

## Studies on Neutralization of Human Serum Bactericidal Activity by Sodium Amylosulfate

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The synthetic anticoagulant sodium amylosulfate (SAS) at concentrations of 125 to 2,000  $\mu\text{g/ml}$  failed to completely neutralize the bactericidal activity of 80 and 50% (by volume) fresh human serum. Furthermore, SAS failed to inhibit the alternative pathway of complement activation in 80% (by volume) fresh human serum that had been chelated with 0.01 M magnesium ions plus 0.01 M ethyleneglycol-bis( $\beta$ -aminoethylether)-*N,N*-tetraacetic acid. However, SAS at 250 to 1,000  $\mu\text{g/ml}$  effectively neutralized the bactericidal activity of 20% (by volume) fresh human serum. Therefore, SAS (at 250 to 1,000  $\mu\text{g/ml}$ ) should be used only in blood samples that have been diluted at least fivefold ( $\leq 20\%$  [by volume]) in suitable broth media.

For decades most clinical microbiology laboratories used the synthetic anticoagulant sodium polyanetholesulfonate (SPS) to neutralize complement-mediated bactericidal activity in human blood samples and/or cultures (7, 16, 18, 23). This substance was shown also to inhibit (complement-dependent) phagocytosis (1, 2). Furthermore, it was recently demonstrated that SPS effectively neutralized both the classical and the alternative pathways (8, 9, 17) of human complement activation (22). Long ago, however, SPS was found to inhibit the growth of anaerobic streptococci, particularly *Peptostreptococcus anaerobius* (10, 12, 24); also, *Neisseria meningitidis* and *Neisseria gonorrhoeae* proved susceptible to SPS (4). The synthetic anticoagulant sodium amylosulfate (SAS), a sulfated polyanion, like SPS, was recently introduced for use in human blood cultures (15). Apart from its anticomplementary activity, SAS was reported to exert no deleterious effects against peptostreptococci (13). As some laboratories adopted SAS for supplementation of blood cultures (11, 14), it was deemed necessary to examine this substance with respect to its usefulness for complete inactivation of both pathways of human complement activation, as endotoxin from gram-negative bacteria (5) and viable *Enterobacteriaceae* previously had been shown to activate both complement pathways (20-22).

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### MATERIALS AND METHODS

**Bacteria.** The "promptly serum-sensitive" strain *Escherichia coli* C was used in all serum assays (19-22).

**Media.** Tryptic soy (TS) broth and agar and brain heart infusion broth and agar were purchased from Difco Laboratories, Detroit, Mich. The bacteria were maintained on TS agar slants at 4°C and in a mixture of 1 volume of brain heart infusion broth plus 1 volume of heat-inactivated bovine serum (Behringwerke, A.G. Marburg/Lahn, West Germany) at -65°C.

**Reagents.** SAS (lot no. M.H.4-24.A) was a gift from G. D. Searle & Co., Chicago, Ill. Aqueous stock solutions of SAS, containing either 5,000  $\mu\text{g/ml}$  (0.5%) or 20,000  $\mu\text{g/ml}$  (2%), were sterilized by autoclaving at 121°C for 15 min. As required, SAS was serially diluted twofold in TS broth. SPS (lot no. K 2710) was received from Hoffmann-La Roche A.G., Basel, Switzerland. Aqueous stock solutions of SPS (5,000  $\mu\text{g/ml}$  = 0.5%) were sterilized as above (22). Ethyleneglycol-bis( $\beta$ -aminoethylether)-*N,N*-tetraacetic acid (EGTA) was obtained from Serva Feinbiochemica GmbH, Heidelberg, West Germany. Aqueous 0.1 M stock solutions of EGTA (pH range, 7.4 to 7.5) were prepared, membrane filter sterilized as previously described (21), and stored at 4°C. Analytical-grade  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  was purchased from E. Merck A.G., Darmstadt, West Germany. Aqueous stock solutions of  $\text{MgCl}_2$  (0.1 M) were sterilized by autoclaving.

**Serum assays.** One healthy adult (W.H.T. = T-serum) repeatedly served as blood donor. The sera were processed and maintained in 5-ml portions at -65°C as previously described (19-22). The portions were heat inactivated at 56°C in a water bath as required. The serum assays (final volume, 2 ml) were performed in sterile polycarbonate tissue cul-

ture tubes (C. A. Greiner und Söhne, Nürtingen, West Germany) which were incubated stationary at 35°C. Fresh T-serum (T-C) was used as such (80, 50, and 20% [by volume] in TS broth), or 80% (by volume) T-C was chelated with 0.01 M MgCl<sub>2</sub> plus 0.01 M EGTA (T-C-Mg/EGTA). Fresh T-C and T-C-Mg/EGTA received SAS at final concentrations of 2,000, 1,000, 500, 250, or 125 µg/ml, unless specified otherwise. Log-phase bacterial inocula (0.2 ml) of control strain *E. coli* C were added to 1.8-ml amounts of T-C, chemically modified T-C, or heat-inactivated T-C. The inocula had been adjusted to yield approximately  $1.5 \times 10^4$  colony-forming units per ml at zero time (20). At 0, 0.3, 1, 2, 4, and 22 h after exposure, samples were removed from the assay tubes and serially diluted 10-fold in TS broth. Two brain heart infusion agar pour plates per dilution served to determine the number of survivors (as colony-forming units per milliliter) after overnight incubation at 35°C.

### RESULTS

In preliminary serum assays, 500, 250, and 125 µg of SAS per ml were added to 80% (by volume) of fresh T-C and 80% (by volume) Mg/EGTA-chelated T-C (T-C-Mg/EGTA). As shown in Table 1, SAS failed to neutralize the

bactericidal activity of T-C against control strain *E. coli* C; within 1 h after exposure, the bacterial inocula had been killed. Different results were obtained with SAS-treated T-C-Mg/EGTA. At a final concentration of 250 µg/ml, SAS neutralized serum bactericidal activity, but the growth of *E. coli* C was retarded during overnight incubation. SAS at 125 µg/ml failed to completely inactivate the bactericidal activity of T-C-Mg/EGTA; the *E. coli* C cells appeared to recover and multiply for several hours, only to be inhibited after prolonged incubation. However, SAS at 500 µg/ml completely failed to block the bactericidal activity of T-C-Mg/EGTA; the killing kinetics of control strain *E. coli* C proved similar to those obtained with control T-C-Mg/EGTA. In the next series of experiments, the concentrations of SAS were raised. As demonstrated in Table 2, SAS at 2,000 µg/ml in 80% (by volume) T-C failed to protect *E. coli* C inocula; there ensued delayed killing. This was true also for T-C that had received 1,000 µg of SAS per ml. In the case of 80% (by volume) T-C-Mg/EGTA, 2,000 and 1,000 µg of SAS per ml blocked prompt killing

TABLE 1. Incomplete inhibition of bactericidal activity in 80% (by volume) fresh human serum by SAS

Sample time (h postexposure)	<i>E. coli</i> C survivors (CFU <sup>a</sup> /ml) with:								
	T-C <sup>b</sup> + SAS <sup>c</sup> (500)	T-C + SAS (250)	T-C + SAS (125)	T-C	T-C-Mg/EGTA + SAS (500)	T-C-Mg/EGTA + SAS (250)	T-C-Mg/EGTA + SAS (125)	T-C-Mg/EGTA	T-56 <sup>d</sup>
0					$2.0 \times 10^4$				
0.3	$1.0 \times 10^4$	$3.5 \times 10^1$	0	0	$1.5 \times 10^4$	$2.0 \times 10^4$	$1.6 \times 10^4$	$2.1 \times 10^4$	$2.0 \times 10^4$
1	0	0	0	0	$5.0 \times 10^0$	$1.8 \times 10^4$	$1.9 \times 10^4$	$7.1 \times 10^3$	$2.9 \times 10^4$
2	0	0	0	0	0	$6.5 \times 10^4$	$4.1 \times 10^4$	0	$8.1 \times 10^4$
4	0	0	0	0	0	$8.5 \times 10^4$	$8.0 \times 10^4$	0	$2.7 \times 10^5$
22	0	0	0	0	0	$4.0 \times 10^4$	$5.5 \times 10^4$	0	$>10^8$

<sup>a</sup> CFU, Colony-forming units.

<sup>b</sup> T-C, 80% (by volume).

<sup>c</sup> SAS had been added to the indicated final concentrations (in micrograms per milliliter) before addition of bacterial inocula.

<sup>d</sup> T-56<sup>d</sup>, 80% (by volume) heat-inactivated (56°C, 30 min) T-C.

TABLE 2. Incomplete neutralization of serum bactericidal activity in 80% (by volume) fresh human serum by SAS<sup>a</sup>

Sample time (h postexposure)	<i>E. coli</i> C survivors (CFU/ml) with:										
	T-C + SAS (2,000)	T-C + SAS (1,000)	T-C + SAS (500)	T-C + SAS (250)	T-C	T-C-Mg/EGTA + SAS (2,000)	T-C-Mg/EGTA + SAS (1,000)	T-C-Mg/EGTA + SAS (500)	T-C-Mg/EGTA + SAS (250)	T-C-Mg/EGTA	T-56 <sup>c</sup>
0						$1.4 \times 10^4$					
0.3	$1.6 \times 10^4$	$1.6 \times 10^4$	$2.1 \times 10^3$	0	0	$1.7 \times 10^4$	$1.7 \times 10^4$	$1.3 \times 10^4$	$1.3 \times 10^4$	$1.4 \times 10^4$	$1.6 \times 10^4$
1	$2.7 \times 10^4$	$1.2 \times 10^4$	0	0	0	$2.2 \times 10^4$	$2.1 \times 10^4$	0	$8.8 \times 10^3$	$1.1 \times 10^4$	$2.4 \times 10^4$
2	$4.6 \times 10^4$	$1.8 \times 10^3$	0	0	0	$6.6 \times 10^4$	$4.0 \times 10^4$	0	$2.1 \times 10^4$	$2.0 \times 10^3$	$4.3 \times 10^4$
4	$5.2 \times 10^4$	0	0	0	0	$1.8 \times 10^5$	$1.2 \times 10^5$	0	$1.3 \times 10^4$	0	$9.0 \times 10^4$
22	0	0	0	0	0	$1.2 \times 10^4$	0	0	$4.2 \times 10^4$	0	$>10^8$

<sup>a</sup> See Table 1 for explanatory footnotes.

of *E. coli* C; there occurred, however, bacteriostatic and bactericidal activity of T-C-Mg/EGTA upon prolonged incubation. Again, 500  $\mu\text{g}$  of SAS per ml failed to protect *E. coli* C in T-C-Mg/EGTA, whereas 250  $\mu\text{g}$  of SAS per ml almost completely neutralized serum bactericidal activity, though a late bacteriostatic effect was apparent after overnight incubation.

The final series of serum assays served to determine whether 500  $\mu\text{g}$  of SAS per ml sufficed for neutralization of the bactericidal activity of smaller percent (by volume) concentrations of fresh human serum. For this purpose, 1,000, 500, 250, and 125  $\mu\text{g}$  of SAS per ml were added to both 50 and 20% (by volume) T-C. A representative experiment is shown in Table 3. Concentrations of SAS as high as 1,000  $\mu\text{g}/\text{ml}$  failed to completely inactivate the bactericidal activity of 50% (by volume) T-C. However, 1,000, 500, and 250  $\mu\text{g}$  of SAS per ml effectively neutralized the bactericidal activity of 20% (by volume) fresh human serum.

### DISCUSSION

In contrast to SPS (22), SAS at 125 to 2,000  $\mu\text{g}/\text{ml}$  failed to completely neutralize the bactericidal activity of 80 and 50% (by volume) fresh T-C (Tables 1 and 2). However, SAS at 250 to 1,000  $\mu\text{g}/\text{ml}$  inhibited complement-mediated killing of the exquisitely serum-sensitive assay strain *E. coli* C when the serum concentrations had been lowered to 20% (by volume) (Table 3). Unexpected was the finding that SAS at 500  $\mu\text{g}/\text{ml}$  was less effective than either lower (125 and 250  $\mu\text{g}/\text{ml}$ ) or higher (1,000 and 2,000  $\mu\text{g}/\text{ml}$ ) concentrations with respect to the neutralization of the bactericidal activity of 80% (by volume) Mg/EGTA-chelated fresh T-C (Tables 1 and 2). Chelation of fresh human serum with Mg/EGTA had been shown by Des Prez et al. (3) and by Fine et al. (6) to render the classical pathway of complement activation nonfunc-

tional via sequestration of calcium ions. Possibly, SAS at 500  $\mu\text{g}/\text{ml}$ , but not at higher or lower concentrations, permitted minute amounts of calcium ions to remain free. This small, unsequestered amount of ionized calcium might have allowed concurrent activation of both pathways of complement, thus accounting for the observed slightly more rapid killing of control strain *E. coli* C in SAS-treated (500  $\mu\text{g}/\text{ml}$ ) Mg/EGTA-chelated serum as compared with control Mg/EGTA-chelated fresh T-C. In any case, SAS, unlike SPS, was ineffective with respect to the neutralization of the alternative pathway of complement activation in 80% (by volume) fresh human serum.

These results warrant caution against the indiscriminate use of SAS in human blood cultures. Although Kocka et al. (14) and Hall et al. (11) observed no significant differences in isolation rates of bacteria from blood cultures to which had been added SAS and SPS, respectively, the latter investigators had noted that certain gram-negative bacteria were recovered less frequently from blood cultures supplemented with SAS. Therefore, when using SAS at the manufacturer's recommended concentration of 500  $\mu\text{g}/\text{ml}$ , blood samples should be diluted at least fivefold ( $\leq 20\%$  [by volume]) so as to ensure effective inactivation of complement (Table 3). In view of the different anticomplementary properties of the two synthetic anticoagulants SAS and SPS, further comparative investigations are called for to elucidate the mechanism(s) whereby these substances interact with components comprising the classical and alternative pathways of human complement activation.

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TABLE 3. Neutralization of bactericidal activity of 20% (by volume) T-C by SAS, and lack thereof in 50% (by volume) fresh T-C<sup>a</sup>

Sample time (h postexposure)	<i>E. coli</i> C survivors (CFU/ml) with:										
	50% T-C + SAS (1,000)	50% T-C + SAS (500)	50% T-C + SAS (250)	50% T-C + SAS (125)	50% T-C	20% T-C + SAS (1,000)	20% T-C + SAS (500)	20% T-C + SAS (250)	20% T-C + SAS (125)	20% T-C	T-56° (80% [by vol])
0											
0.3	$1.4 \times 10^4$	$1.5 \times 10^4$	$1.2 \times 10^4$	0	0	$1.4 \times 10^4$	$1.3 \times 10^4$	$1.5 \times 10^4$	$1.5 \times 10^4$	0	$1.4 \times 10^4$
1	$1.4 \times 10^4$	$3.9 \times 10^2$	0	0	0	$1.7 \times 10^4$	$1.9 \times 10^4$	$1.9 \times 10^4$	$5.7 \times 10^3$	0	$2.1 \times 10^4$
2	$4.5 \times 10^3$	0	0	0	0	$3.7 \times 10^4$	$4.0 \times 10^4$	$2.2 \times 10^4$	$2.1 \times 10^2$	0	$5.8 \times 10^4$
4	0	0	0	0	0	$1.1 \times 10^6$	$8.2 \times 10^5$	$2.6 \times 10^5$	0	0	$1.9 \times 10^5$
22	0	0	0	0	0	$>10^8$	$>10^8$	$>10^8$	0	0	$>10^8$

<sup>a</sup> See Table 1 for explanatory footnotes.

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