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Protease Production by Clinical Isolates of Type III Group B Streptococci

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Six strains of serotype III group B streptococci isolated from confirmed cases of neonatal disease were examined for their ability to produce proteolytic enzymes. Three neuraminidase-producing strains and three non-neuraminidase-producing strains were employed in this study. Protease production was examined in 1,000-fold concentrated filtrates of stationary-phase cells with an insoluble substrate derived from horse hide powder labeled covalently with Remazol brilliant blue. Protease activity was not detected in any cultural supernatant fluids until they were fractionated on Sephadex G-100. After fractionation, the neuraminidase-producing strains were shown to elaborate approximately sixfold more protease than the non-neuraminidase-producing strains. The finding that clinical isolates of group B streptococci that elaborated high levels of neuraminidase also produced elevated levels of extracellular protease may indicate that the production of several different factors may determine the virulence of these organisms.

Renewed interest in the characterization of the potential extracellular virulence factors produced by the group B streptococci (GBS) has occurred recently due to the increased awareness of the role of these organisms in serious neonatal disease (18). Earlier studies by Todd in 1934 (25) described the production of an oxygen-stable, heat-labile, non-immunogenic hemolysin by these organisms. McClean (15), in 1941, demonstrated that the GBS elaborated a hyaluronidase, and this was confirmed in a study 39 years later by Kjems et al. (11). Deibel, in 1963 (4), and Wannamaker, in 1964 (27), demonstrated that several strains of GBS were capable of elaborating enzymes with deoxyribonuclease activity, and Ferrieri et al. (6) showed that GBS strains representing all the five major serotypes were all capable of producing extracellular nucleases. The isolation of the CAMP factor (a protein which causes the rapid lysis of erythrocytes treated with the beta-hemolysin of *Staphylococcus aureus*) of the GBS was reported by Brown et al. (2) in 1974. In 1979, Bernheimer et al. (1) demonstrated that in erythrocytes from sheep, the CAMP factor reacts with the membrane ceramide formed by the activity of the staphylococcal beta-hemolysin. The beta-hemolysin has been shown to be a sphingomyelinase, and the binding of the CAMP factor to the resultant ceramide disrupts the lipid bilayer to such a degree that the result is hemolysis. Hayano and Tanaka (8) and Hayano et al. (9) were able to demonstrate the production of a GBS neuraminidase that possessed enzymatic activity against a sialomucoid preparation from bovine submaxillary mucin. Milligan et al. (16, 17) further characterized the production of this enzyme and demonstrated that type III isolates from diseased infants were more often classified as high neuraminidase producers than strains of GBS of other serotypes from diseased infants. Furthermore, these authors showed that type III strains isolated from neonatal infections were more often high neuraminidase producers than those isolated from asymptomatically colonized infants. Finally, although Deibel (4) examined six strains of GBS for the production of extracellular protease, he was not able to demonstrate proteolytic activity against gelatin. If these organisms could be shown to be capable of protease production, these enzymes must also be considered as potential virulence factors.

As protease activity has been associated with virulence in other bacteria (7, 10, 19), the present study was undertaken to reexamine the proteolytic capability of GBS. Our findings indicated that type III GBS possess proteases, but fractionation is essential to demonstrate activity.

(A preliminary report of this work was presented at the 80th Annual Meeting of the American Society for Microbiology, Miami Beach, Fla., 11-16 May 1980 [D. C. Straus, S. J. Mattingly, T. W. Milligan, and T. I. Doran, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, D47, p. 45].)

MATERIALS AND METHODS

Bacterial cultures and medium. GBS strains 110, 118, 120, 127, 130, and 142 were used in this study. All are type III organisms, and all were clinical isolates from cases of neonatal disease. Strains 110, 120, and 130 are classified as high neuraminidase producers (>140 nmol of sialic acid released per min per mg of cell dry weight), and strains 118, 127, and 142 are nonproducers of this enzyme (<10 nmol of sialic acid released per min per mg of cell dry weight) (16). Before inoculation into the chemically defined medium (FMC), the cells were handled as previously described (16). FMC was prepared by the procedure of Terleckyj et al. (24). Briefly, this medium contained amino acids, vitamins, purine and pyrimidine bases, minerals, 1% glucose, 0.065 M phosphate, and 0.019 M sodium carbonate, with an initial pH of 7.0.

Growth and preparation of culture supernatant fluids. Starter cultures of each of the six strains containing approximately 0.1 of the final volume of FMC were inoculated at an initial density of 25 to 50 adjusted optical density units. All optical densities were measured in a Coleman Junior II spectrophotometer at 675 nm against a water blank in calibrated Corning Pyrex test tubes (18 by 150 mm). Optical densities were multiplied by 1,000 and adjusted to conform with Beer's law. The result was expressed as adjusted optical density units (26). One optical density unit is equivalent to 0.43 μg of cell dry weight per ml. The cells were collected after 18 h of growth (late stationary phase) (0.65 mg of cellular dry weight per ml). In experiments in which the incorporation of ^{3}H-amino acids (Schwartz/Mann) into GBS proteins was examined, the specific activity of the amino acid precursors was increased by decreasing the normal concentration of amino acids in the medium (24) by 0.5. The ^{3}H-amino acid mixture was present at a concentration of 1.0 mCi/ml.

Cells were pelleted by centrifugation at 8,000 × *g* in a Sorvall RC-5 refrigerated centrifuge or a Sorvall SS-3 automatic centrifuge at 4°C for 20 min. The supernatant fluid was then dialyzed for 3 to 4 days against 10 mM acetate (pH 6.5) in dialysis tubing with a 12,000-molecular weight exclusion (Arthor H. Thomas Co.) at 4°C. It was then lyophilized to dryness.

The product was then dissolved in 6 ml of 0.01 M tris(hydroxymethyl)aminomethane buffer (pH 8.0) and dialyzed against the same buffer before gel filtration.

Fractionation of extracellular material. The concentrated supernatant fluid (5 ml) was applied to an upward-flow Sephadex G-100 column (2.6 by 90 cm), and the remaining 1 ml was retained for future analysis. The column was eluted with 0.01 M tris(hydroxymethyl)aminomethane (pH 8.0). Fractions (4 ml each) were collected after 100 ml had passed through the column. The elution of proteins was followed with a Gilford model 250 single-beam spectrophotometer at 280 nm. Fractions in the center of each peak were concentrated by lyophilization, suspended in 2 ml of distilled water, and then dialyzed against 0.01 M tris(hydroxymethyl)aminomethane buffer (pH 8.0). When radiolabeled supernatant fluids were fractionated, 0.5 ml from every third fraction was processed for scintillation spectroscopy as previously described (22).

Physical and chemical determinations. Protein concentrations were measured by the procedure of Lowry et al. (14) with bovine serum albumin as the standard. For the quantitation of protease activity, a modification of the procedure of Rinderknecht et al. (21) was employed. Briefly, scintillation vials containing 20 mg of the horse hide powder blue substrate, 0.01 M phosphate buffer (pH 7.0 at 37°C), and 20 to 50 μl of the enzyme sample in a final volume of 5 ml were incubated for periods of time ranging from 6 to 22 h. Optimum pH determinations on several of the protease pools (data not shown) demonstrated that maximal proteolytic activity was obtained at pH 7.0. Assays were performed in duplicate, and the reaction was terminated by filtration with a Millipore filtration apparatus, which removed any remaining insoluble substrate. Enzyme preparations inactivated by heating at 100°C for 5 min were used as the blanks for each sample. The supernatant fluid was then read at 595 nm (21), and the protease activity was reported as units per minute per milligram of protein. One unit is defined as an optical density increase of 0.001 above the background value.

Protein peaks (10 to 30 μg) were analyzed by slab gel electrophoresis with sodium dodecyl sulfate-polyacrylamide (10%) by the procedure of Laemmli (13). The bands were stained with Coomassie brilliant blue (Bio-Rad Laboratories).

RESULTS

Comparison of the extracellular material produced by three neuraminidase-producing and three non-neuraminidase-producing type III strains of GBS. Because we were unable to detect significant amounts of proteolytic activity in any of the unfractionated GBS culture supernatants (Table 1), we decided to fractionate this material to see if the proteolytic activity could be enhanced. The elution profiles of the six GBS strains on Sephadex G-100 can be seen in Fig. 1. This figure shows that the elution profiles at an absorbance at 280 nm of

TABLE 1. *Protease activity of pools from Sephadex G-100 column chromatography of extracellular material produced by type III strains of GBS*

Fraction pool	Protease activity (U/min per mg of protein) of:					
	High neuraminidase-producing strains			Non-neuraminidase-producing strains		
	110	120	130	118	127	142
Unfractionated	0.04	0.02	0.02	0.02	0.04	ND[a]
I	3.77	0.05	0.91	2.34	ND	0.86
II	4.26	2.35	7.83	0.67	0.68	ND
III	5.22	1.45	6.88	0.62	0.21	0.15
IV	1.94	2.17	0.24	0.38	ND	ND

[a] ND, Not detected.

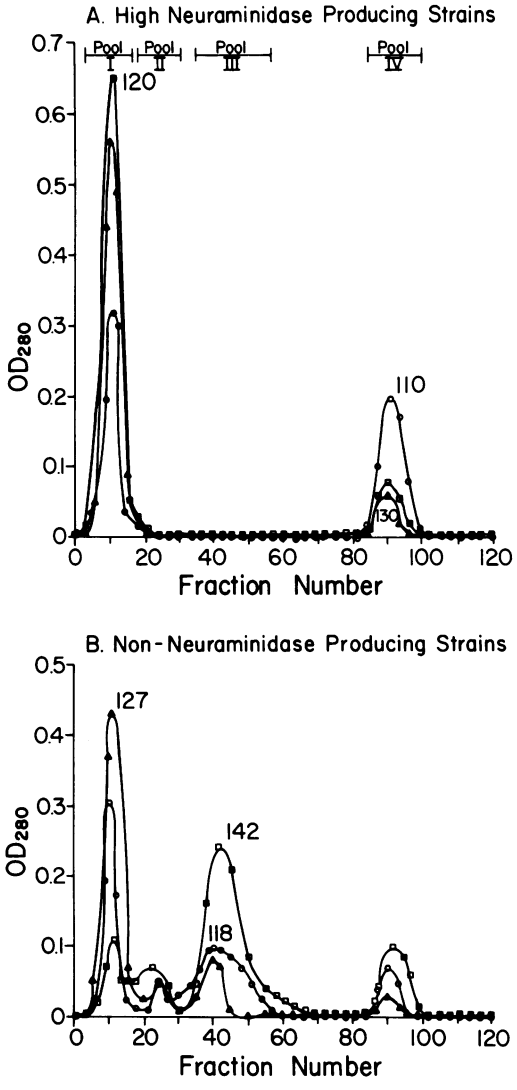

FIG. 1. *Elution profile on Sephadex G-100 of extracellular material produced by six different strains of GBS. Pools I, II, III, and IV represent compounds having elution volumes of 150, 196, 293, and 514 ml, respectively. The void volume of this column was 140 ml, and the salt volume was 540 ml. OD_{280}, Optical density at 280 nm.*

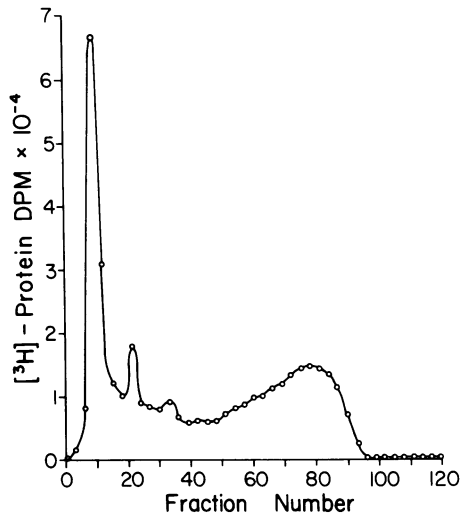

FIG. 2. *Elution profile on Sephadex G-100 of the ^3H-labeled extracellular protein produced by high neuraminidase-producing GBS strain 110. The void and salt volumes of this column were the same as those in Fig. 1.*

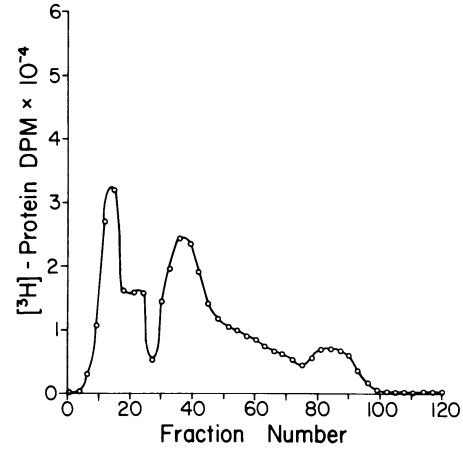

FIG. 3. *Elution profile on Sephadex G-100 of the ^3H-labeled extracellular protein produced by non-neuraminidase-producing GBS strain 118. The void and salt volumes of this column were the same as those in Fig. 1.*

the extracellular material from all three high neuraminidase producers were strikingly similar in that there were two peaks observed in each one, and the corresponding peaks all had the same elution volume. A similar phenomenon was noted when examining the elution profiles at an absorbance at 280 nm of the fractionated supernatant fluids from the non-neuraminidase producers. Here, four peaks were always observed, and the elution volumes of the corresponding peaks were always similar. Even though only two peaks were observed when the supernatant fluids from the high neuraminidase producers were fractionated, the fractions that corresponded to pools II and III from the non-neuraminidase producers were also pooled and examined for protease activity.

An examination of the proteolytic activity found in the various pools is shown in Table 1. The results indicate that although relatively lit-

tle proteolytic activity was seen in each of the six culture supernatant fluids, fractionation of these materials on Sephadex G-100 demonstrated that considerable proteolytic activity was present. With few exceptions, each one of the individual pools contained more protease activity than did the unfractionated supernatant fluids. These data also indicate a relatively wide variation in the amounts and molecular weights of the proteases produced by the GBS. However, one very important generalization can be made when comparing the total proteolytic activity elaborated by the high neuraminidase-producing strains with that excreted by the non-neuraminidase-producing strains; GBS strains 110, 120, and 130 were consistently more proteolytic than GBS strains 118, 127, and 142. The high neuraminidase-producing strains elaborated approximately sixfold (36.98 total U/min per mg of protein versus 5.91 total U/min per mg of protein) more protease than did the non-neuraminidase-producing strains. These data represent an average of two separate determinations, and the difference was shown to be significant at the 95% confidence level by the Student *t* test.

Elution profile of radiolabeled extracellular material produced by GBS strains 110 and 118. As proteolytic activity was detectable in several regions of the elution profile where an absorbance at 280 nm was not observed, the sensitivity of the assay for protein was substantially enhanced by radiolabeling the protein with a ^{3}H-amino acid mixture. Figure 2 demonstrates this elution profile of radiolabeled protein produced by GBS strain 110 and indicates that there was indeed protein in all the areas that were pooled. A similar experiment was performed with GBS strain 118. As can be seen from Fig. 3, the radioactive elution profile corresponded well with the elution profile monitored at 280 nm.

Sodium dodecyl sulfate-polyacrylamide electrophoresis. A 10% sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis was run on the four pools obtained by Sephadex G-100 fractionation of the supernatant fluids of GBS strains 110 and 118. There were many extracellular proteins produced by these strains, and the electrophoretic pattern produced by strain 110 was substantially different from that produced by strain 118 (data not shown).

DISCUSSION

This study represents the first report of protease production by GBS. Although a study by Deibel (4) indicated that proteolytic activity was not a widely distributed characteristic in the genus *Streptococcus,* these data and other studies would seem to dispute this. The group A streptococci have been shown to produce a protease (5) that is capable of hydrolyzing their own antiphagocytic M-protein. In addition, there have been several reports of protease production by the viridans streptococci (3, 20, 22, 23), and a recent study that demonstrated infection with a proteolytic strain of *Streptococcus faecalis* resulted in a more severe clinical picture during experimental endocarditis in rabbits than infection with a non-proteolytic strain of the same organism (7).

It is apparent from the present study that GBS also possess proteolytic activity, although fractionation of the concentrated extracellular material is essential before proteolytic activity can be detected. The use of unfractionated material in previous studies of GBS (4), therefore, explains the lack of detectable proteolytic activity. Although the nature of the inhibition of unfractionated material is not known, the dilution of crude mixtures of inactive proteases has been shown to restore protease activity in other systems (12). Whether this dilution or fractionation of GBS extracellular material results in the removal of natural protease inhibitors or possibly provides for a more suitable ionic environment for protease activity remains to be determined.

The high level of proteolytic activity in strains of type III GBS previously determined to produce high levels of extracellular neuraminidase (16) and release elevated levels of type III antigen (T. I. Doran, T. W. Milligan, D. C. Straus, and S. J. Mattingly, Abstr. Annu. Meet. Soc. Microbiol. 1979, B86, p. 30) suggests that several extracellular factors elaborated by these strains may be important in the virulence of these organisms. These results also indicate that type III GBS can be readily divided into two major physiological groups based on the nature and level of extracellular material secreted. Type III strains producing high levels of extracellular factors (16) are significantly more often isolated from infected infants than strains producing low levels. However, the isolation from infected infants of some non-neuraminidase-producing type III strains (16) which produce decreased levels of protease and type III antigen suggests that other undetermined factors may also play a role in neonatal disease.

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