BIOCHEMICAL AND IMMUNOLOGICAL CHARACTERIZATION OF THE EXTRACELLULAR NUCLEASES OF GROUP B STREPTOCOCCI*

By PATRICIA FERRIERI, ERNEST D. GRAY, and LEWIS W. WANNAMAKER‡

From the Departments of Pediatrics and Biochemistry, University of Minnesota Medical School, Minneapolis, Minnesota 55455

The emergence of the group B *Streptococcus* as a leading agent of neonatal sepsis and meningitis (1) has stimulated interest in furthering understanding of the biology and immunochemistry of this organism. Various antigens of the five major human group B streptococcal serotypes have been under recent investigation, including type-specific antigens (2–4) as well as an intracellular enzyme, hippuricase (5), and also the extracellular enzyme, neuraminidase (6), and CAMP protein (7). Advances in our knowledge of the group B *Streptococcus* have been the subject of recent reviews (8–10).

Our studies were undertaken to explore the possibility that group B streptococci may elaborate extracellular nucleases different from those of group A streptococci, and that these nucleases may be valuable antigenic markers that could be used to follow the immune response to group B streptococcal colonization or infection.

The frequency of production of extracellular DNase had been reported to be 38% in group B streptococci in contrast to 100% in group A streptococci (11). The possibility that these differences might be a result of the production of lower levels of enzyme by group B streptococci led to the examination of methods that are more sensitive for screening group B streptococcal strains for nuclease activity. Because of the high prevalence of nuclease activity detected in the initial screening of many group B streptococcal strains (*vide infra*), studies were continued to isolate the nucleases and to characterize them biochemically and immunologically.

Materials and Methods

Bacteria. The group B streptococci were recent human isolates from the maternal genital tract, and from the mucosal sites, blood, and cerebrospinal fluid of infants. Identification of their serological group was by the hot-acid extraction and capillary precipitin method of Lancefield (12), and typing was done by immunodiffusion in agar (13) and also by capillary precipitin technique using rabbit antisera prepared in our laboratory (grouping) and antisera provided by the Center for Disease Control, Atlanta, Ga. (typing). Streptococcal strains were grown up to log phase and frozen in small aliquots at −20°C.

Preparation of Extracellular Material. Cultures of group B streptococci were grown in vol of 10–30 ml for screening of nuclease activity in DNA-methyl green (MG)[1] agar and in vol up to

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[1] *Abbreviations used in this paper:* CM, carboxymethyl; MG, methyl green; TCMD buffer, 0.001 M Tris-HCl, pH 7, containing 0.001 M $CaCl_2$, 0.001 M $MgCl_2$, and 0.001 M dithiothreitol.

5–10 l for preparative work. Frozen log-phase cultures were transferred to Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) and incubated overnight at 37°C. All subsequent operations were carried out at 4°C. The supernatant fluid was adjusted to 85% saturation with solid $(NH_4)_2SO_4$ and stirred for 1 h. The precipitate was collected by centrifugation at 10,000 *g* for 20 min then dissolved in 1/10 vol 0.001 M Tris, pH 7, containing 0.001 M $CaCl_2$, 0.001 M $MgCl_2$, and 0.0001 M mercaptoethanol, and was dialyzed for 3 h against the same buffer with several changes of buffer. After dialysis, the crude preparations were concentrated to 1/10 of the original vol in dialysis tubing (Fisher Scientific Co., Pittsburgh, Pa.) with Carbowax Polyethylene Glycol 20M (Union Carbide Corp., Carbon Products Div., New York) for 4–5 hours and then dialyzed against the same buffer. The material was then either fractionated or stored at −20°C.

Screening for Nuclease Activity in DNA-MG Agar Plates. An adaptation of a previously described technique was used to screen for nuclease activity (14). Wells of 15-mm Diam were cut in agar plates containing 1.5% Noble agar (Difco Laboratories), 0.5 mg/ml of DNA (calf thymus, Sigma Chemical Co., St. Louis, Mo.) and 0.13 mg/ml of MG dye (Fisher certified stain, Fisher Scientific Co.). As indicated below, 30–70 μl of crude (concentrated but unfractionated) enzyme preparations or 30 μl of eluates of polyacrylamide gel slices were placed in the wells. The plates were incubated at 37°C and readings taken at 2, 4, and 18–24 h. Enzyme activity was evaluated by the presence of zones of decolorization which reflected degradation of the DNA-MG complex. The control enzyme was from a lot of purified standard DNase B from group A streptococci, prepared in our laboratory.

Assays of DNase Activity. Nuclease activity was assayed by measurement of the acid-soluble fraction as described previously by Marker and Gray (14) and also by the microtiter method with DNA-MG as substrate as described by Nelson et al. (15).

Assay of RNase Activity. RNA was prepared from baker's yeast which was pretreated with pancreatic DNase I to remove contaminating DNA. The assay mixture contained 0.01 M Tris-HCl, pH 7, 0.001 M $CaCl_2$, 0.001 M $MgCl_2$, and 0.5 mg RNA in a total vol of 1.0 ml including the enzyme solution. After incubation at 37°C, the samples were processed as for the acid-soluble assay of DNase activity (14).

Protein Assay. Protein was measured by absorbance at 280 nm or by the method of Lowry et al. (16).

DEAE-Cellulose Chromatography. The crude ammonium sulfate precipitates of extracellular products were applied to DEAE-cellulose (Whatman DE-23, H. Reeve Angel & Co., Inc., Clifton, N. J.) columns (2.5 × 30 cm) previously equilibrated with 0.001 M Tris-HCl, pH 7, containing 0.001 M $CaCl_2$, 0.001 M $MgCl_2$, and 0.001 M dithiothreitol (TCMD buffer) and eluted with a linear gradient of 0–0.25 M NaCl in the equilibrating buffer. All chromatographic separations were done at 4°C. Protein elution was monitored by absorbance at 280 nm. Nuclease activity was assayed by the acid-soluble technique. Pooled fractions of nuclease activity were concentrated by the Carbowax method and then dialyzed against TCMD buffer.

Carboxymethyl Cellulose Chromatography. Nuclease fractions from DEAE chromatography were applied to carboxymethyl (CM)-cellulose (Whatman CM-52, H. Reeve Angel and Co., Inc.) columns previously equilibrated with TCMD buffer. The columns were eluted with a linear gradient of 0–0.25 M NaCl in TCMD.

Polyacrylamide Gel Electrophoresis. Electrophoresis in 7.5% polyacrylamide gels was carried out using the method of Davis (17) as adapted by Marker and Gray (14). For some screening experiments, concentrated and dialyzed ammonium sulfate precipitates of extracellular products were subjected directly to electrophoresis in 7.5% polyacrylamide gels. Gel slices were eluted overnight with TCMD buffer, and the gel eluates were assayed for nuclease activity by diffusion in DNA-MG agar and by the acid-soluble method.

Molecular Weights. Molecular weights of the nucleases were examined by the method of Hedrick and Smith (18) using nondenaturing polyacrylamide gel electrophoresis. A series of marker proteins was used for calibrations. These were pepsin, myoglobin, bovine serum albumin, lactate dehydrogenase, and ovalbumin. Active fractions of nucleases from DEAE columns were run with the standards in 4–10% polyacrylamide gels. The values were calculated as the average from six separate experiments.

In addition, molecular weights were determined by gel filtration using Sephadex G-100 (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) in 2.5- × 45-cm

columns equilibrated with TCMD buffer containing 0.2 M NaCl by the method of Andrews (19). The protein standards were bovine serum albumin, ovalbumin, bovine hemoglobin, and myoglobin. Molecular weights of the nucleases were averages of at least two determinations.

Heat Stability and pH Optima of Nucleases. Samples of the nucleases from DEAE chromatography were incubated separately for 30 min at 37, 56, 65, and 80°C. Activity of heated and unheated samples was determined by the microtiter plate method (15).

The relative rates of reaction of the group B nucleases were studied after adjustment of the pH of the assay mixture in increments from pH 6 to 9 in 0.001 M Tris buffer, at pH 6 in 0.001 M KH_2PO_4 buffer, and at pH 9 and pH 10 in 0.001 M glycine buffer. Nuclease activity was measured by the acid-soluble method.

Cationic Requirements. The DNA was dialyzed against 0.05 M EDTA, pH 7, then against 0.01 M Tris buffer, pH 7, to remove any traces of divalent cations. In the absence of any divalent cations, DNase activity was negligible. The single divalent cations studied were calcium, magnesium, and manganese in final concentration of 10^{-1}–10^{-5} M. Combinations of calcium and magnesium, calcium and manganese, and magnesium plus manganese were also examined with the same range of final molarities. Enzyme activity was measured by the acid-soluble method.

Animal Immunizations and Production of Antisera. Initially, as part of another study in this laboratory, guinea pigs (males of 600–900 g) were injected with whole, heat-killed broth cultures of group B streptococci s.c. (0.3 ml of an 18-h culture containing 10^8 colony-forming units/ml and heated at 56°C for 1 h) for two to six injection series. Each series consisted of twice-weekly injections for 2–4 wk with a rest period of 2–2½ wk between each series. Blood was obtained by cardiac puncture from ether-anesthetized animals before beginning injections and at ~4- to 5-wk intervals. In many experiments, animals were later boosted with a crude extracellular preparation containing nuclease activity from the strain used for initial immunizations. Typically, a 0.1- to 0.25-ml vol was injected s.c. twice weekly for 2 wk, the animals were rested for 2–4 wk and a second and/or third booster series was given. Bleedings were taken 3–7 d after each booster series. Groups of 10–20 animals were used for each experiment.

When individual nucleases became available from DEAE-cellulose fractionation, animals were boosted with these enzymatically active preparations. In addition, some animals were given primary immunizations with crude extracellular nuclease preparations.

Adult New Zealand rabbits weighing 2.5 kg, in groups of 10, were also immunized with heat-killed broth cultures and boosted with crude extracellular material exhibiting nuclease activity and/or with active peaks from DEAE chromatography. 1 ml of bacteria was injected and the vol of crude or partially purified enzyme given was 0.3 ml. Schedules of immunization and bleedings were similar to that described for the guinea pigs. Blood was obtained from ear veins.

Initial experiments involved immunization of 19 guinea pigs with strain No. 50 (nontypable) that was later found to elaborate predominantly nuclease I and small amounts of nuclease II. In later immunization experiments, an attempt was made to choose strains that elaborated only one nuclease as documented by ion-exchange chromatography. By this means, it was possible to obtain antisera against nuclease I and nuclease III by immunization with strains No. 16 and No. 17, respectively, and their homologous nucleases. No group B streptococcal strain was found to produce only nuclease II. Therefore, animals were immunized with strain No. 55, that produced nucleases I and II; these animals were boosted with a DEAE peak characteristic of nuclease II.

Specific rabbit antisera to purified group A streptococcal nucleases, A, B, C, and D and the purified group A streptococcal nucleases were available in our laboratory for comparisons with the group B streptococcal nucleases.

Assays of Antibody in Animal and Human Sera. Neutralizing antibodies for the group B streptococcal nucleases were assayed by an adaptation of the microtiter technique as described by Nelson et al. (15). Sera were diluted one to five and inactivated at 63°C. Twofold serial dilutions were made instead of the usual log dilutions. Neutralizing antibody titers were expressed as the reciprocal of the serum dilution, and 10 was the lowest titer expressed. Slightly positive antibody titers meant that partial neutralization of the test enzyme was seen at a final serum dilution of 1:10.

Human sera were obtained from pregnant women in labor, and infant cord sera were obtained from placental vessels. These patients were enrolled in epidemiological studies of group B streptococcal colonization, and written permission was obtained to enlist their participation in the studies. Control sera were from hospitalized and nonhospitalized patients representing various illnesses and age groups.

Results

Screening Group B Streptococcal Strains for Nuclease Activity in DNA-MG Agar Plates. In preliminary experiments, crude extracellular products of the supernates of growth of 64 group B streptococcal strains were assayed for nuclease activity. After a 2-h incubation at 37°C, zones of decolorization around the wells containing 30 μl of material indicated hydrolysis of DNA. 86% of the strains exhibited definite nuclease activity using this agar diffusion method (Table I). There were no significant differences in frequency of production of enzyme among the different serotypes. Subsequently, 75 additional group B streptococcal strains were tested, and 74 of 75 (99%) exhibited enzymatic activity when 60–70 μl were placed in the wells. This technique was more sensitive in detecting enzymatic activity than the microtiter method (15) or spectrophotometric measurement of acid-soluble DNA degradation products (14).

Isolation and Purification of Group B Streptococcal Nucleases. The extracellular products of group B streptococcal strains exhibited one to three peaks of DNase activity when fractionated on DEAE-cellulose. There were three nuclease peaks, eluted from DEAE-cellulose, from strain No. 57, serotype III (Fig. 1). The enzymes have been designated as group B streptococcal nucleases I, II, and III according to the order of their elution from DEAE columns. In this case, the earliest eluted peak contained the greatest activity.

DEAE chromatography was carried out on the extracellular products of 10 group B streptococcal strains. The commonest pattern was detection of two nuclease peaks which corresponded to the earlier eluting two nuclease peaks (I and II) seen in Fig. 1. One strain (No. 16) was found to produce only nuclease I, one strain (No. 17) was found to produce only nuclease III, and the other seven strains produced nucleases I and II.

The peaks from DEAE chromatography, representing good activity of nucleases I, II, and III from three different group B streptococcal strains were chromatographed separately on CM-cellulose at pH 7 (Fig. 2). This procedure added confirmation that the nucleases were three distinct species. As expected, the elution order was the reverse of the pattern seen with elution of nucleases I and III from DEAE-cellulose. The bulk of protein was separated from nucleases I and II during fractionation (Fig. 2C and B, respectively).

Electrophoresis in 7.5% polyacrylamide gels of the three different nucleases from DEAE-cellulose revealed mobilities that were separate and distinct and confirmed the ion-exchange chromatographic findings (Fig. 3). The relative mobility of nuclease I was the lowest with progressively greater mobilities for nucleases II and III.

Molecular Weights of the Nucleases. The molecular weights of the three nucleases were determined by two methods. Migration in polyacrylamide gels of varying concentration gave the following mol wt with their SD: nuclease I: 18,600 (± 2,800); nuclease II: 33,000 (± 8,800); and nuclease III: 26,500 (± 6,700). Using Sephadex

TABLE I

Frequency of Nuclease Production by Group B Streptococcal Serotypes: Detection by Well-Diffusion in DNA-MG Plates

Serotype	Number of strains tested	Number with nuclease activity
Ia	11	9
Ib	5	5
Ic	29	28
II	6	4
III	11	8
Nontypable	2	1
Total	64	55 (86%)

30 μl of the 10-fold-concentrated extracellular preparation were placed in the 15-mm Diam wells cut in the agar plates and incubated at 37°C.

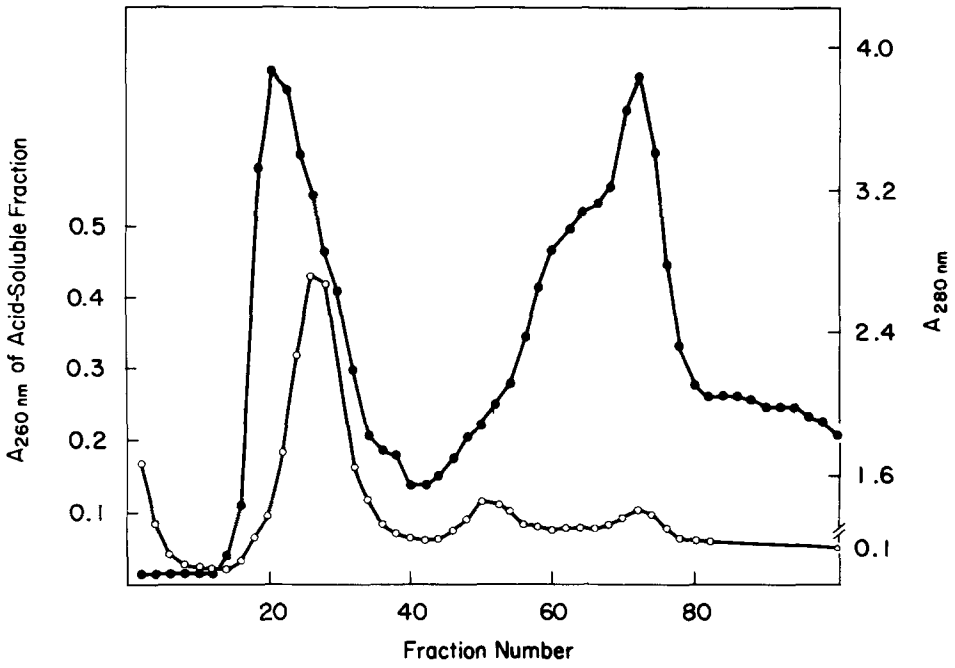

FIG. 1. DEAE-cellulose chromatogram of extracellular products (from group B streptococcal strain (No. 57, serotype III) prepared as described in Materials and Methods. O, nuclease activity ($A_{260\ nm}$/30 min); ●, protein absorbance at 280 nm.

gel filtration the mol wt were: nuclease I: 18,500; nuclease II: 43,000; and nuclease III: 32,500.

Cationic Requirements of Group B Streptococcal Nucleases. In the absence of any divalent cations, DNase activity of the three nucleases was negligible. For all three nucleases calcium plus manganese had the greatest activating effect. Manganese was the most effective of the single divalent cations (Table II). Other effective cationic combinations were magnesium and manganese for nuclease I and calcium and magnesium for nucleases II and III. In all cases, 0.01-M concentrations of the cations exerted optimal

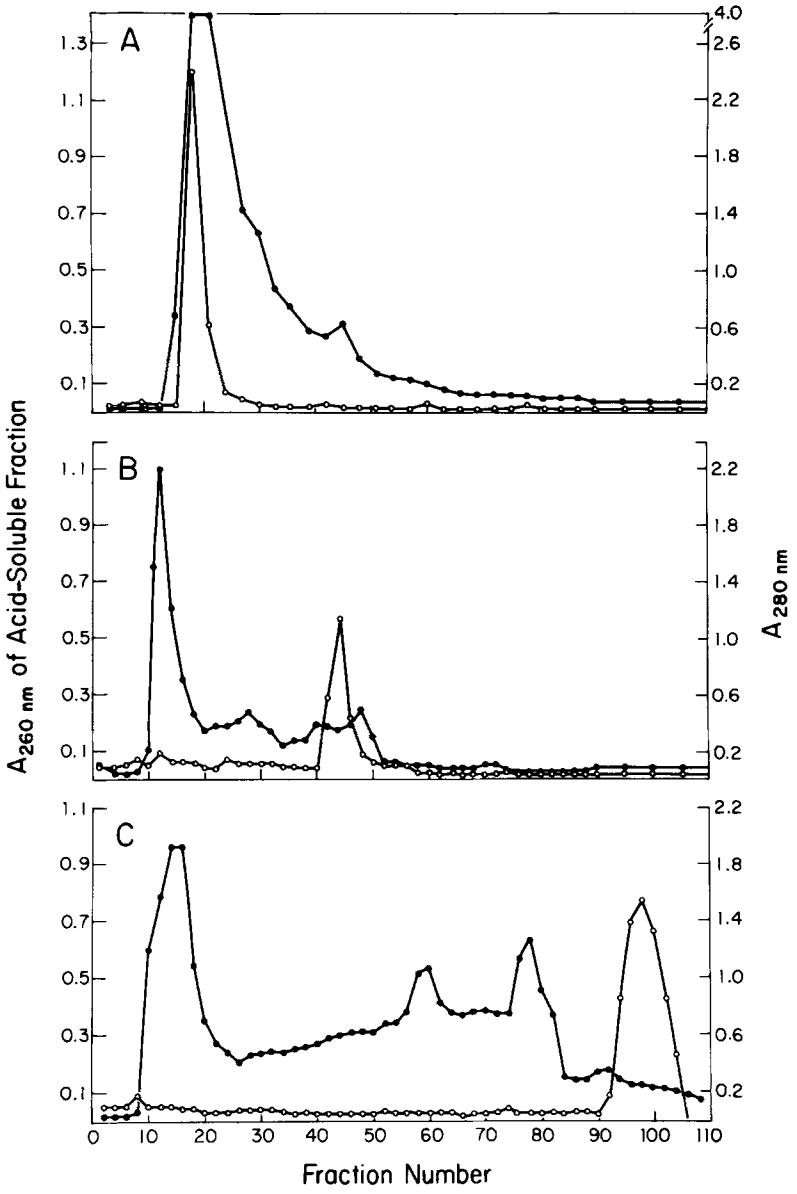

FIG. 2. CM-cellulose chromatograms of nuclease peaks from DEAE-cellulose column eluates. ○, nuclease activity (A_{260}); ●, protein absorbance at 280 nm. (A) Nuclease III; (B) nuclease II; (C) nuclease I. Incubation time for the acid-soluble assay was 30 min in (A) and (B), 10 min in (C).

activating effects. Studies employing divalent cations of cobalt, zinc, copper, mercury, and iron revealed no significant activation of nuclease III.

Other Properties of the Group B Streptococcal Nucleases. All three nucleases actively degraded RNA but the rate of digestion was far less than for hydrolysis of DNA. The pH optima for all of the nucleases were quite broad with highest activity at pH 6 in

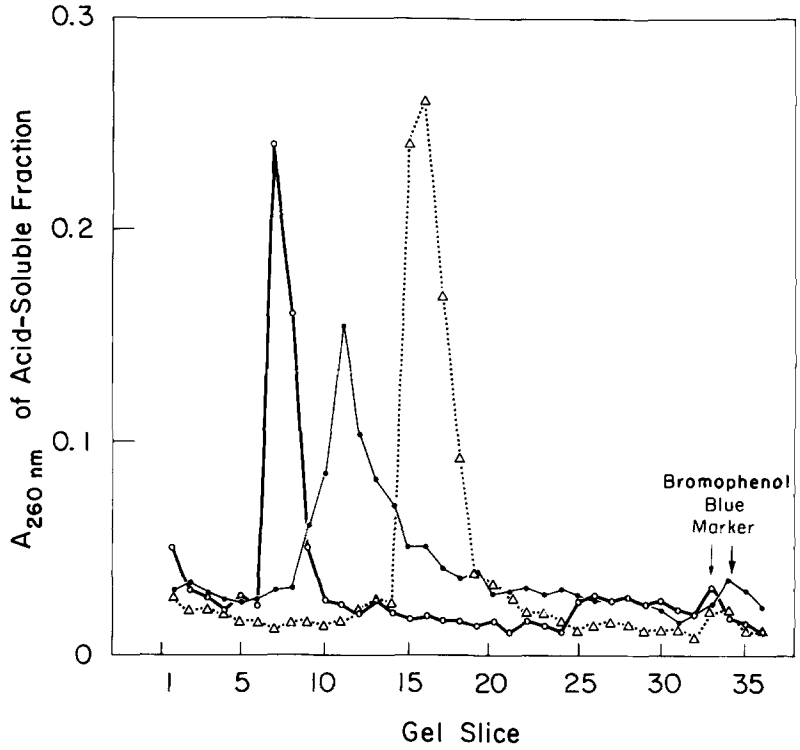

FIG. 3. Polyacrylamide gel electrophoresis of nuclease peaks from DEAE-cellulose chromatography. Nuclease activity was measured in eluates of gel slices by the acid-soluble method. $A_{260\ nm}$ nuclease I (○); nuclease II (●); nuclease III (△).

TABLE II

Cationic Activation of Group B Streptococcal Nucleases

Nuclease	Relative nuclease activity					
	Ca^{++}	Mg^{++}	Mn^{++}	Ca^{++} + Mg^{++}	Ca^{++} + Mn^{++}	Mg^{++} + Mn^{++}
I	0.27	0.61	0.84	0.70	1	0.97
II	0.55	0.41	0.80	0.99	1	0.78
III	0.28	0.44	0.60	0.72	1	0.66

The divalent cations, singly or in combination, were present in final concentration of 0.01 M. Maximum nuclease activity was seen with the combination of Ca^{++} plus Mn^{++}, and it was assigned a value of 1; other values represent fractions of this activity.

KH_2PO_4 buffer and at pH 7–9 in Tris buffer. There was less activity at pH 9 and low activity at pH 10 in glycine buffer.

Nucleases I and III were relatively heat stable with a twofold loss in activity at 56, 65, and 80°C compared to a control incubated at 37°C. In contrast, nuclease II had a fourfold to eightfold reduction in activity with heating at 65 and 80°C.

The Immunogenicity of the Nucleases in Animals. A representative example of a humoral immune response to nuclease-producing group B streptococci was seen in 19 guinea pigs injected with a heat-killed broth culture of strain No. 50. This strain elaborated

TABLE III

Neutralizing Titers of Sera from Animals Injected with Strain No. 50 for Nucleases Produced by Various Group B Streptococcal Strains

Animal number and source of serum	Neutralizing titer for designated nuclease(s) of indicated strains									
	No. 50, nuclease		No. 16, nuclease	No. 55, nuclease		No. 7, nuclease		No. 57, nuclease		No. 17, nuclease
	I	II	I	I	II	I	II	I	II	III
480	10	0	20	10	0	20	0	20	0	0
483	10	0	10	10	0	10	0	10	0	0
486	40	0	40	80	0	80	0	80	0	0
488	10	0	20	20	0	20	0	20	0	0
489	40	0	40	40	0	80	0	40	0	0
492	20	0	40	40	0	40	0	40	0	0
495	20	0	40	20	0	40	0	20	0	0

primarily one nuclease, of the type designated I, but also produced smaller amounts of nuclease II. 12 of the 19 injected animals developed neutralizing titers of 10–40 against nuclease I by 2–4 mo after the beginning of the series of injections. Five uninoculated controls were seronegative. The neutralizing capacity of the post-injection sera of 7 of these 12 animals for the separate nucleases produced by the immunizing strain as well as for the nucleases of five other group B streptococcal strains is summarized in Table III. In this experiment, the hyperimmune sera neutralized nuclease I produced by the immunizing strain and neutralized in similar titers, nucleases I produced by strains No. 16, No. 55, No. 7, and No. 57. However, there was no neutralization of nucleases II and III.

In other experiments in which guinea pigs and rabbits were immunized with strain No. 16 which produced only nuclease I and boosted with crude enzyme, some animals developed neutralizing titers to nuclease I produced by the various strains listed in Table III.

Other animal injection experiments in guinea pigs and rabbits revealed that injection of a strain (No. 17) that produced only nuclease III followed by boosters of partially purified nuclease III, resulted in hyperimmune sera that neutralized nuclease III as well as nuclease II produced by a number of different strains. Conversely, injection of animals with a strain (No. 55) that produced nucleases I and II, followed by boosters with partially purified nuclease II, resulted in antisera which fully neutralized nuclease II as well as nuclease III; some antisera had partial neutralization of nuclease I in low titer.

Clearly, nuclease I was antigenically different from nucleases II and III; nucleases II and III appeared antigenically similar. All three nucleases were immunogenic in the experimental animals.

Immunological Comparison of Nucleases of Group B and Group A Streptococci. Of special interest was whether the group B nucleases were immunologically different from the nucleases of group A streptococci. None of the examples of the three group B nucleases was neutralized by rabbit antisera specific for nucleases A, B, C, and D of group A streptococci. In addition, guinea pig and rabbit antisera with neutralizing activity

against the nucleases of group B strains did not neutralize any of the four nucleases produced by group A streptococci.

Screening and Prevalence of Specific Nucleases Among Group B Streptococcal Strains. In an attempt to screen for the specific types of nucleases produced by 18 other group B streptococcal strains, (demonstrated by diffusion in DNA-MG agar to elaborate DNase) the crude, concentrated extracellular products prepared by ammonium sulfate precipitation were applied directly to polyacrylamide gels. The peaks of activity in the gel eluates were pooled and saved for immunological identification with antisera specific for the group B streptococcal nucleases. Using this screening method, 5 of the 18 strains revealed two peaks of nuclease activity, and the others had only one obvious peak of activity in the gel eluates. There was no pattern of nuclease production in relation to specific serotype. The peaks from three of the five strains found to possess two nucleases were tested and identified tentatively as nuclease I and nuclease II/III by neutralization tests. The single nuclease peaks from 5 of the other 13 strains were tested immunologically and were unequivocally nuclease II/III. Because small volumes of culture supernates were processed in this screening method, less amounts of nucleases were produced, and this may have decreased the chance of detecting all of the nucleases produced by a strain. In contrast, with DEAE fractionation of extracellular products of larger volumes of bacterial growth (*vide supra*), 7 of 10 other strains produced nucleases I and II, and one produced nucleases I, II, and III. This direct screening method on polyacrylamide gels requires further study.

Detection of Anti-Nuclease Activity in Mothers and Infants Harboring Group B Streptococci versus Controls. Studies were carried out to examine maternal/infant immunological responses to the group B nucleases. Inclusion in the test group required that the pregnant women had positive genital tract cultures for group B streptococci during the third trimester of pregnancy or at time of labor or that their newborn infants, cultured immediately after delivery, had mucosal surface cultures positive for group B streptococci. 21 control mothers and their 21 infants were not colonized with group B streptococci at any of the above designated times. 61 other hospitalized and nonhospitalized patients with various illnesses were also studied.

In 216 birthing events that fulfilled the criteria for the test group, 13% of the maternal and/or infant sera exhibited definite neutralizing titers of 1:10 or greater against the nucleases, (Table IV). Commonly, titers of 1:20 to 1:40 were seen, and maternal/infant sera (when both were available) were concordant within one twofold dilution. An additional 12% exhibited partial activity (incomplete neutralization at a serum dilution of 1:10). Among the culture-negative control mother and infant pairs, 5% had definite neutralizing titers and 14% had partial activity. In the group of 61 nonpregnant controls, 5% had definite and 5% had partial neutralizing activity. This latter group had various antibody titers for the group A streptococcal nuclease, DNase B, that did not correlate with the group B anti-nuclease activity.

Analysis for a possible correlation between neutralizing activity and the culture status before delivery (third trimester and labor) revealed an equal prevalence of positive cultures in both the third trimester and at time of labor in the seropositive mothers and infants (52% with positive cultures) and the seronegative mothers and infants (59% with positive cultures). There was no difference in the prevalence of anti-nuclease activity in those women who were culture positive at both times (third trimester and labor) compared to those women with culture conversion (either positive to negative or the reverse) between the third trimester and the time of labor.

TABLE IV

Prevalence of Neutralizing Activity to Group B Streptococcal Nucleases II and III in the Sera of Mothers and Infants and Controls

Patient group	Number	Percentage with neutralizing activity	
		Definite	Partial
		%	
Group B streptococcal-colonized mothers/infants	216 pairs	13	12
Culture-negative mothers/infants	21 pairs	5	14
Hospitalized/clinic patients	61	5	5

It is curious that, with two exceptions, all of the sera with anti-nuclease activity have neutralized, in similar titer, nucleases II and III but not nuclease I. Two adult patients, one with group B streptococcal meningitis and the other with peritonitis, also had neutralizing titers for nucleases II and III.

Discussion

The extracellular nucleases of group A streptococci are produced by essentially all strains (11). There are at least four distinct and purified nucleases designated A, B, C, and D that have been studied in detail for their biochemical and immunological properties (20). These nucleases are endonucleolytic in their action on DNA (20). DNase B is of particular interest because of the high frequency of antibody responses to this antigen in the serum of patients with group A streptococcal infections of the throat and skin as well as in patients with post-streptococcal sequelae of rheumatic fever and acute glomerulonephritis (21).

20 yr ago, using crude whole supernates and alcohol precipitation of DNA, it was found that 38% of group B streptococcal strains elaborated extracellular nuclease activity (11). Until our recent studies were carried out (22), no other significant information had been available on the frequency of production of nucleases by group B streptococci, or on their biochemical and immunological characteristics. Using the agar-well-diffusion techniques in DNA-MG agar plates for screening extracellular material, it was found that 86% of group B streptococcal strains exhibited nuclease activity. When larger volumes of material were placed in the wells, this figure increased to nearly 100%. However, the amounts of DNase produced by group B streptococcal strains were generally significantly less than the amounts produced by group A strains.

Ion-exchange chromatography of the products of one group B streptococcal strain initially revealed only one peak of nuclease activity. When many strains were examined, it was clear that most strains produced two nucleases and one produced three nucleases. Elution patterns of these nucleases from both DEAE-cellulose and CM-cellulose columns revealed that three different nucleases had been isolated, and their migration patterns in 7.5% polyacrylamide gels also substantiated their designation as separate species of nucleases. Although these nucleases have not been purified to homogeneity, preliminary determinations of molecular weights were carried out. The relative molecular weights of the nucleases, determined by two independent methods, were: I < III < II.

All three group B streptococcal nucleases were maximally activated by divalent cations of calcium plus manganese. This is similar to the four nucleases of group A streptococci which exhibit optimum activity in the presence of these two divalent cations.[2]

Of considerable interest was whether the group B streptococcal nucleases were immunogenic. Extensive immunization experiments in animals revealed that after injection of heat-killed, whole broth cultures of bacteria, neutralizing activity to all three nucleases could be provoked, and also that partially purified nucleases boosted the response. The immunologic role of the bacterial cells is not clear, but the cells could have served as a direct source of nuclease or have functioned as an adjuvant for the nuclease antigen. In many cross-neutralization studies it was found that antisera that neutralized nuclease I did not neutralize nucleases II and III. Curiously, antisera to nucleases II and III cross-neutralized these two nucleases in equal titer, suggesting that they may share a common antigenic determinant, despite their distinction on the basis of several other properties detailed here.

In pursuing further the immunological identity of the group B nucleases it was found that they were not neutralized at all by specific antisera for the four group A streptococcal nucleases, and that guinea pig and rabbit antisera for the group B nucleases did not neutralize the purified group A nucleases. This immunological difference between the nucleases of these two serological groups of streptococci was intriguing and fortunate. A biological antigenic marker, such as the extracellular nucleases with their immunological uniqueness, can be useful in confirming the specificity of a serological response for either a group A or group B streptococcal infection.

An extension of this immunological probing was to examine sera from pregnant women harboring group B streptococci and from their newborn infants for neutralizing activity to the group B streptococcal nucleases. In 25% of 216 mother-infant pairs fitting the bacteriologic criteria for study, one or both of them were seropositive, divided equally between definite neutralizing titers and titers of partial neutralization. In contrast, a small group of culture-negative mothers and infants had a 5% prevalence of definite titers and a 14% prevalence of slight neutralizing activity in their serum. Thus, it appeared that recent colonization with group B streptococci may lead to an immune response to the group B streptococcal nucleases. The generally equal titers in paired maternal and cord sera suggest that antibodies to these nucleases readily cross the placenta. Studying the chronology of development of neutralizing activity with several bleedings will be a natural extension of these preliminary findings. Demonstration of neutralizing activity in culture-negative controls may be a result of previous colonization with group B streptococci in a population known to commonly harbor these bacteria (23). The seropositivity in the other heterogeneous controls (culture status unknown) was unexplained but may reflect common exposures to group B streptococci.

Although all three nucleases were immunogenic in animals, human sera, with rare exceptions, neutralized equally well only nucleases II and III. Perhaps nuclease I is a poor immunogen in humans, or is not produced in large amounts by the strains to

[2] Gray, E. D., A. E. Flores, and S. C. Marker. Streptococcal nuclease B: purification and characterization. Submitted for publication.

which humans are ordinarily exposed. This point requires further investigation. The neutralizing activity for the nucleases of group B streptococci probably reflects true antibody, although definitive proof for this is not available at this time.

Summary

Nearly all group B streptococcal strains representing the five major serotypes were found to produce extracellular nucleases by screening with an agar-well-diffusion technique in DNA-methyl green agar plates. Three different nucleases have been isolated and partially purified by DEAE- and carboxymethyl-cellulose chromatography. They possessed different mobilities on polyacrylamide gel electrophoresis and different molecular weights. These nucleases, designated I, II, and III, are optimally activated by cations of calcium and manganese and exhibited RNase as well as DNase activity. Despite differences in their physical and biochemical properties, nucleases II and III appear antigenically similar, but distinct from nuclease I. These group B streptococcal nucleases are immunologically different from the nucleases of group A streptococci. Neutralizing activity, probably antibody, to nucleases II and III was found in human sera, and was most prevalent in sera of pregnant women colonized with group B streptococci and in their newborn infants.

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