INFECTION AND IMMUNITY, Oct. 1992, p. 3986–3993
0019-9567/92/103986-08$02.00/0

Vol. 60, No. 10

Prevention of C3 Deposition by Capsular Polysaccharide Is a Virulence Mechanism of Type III Group B Streptococci

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Received 3 April 1992/Accepted 9 July 1992

Strains of type III group B streptococci isolated from patients with neonatal sepsis are generally resistant to complement-mediated phagocytic killing in the absence of specific antibody. It has been suggested that the resistance of type III group B streptococci to phagocytosis results from inhibition of alternative-complement-pathway activation by sialic acid residues of the type III polysaccharide. To better define the relationship between structural features of the type III capsule and resistance of type III group B streptococci to complement-mediated phagocytic killing, we measured deposition of human C3 on group B streptococcal strains with altered capsule phenotypes. C3 binding was quantified by incubating bacteria with purified human ^{125}I-C3 in 10% serum. Wild-type group B *Streptococcus* sp. strain COH1 bound eightfold fewer C3 molecules than did either of two isogenic mutant strains, one expressing a sialic acid-deficient capsule and the other lacking capsule completely. Similar results were obtained when the incubation with ^{125}I-C3 was performed in serum chelated with Mg-ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (MgEGTA), suggesting that the majority of C3 deposition occurred via the alternative pathway. In contrast to the wild-type strain, which was relatively resistant, both mutant strains were killed by human leukocytes in 10% serum with or without MgEGTA. We also measured C3 binding to 14 wild-type strains of type III group B streptococci expressing various amounts of capsule. Comparison of degree of encapsulation with C3 binding revealed a significant inverse correlation ($r = -0.72$; $P < 0.01$). C3 fragments released by methylamine treatment of wild-type strain COH1 were predominantly in the form of C3bi, while those released from the acapsular mutant were predominantly C3b and those from the asialo mutant represented approximately equal amounts of C3b and C3bi. We conclude from these studies that the sialylated type III capsular polysaccharide inhibits alternative-pathway activation, prevents C3 deposition on group B streptococci, and protects the organisms from phagocytic killing.

Group B streptococci (GBS) are leading causes of septicemia and meningitis among newborn infants. Most strains of GBS isolated from infected infants express one of the four major type-specific GBS capsular polysaccharides, Ia, Ib, II, and III. While the major capsular types are represented in roughly equal proportions among colonizing strains, type III strains account for about two-thirds of the GBS isolates associated with invasive neonatal disease, suggesting that the type III capsule may serve a virulence function (1). Among type III GBS isolates, those strains expressing larger amounts of capsular polysaccharide have been associated with invasive human infection and with virulence in experimental animals. A correlate of animal virulence of these strains is their relative resistance in vitro to phagocytic killing in serum deficient in specific antibody. The ability of the organisms to resist opsonophagocytosis unless specific antibody is available appears to reflect, at least in part, failure of the bacterial surface to efficiently activate the alternative pathway of complement. The failure of type III GBS organisms to activate the alternative pathway appears to be a result of the presence of terminal sialic acid residues on each repeating unit of the type III polysaccharide. Edwards et al. found that removal of the capsular sialic acid residues by growth of the organisms in the presence of neuraminidase rendered type III GBS sensitive to alternative-pathway-mediated opsonophagocytic killing in the absence of specific antibody, while bacteria expressing the native sialylated type III polysaccharide were resistant (6). Subsequent studies demonstrated that neuraminidase-treated type III organisms activated the alternative pathway in antibody-deficient serum, resulting in consumption of C3 and factor B, while intact untreated bacteria did not (5). While these results supported the hypothesis that the sialic acid-rich GBS capsule interfered with phagocytic killing by inhibiting alternative-pathway activation, a more recent study concluded that the type III capsule actually promoted C3 deposition on the bacterial surface (4). Campbell et al. found that the amount of C3 deposited on the encapsulated organisms increased with longer periods of opsonization, while the pattern of deposition on an acapsular strain remained relatively constant (4). The implication that the type III capsule facilitates rather than prevents deposition of opsonic complement fragments is at odds with earlier work that indicated that capsular sialic acid residues inhibited complement activation and interfered with phagocytosis (5, 6). We have reexamined the question of how capsular sialic acid affects C3 binding to GBS and the implications of this interaction for phagocytic killing of GBS in the nonimmune host. Utilizing isogenic mutant strains of type III GBS lacking capsule entirely or lacking only capsular sialic acid, we have quantitatively measured the deposition of C3 during opsonization. These results and those of experiments correlating C3 deposition with amount of capsule in 14 clinical

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isolates of type III GBS show definitively that the sialic acid-rich type III capsule prevents C3 deposition on the bacterial surface, thereby protecting the organisms from phagocytic killing.

MATERIALS AND METHODS

Buffers and reagents. For complement activation experiments, buffers included isotonic Veronal-buffered saline (VBS); VBS with 0.1% gelatin, 0.15 mM $CaCl_2$, and 1.0 mM $MgCl_2$ ($GVBS^+$) or VBS without cations ($GVBS^-$); and VBS with 0.1% gelatin and 0.1 M EDTA. Stock Mg-ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*,-tetraacetic acid (MgEGTA) contained 0.1 M $MgCl_2$ and 0.1 M EGTA at pH 7.5.

Bacterial strains. GBS strains included COH1, a type III strain; COH1-11, a mutant whose capsule lacks sialic acid, and COH1-13, an unencapsulated mutant, both derived from wild-type strain COH1 by transposon Tn*916ΔE* insertional mutagenesis (17a, 22); COH31, a type III strain; and COH31-21, a mutant whose capsule lacks sialic acid, and COH31-15, an unencapsulated mutant, both derived from wild-type strain COH31 by Tn*916* mutagenesis (18, 24). Thirteen other type III clinical isolates, most of which were donated by Carol Baker, Baylor College of Medicine, Houston, Tex., were also used in some experiments. All strains were grown on blood agar plates overnight and then inoculated into 10 ml of Todd-Hewitt broth and grown to an A_{650} of 0.30 before being washed and suspended in the appropriate buffer.

Preparation of ^{125}I-C3. Purified human C3 was isolated from human serum as described previously (10). Functionally active C3 was iodinated by using IODOBEADS (Pierce Chemical Co., Rockford, Ill.) and NaI (Amersham Corp., Arlington Heights, Ill.) to a specific activity of 2.2×10^5 cpm/μg. Each batch of radiolabeled C3 was stored at 4°C for up to 2 weeks.

Complement source. Pooled normal human serum from healthy volunteers was prepared. Serum was adsorbed twice with a freshly harvested highly encapsulated type III GBS strain (M781) on ice for 30 min to remove anti-type III capsular polysaccharide antibody. Adsorbed serum was collected by centrifugation at 3,000 × *g* for 15 min at 4°C, filter sterilized, and immediately frozen in aliquots at −70°C (serum). Adsorption of antibody to the type III polysaccharide was confirmed by specific enzyme-linked immunosorbent assay (ELISA). Heat inactivation was performed at 56°C for 30 min in a water bath as needed. For each experiment, a newly thawed aliquot of serum was combined with ^{125}I-C3 (20 to 24 μg of ^{125}I-C3 per ml of serum). Any precipitate was removed by centrifugation at 13,600 × *g* for 5 min at 4°C immediately before the serum was mixed with bacteria in the reaction tubes.

Antiserum. Type III GBS antiserum was prepared by immunizing rabbits with whole organisms of GBS M732 (6). Antiserum was heat inactivated at 56°C for 30 min and used at a final dilution of 1:100 in opsonization experiments.

Opsonophagocytic assay. The opsonophagocytic assay described by Baltimore et al. (2) was used. Samples contained 1.5×10^6 CFU of GBS previously grown in broth culture to mid-logarithmic phase and 3×10^6 human peripheral blood leukocytes in 500 μl of Eagle's minimum essential medium. Bacteria were preopsonized by incubation in 10% serum with or without 5 mM MgEGTA before being added to the assay mixture. Controls included assay mixtures in which heat-inactivated serum was substituted for normal serum and assay mixtures from which leukocytes were omitted. Assay mixtures were incubated at 37°C with end-over-end rotation for 60 min. Aliquots were removed at time zero and at 60 min, diluted in sterile water, and spread on blood agar plates for quantitative culture. Results were expressed as percent killing and log decrease in CFU.

Capsule index determination. Type III GBS strains were grown in 10 ml of broth to an A_{650} of 0.45. Five milliliters of the culture was spun down, and the pellet was washed in phosphate-buffered saline (PBS) and suspended in 1 ml of mutanolysin (Sigma Chemical Co., St. Louis, Mo.) mixture (0.27 mg/ml in 30 mM phosphate buffer, pH 7.0, containing 40% sucrose). Tubes were then rotated for 60 min at 37°C, protoplasts were pelleted, and supernatants were collected as crude capsule extracts. Capsule extracts were diluted in phosphate buffer at a 1:25 dilution and added to microtiter plates in triplicate before the addition of type III GBS antiserum diluted 1:2,000. Mixtures of capsule extract and antiserum were then transferred to ELISA wells previously coated with GBS type III polysaccharide, and the ELISA was carried out as described elsewhere (18). The amount of capsule produced by each strain was quantified by comparison with a standard curve for purified type III polysaccharide (23) and expressed as a capsule index based on the degree of inhibition produced by the test strain relative to that produced by a well-encapsulated reference strain (M732) assayed simultaneously. Capsule index was calculated by the following formula: capsule index = test strain extract (μg of polysaccharide/ml)/M732 extract (μg of polysaccharide/ml).

C3 binding assay. Binding of ^{125}I-C3 to GBS strains was measured in 10% serum or in MgEGTA–10% serum. In some experiments, type III GBS antiserum was included. Log-phase bacteria were washed twice in isotonic saline and suspended in $GVBS^+$. Appropriate volumes of the suspensions were mixed with 10% serum or MgEGTA–10% serum containing trace-labeled ^{125}I-C3 (ratio of labeled/unlabeled = 1:60) to a final concentration of 2×10^8 CFU/ml in 0.4 ml (total volume) in $GVBS^+$. Assay mixtures were incubated for 30 min at 37°C with end-over-end rotation. Control tubes contained 10 mM EDTA in addition to the other components. In order to separate bound from free ^{125}I-C3, 0.1-ml samples were centrifuged at 13,600 × *g* through 0.3 ml of 15% sucrose–$GVBS^-$ in 0.5-ml Microfuge tubes for 6 min (16). Radioactivity in the pellets and supernatants was counted separately in a gamma counter (Beckman Instruments Inc., Irvine, Calif.). Specific binding was calculated by subtracting the binding in EDTA-serum from total binding in serum or MgEGTA-serum. The number of C3 molecules bound per CFU was calculated according to the following formula.

$$\text{C3 molecules/CFU} = \left(\frac{\text{cpm (total-nonspecific)}}{\text{cpm/molecule of } ^{125}\text{I-C3}} \times \frac{\text{60 molecules of unlabeled C3}}{\text{molecule of } ^{125}\text{I-C3}} \right) \div \text{CFU in pellet}$$

Neuraminidase-treated bacteria. Enzymatic removal of sialic acid residues was accomplished by growing strain COH1 in broth containing 0.43 U of *Clostridium perfringens* neuraminidase (type V; Sigma) per ml. Neuraminidase in the growth medium was replenished once during an incubation period of 3 h. When the A_{650} reached 0.60, 0.3% formalin (Fisher Scientific, Fair Lawn, N.J.) was added to a final concentration of 0.3% (vol/vol), and the mixture was stirred

for 15 min at 4°C. Organisms were harvested by centrifugation, washed in isotonic saline, and suspended in saline containing 0.3% formalin to be stored at 4°C. Control organisms were grown without neuraminidase and treated with formalin in parallel. Subcultures of the formalin-fixed suspensions confirmed loss of viability before the organisms were used in experiments.

Consumption of alternative-pathway components by COH1. Native and neuraminidase-treated COH1 cells were incubated in MgEGTA–42% serum for 30 min at 37°C with tumbling. Controls included serum incubated with zymosan or buffer. Bacteria were removed by centrifugation, and then 50-μl aliquots of each supernatant were serially diluted twofold in MgEGTA-GVBS$^+$ and mixed with an equal volume of a 1% suspension of rabbit erythrocytes in the same buffer in U-shaped microtiter wells. Plates were incubated for 60 min at 37°C and then centrifuged for 5 min at 800 × *g*. Fifty-microliter aliquots of the supernatants were transferred to flat-bottom microtiter wells, and the A_{405} was read in an automated ELISA reader to assess hemoglobin release (complement-mediated lysis). Controls included wells containing erythrocytes in buffer alone (no lysis) or in water (100% lysis). The data were analyzed according to the method of Van Krogh, as described by Mayer (14) to calculate alternative-pathway activity of each sample. The percentage of alternative-pathway activity consumed under each set of conditions was calculated relative to that of serum incubated with buffer, which was considered 100% alternative-pathway activity.

Form of C3 bound to GBS. Bacteria were opsonized as for C3 binding assays and then washed twice in 0.01 M EDTA, suspended in sodium dodecyl sulfate (SDS)-sample buffer containing 2-mercaptoethanol (Sigma), and boiled for 5 min. Residual cellular debris was pelleted in the Microfuge and the supernatants were applied to 4 to 15% gradient SDS gels (Bio Rad, Richmond, Calif.). No radioactivity remained associated with the cell debris pellet, indicating that this procedure effectively solubilized all bound C3 molecules. Radiolabeled C3 was visualized by autoradiography of the SDS gels.

Trypsin treatment of GBS strains. Cell-bound C3bi but not C3b is susceptible to cleavage by doses of trypsin lower than 2.5 μg/ml (9, 17). A dose-response curve for doses of trypsin ranging from 0.002 to 2 μg/ml was constructed by exposing preopsonized ^{125}I-C3-bearing COH1-11 and COH1-13 organisms to L-1-tosylamide-2-phenylethylchloromethyl ketone (TPCK)–trypsin (Sigma) in Hanks balanced salt solution for 10 min at 30°C. C3 fragments released by trypsin treatment were quantified by pelleting the treated bacteria (Microfuge) and then counting the radioactivity in the supernatants and pellets separately in the gamma counter. The percentage of bound radioactivity released by trypsin treatment was calculated as percent C3 released = (cpm in supernatant after trypsin/total cpm bound) × 100. Trypsin-cleaved C3 fragments were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography.

Methylamine treatment of C3-bearing GBS. In order to identify the form of C3 molecules bound via ester bonds to COH1, COH1-11, and COH1-13, opsonized organisms of each strain were washed in 0.01 M EDTA and then incubated for 60 min at 37°C in 0.05 M carbonate-bicarbonate buffer, pH 9.6, containing 25 mM methylamine (Sigma). The bacteria were pelleted (Microfuge), and the supernatants were analyzed by SDS-PAGE and immunoblot. Proteins were transferred to nitrocellulose by electroblotting in 20 mM Tris–150 mM glycine–20% methanol (pH 8.0) for 1 h at 100 mA in a Semi-Dry Transfer Unit (Hoefer Scientific Instruments, San Francisco, Calif.). After being blocked for 2 h at 37°C with 5% skim milk in PBS, blots were exposed to goat antiserum to human C3 (Sigma) at a 1:300 dilution in PBS for 1 h at 37°C. Second antibody was alkaline phosphatase-conjugated rabbit anti-goat immunoglobulin G used at 1:1,000 for 1 h at 37°C. Following several washes, bands were visualized by utilizing the indoxyl phosphate-Nitro Blue Tetrazolium reagent (Sigma).

TABLE 1. Correlation between killing of GBS strains by human leukocytes and C3 binding in vitro[a]

Opsonization mixture and strain	% Killing	Mean log decrease in CFU (range)	No. of C3 molecules/cfu (mean ± SE)
10% serum			
COH1	33	0.40 (−0.1–0.5)	5,983 ± 713
COH1 + antiserum	97	1.60 (1.1–2.0)	33,051 ± 37
COH1-11	86	0.90 (0.6–1.0)	47,786 ± 16,506[b]
COH1-13	92	1.20 (0.9–1.7)	43,939 ± 9,201[c]
MgEGTA–10% serum			
COH1	11	0.05 (0.0–0.1)	2,300 ± 645
COH1-11	90	1.00 (0.7–1.2)	32,482 ± 1,505[d]
COH1-13	95	1.30 (1.2–1.5)	42,128 ± 3,764[e]

[a] Each value represents the mean of at least two separate determinations.
[b] $P = 0.045$ compared with COH1.
[c] $P = 0.014$ compared with COH1.
[d] $P = 0.0001$ compared with COH1.
[e] $P = 0.0005$ compared with COH1.

Statistical analysis. The unpaired (two-tailed) Student *t* test was used to compare C3 molecules bound to the GBS strains.

RESULTS

Opsonophagocytic killing of GBS by human leukocytes. GBS COH1, COH1-11, and COH1-13 were opsonized in 10% serum or MgEGTA–10% serum, washed, and then incubated with human peripheral blood leukocytes. After opsonization of cells in serum, incubation with leukocytes resulted in only 33% reduction in CFU of wild-type strain COH1 (Table 1), while COH1-11 (asialo mutant) and COH1-13 (unencapsulated mutant) were readily killed (86 and 92% decrease in CFU, respectively). When parent strain COH1 was opsonized in serum containing MgEGTA to block complement activation via the classical pathway, there was an 11% decrease in CFU after incubation with phagocytes, but the mutants showed the same susceptibility to killing as in nonchelated serum (86 and 95% decrease in CFU, respectively). The relative resistance of wild-type strain COH1 to opsonophagocytic killing could be overcome by the addition of specific antibody: when COH1 was opsonized in 10% serum with 1% type III GBS antiserum, subsequent incubation with leukocytes resulted in a 97% decrease in CFU, a degree of killing comparable to that seen with the mutant strains in the absence of specific antibody.

C3 binding to COH1, COH1-11, and COH1-13. The difference in susceptibility to opsonophagocytosis between the wild-type and mutant strains suggested that the presence of the sialylated type III capsule interfered with activation and/or deposition of complement on the bacterial surface. To study directly the influences of the capsule and of capsular sialic acid on C3 deposition, direct binding assays were performed to quantify the number of C3 molecules deposited on the surfaces of the wild-type or mutant strains. The

number of C3 molecules bound per CFU was determined by using ^{125}I-trace-labeled human C3 diluted in serum. After opsonization of cells in 10% serum, COH1 bound only 5,983 ± 713 C3 molecules per CFU. In contrast, both the asialo mutant COH1-11 and the acapsular mutant COH1-13 bound approximately eightfold more C3 molecules under these conditions (47,786 ± 16,506 and 43,939 ± 9,201, respectively). COH1 incubated with 1% type III GBS antiserum added to the 10% serum bound 33,051 C3 molecules per CFU. The addition of either type III GBS antiserum or type 14 pneumococcal antiserum (which recognizes the asialo type III polysaccharide [24]) failed to increase the amount of C3 bound to COH1-11. When bacteria were opsonized in MgEGTA-serum, the differences in C3 uptake between wild-type strain COH1 and its mutants were even more striking (Table 1).

Neuraminidase treatment of COH1. That the asialo mutant COH1-11 gave results similar to those obtained with the acapsular mutant COH1-13 suggested that the sialic acid residues of the type III polysaccharide played a central role in the capacity of the capsule to regulate C3 deposition on the bacterial surface. However, while strain COH1-11 expresses a polysaccharide with a repeating structure identical to that of the native type III capsule except for the absence of sialic acid residues, the amount of polysaccharide on the surface of strain COH1-11 is lower than that of the wild-type strain COH1 (22). Since both the reduced capsule expression and the absence of capsular sialic acid could have influenced C3 binding to COH1-11, we carried out additional experiments to define more precisely the effects of capsule sialylation on complement deposition independent of the effects of reduced capsule expression. Wild-type strain COH1 was grown in broth culture with or without neuraminidase. The bacteria were treated with formalin to prevent regeneration of capsular sialic acid during complement binding and activation assays. The abilities of neuraminidase-treated versus untreated organisms to bind C3 were assessed by using the direct C3-binding assay described above. The neuraminidase-treated (i.e., desialylated) COH1 bound two to three times as much C3 as the native COH1, both in serum (18,493 ± 3,607 and 7,205 ± 1,649 molecules per CFU, respectively [P = 0.017]) and in MgEGTA-serum (7,360 ± 2,776 and 2,262 ± 997 molecules per CFU, respectively [P = 0.115]). In addition, the capacities of treated versus untreated bacteria to consume the alternative-pathway activity of serum were estimated by measuring the residual activity of the serum after incubation with bacterial cells to mediate lysis of rabbit erythrocytes. As shown in Fig. 1, although both native and desialylated COH1 consumed alternative-pathway activity of serum, the neuraminidase-treated COH1 removed considerably more activity.

C3 binding to COH31, COH31-21, and COH31-15. As an additional model system for studying the effects of capsular sialic acid on complement deposition, we examined the binding of C3 to isogenic transposon mutants of a different type III strain, COH31. In contrast to strain COH1, COH31 produces relatively little capsular polysaccharide, is susceptible to phagocytic killing in the absence of specific antibody, and has a relatively high 50% lethal dose in neonatal rats compared with strain COH1. Still, COH31 mutants that lacked sialic acid (COH31-21) or capsule (COH31-15) were less virulent than COH31 in the animal model (18, 24). We used these strains to test whether the difference in animal virulence between the mutant and wild-type strains would be reflected in differences in C3 binding, even though the parent strain was poorly encapsulated. Both in 10% serum and in

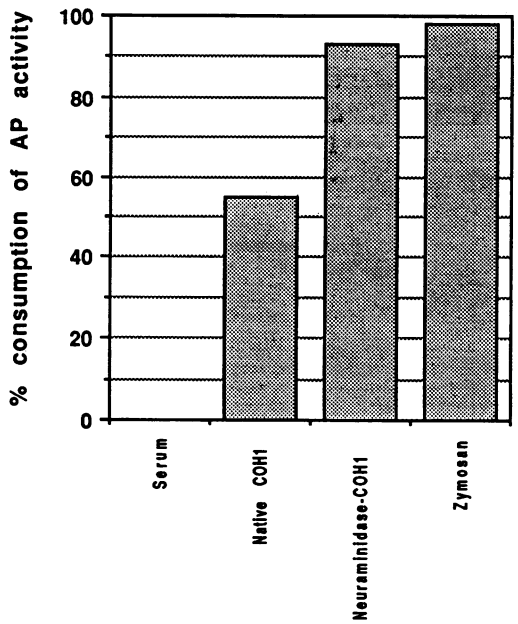

FIG. 1. Consumption of alternative-pathway (AP) activity by native and neuraminidase-treated COH1. Formalinized organisms of GBS type III strain COH1 were incubated in MgEGTA-serum that was subsequently exposed to rabbit erythrocytes. Units of alternative-pathway activity were calculated on the basis of hemoglobin released, and percent consumption of alternative-pathway hemolytic activity was relative to serum alone. Data shown are from a representative experiment.

MgEGTA–10% serum, COH31 bound less C3 than either the asialo or acapsular mutants: 25,213 for COH31 versus 52,754 and 54,766 C3 molecules per CFU for the asialo mutant COH31-21 and the acapsular mutant COH31-15, respectively, in serum and 27,100 versus 39,870 and 46,957 C3 molecules per CFU for COH31, COH31-21, and COH31-15, respectively, in MgEGTA-chelated serum.

C3 binding to strains having different amounts of capsule. The difference in C3 binding to the well-encapsulated strain COH1 versus the poorly encapsulated strain COH31 suggested that the degree of encapsulation of type III strains might be an important determinant of complement deposition on the bacterial surface. To examine this question, we assayed C3 binding to 14 clinical isolates of type III GBS with different amounts of capsular polysaccharide. Utilizing the direct binding assay described above, we measured C3 binding to each strain. Because C3 binding studies utilizing strains COH1, COH31, and their mutants had indicated that most C3 deposition occurred via the alternative pathway, C3 binding in this set of experiments was determined in MgEGTA–10% serum only. The amount of capsule expressed on the surface of each strain was determined by using an inhibition ELISA. We found an inverse correlation between the amount of capsule on the surfaces of these strains and the number of molecules of C3 bound per CFU (r = −0.72; Fig. 2). This result suggests not only that the presence of the capsule regulates complement activation but also that the quantity of capsular polysaccharide has a major influence on the degree of alternative-pathway activation and C3 deposition on the bacterial cell.

Determination of the form of bound C3. Native C3 is composed of two disulfide-linked chains, α (120 kDa) and β (75 kDa). Only the α chain is cleaved upon complement

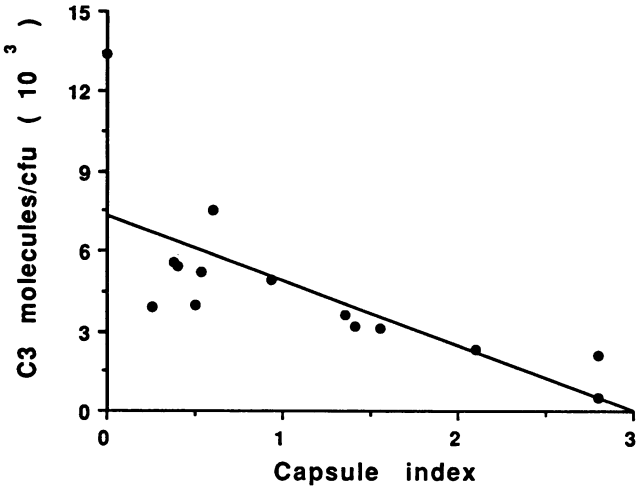

FIG. 2. Correlation of capsule index with C3 binding. Each datum point represents measurements of capsule index and mean of two determinations of C3 binding in MgEGTA–10% serum for individual type III GBS strains ($r = -0.72$; $P < 0.01$).

activation and C3 binding to cellular surfaces. Proteolytic degradation of the α chain of C3 generates additional fragments, including C3b, C3bi, and C3dg (8). We used SDS-PAGE and autoradiography to investigate the form of ^{125}I-C3 bound to GBS COH1, COH1-11, and COH1-13. In these experiments, opsonized bacteria were washed, solubilized in SDS-sample buffer, and applied to SDS gels under reducing conditions. Autoradiography of the gels showed that the majority of the ^{125}I-C3 counts were in a diffuse, high-molecular-weight (MW) band(s), presumably representing C3 complexed with bacterial acceptor molecules (Fig. 3). No clear difference in the pattern of C3 fragments bound was observed for the three strains tested, although the intensities of the bands were much greater for mutant strains COH1-11 and COH1-13 than for wild-type strain COH1 except when COH1 was opsonized with specific antiserum. The intensities of the C3 fragments appeared somewhat greater for COH1 opsonized with specific antibody than for the mutant

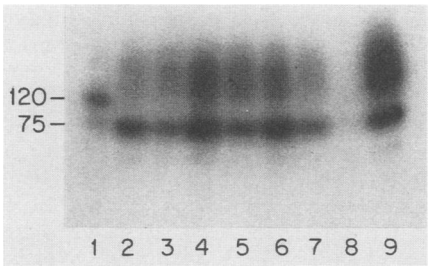

FIG. 3. Form of C3 bound to GBS. Organisms of GBS type III strains COH1, acapsular mutant strain COH1-13, and asialo mutant strain COH1-11 were opsonized with ^{125}I-C3 under various conditions, solubilized, and analyzed by SDS-PAGE (4 to 15% gradient gels). After electrophoresis, the gel was exposed to X-ray film. Lanes: 1, ^{125}I-C3 standard; 2, COH1-11 in 10% serum; 3, COH1-11 in MgEGTA–10% serum; 4, COH1-11 plus type III GBS antiserum; 5, COH1-11 plus type 14 pneumococcus antiserum; 6, COH1-13 in 10% serum; 7, COH1-13 in MgEGTA–10% serum; 8, COH1 in 10% serum; 9, COH1 plus type III GBS antiserum. Molecular sizes are in kilodaltons.

strains opsonized in adsorbed serum, although similar amounts of C3 were detected in the C3-binding experiments (Table 1). Differences in the rate of release of bound C3 may account for this apparent discrepancy between total C3 deposition (measured after sucrose centrifugation; Table 1) and the intensities of the C3 fragment bands by autoradiography (determined after the bacteria were washed; Fig. 3). In particular, the apparently greater amount of C3 seen by autoradiography of the wild-type strain COH1 in the presence of specific antibody may reflect the contribution to total C3 binding by C3b-immunoglobulin G complexes, as immunoglobulin G bound to the bacteria provides a stable site for the attachment of C3 (20).

Proportion of C3b to C3bi molecules bound to COH1-11 and COH1-13. To better define the C3 fragment(s) represented by the high-MW band(s) and to determine the relative amounts of C3b and C3bi on the surface of the mutant strains, we took advantage of the sensitivity of cell-associated C3bi to low concentrations of trypsin. COH1-11 and COH1-13 organisms were opsonized with ^{125}I-C3 in 10% serum or MgEGTA–10% serum, washed, and then incubated in various concentrations of trypsin. The results showed that for both COH1-11 and COH1-13, the number of cell-associated C3 molecules sensitive to cleavage by trypsin reached a plateau at concentrations as low as 0.2 μg/ml, increasing minimally with a 10-fold-higher concentration of trypsin. About 50% of C3 molecules bound to the asialo mutant COH1-11 in serum or MgEGTA-serum were released by 2 μg of trypsin per ml, indicating that approximately half the bound C3 molecules were in the form of C3bi. Similarly, 42% of the C3 molecules bound to the acapsular mutant COH1-13 were released by this concentration of trypsin. SDS-PAGE analysis and autoradiography of the fragments released by trypsin confirmed the presence of the 22- 23-kDa (α'_3 fragment) band consistent with the anticipated cleavage by trypsin of C3bi to C3c (Fig. 4). In the same figure, the high-MW band(s) (above 110 kDa) probably represents the complexes of ^{125}I-C3 bound to bacterial acceptors. These complexes appear to be released from the cell surface spontaneously rather than as a result of trypsin treatment, as they were also seen in samples not subjected to trypsin treatment (data not shown). The decrease in intensity of the high-MW band(s) with increasing concentrations of trypsin and the corresponding increase in the 22- to 23-kDa band suggests that the form of C3 in the high-MW complexes is C3bi, which is cleaved by trypsin to C3c. The faint band at 110 kDa represents the α′ chain of C3b, which remained unchanged with increasing concentrations of trypsin because C3b is not susceptible to cleavage at these concentrations. The 75-kDa band is the intact β chain of C3. The fragment that migrates just below the β chain is the α'_1 fragment of C3bi (68 kDa). As anticipated, the intensity of this band diminishes with increasing concentrations of trypsin, as C3bi is cleaved to C3c. The faint reappearance of the band at the highest trypsin concentration may reflect release of free C3bi from high-MW complexes or minimal cleavage of C3b to C3bi by trypsin.

Determination of ester-linked C3. Activation of C3 exposes a reactive thiolester group that may covalently bind either to a hydroxyl group via an ester linkage or to an amino group via an amide bond, depending on the groups available on the acceptor surface. The use of a nucleophilic agent such as methylamine permits determination of the form of C3 molecules that are ester bound because they are sensitive to cleavage. Because the total amount of C3 bound to the encapsulated parent strain COH1 was very reduced com-

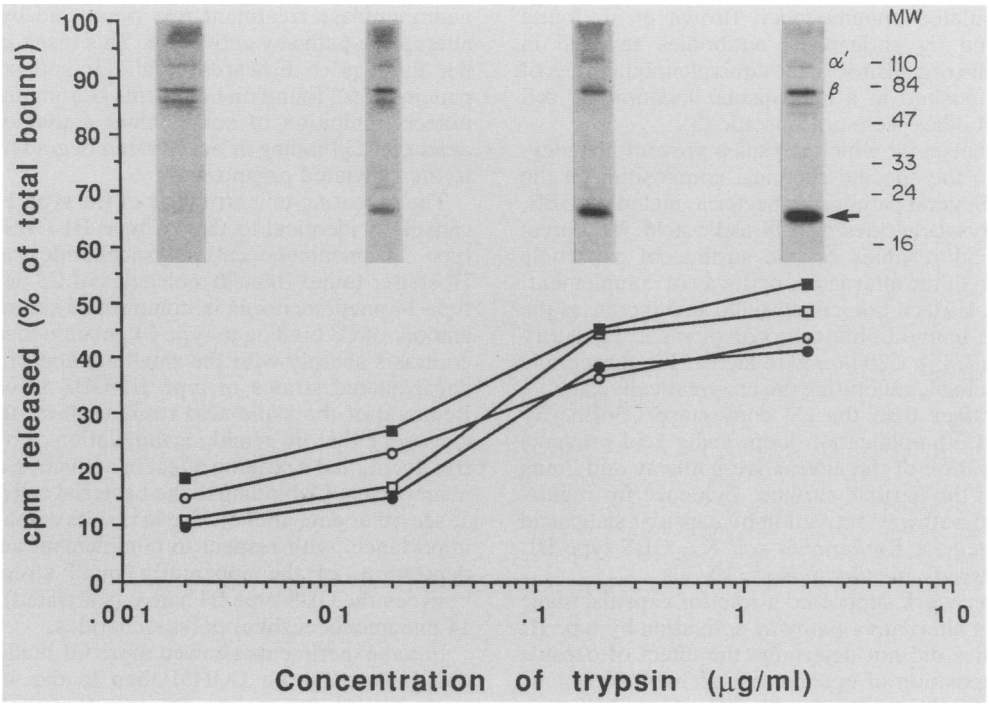

FIG. 4. Release of ^{125}I-C3 from COH1-11 and COH1-13 by trypsin. Mutant strains were opsonized in 10% serum or MgEGTA–10% serum, washed, and then incubated with various concentrations of trypsin. Datum points represent the mean percentages of bound radioactivity released by trypsin treatment in two experiments (variations among individual determinations for each datum point were less than 10%). Closed squares, COH1-11 opsonized in 10% serum; open squares, COH1-11 in MgEGTA–10% serum; closed circles, COH1-13 in 10% serum; open circles, COH1-13 in MgEGTA–10% serum. Inserts show autoradiography of SDS-PAGE of the C3 fragments released in the supernatants at each trypsin concentration (from COH1-11 opsonized in serum). The calculated molecular mass of the band indicated by the arrow is 22 to 23 kDa, confirming that it is the α'_3 fragment of C3bi. The pattern of C3 fragments released was the same for COH1-11 and COH1-13 both in 10% serum and in MgEGTA–10% serum. Migrations of MW standards are shown on the right.

pared with amounts bound by the acapsular or asialo mutants, we analyzed the methylamine-released fragments by immunoblot rather than simply by opsonization in serum containing ^{125}I-C3 and autoradiography. Although immunoblotting is not a quantitative method, it clearly showed that the intensity of the band at 110 kDa (α' fragment of C3b) relative to that of the band at 68 kDa (α'_1 fragment of C3bi) was different for COH1 and its mutants. C3 molecules released by methylamine treatment of COH1 organisms were mostly C3bi, while the bands representing C3b and C3bi released from the asialo mutant COH1-11 appeared of similar intensities, and the C3 fragments released from the acapsular mutant COH1-13 were predominantly C3b (Fig. 5).

DISCUSSION

Immune defense against gram-positive bacteria, particularly upon their entry into the bloodstream, is dependent on phagocytosis and killing by fixed and circulating leukocytes. For GBS, opsonization of the organisms by serum complement is essential to phagocytic killing (2, 19). Without complement, phagocytic cells do not ingest and kill these organisms. The current studies were designed to better define the interaction between GBS and the complement system. The results of these studies clearly establish that the sialic acid-rich type III capsular polysaccharide prevents C3 deposition on the bacterial cell.

Many pathogenic bacteria are surrounded by capsules composed of high-MW polysaccharides. These capsular polysaccharides have been shown to play an important role in virulence by rendering the organisms more resistant to phagocytosis. For certain organisms, the capsular polysaccharide appears to function as a physical barrier: the presence of a layer of capsular polysaccharide extending for some distance from the cell wall may impede access of phagocytic leukocytes to opsonic fragments bound to subcapsular structures. Such a mechanism appears to account for the failure of cell wall-specific antibodies to effectively

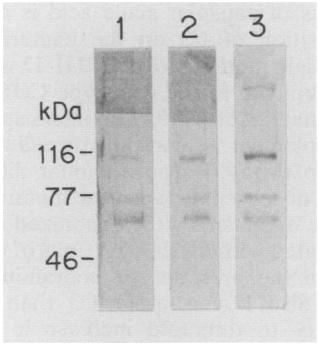

FIG. 5. C3 fragments released by methylamine. SDS-PAGE and Western blot with C3-specific antibody were used to detect C3 fragments released by methylamine from type III GBS strains after opsonization in 10% serum. Lanes: 1, C3 from encapsulated strain COH1; 2, C3 from asialo mutant strain COH1-11; 3, C3 from acapsular mutant strain COH1-13.

opsonize encapsulated pneumococci. Brown et al. found that C3 deposited by anticapsule antibodies resulted in phagocytosis of the organisms, while equivalent quantities of C3 molecules deposited in a subcapsular location by cell wall-specific antibodies were not opsonic (3).

Another mechanism by which capsules prevent phagocytosis is related to the specific chemical composition of the polysaccharide. Several pathogenic bacteria, including GBS, have surface polysaccharides rich in sialic acid. The presence of sialic acid residues on the surface of a particle inhibits activation of the alternative pathway of complement. On erythrocytes, surface-associated sialic acid increases the affinity of surface-bound C3b for the complement regulatory protein factor H (7, 15). C3b bound to factor H is susceptible to cleavage by factor I, generating the enzymatically inactive fragment C3bi rather than the C3 convertase C3bBb. By interrupting the C3 amplification loop, sialic acid prevents the efficient activation of the alternative pathway and limits C3 deposition on the particle surface. Evidence for regulation of alternative-pathway activation by capsular sialic acid has been presented for *Escherichia coli* K1, GBS type III, and group B *Neisseria meningitidis* (5, 12, 21).

Although earlier work supported a role for capsular sialic acid in preventing alternative-pathway activation by type III GBS, those studies did not determine the effect of capsule sialylation on deposition of opsonic complement fragments on the bacterial cell. In a more recent study, Campbell et al. (4) used C3-specific antibodies in Western blots (immunoblots) of SDS-PAGE gels to compare the patterns of C3 deposition on acapsular and asialo mutants of type III GBS with that on a genetically unrelated, well-encapsulated type III strain. Those authors concluded that the type III capsule promoted C3 deposition on the bacterial surface because for the encapsulated strain, the C3 bands became more intense as the time of opsonization increased from 5 to 90 min, while the C3 bands for the mutant strains remained at a relatively constant intensity. As no quantitative measurements of the amount of C3 deposited on either strain were made, it seems to us more likely that the results are explained by the rapid and efficient deposition of C3 on the mutants, with only modest and slow deposition on the encapsulated strain with prolonged opsonization. This interpretation is consistent both with the data of Campbell et al. and with the results of our current studies, which show conclusively that the sialylated type III capsule prevents C3 deposition on the bacterial surface.

In the present investigation, direct C3-binding assays showed that loss of capsular sialic acid is associated with increased deposition of C3 on the bacterial surface. C3 binding to the asialo mutant strain COH1-11 was increased 8- to 16-fold over binding to the wild-type COH1, an increase comparable to that observed for the unencapsulated mutant COH1-13. Complement activation proceeded primarily via the alternative pathway, because similar differences in C3 deposition were observed between the mutant and wild-type strains when the organisms were opsonized in whole serum or in serum chelated with MgEGTA. Cells of wild-type strain COH1 grown in the presence of neuraminidase, like the asialo mutant, also bound more C3 than did untreated COH1. The two- to threefold increase in C3 binding to COH1 after neuraminidase treatment was somewhat less than that observed with the asialo mutant, a fact consistent with either incomplete removal of sialic acid residues by neuraminidase treatment or with some additive effect in the asialo mutant COH1-11 of reduced capsule expression and loss of capsular sialic acid. The increase in C3 binding after neuraminidase treatment was paralleled by an increase in alternative-pathway activation. This result confirms the earlier findings of Edwards et al. (5) and suggests that the paucity of C3 found on organisms bearing the native capsule reflects inhibition of complement activation rather than a defect in C3 binding or accelerated degradation of C3 bound to the sialylated organisms.

The repeating-unit structure of the type 14 pneumococcal capsule is identical to that of type III GBS except that the type 14 pneumococcal polysaccharide lacks sialic acid. Hostetter found 108,000 molecules of C3 per CFU bound to type 14 pneumococcus in nonimmune serum (11). The large amount of C3 binding to type 14 pneumococcus in that study contrasts sharply with the small amount of binding to well-encapsulated strains of type III GBS in our experiments. Removal of the sialic acid residues from the type III GBS capsule, either by genetic manipulation or by neuraminidase treatment, had a striking effect in activating complement and in increasing C3 binding to the bacterial cell (Table 1 and Fig. 1; see other data above). These results emphasize the critical importance, with respect to complement activation and C3 deposition, of the apparently small structural difference between the GBS type III native (sialylated) and asialo (type 14 pneumococcuslike) polysaccharides.

Initial experiments showed more C3 binding to the poorly encapsulated strain COH31 than to the well-encapsulated strain COH1, suggesting not only that the presence of the type III capsule prevented C3 deposition but also that the degree of inhibition of C3 deposition might be related to the amount of capsule on the bacterial surface. This hypothesis was confirmed by the results of experiments comparing capsule expression with alternative-pathway-mediated C3 binding to 14 different type III GBS strains. A significant inverse correlation between amount of type III capsule and C3 binding to the organisms was found. Thus, for type III GBS, the amount of capsular polysaccharide on the bacterial surface is an important determinant of C3 deposition on the bacterial cell. This effect is independent of potential effects of the capsule in blocking access of phagocytes to subcapsular opsonins bound to the cell surface.

C3 binds covalently to acceptor molecules on the cell surface through an internal thiolester that is exposed upon complement activation. Autoradiography of labeled C3 molecules solubilized from opsonized GBS cells and fractionated by SDS-PAGE showed that a large proportion of C3 was part of high-MW complexes. One method of determining the form of C3 present in these complexes is treatment of the opsonized bacteria with methylamine. This treatment releases ester-linked but not amide-linked C3. C3 fragments released by methylamine from COH1 were mostly C3bi, while both C3b and C3bi in similar proportions were released from COH1-11 and COH1-13. The predominance of C3bi over C3b on the surface of the encapsulated strain COH1 is consistent with the hypothesis that the sialylated type III capsule inhibits alternative-pathway activation and C3 deposition. On erythrocytes, the affinity of H for C3b is low on activating surfaces and high on nonactivating (sialic acid-rich) surfaces, leading to the proteolytic cleavage of C3b to C3bi on the sialic acid-rich particles (13). The proportion of C3b compared with C3bi appeared greater for the acapsular mutant COH1-13 than for the asialo mutant COH1-11, suggesting that even the asialo capsule may mask, to some degree, cell wall components that activate the alternative pathway.

Our studies are the first to rigorously examine the relationship between particular structural features of the type III

GBS capsule and C3 deposition. Direct-binding studies demonstrated that the presence of capsular sialic acid had a marked effect in reducing the deposition of C3 on the bacterial cell. This regulation of C3 deposition was affected not only by the presence or absence of capsular sialic acid but also by the degree of encapsulation, with more highly encapsulated strains binding less C3 than poorly encapsulated ones. We conclude that the antiphagocytic effect of the type III capsule of GBS is not due solely to physical blockade of phagocytosis but rather to a highly specific interaction between capsular sialic acid residues and components of the alternative pathway of complement, an interaction that prevents efficient complement activation and deposition of opsonic fragments on the bacterial cell.

ACKNOWLEDGMENTS

This work was supported in part by grants AI 23339 and AI 28040 from the National Institutes of Health and by a grant-in-aid from the American Heart Association.

We thank Ming-Jane Liu and Thomas J. DiCesare for technical assistance, Carol Baker for providing bacterial strains, and Albert Liou for statistical analysis.

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