjugate	Trivalent PS + TT	P
Ia, 515	19/29 (66)	5/11 (45)	0.147	24/28 (86)	6/13 (46)	0.011
Ib, H36B	1/15 (7)	0/10 (0)	0.600	10/27 (37)	7/13 (54)	0.160
II, 18RS21	20/27 (74)	4/11 (36)	0.030	21/21 (100)	8/14 (57)	0.002
III, M781	15/23 (65)	2/11 (18)	0.011	26/26 (100)	2/6 (33)	<0.001

[a] Percent surviving is given in parentheses.
[b] PS, polysaccharide.

Duration of protection by GBS III-TT vaccine in mice. The duration of maternally derived protection was tested by challenge of successive litters of pups born to dams vaccinated with TT-conjugated or uncoupled type III polysaccharide. Female mice received intraperitoneally 2 μg in a total volume of 0.5 ml of either III-TT or type III polysaccharide (with alum) on day 0 and a booster dose on day 21. Mice were bred 6 days after the primary vaccination. First, second, and third sets of litters (born 30, 108, and 185 days, respectively, after the primary dose) were challenged intraperitoneally with 1.40×10^7 to 1.65×10^7 CFU of GBS strain M781 per pup in 0.05 ml. Survival of pups was assessed 48 h after challenge.

Statistics. Fisher's exact test was used to compare the protective efficacies of GBS vaccines. InStat version 2.0 software (Graphpad Software, Inc., San Diego, Calif.) was used in these analyses.

RESULTS

Protective efficacy of trivalent GBS conjugate vaccine in mice. We recently reported protection of adult mice against either GBS type Ia strain 515 or GBS type Ib strain H36B by rabbit antisera to Ia-TT vaccine (33). This observation led us to explore the potential of inducing protection against GBS types Ia, Ib, II, and III by active vaccination with a trivalent GBS conjugate preparation including Ia-TT, II-TT, and III-TT vaccines. In the first set of litters born to dams that received this vaccine, at least 65% of pups survived challenge with type Ia, II, or III GBS, while protection against a GBS type Ib challenge was clearly lacking (Table 2). After challenge with type Ia or Ib GBS, the survival of pups born to dams that received the trivalent conjugate vaccine did not differ significantly from that of pups born to dams given a mixture of the trivalent capsular polysaccharide and TT. Without additional booster doses of vaccine, these female mice were bred again and their offspring were challenged with GBS. The rates of survival among the second set of litters born to trivalent conjugate vaccine recipients were 100, 100, and 86% after challenge with either GBS type III, II, or Ia, respectively (Table 2). This increase in protection against GBS infections, particularly against the type Ia challenge, in the second set of litters was not observed with GBS type Ib challenge (Table 2). This result suggested that although protective antibodies were raised to type Ia polysaccharide as a component of a Ia-TT vaccine, these antibodies did not provide cross-protection against type Ib organisms in this animal model. Therefore, a Ib-TT conjugate vaccine was synthesized as an additional component for a multivalent GBS vaccine.

Composition of GBS tetravalent conjugate vaccine. Seen in Fig. 1 are the repeating-unit structures of the type Ia, Ib, II, and III polysaccharides. The common structural feature of the terminal side chain sialic acid residues made it possible to use the same basic strategy to synthesize a GBS type Ib polysaccharide-TT conjugate that had been used previously to synthesize Ia-TT, II-TT, and III-TT vaccines. The GBS type Ib-TT vaccine was prepared from native type Ib polysaccharide with an M_r of $>10^6$. A portion of the sialic acid residues of the Ib polysaccharide was oxidized with sodium periodate to introduce aldehyde groups for coupling to TT. Gas chromatography-mass spectrometry spectra of trimethylsilyl derivatives of oxidized type Ib polysaccharide showed signals corresponding to the native sialic acid (C_9) derivative and the partially oxidized 8-carbon (C_8) derivative, which had relative areas of 88 and 12%, respectively. Thus, 12% of the type Ib polysaccharide sialic acid residues were converted to the C_8 analog and were available for coupling to TT. The oxidized polysaccharide was coupled to TT by reductive amination. Analysis of the purified Ib-TT conjugate showed 68% (wt/wt) carbohydrate and 32% (wt/wt) protein with a molar ratio of carbohydrate to protein of 2.1 (Table 1).

Immunogenicity of Ib-TT vaccine in rabbits. The immunogenicities of Ib-TT vaccine and an uncoupled mixture of Ib_{ox}+TT in rabbits were compared. Type Ib-specific IgG titers

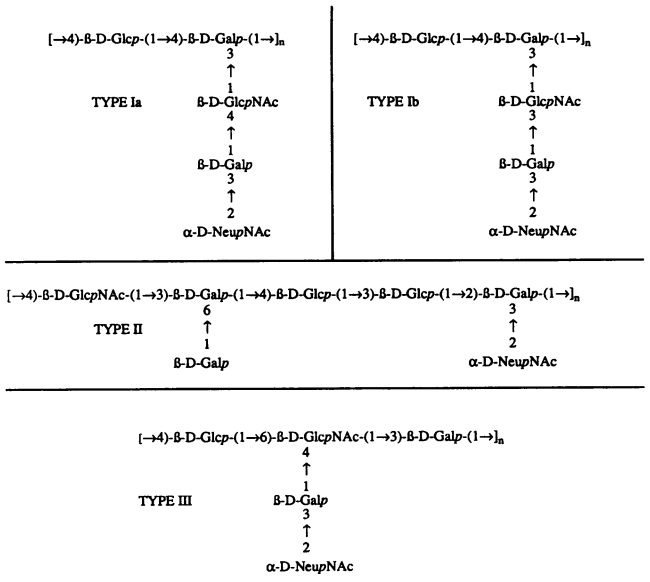

FIG. 1. Repeating-unit structures of the capsular polysaccharides of GBS types Ia and Ib (18), type II (19), and type III (34).

TABLE 3. Titers of GBS type Ib polysaccharide-specific IgG in rabbits given Ib-TT vaccine or a mixture of Ib_{ox}+TT

Vaccine	ELISA antibody titer[a] on indicated day					
	0[b]	20[c]	34	41[b]	55	69
Ib-TT						
Rabbit 1	100[d]	400	51,200	25,600	51,200	51,200
Rabbit 2	100	100	800	3,200	12,800	12,800
Rabbit 3	100	200	6,400	12,800	51,200	51,200
Ib_{ox}+TT						
Rabbit 1	100	100	100	100	100	100

[a] Values are means of duplicate determinations.
[b] Primary dose was administered with CFA.
[c] Booster doses were administered with incomplete Freund's adjuvant.
[d] A value of 100 indicates an antibody titer of ≤100.

rose in all of three rabbits given two doses of Ib-TT vaccine (Table 3). A third dose of Ib-TT vaccine further increased the Ib polysaccharide-specific antibody titer in all of three rabbits. Titers peaked at 12,800 and 51,200 in these three animals 2 weeks after the third dose. An unconjugated mixture of Ib_{ox}+TT failed to elicit type-specific IgG (Table 3).

In vitro functional activity of Ib-TT vaccine-induced rabbit antisera. We previously showed that antibodies elicited in rabbits by Ia-TT, II-TT, and III-TT vaccines opsonized GBS of the homologous serotype for phagocytic killing by human blood leukocytes (25, 32, 33). In the present study, serum (diluted 1:100) from each of the three rabbits immunized with Ib-TT vaccine, in combination with complement and human blood leukocytes, reduced counts of GBS type Ib strain H36B by >2 $\log_{10}$ CFU after 60 min of incubation (Table 4). In contrast, neither pooled preimmunization serum from rabbits that were later immunized with the Ib-TT vaccine nor serum from the rabbit immunized with Ib_{ox}+TT opsonized GBS type Ib cells for killing by leukocytes (Table 4).

Protective efficacy of GBS tetravalent vaccines in mice. Transplacentally acquired immunity to four GBS serotypes in mice born to dams vaccinated with GBS tetravalent conjugate vaccine (Ia-TT, Ib-TT, II-TT, and III-TT), GBS tetravalent polysaccharide vaccine (Ia, Ib, II, and III), or uncoupled TT was evaluated. These vaccines were administered to dams in alum rather than in CFA because the former is acceptable for human use. Protection of neonatal pups against challenge with GBS (within 36 h of birth) was evaluated in two successive sets of litters. In the first set of litters born to dams given GBS tetravalent conjugate vaccine, all pups challenged were protected against type Ia strain 515, type Ib strain H36B, and type III strain M781 (Table 5). The survival rates in these groups were statistically higher than those in litters born to dams that received either uncoupled TT or GBS tetravalent polysaccharide vaccine (Table 5). However, the rates of survival of pups in the three vaccine groups challenged with GBS type II strain 18RS21 did not differ statistically (Table 5).

Without further vaccination, the same female mice were rebred, and the second set of litters was challenged with GBS. As in the first set of litters, there was 100% protection against challenge with GBS types Ia and III (Table 6). The survival rate was 76% among pups challenged with type Ib and 97% among those challenged with type II (Table 6). The level of protection afforded to all groups of second-litter pups born to dams that received the GBS tetravalent conjugate vaccine was significantly higher ($P < 0.0001$) than that conferred on pups born to dams that received either the GBS tetravalent polysaccharide vaccine or uncoupled TT (Table 6).

Opsonophagocytosis of GBS vaccine-induced mouse anti-

TABLE 4. Opsonophagocytic killing of GBS type Ib strain H36B by pooled preimmunization rabbit serum and by rabbit antisera raised to Ib-TT or to Ib_{ox}+TT vaccine[a]

Vaccine	$\log_{10}$ CFU at indicated time		$\log_{10}$ decrease (0 − 60 min)
	0 min	60 min	
PRS[b]			
Pooled	6.58 ± 0.01	6.62 ± 0.10	−0.04 ± 0.09
Ib-TT			
Rabbit 1	6.39 ± 0.12	3.75 ± 0.26	2.64 ± 0.15
Rabbit 2	6.51 ± 0.02	4.08 ± 0.16	2.43 ± 0.16
Rabbit 3	6.49 ± 0.12	3.99 ± 0.08	2.50 ± 0.06
Ib_{ox}+TT			
Rabbit 1	6.60 ± 0.07	6.62 ± 0.03	−0.02 ± 0.04

[a] Values are means and standard deviations of triplicate determinations.
[b] PRS, preimmunization rabbit serum.

TABLE 5. Efficacy of GBS tetravalent conjugate vaccine (Ia-TT, Ib-TT, II-TT, and III-TT), GBS tetravalent polysaccharide vaccine (Ia, Ib, II, and III), and TT in protection of neonatal mice against GBS challenge (first litter)

Challenge GBS serotype (strain)[a]	Protection (no. of animals surviving/no. challenged) with indicated vaccine[b]		
	Tetravalent conjugate	TT	Tetravalent polysaccharide
Ia (515)	25/25 (100)[c]	4/21 (19)	4/38 (10)
Ib (H36B)	21/21 (100)[d]	10/19 (53)	9/24 (37)
II (18RS21)	13/37 (35)[e]	9/23 (39)	6/40 (15)
III (M781)	11/11 (100)[f]	11/22 (50)	14/36 (39)

[a] Challenge doses per pup were as follows: 1.0×10^7 to 5.1×10^7 CFU for strain 515, 0.28×10^7 to 5.8×10^7 CFU for strain H36B, 1.8×10^7 to 6.5×10^7 CFU for strain 18RS21, and 3.0×10^7 to 3.6×10^7 CFU for strain M781.
[b] Percent surviving is given in parentheses.
[c] $P < 0.0001$ compared with TT or tetravalent polysaccharide vaccines.
[d] $P = 0.0003$ compared with TT vaccine; $P < 0.0001$ compared with tetravalent polysaccharide vaccine.
[e] $P = 0.7884$ compared with TT vaccine; $P < 0.063$ compared with tetravalent polysaccharide vaccine.
[f] $P = 0.005$ compared with TT vaccine; $P = 0.0003$ compared with tetravalent polysaccharide vaccine.

TABLE 6. Efficacy of GBS tetravalent conjugate vaccine (Ia-TT, Ib-TT, II-TT, and III-TT), GBS tetravalent polysaccharide vaccine (Ia, Ib, II, and III), and TT in protection of neonatal mice against GBS challenge (second litter)[a]

Challenge GBS serotype (strain)[b]	Protection (no. of animals surviving/no. challenged) with indicated vaccine[c]		
	Tetravalent conjugate	TT	Tetravalent polysaccharide
Ia (515)	15/15 (100)[d]	0/10 (0)	0/10 (0)
Ib (H36B)	22/29 (76)[d]	7/31 (22)	1/39 (2)
II (18RS21)	31/32 (97)[d]	3/27 (11)	3/15 (20)
III (M781)	42/42 (100)[d]	11/46 (24)	22/74 (30)

[a] Dams whose first litters are characterized in Table 5 were rebred, and their offspring were challenged with GBS.
[b] Challenge doses per pup were as follows: 1.7×10^7 CFU for strain 515, 3.3×10^7 to 3.7×10^7 CFU for strain H36B, 1.2×10^7 CFU for strain 18RS21, and 1.9×10^7 to 3.0×10^7 CFU for strain M781.
[c] Percent surviving is given in parentheses.
[d] $P < 0.0001$ compared with TT or tetravalent polysaccharide vaccine.

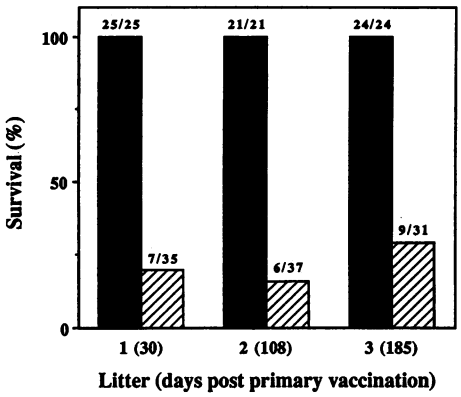

FIG. 2. Survival of three successive litters of mice born to dams given either GBS type III polysaccharide-TT vaccine (solid bars) or GBS type III polysaccharide (hatched bars). Numbers above each bar indicate the number of pups that survived per the number of pups challenged.

sera. Serum collected from dams 1 week after the birth of the first set of litters (i.e., day 46 after primary vaccination) was tested in the opsonophagocytic killing assay for opsonic antibody to GBS types Ia, Ib, II, and III. Pooled serum from mice vaccinated with GBS tetravalent conjugate vaccine (diluted 1:50) reduced counts of GBS types Ia, Ib, II, and III by 1.25, 1.37, 1.77, and 0.86 $\log_{10}$ CFU, respectively. In contrast, pooled antiserum to GBS tetravalent polysaccharide vaccine or to TT (also diluted 1:50) reduced counts of all four serotypes of GBS by $\leq$0.46 $\log_{10}$ CFU.

Mice given the GBS tetravalent conjugate vaccine or TT alone had TT-specific IgG ELISA titers of 6,400 and 3,200, respectively, whereas those given the GBS tetravalent polysaccharide vaccine had a TT-specific titer of 200.

Duration of protection after active vaccination of female mice. The persistence of protective levels of type III-specific IgG in adult mice was examined by giving female mice two doses of III-TT vaccine and challenging each of three successive litters with an ordinarily lethal dose of GBS type III strain M781. Complete (100%) protection was afforded to three successive sets of litters born 30, 108, and 185 days after primary immunization of dams (Fig. 2). In contrast, in each of the three successive litters born to dams vaccinated with type III capsular polysaccharide, fewer than 30% of pups survived challenge with an ordinarily lethal dose of GBS (Fig. 2).

DISCUSSION

Susceptibility to GBS infection in the neonate is correlated with the presence of low levels, or the absence, of capsular polysaccharide-specific antibody in the mother (4). This observation, together with experimental evidence for a protective role of polysaccharide-specific antibodies, provides the rationale for development of a maternal GBS vaccine based on the type-specific GBS polysaccharides. Like those to other bacterial polysaccharides, the human antibody response to purified GBS capsular polysaccharides is variable and depends on the immune status of the individual (5, 6). Therefore, the effective use of GBS polysaccharides as vaccines hinges on the development of polysaccharide vaccine formulations with increased immunogenicity. Several studies have demonstrated that the coupling of GBS capsular polysaccharide to immunogenic protein carriers enhances the immunogenicity of the polysaccharide in animals (20, 25, 26, 32, 33).

The current studies were designed to formulate a multivalent GBS vaccine and test it in an animal model. A maternal vaccination model in mice was utilized to simulate the intended use of such a vaccine in humans. The model has several features that make it attractive for testing the potential efficacy of a maternal vaccine. Demonstration of efficacy in this model requires that the vaccine elicit specific maternal antibodies, that vaccine-stimulated IgG antibodies cross the placenta, and that the antibodies function in the neonatal animal to mediate opsonophagocytic killing of GBS, thereby providing protection against an ordinarily lethal GBS challenge.

In our studies, immunity conferred by breast milk was not excluded. However, others have shown that while naive rat pups were only partially protected by breast milk from dams immunized with a GBS whole-cell vaccine, pups born to immunized dams were completely protected, even when nursed by nonimmune dams (16). These data suggest that transplacental transfer is likely to be the major mechanism of acquisition of maternal antibodies.

Because the vast majority of GBS clinical isolates from ill infants are of capsular type Ia, Ib, II, or III, an immunogenic multivalent vaccine that stimulated antibodies active against these four serotypes would be expected to provide protective immunity against more than 90% of disease-causing strains. However, it was necessary to establish that the simultaneous administration of vaccines against several serotypes did not interfere with the antibody response to the other component polysaccharides. The results of these studies with mice showed that administration of a multivalent GBS polysaccharide-TT conjugate vaccine stimulated maternal antibodies that were effectively transferred to the fetus in utero and that these antibodies were highly effective in protecting the neonate against challenge with the serotypes of GBS prevalent in human neonatal infections.

Our previous studies of the GBS Ia-TT vaccine provided evidence that rabbit antibodies specific for the type Ia polysaccharide also recognized the structurally related type Ib polysaccharide, albeit with a 100-fold lower affinity. That this cross-reaction might be functionally significant was suggested by the findings that Ia-TT antiserum opsonized type Ib organisms for phagocytic killing in vitro and protected mice against challenge with type Ib GBS (33). Since antibodies raised to Ia-TT in rabbits were functionally active against type Ib GBS, our first formulation of a multivalent GBS polysaccharide-protein conjugate vaccine included just three components, Ia-TT, II-TT, and III-TT, and was used to test the hypothesis that antibodies induced in mice by active vaccination with Ia-TT would protect against Ib challenge. However, pups of dams vaccinated with the trivalent formulation were not protected against Ib challenge. Failure of the Ia-TT component to elicit Ib protection in this model suggests that the lower binding affinity of Ia-TT-induced antibodies for Ib than for Ia polysaccharide was not sufficient to mediate efficient opsonophagocytic killing of type Ib organisms in vivo. The apparent discrepancy between these results and those of Ia-TT immunogenicity testing in rabbits may reflect species-related differences in the relative functional activity or antigenic fine specificity of the vaccine-induced antibodies.

Synthesis of the Ib-TT conjugate permitted the testing of a tetravalent conjugate vaccine in the mouse maternal immunization model. In contrast to the trivalent preparation, the tetravalent conjugate vaccine (including Ib-TT) elicited antibodies in the dams that protected their pups against challenge with type Ib, as well as with types Ia, II, and III. Although we did not directly test the kinetics of antibody responses to booster vaccination in mice, the enhanced protection against type II challenge in the second-litter pups born to dams

vaccinated with the tetravalent conjugate vaccine suggests that peak antibody responses may not have developed until more than 21 days after booster vaccination. A significant rise in protection against challenge with types II ($P = 0.0136$) and III ($P = 0.0011$) was also observed for second-litter versus first-litter pups born to dams vaccinated with the trivalent conjugate vaccine. The duration of protective levels of antibody was not addressed in studies with the GBS tetravalent conjugate vaccine; however, in each of three successive mouse litters, we observed complete (100%) protection by III-TT vaccine that spanned 185 days.

The results of our present studies demonstrate the induction of protective immunity to multiple serotypes of GBS by maternal vaccination of mice with a tetravalent GBS polysaccharide-protein conjugate vaccine. The individual capsular polysaccharides used in these studies were of various sizes and were oxidized to various degrees before coupling. As a result, the individual conjugates in the tetravalent vaccine differed in carbohydrate loading and in the degree of cross-linking of polysaccharide and protein. The influence of these physical properties on the immunogenicity of GBS conjugate vaccines has not been defined.

Should epidemiologic surveillance indicate that GBS type V or other serotypes are becoming more prevalent in neonatal infections, the structural similarity of all GBS capsular polysaccharides will permit the addition of one or more additional or alternative GBS polysaccharide-TT conjugates to the multivalent formulation. Our findings suggest that a multivalent GBS conjugate vaccine of the design described in this report might be an effective maternal vaccine to protect human infants against neonatal GBS infection.

ACKNOWLEDGMENTS

This work was accomplished with the superb technical assistance of April Blodgett, Julieanne Pinel, and Ken Johnson.

This work was funded by National Institutes of Health (NIH) grants AI-23339 and AI-30628 and NIH contract AI-25152. M.R.W. is an Established Investigator of the American Heart Association.

REFERENCES

1. **Anthony, B. F.** 1976. Immunity to the group B streptococci: interaction of serum and macrophages with types Ia, Ib, and Ic. J. Exp. Med. **143:**1186–1198.
2. **Baker, C. J., and M. S. Edwards.** 1990. Group B streptococcal infections, p. 742–811. *In* J. S. Remington and J. O. Klein (ed.), Infectious diseases of the fetus and newborn infant. W. B. Saunders Co., Philadelphia.
3. **Baker, C. J., M. S. Edwards, and D. L. Kasper.** 1981. Role of antibody to native type III polysaccharide of group B *Streptococcus* in infant infection. Pediatrics **68:**544–549.
4. **Baker, C. J., and D. L. Kasper.** 1976. Correlation of maternal antibody deficiency with susceptibility to neonatal group B streptococcal infection. N. Engl. J. Med. **294:**753–756.
5. **Baker, C. J., and D. L. Kasper.** 1985. Group B streptococcal vaccines. Rev. Infect. Dis. **7:**458–467.
6. **Baker, C. J., M. A. Rench, M. S. Edwards, R. J. Carpenter, B. M. Hays, and D. L. Kasper.** 1988. Immunization of pregnant women with a polysaccharide vaccine of group B *Streptococcus*. N. Engl. J. Med. **319:**1180–1220.
7. **Baltimore, R. S., C. J. Baker, and D. L. Kasper.** 1981. Antibody to group B *Streptococcus* type III in human sera measured by a mouse protection test. Infect. Immun. **32:**56–61.
8. **Baltimore, R. S., D. L. Kasper, C. J. Baker, and D. K. Goroff.** 1977. Antigenic specificity of opsonophagocytic antibodies in rabbit anti-sera to group B streptococci. J. Immunol. **118:**673–678.
9. **Boyer, K. M., L. S. Kendall, C. K. Papierniak, M. E. Klegerman, and S. P. Gotoff.** 1984. Protective levels of human immunoglobulin G antibody to group B Streptococcus type Ib. Infect. Immun. **45:**618–624.
10. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for the determination of sugars and related substances. Anal. Chem. **28:**350–356.
11. **Edwards, M. S., C. J. Baker, and D. L. Kasper.** 1979. Opsonic specificity of human antibody to the type III polysaccharide of group B streptococci. J. Immunol. **140:**1004–1008.
12. **Farley, M. M., R. C. Harvey, T. Stull, J. D. Smith, A. Schuchat, J. D. Wenger, and D. S. Stephens.** 1993. A population-based assessment of invasive disease due to group B *Streptococcus* in nonpregnant adults. N. Engl. J. Med. **328:**1807–1811.
13. **Givner, L. B., M. S. Edwards, and C. J. Baker.** 1988. A polyclonal human IgG preparation hyperimmune for type III, group B *Streptococcus*: in vitro opsonophagocytic activity and efficacy in experimental models. J. Infect. Dis. **158:**724–730.
14. **Gray, B. M.** 1979. ELISA methodology for polysaccharide antigens: protein coupling of polysaccharides for adsorption to plastic tubes. J. Immunol. Methods **28:**187–192.
15. **Greenberg, D. N., D. P. Ascher, B. A. Yoder, H. S. Heiman, and J. F. Keith III.** 1993. Group B *Streptococcus* serotype V. J. Pediatr. **123:**494–495. (Letter.)
16. **Heiman, H. S., and L. E. Weisman.** 1989. Transplacental or enteral transfer of maternal immunization-induced antibody protects suckling rats from type III group B streptococcal infection. Pediatr. Res. **26:**629–632.
17. **Hervas, J. A., L. Gonzalez, J. Gil, L. C. Paoletti, L. C. Madoff, and V. J. Benedi.** 1993. Neonatal group B streptococcal infection in Mallorca, Spain. Clin. Infect. Dis. **16:**714–718.
18. **Jennings, H. J., E. Katzenellenbogen, C. Lugowski, and D. L. Kasper.** 1983. Structure of the native polysaccharide antigens of type Ia and type Ib group B *Streptococcus*. Biochemistry **22:**1258–1263.
19. **Jennings, H. J., K. G. Rosell, E. Katzenellenbogen, and D. L. Kasper.** 1983. Structural determination of the capsular polysaccharide antigen of type II group B *Streptococcus*. J. Biol. Chem. **258:**1793–1798.
20. **Lagergard, T., J. Shiloach, J. B. Robbins, and R. Schneerson.** 1990. Synthesis and immunological properties of conjugates composed of group B *Streptococcus* type III capsular polysaccharide covalently bound to tetanus toxoid. Infect. Immun. **58:**687–694.
21. **Larson, E., B. Howlett, and A. Jagendorf.** 1986. Artificial reductant enhancement of the Lowry method for protein determination. Anal. Biochem. **155:**243–248.
22. **Madoff, L. C., J. L. Michel, E. W. Gong, A. K. Rodewald, and D. L. Kasper.** 1992. Protection of neonatal mice from group B streptococcal infection by maternal immunization with beta C protein. Infect. Immun. **60:**4989–4994.
23. **Paoletti, L. C., D. L. Kasper, F. Michon, J. DiFabio, K. Holme, H. J. Jennings, and M. R. Wessels.** 1990. An oligosaccharide-tetanus toxoid conjugate vaccine against type III group B *Streptococcus*. J. Biol. Chem. **265:**18278–18283.
24. **Paoletti, L. C., D. L. Kasper, F. Michon, J. DiFabio, H. J. Jennings, T. D. Tosteson, and M. R. Wessels.** 1992. Effects of chain length on the immunogenicity in rabbits of group B *Streptococcus* type III oligosaccharide-tetanus toxoid conjugates. J. Clin. Invest. **89:**203–209.
25. **Paoletti, L. C., M. R. Wessels, F. Michon, J. DiFabio, H. J. Jennings, and D. L. Kasper.** 1992. Group B *Streptococcus* type II polysaccharide-tetanus toxoid conjugate vaccine. Infect. Immun. **60:**4009–4014.
26. **Que, J., and J. S. Cryz.** 1993. Immunogenicity of a trivalent conjugate vaccine against group B *Streptococcus* capsular polysaccharide, abstr. E-81, p. 156. Abstr. 93rd Gen. Meet. Am. Soc. Microbiol. 1993. American Society for Microbiology, Washington, D.C.
27. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. **27:**493–497.
28. **Rench, M., and C. Baker.** 1993. Neonatal sepsis caused by a new group B streptococcal serotype. J. Pediatr. **122:**638–640.
29. **Rodewald, A. K., A. B. Onderdonk, H. B. Warren, and D. L. Kasper.** 1992. Neonatal mouse model of group B streptococcal infection. J. Infect. Dis. **166:**635–639.
30. **Warren, L.** 1959. The thiobarbituric acid assay of sialic acids. J. Biol. Chem. **234:**1971–1975.
31. **Weisman, L., B. Anthony, V. Hemming, and G. Fischer.** 1993.

Comparison of group B streptococcal hyperimmune globulin and standard immune globulin in neonates. J. Pediatr. **122:**929–937.

32. **Wessels, M. R., L. C. Paoletti, D. L. Kasper, J. L. DiFabio, F. Michon, K. Holme, and H. J. Jennings.** 1990. Immunogenicity in animals of a polysaccharide-protein conjugate vaccine against type III group B *Streptococcus*. J. Clin. Invest. **86:**1428–1433.
33. **Wessels, M. R., L. C. Paoletti, A. K. Rodewald, F. Michon, J. DiFabio, H. J. Jennings, and D. L. Kasper.** 1993. Stimulation of protective antibodies against type Ia and Ib group B streptococci by a type Ia polysaccharide-tetanus toxoid conjugate vaccine. Infect. Immun. **61:**4760–4766.
34. **Wessels, M. R., V. Pozsgay, D. L. Kasper, and H. J. Jennings.** 1987. Structure and immunochemistry of an oligosaccharide repeating unit of the capsular polysaccharide of type III group B *Streptococcus*. A revised structure for the type III group B streptococcal polysaccharide antigen. J. Biol. Chem. **262:**8262–8267.