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Inhibition of *Neisseria gonorrhoeae* by Sodium Polyanetholesulfonate

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Sodium polyanetholesulfonate (SPS), in concentrations commonly used in blood culture media, inhibited the growth of a significant number of isolates of *Neisseria gonorrhoeae* in an agar dilution system. This SPS toxicity, shown to be bactericidal when examined in broth culture, could be reversed by hemoglobin and gelatin. Gelatin in 1% conentration allowed optimal growth of SPS-sensitive isolates in the presence of 0.025% SPS. Of 50 clinical isolates of *N. gonorrhoeae* tested under simulated blood cultures conditions with SPS, 16 isolates failed to grow on subculture at days 1, 3, and 10 after inoculation. Recovery was delayed with eight isolates as compared to controls. Early subcultures at 4, 8, and 12 h failed to recover SPS-sensitive isolates, whereas 1% gelatin, added even as late as 8 h after inoculation, reversed the SPS toxicity. The data reported suggest that SPS at concentrations routinely used in blood cultures can delay or prevent isolation of *N. gonorrhoeae*, but 1% gelatin can eliminate this adverse effect.

Recognition of the presence of *Neisseria gonorrhoeae* in the blood is an important component in the diagnosis of disseminated gonococcal infection. At our institution, recovery of this organism from the blood of patients with a history and clinical presentation strongly suggesting disseminated gonococcal infection has been erratic. Graves et al. (5) have shown sodium polyanetholesulfonate (SPS), a common component of commercial blood culture systems, to exert a toxic effect on anaerobic cocci. The work of Eng (1) demonstrates a similar detrimental effect of SPS on the recovery of *N. meningitidis* from blood culture. The study of Eng and Iveland (3), which used an agar disk diffusion technique and suggested a variable response to SPS by *N. gonorrhoeae*, prompted us to examine more closely the growth activity of clinical isolates of *N. gonorrhoeae* in the presence of SPS, particularly at concentrations and under conditions routinely used for blood culture. The reported ability of gelatin to protect organisms against SPS toxicity (2, 8) was also evaluated.

MATERIALS AND METHODS

Bacterial cultures. Organisms used in this study were genital or rectal isolates of *N. gonorrhoeae* provided by the Cincinnati Health Department Microbiology Laboratory and the Clinical Microbiology Laboratory, University of Cincinnati Hospital. Identification was carried out using direct fluorescent-antibody staining (Difco Laboratories, Detroit, Mich.). Isolates of *N. gonorrhoeae*, once obtained in pure culture, were frozen in an aqueous solution containing 1% proteose peptone and 10% glycerol at −70°C until used.

Media and components. Chocolate blood agar (CBA) and Columbia broth were purchased from GIBCO Diagnostics (Columbus, Ind.). Mueller-Hinton agar (MHA) and broth (MHB) were prepared from dehydrated media (Difco). SPS was obtained from Sigma Biochemicals, St. Louis, Mo.; gelatin and pancreatic hydrolysate of gelatin were purchased from BBL Microbiology Systems, Cockeysville, Md. The latter three medium additives were either autoclaved separately and added to the base medium or autoclaved with it. Hemoglobin (BBL), sheep erythrocytes (SRBC) preserved in Alsever solution (Colorado Serum Co. Labs, Denver, Colo.), and IsoVitaleX (BBL) were purchased sterile and added aseptically to base medium. Human blood was obtained from volunteers by aseptic venipuncture and added directly to the medium. The final pH of all media used in this study ranged from 7.1 to 7.3.

Inoculum preparation. The majority of experiments were carried out using a second subculture of *N. gonorrhoeae* isolates previously frozen at −70°C. Growth on CBA after 18 to 24 h of incubation was suspended in MHB to match the turbidity of a 0.5 MacFarland standard, giving approximately 10^8 viable colony-forming units (CFU) per ml. Appropriate dilutions were made using MHB to achieve the desired inoculum size for experiments.

Agar dilution. Medium for the agar dilution experiments consisted of MHA with 1% IsoVitaleX, 5% SRBC, and concentrations of SPS ranging in twofold dilution steps from 0.0015% to 0.2%; the latter three components were aseptically added to molten MHA before plate pouring. An inoculum of 10^6 CFU of each test isolate per ml was prepared in MHB and was delivered using a Steers replicating apparatus (6). Each inoculation prong delivered approximately 0.001 ml per application, resulting in the seeding of the plate

surface with approximately 10^3 CFU of each isolate. The minimum inhibitory concentration of SPS for each isolate was defined as the lowest concentration of SPS (percent weight/volume) that totally inhibited growth on CBA after 48 h of incubation at 35°C under 7 to 10% CO_2.

Broth culture experiments. Base medium consisted of 10 ml of MHB with final concentrations of 1% IsoVitaleX and 5% SRBC. In those instances where 1% hemoglobin was an additive, the SRBC were omitted. The additives, consisting of SPS at 0.025%, gelatin at various concentrations, 2% peptic hydrolysate of gelatin, and 2% proteose peptone, were autoclaved with the MHB. Each test isolate was prepared and added such that a starting inoculum of 10^4 CFU/ml was achieved. After 24 h of incubation at 35°C under 7 to 10% CO_2, each tube was subcultured to CBA by means of a 0.01-ml calibrated loop, incubated at 35°C under 7 to 10% CO_2, and read for growth after 24 h. For those experiments in which varying concentrations of gelatin were used, quantitative growth yields were determined by plating 10-fold dilutions of each culture with the calibrated loop after 24 h of incubation of the broth culture.

Simulated blood culture. Columbia broth base (GIBCO) with 10% (vol/vol) freshly drawn human blood was distributed into culture tubes (16 by 100 mm) to a final volume of 5 ml. Those tubes containing SPS had a final concentration of 0.025% before blood was added. A final gelatin concentration of 1% was used where appropriate. For each experiment, a starting inoculum size of 10^2 CFU/ml was used to mimic the usually low bacterial concentrations present during bacteremia. Cultures were incubated at 35°C under 7 to 10% CO_2 and subcultured to CBA with a 0.01-ml calibrated loop on days 1, 3, and 10 after inoculation, unless otherwise noted. Subcultured plates were scored as either growth or no growth after 48 h of incubation at 35°C under 7 to 10% CO_2.

RESULTS

Agar dilution experiments. Results of adding various concentrations of SPS to 85 randomly selected clinical isolates of *N. gonorrhoeae* (Table 1) demonstrated that at least one-third of the isolates were inhibited at levels of SPS commonly employed in blood culture medium (0.025 to 0.05%). Although complete inhibition of growth was used to determine the quantitative endpoint, some degree of growth inhibition, as evidenced by either lighter lawns or decreased numbers of discrete colonies per application, was noted with almost all isolates tested. The substitution of 1% hemoglobin for 5% SRBC appeared to decrease the SPS toxicity significantly.

Broth culture experiments with additives. Experiments carried out in MHB with 0.025% SPS determined that the SPS was bactericidal for isolates inhibited in the agar dilution system because subculture after incubation failed to recover the organisms (Table 2). The effect of various additives on SPS toxicity is also shown in Table 2. It was apparent that 2% gelatin and 1% hemoglobin, but not 2% peptic hydrolysate of gelatin, were protective against SPS toxicity. Proteose peptone protected only half of the isolates tested. Further studies in broth with 0.025% SPS and various concentrations of gelatin indicated that 1% gelatin was sufficient to completely neutralize the SPS toxicity and to allow for growth yields equal to those in the absence of SPS (Table 3).

Simulated blood culture experiments. Fifty randomly collected clinical isolates of *N. gonorrhoeae* different from those used in the agar dilution series were inoculated into simulated blood culture conditions using Columbia broth base without SPS, with 0.025% SPS, and with 0.025% SPS plus 1% gelatin. Small inocula of 10^2 CFU/ml were used. The results of subcultures to CBA at days 1, 3, and 10 after inoculation are shown in Table 4.

Since some clinical laboratories have adopted a subculture schedule for blood bottles involving early or same-day subculture 3 to 18 h after inoculation (8), it was of interest to determine whether SPS-sensitive isolates of *N. gonorrhoeae* could be "rescued" from simulated blood cultures by early subculture. Therefore, five isolates known to be sensitive to SPS at 0.025% were inoculated as described above but subjected to subculture at 4, 8, and 12 h after inoculation as well as at 1, 3, and 10 days. None

TABLE 1. *Minimum inhibitory concentration of SPS for N. gonorrhoeae as determined by agar dilution*

Medium	No. of isolates tested	No. of isolates with SPS MIC[a] of:							
		0.0015%	0.003%	0.006%	0.012%	0.025%	0.05%	0.1%	0.2%
MHA-1% IsoVitaleX-5% SRBC	85	0	0	3 (4)[b]	6 (11)	13 (26)	9 (36)	5 (42)	4 (47)
MHA-1% IsoVitaleX-1% hemoglobin	72	0	0	0	0	0	0	9 (13)	12 (29)

[a] Minimum inhibitory concentration (MIC) is expressed as percent (wt/vol) SPS.
[b] Parentheses indicate cumulative percent inhibition of the total number of isolates tested.

TABLE 2. *Growth of N. gonorrhoeae (sensitive to 0.025% SPS) in broth culture with SPS and additives*[a]

Additive	No. of isolates tested	No. of isolates recovered
None	8	0
2% gelatin	8	8
2% peptic hydrolysate of gelatin	8	0
2% protease peptone	8	4
1% hemoglobin	8	8

[a] Base medium was MHB with 1% IsoVitaleX, 5% SRBC, and 0.025% SPS. Inoculum size was 10^4 CFU/ml. Incubation was for 24 h at 35°C.

TABLE 3. *Growth of six N. gonorrhoeae isolates (sensitive to 0.025% SPS) in SPS plus gelatin*[a]

Gelatin concn (%)	No. of isolates recovered	Avg growth yield (CFU $\times 10^6$ per ml)
Control[b]	6	2.4
0	0	
0.125	0	
0.25	6	0.5
0.5	6	1.0
1.0	6	2.8
2.0	6	1.7

[a] Base medium was MHB with 1% IsoVitaleX, 5% SRBC, and 0.025% SPS. Inoculum size was 10^4 CFU/ml. Incubation was for 24 h at 35°C.

[b] Control: No SPS added.

TABLE 4. *Recovery of 50 clinical isolates of N. gonorrhoeae from simulated blood culture*[a]

No. of isolates	Growth in culture		
	Columbia broth–10% human blood	Columbia broth–10% human blood–0.025% SPS	Columbia broth–10% human blood–0.025% SPS–1% gelatin
33	+	+[b]	+
15	+	−	+
1	−	−	+
1	−	−	−

[a] Initial inoculum was 10^2 CFU/ml; subculture was performed at days 1, 3, and 10.

[b] Growth was detected initially upon day 3 subculture for 8 of 33 isolates. All other isolates in this table were detected upon day-1 subculture.

of the isolates was recovered at the early subculture times, whereas recoveries were made at the 4-h subculture in control cultures containing 1% gelatin.

Of equal interest was the time interval after inoculation into SPS-containing medium during which the addition of gelatin to the blood bottle would rescue SPS-sensitive organisms. Five SPS-sensitive isolates were inoculated into parallel rows under simulated blood culture conditions. Gelatin was added to the appropriate row to allow for a final concentration of 1% at 2, 4, 8, and 20 h after inoculation. Subcultures of all tubes followed at 1, 3, and 10 days. The interval between the 20-h gelatin addition and the 1-day subculture was approximately 6 h. All five isolates were recovered from the 1-day subculture when gelatin was added as late as 8 h after inoculation. Addition of gelatin at 20 h permitted the recovery of one of five isolates. Subsequent subculture at days 3 and 10 failed to recover the remaining four isolates to which gelatin had been added at 20 h.

Five isolates determined to be sensitive to 0.025% SPS under simulated blood culture conditions were subcultured daily onto CBA for 8 consecutive days and reinoculated into simulated blood culture tubes containing 0.025% SPS. All five isolates were successfully recovered at the 1-day subculture, indicating that the SPS sensitivity of each isolate was lost during the subculture on CBA.

DISCUSSION

It is apparent from these studies that SPS possesses a definite toxic effect for a significant number of randomly selected clinical isolates of *N. gonorrhoeae*, whether tested in agar, broth, or simulated blood culture media. Of particular concern is the finding that levels of 0.025% SPS that are routinely used in commercial blood culture systems can either delay or prevent recovery of certain isolates. Gelpi (4), in summarizing the literature on gonococcal sepsis, pointed out that among 161 patients with clinical presentation and adjunctive culture data highly suggestive of gonococcal sepsis, only 37 patients had positive blood cultures for *N. gonorrhoeae*. Specifics of the blood culture techniques are not available, and we cannot help wondering what effect SPS toxicity had on this statistic.

Previous reports of others also describe SPS toxicity for *Peptostreptococcus anaerobius* (5) and both *Neisseria meningitidis* and *N. gonorrhoeae* (1, 3) which was significantly decreased in the presence of gelatin (2, 8). The SPS toxicity for these organisms is poorly understood and appears to be influenced by medium additives and, at least in the case of *N. gonorrhoeae*, the number of subcultures on laboratory medium that a particular clinical isolate has undergone. Whether in vitro passage of clinical isolates selects for SPS-resistant members of the population, or whether the increased resistance represents an adaptation of the entire cell population

to a laboratory environment, has not been investigated. However, the importance seems to lie in the fact that fresh clinical isolates are either inhibited or killed by the presence of low levels of SPS. Equally undefined is the mechanism of the reversal of SPS toxicity by gelatin and also, in our studies, by hemoglobin and, to some degree, proteose peptone. That gelatin provides some type of nutritional requirement is unlikely since gelatin hydrolysate failed to exert a protective effect. Wilkins and West (8) suggest that gelatin or the peptides of proteose peptone might act in some manner to bind the SPS and presumably to detoxify the substance. Perhaps hemoglobin also functions in a similar manner to protect gonococci. Wilkins and West point out that the addition of gelatin, in concentrations close to those we are suggesting, fortunately does not significantly alter the neutralization of human serum bactericidal effects by SPS, which is certainly a benficial aspect of SPS.

The degree of SPS sensitivity of *N. gonorrhoeae* at first appears, from our work, to be more variable than that of *Peptostreptococcus* in that Wilkins and West (8) demonstrated inhibition at 0.05% SPS with 24 of 24 *Peptostreptococcus* strains tested, whereas in our studies only 31 of 85 *N. gonorrhoeae* isolates tested at 0.05% SPS were inhibited. However, in the *Peptostreptococcus* report, a strain was considered sensitive to SPS in an agar dilution system if the isolate failed to grow or showed diminished growth compared to a control at 48 h. In our agar dilution study, an isolate was considered sensitive only if growth was completely inhibited at any given SPS concentration. Had we applied the criterion of Wilkins and West, almost all isolates examined would have shown some degree of "sensitivity" to SPS at 0.05%. This variation in SPS sensitivity of the gonococcus concurs with the report of Eng and Iveland (3), who, using an agar disk diffusion assay, noted differences in the diameter of zones of inhibition among isolates of both *N. meningitidis* and *N. gonorrhoeae*. It is curious that in the same publication of Eng and Iveland, some degree of SPS sensitivity existed with isolates of *Streptococcus pneumoniae*, alpha-hemolytic streptococci, and both groups A and B beta-hemolytic streptococci.

The reversal of SPS toxicity by certain additives such as gelatin, hemoglobin, and proteose peptone has obvious application to blood culture methodology. Whereas hemoglobin is an impractical additive to blood culture broth and proteose peptone appears to protect only certain isolates, gelatin is an excellent candidate as an additive in that it is readily available and inexpensive, imparted 100% protection in this study, and retains its protective ability through autoclaving. Wilkins and West attribute the growth of *Peptostreptococcus* in supplemented peptone broth (Becton-Dickinson Co., Rutherford, N.J.) with SPS to the gelatin component of the medium (8). Although it appears that early or same-day subculture techniques for blood cultures might not be fruitful for the recovery of low numbers of SPS-sensitive organisms, the addition of gelatin to blood culture broth even 8 h after inoculation may rescue organisms that might otherwise have remained undetected. Currently in our laboratory blood culture system, Columbia broth with SPS is used. It is standard procedure to inject 5 ml of a 20% gelatin stock solution into the 100-ml aerobic blood bottle for those culture sets drawn from patients who by history and clinical presentation may have either meningococcal or gonococcal bacteremia.

The evidence continues to mount that certain organisms commonly responsible for bacteremia show sensitivity to SPS. The SPS component of blood culture medium is highly valued for its anticoagulant activity and its dramatic enhancement of recovery, particularly of gram-negative bacilli, through inactivation of serum factors and certain antibiotics. Although it makes little sense to relinquish these beneficial properties of SPS to enhance the recovery of organisms such as *Neisseria* and *Peptostreptococcus*, which are less frequently encountered than the gram-negative bacilli, it seems quite appropriate to employ additives such as gelatin which not only appear to offer protection to SPS-sensitive organisms but also fail to interfere with the beneficial properties of SPS.

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