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Production and Degradation of Serogroup B *Neisseria meningitidis* Polysaccharide

PETER C. MALONEY,[1] HERMAN SCHNEIDER, AND BRENDA L. BRANDT

Department of Bacterial Diseases, Walter Reed Army Institute of Research, Washington, D.C. 20012

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Polysaccharide produced from cultures of serogroups A, B, and C *Neisseria meningitidis* was assayed by the serogroup-specific hemagglutination inhibition (HAI) test. The polysaccharide produced by all serogroups was found to increase during the exponential phase of growth. For serogroups A and C, the HAI activity was stable during the stationary phase; for serogroup B, however, the HAI decayed rapidly. The degradation of the serogroup B polysaccharide was not caused by enzymatic degradation, but was due to acid accumulation in the culture medium. The large-molecular-size serogroup B polysaccharide was stabilized by increasing the buffering capacity of the medium, which also increased the yield of this antigen.

The serogroup-specific capsular polysaccharides of *Neisseria meningitidis* have been shown to be both chemically and immunologically distinct. The polysaccharide of serogroup A is a polymer of *N*-acetyl, *O*-acetyl mannosamine phosphate (5, 9). The serogroup B and C polysaccharides are both homopolymers of sialic acid (*N*-acetyl neuraminic acid), but are chemically distinct because the serogroup C polysaccharide also contains *O*-acetyl groups (5, 8).

Studies with serogroup A polysaccharide have shown that, for it to be immunogenic in man, a large-molecular-weight preparation is required (4–6), which is similar to the experience of Kabat with dextrans (7). Serogroup C polysaccharide preparations, which were larger in molecular size than the serogroup A polysaccharide, have also been immunogenic in man (2). Preparation of serogroup B polysaccharides by methods similar to those used for serogroups A and C did not consistently result in large-molecular-weight products (4). Extraction of polysaccharide from very young (6 to 8 hr) serogroup B cultures resulted in high-molecular-weight preparations, but antigen yields from such young cultures were low (Wyle and Matucik, *in press*).

Studies of Liu et al. (9) have shown that the serogroup B polysaccharide is degraded to sialic acid by incubation with neuraminidase. Therefore, experiments were undertaken to examine cultures of serogroup B meningococci for the presence of neuraminidase-like activity. The studies reported here demonstrate that serogroup B polysaccharide is labile under standard growth conditions, and that this lability is almost certainly due to acid production alone, there being no evidence of enzyme activity.

[1] Present address: Department of Physiology, Harvard Medical School, Boston, Mass. 02115.

MATERIALS AND METHODS

Bacterial strains. Strains of *N. meningitidis* were from the culture collection of the Walter Reed Army Institute of Research and included strains A-1 (serogroup A), C-11 (serogroup C), B-11, 690214, 26-II, 29-II, 130-V, 85-IV, 90-VI, 130 Eur, and 6155 (serogroup B), all originally isolated from clinical cases of meningitis. Bacterial strains were stored lyophilized at 4 C.

Media and growth conditions. Initially, the liquid medium used in these experiments was a modified Frantz medium (13) which contained (per liter) 2.5 g of Na_2HPO_4, 0.09 g of KCl, 0.06 g of $MgSO_4 \cdot 7H_2O$, 12 mg of cysteine, 10 g of Difco Casamino Acids (technical grade), and 5 g of glucose, at a final *p*H of 7.4. This will be referred to as the standard medium. For certain experiments, this medium was further modified by omitting cysteine and increasing the Na_2HPO_4 concentration fourfold.

Liquid cultures were grown in side-arm flasks at 37 C in a rotary shaker bath. These flasks were inoculated with the growth from a 4- to 6-hr culture grown on Mueller-Hinton agar. The initial concentration of organisms was about 2×10^7 to 5×10^7 ml, and subsequent growth was monitored by measuring the optical density at 650 nm by use of a Bausch & Lomb Spectronic 100 spectrophotometer. An optical density of 1.0 at 650 nm corresponded to about 10^9 viable organisms/ml. Under the above conditions, exponential growth was attained within one generation and ceased after 4 to 6 hr, at which time a cell density of about 10^9 organisms/ml had been attained. The doubling time of *N. meningitidis* in either modification of Frantz medium was 40 min.

Colorimetric assays. Total *N*-acetyl-neuraminic acid (sialic acid) was determined by the resorcinol method (11); correction was made for neutral hexose interference with glucose as a standard. Free sialic acid was measured as described by Warren (12). Protein was assayed according to the method of Lowry et al. (10) with bovine serum albumin as a standard.

Hemagglutination inhibition (HAI) assay. Serogroup-specific polysaccharide was detected by the inhibition of a standardized hemagglutination system (1). This method utilized polysaccharide-coated erythrocytes (Wyle et al., *in preparation*) and two units of serogroup-specific rabbit antibody. The HAI assay has been shown to measure primarily large-molecular-weight polysaccharide. Pure sialic acid does not inhibit either a serogroup B or C hemagglutination assay.

Neuraminidase assay. The procedure of French and Ada (3) was used to test for neuraminidase activity in culture filtrates. A sample of a culture filtrate (adjusted to *p*H 5.6) was added to an equal volume of a solution containing buffer (0.2 M sodium acetate, *p*H 5.60), substrate (0.45 mM *N*-acetyl-neuraminyl-lactose), and 0.02% bovine serum albumin. At zero time and after 24 hr of incubation at 37 C, samples were withdrawn for the determination of free sialic acid.

Preparation of serogroup B polysaccharide. Serogroup B polysaccharide for use as the hemagglutination antigen was prepared by the Cetavlon method (5) and was generously provided by S. Berman and J. Lowenthal of the Department of Biologics Research, Walter Reed Army Institute of Research. A radioactive serogroup B polysaccharide was prepared by growing the organisms in the increased phosphate-buffered Frantz medium in the presence of 10 μCi of ^{14}C-glucose/ml (180 mCi/mmole, Calbiochem, grade B). After 24 hr of growth, cells were removed by centrifugation and the culture supernatant fluid was sterilized by membrane filtration (0.45 μm pore size, Millipore Corp.). The polysaccharide was then purified from the culture filtrate by the Cetavlon method of Gotschlich et al. (5).

Polyacrylamide-gel electrophoresis. Polyacrylamide-gel electrophoresis was performed at room temperature by the method of Weber and Osborne (14). Samples for the electrophoresis were analyzed as described by Zollinger (*in preparation*).

RESULTS

A comparison of the polysaccharide (sss) produced by growing cultures of three strains of *N. meningitidis* representing serogroups A, B, and C is shown in Fig. 1A. Each organism was grown in liquid culture in a volume of 50 ml of standard medium. Samples (1 ml) were removed at 1-hr intervals, filtered through 0.45-μm membrane filters, and tested for HAI activity. (Cell-free polysaccharide has been shown to represent about 60% of the total polysaccharide during all phases of the growth cycle.) From Fig. 1A, it is apparent that HAI activity of all three strains increased rapidly during logarithmic growth but that serogroup B HAI activity alone decayed rapidly during stationary phase. Acid production by these same cultures is shown in Fig. 1B. The *p*H in culture fell below 7 at 6 hr of growth and dropped to *p*H 5.5 at 8 hr, remaining below 5.5 thereafter.

To determine whether the lability of serogroup Bsss was due to neuraminidase activity, the culture filtrates were tested for free sialic acid; none was found. Testing of culture filtrates with

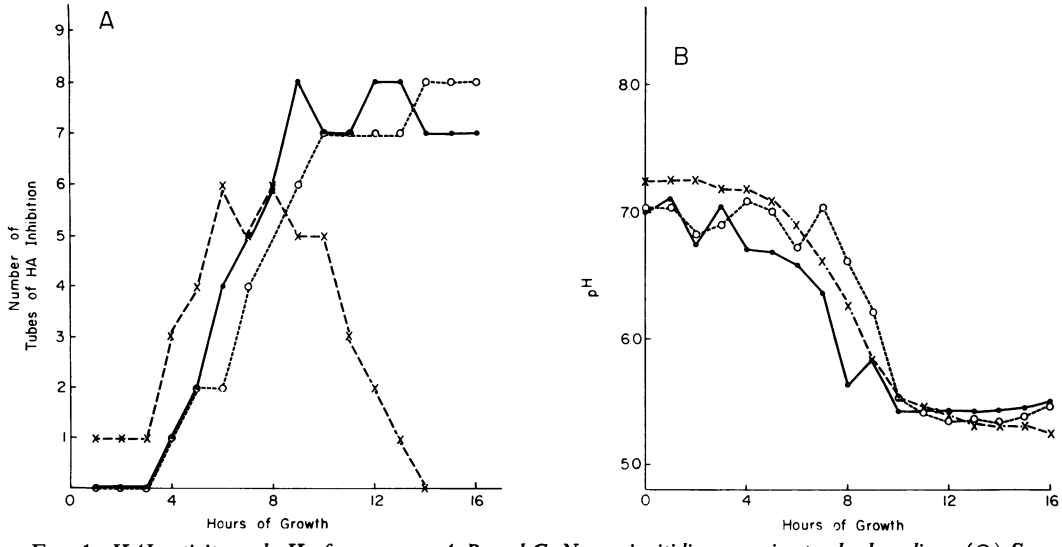

FIG. 1. *HAI activity and pH of serogroups A, B, and C. N. meningitidis grown in standard medium.* (○) *Serogroup A,* (×) *serogroup B,* (●) *serogroup C.*

neuraminyl-lactose as substrate also failed to demonstrate neuraminidase activity. Finally, it was shown that culture filtrates were capable of degrading purified serogroup Bsss even after the filtrate was autoclaved or treated with Pronase. Thus, the HAI degrading activity could not have been enzymatic in nature.

An experiment was performed to demonstrate that acidity alone was responsible for loss of serogroup Bsss activity. Purified serogroup Bsss was incubated at 37 C with an equal volume of either a 24-hr group B sterile culture filtrate, *p*H 5.2, or fresh sterile growth medium adjusted to *p*H 5.2. Results shown in Fig. 2 indicate that HAI activity was reduced in an identical fashion in each mixture.

In other experiments of this kind, purified serogroup Bsss was added to unused growth medium, which had been adjusted to a *p*H between 5.0 and 8.0, and HAI activity was determined after 16 hr of incubation at 37 C. Progressively lower levels of HAI activity were recovered as the *p*H of the medium decreased below 6.5. At *p*H values above 6.5, however, HAI activity was not significantly altered. Release of free sialic acid was not detected in these experiments.

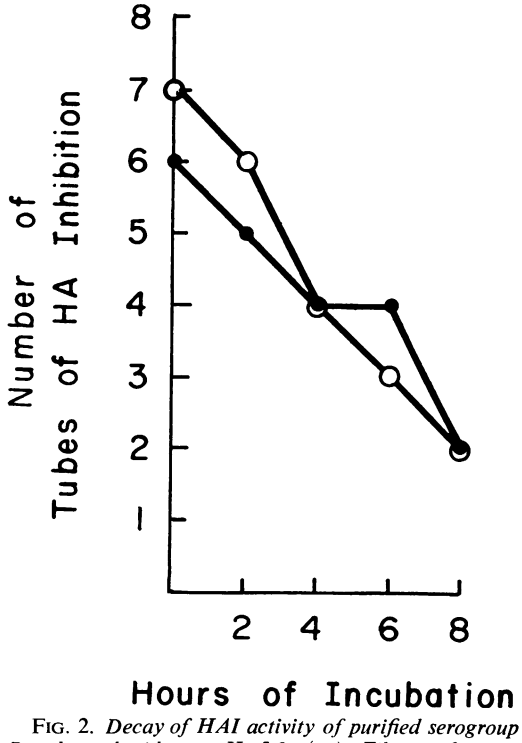

FIG. 2. *Decay of HAI activity of purified serogroup B polysaccharide at pH 5.2. (○) Filtrate from a 24-hr serogroup B culture, pH 5.2; (●) fresh sterile growth medium adjusted to pH 5.2.*

It is concluded, therefore, that at acid *p*H serogroup Bsss is degraded to smaller-molecular-weight polymers but not to free sialic acid. A further experiment to demonstrate the acid lability of serogroup Bsss was performed. Polyacrylamide-gel electrophoresis was used to follow the change in size of radioactively labeled serogroup Bsss after incubation for 18 hr at either *p*H 5 or 7.2. As shown in Fig. 3, incubation at *p*H 5.0 resulted in a shift of the labeled material to the right, a position corresponding to lower molecular size. Similar results were obtained when these same samples were analyzed by use of Sepharose 4B column chromatography.

Studies on eight additional serogroup B strains gave similar results in terms of degradation of HAI activity during the stationary phase of growth.

Experiments were undertaken to prevent Bsss depolymerization during growth of the culture by preventing acid accumulation. Two different modifications of the standard medium have been tested. In the first, acid production was limited by decreasing the concentration of glucose in the medium. In the second, the buffering capacity of the medium was increased by raising the concentration of phosphate fourfold. Both changes served to stabilize peak titers of HAI activity during the stationary phase of growth. For subsequent studies, the second modification has been utilized, since at the lower glucose concentrations the yields of cell mass were slightly lower.

A comparison of yields of high-molecular-weight Bsss from cultures grown in standard medium and high phosphate-buffered Frantz medium is shown in Fig. 4. Samples were removed

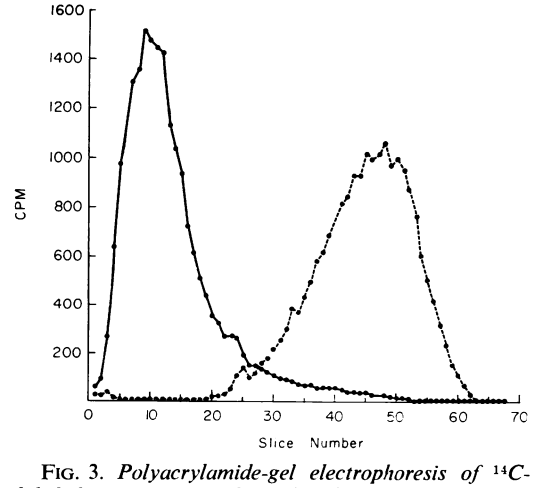

FIG. 3. *Polyacrylamide-gel electrophoresis of ^{14}C-labeled serogroup B polysaccharide after incubation at pH 7.2 or 5.0. Solid line: pH 7.2. Dashed line: pH 5.0.*

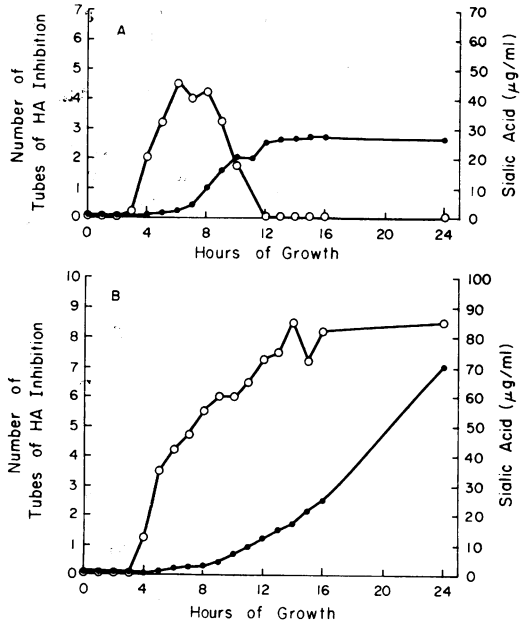

FIG. 4. *Appearance of HAI activity in cultures of serogroup B N. meningitidis. (A) Standard medium; (B) high-phosphate medium. (○) HAI activity; each point represents the average of the number of tubes of HAI for three experiments. (●) Sialic acid; polysaccharide precipitated from culture filtrates by Cetavlon and dissolved in 1 M NaCl prior to assay for total sialic acid. Neither Cetavlon nor 1 M NaCl was found to interfere with sialic acid determinations.*

at 1-hr intervals, filtered, and tested for both HAI activity and total polysaccharide content (as total sialic acid). In both cultures, the appearance of HAI activity followed similar kinetics. As expected, in the standard medium (Fig. 4A) HAI activity had disappeared by 12 hr, whereas in the highly buffered medium (Fig. 4B) HAI activity increased steadily until 16 hr and maintained the peak level throughout 24 hr. In the standard medium, the amount of total polysaccharide in culture filtrates rose to a maximum somewhat earlier than in the highly buffered medium, but did not increase further after 8 hr of incubation (Fig. 4A). However, in the highly buffered medium, the concentration of total polysaccharide continued to increase in stationary phase, finally reaching a level three times greater than that in the original medium (Fig. 4B). The increased concentration of sialic acid at 24 hr of culture without a striking increase in HAI activity suggested that this late polysaccharide was of smaller molecular size. The relationship of HAI activity and sialic acid production, expressed as the ratio 2^n:micrograms of sialic acid per milliliter, where n = number of tubes of HAI, was determined for each of the timed samples from the culture in highly buffered medium. These ratios varied twofold from an average of 10 throughout the experiment, a variation considered inherent in the HAI assay. In addition, acrylamide-gel disc electrophoresis of ^{14}C-labeled polysaccharide recovered from 22.5-hr cultures in highly buffered medium yielded a pattern similar to the *p*H 7.2-treated 18-hr polysaccharide shown in Fig. 3, indicating continuing synthesis of large-molecular-size polysaccharide during stationary phase.

It was noted in this and in other experiments that the numbers of viable cells in stationary cultures were markedly different in the two media. In the culture described in Fig. 4A, the number of viable cells reached a peak of about 10^9/ml after exponential growth had ceased (about 6 hr) and fell to less than 10^7/ml by the time the experiment was terminated (24 hr). However, in the highly buffered medium (Fig. 4B), viability was maintained throughout stationary phase at a level of 10^9 to 2×10^9 ml. Thus, it is likely that the increased yield of polysaccharide in the highly buffered medium was attributable to a greater number of organisms synthesizing this molecule.

DISCUSSION

Serogroup B polysaccharide of *N. meningitidis* was degraded rapidly in culture fluids after the period of exponential growth, unlike the serogroup A and C polysaccharides which were stable during this period. It was shown by a variety of methods that the degradation of the serogroup B polysaccharide is a reflection of an acid lability of the molecule. It was possible to increase the production and yield of antigenically active serogroup B polysaccharide by appropriate modifications of the standard growth medium.

By use of these principles, it was possible to produce relatively large quantities of high-molecular-weight serogroup B polysaccharides for studies of an immunogenicity in animals and human volunteers (Wyle et al., *in preparation*).

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