Inactivation of Classical and Alternative Pathway-Activated Bactericidal Activity of Human Serum by Sodium Polyanetholsulfonate

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Sodium polyanetholsulfonate (SPS) at a final concentration of at least 250 $\mu\text{g/ml}$ (0.025%) was required for inhibition of the bactericidal activity of 80% (vol/vol) of fresh human serum against "promptly serum-sensitive" strains of *Serratia marcescens* and control strain *Escherichia coli* C, i.e., for inhibition of the classical pathway of complement activation. In contrast, SPS at 125 $\mu\text{g/ml}$ (0.0125%) was sufficient for neutralization of the bactericidal activity of 80% (vol/vol) fresh human serum against "delayed serum-sensitive" strains of *S. marcescens* known to activate the alternative pathway of human complement. Addition of up to 500 μg of SPS per ml to 80% (vol/vol) fresh human serum failed to neutralize transferrin-mediated, "late" bacteriostasis against control strain *E. coli* C, an effect that was demonstrable only after prolonged, i.e., overnight, incubation of the test strain. However, this late inhibitory effect against *E. coli* C was not observed in SPS-treated 20% (vol/vol) fresh human serum or in 10 or 20% (vol/vol) conventionally heat-inactivated human serum. Immunoelectrophoretic examination disclosed that SPS did not precipitate transferrin from either fresh or heat-inactivated human serum. Thus, SPS, at 250 $\mu\text{g/ml}$, was demonstrated to be sufficient for the inhibition of both classical and alternative complement pathway-activated bactericidal activity of 80% (vol/vol) human serum. However, SPS at a concentration of 500 $\mu\text{g/ml}$ failed to antagonize one antimicrobial system of 80% (vol/vol) human serum, namely transferrin-mediated bacteriostasis.

Recent studies in our laboratory concerning the susceptibility of clinical isolates of *Serratia marcescens* to the bactericidal activity of fresh human serum revealed that over 80% of the isolates examined proved "delayed serum-sensitive" (DSS), i.e., were killed by 80% (vol/vol) fresh human serum only after several hours of exposure. "Promptly serum-sensitive" (PSS) isolates of *S. marcescens*, on the other hand, were killed within minutes, i.e., in a manner analogous to control strain *Escherichia coli* C (16). Further experiments utilizing fresh human serum that had been chelated with 0.01 M MgCl_2 plus 0.01 M ethyleneglycoltetraacetic acid (EGTA), a procedure (2, 4) known to render the classical pathway of complement activation nonfunctional, permitted the tentative conclusion that DSS strains of *S. marcescens* were killed by the activation of the alternative pathway of human complement (5, 6, 14), whereas PSS isolates and control strain *E. coli*

C were killed through classically activated serum bactericidal activity (17). The synthetic anticoagulant sodium polyanetholsulfonate (SPS) has been used for decades to neutralize serum bactericidal activity in blood samples for bacteriological culture (19). The present study examined SPS inhibition of both pathways of human complement activation at various concentrations. Furthermore, the study determined that SPS, at the usual concentrations used in blood samples/cultures (0.025 to 0.05%), failed to neutralize transferrin-mediated bacteriostasis of 80% (vol/vol) human serum against control strain *E. coli* C, a finding substantiated by the observation that SPS did not precipitate transferrin either from fresh or heat-inactivated human serum.

MATERIALS AND METHODS

Bacterial strains. The DSS *S. marcescens* isolates no. SE 142 and SE 143-b and the PSS *S. marcescens* strain no. SM 29 were the same as previously used (16). The other PSS *S. marcescens* isolate, strain

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CDC O13:H4, was a gift from Betty R. Davis, Enterobacteriology Unit, Center for Disease Control, Atlanta, Ga. The PSS strain *E. coli* C served as a control in all serum assays (15).

Media. Tryptic soy agar and broth, brain heart infusion broth and agar, and Noble special agar were purchased from Difco Laboratories, Detroit. The test strains were maintained on tryptic soy 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 AG, Marburg, West Germany) at -65°C.

Chemical and immunological reagents. SPS was obtained through the courtesy of Hoffmann-La Roche AG, Basel, Switzerland (lot no. K 2710). Aqueous stock solutions of SPS containing 5,000 µg/ml (0.5%) were sterilized by autoclaving at 121°C for 15 min. As required, SPS was serially diluted two-fold in tryptic soy broth. EGTA was purchased from Serva Feinbiochemica GmbH, Heidelberg, West Germany; aqueous 0.1 M stock solutions of EGTA (pH range 7.4 to 7.5) were prepared and sterilized as described previously (17) and stored at 4°C. Analytical grade MgCl₂·6H₂O was obtained from E. Merck, Darmstadt, West Germany; aqueous 0.1 M stock solutions of MgCl₂ were sterilized by autoclaving. Lyophilized human transferrin (lot no. 1627 B) and rabbit anti-human transferrin immune serum (batch no. 2699 D) were procured from Behringwerke.

Serum assays. Fresh human serum (T-serum) was obtained repeatedly from one healthy adult volunteer (W.H.T.); the serum was processed and maintained at -65°C as previously described (16, 17). As needed, aliquots were heat-inactivated at 56°C for 30 min in a water bath. All serum assays (final volume = 2 ml) were performed in sterile, polycarbonate tissue culture tubes (C. A. Greiner und Söhne, Nürtingen) which were incubated without shaking at 35°C. As required, fresh T-serum (T-C) was either supplemented with SPS at final concentrations of 500, 250, 125, and 63 µg/ml, or chelated with 0.01 M MgCl₂ plus 0.01 M EGTA as described before (17). Log-phase bacterial inocula adjusted to yield approximately 1.5×10^4 colony-forming units (CFU)/ml at zero time (16, 17) were exposed to 80% (vol/vol) T-C, heat-inactivated (T-56°), or chemically modified T-serum. At 0, 0.3, 1, 2, 4, 6, and 22 h after exposure, unless stated otherwise, samples were removed from the assay tubes and serially diluted 10-fold in tryptic soy broth. Two brain heart infusion agar pour plates per dilution, which were incubated at 35°C overnight, served to determine the number of survivors (CFU per milliliter).

Immunoelectrophoresis. Immunoelectrophoretic experiments were carried out on glass slides with 1.5% Noble agar plus high resolution buffer (Gelman) at 4 mA per frame for 2 h, using Gelman equipment (CAMAG AG, Berlin). Wells received 1 µl of fresh T-serum, heat-inactivated T-serum, and a control stock solution of human transferrin (5 mg/ml of phosphate-buffered saline, pH 7.5), with and without 500 µg of SPS per ml, respectively, and

1 µl of the SPS precipitate from fresh T-serum that had been washed once in 500 µg of aqueous SPS per ml. The troughs were charged with 20 µl of rabbit anti-human transferrin immune serum. The immunoelectrophoretic slides were incubated in humidity chambers for 24 h at room temperature, stained with amido black 10 B, and decolorized in accordance with the instructions of the Gelman Instrument Co.

RESULTS

The first series of experiments established the amount (in terms of percentage [volume/volume]) of fresh T-C required for complete killing of bacterial inocula of both PSS and DSS strains of *S. marcescens*. A representative experiment is shown in Table 1. As before (10), 10% (vol/vol) T-C sufficed for prompt killing of control strain *E. coli* C. Similarly, 10% (vol/vol) T-C effected killing of the PSS *S. marcescens* strain no. SM 29, although at a slightly reduced kinetic rate. In contrast, the DSS *S. marcescens* isolate no. SE 142 required at least 50% (vol/vol) T-C for effective killing; in the case of 20% (vol/vol) T-C, a rebound in growth occurred during overnight incubation, and 10% (vol/vol) T-C resulted merely in a transient reduction in the number of surviving CFU per milliliter, with rebound growth occurring as early as 6 h after exposure. As noted before (17), 80% (vol/vol) heat-inactivated T-serum (T-56°) exerted a bacteriostatic effect against control strain *E. coli* C after prolonged, i.e., overnight, incubation, but not against any of the *S. marcescens* isolates tested.

The next series of experiments determined the number of CFU of DSS and PSS isolates of *S. marcescens* per milliliter killed by 80% (vol/vol) T-C, as contrasted with control strain *E. coli* C. As demonstrated in Table 2, 80% (vol/vol) T-C promptly killed 1.5×10^7 CFU of *E. coli* C per ml as well as 1.9×10^7 CFU of the PSS *S. marcescens* strain SM 29 per ml, the latter though at a slightly reduced kinetic rate. On the other hand, 80% (vol/vol) T-C resulted in killing of 1.3×10^6 CFU of the DSS *S. marcescens* isolate no. SE 143-b per ml, but failed to completely kill the inoculum of 1.1×10^7 CFU/ml. Interestingly, T-56° serum, which had been stored at 4°C overnight and reactivated at 56°C for 10 min, exerted no bacteriostatic effect against control strain *E. coli* C upon overnight incubation.

Following these preliminary experiments, the amount of SPS necessary to inhibit the classical and the alternative pathway of complement activation in fresh human serum was assessed. For this purpose, 500, 250, 125, and 63

TABLE 1. Determination of percentage (volume/volume) of fresh human serum required for killing of PSS *S. marcescens* isolate no. SM 29 and of DSS *S. marcescens* isolate no. SE 142

Time that tube samples were assayed (h)	PSS <i>S. marcescens</i> SM 29 (CFU/ml)					DSS <i>S. marcescens</i> SE 142 (CFU/ml)					PSS <i>E. coli</i> C (control) (CFU/ml)		
	T-C ^a (10%, vol/vol)	T-C (20%, vol/vol)	T-C (50%, vol/vol)	T-C (80%, vol/vol)	T-56 ^b (80%, vol/vol)	T-C (10%, vol/vol)	T-C (20%, vol/vol)	T-C (50%, vol/vol)	T-C (80%, vol/vol)	T-56 ^b (80%, vol/vol)	T-C (10%, vol/vol)	T-56 ^b (80%, vol/vol)	
	1.8 × 10 ⁴ 4.3 × 10 ²	1.8 × 10 ⁴ 2.5 × 10 ²	1.8 × 10 ⁴ 5.0 × 10 ¹	1.8 × 10 ⁴ 0	1.8 × 10 ⁴ 1.7 × 10 ⁴	1.7 × 10 ⁴ 1.7 × 10 ⁴	1.7 × 10 ⁴ 2.3 × 10 ³	1.7 × 10 ⁴ 1.6 × 10 ³	1.7 × 10 ⁴ 3.0 × 10 ²	1.7 × 10 ⁴ 2.2 × 10 ⁴	1.5 × 10 ⁴ 0	1.5 × 10 ⁴ 0	
0	1.8 × 10 ⁴	1.8 × 10 ⁴	1.8 × 10 ⁴	1.8 × 10 ⁴	1.8 × 10 ⁴	1.7 × 10 ⁴	2.3 × 10 ³	1.6 × 10 ³	1.5 × 10 ³	1.3 × 10 ⁴	1.5 × 10 ⁴	1.5 × 10 ⁴	1.5 × 10 ⁴
0.3	4.3 × 10 ²	2.5 × 10 ²	5.0 × 10 ¹	0	1.7 × 10 ⁴	1.7 × 10 ⁴	2.3 × 10 ³	1.6 × 10 ³	1.5 × 10 ³	1.3 × 10 ⁴	1.5 × 10 ⁴	1.5 × 10 ⁴	1.5 × 10 ⁴
1	0	0	0	0	1.6 × 10 ⁴	1.7 × 10 ⁴	4.9 × 10 ²	3.6 × 10 ²	3.0 × 10 ²	2.2 × 10 ⁴	0	0	2.4 × 10 ⁴
2	0	0	0	0	2.0 × 10 ⁴	1.5 × 10 ³	4.9 × 10 ²	3.6 × 10 ²	3.0 × 10 ²	2.2 × 10 ⁴	0	0	5.5 × 10 ⁴
4	0	0	0	0	3.5 × 10 ⁴	8.6 × 10 ²	1.0 × 10 ²	7.0 × 10 ¹	8.0 × 10 ¹	8.5 × 10 ⁴	0	0	2.3 × 10 ⁵
6	0	0	0	0	>10 ⁸	5.2 × 10 ³	6.0 × 10 ¹	5.0 × 10 ¹	2.0 × 10 ¹	7.4 × 10 ⁵	0	0	2.0 × 10 ⁶
22	0	0	0	0	>10 ⁸	>10 ⁸	2.4 × 10 ⁶	0	0	>10 ⁸	0	0	2.0 × 10 ⁶

^a Serum received by assay tubes.

^b 80% (vol/vol) heat-inactivated (56°C, 30 min) human serum, which had been stored at 4°C overnight and reactivated at 56°C for 10 min.

μg of SPS per ml were added to 80% (vol/vol) T-C before the addition of the bacterial inocula. As shown in Table 3, at least 250 μg of SPS per ml was required to inactivate the bactericidal activity of T-C against control strain *E. coli* C. However, the number of viable cells diminished markedly during overnight incubation at this particular concentration of SPS. The addition of 500 μg of SPS per ml to T-C completely neutralized serum bactericidal activity; but as with T-56° serum, a late bacteriostatic effect occurred after prolonged, i.e., overnight, incubation. In contrast, only half as much, namely, 125 μg of SPS per ml, was sufficient to neutralize the bactericidal activity of 80% (vol/vol) T-C against the DSS *S. marcescens* isolate no. SE 142. Furthermore, neither T-56° nor SPS-treated T-C exerted a late bacteriostatic effect during overnight incubation. In analogy to control strain *E. coli* C, at least 250 μg of SPS per ml was required to protect the PSS *S. marcescens* strain no. CDC O13:H4 from the lethal activity of T-C (Table 4). Again, this strain was not inhibited by either T-56° or by SPS-treated T-C after prolonged incubation.

With regard to the alternative pathway of complement, 125 μg of added SPS per ml sufficed to inhibit the bactericidal activity of Mg-EGTA-chelated T-C serum against control strain *E. coli* C, whereas at least 250 μg of SPS per ml was required for the same purpose in the case of control fresh human serum, as demonstrated in Table 5. Again, T-C with 250 μg of added SPS per ml effected a diminution of viable *E. coli* C cells after prolonged incubation, and T-56°, as well as Mg-EGTA-chelated, SPS-treated T-C, and control T-C treated with 500 μg of SPS per ml, exerted a late bacteriostatic effect against *E. coli* strain C. However, this late bacteriostatic effect of SPS-treated 80% (vol/vol) T-C was no longer demonstrable when the amount of SPS-treated T-C had been reduced to 20% (vol/vol) (Table 6), or when the amount of T-56° serum had been decreased to 20% (vol/vol).

Finally, previous experiments in this laboratory had shown that the late bacteriostatic effect of 80 and 50% (vol/vol) heat-inactivated T-serum against control strain *E. coli* C was due to serum transferrin. Briefly, addition of either 50 to 100 μM ferrous or 25 to 50 μM ferric ions to 80% (vol/vol) T-56° serum prevented this late onset bacteriostatic effect. Heat-inactivation of 50% (vol/vol) T-serum at 65°C for 30 min also resulted in unretarded growth of control strain *E. coli* C. Therefore, it was of interest to determine whether SPS precipitated this particular serum protein from fresh human serum.

TABLE 2. Determination of the number of CFU of DSS and PSS strains of *S. marcescens* killed by 80% (vol/vol) fresh human serum

Time that assay tubes were sampled (h)	DSS <i>S. marcescens</i> SE 143-b (CFU/ml)				PSS <i>S. marcescens</i> CDC O13:H4 (CFU/ml)				PSS <i>E. coli</i> C (CFU/ml)	
	T-C ^a	T-C	T-C	T-56 ^b	T-C	T-C	T-C	T-56 ^b	T-C ^a	T-56 ^b
0	1.4×10^4	1.3×10^6	1.1×10^7	1.4×10^4	2.3×10^4	1.9×10^6	1.9×10^7	2.3×10^4	1.5×10^7	2.1×10^4
0.3					5.0×10^6	2.0×10^2	1.7×10^4	2.4×10^4	0	2.0×10^4
1	2.7×10^2	2.4×10^4	1.4×10^5	9.6×10^3	0	5.0×10^6	3.5×10^1	2.6×10^4	0	2.3×10^4
2	2.5×10^1	1.4×10^3	2.7×10^4	2.9×10^4	0	0	0	3.7×10^4	0	3.8×10^4
4	0	2.4×10^2	3.1×10^3	4.5×10^5	0	0	0	8.0×10^5	0	3.6×10^5
6	0	6.5×10^1	1.1×10^3	4.8×10^6						
22	0	0	9.2×10^1	$>10^8$	0	0	0	$>10^8$	0	5.2×10^7

^a 80% (vol/vol) fresh T-serum.^b 80% (vol/vol) heat-inactivated (56°C, 30 min) T-serum, which had been stored at 4°C overnight and reactivated at 56°C for 10 min.

For this purpose, 500 μ g of SPS per ml was added to undiluted T-C, T-56°, and to a 5-mg/ml stock solution of transferrin. The SPS-treated T-C turned turbid, whereas T-56° and the transferrin solution remained clear after the addition of SPS. After centrifugation at $2,500 \times g$ for 20 min at 4°C, only SPS-treated T-C yielded a visible sediment. This sediment was washed once more with 500 μ g of SPS per ml in phosphate-buffered saline, pH 7.5. Immunoelectrophoretic examination revealed identical amounts of immunoprecipitable transferrin in control T-C and in the supernatant fluid of SPS-treated T-C. The SPS precipitate derived from T-C lacked detectable transferrin. Similarly, the amount of transferrin was identical in control T-56° and in SPS-treated T-56°, as well as in the control and in the SPS-treated transferrin stock solution.

DISCUSSION

The preliminary experiments shown in Tables 1 and 2 served to emphasize several points. First, small amounts of fresh human serum, i.e., 10% (vol/vol), sufficed to kill exquisitely serum-sensitive, gram-negative rod bacteria, such as control strain *E. coli* C, as has been shown earlier (10) and has been confirmed recently by several authors (1, 21). In contrast, those *Enterobacteriaceae* that were killed via activation of the alternative pathway of complement by the lipopolysaccharide-endotoxin moiety of the outer membrane (3) required a considerably larger concentration of serum for efficient killing to ensue. Eighty percent (vol/vol) fresh human serum effected rapid killing of up to 10^7 CFU of PSS isolates of *S. marcescens* and of *E. coli* C per ml. However, 80% (vol/vol) fresh human serum that had been chelated with 0.01 M Mg + EGTA was capable only of

killing up to 10^4 to 10^5 CFU of DSS strains of *S. marcescens* per ml within the first 6 h of exposure (Table 2). These data substantiated those of Root et al. (12) who had noted that C4-deficient guinea pig serum was not nearly as efficient as normal guinea pig serum with respect to bacterial killing.

Thus, it was not surprising that 125 μ g of SPS per ml was found to completely neutralize the bactericidal activity of 80% (vol/vol) human serum against DSS strains of *S. marcescens* and that of Mg-EGTA-chelated fresh human serum against control strain *E. coli* C. On the other hand, 250 μ g of added SPS per ml was required to inhibit the bactericidal activity of 80% (vol/vol) fresh human serum against PSS strains of *S. marcescens* and *E. coli* C, which previously had been shown to be killed via "classically" activated serum bactericidal activity (17). Therefore, SPS at a final concentration of 250 μ g/ml (0.025%) sufficiently neutralized both classically and alternatively generated bactericidal activity of 80% (vol/vol) fresh human serum. In the case of control strain *E. coli* C, 80% (vol/vol) fresh human serum with 250 μ g of added SPS per ml (Table 3) revealed renewed, though limited, bactericidal activity during prolonged incubation. This effect was not seen with Mg-EGTA-chelated 80% (vol/vol) fresh human serum plus 250 μ g of SPS per ml (Table 5). Possibly, the anti-classical-complementary effect of SPS failed to persist during overnight incubation. In this context, Klein and Lange (8) years ago had observed that the anti-complementary effect of SPS was reversible after the addition of egg white.

The precise nature of the inhibitory effect of SPS against the activation of human complement via the classical and alternative pathways remains to be elucidated. It has been demonstrated that SPS interacted with the first

TABLE 3. Inhibition of the bactericidal activity of 80% (vol/vol) fresh human serum by SPS against the DSS *S. marcescens* isolate no. SE 142 and control strain *E. coli* C

Time that assay tubes were sampled (h)	DSS <i>S. marcescens</i> SE 142 (CFU/ml)						PSS <i>E. coli</i> C (CFU/ml)					
	T-C ^a (500) ^c	T-C (250)	T-C (125)	T-C (63)	T-C	T-56 ^b	T-C (500)	T-C (250)	T-C (125)	T-C (63)	T-C	T-56 ^c
0	2.1 × 10 ⁴	2.1 × 10 ⁴	2.1 × 10 ⁴	2.1 × 10 ⁴	2.1 × 10 ⁴	2.1 × 10 ⁴	1.5 × 10 ⁴	1.5 × 10 ⁴	1.5 × 10 ⁴	1.5 × 10 ⁴	1.5 × 10 ⁴	1.5 × 10 ⁴
0.3												
1	2.0 × 10 ⁴	1.8 × 10 ⁴	1.6 × 10 ⁴	4.4 × 10 ³	3.2 × 10 ³	2.1 × 10 ³	1.8 × 10 ⁴	1.7 × 10 ⁴	0	0	0	1.7 × 10 ⁴
2	2.3 × 10 ⁴	2.3 × 10 ⁴	2.5 × 10 ⁴	1.8 × 10 ³	1.1 × 10 ³	3.6 × 10 ⁴	2.5 × 10 ⁵	2.0 × 10 ⁴	0	0	0	2.3 × 10 ⁴
4	7.5 × 10 ⁴	8.0 × 10 ⁴	1.1 × 10 ⁵	6.5 × 10 ²	3.3 × 10 ²	2.1 × 10 ⁵	5.3 × 10 ⁴	3.1 × 10 ⁴	0	0	0	6.4 × 10 ⁴
6	1.0 × 10 ⁶	1.8 × 10 ⁶	6.7 × 10 ⁵	2.9 × 10 ²	7.5 × 10 ¹	2.2 × 10 ⁶	3.3 × 10 ⁵	7.5 × 10 ⁵	0	0	0	3.5 × 10 ⁵
22	>10 ⁸	>10 ⁸	>10 ⁸	0	0	>10 ⁸	2.0 × 10 ⁶	6.5 2 10 ²	0	0	0	1.2 × 10 ⁵

^a 80% (vol/vol) fresh T-serum.^b 80% (vol/vol) heat-inactivated (56°C, 30 min) T-serum.^c SPS was added to fresh T-serum at the indicated final concentrations (micrograms per milliliter) before the addition of bacterial inocula.

(C1) and third (C3) components of human complement (11, 13, 18, 22). Furthermore, Klein (7) had observed the inactivation of "C_M," a heat-labile, ammonium-resistant component of complement. Very recently, it was shown that, among other polyanions tested, SPS prevented the uptake of C2 by EAC4b (9), an effect which could be circumvented by the addition of Mg ions, suggesting that SPS acted through sequestration of Mg ions. The interaction of SPS with C3 might explain the suitability of this synthetic anticoagulant for the inactivation of both pathways of complement activation. However, it is conceivable that certain factors comprising the so-called properdin system might be affected by SPS as well.

Of significance was the finding that addition of 125 to 500 µg of SPS per ml to 20% (vol/vol) fresh human serum completely and, more important, throughout overnight incubation, neutralized serum bactericidal activity against control strain *E. coli* C (Table 6). At this lower percentage (volume/volume) concentration of either SPS-treated fresh human serum or heat-inactivated serum per se, no late bacteriostatic, i.e., transferrin-mediated, effect against this test strain was demonstrable. After 10 ml of blood samples are inoculated into culture vessels with 50 ml or more of broth plus 0.05 to 0.025% of added SPS, blood is diluted at least fivefold, and at this blood dilution the percentage (volume/volume) concentration of serum in the blood culture vessel certainly amounts to less than 20% (vol/vol). Thus, at this latter serum concentration, the transferrin-mediated bacteriostatic effect were simply diluted out. If, on the other hand, undiluted blood samples with added 0.05 or 0.025% SPS (500 or 250 µg/ml) were for some reason or another incubated as such for prolonged periods of time, the anti-complementary effect of 250 µg of SPS per ml might no longer be operative. At 500 µg of added SPS per ml, the growth of bacterial isolates subject to transferrin-mediated iron-deprivation might be inhibited, particularly in those instances where the isolates were weak or nonproducers of siderophores and the like (20). Of relevance in this regard was the finding that SPS at 500 µg/ml failed to precipitate transferrin from either fresh or heat-inactivated human serum.

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LITERATURE CITED

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TABLE 4. Inhibition of bactericidal activity of 80% (vol/vol) fresh human serum by SPS against the PSS *S. marcescens* strain CDC O13:H4^a

Time that assay tubes were sampled (h)	PSS <i>S. marcescens</i> CDC O13:H4 (CFU/ml)						PSS <i>E. coli</i> C	
	T-C (500)	T-C (250)	T-C (125)	T-C (63)	T-C	T-56°	T-C	T-56°
0	1.7 × 10 ⁴	1.7 × 10 ⁴	1.7 × 10 ⁴	1.7 × 10 ⁴	1.7 × 10 ⁴	1.7 × 10 ⁴	1.2 × 10 ⁴	1.2 × 10 ⁴
0.3	1.6 × 10 ⁴	1.6 × 10 ⁴	1.1 × 10 ⁴	6.0 × 10 ³	0	1.5 × 10 ⁴	0	1.2 × 10 ⁴
1	1.9 × 10 ⁴	1.8 × 10 ⁴	7.2 × 10 ³	0	0	1.7 × 10 ⁴		
2	2.5 × 10 ⁴	2.2 × 10 ⁴	7.1 × 10 ³	0	0	3.3 × 10 ⁴		
4	1.3 × 10 ⁵	1.2 × 10 ⁵	7.6 × 10 ³	0	0	5.4 × 10 ⁵	0	2.4 × 10 ⁵
22	>10 ⁸	>10 ⁸	7.5 × 10 ⁴	0	0	>10 ⁸	0	7.5 × 10 ⁴

^a See Table 3 for explanatory footnotes.

TABLE 5. Inhibition of bactericidal activity of fresh and Mg-EGTA-chelated fresh human serum by SPS against control strain *E. coli* C

Time that assay tubes were sampled (h)	PSS <i>E. coli</i> C (CFU/ml)								
	T-C Mg-EGTA ^a (500) ^b	T-C Mg-EGTA (250)	T-C Mg-EGTA (125)	T-C ^c (500)	T-C (250)	T-C (125)	T-C Mg-EGTA	T-C	T-56° ^d
0	1.0 × 10 ⁴	1.0 × 10 ⁴	1.0 × 10 ⁴	1.0 × 10 ⁴	1.0 × 10 ⁴	1.0 × 10 ⁴	1.0 × 10 ⁴	1.0 × 10 ⁴	1.0 × 10 ⁴
0.3	1.2 × 10 ⁴	1.2 × 10 ⁴	1.2 × 10 ⁴	1.2 × 10 ⁴	9.6 × 10 ³	0	9.9 × 10 ³	0	1.3 × 10 ⁴
1	1.6 × 10 ⁴	1.5 × 10 ⁴	1.5 × 10 ⁴	1.5 × 10 ⁴	1.4 × 10 ⁴	0	4.5 × 10 ³	0	1.9 × 10 ⁴
2	4.0 × 10 ⁴	4.1 × 10 ⁴	2.3 × 10 ⁴	4.5 × 10 ⁴	1.4 × 10 ⁴	0	5.5 × 10 ³	0	4.8 × 10 ⁴
4	7.0 × 10 ⁴	9.5 × 10 ⁴	5.4 × 10 ⁴	2.5 × 10 ⁵	4.5 × 10 ⁴	0	0	0	2.5 × 10 ⁴
22	7.0 × 10 ⁴	6.0 × 10 ⁴	4.1 × 10 ⁴	8.5 × 10 ⁵	3.5 × 10 ⁵	0	0	0	7.5 × 10 ⁴

^a 80% (vol/vol) fresh T-serum that had been chelated with 0.01 M MgCl₂ + 0.01 M EGTA.

^b SPS was added to fresh T-serum at the indicated final concentrations (micrograms per milliliter) before the addition of bacterial inocula.

^c 80% (vol/vol) fresh T-serum.

^d 80% (vol/vol) heat-inactivated (56°C, 30 min) T-serum.

TABLE 6. Inhibition of bactericidal activity of 20 and 80% (vol/vol) fresh human serum by SPS against control strain *E. coli* C

Time that assay tubes were sampled (h)	PSS <i>E. coli</i> C (CFU/ml)							
	T-C ^a (80%, vol/vol) (500) ^b	T-C (80%, vol/vol)	T-56° (80%, vol/vol)	T-C (20%, vol/vol) (500)	T-C (20%, vol/vol) (250)	T-C (20%, vol/vol) (125)	T-C (20%, vol/vol)	T-56° (20%, vol/vol)
0	2.2 × 10 ⁴	2.2 × 10 ⁴	2.2 × 10 ⁴	2.2 × 10 ⁴	2.2 × 10 ⁴	2.2 × 10 ⁴	2.2 × 10 ⁴	2.2 × 10 ⁴
0.3	2.2 × 10 ⁴	0	2.0 × 10 ⁴	2.2 × 10 ⁴	2.2 × 10 ⁴	2.1 × 10 ⁴	0	1.8 × 10 ⁴
6	6.5 × 10 ⁵	0	4.5 × 10 ⁵	6.5 × 10 ⁶	7.0 × 10 ⁶	1.2 × 10 ⁷	0	1.8 × 10 ⁷
22	9.0 × 10 ⁶	0	8.4 × 10 ⁶	>10 ⁹	>10 ⁹	>10 ⁹	0	>10 ⁹

^a Serum received by assay tubes.

^b Serum had received SPS at the indicated final concentrations (micrograms per milliliter) before the addition of bacterial inocula.

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