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Research Article

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Immunogenicity in Animals of a Polysaccharide-Protein Conjugate Vaccine against Type III Group B *Streptococcus*

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Abstract

The native capsular polysaccharide of type III group B *Streptococcus* elicits a specific antibody response in only 60% of nonimmune human subjects. To enhance the immunogenicity of this polysaccharide, we coupled the type III polysaccharide to tetanus toxoid. Prior to coupling, aldehyde groups were introduced on the polysaccharide by controlled periodate oxidation, resulting in the conversion of 25% of the sialic acid residues of the polysaccharide to residues of the 8-carbon analogue of sialic acid, 5-acetamido-3,5-dideoxy-D-galactosyloctulosonic acid. Tetanus toxoid was conjugated to the polysaccharide by reductive amination, via the free aldehyde groups present on the partially oxidized sialic acid residues. Rabbits vaccinated with the conjugate vaccine produced IgG antibodies that reacted with the native type III group B streptococcal polysaccharide (3/3 rabbits), while rabbits immunized with the unconjugated type III polysaccharide failed to respond (0/3 rabbits). Sera from animals receiving conjugate vaccine opsonized type III group B streptococci for phagocytic killing by human peripheral blood leukocytes, and protected mice against lethal challenge with live type III group B streptococci. The results suggest that this method of conjugation to a carrier protein may be a useful strategy to improve the immunogenicity of the type III group B *Streptococcus* polysaccharide in human subjects. (*J. Clin. Invest.* 1990. 86:1428–1433). Key words: immunity • antibody • sialic acid • capsule • neonatal sepsis

Introduction

Group B *Streptococcus* (GBS)[1] is a major cause of neonatal sepsis and meningitis in the United States, with a nationwide attack rate estimated at 2–3 per 1,000 live births (1–3). Despite advances in diagnosis and improved supportive care, neonatal

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1. *Abbreviations used in this paper:* GBS, group B *Streptococcus;* GC-MS, gas chromatography-mass spectrometry; NMR, nuclear magnetic resonance spectroscopy.

GBS infection still carries a 10–20% mortality, and an estimated 30–50% incidence of serious long term neurologic sequelae among survivors of GBS meningitis (4–6). Organisms of capsular type III are most prevalent among GBS strains causing invasive neonatal disease, accounting for approximately 60% of all GBS isolates from neonatal sepsis and meningitis cases (2, 7). A large body of experimental evidence has indicated that one of the major determinants of immunity to GBS infection is antibody directed against the type-specific capsular polysaccharide of the organism. Baker and Kasper demonstrated that human infants with type III GBS sepsis and meningitis uniformly had low or undetectable levels of anticapsular antibodies (8). As IgG class antibodies cross the placenta and could achieve protective levels in the fetus before delivery, immunization of women before or during pregnancy has been proposed as a strategy to prevent neonatal GBS infection (9, 10). Purified preparations of three of the four major capsular polysaccharides (Ia, II, III) have been given as experimental vaccines both to animals and to human volunteers (9–13). While maternal vaccination appears to be a promising approach for preventing neonatal GBS disease, the native type III polysaccharide vaccine elicits a specific antibody response in only 60% of nonimmune recipients (10, 12, 13).

Conjugation to a carrier protein is a strategy which has been used successfully to enhance the immunogenicity of several polysaccharide antigens, including such bacterial capsular polysaccharides as the type b capsule of *Haemophilus influenzae,* groups A and C meningococcus, and others (14–16). A variety of coupling methods have been utilized to link polysaccharides to protein carriers directly or via a synthetic spacer molecule (17). In some cases the efficiency of the coupling reaction has been increased by the introduction of functional groups at specific or random sites on the polysaccharide. Such modifications of the carbohydrate, however, may alter the structure of the polysaccharide sufficiently to affect epitope expression, in which case the polysaccharide-protein conjugate may not elicit antibodies reactive with the native antigen. Antibodies directed against the GBS type III capsular polysaccharide appear to recognize a conformational epitope. The epitope is influenced both by the charged sialic acid residues present as side chain termini of the polysaccharide, and by the polymeric state of the polysaccharide (18–20). Because of the conformational nature of the epitope of the type III polysaccharide, we sought to develop a conjugation method that would preserve both the native repeating unit structure and the conformation of the polysaccharide, yet would result in efficient coupling to a carrier protein. Treatment of the polysaccharide with controlled periodate oxidation permitted the introduction of free aldehyde groups on a portion of the side chain sialic acid residues, while preserving the native epitope. The polysaccharide was then covalently coupled to tetanus

toxoid, via the free aldehyde groups, by reductive amination using sodium cyanoborohydride. In contrast to the purified polysaccharide alone, the polysaccharide-protein conjugate elicited high-titered IgG class antibodies in rabbits. These antibodies were opsonic in vitro, and protected mice against lethal challenge with type III GBS organisms.

Methods

Bacterial strain. GBS type III strain M781, originally isolated from a human neonate with GBS meningitis, was donated by Carol J. Baker, Baylor College of Medicine, Houston, TX. This strain was used as a source of type III GBS capsular polysaccharide, as the target strain for in vitro opsonophagocytic assays, and as the challenge strain for mouse protection assays.

Antiserum. Antiserum raised in a rabbit as described previously (19, 21), against formalinized whole organisms of type III GBS, was used in the ELISA inhibition assays for purification of GBS type III polysaccharide, and as a reference serum in ELISA assays for measurement of vaccine-induced antibodies.

Isolation and purification of type III GBS capsular polysaccharide. GBS type III strain M781 was grown in a dialysate (10,000 mol wt cutoff membrane, Pellicon Cassette System; Millipore Corp., Bedford, MA) of Columbia broth (Difco Laboratories, Inc., Detroit, MI) supplemented with glucose, 80 g/liter. An 8-h culture in a volume of 6 liters was used to inoculate 60 liters of broth in a 100-liter fermentor (New Brunswick Scientific Co., Inc., Edison, NJ). The organisms were grown at 37°C for 16 h, with continuous stirring (200 rpm), aeration (5 liters/min), and pH maintained at 7.0 with 10 M KOH. Bacterial cells were removed using a membrane filtration system (Prostak; Millipore), and the culture fluid was concentrated over a 10,000 mol wt cutoff pore size membrane (Pellicon) to a volume of 2 liters. The solution was brought to 30% (vol/vol) ethanol, and the precipitate formed was removed by centrifugation and discarded. Crude capsular polysaccharide was precipitated from the remaining supernatant fluid by bringing the solution to a concentration of 80% (vol/vol) ethanol. The precipitate was dried, dissolved in 10 mM Tris-HCl, pH 7.0, and treated with RNase (250,000 U; Worthington Biochemical Corp., Freehold, NJ) and DNase (100,000 U; Worthington), then Pronase (5,000 U; Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA), each overnight at 37°C. Contaminating group B polysaccharide was depolymerized by treatment of the solution with 1 N NaOH overnight at 37°C (22). The preparation was dialyzed extensively, then applied to a 2.6 × 94 cm column of DEAE-Sephacel equilibrated in 10 mM Tris-HCl, pH 7.2, and eluted with a 2-liter gradient of 0 to 0.2 M NaCl in the same buffer. Column fractions were tested by ELISA inhibition using specific rabbit antiserum for the presence of type III polysaccharide (22). Fractions showing inhibitory activity in the type III ELISA were pooled, dialyzed against water, and lyophilized. Because base treatment results in partial de-N-acetylation of sialic acid residues on the polysaccharide, this material was re-N-acetylated by treatment with acetic anhydride, as described previously (22). Contaminating high relative molecular mass mannan (from the growth medium) was removed by passing the polysaccharide over a 2.6 × 28 cm column of Concanavalin A linked to Sepharose 4B (Sigma Chemical Co., St. Louis, MO) in 20 mM Tris-HCl, pH 7.4 containing 0.5 M NaCl, 1 mM MnCl₂, 1 mM CaCl₂. The polysaccharide was size fractionated by passage over a 2.6 × 90 cm column of Ultragel ACA 44 (IBF Biotechnics, Inc., Savage, MD) in PBS. The material eluting at the center of the major peak was pooled, dialyzed against water, and lyophilized to yield purified capsular polysaccharide of estimated 200,000 M_r. Analysis of this material by gas chromatography-mass spectrometry (GC-MS) after conversion of the component sugars to alditol acetates, and by ¹H-nuclear magnetic resonance spectroscopy (¹H-NMR), confirmed the native type III polysaccharide structure and the absence of contaminating group B polysaccharide (19).

Periodate oxidation of type III polysaccharide. GBS type III poly-

saccharide (16 mg) was incubated with 4 μM of sodium periodate in 1.6 ml of water, in the dark, at 23°C for 1 h. Ethylene glycol was added to consume any residual periodate, the mixture was dialyzed against water, and lyophilized. Degree of oxidation of the sialic acid residues was assessed by GC-MS of trimethylsilyl derivatives: a 1-mg sample of the oxidized polysaccharide was treated with sodium borohydride, 10 mg in 0.5 ml water, at 23°C for 1 h, then deionized over Rexyn 101(H) resin (Fisher Scientific Co., Fair Lawn, NJ), and evaporated three times on a rotary evaporator with 5-ml amounts of methanol. Sialic acid residues and their oxidized derivatives were hydrolyzed from the polysaccharide and converted to methyl esters/methyl glycosides by treatment with 2 N methanolic HCl, 1 ml, at 80°C for 2 h. HCl was removed by evaporation with methanol, and trimethylsilyl derivatives were prepared, as described (23). Analysis of this sample by GC-MS was carried out as described (24).

Purification of tetanus toxoid. Tetanus toxoid (Institute Armand Frappier, Montreal, Canada) was purified by gel filtration chromatography before use. 15 ml of a liquid preparation containing 1 mg/ml protein nitrogen was loaded onto a 2.6 × 90 cm column of Ultragel ACA44 equilibrated in PBS and eluted with the same buffer. Protein eluted from the column in two peaks: the earlier eluting peak contained polymerized toxoid, and the later eluting peak, corresponding to ~ 150,000 M_r, contained tetanus toxoid monomer. Fractions corresponding to the later (monomer) peak were pooled, dialyzed against water, and lyophilized.

Coupling of type III polysaccharide to tetanus toxoid. Periodate-treated type III polysaccharide (11 mg) and purified tetanus toxoid monomer (11 mg) were dissolved in 0.1 M sodium bicarbonate, pH 8.1. Sodium cyanoborohydride (20 mg) was added, and the mixture was incubated at 37°C for 10 d. The progress of the conjugation was monitored by removing aliquots at various time points for analysis by fast performance liquid chromatography (FPLC), using a Superose 6, HR 10/30 column (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, NJ), with PBS as eluant at a flow rate of 0.5 ml/min. Conjugation of polysaccharide to tetanus toxoid was indicated by a progressive increase in a broad peak (monitored by measurement of UV absorbance at 214 nm) eluting soon after the void volume of this column. After conjugation was complete, sodium borohydride (10 mg) was added to the reaction mixture to reduce any remaining free aldehyde groups. The conjugate was purified by passage over a 1.6 × 85 cm column of Biogel A, 0.5 M (Bio-Rad Laboratories, Richmond, CA), equilibrated in PBS, and eluted with the same buffer. Fractions corresponding to the void volume peak were pooled, dialyzed against water, and lyophilized.

Chemical analysis of the conjugate. Carbohydrate content was measured by the phenol-sulfuric acid method, using the native type III polysaccharide as a standard (25). Protein content was measured by the method of Lowry et al., using bovine serum albumin as a standard (26). Sialic acid content was measured by the resorcinol method, using sialic acid as a standard (27).

Immunization of rabbits. Female New Zealand White rabbits weighing ~ 2 kg were injected with 50 μg s.c. of native type III polysaccharide or 50 μg of conjugate in complete Freund's adjuvant. Booster doses of 50 μg of the same vaccine were given subcutaneously in incomplete Freund's adjuvant 3 and 6 wk after the first dose.

Measurement of antibodies against type III GBS polysaccharide. Antibodies against the native type III GBS polysaccharide were measured by ELISA. Native type III polysaccharide was coupled to poly-L-lysine and coated onto 96-well flat bottom plastic plates at a concentration of 1 μg/ml, as previously described (28). Test serum at several dilutions was added, and subsequent steps of addition of alkaline phosphatase coupled antirabbit IgG and substrate carried out as described previously (29). Each assay was standardized using a reference serum (raised against formalinized whole type III GBS) on the same plate. Plates were read when duplicate wells developed with the reference serum at a dilution of 1:80,000 reached absorbance at 405 nm = 0.5. Antibody titer was defined as the reciprocal of the highest serum dilution giving absorbance at 405 nm > 0.3.

Opsonophagocytic assays. Opsonophagocytic assays were performed essentially as described by Baltimore et al. (29). Early log phase organisms of type III GBS strain M781 (4×10^6 CFU) were incubated with 3×10^6 human peripheral blood leukocytes in 0.5 ml of Eagle's minimal essential medium. Each assay contained, in addition, 10% human serum (previously absorbed with GBS type III organisms and stored at $-80°C$ to preserve complement activity) and the rabbit serum to be tested, at a final concentration of 1% vol/vol (i.e., 1:100 dilution). Aliquots were removed, diluted in sterile water, and spread on blood agar plates for quantitative culture at time zero and after 1 h of end-over-end rotation at 37°C.

Mouse protection assay. Female CD-1 mice (Charles River Breeding Laboratories, Inc., Wilmington, MA) weighing $\sim$ 20–25 g were given a single intraperitoneal injection of undiluted antiserum or antiserum diluted in PBS in a total volume of 0.2 ml. 24 h later, the mice were challenged with an LD_{90} dose (6.6×10^7 CFU) of strain M781, administered by intraperitoneal injection in a total volume of 1 ml of Todd Hewitt broth. Dead animals were removed from the cages at 24-h intervals, and the overall mortality for each group was assessed at 3 d after challenge.

Results

Preparation of the conjugate vaccine. The presence of $\alpha(2 \rightarrow 3)$ linked sialic acid residues as side chain termini provides a unique site for introduction of free aldehyde groups on the GBS type III polysaccharide, without disrupting the native epitope. Controlled periodate oxidation results in cleavage of the polyhydroxylated tail of sialic acid, leaving the 8-carbon analogue (5-acetamido-3,5-dideoxy-D-galactosyloctulosonic acid) or 7-carbon analogue (5-acetamido-3,5-dideoxy-D-galactosylheptulosonic acid), with a free terminal aldehyde group at the site of periodate cleavage (30). Immunochemical and NMR studies of the type III GBS polysaccharide have indicated that oxidation of the sialic acid residues of the polysaccharide does not alter the antigenicity (i.e., reactivity with antibodies raised against organisms bearing the native polysaccharide) or physical conformation of the polysaccharide (18). As this modification appears not to alter the antibody binding characteristics of the native epitope, we utilized limited periodate oxidation to cleave carbon 9 from 25% of the sialic acid residues of the type III polysaccharide, leaving a free aldehyde at the carbon-8 position of the oxidized residues available as sites for coupling to a carrier protein by reductive amination (Fig. 1). The periodate-treated polysaccharide was analyzed by GC-MS after reduction, methanolysis, and preparation of trimethylsilyl derivatives of the native or partially oxidized sialic acid residues (Fig. 2). The signals representing the native sialic acid (C9) derivative and the partially oxidized 8-carbon (C8) derivative had relative abundances of 75.5% and 24.5%, respectively, confirming that $\sim$ 75% of the sialic acid residues remained in the native state, while 25% had been converted to the C8 analogue (Fig. 2). Under these conditions, further oxidation to the C7 analogue did not occur to an extent detectable by GC-MS analysis.

The partially oxidized polysaccharide was covalently linked to tetanus toxoid by reductive amination using sodium cyanoborohydride. The progress of the conjugation was monitored by FPLC analysis, on a Superose 6 column, of small aliquots of the reaction mixture. Polysaccharide-tetanus toxoid conjugate eluted from the column in a broad peak that began at 11 min, just after the void volume, and extended into the beginning of a second peak (Fig. 3). The second peak, eluting at 20 min, contained excess unconjugated tetanus tox-
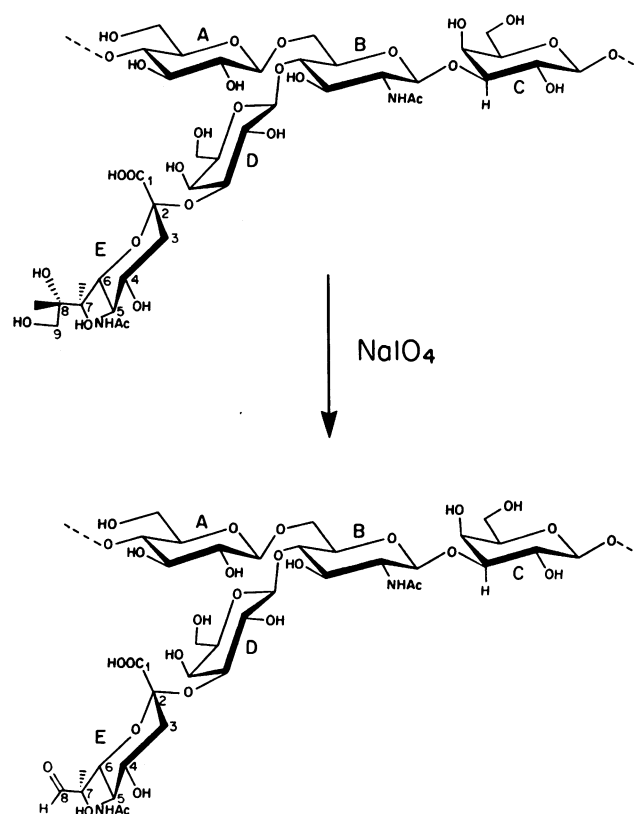

Figure 1. Limited periodate oxidation of the GBS type III polysaccharide. Approximately 25% of the sialic acid residues (E) were converted to the 8-carbon analogue (5-acetamido-3,5-dideoxy-D-galactosyloctulosonic acid) with a free aldehyde group at carbon 8.

oid (Fig. 3). After 7 d of incubation, no further increase was observed in the size of the first peak, indicating that conjugation was complete. The high molecular weight conjugate was purified from the reaction mixture by gel filtration chromatography on a column of Biogel A, 0.5 M. The void volume peak was collected, dialyzed against water, and lyophilized to yield 7.3 mg of purified conjugate vaccine. The purified conjugate contained 59% protein, 41% polysaccharide, and 8% sialic acid, by weight. These values indicate that the coupled polysaccharide retained essentially the same sialic acid content as the native polysaccharide (20% in the coupled polysaccharide vs. 22% in the native).

Immunogenicity of the conjugate vaccine. Groups of three rabbits were vaccinated with the native polysaccharide or with the conjugate vaccine. The animals were given booster doses of the same vaccine at 3 and 6 wk after the initial vaccination. Because the ultimate goal of a GBS type III conjugate vaccine is to induce antibodies in women which will cross the placenta, that is, IgG class antibodies, we used an IgG-specific alkaline phosphatase conjugated anti-rabbit antibody to develop the type III ELISA. None of the animals given the native polysaccharide had a rise in specific IgG antibody titer above 200, while all three rabbits given the conjugate vaccine had increases in type III polysaccharide specific antibody titer after a single dose, which increased further after each of two booster doses (Table I). An additional group of three rabbits was vaccinated with the same dose and schedule of conjugate vaccine
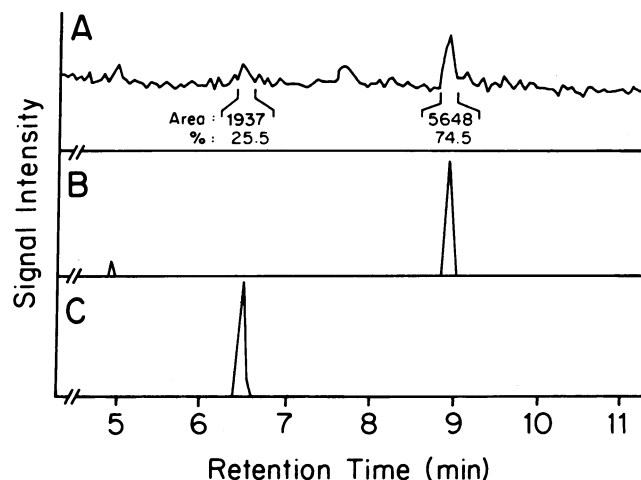
Figure 2. GC-MS analysis of periodate oxidized GBS type III polysaccharide. Residues of sialic acid (C9) and the partially oxidized 8-carbon derivative (C8) were cleaved from the polysaccharide by methanolysis to form their respective methyl esters/methyl glycosides, which were converted to trimethylsilyl derivatives for GC-MS analysis. (A) Total ion current. (B) Selective ion monitoring at 568 m/e (mass corresponding to the C9 derivative molecular ion minus O-trimethylsilane). (C) Selective ion monitoring at 466 m/e (mass corresponding to the C8 derivative molecular ion minus O-trimethylsilane). Relative abundance of the C9 and C8 derivatives is expressed as area under the curve (area) and as relative area % (%).

without Freund's adjuvant. Peak antibody titers of these three rabbits were 400, 1,600, and 1,600, reached at day 34, 34, and 55, respectively. Thus, even in the absence of adjuvant, the conjugate elicited specific IgG antibodies, while the unconjugated polysaccharide was nonimmunogenic. Sera from rabbits immunized without adjuvant were not used in opsonophagocytic assays or mouse protection studies.

Opsonic capacity of vaccine-induced antibodies. We tested the ability of antiserum from rabbits vaccinated with either the native GBS type III polysaccharide or the polysaccharide-protein conjugate vaccine to opsonize type III GBS organisms. Incubation of type III GBS with human peripheral blood leukocytes, a complement source, and sera from rabbits vaccinated with the conjugate vaccine resulted in a 1.8–2.0 log decrease in CFU after 1 h, compared with the input inoculum (Table II). The same degree of killing was observed when a purified IgG fraction, prepared by Protein A-Sepharose affinity chromatography of conjugate-induced immune serum, was
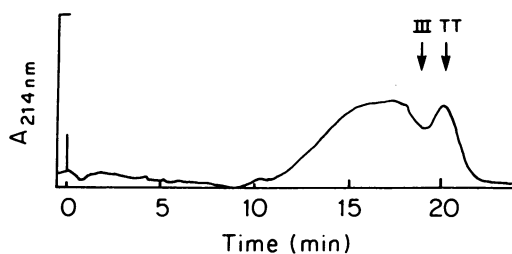

Figure 3. FPLC elution profile of conjugation reaction mixture after 7 d of incubation. Elution times of unconjugated GBS III polysaccharide (III) and of unconjugated tetanus toxoid (TT) are indicated by arrows.

Table I. Titers of GBS Type III Polysaccharide-Specific Antibodies, Measured by ELISA, in Sera of Rabbits Vaccinated with Native GBS Type III Polysaccharide (III Native) or with GBS Type III Polysaccharide-Tetanus Toxoid Conjugate Vaccine (III-TT)

Vaccine	Antibody titer				
	Day 0	Day 20	Day 34	Day 55	Day 75
III native					
Rabbit 1	100*	100	100	100	100
Rabbit 2	100	100	200	100	100
Rabbit 3	100	100	100	100	100
III-TT					
Rabbit 1	100	1,000	16,000	64,000	128,000
Rabbit 2	100	4,000	16,000	64,000	128,000
Rabbit 3	100	500	32,000	256,000	256,000

Values are means of duplicate determinations. Rabbits were vaccinated on days 0, 20, and 41. * A value of 100 indicates an antibody titer of 100 or less.

substituted for whole serum (data not shown). Neither preimmune sera from rabbits receiving the conjugate vaccine, nor postvaccination sera from animals receiving the native polysaccharide produced more than a 0.15 log reduction in CFU, under the same conditions (Table II).

Mouse protection by vaccine-induced antibodies. To further establish the functional capacity of vaccine-induced antibodies, groups of mice were injected with pooled serum from rabbits vaccinated either with the native polysaccharide or with the conjugate vaccine. 24 h later, the mice were given an LD$_{90}$ dose of live organisms of type III GBS strain M781. Neither preimmune serum from rabbits receiving the conjugate vaccine, nor postvaccination serum from rabbits receiving the native polysaccharide alone provided any protection, while postvaccination serum from rabbits receiving the conjugate vaccine was highly protective at both doses tested: 100% sur-

Table II. Opsonophagocytic Killing of Type III GBS by Human Peripheral Blood Leukocytes in the Presence of Serum from Rabbits Vaccinated with Native GBS Type III Polysaccharide (III Native) or GBS Type III Polysaccharide-Tetanus Toxoid Conjugate (III-TT)

Serum source	$t = 0$ h (CFU)	$t = 1$ h (CFU)	Log kill
III native, day 34 bleed			
Rabbit 1	4.8×10^6	4.4×10^6	0.03
Rabbit 2	4.6×10^6	3.6×10^6	0.10
Rabbit 3	4.8×10^6	5.7×10^6	−0.08
III-TT, prevaccination			
Rabbit 1	4.5×10^6	3.2×10^6	0.15
Rabbit 2	4.7×10^6	3.5×10^6	0.13
Rabbit 3	3.4×10^6	4.0×10^6	−0.07
III-TT, day 34 bleed			
Rabbit 1	3.3×10^6	6.8×10^4	1.7
Rabbit 2	3.7×10^6	4.0×10^4	2.0
Rabbit 3	3.0×10^6	4.6×10^4	1.8

Values are means of duplicate determinations.

Table III. Survival of Mice Challenged with an LD₉₀ Dose of Live Type III GBS, Following Passive Immunization with Pooled Antiserum of Rabbits Vaccinated with Native GBS Type III Polysaccharide (III Native) or GBS Type III Polysaccharide-Tetanus Toxoid Conjugate (III-TT)

| | No. mice surviving/no. mice challenged | | |
Antiserum	Day 1	Day 2	Day 3
III native, day 34 (undiluted)	0/10	0/10	0/10
III-TT, day 0 (undiluted)	0/10	0/10	0/10
III-TT, day 34 (undiluted)	10/10	10/10	10/10
III-TT, day 34 (1:10 dilution)	10/11	9/11	9/11

vival for the group receiving undiluted antiserum and 83% protection for the group receiving antiserum diluted 1:10 (Table III).

Discussion

A recent study by Baker et al. showed that women vaccinated in the third trimester of pregnancy with a single dose of native GBS type III polysaccharide developed IgG antibodies that were efficiently transferred to their infants (10). The major limitation of maternal immunization with the isolated polysaccharide, however, is that the type III polysaccharide elicits an antibody response in only about 60% of previously nonimmune pregnant or nonpregnant women. The results of the current study suggest that the immunogenicity of the native polysaccharide may be enhanced greatly by conjugation to a protein carrier. Bacterial polysaccharides are considered thymus independent (TI) antigens because, in contrast to thymus dependent (TD) antigens, they are able to elicit an antibody response in athymic nude mice deficient in T helper cells. Conjugation of a variety of polysaccharides or oligosaccharides to carrier proteins has produced immunogens with TD properties including enhanced immunogenicity, predominant IgG isotype antibodies, and a booster or anamnestic response to subsequent vaccinations (14–16, 31). In the case of the GBS type III polysaccharide, the goal of conjugation is simply to increase the immunogenicity of the polysaccharide antigen. In contrast to *Haemophilus influenzae* type b, for example, the target population for immunization with a GBS vaccine is young adults, so the delayed ontogeny of the immune response to polysaccharide antigens is not an issue. Likewise, manipulation of the immune response to induce IgG class antibodies does not appear to be necessary, as the isotype of antibody elicited by the isolated GBS type III polysaccharide in human subjects is predominantly IgG (10, 32). Lagergard et al. have described the preparation of a GBS type III conjugate vaccine, in which the polysaccharide was activated at random sites with cyanogen bromide, then linked via a spacer molecule to tetanus toxoid (33). That conjugate elicited a greater antibody response in mice than did the native polysaccharide, but animal protection studies were not reported.

Several considerations went into the design of the conjugate vaccine used in our studies. A well characterized antigen of defined molecular size was utilized: purity of the polysaccharide was confirmed by GC and ¹H-NMR analysis, and the saccharide used for coupling was size fractionated by gel filtration chromatography. Functional groups were introduced at defined sites on the molecule to increase the efficiency of coupling. The presence of $\alpha(2 \rightarrow 3)$ linked sialic acid residues in a terminal position on the side chains of the GBS type III polysaccharide permitted the introduction of free aldehyde groups at specific sites on the saccharide. This selective modification of the polysaccharide structure has been shown not to alter the conformational and antigenic properties of the polysaccharide (18). Coupling was done by reductive amination under mild conditions without potentially toxic spacer molecules. The efficacy of this coupling method has been demonstrated for linking other bacterial polysaccharide antigens to proteins (16). In view of potential future use in human subjects, tetanus toxoid was used as the carrier protein as this preparation has an established record of safety for immunization of pregnant women (34).

Immune defense against GBS, like that against other Gram positive bacteria, depends on clearance of the organisms by phagocytic leukocytes. Type III GBS isolates from infants with invasive GBS disease are generally resistant to phagocytic killing by peripheral blood leukocytes in the presence of nonimmune serum (29). Antibodies directed against the type III capsular polysaccharide, in concert with serum complement, effectively opsonize type III GBS for phagocytic killing, in vitro (29). The GBS type III polysaccharide-tetanus toxoid conjugate vaccine elicited high titered, functional, IgG class antibodies in rabbits. The isolated native type III polysaccharide was completely nonimmunogenic, while the conjugate vaccine elicited IgG class antibodies after a single dose. Moreover, the titer rose in all animals after each of two booster doses of conjugate vaccine given 3 and 6 wk after the first injection. The antibodies elicited by the conjugate opsonized type III GBS organisms for phagocytosis in vitro, and protected mice against lethal challenge with type III GBS organisms.

While characterization of the T cell dependency of various antigens has been done primarily in mice, the rabbit model may be a better predictor of the response of human subjects to a GBS conjugate vaccine. TI antigens such as bacterial polysaccharides classically elicit a weak, predominantly IgM antibody response in mice, which cannot be augmented further by booster vaccination. In contrast, rabbits, like 40% of adult humans, uniformly fail to develop any antibody response to vaccination with the native GBS type III polysaccharide. Our data on immunogenicity of the GBS type III conjugate vaccine in rabbits suggest that conjugation of the GBS type III polysaccharide to tetanus toxoid may greatly enhance the immunogenicity of this antigen in those human subjects who fail to respond to the native polysaccharide. These results establish the immunogenicity in animals of a conjugate vaccine against type III GBS, and provide a rationale for testing a vaccine of this design in human subjects.

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