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Influence of Serotype of Group B Streptococci on C3 Degradation

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Serotype III strains of group B streptococci (GBS) are isolated from the majority of young infants with bacteremia or meningitis. We hypothesized that serotype-associated differences in structure of the type-specific capsular polysaccharide or the presence of c protein would influence the extent to which C3 degradation occurs on GBS and that type-specific antibody would alter C3 deposition or degradation patterns. When clinical isolates of GBS representing serotypes Ia, Ib/c, II (with or without c protein), and III were employed with hypogammaglobulinemic serum as an opsonic source, a remarkable similarity was observed in patterns of C3 deposition and degradation for each of the four GBS serotypes and between strains with or without c protein. Both C3b and iC3b were detected by 5 min and throughout a 90-min opsonization interval. Less deposition occurred at 5 min on serotypes Ia and Ib/c than on types II and III GBS. Minimal degradation to C3d or smaller fragments was observed. Type-specific antibody facilitated C3b deposition on GBS and C3b degradation to iC3b early in opsonization. Possibly, accessibility of C3 fragments to neutrophil receptors, rather than the extent to which the surface permits C3 degradation, accounts for differential virulence among GBS serotypes.

Group B streptococci (GBS) are leading causes of life-threatening infection in neonates and young infants. Although bacteremia, meningitis, or localized infection may be associated with any serotype, type III is isolated from 80 to 90% of infants with meningitis or onset of infection after the first week of life (2). One proposed cause of differential virulence among GBS serotypes is the composition of the capsular polysaccharide (19). Each of the five major serotypes of GBS (Ia, Ib/c, Ia/c, II, and III) has a polysaccharide capsule consisting of repeating subunits of the monosaccharides galactose, glucose, *N*-acetylglucosamine, and sialic acid (10–12, 22). Types Ia and Ib/c GBS have identical backbone and side chain sequences, differing only in the linkage of their branch galactose-to-glucosamine residues. Type III GBS have a molar ratio of these monosaccharides that is identical to that of types Ia and Ib GBS but differs in its side chain linkage of galactose and sialic acid to the backbone. Serotype II GBS strains have two monosaccharide side chains, galactose and sialic acid, each extending directly from the repeating backbone.

In addition to structural differences in polysaccharide antigens, some serotypes of GBS possess protein c, a surface-localized protein found in all Ib/c and Ia/c strains and in the majority of type II strains (13). The presence of the trypsin-sensitive or trypsin-resistant components of c protein in type II strains was associated in vitro with resistance to opsonization and killing by human sera (17).

Previously, we have shown that an encapsulated strain of type III GBS permitted C3 deposition and degradation to iC3b and that antibody with specificity for the capsular polysaccharide facilitated C3 fragment deposition in the early phases of opsonization (6). This observation is consistent with that of Hostetter (8), who showed previously that iC3b is the C3 degradation product borne by pneumococcal serotypes, such as types 6A or 14 *Streptococcus pneumoniae*, that frequently cause infection in childhood. Conversely, pneumococcal serotypes less virulent for children bear C3b preferentially on their surfaces. We hypothesized that structural differences in capsular polysaccharide or the presence of c protein might similarly affect C3 fragment deposition by GBS and that serotypes less virulent than type III might bear C3b in preference to iC3b. We also sought to determine the influence of type-specific antibody upon the timing of C3 deposition and the specific C3 cleavage fragments borne by GBS.

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MATERIALS AND METHODS

Bacterial strains and growth. GBS clinical isolates representing types Ia (strain 515), Ib/c (strain 710), II (strains 612 and 601 with and without, respectively, trypsin-sensitive and -resistant components of c protein), and III (strain M861) were employed. Each isolate was recovered originally from blood or cerebrospinal fluid of an infant and stored at −70°C in aliquots. These strains were typical of serotypes previously assessed for opsonization and phagocytosis in vitro (3, 4, 16). For each experiment, a frozen aliquot was plated on blood agar and incubated at 37°C overnight, and colonies were inoculated into Todd-Hewitt broth and incubated at 37°C for approximately 2 h to mid-log phase. After centrifugation, bacteria were resuspended in Dulbecco's phosphate-buffered saline (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 0.2% glucose to a concentration of $\sim 2 \times 10^8$ CFU/ml in the reaction mixture as confirmed by plating aliquots of serial dilutions of the bacterial suspension on blood agar.

Complement and serum sources. After informed consent was obtained, blood processed to preserve endogenous complement activity as described previously (6) was collected from a child with common variable hypogammaglobulinemia (immunoglobulin G [IgG], 25 mg/dl; IgM, 39 mg/dl; IgA, 8 mg/dl). This serum had normal complement function (50% hemolytic complement, 626 U) but lacked detectable

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antibody to capsular polysaccharides of types Ia, II, and III GBS by radioactive antigen-binding assay (RABA) and to type Ib/c GBS by enzyme-linked immunosorbent assay (ELISA). To determine the influence of type-specific antibody, sera from adults responding to immunization with purified GBS capsular polysaccharides (Ia, II, or III) or convalescing from bacteremia (Ib/c) were employed. Sera were selected that contained moderately high levels (21.3 to 43.7 μg/ml by RABA for Ia, II, and III and 0.22 μg/ml by ELISA for Ib) of antibody to the specific serotype but less than 2.0 μg/ml by RABA to each of the other GBS serotypes. Each of these sera had a normal 50% hemolytic complement.

Opsonization and experimental procedure. GBS were opsonized in a total volume of 600 μl, consisting of 150 μl of the bacterial suspension (~2×10^8 CFU/ml) and 450 μl of serum diluted in Dulbecco's phosphate-buffered saline to give a final serum concentration of 30%. Opsonization was carried out at 37°C in a Thermomixer (model 5436, Eppendorf). After opsonization for 5 to 90 min, the reaction was stopped by cooling to 4°C on ice. Vials were centrifuged at 13,000 × *g* (model 235B, Fisher), and the pellets were washed with phosphate-buffered saline–1% sodium dodecyl sulfate (SDS) (Bio-Rad Laboratories, Richmond, Calif.) to remove noncovalently bound C3. The pellet was suspended in 1 M hydroxylamine (Sigma Chemical Co., St. Louis, Mo.)–1% SDS (pH 9.0 in $NaHCO_3$-$NaCO_3$) to disrupt ester bonds (8, 9, 15). After centrifugation, the supernatant contained C3 fragments released by hydroxylamine; amide-bound C3 fragments remained on the bacterial pellet. Two hundred microliters of the C3 fragment suspension was reduced with 10 mM dithiothreitol (Sigma)–1% SDS at 37°C for 60 min and then alkylated with 22 mM iodoacetamide (Sigma) at 37°C for 60 min (8, 9). Fragments then were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) or were frozen at −70°C until use (14).

Analysis of C3 fragments. Aliquots of the C3 fragment suspension were diluted 1:2 in sample buffer and subjected to SDS-PAGE with a 7.5% polyacrylamide gel under reducing conditions (14). After electrophoresis (175 mV, 35 min) of the samples in a mini-PROTEAN II apparatus (Bio-Rad), the gel was equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). Proteins were transferred electrophoretically to nitrocellulose paper (100 mV) for 1 h in a Mini-Transblot apparatus (Bio-Rad). After nonspecific binding sites were blocked with phosphate-buffered saline–1% bovine serum albumin overnight, the nitrocellulose paper was incubated in a 1:250 dilution of goat anti-human C3 (Jackson ImmunoResearch, West Grove, Pa.) for 2 h, washed, and incubated with alkaline phosphatase-conjugated rabbit anti-goat IgG (1:500 dilution) (Cappel, West Chester, Pa.). The Western blot (immunoblot) was developed by using bromochloroindolyl phosphate-Nitro Blue Tetrazolium (Calbiochem, La Jolla, Calif.) for 2 min (6).

Two C3 preparations were used as controls. One contained intact C3 alpha chain (115 kDa) and C3b, consisting of an alpha chain of 105 kDa and a beta chain of 75 kDa (8, 9). The other preparation, provided by David Gordon (Flinders Medical Center, South Australia, Australia), contained the intact beta chain (75 kDa) common to both C3b and iC3b, a 40-kDa alpha subunit component of iC3b, a 33-kDa band representing C3d, and several smaller degradation subunits.

RESULTS

Influence of serotype on C3 deposition by GBS. The C3 fragments released from type Ia GBS, after opsonization for 5 to 90 min with hypogammaglobulinemic serum, are shown in Fig. 1A. After 5 min of opsonization, both C3b, consisting of an alpha chain of 105 kDa and a beta chain of 75 kDa, and iC3b, indicated by alpha chains of 67 and 40 kDa in addition to the beta subunit shared with C3b, were detectable. Both C3b and iC3b were detectable at all opsonization times. This pattern of C3 deposition mirrored that described previously for type III GBS strain M861 (6). No detectable difference in the fragments was observed between type Ia and type Ib/c GBS (Fig. 1B). By using the same experimental conditions, C3 fragment deposition was compared for isolates of type II GBS possessing both trypsin-sensitive and -resistant components of c protein (II/c) or lacking c protein (II) (Fig. 2). Again, bands representing C3b and iC3b were evident at 5 min and throughout the 90-min opsonization interval. For the strain lacking c protein, a faint 33-kDa band also was present at 60 and 90 min, consistent with C3 degradation to C3d (Fig. 2B).

The Western blot shown in Fig. 3 compares the C3 fragments released from each of four major serotypes of GBS in the early phase of opsonization (5 min) with hypo-

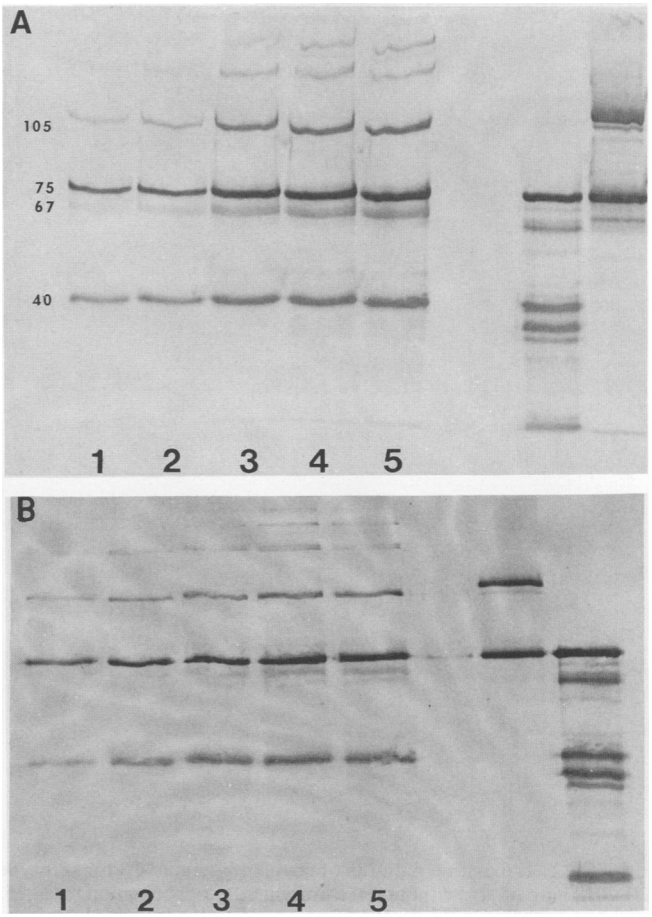

FIG. 1. Degradation patterns of covalently bound C3 fragments released from serotypes Ia (A) and Ib/c (B) GBS after opsonization for 5, 15, 30, 60, and 90 min (lanes 1 through 5, respectively) in hypogammaglobulinemic serum. The presence of the 105-kDa chain indicates C3b. The 67- and 40-kDa chains indicate iC3b. The 75-kDa band is the beta chain common to both C3b and iC3b. Preparations of C3b and iC3b with further degradation fragments of C3 are shown on the right.

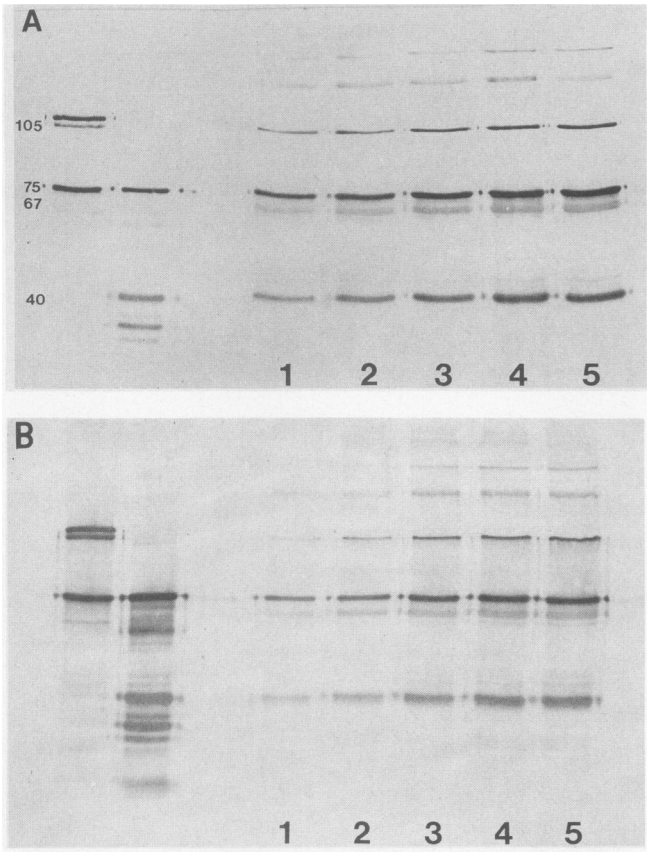

FIG. 2. Deposition patterns of covalently bound C3 for serotype II GBS with (A) or without (B) c protein after opsonization for 5, 15, 30, 60, and 90 min (lanes 1 through 5, respectively) with hypogammaglobulinemic serum. C3 degradation controls are shown on the left.

gammaglobulinemic serum. Although the pattern is similar for each serotype, there is qualitatively less C3 deposited on types Ia and Ib/c when compared with that detectable on types II and III GBS.

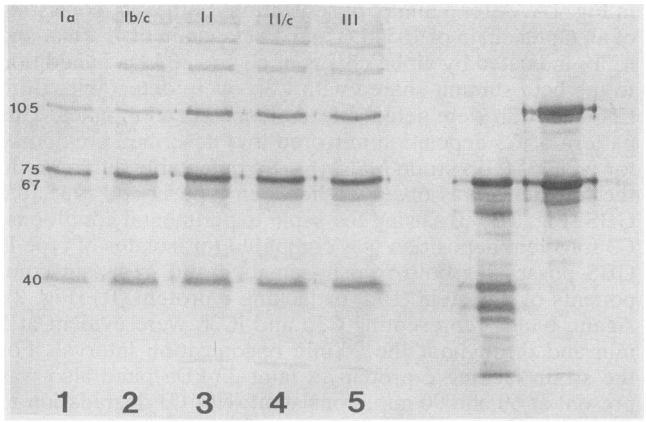

FIG. 3. Comparison of C3 deposition by 10^8 CFU ml of serotypes Ia, Ib/c, II, II/c, and III (lanes 1 through 5) per ml after opsonization for 5 min in hypogammaglobulinemic serum. Degradation controls for C3 are shown on the right.

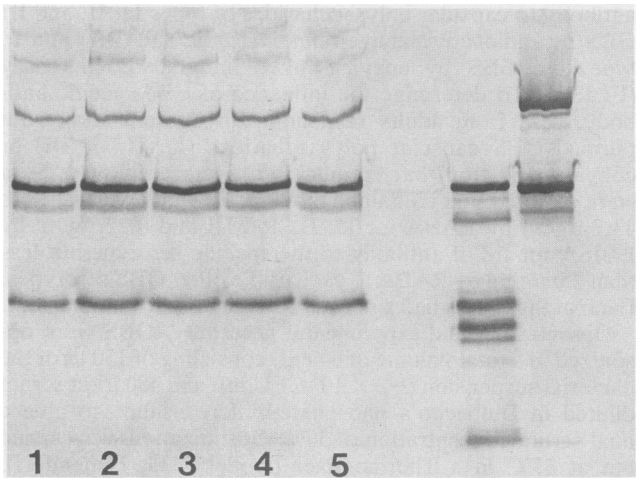

FIG. 4. Deposition pattern of covalently bound C3 fragments released after opsonization of serotype Ia GBS in serum containing specific antibody to Ia capsular polysaccharide for 5, 15, 30, 60, and 90 min (lanes 1 through 5, respectively). Note the intensification of the 105- and 67-kDa bands at 5 and 15 min (lanes 1 and 2) in comparison with those bands in Fig. 1A.

Influence of antibody upon C3 deposition by GBS. Experiments were then performed in which adult sera that contained moderately high levels of antibody to capsular polysaccharides of types Ia, Ib/c, II, or III GBS rather than hypogammaglobulinemic serum were employed for opsonization. After opsonization for 5 to 90 min in these immune sera, both C3b and iC3b were detected for each GBS serotype. The presence of antibody did not alter the character of the C3 fragments present. However, when compared with hypogammaglobulinemic serum (Fig. 1A), C3 fragments detected early in opsonization, especially the 67-kDa band, were intensified with immune sera (Fig. 4). Thus, when specific antibody directed to capsular polysaccharide is present, the C3 degradation pattern is not altered. However, C3 fragments formed in the presence of antibody are visualized in the early as well as the later phases of opsonization.

DISCUSSION

A twofold rationale formed the basis for the preceding experiments. First, drawing from the observations of Hostetter (8) concerning C3 deposition patterns on pathogenic pneumococci, we proposed that serotypic differences in deposition and degradation of ester-bound C3 might account for the particular virulence of serotype III versus other serotypes of GBS. Hostetter (8) found that *S. pneumoniae* types 3 and 4, which are highly resistant to phagocytosis, released C3b, iC3b, and C3d from their capsular surfaces by hydroxylamine after opsonization in agammaglobulinemic serum. Pneumococcal serotypes 6A and 14, which are less resistant to phagocytosis, bore only iC3b on their surfaces. Hostetter proposed that "the antiphagocytic nature of the pneumococcal capsule may well reside not in its inhibition of C3b deposition, but rather in its capacity to permit both the deposition and the proteolytic degradation of capsule-bound C3b to fragments that fail to serve as ligands for phagocytic receptors."

In contrast to the variations in C3 deposition observed among pneumococcal serotypes, we observed remarkable

similarity in the degradative processing of ester-bound C3 among four major serotypes of GBS. After 5 min of opsonization, there was qualitatively less deposition on GBS serotypes Ia and Ib/c than on serotypes II or III but the character of the C3 fragments bound did not differ. For each, C3b and iC3b were detectable by 5 min of opsonization and throughout the 90-min interval evaluated. Minimal degradation to C3d, which may be important in triggering antibody production from the B lymphocyte (8), was observed. Similarly, smaller degradation fragments that have no defined neutrophil membrane receptors were notably absent. These observations indicate that C3 binds to GBS in nonimmune serum and that degradation to fragments recognized by neutrophil membrane complement receptors (CR), including CR1 (CD35) and CR3 (CD11b/CD18), occurs in this setting. Although not evaluated in this in vitro assay, GBS opsonized with C3b also can bind to erythrocytes via CR1 and therefore be transported from extravascular tissue sites to the reticuloendothelial system. This is another important regulatory event that occurs in vivo (20).

Our second hypothesis concerning differential virulence among GBS serotypes related to the effect that type-specific antibody might have upon C3 degradation patterns. IgG is an acceptor surface for nascent C3b (1). It has been shown that complement activation by IgG-sensitized bacteria results in substantial deposition of C3b onto antibody molecules (5) and that C3b covalently bound to IgG demonstrates a reduced rate of cleavage by factors H and I to iC3b (7). We proposed that specific antibody might prevent degradation of C3b to a greater extent by the less-virulent GBS serotypes. These long-lived C3b-IgG complexes could promote ingestion by presenting ligands to neutrophils both for CR1 and IgG-Fc receptors.

Regardless of serotype, opsonization by immune sera did not alter the character of C3 fragments detected. However, C3b and iC3b bands detected early in opsonization were intensified in comparison with those seen after opsonization with hypogammaglobulinemic serum. There was no suggestion for any GBS serotype that specific antibody retarded inactivation of C3b by providing a protected binding site on IgG. These experiments do not, however, exclude the possibility that IgG, when available, functions as a docking site for C3b.

In summary, in the setting of antibody deficiency, structural differences in GBS capsular polysaccharides and the presence or absence of c protein did not affect the character of C3 fragments bound covalently by ester bonds to GBS. The addition of antibody with specificity for capsular polysaccharide accelerated C3 fragment deposition for each serotype. These findings are in accord with a recent observation by Smith and Gray (21). These investigators, using a whole-bacterial-cell ELISA, demonstrated rapid C3 fixation on serotypes Ia, Ib, II, and III GBS that was maximal by 20 min after opsonization with normal human serum. When deposition was limited to that mediated by the alternative complement pathway, serotypes Ia and Ib GBS bound less total C3 than strains representing serotypes II and III GBS. Our findings also corroborate the observation of Robinson and Lassiter (18) that the addition of IgG to neonatal serum did not uniformly enhance C3 deposition on a type III GBS strain. Additional explanations for the enhanced virulence of serotype III GBS, including the potential of differential accessibility of bound C3 fragments to neutrophil receptors, await further study.

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