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Inactivation of the Polyanionic Detergent Sodium Polyanetholsulfonate by Hemoglobin

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Sodium polyanetholsulfonate (SPS) has been added to blood culture media for many years. Its incorporation results in a higher yield of positive blood cultures due to its inactivation of antimicrobial cationic compounds. The most active of these cations include complement components, aminoglycoside-aminocyclitol antibiotics, and receptors on polymorphonuclear leukocytes. There have been reports from studies conducted outside patient blood culture bottles that SPS itself may possess antibacterial activity against some isolates of *Neisseria meningitidis*, *Neisseria gonorrhoeae*, and *Peptostreptococcus anaerobius*. Conversely, in patient clinical trials there has been no significant difference in pathogen isolation rates in the presence or absence of SPS. In an attempt to explain this in vitro/in vivo disparity, a search was undertaken to elucidate which variable constituent in blood, heretofore not studied quantitatively, might have a major effect on modulating the activity of SPS. It was found that hemoglobin combined stoichiometrically with SPS with a K_d of approximately 10^{-7} mol/liter. Optimum SPS inactivation occurred at an SPS/hemoglobin ratio of 1:6 (wt/wt). SPS-sensitive isolates of *N. gonorrhoeae* and *N. meningitidis* were protected by the addition of hemoglobin from the antimicrobial effects of this polyanion in time-kill studies. This protection was directly related to the amount of SPS combined in solution. Therefore, the amount of free hemoglobin in solution must be measured when studying the antimicrobial activity of polyanions or when evaluating the effect of different polyanions on the recovery rates of pathogens in patient blood culture clinical trials.

Sodium polyanetholsulfonate (SPS) is a polyanionic detergent that has been incorporated into blood culture media for many years (6, 10). SPS exerts its action by obviating the activity of cationic compounds. Among its most important activities are the prevention of blood clotting, inhibition of phagocytosis, inactivation of complement, removal of cationic proteins, and elimination of aminoglycoside-aminocyclitol and polymyxin antibiotics (1, 2, 4, 8, 10). Positively charged antibiotics and proteins are particularly affected (2). SPS combines directly with multivalent cationic ligands to form insoluble polymers (4). This reaction is a stoichiometric one (2, 8).

It has been reported that SPS can deleteriously affect the growth of *Neisseria meningitidis*, *Neisseria gonorrhoeae*, and *Peptostreptococcus anaerobius* from blood cultures (5). However, SPS exhibited little antibacterial activity against other bacteria, with the family *Enterobactericeae* and the genus *Staphylococcus* unaffected by concentrations as high as 1.25% (1). Sodium amylosulfate, which has also been incorporated into blood culture media, was not as effective as SPS in neutralizing serum bacteriocidal activity (9), was no better in clinical blood culture trials than SPS, and may be inhibitory toward gram-negative rods (6). Little is known regarding the antimicrobial activity of other polyanionic detergents in blood culture systems.

The antimicrobial activity of SPS appears to be specific for a particular microbial isolate and also concentration dependent. There is a lack of correspondence regarding the antimicrobial activity of SPS in studies performed inside or outside of clinical blood culture bottles. For example, Enj and Iveland (5) reported an inhibitory effect of SPS against *Neisseria meningitidis* in a disk diffusion test on plate media, whereas Hall et al. found no statistically significant difference between SPS and sodium amylosulfate in a patient blood culture evaluation (6).

In an attempt to explain the disparity of SPS activities in various media and in blood culture bottles, a study was undertaken to examine which serum elements might have the greatest modulating effect on SPS. The role of hemoglo-

bin was investigated in the inactivation of SPS because it had the properties of a cation, was present in blood culture bottles in a wide range of concentrations, was incorporated into some plate media but not others, and was not studied as a major uncontrolled variable from reports in the literature.

MATERIALS AND METHODS

Interaction of hemoglobin and SPS. The amount of hemoglobin bound to SPS was determined by an ultrafiltration equilibrium method. A magnetically stirred 450-ml Teflon-coated ultrafilter cell was employed (Amicon Corp., Lexington, Mass.), and cellulose acetate membranes designed to exclude molecules above molecular weight 20,000 were made and fitted into the ultrafilter cell (3, 11). The membranes were washed with 1% SPS and rinsed in normal physiological saline to eliminate nonspecific binding. The grams of SPS bound per gram of hemoglobin were measured by adding to 0.1% SPS (Hoffmann-LaRoche Inc., Nutley, N.J.) in normal physiological saline concentrations of hemoglobin (Sigma Chemical Co., St. Louis, Mo.) from 0.01 to 2%. In each reaction vessel, the 0.1% SPS solution was allowed to react with an individual hemoglobin solution for 6 h at 22°C. Free SPS was separated from SPS bound to hemoglobin by activating the ultrafiltration mechanism. The contents of the reaction vessel were stirred at 250 rpm to thoroughly disperse the reactants and reduce the surface polarization of the membrane. SPS was quantified in the ultrafiltrate by absorption at 275 nm. Because the molecular weight of polymer SPS was not known exactly, concentrations were expressed as weight per volume.

Effect of hemoglobin on the antimicrobial activity of SPS. The effect of hemoglobin on the antimicrobial activity of SPS was determined by utilizing SPS-sensitive and SPS-resistant isolates of *N. meningitidis* and *N. gonorrhoeae*. *Neisseria* species were grown in brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 1% IsoVitaleX (BBL). Time-kill (CFU per hour) studies were performed with SPS at a constant concentration of 0.1%, with hemoglobin concentrations from 0.01 to 2.0% (see Table 1). The initial inoculum for each test isolate was 10^5 CFU/ml, and the inocula from growth controls were determined in broth with no additives. The organisms were grown in each SPS: hemoglobin ratio for 24 h at 36°C in 6% carbon dioxide. Incubation took place on a reciprocal shaker, with samples taken at 2, 4, 6, 18, and 24 h to determine viable numbers. Viable counts and determinations of CFUs per milliliter were performed by the method of Koch (7). Five SPS-sensitive *N. meningitidis* and five SPS-sensitive *N. gonorrhoeae* isolates were compared with five each of SPS-resistant species.

RESULTS

Effect of hemoglobin on SPS. Figure 1 displays the stoichiometry of the reaction between hemoglobin and SPS. SPS and hemoglobin combine in a first-order kinetic interaction to form an insoluble precipitate. The optimum ratio occurs when 1 g of SPS binds to 6 g of hemoglobin. At saturation, if one assumes for purposes of calculation that SPS has an average molecular weight of 10,000 (L. J. Sorensen, Hoffmann-LaRoche, personal communication), an affinity equilibrium dissociation constant of approximately 10^{-7} mol/liter was computed.

Protective effect of hemoglobin on SPS-sensitive *Neisseria* isolates. Figure 2 presents the time-kill studies of SPS-sensitive and SPS-resistant isolates of *N. meningitidis* and *N. gonorrhoeae* in

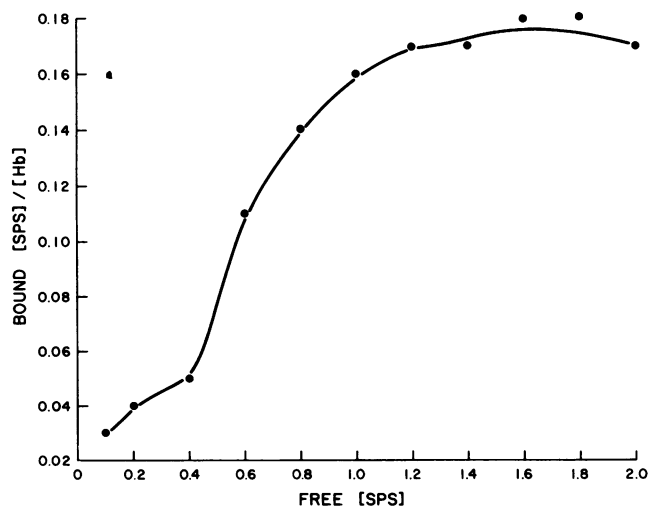

FIG. 1. Binding of SPS to hemoglobin. The reaction took place in an ultrafilter cell above a cellulose acetate membrane designed to exclude molecules of molecular weight ≥20,000. Concentrations of hemoglobin from 0.01 to 2% (wt/vol) were added to 0.1% SPS (wt/vol). Free SPS was determined by activating the ultrafilter device and assaying SPS in the effluent.

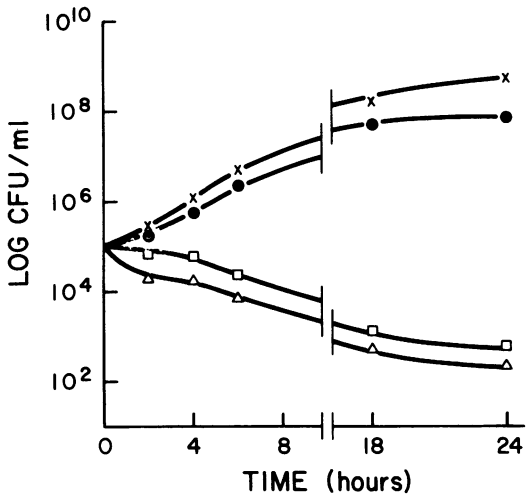

FIG. 2. Effect of hemoglobin on the anti-*Neisseria* activity of SPS. Microbes were grown in 0.5% SPS alone and at the optimum combining ratio of 1:6. Shown are *N. meningitidis* in 0.5% SPS (□), *N. gonorrhoeae* in 0.5% SPS (△), *N. meningitidis* in 0.5% SPS plus 3.0% hemoglobin (×), and *N. gonorrhoeae* in 0.5% SPS plus 3.0% hemoglobin (●).

the presence of the optimum SPS:hemoglobin ratio, 1:6 (wt/wt). Table 1 demonstrates the modulating effect hemoglobin has on the anti-*Neisseria* activity of SPS.

Hemoglobin exerted a protective effect on SPS-sensitive isolates. The effect was maximum at a ratio of 1:6 (SPS:hemoglobin). This corresponded with the maximum binding of SPS to hemoglobin (see Fig. 1). In the presence of SPS, all sensitive *N. meningitidis* and *N. gonorrhoeae* isolates demonstrated a decrease in log CFU per milliliter over time, with a major decrease occurring after 4 h of incubation. The protective effect of hemoglobin on SPS occurred at the first sampling time (2 h). This observation was in keeping with a chemical reaction between SPS and hemoglobin rather than an indirect effect through the bacterial cell metabolism. At an SPS-hemoglobin ratio of 1:6 (wt/wt), there was no difference between growth in the control tubes or among the SPS-resistant and SPS-sensitive species tested (see Table 1). There was little variation in the growth of isolates of each species. The inhibition of SPS on sensitive isolates was directly related to the amount of unbound SPS in solution.

DISCUSSION

It is known that SPS can combine with polycationic compounds in a stoichiometric manner governed by first-order kinetics to render each reactant inactive. The reaction is rapid, and the insoluble polymer formed removes each constituent from solution and effectively obviates their activity. In blood, the most commonly encountered polycationic ligands are beta-lipoproteins, complement components, clotting factors, and polymorphonuclear neutrophils. In addition, in blood culture bottles, one may encounter aminoglycoside-aminocyclitol and polymyxin antibiotics.

There has been incongruity concerning the reported activity of SPS against *Neisseria* isolates. Studies with plate media and non-blood culture techniques found that SPS at concentrations as low as 0.05% may inhibit *Neisseria*. There was considerable variation in the sensitivity of individual strains of a species. Conversely, studies in which blood culture bottles were used in a clinical setting showed essentially no difference between the isolation of bacteria, with or

TABLE 1. Modulation of the anti-*Neisseria* activity of SPS by hemoglobin

Medium or SPS/hemoglobin mixture ratio	SPS-sensitive isolates										SPS-resistant isolates									
	N. meningitidis (log CFU/ml) after (h):					*N. gonorrhoeae* (log CFU/ml) after (h):					*N. meningitidis* (log CFU/ml) after (h):					*N. gonorrhoeae* (log CFU/ml) after (h):				
	2	4	6	18	24	2	4	6	18	24	2	4	6	18	24	2	4	6	18	24
Broth control	5.5	6.0	6.5	8.1	8.4	5.3	5.8	6.4	7.6	7.8	5.4	6.1	6.4	8.3	8.7	5.4	5.8	6.4	7.7	7.9
Broth + 0.5% SPS	4.8	4.7	4.3	3.2	2.8	4.4	4.2	3.9	2.7	2.4	5.3	5.9	6.2	7.9	8.3	5.5	5.8	6.2	7.6	7.8
Broth + 1.0% hemoglobin	5.3	5.9	6.6	8.0	8.4	5.4	5.9	6.5	7.9	8.1	5.5	6.0	6.2	7.8	8.3	5.5	5.9	6.3	7.8	8.0
1:33	4.7	4.7	4.2	3.0	2.9	4.5	4.0	4.0	2.9	3.0	5.3	5.8	6.4	7.7	8.1	5.4	5.6	6.3	7.5	8.0
1:25	4.8	4.8	4.7	4.2	4.1	4.6	3.8	3.6	3.1	3.0	5.3	6.0	6.6	8.1	8.5	5.5	5.9	6.3	7.5	7.9
1:20	5.1	5.3	5.4	5.5	5.4	5.0	5.1	5.1	5.0	4.9	5.4	6.0	6.3	8.3	8.6	5.2	5.7	6.3	8.0	8.1
1:10	5.0	5.4	6.0	7.2	7.3	4.8	5.1	5.8	6.9	7.1	5.3	5.7	6.5	7.8	8.3	5.2	5.4	6.1	7.5	8.0
1:6	5.3	5.8	6.5	8.0	8.4	5.3	5.7	6.3	7.6	7.8	5.3	6.0	6.1	7.9	8.3	5.4	5.8	6.4	7.8	8.0

[a] Average of test results for five isolates.

without SPS present. One variable not taken into account in these investigations was the concentration of hemoglobin in the media.

Hemoglobin may be found in both plate media and blood culture bottles. Hemoglobin is present in blood-containing plate media, not as an additive but with the blood. As a result, the exact concentration of hemoglobin in blood-containing agar plates is not measured. The amount of free hemoglobin found in blood culture bottles can vary much more widely than the concentration found in agar plates. In blood culture bottles, the free hemoglobin enters the system with the blood and can change with the amount of hemolysis occurring inside the bottle. Hemoglobin concentrations can vary from less than 0.01% to over 1% just by adding blood to the blood culture bottle.

That hemoglobin can combine with SPS to render it inactive can partly explain the difference in antimicrobial activity observed in various test systems. The affinity constants of SPS for neutrophils, complement components, and beta-lipoproteins are not known, but the calculated approximate K_d of 10^{-7} mol of SPS per liter for hemoglobin is not strong. If the affinity constants for other serum components and clotting factors are greater than hemoglobin, those with the highest K_d would be affected preferentially. Therefore, the activity of some serum components, such as neutrophil function, can be affected, whereas other activities, such as inactivation of complement, may be only partial. We postulate that it is only the residual SPS in the blood culture bottle after all antibiotics and serum components have combined with the polyanion that is available to interact with potentially sensitive microbes, and the residual polyanion concentration can vary widely.

Any comparison among polyanionic detergents must control for all possible ligands to which the detergent can combine. Accordingly, comparisons between SPS and sodium amylosulfate or other polyanions must be made under the same conditions; clinical studies in which human blood in inexact amounts is added to blood culture media will not provide reproducible results. Furthermore, when other polyanionic compounds such as dextran sulfate (8) are considered, each compound must be tested under exactly the same conditions. This will permit one to equalize results to unit charge per length of detergent molecule.

One must also consider the construction of the receptor on the surface of sensitive microorganisms. We currently do not know the nature of these receptors or if they are constitutive or inducible. The type of microbe response to the polyanionic detergent must be regarded when examining potentially new agents.

In conclusion, we showed that hemoglobin can inactivate SPS at an optimum SPS/hemoglobin ratio of 1:6. This finding must be taken into account whenever the effects of polyanionic detergents against microbes are considered. Because hemoglobin is one of the major constituents inside blood culture bottles, and since by lysis its concentration can change over time, it is extremely difficult to predict whether the amount of free hemoglobin will be enough to inhibit a particular isolate of *Neisseria* in an individual blood culture bottle.

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