Effects of Two Blood Culture Anticoagulants on Growth of *Neisseria meningitidis*

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Tests of 25 strains of *Neisseria meningitidis* for sensitivity to sodium polyanethol sulfonate (SPS) showed that the sensitivity of strains varied with both inoculum size and SPS concentration. In Trypticase soy broth (TSB), 2 out of 13 strains were sensitive to 0.05% SPS, whereas 8 out of 13 strains were sensitive to the same concentration of SPS in brain heart infusion (BHI). In artificial blood cultures with six strains of meningococci, the addition of 10% defibrinated blood was found to eliminate the sensitivity of all six strains to SPS in BHI, but not of the two strains in TSB. Addition of 1.2% gelatin to artificial blood cultures eliminated the inhibitory effect of 0.05% SPS, whereas the addition of 1% yeast extract to blood cultures containing 0.025% or 0.05% SPS enhanced the inhibitory effect of this anticoagulant. None of the 13 strains tested was inhibited by 0.05% sodium amylosulfate in TSB or BHI alone or in artificial blood cultures with these media.

The value of sodium polyanethol sulfonate (SPS) as an additive to blood culture media is widely recognized. Several studies of both artificially inoculated blood culture systems (1, 17) and clinical blood cultures (3, 15) have demonstrated improved survival, increased frequency of isolation, and more rapid recovery of most species of medically important bacteria in the presence of small concentrations of SPS, compared with media lacking this additive. The effects of SPS are attributed to its activity as an anticoagulant, its ability to inhibit lysozyme and complement, its interference with phagocytosis, and its partial inactivation of several antibiotics (2, 11, 13, 16).

Not all species of bacteria show enhanced growth in the presence of SPS in the concentrations usually employed (0.025 to 0.05%). Inhibition of *Peptostreptococcus anaerobius* by SPS at these concentrations has been well documented (6, 8, 9), and has been shown to be medium dependent (18). A newly developed anticoagulant, sodium amylosulfate (SAS), has been introduced commercially in blood culture media to avoid the problem of the inhibition of the growth of this species. Broth cultivation experiments have confirmed the lack of any inhibitory effect of SAS on peptostreptococci (12), and clinical evaluations have shown SAS to be as effective as SPS in enhancing the isolation of many bacterial species from blood cultures (7, 10).

Two recent reports by Eng (3) and Eng and

Iveland (5) demonstrated inhibition of another species, *Neisseria meningitidis*, by SPS. In the first of these reports, 10 out of 11 clinical blood cultures of meningococci showed delayed and diminished growth in media with 0.05% SPS. In the second, broth cultivation studies with 24 strains of meningococci indicated that 50% of the strains tested were inhibited by 0.05% SPS in nutrient serum broth. These findings were in contrast to those in previous reports in which meningococci inoculated into whole blood were found to survive better in the presence of 0.05% SPS (1, 17), and in which the isolation of meningococci from both artificially inoculated and actual clinical blood cultures was enhanced by the addition of concentrations of SPS similar to that described by Eng (1, 14, 15).

The present study was undertaken to examine the effects of SPS and SAS on the growth of a number of strains of meningococci in media commonly used in clinical blood cultures which differed from that described by Eng (3) and Eng and Holten (4). Selected strains of meningococci were first screened for sensitivity to SPS by a broth cultivation technique. Several isolates were then tested for sensitivity to both SPS and SAS in two different broth media to detect any medium-dependent influence on inhibition. Finally, the effects of SPS and SAS were examined in several blood culture systems artificially inoculated with selected strains of meningococci. The effects of the addition of gelatin and yeast were studied and found to influence the system.

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MATERIALS AND METHODS

Bacterial strains. A total of 25 strains of *N. meningitidis* were included. Isolates were obtained from the clinical laboratories of Harborview Medical Center and University Hospital, Seattle, Washington, Seattle-King County Health Department Laboratory, through the courtesy of Evelyn Tronca, Jane Ballard of the Washington State Department of Social and Health Services Laboratory, and Jan Eng and Eirik Holten who provided eight SPS-sensitive strains from the Kaptein W. Wilhelmsen og Frues Bacteriological Institute, Oslo, Norway. Ten of the strains obtained within the state of Washington were clinical isolates from blood or cerebrospinal fluid, four strains were isolated from throat cultures, and three strains were from unknown sources. The strains included serogroups A, B, and C, as well as some nongroupable isolates.

• **SPS and SAS.** SPS was purchased from Roche Diagnostics (Nutley, N.J.) in a 5% aqueous solution (Grobax). SAS was obtained in powdered form through the courtesy of F. Tracy Wright of Becton-Dickinson Co. (Cockeysville, Md.).

Screening for SPS sensitivity. Twenty-five strains were grown on blood agar base with 5% sheep blood and incubated for 18 to 24 h in candle jars at 35°C. Organisms were removed from the plates with dry cotton-tipped applicator sticks and suspended in 0.85% saline. After the suspension was blended in a Vortex mixer for 30 s to disperse the clumps, the turbidities were adjusted to equal that of a 0.5 MacFarland nephelometer standard. The suspensions were remixed and serially diluted in 10-fold steps in Trypticase soy broth (TSB) (Bioquest) through a 10^{-7} dilution. One-tenth milliliter was removed from each of the last four tubes in each dilution series and inoculated into each of five tubes containing 2 ml of TSB and 2 ml each of TSB plus 0.025, 0.05, or 0.1% SPS blood agar base with 5% sheep blood and onto plates in triplicate to determine the number of colony-forming units (CFU) per milliliter in the inocula. The broth cultures were incubated in 5 to 10% CO₂ for 25 h at 35°C. After incubation, each broth culture was blended in a Vortex mixer for 30 s, and 0.001-ml portions were removed with a calibrated loop and plated onto each of three blood agar plates for quantitation of growth.

Strains were considered sensitive to a given concentration of SPS if the growth, in CFU per milliliter, in media with SPS differed by a factor of 2 logs when compared with controls without SPS. The probability of such a difference occurring in this test system due to chance in triplicate trials is $P < 0.001$.

Comparison of SPS and SAS sensitivity in two different culture media. Dilutions of 13 meningococcal strains were prepared as described above and inoculated into 2 ml each of TSB, TSB plus 0.05% SPS, TSB plus 0.05% SAS, brain heart infusion (BHI) (Difco), BHI plus 0.05% SPS, and BHI plus 0.05% SAS. Cultures were incubated and growth was quantitated as above, except that subcultures were made at both 24 and 48 h. Following the criteria for sensitiv-

ity described above, SPS and SAS sensitivities were determined for each strain by comparing growth in a medium containing SPS and SAS with growth in the same medium without either compound.

Artificial blood cultures for growth curves. Human blood was obtained from volunteers by venipuncture and defibrinated with glass beads in a sterile flask. A 5-ml portion of defibrinated blood was added to 50 ml of each of the media to be tested. To quantitate the inocula, dilutions of meningococci were prepared in 0.85% saline and adjusted to contain approximately 20 to 30 CFU per 0.1 ml, which was inoculated into each of the broth cultures after addition of the blood and onto blood agar base with 5% sheep blood plates in triplicate. The broth cultures were then incubated at 35°C in 5 to 10% CO₂. At intervals of 4, 8, and 12 h, 0.1- and 0.01-ml portions were removed and plated for quantitation. At 24 h, triplicate dilutions of 10^{-2} , 10^{-4} , and 10^{-6} were prepared from each broth culture and plated for quantitation.

RESULTS

Screening for SPS sensitivity. The number of SPS-sensitive strains was dependent on both inoculum size and SPS concentration. None of the 25 strains tested demonstrated sensitivity to 0.025% SPS in 2 ml of TSB when the inoculum contained ≥ 300 CFU, whereas one strain did so at an inoculum level of 1 to 30 CFU. With 0.05% SPS, however, one strain was sensitive with an inoculum of ≥ 300 CFU, and two strains were sensitive with inocula of 1 to 30 CFU. These two strains were from SPS-sensitive strains from Norway. In 0.1% SPS, one strain was sensitive with an inoculum of ≥ 300 CFU, whereas three strains were sensitive with inocula of 1 to 30 CFU (Table 1).

Comparison of SPS and SAS sensitivity in two different culture media. More strains of meningococci were sensitive to 0.05% SPS in BHI than in TSB. After incubation for 24 h, only 1 out of 13 strains was sensitive to this concentration of SPS in TSB when the inoculum was ≥ 300 CFU, and 2 out of 13 strains appeared sensitive when inocula of 1 to 30 CFU were used. After 48 h, no sensitive strains could be detected in TSB plus 0.05% SPS, indicating a growth delay in the presence of SPS of the single susceptible strain.

In BHI without SPS, 6 out of 13 strains failed to reach detectable numbers (at least 1,000

TABLE 1. Inhibition of *N. meningitidis* strains by various concentrations of SPS at different inoculum sizes

Inoculum (CFU)	No. of strains	No. of strains sensitive at SPS concn		
		0.025%	0.05%	0.10%
300	25	0	1	1
1-30	25	1	2	3

CFU/ml) after incubation for 24 h. Six of the seven strains that could be quantitated after 24 h were sensitive to 0.05% SPS. After 48 h, all 13 strains grew to detectable numbers. When growth was quantitated at this time, 8 out of 13 strains were sensitive to 0.05% SPS. Inocula of 1 to 30 CFU frequently failed to reach detectable numbers in BHI, so only figures from inocula ≥ 300 CFU were included in the data (Table 2).

None of the strains tested was sensitive to 0.05% SAS in either TSB or BHI after either 24 or 48 h (Table 2).

Growth rates in artificial blood cultures.

In the first series of experiments, six strains of meningococci were selected from the results of the previous experiments. Two of these strains were sensitive to 0.05% SPS in TSB without blood, with inocula of 1 to 30 CFU, whereas all six were sensitive to 0.05% SPS in BHI without blood. In a comparison of parallel blood cultures using BHI and TSB with and without 0.05% SPS or SAS, only the two strains previously identified as sensitive to 0.05% SPS in TSB showed delayed and diminished growth. The diminished growth occurred only in TSB and not in BHI. After 24 h, the two SPS-sensitive cultures reached levels of approximately 10^3 to 10^4 CFU/ml, compared with levels of 10^7 to 10^8 CFU/ml in control cultures without SPS. None of the six strains was inhibited in BHI in blood cultures with 0.05% SPS, even though all six were previously found to be sensitive to the same concentration of SPS in BHI alone (Table 2). Growth rates in blood cultures with 0.05% SAS were essentially the same as the controls for all strains for both media.

In the second series of artificial blood cultures, the two strains identified as SPS sensitive in TSB and TSB with blood in the previous experiments were found to be insensitive to 0.025% SPS in TSB with blood, based on triplicate trials. The same two strains were also found to be insensitive to 0.05% SPS when 1.2% gelatin (Difco) was added to the medium, also based on triplicate trials.

In the third series, the addition of 1% yeast

extract (Difco) was found to increase the sensitivity of both strains to 0.025% SPS and 0.05% SPS in TSB with blood cultures. This result was confirmed by four replicate cultures of each of the two strains. With one strain, no growth was detectable within 24 h in media containing 1% yeast extract and either concentration of SPS (Fig. 1). The other strain showed growth in media containing 1% yeast extract and 0.025% SPS, which was diminished by approximately 6 logs, as compared with control cultures without either SPS or yeast extract. No growth was detectable with this strain in media containing 1% yeast extract and 0.05% SPS (Fig. 1). In the cases of both strains, control cultures containing 1% yeast extract alone, without SPS, showed no inhibition of growth when compared with the

FIG. 1. Analysis of the effect of yeast extract on two SPS-sensitive strains of *N. meningitidis* in (○) TSB, (□) TSB + 0.05% SPS, and (△) TSB + 0.025% SPS. Closed symbols represent media with yeast extract. The strains are (A) Nm 8; (B) Nm 20.

TABLE 2. Sensitivity of *N. meningitidis* strains to 0.05% SPS and SAS in two different broth media

Inoculum (CFU)	No. of strains inhibited by 2 or more logs							
	24 h of incubation ^a				48 h of incubation ^b			
	TSB + SPS	TSB + SAS	BHI + SPS	BHI + SAS	TSB + SPS	TSB + SAS	BHI + SPS	BHI + SAS
300	1	0	6 ^c	0	0	0	8	0
1-30	2	0	— ^c	— ^c	0	0	— ^c	— ^c

^a Results from 13 strains in TSB and from 7 strains in BHI. Six strains failed to reach detectable numbers in BHI after incubation for 24 h.

^b Results from all 13 strains.

^c —, Inocula of 1 to 30 CFU frequently failed to reach detectable numbers in BHI at 24 h.

TSB in blood cultures alone. Cultures containing 0.025% SPS and 0.05% SPS without yeast extract gave results similar to those of the previous series. In addition, yeast extract did not increase the SPS sensitivity of four strains, which were otherwise insensitive to this compound.

DISCUSSION

The number of SPS-sensitive strains identified in this study differs markedly from the findings of Eng and Iveland (5), who described 12 out of 24 strains of meningococci that were sensitive to 0.05% SPS. Eight of the strains included in the present study were among those considered SPS sensitive in that study, but only two of these strains met our criterion for SPS sensitivity in the first set of experiments described (Table 1). None of our own isolates demonstrated sensitivity. Two differences in the studies may account for the discrepancies. First, in the work of Eng and Iveland, sensitivity was defined as a difference in the ability of inocula of different sizes to grow in media with and without 0.05% SPS. A difference in the growth of five 1:33 dilution steps indicated sensitivity to SPS; however, in the present study sensitivity was defined on the basis of a comparison of the quantity of growth in media with and without SPS, using the same inoculum size in each medium. The differences in definitions and inocula may account for some of the discrepancies since our study demonstrates the effect of inoculum size on SPS sensitivity (Table 1).

Second, the medium used in Eng and Iveland's experiments was a serum-supplemented nutrient broth (5); whereas, TSB was used in screening strains for SPS sensitivity in this study. This difference, along with the increased sensitivity of meningococci to SPS observed in BHI, suggests that the medium composition may also have contributed to the differences. Eng's report of the inhibition of meningococci by SPS in a clinical blood culture study (3) may also be accounted for, in part, by the type of broth used since several studies of both artificial and clinical blood cultures (1, 14, 15, 17) have indicated a favorable effect of this anticoagulant on the isolation of meningococci with the addition of 0.03 to 0.05% SPS using brucella, thioglycolate, or TSB.

Wilkins and West (18) reported the medium dependence of the inhibition of *P. anaerobius* by SPS. Their experiments indicated that some proteins (casein or gelatin), when added to a peptone basal medium to a concentration of 1.2% or greater, provided protection from inhibition by SPS. The results of our study indicate a similar effect in the case of meningococci. Ad-

dition of 10% defibrinated blood to BHI reversed the SPS sensitivity of all six strains in the blood culture experiments; whereas, addition of blood to TSB did not alter the sensitivity of the two strains that were inhibited by SPS in TSB alone. The addition of gelatin to this blood culture system, in a manner similar to that in the results reported by Eng and Holten (5), did, however, reverse the toxicity of SPS in TSB. Wilkins and West have suggested that proteins or large protein fragments with a proper tertiary structure to allow binding to SPS molecules may, when present in sufficient concentration, protect against the inhibitory effect of SPS (18). BHI, an infusion of brain and heart tissue, would be expected to contain predominantly intact proteins or large protein fragments, which may allow the greater binding of SPS molecules and protect against the inhibitory effect (18). The addition of blood to this medium may bring the larger protein concentration to a level that may be capable of inhibiting SPS activity. TSB, an enzymatic digest of proteins, contains predominantly small peptone fragments, and the addition of the same amount of blood to this medium may fail to provide a protective level of large protein molecules. Wilkins and West also found that the addition of blood to a peptone medium failed to confer protection against inhibition of peptostreptococci by SPS (18).

As has been previously reported for peptostreptococci (9), 0.05% SAS had no measurable inhibitory effect on any of the *N. meningitidis* strains tested.

Enhancement of the inhibitory effect of SPS on the two sensitive strains in the presence of 1% yeast extract may have important implications for clinical blood culture media. The effect of yeast extract could not be explained on the basis of the activity of this substance alone since cultures without SPS, which contained yeast extract, showed growth comparable to that of controls. SPS may interfere with the recovery of some strains from actual blood cultures by preventing them from reaching sufficient numbers to be detected by the routine blind subculture methods commonly used in many clinical laboratories.

Several modifications of blood culture media systems may improve the recovery of *N. meningitidis*; however, the number of SPS-sensitive strains is not known. Reduction of SPS concentration to 0.025%, as has been recommended for peptostreptococci, would reduce or eliminate the inhibitory effect. Our data also indicate that addition of 1.2% gelatin to media containing SPS, as suggested by Eng and Holten (4), or substitution of 0.05% SAS for SPS, would also

favor the isolation of SPS-sensitive strains. Our data also indicate that the presence of yeast products in several blood culture media also needs reevaluating.

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