INFECTION AND IMMUNITY, Nov. 1981, p. 448-454
0019-9567/81/110448-07$02.00/0

Vol. 34, No. 2

Correlation Between the Production of Extracellular Substances by Type III Group B Streptococcal Strains and Virulence in a Mouse Model

DAVID L. DURHAM, STEPHEN J. MATTINGLY, TERENCE I. DORAN, THOMAS W. MILLIGAN, AND DAVID C. STRAUS†*

Department of Microbiology, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284

Received 27 April 1981/Accepted 15 July 1981

Twelve strains of serotype III group B streptococci (8 isolated from cases of neonatal disease, 3 isolated from asymptomatically colonized infants, and 1 laboratory reference strain) were examined for in vitro production of three potential extracellular virulence products: type-specific antigen, neuraminidase, and protease. In addition, virulence in a mouse model, expressed as 50% lethal dose, was determined for the 12 strains to determine whether a relationship existed between the production of any of the three extracellular products and virulence. Only production of extracellular type-specific antigen showed a correlation with virulence in the mouse model. The high producers of extracellular type-specific antigen were an average of 166-fold more virulent for mice than low producers of the same component. There was no correlation between virulence and either neuraminidase or protease production, nor was there a correlation between either of these two extracellular products and the levels of extracellular type-specific antigen. When levels of group B streptococci of each type (a high and low producer of extracellular type-specific antigen) in organs of infected mice were examined, comparable levels of organisms were found in the brain, spleen, and lungs of mice near death regardless of the initial inoculum. However, the high producer of extracellular type-specific antigen caused death in mice with a 2 to 3 log lower inoculum than the low producer, suggesting that these strains may be more invasive.

The group B streptococci (GBS) are now recognized as major bacterial pathogens for infants (2), and although several potential virulence products have been described in the literature, there is little definitive information on the actual factors involved in virulence. In 1934, Todd (39) described an oxygen-stable, non-immunogenic hemolysin produced by GBS. A deoxyribonuclease was first reported by Ferrieri et al. (P. Ferrieri, E. D. Gray, and L. W. Wannamaker, Abstr. VI Int. Symp. *Streptococcus pyogenes*, Liblice-Prague, 1975, p. 9) in GBS, and later Ferrieri et al. (14) demonstrated extracellular nuclease production by all five major serotypes. In 1941, McClean (25) demonstrated that GBS elaborated a hyaluronidase. Kjems et al. (22) recently determined that 75% of 252 strains of various serotypes of GBS produced hyaluronidase, although 57% of serotype III did not produce the enzyme. Hayano et al. (16, 17) demonstrated the production of a group B streptococcal neuraminidase which was active on a sialomucoid preparation from bovine submaxillary glands. The production of this enzyme was further characterized by Milligan et al. (26), who showed a correlation between high levels of extracellular neuraminidase and serotype III GBS organisms isolated from diseased infants. A more recent study by Mattingly et al. (24) examining 73 clinical isolates of GBS from diseased infants in 23 states demonstrated that although the majority of type III GBS from infected neonates produced elevated levels of neuraminidase, enzyme production was not an absolute requirement for infection in infants. It has recently been demonstrated that large amounts of type-specific antigen (TSA) from type III GBS are produced extracellularly in in vitro cultures in a chemically defined medium (11, 12) or in a chemically defined broth supplemented with acid-hydrolyzed casein (8). The amount of TSA recoverable from the supernatant fluid was approximately 20-fold greater than amounts recovered from neutral buffer extraction of whole cells (11). Finally,

† Present address: Department of Microbiology, Texas Tech University Health Sciences Center, Lubbock, TX 79430.

Straus et al. (37) recently demonstrated that the extracellular supernatant fluid from six serotype III GBS strains possessed protease activity.

In addition to the reports characterizing extracellular products of the GBS, there have been several studies demonstrating a variation in the 50% lethal dose (LD_{50}) of type III GBS for certain animal models. Baltimore et al. (5) demonstrated greater than a 100-fold variation in the LD_{50} among six type III strains in a mouse model. Ferrieri et al. (13), working with an infant rat model, showed that three type III strains, when given in a range of 10^2 to 10^4 colony-forming units (CFU), produced a variation in mortality ranging from 73 to 94%. Also, Tieffenberg et al. (38) showed a significant difference in mortality for 12-day-old chicken embryos challenged with two different type III GBS strains. Unfortunately, none of the above studies attempted to correlate the observed variation in virulence with any of the previously characterized potential virulence products.

Since correlations have been shown in other bacteria between virulence and protease (15, 19, 32) and neuraminidase (31) production as well as between severity of infection and circulating capsular polysaccharide antigens (33), the present study was undertaken to examine whether elevated levels of any of these extracellular products from type III GBS correlated with virulence in a mouse model.

MATERIALS AND METHODS

Bacterial strains. Serotype III GBS strains 110, 118, 120, 122, 124, 127, 130, 150, 180, 182, and D136C were used in this study. The strains were kindly supplied by Carol J. Baker, Baylor College of Medicine, Houston, Tex., with the exception of D136C, which was supplied by Hazel Wilkinson, Centers for Disease Control, Atlanta, Ga. All were clinical isolates (8 from cases of neonatal disease and 3 from asymptomatically colonized infants) except strain D136C, which is a standard Lancefield immunizing strain. The organisms were stored in Todd-Hewitt broth (THB) at −70°C until needed. Before use, the cultures were streaked on 5% sheep blood agar plates and incubated at 37°C for 18 to 24 h.

Mice. All virulence studies utilized ICR male mice, 6 weeks of age, which were obtained from Simonsen Laboratories, Gilroy, Calif.

Media. THB (Difco Laboratories, Detroit, Mich.), prepared according to the manufacturer's directions, was used for growing and diluting the organisms involved in all virulence studies.

A chemically defined medium, FMC, which was previously described by Milligan et al. (27), was used to grow the organisms for the determination of extracellular TSA (11) as well as for extracellular levels of protease (37). Briefly, this medium contains amino acids, vitamins, purine and pyrimidine bases, minerals, 1% glucose, 0.065 M phosphate, and 0.019 M sodium carbonate at an initial pH of 7.0. FMC, supplemented with THB and human serum albumin, was used for determination of neuraminidase activity (28).

Determination of neuraminidase and protease activity. The growth conditions and assay procedure for neuraminidase activity were performed as previously described by Milligan et al. (28), and the growth conditions and assay procedure for protease activity were performed as previously described by Straus et al. (37) except that the protease reaction was terminated after 6 h of incubation.

Determination of extracellular TSA. The growth conditions and assay procedure for determination of extracellular TSA levels have been previously described by Doran et al. (11). Of the two sialic acid-containing type-specific polysaccharide peaks obtained after Sepharose 4B column chromatography (a high-molecular-weight [HMW] antigen and a low-molecular-weight [LMW] antigen), only the HMW antigen was quantitated for each strain. Quantitation was initially achieved for six strains by rocket immunoelectrophoresis procedures similar to that described by Weeke (41). TSA quantities for these six strains were then compared with planimeter tracings of pool 1 elution profiles (HMW antigen) of their extracellular antigens from a Sepharose 4B column (see Fig. 1) in order to perform a least-squares linear regression (correlation coefficient = 0.876; r_{test} = 95 to 99% valid). This linear regression was utilized to quantitate extracellular TSA from appropriate planimeter tracings for other GBS strains.

Mouse lethality tests. Bacteria from a single GBS colony on a blood agar plate were inoculated into 10 ml of THB. This was incubated in a 37°C water bath until an adjusted optical density reading of ~490 at 675 nm (early stationary phase) was achieved on a Coleman Junior model 620 spectrophotometer. Optical densities were multiplied by 1,000 and converted to adjusted optical density units so that values would be in accordance with Beer's Law and be proportional to bacterial mass (40). One adjusted optical density unit is equivalent to 0.43 μg of cellular dry weight per ml (28). This THB culture contained approximately 3.5×10^8 CFU/ml (mean number of CFU [±standard deviation] of 40 tests = $3.47 \times 10^8 \pm 1.19 \times 10^8$). Cultures were removed and chilled on ice. Appropriate 10-fold dilutions were made in THB, and groups of five mice were injected intraperitoneally with 1.0 ml of each dilution. Dead mice were counted and removed from their cages at 24-h intervals for 72 h.

Determination of levels of GBS in various organs of infected mice. ICR mice were injected intraperitoneally with 1.0 ml of THB containing various concentrations of either GBS 127 or 130. Pairs of mice were sacrificed by cervical dislocation at various times, and their brains, lungs, and spleens were aseptically removed, weighed, and placed in 3.0 ml of sterile saline. The tissues were homogenized with a Kinematica GmbH Polytron tissue grinder (Brinkmann), and final volumes were determined. The tissue suspensions were diluted in sterile saline and plated on sheep blood agar plates. The plates were incubated at 37°C for 18 to 24 h, and the CFU per milliliter were enumerated. The CFU per gram of tissue were then determined.

Statistical analysis. LD_{50} values were calculated by the method of Reed and Muench (34). Comparison

of significance of differences between LD_{50} values was by the Mann-Whitney test (9). Significance of differences between mortality values per single dilution was determined by chi-square analysis (9). The paired and unpaired Student's t tests (35) were used to compare all assayed levels of extracellular products for significant differences.

RESULTS

Quantitative comparison of extracellular type III antigen produced by type III GBS strains. The supernatant fluids from 10-liter cultures of various strains of type III GBS, grown in a chemically defined medium to early stationary phase, were chromatographed on a Sepharose 4B column to separate the group B and type III antigens. The eluate fractions were collected and assayed for sialic acid content as well as for type III specificity by the capillary precipitin reaction. The results are expressed as micrograms of sialic acid per milligram of cell dry weight to allow comparisons of samples from different experiments based on equivalent cell mass.

The elution profile of two GBS strains (Fig. 1) demonstrates two sialic acid-containing peaks, a HMW antigen peak centered at fraction 40 and a LMW antigen peak centered about fraction 75. The HMW fraction, which gave a strong capillary precipitin reaction with type III antiserum and a negative reaction with group B antiserum, has been demonstrated by Doran et al. to be immunologically (11) and chemically (12) identical to the "native" type III antigen described by Baker et al. (3). The LMW antigen, which has been shown to give a line of complete identity with the HMW antigen in immunodiffusion studies (11), has not been completely characterized at this time.

Quantitation of the extracellular HMW antigen produced by these strains allowed them to be separated into two groups, "high" and "low" producers. The elution profile in Fig. 1 of strain 122, with a peak height of 0.085 μg of sialic acid per mg of cell dry weight, is representative of a high producer, and the profile of 127, with a peak height of 0.015 μg of sialic acid per mg of cell dry weight, is typical of a low producer. All high producers have extracellular HMW type antigen peak levels above 0.035 μg/mg of cell dry weight (with most above 0.04), whereas the low producers' extracellular HMW type antigen peak levels are less than 0.02 μg/mg of cell dry weight.

When the total amount of HMW type antigen from each of the 12 strains was quantitated by rocket immunoelectrophoresis utilizing known TSA standards, the groups were significantly different ($P = 0.005$) as demonstrated in Table 1. Based on these data, strains 110, 120, 130, 122, 124, 150, 180, 181, and 182, with levels of extracellular TSA ranging from 3.73 to 16.89 μg/ml of cell dry weight, can be grouped as high producers, and strains D136C, 118, and 127, with levels of extracellular TSA of 1.07 μg/ml of cell dry weight or less, are low producers.

Quantitative comparison of other extracellular products of type III GBS strains. The supernatant fluids from 50-ml and 6-liter cultures of the 12 strains were assayed for neuraminidase and protease activity, respectively (Table 1), to determine whether enzymatic activity correlated with the amount of type antigen produced. Neuraminidase production by type III strains can be separated into high and nonpro-

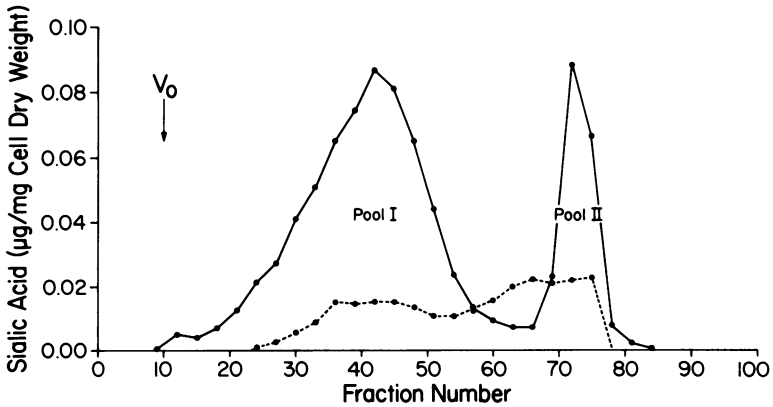

FIG. 1. *Sepharose 4B elution profile of extracellular sialic acid-containing type III antigens from 1-liter cultures of two GBS strains. Fractions were assayed for sialic acid by the thiobarbituric acid assay (1) and tested for type III specificity by the capillary precipitin test with rabbit antiserum against type III antigen. (——), Strain 122; (-----) strain 127; V_0, void volume.*

TABLE 1. *Relationship between in vitro levels of extracellular products and virulence in a mouse model of various strains of type III GBS*

Strain of type III GBS	Extracellular TSA (μg/mg of cell dry wt)[a]	Neuraminidase (nmol of sialic acid released per min per mg of cell dry wt)	Protease activity (units/min per mg of protein)	LD_{50} (CFU) in a mouse model[b]
High TSA producers				
110	3.73	248.8[c]	15.19[d]	6.3×10^4
120	8.28	307.1[c]	5.85[d]	7.9×10^4
122	16.89	355.4[c]	55.19	1.3×10^4
124	6.13	328.3[c]	ND[e]	1.1×10^5
130	7.49	327.0[c]	15.46[d]	1.9×10^5
150	7.04	309.9[c]	2.92	5.7×10^4
180	7.14	<10[c]	ND	1.1×10^5
181	5.54	<10[c]	0.21	2.2×10^5
182	5.78	<10[c]	0.38	8.5×10^4
Low TSA producers				
D136C	<0.02	<10[c]	1.61	2.5×10^7
118	0.43	<10[c]	4.01[d]	1.3×10^6
127	1.07	<10[c]	0.84[d]	2.5×10^7

[a] Unpaired Student's t test: $t = 3.64$; $P = 0.005$.
[b] Mann-Whitney Test: $P = 0.0091$.
[c] Results from Milligan et al. (26).
[d] Results from Straus et al. (37).
[e] ND, Not detected.

ducers as defined by Milligan et al. (26). Six of the strains tested were high neuraminidase producers, and six had no detectable enzyme activity. When the levels of neuraminidase activity for high TSA producers were compared with those of nonproducers, six of the high TSA producers (GBS 110, 120, 122, 124, 130, and 150) were indeed high neuraminidase producers, but a significant difference could not be demonstrated since three of the high TSA producers (GBS 180, 181, and 182) were also non-neuraminidase producers (Table 1). The same holds true for protease activity demonstrated by these strains. Although Straus et al. (37) were able to show that certain high neuraminidase producers (GBS 110, 120, and 130) also had significantly higher protease activity than several non-neuraminidase producers (GBS 118, 127, and 142), there was not a significant difference in protease activity between high and low TSA producers when additional strains (GBS 122, 124, 150, 180, 181, 182, and D136C) were examined.

Mouse lethality tests. Virulence studies with the 12 GBS strains were performed on mice to determine wehther the organisms could be separated into groups based on degrees of virulence and also to determine whether these groups correlate with levels of extracellular products.

The test conditions for determination of virulence and subsequent calculation of an LD_{50} value were similar to those described by Baltimore et al. (5). The LD_{50} values of the 12 strains for the mouse model can be seen in Table 1. When the strains were grouped on the basis of levels of extracellular TSA, there was a 10- to 3,000-fold difference in LD_{50} between the high producers (GBS 110, 120, 122, 124, 130, 150, 180, 181, and 182) and the low producers (GBS D136C, 118, and 127). Only when the 12 strains were grouped in this manner (based on TSA levels) was there any significant difference in virulence (LD_{50}) when assessed by the Mann-Whitney Test ($P = 0.0091$). There were no significant differences in virulence (LD_{50}) when the strains were classified by levels of neuraminidase production or protease activity.

Table 2 demonstrates the virulence data based on percentage killed by the various strains at two different concentrations of bacteria, either 3.5×10^4 or 3.5×10^5 CFU/ml. The strains were again classified according to the levels of extracellular TSA. When chi-square analysis was applied to these data, a highly significant difference ($P = <<0.001$) was observed between the two groups, with 68.8% of the mice killed by the high producers versus only 1.8% killed by the low producers.

Levels of GBS in various organs of infected mice. Since it had been previously determined that a high TSA producer required fewer organisms for mouse lethality than a low TSA producer, it was necessary to determine whether high TSA producers were actually able to cause death with fewer resultant organisms in these organs than could be found in these same organs of a mouse infected with a low TSA producer. Therefore, the number of GBS in the brain, lungs, and spleens of mice infected with either a high or low extracellular TSA producer was determined at various times. The brain and

TABLE 2. *Comparison of virulence in mice for strains of type III GBS*

Type III GBS strains[a]	No. of mice dead/no. injected[b] (%)[c]
High TSA producers	148/215 (68.8)
Low TSA producers	1/55 (1.8)

[a] High TSA producers: GBS strains 110, 120, 130, 122, 124, 150, 180, 181, and 182. Low TSA producers: GBS strains D136C, 118, and 127.
[b] ICR mice were given either 3.5×10^4 or 3.5×10^5 organisms of the indicated strains, injected intraperitoneally in 1.0 ml of THB.
[c] Chi-square: 2×2 contingency table; $t = 79.5$; $P = \ll 0.001$.

lungs were selected because these have been shown to be the major sites of involvement in infected neonates. Table 3 demonstrates the levels of GBS in organs from mice infected with various inocula of a low TSA producer (GBS 127) and a high TSA producer (GBS 130) at 6, 12, and 24 h postinfection. Normally, 100% mortality occurred between 12 and 24 h in mice infected with 3.5×10^8 CFU of strain 127 or 3.5×10^6 CFU of strain 130 per ml. When levels of both strains of GBS in the three organs of mice given the above inocula were examined at 12 h postinoculation, the CFU per gram of tissue were approximately the same for the two strains. Thus, it appeared that mice which would have eventually succumbed had high but comparable levels of organisms in the brain, spleen, and lungs after 12 h post-inoculation regardless of the initial inoculum or the amount of extracellular TSA produced by the infecting GBS strain, indicating that once infection is established, high and low producers appear to be equally virulent.

DISCUSSION

Although numerous potential extracellular virulence products of the GBS and differences in virulence in animal models among strains of the same type GBS have been described in the literature, there has been no attempt to correlate any extracellular GBS product with virulence. Since the only virulence product described to date is the cell-associated TSA (23), this was a logical product to investigate. There have been several studies with the pneumococcus (6, 7, 10, 21) attempting to correlate the release of specific soluble substance (capsular polysaccharide) in vivo with virulence. In addition, clinical studies have indicated that the in vivo release of capsular polysaccharide antigens from other organisms may also play a role in virulence. For example, O'Reilly et al. (30) suggested that the levels and mechanism of clearance of polyribophosphate may influence the development and intensity of the humoral immune response in infants and children with *Haemophilus influenzae* meningitis. Pollack (33) demonstrated that the presence of circulating klebsiella capsular antigen in the sera of patients infected with *Klebsiella pneumoniae* appeared to correlate with the severity and duration of infection. He postulated that the circulating antigen could neutralize antibodies before opsonization occurred.

Previous investigations have also demonstrated antigens of the GBS in body fluids, lending support to the concept of antigenemia influencing the level of circulating protective antibody. Wilkinson (42) was able to demonstrate by counterimmunoelectrophoresis the presence of type-specific carbohydrates of GBS in the sera of patients with GBS infections, although no correlation with severity of infection was made. Other investigators have also found antigens of GBS in body fluids (4, 18, 36). In the present study, we examined in vitro production of extracellular TSA as well as extracellular levels of neuraminidase and protease from 12 strains of type III GBS. We also determined the virulence, expressed as LD_{50}, of these strains in a mouse model to look for any correlations between the production of extracellular products and virulence. The 12 strains were divided into 9 high and 3 low in vitro producers of extracellular TSA (Table 1). The high TSA producers were significantly different ($P = 0.005$) from the low producers. The LD_{50} values in a mouse model for the nine high extracellular TSA producers were an average of 166-fold more virulent

TABLE 3. *Levels of GBS in organs of infected mice at various times*

GBS strain	GBS inoculum (CFU)[a]	GBS levels[b] (CFU/g of tissue) at time post-inoculation:								
		6 h			12 h			24 h		
		Brain	Lungs	Spleen	Brain	Lungs	Spleen	Brain	Lungs	Spleen
127 (low TSA producer)	3.5×10^8	8.2×10^2	1.9×10^8	5.4×10^8	4.1×10^6	1.7×10^9	8.4×10^8	NS[c]	NS	NS
		2.4×10^5	3.9×10^7	1.8×10^8	1.1×10^7	8.6×10^8	2.1×10^9	NS	NS	NS
	3.5×10^6	ND[d]	ND	5.5×10^4	ND	ND	1.5×10^4	5.2×10^3	4.7×10^4	1.1×10^4
		ND	ND	ND	ND	2.8×10^4	ND	ND	ND	7.8×10^3
	3.5×10^4	ND	ND	2.5×10^4	ND	ND	ND	ND	ND	ND
		ND	ND	ND	ND	ND	ND	ND	ND	ND
130 (high TSA producer)	3.5×10^6	1.1×10^5	3.3×10^7	5.7×10^7	2.4×10^5	3.1×10^8	1.9×10^8	2.1×10^7	3.0×10^9	2.5×10^9
		1.4×10^6	3.7×10^7	2.7×10^8	3.1×10^5	3.5×10^8	5.6×10^8	NS	NS	NS
	3.5×10^4	9.1×10^2	ND	1.2×10^4	1.7×10^5	2.5×10^7	8.6×10^7	ND	1.2×10^4	5.7×10^3
		9.4×10^4	1.0×10^7	1.6×10^7	1.5×10^6	5.0×10^7	1.2×10^8	ND	9.6×10^3	7.8×10^3

[a] Given intraperitoneally in 1.0 ml of THB.
[b] Levels calculated for two different mice each time.
[c] NS, No survivors.
[d] ND, Not detected. No growth was detected when 10 μl of undiluted tissue suspension was plated. Concentration limits were as follows: $<3 \times 10^3$ CFU/g of spleen, $<1.5 \times 10^3$ CFU/g of lungs, and $<8.5 \times 10^2$ CFU/g of brain.

than the low producers. The difference in virulence of these two groups was significant at the 99% confidence level. It should be noted that the grouping of these strains into high and low TSA producers is based on in vitro differences in quantity of HMW antigen. It is not clear at the present time whether the same phenomenon occurs in vivo, but preliminary studies employing rocket immunoelectrophoresis of mouse sera from animals infected with high and low TSA producers demonstrate that this may be the case (data not shown).

There are several possible explanations as to how elevated levels of extracellular TSA could increase the virulence of GBS. In addition to neutralizing protective type-specific antibodies in the host, it is also possible that the extracellular TSA might activate complement via the alternative pathway since activation of complement by carbohydrate antigens such as lipopolysaccharide has been documented (29). Another possibility is that extracellular TSA could have a suppressive effect on the maturation of macrophages or recruitment of polymorphonuclear leukocytes. Yokochi et al. (43, 44) demonstrated that the capsular polysaccharide from *K. pneumoniae* markedly suppressed the generation of macrophages from precursor cells, although no direct toxic effect on mature macrophages was shown. Also, Kato et al. (20) demonstrated that in mice treated with *K. pneumoniae* TSA, the migration of polymorphonuclear leukocytes into the peritoneal fluid after challenge with *Salmonella enteritidis* was less marked than in untreated control animals.

Although protease (15, 19, 32) and neuraminidase (31) activity have been shown to be involved in virulence in other bacteria, attempts to demonstrate any correlation between these enzymes from the GBS and virulence in a mouse model were unsuccessful under the present assay conditions. These data, showing neither enzyme correlating with virulence or with extracellular TSA levels, failed to support any role of these enzymes in virulence for the mouse model.

In the experiment involving the level of GBS in various organs of infected mice, it was of interest that in all three organs assayed (spleen, lungs, and brains) the levels of GBS (high or low producers) were comparable, regardless of the initial inoculum, in mice which would ultimately succumb to the infectious process. These data suggest that high producers of extracellular TSA may have a selective advantage in initiating infection at a lower inoculum, due perhaps to the production of high levels of TSA, but once the infection process is established, either class of type III GBS has the same potential to invade tissue.

Although these data support the concept that increased levels of extracellular HMW TSA are related to virulence, it is possible that other undefined extracellular or cell-bound products could also contribute to virulence. Further studies are therefore needed to determine whether the production of factors previously described for the GBS correlates with virulence in the various animal models. And finally, the mechanism of how the extracellular TSA contributes to virulence, if indeed it does, remains to be determined.

ACKNOWLEDGMENTS

This work was supported by Public Health Service research grant AI-13836 from the National Institute of Allergy and Infectious Diseases.

We thank Elizabeth Kay Eskew, Alice Ann Pierpont, and Alonzo Guzman, Jr., for their very capable assistance in the laboratory.

LITERATURE CITED

1. **Aminoff, D.** 1961. Methods for quantitative estimation of N-acetyl-neuraminic acid and their application to hydrolysates of sialomucoids. Biochem. J. **81**:384–392.
2. **Baker, C. J.** 1977. Summary of the workshop on perinatal infections due to group B streptococcus. J. Infect. Dis. **136**:137–152.
3. **Baker, C. J., D. L. Kasper, and C. E. Davis.** 1976. Immunochemical characterization of the "native" type III polysaccharide of group B streptococcus. J. Exp. Med. **143**:258–270.
4. **Baker, C. J., B. J. Webb, C. V. Jackson, and M. S. Edwards.** 1980. Counter-current immunoelectrophoresis in the evaluation of infants with group B streptococcal disease. Pediatrics **65**:1110–1114.
5. **Baltimore, R. S., D. L. Kasper, and J. Vecchitto.** 1979. Mouse protection test for group B streptococcus type III. J. Infect. Dis. **140**:81–88.
6. **Blake, F. G.** 1981. Antigen-antibody balance in lobar pneumonia. Arch. Intern. Med. **21**:779–790.
7. **Bukantz, S. C., P. F. DeGara, and J. G. M. Bullowa.** 1942. Capsular polysaccharide in the blood of patients with pneumococcic pneumonia. Arch. Intern. Med. **69**: 191–212.
8. **Carey, R. B., T. K. Eisenstein, G. D. Shockman, T. F. Greber, and R. M. Swenson.** 1980. Soluble group- and type-specific antigens from type III group B streptococcus. Infect. Immun. **28**:195–203.
9. **Conover, W. J.** 1971. Practical nonparametric statistics. John Wiley and Sons, Inc., New York.
10. **Dochez, A. R., and O. T. Avery.** 1971. The elaboration of specific soluble substance by pneumococcus during growth. J. Exp. Med. **26**:477–493.
11. **Doran, T. I., D. C. Straus, and S. J. Mattingly.** 1980. Extracellular antigens of serotype III group B streptococci. Infect. Immun. **30**:890–893.
12. **Doran, T. I., D. C. Straus, and S. J. Mattingly.** 1981. Factors influencing release of type III antigens by group B streptococci. Infect. Immun. **31**:615–623.
13. **Ferrieri, P., B. Burke, and J. Nelson.** 1980. Production of bacteremia and meningitis in infant rats with group B streptococcal strains. Infect. Immun. **27**:1023–1032.
14. **Ferrieri, P., E. D. Gray, and L. W. Wannamaker.** 1980. Biochemical and immunological characterization of the extracellular nucleases of group B streptococci. J. Exp. Med. **151**:56–68.
15. **Gutschik, E., S. Motles, and N. Christensen.** 1979. Experimental endocarditis in rabbits. 3. Significance of

proteolytic capability of the infecting strains of *Streptococcus faecalis*. Acta Pathol. Microbiol. Scand. Sect. B **87**:353–362.

16. **Hayano, S., and A. Tanaka.** 1969. Sialidase-like enzymes produced by group A, B, C, G, and L streptococci and by *Streptococcus sanguis*. J. Bacteriol. **97**:1328–1333.
17. **Hayano, S., A. Tanaka, and Y. Okuyama.** 1969. Distribution and serological specificity of sialidase produced by various groups of streptococci. J. Bacteriol. **100**:354–357.
18. **Hill, H. R.** 1975. Rapid detection and specific identification of infections due to group B streptococci by counterimmunoelectrophoresis, p. 84–88. *In* D. Schlessinger (ed.), Microbiology—1975. American Society for Microbiology, Washington, D.C.
19. **Holder, I. A., and C. F. Haidaris.** 1979. Experimental studies of the pathogenesis of infections due to *Pseudomonas aeruginosa*: extracellular protease and elastase as *in vivo* virulence factors. Can. J. Microbiol. **25**: 593–599.
20. **Kato, N., O. Kato, I. Nakashima, S. Naito, and J. Asai.** 1979. Effect of capsular polysaccharide of *Klebsiella pneumoniae* on host resistance to bacterial infection. III. Further study of its effects on interactions between peritoneal leukocytes and virulent *Salmonella enteritidis*. Microbiol. Immunol. **23**:369–382.
21. **Kenny, G. E., B. B. Wentworth, R. P. Beasley, and H. M. Foy.** 1972. Correlation of circulating capsular polysaccharide with bacteremia in pneumoccal pneumonia. Infect. Immun. **6**:431–437.
22. **Kjems, E., B. Perch, and J. Henrichsen.** 1980. Serotypes of group B streptococci and their relation to hyaluronidase production and hydrolysis of sialicin. J. Clin. Microbiol. **11**:111–113.
23. **Lancefield, R. C.** 1938. Two serological types of group B hemolytic streptococci with related, but not identical, type specific substances. J. Exp. Med. **67**:25–40.
24. **Mattingly, S. J., T. W. Milligan, A. A. Pierpont, and D. C. Straus.** 1980. Extracellular neuraminidase production by clinical isolates of group B streptococci from infected neonates. J. Clin. Microbiol. **12**:633–635.
25. **McClean, D.** 1941. The capsulation of streptococci and its relation to diffusion factor (hyaluronidase). J. Pathol. Bacteriol. **53**:13–27.
26. **Milligan, T. W., C. J. Baker, D. C. Straus, and S. J. Mattingly.** 1978. Association of elevated levels of extracellular neuraminidase with clinical isolates of type III group B streptococci. Infect. Immun. **21**:738–746.
27. **Milligan, T. W., T. I. Doran, D. C. Straus, and S. J. Mattingly.** 1978. Growth and amino acid requirements of various strains of group B streptococci. J. Clin. Microbiol. **7**:28–33.
28. **Milligan, T. W., D. C. Straus, and S. J. Mattingly.** 1977. Extracellular neuraminidase production by group B streptococci. Infect. Immun. **18**:189–195.
29. **Morrison, D. C., and L. F. Kline.** 1977. Activation of the classical and properdin pathways of complement by bacterial lipopolysaccharides (LPS). J. Immunol. **118**: 362–368.
30. **O'Reilly, R. J., P. Anderson, D. L. Ingram, G. Peter, and D. H. Smith.** 1975. Circulating polyribophosphate in *Hemophilus influenzae* type B meningitis. J. Clin. Invest. **56**:1012–1022.
31. **Pardoe, G. I.** 1974. The inducible neuraminidases of pathogenic microorganisms. Behring Inst. Mitt. **55**:103–122.
32. **Pavlovskis, O. R., and B. Wretlind.** 1979. Assessment of protease (elastase) as a *Pseudomonas aeruginosa* virulence factor in experimental mouse burn infection. Infect. Immun. **24**:181–187.
33. **Pollack, M.** 1976. Significance of circulating capsular antigen in klebsiella infections. Infect. Immun. **13**:1543–1548.
34. **Reed, L. J., and H. A. Muench.** 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. **27**: 493–497.
35. **Snedecor, G. W., and W. G. Cochran.** 1967. Statistical methods, p. 102–106. The Iowa State University Press, Ames.
36. **Stechenberg, B. W., R. L. Schreiver, S. M. Grass, and P. G. Shackelford.** 1970. Counter current immunoelectrophoresis in group B streptococcal disease. Pediatrics **64**:632–634.
37. **Straus, D. C., S. J. Mattingly, T. W. Milligan, T. I. Doran, and T. J. Nealon.** 1980. Protease production by clinical isolates of type III group B streptococci. J. Clin. Microbiol. **12**:421–425.
38. **Tieffenberg, J., L. Vogel, R. R. Kretschmer, D. Padnos, and S. P. Gotoff.** 1978. Chicken embryo model for type III group B beta-hemolytic streptococcal septicemia. Infect. Immun. **19**:481–485.
39. **Todd, E. W.** 1934. A comparative serological study of streptolysins derived from human and animal infection with notes on pneumococcal hemolysins, tetanolysin and staphylococcus toxin. J. Pathol. Bacteriol. **39**:299–321.
40. **Toennies, G., and D. L. Gallant.** 1949. The relationship between photometric turbidity and bacterial concentration. Growth **13**:7–20.
41. **Weeke, B.** 1972. A manual of quantitative immunoelectrophoresis methods and applications. Scand. J. Immunol. **2**(Suppl. 1):15–46.
42. **Wilkinson, H. W.** 1978. Detection of group B streptococcal antibodies in human sera by radioimmunoassay: concentrations of type-specific antibodies in sera of adults and infants infected with group B streptococci. J. Clin. Microbiol. **7**:194–201.
43. **Yokochi, T., I. Nakashima, and N. Kato.** 1977. Effects of capsular polysaccharide of *Klebsiella pneumoniae* on the differentiation and functional capacity of macrophages cultured *in vitro*. Microbiol. Immunol. **21**:601–610.
44. **Yokochi, T., I. Nakashima, and N. Kato.** 1979. Further studies on generation of macrophages in *in vitro* cultures of mouse spleen cells and its inhibition by the capsular polysaccharide of *Klebsiella pneumoniae*. Microbiol. Immunol. **23**:487–499.