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# Isolation and Characterization of Type III Group B Streptococcal Mutants Defective in Biosynthesis of the Type-Specific Antigen

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Four classes of mutants of type III group B streptococcus were isolated by serial subculture of the wild-type strain in the presence of type III-specific rabbit antiserum. Class I mutants no longer synthesized sialic acid but still elaborated the core antigen. Class II mutants maintained the ability to synthesize sialic acid but could not attach it to the core antigen. Class III mutants did not produce the core antigen but still synthesized intracellular sialic acid. Class IV mutants synthesized the complete antigen; however, only ~4% of the antigen synthesized was found associated with the cell wall peptidoglycan (in the wild-type strain >85% of the antigen synthesized is covalently attached to the cell wall peptidoglycan), whereas >90% of the antigen was secreted into the growth medium. Production of other components (CAMP factor, group B antigen, β-hemolysin, neuraminidase) by these mutants appeared similar to those of the wild-type strain. Mouse lethality studies of these strains indicated that all four classes have >3 $\log_{10}$-higher 50% lethal dose values than that of the wild-type strain. To understand the basis for this variation, the invasive ability of the wild-type strain and the sialic acid-deficient mutant strain M-10 (class I) was examined. Mice received $10^5$ CFU of each organism; they were then sacrificed at various times postinoculation, and viable group B streptococci from different organs were enumerated. Mice were able to clear M-10 more efficiently, with >80% of M-10 cells being phagocytized by macrophages within 1 h, whereas the wild-type strain was able to evade phagocytic killing and disseminate to other tissues. These data, therefore, strongly indicate that the sialic acid moiety greatly enhances the virulence of the type III antigen. In addition, the level of cell-associated type-specific antigen appears to contribute significantly to the pathogenicity of the organism.

The cell surface type-specific antigen (TSA) of group B streptococcus (GBS), previously identified as capsular polysaccharide (24), has recently been confirmed to be a cell wall polysaccharide by virtue of its covalent association with the cell wall peptidoglycan (10, 11), and its role as a virulence determinant is well documented (28). The TSA is negatively charged due to the presence of sialic acid residues which account for ~25% of the total purified TSA by weight (12, 22). Since the cell surfaces of macrophages and polymorphonuclear neutrophils are also negatively charged (44), a possible role played by TSA in evasion of phagocytosis has been postulated. Indeed, resistance to phagocytosis of some strains of GBS has been reported (8, 16, 18, 19). Kasper and co-workers have proposed that the sialic acid moiety represents a primary virulence determinant of TSA (25). To prove the latter speculationn, Edwards et al. (14) studied complement consumption, utilizing Formalin-killed type III GBS with complete TSA on the cell surface and Formalin-killed GBS previously grown in the presence of neuraminidase, which enzymatically cleaves the glycosidic linkage to sialic acid. Although similar strategies have been adopted in eucaryotic cell systems, only 20 to 75% of the sialic acid residues can be cleaved depending upon the specificities of the enzyme and substrates used (2, 5, 20, 21). It appears then that the best way to elucidate the role of TSA, especially that of the sialic acid moiety, would be to use mutants that are defective at various levels in the biosynthesis of the polysaccharide antigen.

By the technique of serial subculture of a wild-type strain of GBS in the presence of type-specific antiserum, four classes of mutants blocked at major stages of TSA synthesis have been isolated. This technique was quite similar

to that used for the isolation of strain 090R which lacked the type-specific antigen (9, 28). The unique feature of the present study, however, is that subtle changes in the composition of the type III antigen have been delineated so that a spectrum of mutants has been obtained ranging from those that have lost only a single sugar residue to mutants that have lost the entire antigen and are nontypable. The characterization of the mutants was investigated and their virulence in mice was examined. Data obtained from the present study strongly suggested that only the sialylated cell surface high-molecular-weight (HMW) TSA appeared to play a significant role in virulence of the organism based on the following: (i) mutant classes I, II, and III which lacked sialic acid on the cell surface TSA were less virulent than the wild-type strain; (ii) a class IV mutant which secreted elevated levels of low-molecular-weight sialylated TSA but possessed only a negligible amount of cell-associated HMW TSA was also less virulent than the wild-type strain; and (iii) a revertant obtained after one passage in mice (strain 5.4, a class IV mutant) was demonstrated to have a comparable level of cell-associated TSA compared with that of the wild-type strain and a similar 50% lethal dose ($LD_{50}$) value.

## MATERIALS AND METHODS

**Isolation of mutants of type III GBS.** GBS strain 122 (a clinical isolate from late-onset meningitis [33]) was obtained from a frozen stock culture, streaked onto a blood agar plate, and incubated overnight at 37°C. Twelve colonies were randomly picked and transferred to tubes containing 10 ml of a chemically defined medium (FMC; 41). The organisms were grown to stationary phase (0.56 mg of cellular dry weight per ml), harvested by centrifugation, and washed three times with phosphate-buffered saline (PBS), pH 7.4. The washed cell pellets were divided into two portions. One portion was subjected to Lancefield's hot HCl extraction method (27) to obtain cell surface antigen for serotyping, and the other portion was suspended in 0.5 ml of PBS and hydrolyzed with 0.1 N HCl (final pH, <2.0) at 84°C for 20 min before analysis for sialic acid by the method of Aminoff (1). Of the 12 colonies selected, all contained extractable cell surface group- and type-specific antigens as well as sialic acid, eliminating the possible existence of a significant level of naturally occurring mutants in the stock culture. A single colony was selected from a fresh blood agar plate, inoculated into 10 ml of FMC, and allowed to grow to stationary phase. Washed whole cells were analyzed for hot HCl-extractable TSA and sialic acid as described above to define the characteristics of the initial colony (wild-type). Cells from the same colony were also inoculated into three tubes containing 5 ml of FMC plus 1 ml of rabbit anti-type III, anti-type II (specificity control), and normal rabbit sera, all prepared according to the method of Lancefield et al. (28). After 8 to 12 h a smooth cell suspension was observed in tubes containing anti-type II serum and normal rabbit serum, as well as the FMC medium control. The whole cells were also analyzed for TSA and sialic acid, and results confirmed the presence of type III antigen containing the sialic acid moiety. In the tube containing anti-type III serum a solid clump was observed at the bottom of the tube, confirming that agglutination is specific for type III antigen and anti-type III serum. Upon light agitation, some cells were dislodged from the clump and remained free in the nonagglutinated phase of the cell culture. A 5-μl amount of cell suspension from this phase was transferred to 5 ml of FMC containing 250 μl of anti-type III serum. After overnight incubation, clumps were again observed and cells from the nonagglutinated phase were transferred to fresh FMC containing anti-type III serum. This serial subculture procedure continued for as many as 16 passages. Upon each transfer, cells from the nonagglutinated phase were streaked onto a blood agar plate. Ten isolated colonies from each passage were then inoculated into 10 ml of FMC, and the characteristics (type III reactivity and sialic acid moiety) of these cells were determined.

**Extraction and isolation of the intracellular sialic acid pool from GBS.** Organisms (GBS 122 wild-type strain and mutants) were grown in 500 ml of FMC to the late exponential phase of growth. Cells were harvested and washed three times with PBS before suspension in 50 ml of cold 10% trichloroacetic acid. The cells were extracted with trichloroacetic acid overnight at 4°C with constant stirring. The mixtures were then centrifuged at 10,000 × *g* for 30 min (Sorvall RC5) to remove cell debris from the trichloroacetic acid-soluble materials. Trichloroacetic acid was removed by extraction with an equal volume of diethyl ether (three times), and the aqueous phases were concentrated by lyophilization. The concentrated materials were then applied to a Sephadex G-100 gel permeation column (90 by 1.5 cm) and eluted with 10 mM sodium acetate, pH 6.5. Fractions were monitored for sialic acid and type III reactivity.

**Isolation and purification of extracellular TSA.** The methods for isolation and purification of extracellular TSA have been described (12). For the present study, only the anionic-exchange chromatography procedure, using a DEAE-Sephacel column (28 by 2.5 cm), was used. Dialyzed, concentrated supernatant fluids were eluted with a linear gradient of 0.05 to 0.25 M $(NH_4)_2CO_3$ (700 ml volume of each molarity). Fractions were monitored for sialic acid as described above.

**Preparation of M-1 mutanolysin lysates of whole cells.** Soluble cell wall fractions of GBS 122 and mutant strains were prepared by digesting whole cells in sucrose-stabilized medium (protoplast medium) described previously for preparation of GBS protoplasts (47). Late stationary-phase cells from 500-ml cultures were collected, washed, and suspended in 50 ml of protoplast medium. After addition of M-1 mutanolysin (1.5 mg total), the cell suspensions were incubated for 3 h at 37°C with minimal agitation. The protoplast mixtures were then centrifuged at 3,000 × *g* for 45 min, and the supernatant fluids were processed in a manner similar to that described for purification of extracellular supernatant fluids.

To obtain materials containing both soluble cell wall fractions and cytoplasmic material, in particular the

intracellular sialic acid pool, the following procedure was utilized. Late stationary-phase cells from 500-ml cultures were washed and suspended in 25 ml of Tris-hydrochloride (0.03 M), pH 7.0, containing 0.01 M $MgCl_2$ and DNase and RNase (Sigma Chemical Co., St. Louis, Mo.) each at 50 μg/ml. After addition of mutanolysin (1.5 mg total), the cell suspensions were incubated for 3 h at 37°C with constant agitation to ensure complete lysis of cells. The whole-cell lysates were then centrifuged at 10,000 × *g* and 4°C for 1 h. The soluble whole-cell lysates were further purified by G-100 Sephadex gel permeation chromatography, using conditions similar to those described for purification of trichloroacetic acid extracts.

**Mouse lethality test.** The procedure adopted for the mouse lethality test was similar to that described by Durham et al. (13). Briefly, bacteria (wild type and mutants) from blood agar plates were inoculated into 10 ml of Todd-Hewitt broth (THB) and allowed to grow to the stationary phase of growth (adjusted optical density at 675 nm, ~490) (42). The cultures were chilled on ice and serial 10-fold dilutions in THB were made. Groups of five mice (ICR strain; 6-week-old males obtained from Harlan Sprague-Dawley, Madison, Wis.) were injected intraperitoneally with each strain in 1.0 ml of THB at various dilutions. The test continued for 72 h, and $LD_{50}$ values were calculated by the method of Reed and Muench (37).

**Invasive characteristics of the virulent wild-type strain (GBS 122) and mutant strain (M-10) defective in sialic acid production.** Mice were injected intraperitoneally with $10^5$ CFU of each strain in 1.0 ml of THB. Paired mice of each strain were sacrificed by cervical dislocation at 1, 8, 24, 48, and 72 h postinoculation. Blood and peritoneal washes were obtained first. Then various organs, spleen, kidney, liver, lung, and brain, were removed (in the order listed) aseptically and placed in 10 ml of sterile THB. The peritoneal washes were first centrifuged at 500 × *g* for 6 min to separate the supernatant from the macrophages, which were then washed twice with sterile THB medium followed by sonication on ice to achieve cell disruption. Both the supernatant and disrupted macrophage portions were serially diluted and then plated on THB agar plates. The tissues were minced to free bacteria, and the homogenized tissue mixtures centrifuged at 500 × *g* for 6 min to remove the mammalian tissues and cell debris. The supernatant fluids containing bacteria were then subjected to centrifugation at 3,000 × *g* for 20 min. The bacterial pellets were suspended in THB and serial dilutions were made before plating on THB agar plates. The plates were incubated at 37°C and the CFU per milliliter of homogenized tissue were enumberated. The CAMP factor (7) test was performed to confirm the isolation of GBS.

**In vivo killing of strain M-10 and GBS 122 by peritoneal macrophages.** Equal amounts of M-10 and GBS 122 cells determined by dry-weight measurements were combined to give a final theoretical value of $10^5$ CFU/ml. One milliliter of the mixed culture in THB was used as the standard inoculum. At 15, 30, 60, and 120 min postinoculation, paired mice were sacrificed by cervical dislocation and peritoneal washes were processed as described above. Sixty isolated colonies from macrophages and supernatant portions of the washes at each time point were transferred to 10 ml of FMC and allowed to grow to the stationary phase of growth. The cells were washed and then subjected to mild acid hydrolysis with 0.1 N HCl at 84°C for 20 min for sialic acid determination. The original inoculum before inoculation was also plated on blood agar plates, and 60 isolated colonies were randomly picked for sialic acid determination as described above. To eliminate the possibility that one strain may outgrow the other when combined together, an in vitro control as described below was included simultaneously. The 50:50 mixed culture was inoculated into 10 ml of THB on ice to give a final concentration of $10^5$ CFU/ml. Before transfer to a 37°C water bath, a aliquot of the initial culture was plated on agar plates. The culture was then incubated at 37°C, and at 15, 30, 60, and 120 min aliquots were removed, serially diluted in cold THB, and plated on agar plates. Again, 60 isolated colonies from each time point were examined for cell surface sialic acid.

**Chemical analysis.** The chemical compositions of sialic acid-containing materials after purification by column chromatography were analyzed by gas-liquid chromatography (12). Free sialic acid was determined by the method of Warren and Blacklow (45) to distinguish free from bound sialic acid. Extracellular neuraminidase activity was determined by the method described by Milligan et al, utilizing bovine submaxillary mucin (Sigma) as a substrate (33).

## RESULTS

**Isolation of mutants of a type III GBS.** To facilitate the identification of mutants defective in the synthesis of TSA, a screening procedure was developed. Table 1 summarizes the level of sialic acid, a marker for complete TSA, released from whole cells of GBS 122 (parent strain) by various extraction methods. Whereas cold trichloroacetic acid extraction can dissociate noncovalently bound TSA and rupture the cell membrane, M-1 mutanolysin has been shown to be effective in releasing covalently attached TSA from the cell walls of GBS (10, 11). In an osmotically protected medium, digestion of whole cells with M-1 enzymes resulted in soluble cell wall lysates (47). In an osmotically unprotected medium, however, digestion of whole cells with M-1 enzyme yielded soluble fractions consisting of both cell wall and intracellular materials. It should be noted in Table 1 that mild acid hydrolysis, which is commonly used to release terminal sialic acid from purified TSA, is also effective in removing >95% sialic acid from the cell-associated (cell surface) TSA of whole cells. The level of sialic acid released from the mild HCl hydrolysis step was comparable to that released by M-1 treatment in an osmotically protective environment. Thus, mild acid hydrolysis of whole cells was utilized as a screening test for the presence or absence of cell surface complete TSA in the mutant isolation process.

The first notable finding in the present study was the frequent isolation of strains capable of

TABLE 1. Sialic acid released from GBS 122 whole cells after various treatments[a]

| Treatment condition | Sialic acid released (μg)[b] |
|---|---|
| Mild acid hydrolysis[c] | 3,575[d] |
| M-1 mutanolysin digestion after mild acid hydrolysis[e] | <5 |
| M-1 mutanolysin digestion in lysis buffer[f] | 446[d] |
| M-1 mutanolysin digestion in protoplast medium[g] | <5 |
| TCA extraction[h] | 473[d] |
| M-1 mutanolysin digestion after TCA extraction[i] | <5 |

[a] Approximately 280 mg (dry weight) of stationary-phase cells was used for the various extractions.

[b] Determined by the thiobarbituric acid (TBA) assay of Aminoff (1).

[c] Whole cells were treated with 0.1 N HCl (pH 2.0) at 84°C for 20 min. The cell debris was then removed from the soluble hydrolysate by centrifugation.

[d] Free sialic acid was reducible by $NaBH_4$ (45) and became nonreactive in the TBA assay (1).

[e] The cell pellet after mild acid hydrolysis was washed once with PBS and then suspended in lysis buffer (0.03 M Tris-hydrochloride, 0.01 M $MgCl_2$). The washed pellet was digested with M-1 enzyme at 37°C for 3 h. Sialic acid was not detected after mild acid hydrolysis.

[f] Whole cells were suspended in lysis buffer and digested with M-1 enzyme at 37°C for 3 h. Sialic acid (4,080 μg) was detected when the lysate was hydrolyzed with 0.1 N HCl before the TBA assay.

[g] Whole cells were suspended in protoplast medium (47) and digested with M-1 mutanolysin at 37°C for 3 h. Sialic acid (3,797 μg) was detected when the lysate was hydrolyzed before the TBA assay.

[h] Whole cells were extracted with cold 10% trichloroacetic acid (TCA) overnight and processing of TCA-soluble material was described in the text. Sialic acid (261 μg) was detected when the extract was first acid hydrolyzed before the TBA assay.

[i] Cell pellet after treatment in footnote *h* was collected after centrifugation, washed twice with PBS, and treated with M-1 mutanolysin in lysis buffer. Sialic acid (3,276 μg) was detected by the TBA assay after acid hydrolysis.

synthesizing only the core TSA (25) after passage under selective pressure. Identification of these strains were confirmed based on the following: (i) sialic acid was not detected when whole cells were subjected to mild acid hydrolysis (0.1 N HCl at 84°C for 20 min) to release sialic acid from the cell surface TSA; (ii) positive reactions with anti-type III serum were observed when the hot HCl extracts of these strains were used for serotyping with rabbit anti-type III serum in a capillary precipitin test. The frequency of isolating strains that did not synthesize the sialylated complete TSA was estimated at one in five colonies examined after the second subculture and increased as further subculture continued. By passage 6, organisms lacking the core TSA (nontypable) were detected (frequency, 1 in 20 colonies examined), and the frequency of isolating nontypable strains increased to one in four colonies examined (passage 10 to 15 ).

**Characterization of the type III strains.** Loss of the sialic acid moiety from the complete TSA molecule could be due to one of two possibilities: (i) sialic acid was not synthesized, or (ii) the transferase activity responsible for adding sialic acid to the core oligosaccharide was not functional. Therefore, these mutants were further examined for the ability to elaborate free intracellular sialic acid. To achieve this goal, stationary-phase whole cells (wild-type strain and 12 mutants) were extracted with cold 10% trichloroacetic acid and processed as described in Materials and Methods. Figure 1 shows the elution profile of the trichloroacetic acid-extractable material from wild-type strain GBS 122. The sialic acid-containing peak eluting in the void volume was found to react with type III antiserum, and Sepharose 4B chromatography confirmed the HMW nature of this material (12).

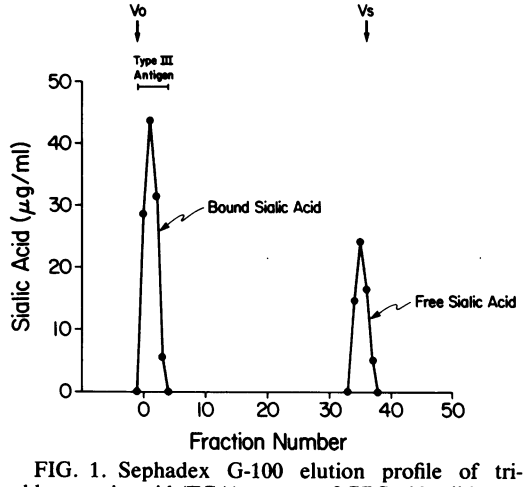


FIG. 1. Sephadex G-100 elution profile of trichloroacetic acid (TCA) extract of GBS 122 wild-type strain. Approximately 280 mg of cell dry weight was extracted with cold 10% TCA at 4°C overnight with constant stirring. The TCA-soluble material was further extracted with diethyl ether (three times) to remove TCA before lyophilization. The concentrated material was then applied to a Sephadex G-100 column (90 by 1.5 cm) and eluted with 10 mM sodium acetate, pH 6.5. The fractions were monitored for bound sialic acid confirmed by its resistance to reduction with sodium borohydride (45), as well as for free sialic acid determined by its sensitivity to reduction with sodium borohydride and negative reaction in the thiobarbituric acid assay (1). Vo, Void volume (56 ml); Vs, total or salt volume (182.6 ml).

Another sialic acid-containing peak, which eluted within the salt volume, did not react with the type-specific antiserum. This sialic acid-containing peak was confirmed to be free sialic acid based on the procedure of Warren and Blacklow (45) and therefore represents the free intracellular sialic acid pool present in the organism.

To confirm the cytoplasmic origin of the free sialic acid peak, whole cells of GBS 122 were enzymatically treated with M-1 mutanolysin in osmotically protected and unprotected media (Table 2). Since M-1 mutanolysin is capable of hydrolyzing the β-1,4 linkage between *N*-acetylglucosamine and *N*-acetylmuramic acid only, the presence of free sialic acid in the cell lysate fraction provided additional support for an intracellular sialic acid pool obtained from the trichloroacetic acid extract. The G-100 Sephadex elution profile (not shown) of the cell lysate was similar to that presented in Fig. 1. However, the level of bound sialic acid present in the cell lysate was substantially higher than that from the trichloroacetic acid extract since the M-1 enzyme can release covalently bound cell-surface TSA, which accounts for >85% of total TSA synthesized (11). The trichloroacetic acid extracts of the mutants were also purified by G-100 Sephadex column chromatography. The bound sialic acid peak as indicated in Fig. 1 was absent in the trichloroacetic acid extracts of all mutants examined. However, the free intracellular sialic acid peak was present in trichloroacetic acid extracts of several mutants. Thus, whereas some mutants maintained the ability to synthesize intracellular sialic acid, others appeared to lose that capacity altogether (Table 3).

**Purification of culture supernatant fluids from mutants.** Since GBS is known to release both group-specific antigens and TSA into the growth medium (6, 10), the culture supernatant fluids of the mutants were also examined. The dialyzed, concentrated supernatant materials were first screened for the presence of sialic acid by the method of Aminoff (1) after mild acid hydrolysis. No detectable sialic acid-containing polymers were found in the supernatant fluids of class I, II, III mutants (Table 3). However, strain 5.4, a class IV mutant, released a high level of the low-molecular-weight form of the type antigen (>90% of the total antigen synthesized [Fig. 2]). An additional sialic acid-containing peak (fractions 33 to 40), not found in the wild-type strain, was also noted. The low-molecular-weight nature (between 5,000 and 10,000) of these two sialic acid-containing peaks was confirmed by gel filtration on Sepharose 4B, Sephadex G-50, and Sephadex G-25 columns (Fig. 3). Both peaks eluted in the low-molecular-weight TSA area on the Sepharose 4B column (fractions 33 to 40, not indicated in the profile). The chemical compositions of both pools were also determined. Based on gas-liquid chromatography analysis, both polymers were composed of galactose, glucose, and *N*-acetylglucosamine in addition to sialic acid. Although no extracellular HMW form of the type antigen was found, this strain did synthesize a small amount of the HMW antigen associated with the cell surface. This conclusion was based on an experiment in which a cell wall lysate of strain 5.4 obtained from M-1 mutanolysin digestion and purified by DEAE-Sephacel and Sepharose 4B column chromatography exhibited a small amount of covalently bound HMW antigen.

TABLE 2. Distribution of bound and free sialic acid from trichloroacetic acid extracts and M-1 mutanolysin lysates of GBS 122

| Treatment | Sialic acid content (μg) | |
|---|---|---|
| | Bound[a] | Free[b] |
| Cold trichloroacetic acid extraction[c] | 263 | 473 |
| M-1 digestion in osmotically protected medium[d] | 3,797 | <5 |
| M-1 digestion in osmotically unprotected medium[e] | 3,702 | 446 |

[a] Represents sialic acid from the type-specific antigen. Mild acid hydrolysis was necessary to release sialic acid before the thiobarbituric acid (TBA) assay (1) and remained reactive in TBA reaction after alkaline treatment with the $NaBH_4$-acetone procedure (45).

[b] Sensitive to the $NaBH_4$-acetone procedure (45) and became nonreactive in TBA reaction; determination achieved without initial mild acid hydrolysis step.

[c] Approximately 280 mg (dry weight) of whole cells was extracted by 10% cold trichloroacetic acid (see test).

[d] Approximately 280 mg (dry weight) of whole cells in protoplast medium was enzymatically digested with M-1 mutanolysin. The soluble cell wall lysates, after separation from intact protoplasts, were extensively dialyzed and concentrated.

[e] Approximately 280 mg (dry weight) of whole cells in hypotonic medium was enzymatically digested with M-1 mutanolysin as described in the text.

The properties of the four classes of mutants isolated by serial subculture of the wild-type strain in the presence of type III-specific antiserum are summarized in Table 3. To further confirm that the mutants isolated were defective only in the biosynthesis of TSA, extracellular neuraminidase which has been shown to be produced in elevated levels by this strain (33) was examined, and the levels were comparable to that of the wild type (Table 3). Also, the hemolytic pattern (31), CAMP factor (7), and group B antigen (9) were also examined, and no distinguishable change in appearance or level

TABLE 3. Summary of mutants defective in the biosynthesis of TSA

| Mutant class | Strain no. | Bound sailic acid (μg) associated with complete TSA[a] | | Free intracellular sialic acid (μg)[b] | Complete type III antigen[c] | Core type III antigen[c] | Total neuraminidase activity[d] |
|---|---|---|---|---|---|---|---|
| | | Extracellular | Cell surface | | | | |
| I | M-10 | ND[e] | ND | ND | − | + | 180 |
| | M-12 | NT[f] | ND | ND | − | + | 183 |
| | M-15 | NT | ND | ND | − | + | 181 |
| II | 5.5 | ND | ND | 756 | − | + | 181 |
| | #5 | NT | ND | 488 | − | + | 178 |
| | #20 | NT | ND | 640 | − | + | 221 |
| | #24 | NT | ND | 486 | − | + | 179 |
| III | 4.5 | ND | ND | 497 | − | − | 243 |
| | 4.9 | NT | ND | 384 | − | − | 167 |
| | 4.10 | NT | ND | 497 | − | − | 211 |
| | 5.9 | NT | ND | 88 | − | − | 175 |
| IV | 5.4 | 1,564 (96%)[g] | 63 (4%)[h] | 224 | + | + | 279 |
| Wild-type | 122 | 505 (12%)[i] | 3,702 (88%)[h] | 473 | + | + | 193 |
| Revertant | $5.4_6$ | 473 (11%)[i] | 3,797 (89%)[h] | 528 | + | + | 215 |

[a] Sialic acid was covalently bound to extracellular or cell surface TSA as demonstrated by its resistance to $NaBH_4$ reduction (45). Mild acid hydrolysis (0.1 N HCl, 84°C, 20 min) was used to detect sialic acid by the Aminoff procedure (1).

[b] Free sialic acid was demonstrated by its susceptibility to $NaBH_4$ reduction (1, 45) and required no initial hydrolysis step before the thiobarbituric acid assay.

[c] Determined by Ouchterlony immunodiffusion with type III-specific rabbit antiserum. The composition of the polysaccharides was determined by gas-liquid chromatography (galactose–glucose–*N*-acetylglucosamine, 2:1:1 molar ratio) (12). Sialic acid was determined by the method of Aminoff (1) after mild acid hydrolysis.

[d] Expressed as nanomoles of sialic acid released per minute per milligram of cell dry weight, using bovine submaxillary mucin as a substrate (33).

[e] ND, Less than 5 μg of sialic acid detected.

[f] NT, Not tested.

[g] Indicates the percentage of total sialic acid contained in the extracellular low-molecular-weight form of the TSA (Fig. 2 [12]).

[h] Indicates the percentage of total sialic acid contained in the HMW form of the TSA.

[i] Indicates the percentage of sialic acid contained in both the HMW and low-molecular-weight forms of the extracellular TSA (Fig. 2, I and III).

was observed. Physiologically, all mutants had doubling times (30 to 33 min) quite similar to that of the wild-type strain under identical growth conditions.

The stability of the mutants was also determined. No reversion to the wild-type characteristic (presence of sialylated TSA on the cell surface) was observed after six or seven in vitro passages in the nonselective chemically defined medium. Nevertheless, for all studies using any of these strains, the phenotype was examined first to assure the characteristics of the mutants used.

**Mouse lethality study.** Since the TSA is believed to be an important virulence determinant of the GBS, these mutants offered a unique opportunity for examining the role of TSA more specifically. The virulence of these mutants was assessed in a mouse model, and all four classes had $LD_{50}$ values $>3$ $\log_{10}$ higher than that of the wild-type strain (Table 4). The data, therefore, confirmed the role of TSA in the pathogenicity of GBS because the only difference between the parent strain and the mutants was in TSA synthesis, whether it was absent (class III), present as an incomplete form (class I and II), or found in minute concentrations on the cell surface (class IV).

Organisms recovered from blood of lethally infected mice were also examined for revertants after in vivo passage (revertants are defined as those strains that have regained the ability to synthesize complete TSA and attach it to the cell surface as the HMW form of the TSA). About 50% of isolated colonies from mice infected with strains 5.4 and 5.5 reverted to the wild-type phenotype, but no revertants were isolated from mice inoculated with strains M-10 and 4.5 in the small random samplings. Detailed characterization of one revertant (strain $5.4_6$) was pursued, and the level of cell-associated and extracellular TSA was quite comparable to that of the wild-type strain (Table 3; Fig. 2). Importantly, the $LD_{50}$ value of this revertant was similar to that of the wild-type strain (Table 4). Data obtained from this revertant and the other four classes of mutants strongly indicated that only the sialylated HMW form of the type antigen contributes

significantly to virulence in this experimental system.

**Comparative study of the invasive property of the wild-type strain and strain M-10.** To examine the role of the sialic acid moiety in contributing to virulence of the organism, a sialic acid-deficient strain (M-10, class I mutant) and the wild-type strain GBS 122 were used in a study to compare the dissemination of the organisms into various tissues in the host. Figure 4 demonstrates a typical comparative profile of these studies. The notable difference was the observation that whereas the wild-type strain was capable of disseminating into various tissues within 1 h postinoculation, the sialic acid-deficient mutant appeared to be quite noninvasive. Furthermore, >80% of the M-10 cells phagocytized were killed within a short period of time, whereas the wild-type strain appeared to be able to evade phagocytic killing. For GBS 122, high levels of bacteria in various hematogenous tissues were observed at 8 and 24 h postinoculation, but declined at 48 h. Detectable levels of bacteria were only found in the kidneys at 72 h (data not shown). For strain M-10, however, organisms were recovered at 48 h postinoculation. The level of cell-associated, sialylated, HMW TSA from these organisms was found to be similar to that in the wild-type strain.

The recovery of the wild-type strain probably represented in vivo selection for wild-type characteristics and as such provides indirect evidence that the mutants isolated were the result of a single mutation. The significance of these experiments, therefore, was that the sialic acid moiety of the cell surface TSA facilitated efficient dissemination of the organism and was a critical determinant for virulence of the wild-type strain.

**In vivo killing of strains M-10 and GBS 122 by peritoneal macrophages.** Data obtained from the previous experiment seemed to indicate that a factor(s) responsible for selective clearance of the sialic acid-deficient strain M-10 resided in the peritoneal cavity of the host. To confirm the selective killing of the sialic acid-deficient strain M-10 and to determine whether strain M-10 could also augment killing of the wild-type strain in vivo, mice were inoculated with $10^5$ CFU containing mixed cultures of strain M-10 and GBS 122 wild type at a 1:1 ratio. At 15, 30, 60, and 120 min postinoculation, paired mice were sacrificed, and peritoneal washes were collected and cultured as described in Materials and Methods. The ratio of M-10 (sialic acid deficient) to GBS 122 (with sialic acid) remained constant throughout the 2-h incubation period in vitro (Table 5). However, only strain M-10 was selectively killed in vivo since almost 100% recovery of GBS 122 was obtained at 1 h postinoculation.

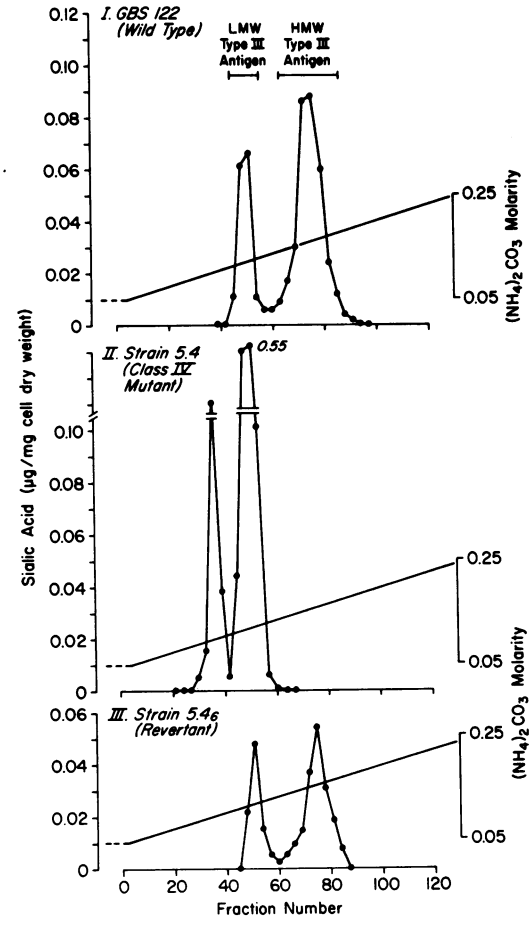


FIG. 2. DEAE-Sephacel elution profile of culture supernatant materials. Wild-type strain GBS 122, class IV mutant strain 5.4, and revertant strain $5.4_6$ were grown in a chemically defined medium (FMC) to stationary phase. The supernatant materials were collected and dialyzed against 10 mM sodium acetate buffer at 4°C for 2 days followed by concentration by lyophilization. The concentrated supernatant materials were then applied to a DEAE-Sephacel column (28 by 2.5 cm) eluted with a continuous gradient of 0.05 to 0.25 M $(NH_4)_2CO_3$. A total of five ml per fraction was collected. The fractions were monitored for sialic acid (method of Aminoff after mild acid hydrolysis at 84°C for 20 min) and type III reactivity.

One of the unavoidable difficulties was the tendency of GBS 122 to form shorter chains than strain M-10, thereby yielding more CFU, although an equal concentration of both cell types based on dry-weight measurements was utilized in the experiment. Nevertheless, data obtained from this and the previous experiment indicated an almost all-or-none recovery of one strain over the other, strongly confirming the selective killing of cells that lacked the sialic acid moiety on the cell surface TSA. Although the peritoneal

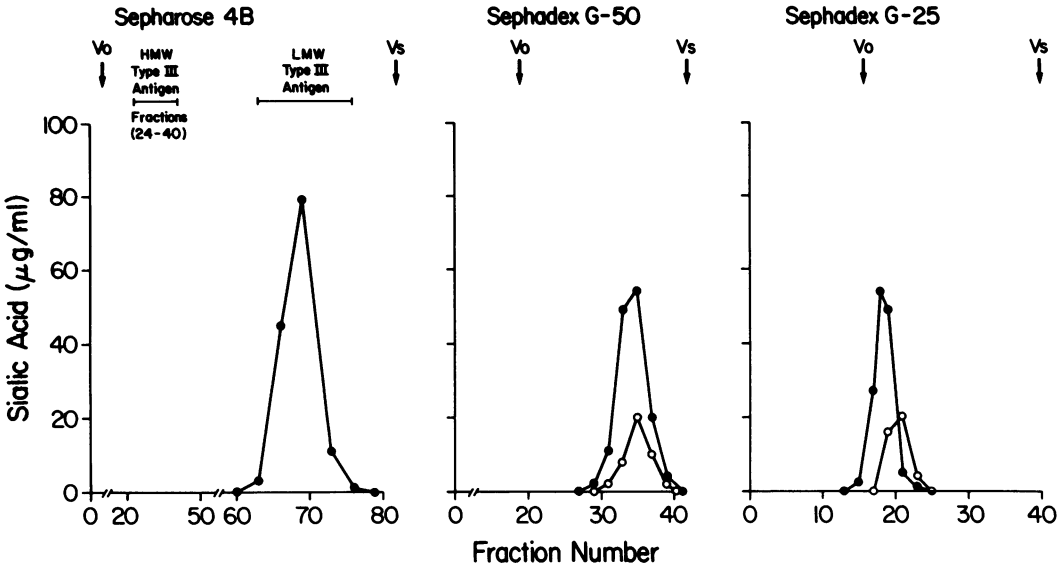


FIG. 3. Gel filtration profiles of the sialic acid-containing peaks (shown in Fig. 2) resolved from supernatant fluid materials of strain 5.4. Fractions 30 to 44, pool I (○), and fractions 47 to 58, pool II (●), were pooled, concentrated, and applied sequentially to Sepharose 4B (90 by 2.5 cm; Vo = 130.5 ml, Vs = 459 ml), Sephadex G-50 (80 by 1.8 cm; Vo = 95 ml, Vs = 210 ml), and Sephadex G-25 (85 by 1.0 cm; Vo = 26 ml, Vs = 60 ml). The bars in the Sepharose 4B profile indicate the location of the HMW and low-molecular-weight type-specific antigen previously described (12). The samples were eluted with 10 mM sodium acetate. Five-milliliter fractions were collected from the Sepharose 4B and Sephadex G-50 columns, and 1.5-ml fractions were collected from Sephadex G-25. Aliquots were then assayed for sialic acid by the thiobarbituric acid assay (1) after mild acid hydrolysis in 0.1 N HCl at 84°C for 20 min.

macrophages probably played an essential role in clearing the sialic acid-deficient strain, the role of complement could not be excluded since complement also enhances phagocytosis (23).

## DISCUSSION

Sialic acid is present ubiquitously on the surfaces of various mammalian cell types (2, 3, 15, 17, 20, 21, 29) and is also frequently detected on many naturally occurring glycoproteins (3, 35). The sialic acid residue of many, if not all, glycoproteins is believed to play a complex and mutually exclusive role in determining the survival of the molecules in the circulation (3). This conclusion was based upon studies by Morell and colleagues (34) and others who demonstrated that the asialoglycoproteins were promptly removed from the circulation whereas the fully sialylated glycoproteins exhibited a normal serum survival time. Evidence was presented that the terminal sialic acid functioned to mask the penultimate galactosyl residue which the hepatocytes recognize. At the same time, while identifying hepatocytes as the major machinery for removal of these desialylated molecules, it was shown that the presence of sialic acid on the receptor site of the plasma membrane of the hepatocytes was essential for the initial binding process, leading to subsequent uptake and intracellular lysosomal catabolism (36). The biological significance of sialic acid has also been demonstrated to play a role in regulating survival of erythrocytes (2) and platelets (17), as well as in influencing the route of circulation of small lymphocytes in the body (46). The sialomucoid layer of certain tumor cells (39) has been implicated in the metastiatic process, and removal of sialic acid from these cells resulted in increased

TABLE 4. $LD_{50}$ determinations of GBS 122 and mutants

| Strain | $LD_{50}$ (CFU)[a] |
|---|---|
| GBS 122 (Wild type) | $2.0 (\pm 1.3) \times 10^4$ |
| $5.4_6$ (revertant)[b] | $2.6 (\pm 0.1) \times 10^4$ |
| M-10 (class I mutant) | $6.0 (\pm 0.2) \times 10^7$ |
| 5.5 (class II mutant) | $4.2 (\pm 1.3) \times 10^7$ |
| 4.5 (class III mutant) | $4.1 (\pm 0.1) \times 10^7$ |
| 5.4 (class IV mutant) | $2.8 (\pm 0.2) \times 10^7$ |

[a] Mice received organisms in 1.0 ml of THB by intraperitoneal inoculation. $LD_{50}$ was determined by the method of Reed and Muench (37). All values were averages of three separate determinations ± standard deviation.

[b] Strain recovered from blood of lethally infected mice inoculated with strain 5.4. Strain $5.4_6$ was demonstrated to synthesize the sialylated HMW form of the TSA (covalently bound and extracellular forms).

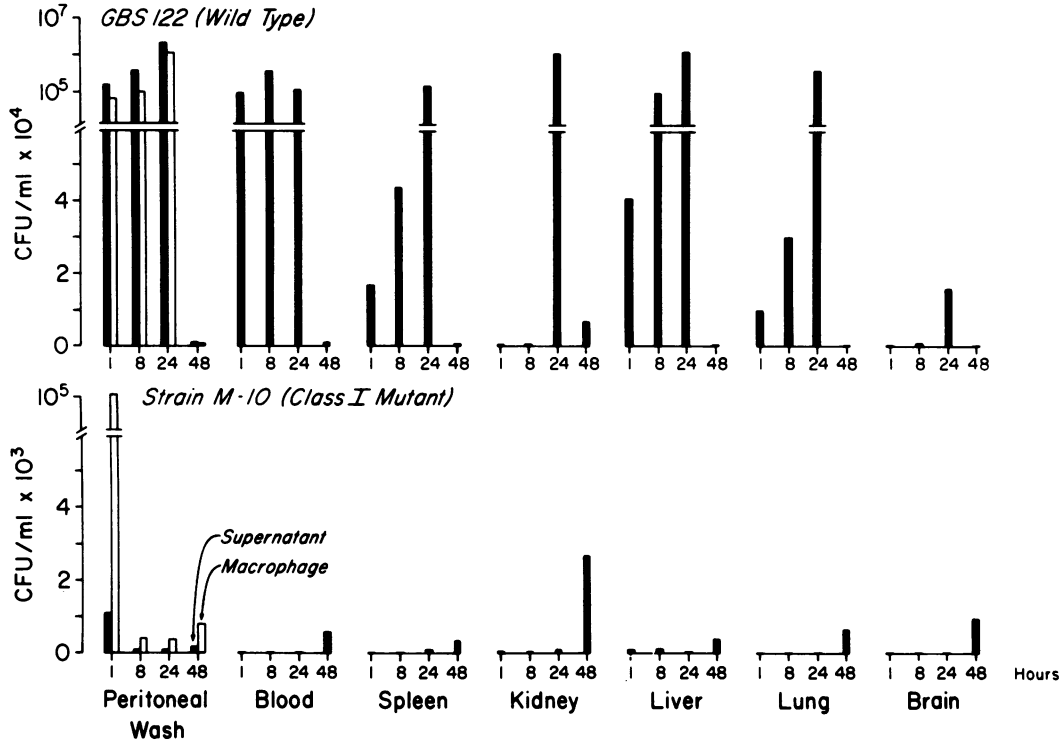


FIG. 4. Levels of viable GBS in tissues from infected mice at various times postinoculation. Paired mice which received $10^5$ CFU in 1.0 ml of THB of GBS 122 wild-type strain and strain M-10 (class I mutant) were sacrificed at 1, 8, 24, and 48 h postinoculation. Blood, peritoneal wash, and various organs were collected aseptically. The peritoneal wash was contrifuged at 500 × *g* for 6 min. Macrophages and supernatant fluid portions were then serially diluted and spread on Todd-Hewitt agar plates for colony enumeration. The various organs were minced to free bacteria, which were also enumerated after serial dilution and subsequent plating on Todd-Hewitt agar plates. The CAMP test was performed to confirm recovery of GBS.

susceptibility to cytotoxic killing by serum immunoglobulins (21). A recent study by McSharry et al. (32) indicated that influenza viruses that lacked sialic acid appeared to be better activators of the alternate complement pathway, and their results, therefore, agreed with the findings by Fearon and co-workers (15, 26), who demonstrated that sialic acid on sheep erythrocytes might be a key component in modulating activation of C3 via the alternate pathway.

The presence of sialic acid-containing macromolecules on the surfaces of bacterial cells is less common. The notable examples have been the K1 antigen of *Escherichia coli* (4) and the capsular polysaccharide of *Neisseria meningitidis* (30) (both polysialyl acid polymers), as well as the TSA of GBS (heteropolysaccharide polymer with terminal sialic acid residues) (22). Interestingly, all three organisms have frequently been implicated in meningitis, and the similarity may be more than coincidental. Analogous to what has been described for eucaryotic cells, these sialic acid-containing polysaccharides might very well play an essential role in masking recognition sites, thereby protecting the microorganisms from the host defense armamentarium. Simultaneously, the negatively charged bacterial particles may encounter little hindrance in their traffic and distribution in the host since the majority of the host defense tissue surfaces are also negatively charged due to the presence of sialic acid (29, 44).

In *E. coli*, studies have shown that the size of the capsules was directly associated with increased resistance to killing by complement and phagocytosis (38, 40). For GBS, the exact mechanism of how TSA contributes to virulence of the organism has not been resolved. Data obtained from the present experimental system, however, appeared to suggest that the sialylated TSA may function to facilitate efficient dissemination of the organism in the host. Therefore, strains that have the cell surface sialylated HMW TSA are more virulent than strains that lack the sialylated antigens (Table 4). The present study also suggested that the level of cell-associated HMW TSA is a better indicator of

TABLE 5. In vivo killing of strain M-10 and wild-type strain 122[a]

| Sample | M-10/GBS 122 recovered postinoculation | | | |
|---|---|---|---|---|
| | 15 min | 30 min | 60 min | 120 min |
| Supernatant[b] | 9/51 | 12/48 | 1/59 | 0/60 |
| Macrophages[c] | 17/43 | 28/32 | 9/51 | 0/60 |
| In vitro mixed culture of M-10 and GBS 122[d] | NT[e] | NT | 16/44 | 17/43 |

[a] A 1.0-ml amount of THB containing $10^5$ CFU of a mixed culture of M-10 and GBS 122 at a 1:1 ratio was inoculated intraperitoneally into ICR mice which were then sacrificed at various times. A total of 60 isolated colonies were randomly picked for sialic acid determination. Results were expressed as ratios of M-10/GBS 122 colonies based on the absence or presence of sialic acid, respectively. The original mixed culture was also plated on blood agar plates. Sixty isolated colonies were selected randomly for sialic acid determination. At zero time before inoculation, the ratio of M-10 to GBS 122 was 17/43.

[b] Liquid phase obtained after the peritoneal wash was centrifuged at 500 × *g* for 6 min at 4°C.

[c] Peritoneal macrophages present in the cell pellet after centrifugation of the peritoneal wash at 500 × *g* for 6 min at 4°C.

[d] A mixed culture was obtained by adding equal concentrations of M-10 and GBS 122 cells, inoculated into 10 ml of THB to give a theoretical $10^5$-CFU/ml final concentration, and allowed to grow at 37°C. At 15, 30, 60, and 120 min, aliquots were removed and chilled on ice immediately. For sialic acid determination, colonies from zero time (base-line value) and 60 and 120 min were used. At zero time, the ratio of M-10/GBS 122 was also 17/43.

[e] NT, Not tested.

virulence of the organism. This conclusion, however, is not in conflict with the results obtained by Durham et al. (13), who demonstrated a direct correlation between level of extracellular (HMW form) TSA and virulence in a mouse model. Quantitative studies in our laboratory (M. K. Yeung and S. J. Mattingly, manuscript in preparation) showed that strains that synthesize a high level of cell-associated TSA also secrete a high level of extracellular TSA into the growth medium. Studies from other laboratories have emphasized the importance of the complete sialylated TSA because antibody directed against the core antigen failed to neutralize the complete antigen (25). Whereas the important role of the sialylated complete TSA in GBS infection cannot be overemphasized, the present study also indicated that the core TSA was not involved in virulence since the $LD_{50}$ values of the sialic acid-deficient mutants were comparable to those of mutants with no TSA (nontypable).

Finally, it is important to note that in the present animal model, antibody does not play any role in eliminating the invading organism from the host since clearance of the organisms occurred in less than 2 h. Although rapid clearance of strain M-10 cells was demonstrated to be the result of efficient intracellular killing by peritoneal macrophages, the possibility that alternate complement factors and perhaps another unknown cytotoxic factor(s) acting in concert cannot be excluded.

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