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Functional Activity of Antibodies to the Group B Polysaccharide of Group B Streptococci Elicited by a Polysaccharide-Protein Conjugate Vaccine

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Group B streptococci (GBS) are a major cause of sepsis and meningitis in infants. While antibodies directed to the type-specific GBS capsule have been shown to be protective, it is less clear whether antibodies to the group B polysaccharide, a noncapsular, cell wall-associated antigen, may play a role in immunity. To investigate the functional activity of group B polysaccharide-specific antibodies, we tested sera from rabbits vaccinated with group B polysaccharide coupled to tetanus toxoid (B-TT). Anti-B-TT was weakly opsonic in vitro for a highly encapsulated type III strain, while antiserum elicited by vaccination with type III capsular polysaccharide linked to tetanus toxoid (III-TT) was a very effective opsonin. In contrast to anti-III-TT, anti-B-TT given before or after bacterial challenge was only marginally effective in protecting newborn mice against lethal infection with type III GBS. The number of C3 molecules bound to type III GBS was augmented by anti-III-TT but not by high antibody concentrations of anti-B-TT. These results suggest that the difference in opsonic activity between anti-B-TT and anti-III-TT may be due to a difference in their ability to deposit C3. In addition, the maximum number of antibody molecules bound to the bacterial surface was greater for anti-III-TT than for anti-B-TT. That anti-B-TT binds to fewer sites than anti-III-TT may explain the differences in complement activation and in opsonic and protective efficacy of antibodies to group B polysaccharide compared with antibodies to the type-specific capsular polysaccharide.

Group B streptococci (GBS) are the leading cause of meningitis and sepsis in neonates in the United States (2). Host immunity to GBS depends on opsonization of the organisms by antibodies and complement, with subsequent killing by phagocytic leukocytes. Opsonic activity and protective activity have been demonstrated for antibodies to the capsular polysaccharides. However, GBS of at least six capsular types have been associated with human disease, and except for potential cross-reactivity between types Ia and Ib, protective antibodies directed toward the individual capsular polysaccharides are type specific. Also, evidence of protection in animals has been presented for antibodies directed to the C proteins, surface proteins expressed by about half of GBS strains (2, 8, 14, 15, 17, 18, 21). The group B polysaccharide is a noncapsular, cell wall-associated antigen which is shared by all serotypes (13, 22, 23). Therefore, if antibodies to the group B polysaccharide conferred protection, they could protect against clinical isolates of all capsular serotypes.

In 1975, Lancefield demonstrated that antisera raised in rabbits against whole GBS organisms contained antibodies to both the capsular and the group B polysaccharides. However, the antisera only conferred protection in mice against challenge with strains of the homologous capsular type, leading her to conclude that group B polysaccharide-specific antibodies were not protective (15). More recently, though, Raff et al. (24, 25) showed a protective effect of immunoglobulin M (IgM) monoclonal antibodies which reacted with the group B polysaccharide. Previously, other investigators found that a murine monoclonal antibody to the group B polysaccharide had no protective effect (28). It has been difficult to draw any firm conclusion about the potential of the group B polysaccharide as a protective antigen on the basis of these apparently conflicting results. Studies utilizing antisera against whole organisms suffer from the undefined specificity of the antibodies. Monoclonal antibodies have defined specificity, but the particular clones studied may not be representative of antibody responses in vivo following immunization or infection. An alternative means of eliciting antibodies of defined specificity in an intact animal is vaccination with a purified, well-defined vaccine. In this report, we utilized high-titer specific antisera elicited in rabbits by polysaccharide-protein conjugates to define the relative functional activity in vitro and in vivo of antibodies directed to the group B polysaccharide compared with antibodies directed to the type III capsular polysaccharide.

MATERIALS AND METHODS

GBS strains. The strains used for these studies were M781, a highly encapsulated type III GBS clinical isolate (donated by Carol Baker, Baylor College of Medicine, Houston, Tex.); COH1, another type III clinical isolate (donated by Craig Rubens, Children's Hospital, Seattle, Wash.); and an unencapsulated mutant strain, COH1-13, derived from COH1 by transposon insertional mutagenesis (27). The amount of cell-

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associated capsular polysaccharide on strain M781 is three to four times the amount on COH1 (19).

Preparation of conjugates and antisera. Isolation of the group B polysaccharide and conjugation to tetanus toxoid (B-TT) or human serum albumin, as well as the analysis of the B-TT and B-human serum albumin conjugates, has been reported (22). Rabbit antisera to B-TT were raised in female New Zealand White rabbits. Rabbits were vaccinated according to one of two schedules: schedule 1, 50 μg of B-TT subcutaneously (s.c.) in complete Freund's adjuvant on day 1, followed by 50 μg in incomplete Freund's adjuvant, s.c., 3 and 5 weeks later; or schedule 2, 50 μg of B-TT in complete Freund's adjuvant, s.c., on day 1, and in incomplete Freund's adjuvant on days 11 and 18. On days 4, 8, and 14, 50 μg of B-TT was given intravenously without adjuvant.

Purification of type III capsular polysaccharide, conjugation to tetanus toxoid (III-TT), and immunization of rabbits have been described previously (30). All antisera were heat inactivated at 56°C for 30 min prior to use.

Group B polysaccharide ELISA. Microtiter wells were coated with B-human serum albumin at 1 μg/ml in 0.05 M carbonate–bicarbonate buffer (pH 9.6) for 1 h at 37°C. Nonspecific protein-binding sites were blocked with 5% skim milk in phosphate-buffered saline (PBS), and serial dilutions of anti-B-TT were added to duplicate wells. After incubating for 1 h at 37°C, the plate was washed three times; alkaline phosphatase-conjugated goat anti-rabbit IgG at 0.1% (Tago, Inc., Burlingame, Calif.) was added, and the plates were incubated for 1 h at 37°C. In some experiments, peroxidase-conjugated goat anti-rabbit IgM (Cappel, Durham, N.C.) was used. Mouse antibodies to group B polysaccharide were detected with alkaline phosphatase-conjugated goat anti-mouse IgG (Cappel) as the secondary antibody. Following several washes with PBS, the enzyme substrate was added, the plate was incubated at 37°C, and the A_{405} readings were obtained in an automated enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Tek Instruments, Winooski, Vt.). The concentration of specific antibodies in individual sera was estimated by comparison in ELISA with a simultaneously tested reference serum. The concentration of group B polysaccharide-specific antibodies in the reference serum was 2 mg/ml, determined by quantitative precipitin analysis with purified group B polysaccharide (12). In ELISA inhibition experiments, a fixed dilution of each antiserum was added to the wells containing soluble purified group B polysaccharide (0.016 to 2.0 μg per well) or buffer. The remainder of the assay was performed as described above.

Complement sources. Pooled normal human serum from healthy volunteers was adsorbed with freshly harvested M781 organisms to remove type III GBS-specific antibodies, as described previously (19). The adsorbed serum pools served as the complement source for the experiments reported here.

Opsonophagocytic assays. The opsonophagocytic assay was a modification of the assay described by Baltimore et al. (3). Reaction tubes contained 4×10^6 human peripheral blood leukocytes, 2×10^6 mid-logarithmic-phase bacterial cells, 50 μl of adsorbed human serum, 50 μl of diluted rabbit serum, and Dulbecco's minimal essential medium to bring the total volume to 500 μl. Results are expressed as the mean decrease in CFU per milliliter after 1 h at 37°C.

Preparation of ^{125}I-C3 and C3 binding assay. Purified human C3 was a gift of Michael Pangburn, University of Texas Health Sciences Center, Tyler. C3 was iodinated and stored as described previously (19). C3 binding to type III GBS strains was determined in 10% adsorbed serum, with or without addition of anti-B-TT or anti-III-TT (19).

Form of C3 on the GBS surface. Organisms of GBS strain M781 were opsonized either in 10% adsorbed serum alone or with anti-B-TT or anti-III-TT for 30 min at 37°C. Following three washes in saline, half of each bacterial pellet was resuspended in Dulbecco's minimal essential medium for 1 h at 37°C before being pelleted again at 13,600 × *g* for 5 min. The supernatants were collected, combined with sodium dodecyl sulfate (SDS)-sample buffer containing 10% 2-mercaptoethanol, and boiled for 5 min. The other half of each pellet was immediately solubilized in SDS-sample buffer, boiled, and centrifuged to remove insoluble cellular debris. Both Dulbecco's minimal essential medium supernatants and whole solubilized organisms were subjected to electrophoresis on 4 to 15% gradient SDS-polyacrylamide gels (Bio-Rad Laboratories, Hercules, Calif.) and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, N.H.). Membranes were incubated with 5% skim milk–PBS to block protein-binding sites and then with goat antiserum to human C3 (Cappel) and alkaline phosphatase-conjugated rabbit anti-goat IgG (Cappel). C3 bands were developed with the phosphatase substrate indoxyl phosphate-Nitro Blue Tetrazolium reagent (Sigma Chemical Co.). The identification of the C3 fragments was based on the calculated molecular weight of each band, compared with the migration of C3 from fresh serum and with prestained molecular weight markers (Bethesda Research Laboratories, Gaithersburg, Md.).

Binding of anti-B-TT and anti-III-TT to M781. GBS strain M781 was grown to mid-logarithmic phase, washed, and resuspended in PBS–1% bovine serum albumin–0.05% Tween 20 to A_{650} = 0.3. Bacterial suspensions (50 μl) and 1 ml of serial dilutions of anti-B-TT, anti-III-TT, or nonimmune rabbit serum were added to duplicate 1.5-ml microcentrifuge tubes. After incubation at 37°C for 30 min, the bacterial cells were collected by centrifugation and washed twice in buffer. The cells were resuspended in buffer containing 0.1% alkaline phosphatase-conjugated goat anti-rabbit IgG (Tago, Inc.) and incubated at 37°C for 1 h. After four washes in buffer, the cells were resuspended in 500 μl of carbonate-bicarbonate buffer, pH 9.6; then duplicate 50-μl aliquots from each tube were added to microtiter wells. The same volume of alkaline phosphatase substrate 104 (Sigma Chemical Co.) at 2 mg/ml was added, and the contents were gently mixed. When the A_{405} values of wells containing bacterial cells opsonized with the highest concentrations of anti-III-TT reached 1.0, the results were read in an automated ELISA reader.

Adsorption of antibodies from anti-B-TT with type III GBS strains. GBS strains M781, COH1, and COH1-13 were grown to A_{650} = 0.3 in 35 ml of broth, harvested, and washed in saline. The bacterial pellets containing ~10^{10} CFU of each strain were mixed with anti-B-TT diluted 1:100 and were incubated on ice for 1 h with occasional agitation. After centrifugation, the supernatants were collected and tested in the group B polysaccharide ELISA. The A_{405} value of control wells containing the same dilution of serum not incubated with the bacterial pellets was considered 100%, and the A_{405} values of the supernatants were expressed as a percentage of the control.

Transfer of passive protection to mouse pups. Groups of three pregnant CD-1 mice, weighing 20 to 25 g, received a single intraperitoneal injection of rabbit serum 2 to 4 days before delivery. Each mouse received either 1 ml of anti-B-TT containing 2 mg of group B polysaccharide antibodies per ml or 0.5 ml of anti-III-TT containing 1.7 mg of type III polysaccharide antibodies per ml or 0.5 ml of nonimmune rabbit serum. Their 1-day-old pups were challenged with an intraperitoneal injection of a lethal dose of strain M781. Survival was assessed 48 h after challenge (26).

TABLE 1. Group B polysaccharide antibody concentration in sera of rabbits vaccinated with B-TT

Vaccination group	Group B polysaccharide antibody level (mg/ml)[a]	
	Day 1	Day 55
Schedule 1, s.c. injections only		
Rabbit 1	<0.005	1.6
Rabbit 2	<0.005	1.2
Rabbit 3	<0.005	1.2
Schedule 2, s.c. and i.v. injections[b]		
Rabbit 4	<0.005	0.45
Rabbit 5	<0.005	1.1
Rabbit 6	<0.005	2.0

[a] Results were calculated from mean ELISA titers for each antiserum tested twice in duplicate.
[b] i.v., intravenous.

Treatment of infected mouse pups. In order to assess the ability of anti-B-TT and anti-III-TT to enhance survival of pups infected with GBS, 1-day-old mouse pups of naive dams were injected intraperitoneally with a lethal dose of strain M781. Four hours later, they were given either a low (5 μl) or a high (50 μl) dose of anti-B-TT containing 4 mg of group B polysaccharide antibodies per ml, a low (5 μl) or a high (50 μl) dose of anti-III-TT containing 1.7 mg of type III polysaccharide antibodies per ml, or a low (5 μl) or a high (50 μl) dose of anti-TT by the same route. The pups were left with their mothers for the next 48 h, at which time percent survival was determined.

Active immunization of mice with B-TT or III-TT conjugates. Adult female CD-1 mice were vaccinated intraperitoneally with B-TT. Preliminary experiments demonstrated that 1- and 2-μg doses of B-TT were poorly immunogenic in mice. Thus, we vaccinated two mice with 5 μg and two mice with 10 μg of B-TT in complete Freund's adjuvant. As controls, two mice were vaccinated with 2.5 μg of TT. In a separate experiment, three mice were vaccinated with 2 μg of III-TT or 1 μg of TT, both in complete Freund's adjuvant. Two to 3 weeks after the first dose of vaccine, all mice received the same dose of the initial vaccine in incomplete Freund's adjuvant and were bred. Prior to delivery, blood was drawn again, and the sera were tested in an ELISA along with the prebooster serum samples. Within the first 24 h after birth, all pups were challenged with a lethal dose of strain M781. The ability of maternal antibodies to B-TT, III-TT, or TT to protect pups from death was assessed 48 h after the bacterial challenge.

Statistical analysis. Results of opsonophagocytic and C3 binding assays were analyzed for statistical significance by two-tailed unpaired Student's *t* test (Statview 512+, The Interactive Statistics and Graphics Package; BrainPower, Inc., Calabasas, Calif.). The comparison between groups with regard to survival in the in vivo studies was made by Fisher's exact test (Instat; GraphPad Software, San Diego, Calif.).

RESULTS

Titer and specificity of anti-B-TT. All rabbits vaccinated with B-TT developed high titers of group B polysaccharide-specific IgG. The concentrations of group B polysaccharide IgG class antibodies in sera collected 55 days after the primary vaccination ranged from 0.45 to 2.0 mg/ml (Table 1). The specificity of the antibodies for group B polysaccharide was demonstrated by inhibition of antibody binding to the immobilized antigen by soluble group B polysaccharide (data not shown). IgM antibodies to group B polysaccharide were not detected in any of the rabbit sera.

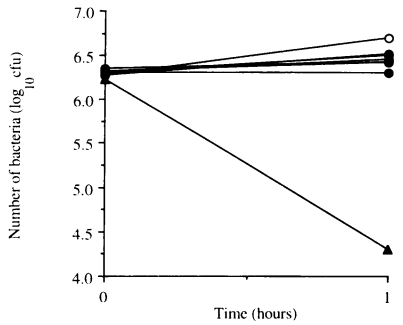

FIG. 1. Opsonophagocytic killing of strain M781. Data points represent initial $\log_{10}$ CFU or $\log_{10}$ CFU after 1 h of incubation with human peripheral blood leukocytes, 10% adsorbed human serum as a complement source, and 1% rabbit antiserum. Rabbit antisera included normal rabbit serum (open circles), anti-B-TT (seven serum samples; closed circles), or anti-III-TT (closed triangles). Each point represents the mean of two experiments done in duplicate. Standard deviations of the means were ≤0.2 log for all values.

Opsonophagocytic killing of M781. GBS strain M781 was incubated with adsorbed human serum, human leukocytes and normal rabbit serum, anti-B-TT, or anti-III-TT. In the absence of specific antibodies, there was significant growth after 1 h of incubation (0.4 log increase in CFU). None of the anti-B-TT sera tested at a 1% concentration mediated sufficient bacterial killing to result in a net decrease in the input inoculum, although in the presence of anti-B-TT there was less net growth of GBS than in nonimmune rabbit serum. As a control, anti-III-TT diluted to 0.1% mediated a 2.0-log decrease in CFU (Fig. 1).

Passive protection of neonatal mice by anti-B-TT and anti-III-TT. We determined the ability of anti-B-TT and anti-III-TT to protect mouse pups from lethal infection in a neonatal mouse model of GBS disease. In two separate experiments, pregnant mice received rabbit anti-B-TT, anti-III-TT, or nonimmune rabbit serum 2 to 4 days before delivery; then the pups were challenged with a lethal dose of GBS. The survival values for pups from each group were 63 and 47% (anti-B-TT), 100 and 100% (anti-III-TT), and 42 and 22% (nonimmune rabbit serum) in experiments 1 and 2, respectively (Table 2). The higher rate of survival in the anti-B-TT-treated compared with the nonimmune rabbit serum group did not reach statistical significance in either experiment ($P = 0.11$ and 0.09, respectively). As reported before, however, anti-III-TT was highly protective in this model (26).

To confirm that rabbit antibodies crossed the placenta in this model, group B polysaccharide antibody levels were measured in three dams after administration of anti-B-TT and in 11 of their pups. As measured in the group B polysaccharide ELISA, the average A_{405} value of rabbit IgG in the dams' sera diluted 1:100 was 0.66 ± 0.03 ($n = 3$), and the A_{405} value for the pups was 0.74 ± 0.02 ($n = 11$), confirming that anti-B-TT reached the maternal circulation and was transferred across the placenta.

Active immunization of mice with B-TT or III-TT. One-day-old pups of dams previously vaccinated with B-TT, III-TT, or TT were challenged with a lethal dose of strain M781. Pups of dams vaccinated with B-TT did not have significantly increased survival compared with pups of dams vaccinated with TT (20 and 0% survival, respectively). All dams' sera in the B-TT-

TABLE 2. Survival of mouse pups of passively immunized dams[a]

Rabbit serum[b]	No. of survivors/ no. challenged	% Survival
Expt 1		
Anti-B-TT[c]	25/40	63[d]
Anti-III-TT[e]	41/41	100
Normal rabbit serum	15/36	42
Expt 2		
Anti-B-TT[c]	15/32	47[f]
Anti-III-TT[e]	30/30	100
Normal rabbit serum	5/23	22

[a] Pups were challenged with a 90% lethal dose of strain M781 1 day after birth.
[b] The dose of each antiserum per dam was 1 ml of anti-B-TT or 0.5 ml of anti-III-TT or normal rabbit serum.
[c] The anti-B-TT serum contained 2.0 mg of group B polysaccharide antibodies per ml.
[d] $P < 0.0001$ for comparison to anti-III-TT; $P = 0.11$ for comparison to normal rabbit serum.
[e] The anti-III-TT serum contained 1.7 mg of type III polysaccharide antibodies per ml.
[f] $P < 0.0001$ for comparison to anti-III-TT; $P = 0.09$ for comparison to normal rabbit serum.

treated group had group B polysaccharide-specific IgG at the time of delivery (Table 3). In a separate experiment, 94% of pups of dams vaccinated with III-TT survived GBS infection, while none of the pups of TT-immune dams survived (data not shown).

Treatment of infected pups with anti-B-TT or anti-III-TT. We also tested the effectiveness of rabbit anti-B-TT and anti-III-TT to treat pups (of naive mothers) infected 4 h earlier with a lethal dose of M781. A 5-μl dose of anti-B-TT containing 20 μg of group B polysaccharide antibodies (low dose) rescued only 8% of the pups from death, and 10 times more antibody (high dose) did not significantly improve survival (17%; Table 4). In contrast, 8.5 μg of anti-III-TT (low dose) resulted in survival of 69% of the pups; a dose of 85 μg (high dose) did not further enhance survival. Anti-TT also failed to improve survival in this model (8 and 0% survival after injection of 5 and 50 μl of serum per mouse, respectively).

C3 binding to M781. Because anti-B-TT and anti-III-TT differed in opsonic activity, we hypothesized that this difference might reflect different degrees of complement activation. Logarithmic-phase bacteria were incubated in adsorbed human serum alone or with anti-B-TT or anti-III-TT at increasing concentrations. As compared with serum alone, anti-III-TT at 1% significantly increased the number of C3 molecules bound to the organisms, while anti-B-TT did not result in detectable augmentation of C3 deposition (Fig. 2). This result suggested that anti-B-TT was not opsonic because it did not activate

TABLE 3. IgG response of dams vaccinated with B-TT and survival of their pups challenged with M781 on the first day of life

Vaccine	Dose (μg)	Group B polysaccharide-specific IgG[a]		No. of live pups/ no. of infected pups (% survival)
		Prebooster	Predelivery	
TT	2.5	0.00 ± 0.00	0.00 ± 0.00	0/13 (0)
B-TT	5	0.12 ± 0.07	0.78 ± 0.06	4/19 (21)[b]
B-TT	10	0.19 ± 0.12	0.40 ± 0.07	3/15 (20)[c]

[a] Data represent means ± standard deviations of A_{405} values in group B polysaccharide-ELISA, using mouse serum diluted 1:100.
[b] $P = 0.13$ for comparison to survival in the TT-vaccinated group.
[c] $P = 0.23$ for comparison to survival in the TT-vaccinated group.

TABLE 4. Survival of mouse pups after challenge with GBS strain M781 and treatment with rabbit antiserum

Rabbit antiserum	Dose (μl)	No. of survivors/ no. challenged	% Survival
Anti-B-TT[a]	5	1/13	8[b]
	50	7/42	17[c]
Anti-III-TT[d]	5	9/13	69
	50	6/10	60
Anti-TT	5	1/12	8
	50	0/12	0

[a] The anti-B-TT serum contained 4 mg of group B polysaccharide antibodies per ml.
[b] $P = 0.004$ for comparison to low-dose anti-III-TT.
[c] $P = 0.01$ for comparison to high-dose anti-III-TT; $P = 0.0007$ for comparison to low-dose anti-III-TT.
[d] The anti-III-TT serum contained 1.7 mg of type III polysaccharide antibodies per ml.

complement efficiently on the surface of encapsulated type III strains. Of note, with anti-III-TT at 0.1% as used in the opsonophagocytic assays, we also could not detect an increase in the number of C3 molecules bound per CFU of M781. At that concentration, however, anti-III-TT mediated a 2.0-log decrease in CFU of strain M781.

Another possibility to explain the difference in opsonization mediated by anti-B-TT and anti-III-TT was that they bound different forms of C3 to the bacteria. Analysis by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot (immunoblot) demonstrated that the pattern of C3 bound to M781 was the same in serum with or without either antiserum (Fig. 3). The intensity of the bands corresponding to the total C3 molecules solubilized from the bacteria (lanes 1, 2, and 3) was greater than the intensity of the fragments shed by the organisms (lanes 4, 5, and 6). In lanes 1 to 3, there were high-molecular-weight bands, the most prominent at 140 kDa, which probably represented C3 bound to bacterial components. A faint band that comigrated with the intact α chain of C3 was seen at 120 kDa, as well as a prominent band at 75 kDa that comigrated with the β chain of C3. A prominent band at 42 kDa likely represents the α'_3 fragment of C3bi. The forms of C3 spontaneously released by the organisms (lanes 4 to 6) were very similar to all fragments bound, except for the presence of a fragment at 110 kDa, likely to be the α′ chain of C3b. These experiments showed that both C3b and C3bi were bound to the bacteria in the presence of anti-B-TT or anti-

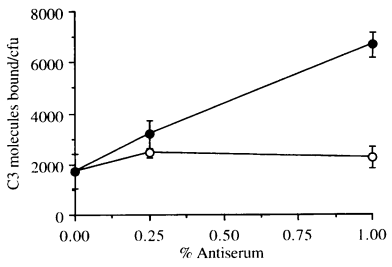

FIG. 2. C3 binding to organisms of strain M781. Binding of C3 to strain M781 was determined in adsorbed human serum with no antiserum (line origin) or with anti-B-TT (open circles) or anti-III-TT (closed circles) at two concentrations. Results are expressed as mean ± standard error of C3 molecules bound per CFU.

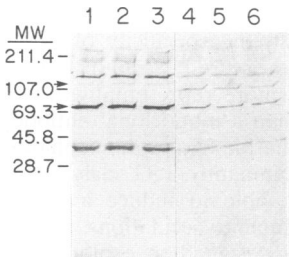

FIG. 3. Form of C3 bound to strain M781. Western blot of SDS-PAGE probed with C3-specific antibodies to demonstrate C3 fragments bound to strain M781. Lanes 1, 2, and 3, total C3 fragments bound after opsonization in human serum with anti-B-TT, anti-III-TT, or no added antiserum, respectively; lanes 4, 5, and 6, C3 fragments released from the organisms during incubation in Dulbecco's minimal essential medium after opsonization in human serum with anti-B-TT, anti-III-TT, or no added antiserum, respectively. Arrows indicate the migration of the α chain (120 kDa) and β chain (75 kDa) of C3 in a standard of fresh human serum.

III-TT and that C3 formed covalent complexes with the bacterial components that persisted after boiling.

Binding of anti-B-TT and anti-III-TT to M781. We quantified anti-B-TT and anti-III-TT binding to the GBS strains in the fluid phase by sequential incubation with either anti-B-TT or anti-III-TT, an enzyme-conjugated second antibody, and the enzyme substrate. The intensity of the color was proportional to the amount of anti-B-TT or anti-III-TT bound to the organisms. These experiments showed that the number of type III capsular polysaccharide-specific antibody molecules bound to the organisms was much higher than the number of group B polysaccharide-specific antibody molecules bound (Fig. 4). Furthermore, the slopes of the binding curves suggested that the affinity of anti-III-TT was higher than that of anti-B-TT. These results strongly suggested that anti-III-TT was more efficient in promoting opsonization because capsular polysaccharide-specific antibodies bind with higher affinity and to many more sites on the surface of the encapsulated strains of GBS than do group B polysaccharide-specific antibodies.

Adsorption of anti-B-TT by type III GBS strains. To assess the influence of the quantity of capsular polysaccharide expressed by each strain on the binding of anti-B-TT to the whole organisms, we determined loss of titer of anti-B-TT by ELISA after incubation with M781 or COH1 (capsule-positive strains) or with COH1-13 (capsule-negative mutant of COH1). Figure 5 demonstrates that group B polysaccharide-specific antibodies bound to all three strains, since incubation of anti-B-TT with

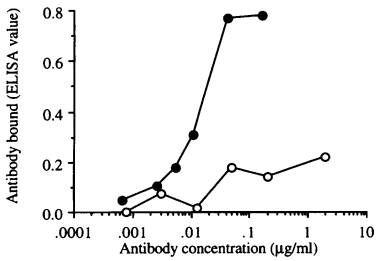

FIG. 4. Binding of anti-B-TT (open circles) and anti-III-TT (closed circles) to strain M781 in fluid-phase ELISA. Values represent net A_{405} for the binding of each antiserum corrected for background values obtained for normal rabbit serum at equivalent serum dilutions. Data are from a representative experiment done in duplicate.

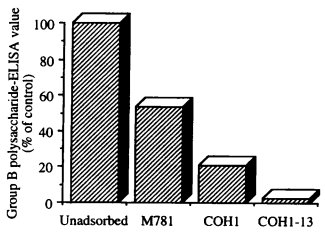

FIG. 5. Adsorption of anti-B-TT by strains of GBS. ELISA values of anti-B-TT before and after adsorption with three GBS strains: M781 (highly encapsulated), COH1 (moderately encapsulated), and COH1-13 (acapsular mutant of COH1). The value for each adsorbed serum sample is expressed as a percentage of the A_{405} value for unadsorbed antiserum tested at the same dilution. Data are from a representative experiment.

each strain reduced the group B polysaccharide antibody titer. However, the unencapsulated strain COH1-13 adsorbed considerably more group B polysaccharide-specific antibodies than COH1 or M781, suggesting that binding of group B polysaccharide-specific antibodies to the intact organisms is impaired by the presence of the capsular polysaccharide.

DISCUSSION

The capacity of antibodies to activate complement is an important determinant of their opsonic activity. IgG-mediated complement activation via the classical pathway occurs only when at least two IgG molecules are in close proximity to allow binding of C1q (5). Therefore, both the distribution of the binding sites and the concentration of antibody molecules on the bacterial surface influence complement activation and deposition of opsonic complement fragments on the bacterial cell. However, not all antibodies that activate complement are opsonic. For example, antibodies to the cell wall of pneumococci bound C3b underneath the capsule, a site not accessible to C3b receptors, and thus did not mediate killing (7).

The opsonic capacity of antibodies to noncapsular epitopes on gram-positive bacteria has not been as clearly defined as the opsonic capacity of anticapsular antibodies. Antibodies to pneumococcal surface protein A have been shown to be opsonic (6, 20). However, antibodies to the pneumococcal cell wall polysaccharide elicited by a polysaccharide-protein conjugate were not protective against encapsulated pneumococci (30). Similarly, antibodies to the cell wall of *Staphylococcus aureus* were opsonic for microencapsulated strains in vitro (33) but did not seem to be protective in vivo (9, 10, 11, 31). Strains of GBS expressing surface C proteins were killed by specific antibodies in opsonophagocytic assays, and the antibodies were protective in vivo in the same neonatal mouse model of infection used in this report (8, 17, 18, 21). However, although R proteins are also found on the surface of some strains of GBS, rabbit antibodies to the R proteins have not been consistently opsonic (8, 16).

Studies of the opsonic and protective capacity of antibodies to the group B polysaccharide, the species-specific cell wall antigen of GBS, have been hampered by lack of specific polyclonal sera. Antisera used in earlier studies were made monospecific by cross-adsorption with GBS antigens or were monoclonal antibodies which may not reflect the "normal" immune response to an antigen. In this report, we compared the properties of antibodies to the group B polysaccharide with those of antibodies to the capsular polysaccharide of type III strains. These antisera were prepared in rabbits vaccinated

with group B polysaccharide or type III capsular polysaccharide covalently coupled to TT. In vitro, anti-B-TT was not efficient in opsonizing GBS for phagocytic killing, while anti-III-TT was very effective. In vivo, transplacentally transferred rabbit group B polysaccharide antibodies were not as effective as rabbit anti-type III capsular polysaccharide in preventing death of neonatal mice challenged with GBS. Indeed, antibodies to group B polysaccharide were, at most, only slightly better opsonins than nonimmune rabbit serum in the neonatal mouse protection model. Anti-B-TT given after bacterial challenge also failed to improve the survival of pups infected with a virulent type III GBS strain, while administration of even a low dose of anti-III capsular polysaccharide resulted in more than 60% survival of infected animals. We also tested the protective ability of group B polysaccharide antibodies and of type III capsular polysaccharide antibodies induced by vaccinating adult female mice with B-TT or III-TT, subsequently challenging their pups with type III GBS. While only 20% of the pups of group B polysaccharide-immune mothers survived challenge with GBS, 94% of the pups of type III capsular polysaccharide-immune mothers lived.

Lancefield found that rabbit antisera raised by vaccination with GBS organisms were not protective when injected into mice challenged with organisms of another capsular polysaccharide type, suggesting that antibodies to the group B polysaccharide, although broadly reactive among serotypes, were not protective (15). Reports on the functional capacity of monoclonal antibodies to the group B polysaccharide have varied. Shigeoka et al. (28) reported that an IgM mouse monoclonal antibody to group B polysaccharide did not protect neonatal rats challenged with type III GBS, while a monoclonal antibody to the capsular polysaccharide was protective. Other investigators (24, 25, 29) reported that human IgM monoclonal antibodies to group B polysaccharide were opsonic in vitro and protective in vivo against strains of several GBS serotypes. Evidence from an epidemiological survey suggested that the presence of group B polysaccharide maternal antibodies did not protect neonates against early-onset GBS disease (1).

Several factors could explain these apparently contradictory reports of group B polysaccharide functional activity. (i) Antisera raised to whole bacteria may have only low concentrations of group B polysaccharide antibodies. This explanation does not appear likely because sera prepared by the Lancefield method give high ELISA titers and strong precipitin lines with purified group B polysaccharide in vitro. (ii) The presence of blocking antibodies in sera obtained from rabbits immunized with whole bacteria could impair the ability of group B polysaccharide antibodies to opsonize. Our data do not rule out this possibility, but the experiments reported here suggest rather that the lack of protective activity reflects the relatively weak opsonic capacity of group B polysaccharide antibodies. (iii) Although certain group B polysaccharide-specific monoclonal antibodies have been shown to have functional activity against GBS, they are not necessarily representative of the normal host response to the group B polysaccharide antigen. A selected clone may be found only rarely among those B cells stimulated by vaccination. Furthermore, the functional monoclonal antibodies to group B polysaccharide were protective to neonatal rats only at very high doses and lost activity upon class switching from IgM to IgG1 (24, 25). An IgM antibody may be a better opsonin since a single bound molecule can activate complement whereas at least two molecules of bound IgG are required for complement activation.

Antibodies raised to the group B polysaccharide by immunization with a conjugate vaccine offer certain advantages in understanding the host immune response to this polysaccharide antigen. First, the specificity of the antibodies for the defined antigen is assured. Second, the polyclonal antibody response may be more representative of the intact host immune response than a monoclonal antibody. Third, conjugation of a polysaccharide to a protein enhances immunogenicity and elicits predominantly IgG class antibodies. Conjugate vaccines are also able to induce a booster or anamnestic response to subsequent vaccinations.

In experiments designed to explore the mechanisms that might explain why anti-B-TT and anti-III-TT were so different functionally, we found that anti-B-TT activated complement poorly on the surface of the organisms. We could not detect any augmentation of C3 deposition on the highly encapsulated strain M781 opsonized by anti-B-TT, while anti-III-TT mediated an increase in C3 binding proportional to the concentration of antibody used. Because the complement-activating properties of antibodies depend on their binding characteristics, we compared the binding of anti-B-TT with that of anti-III-TT to the intact organisms in the fluid phase. The results of these experiments demonstrated that significantly fewer molecules of anti-B-TT than of anti-III-TT bound to encapsulated GBS organisms. Thus, the inability of anti-B-TT to facilitate complement deposition may be due, at least in part, to the few antibody binding sites available on the bacteria relative to the number available for anti-III-TT. We also found an inverse relationship between the amount of capsular polysaccharide expressed by each strain and the ability of that strain to bind anti-B-TT. These data strongly suggest that the capsule interferes with binding of group B polysaccharide antibodies by preventing access of those antibodies to the underlying cell wall. This formulation is consistent with a similar hypothesis proposed by Bohnsack et al. to explain the observation that an intravenous immunoglobulin preparation could inactivate C5a-ase on the surface of unencapsulated GBS but not on the surface of encapsulated strains (4).

The results of these studies demonstrate that group B polysaccharide-specific antibodies elicited by a polysaccharide-protein conjugate vaccine are only weakly opsonic both in vitro and in vivo. The poor functional activity of group B polysaccharide antibodies appears to reflect both limited binding to the bacterial surface and failure to activate complement, properties attributable, at least in part, to the presence of the capsular polysaccharide.

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