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Inactivation of the Bactericidal Activity of Human Serum by Liquoid (Sodium Polyanetholsulfonate)

BETTY L. LOWRANCE AND WALTER H. TRAUB
Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, North Carolina 27103

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Four serum-sensitive strains of *Escherichia coli* were exposed to 10, 20, and 50% fresh, heat-inactivated, and fresh human serum to which had been added Liquoid at a final concentration of 0.05, 0.025, 0.0125 and 0.006%. It was found that 50% fresh serum (in nutrient, Mueller-Hinton, thioglycolate, or Trypticase Soy Broth) killed more than 10^4 organisms/ml within 3 min, whereas 20 and 10% fresh serum required up to 20 and 40 min, respectively, to kill a comparable number of organisms. To neutralize the activity of 50% fresh serum, 0.0125% Liquoid had to be added, whereas an 0.006% final concentration of Liquoid was sufficient to antagonize the activity of 10 and 20% serum. However, when exposing extremely small bacterial inocula to fresh serum, at least 0.025% Liquoid was necessary to abolish the serum-bactericidal activity of 20 and 50% fresh serum. Liquoid had to be added to 50% fresh serum within seconds to prevent the killing of the majority of test organisms derived from small inocula. It is recommended that blood samples drawn from septicemic or bacteremic patients be aseptically added to a suitable broth which contains at least 0.025% Liquoid in order to improve the chances of isolating pathogens present in small numbers.

It has been known for a long time that Liquoid, a synthetic polyanionic anticoagulant, inhibits phagocytosis (1) as well as neutralizes the bactericidal activity of fresh human serum in vitro (3, 4, 9). For these reasons, Liquoid is being widely used in human blood cultures. However, information is scanty with regard to how rapidly and to what extent Liquoid is capable of abolishing the activity of serum, since fresh serum is known to kill serum-sensitive organisms within a matter of minutes (2, 5). The present study serves to determine what amounts of Liquoid are sufficient to abolish the bactericidal activity of various concentrations of fresh serum.

MATERIALS AND METHODS

Bacteria. Four serum-sensitive strains of *Escherichia coli* served as the test organisms. *E. coli* C was a gift from S. D. Davis, Department of Pediatrics, University of Washington, Seattle. *E. coli* 2 was isolated from clinical material. *E. coli* strains 0111 and 0127 were laboratory stock strains.

Media. Difco Nutrient Broth (NB), Mueller-Hinton broth (MHB), thioglycolate broth with added glucose (TGCB), and BBL Trypticase Soy Broth (TSB) were used in the serum bactericidal experiments. Pour plates contained BBL Trypticase Soy Agar (TSY) enriched with 2% yeast extract (Difco). Nutrient Broth served as the diluent in all experiments.

Liquoid. Liquoid was obtained through the courtesy of Hoffmann-La Roche, Inc., Nutley, N.J.

Serum. Two normal adult donors, who had not received any antibiotic for at least 1 month, were bled. The collected blood was allowed to clot at room temperature for 1 hr and then held at 4 C for 1 hr. The sera were separated by centrifugation at 3,020 × *g* for 20 min in the cold. The sera (L serum and T serum) were dispensed in 2.5-ml samples into screw-cap vials which were frozen and kept stored at −65 C. As needed, vials were rapidly thawed in a 37 C water bath. As required, serum was heat-inactivated at 56 C for 1 hr.

Serum bactericidal experiments. Fresh and heat-inactivated L or T sera were mixed with NB, MHB, TGCB, or TSB to yield 50, 20, or 10% serum. These served as the fresh and heat-inactivated serum controls, respectively. The assay tubes which contained 50, 20, or 10% fresh serum also received Liquoid at a final concentration of 0.05, 0.025, 0.0125, and 0.006%, which was added either immediately prior to the inoculation of test organisms or immediately thereafter. Organisms in the exponential phase of growth were added to yield approximately 10^3 to 10^4 organisms/ml at zero time. The assay tubes which had a total volume of 2.0 ml were held at 37 C. At appropriate intervals, samples were withdrawn and diluted 10-fold and 100-fold, and each dilution was incorporated into duplicate TSY-agar pour plates. Pour plates were counted after overnight incubation at 37 C. Both sera were tested against all serum-sensitive strains of *E. coli* in all of the four broth media used in the present study.

TABLE 1. *Kinetics of serum-bactericidal activity: T-serum versus E. coli C in Nutrient Broth*

Time after exposing organisms to serum	6.4 × 10^4 Organisms/ml at 0 time			4.6 × 10^4 Organisms/ml at 0 time	
	10% C'[a]	50% C'	50% 56 C[b]	20% C'	20% 56 C
min					
5	5.2 × 10^4	3.5 × 10^2		3.9 × 10^4	
10	4.6 × 10^4	0[c]	6.6 × 10^4	5.5 × 10^2	5.0 × 10^4
20	3.5 × 10^4	0		0	5.5 × 10^4
30	4.2 × 10^3	0	6.7 × 10^4		
40	2.0 × 10^2	0			
50	8.5 × 10^1	0			
60	5.0 × 10^1	0	7.2 × 10^4		

[a] Fresh serum, C'.
[b] Heat-inactivated serum, 56 C.
[c] Less than 10 organisms/ml, 0.

TABLE 2. *Serum-bactericidal activity of L serum versus E. coli C and 0111 in TGCB*[a]

Time after exposure of organisms to serum	*E. coli* C (organisms/ml at 0 time)						*E. coli* 0111			
	3.2 × 10^4		4.7 × 10^3		3.2 × 10^4		(6.5 × 10^3 organisms /ml at 0 time)			
	50% C'	50% 56C	20% C'	20% 56 C	10% C'	10% 56 C	20% C'	20% 56 C	10% C'	10% 56 C
min										
5	0	4.2 × 10^4								
20			0	5.3 × 10^3			0	6.4 × 10^3		
40					0	3.9 × 10^4			3.0 × 10^1	9.8 × 10^3

[a] See footnotes, Table 1.

TABLE 3. *Serum-bactericidal activity of L-serum versus E. coli C and 0111 in TSB*[a]

Time after exposure of organisms to serum	*E. coli* C (3.7 × 10^3 organisms/ml at 0 time)				*E. coli* 0111 (6.5 × 10^3 organisms/ml at 0 time)			
	20% C'	20% 56 C	10% C'	10% 56 C	20% C'	20% 56C	10% C'	10% 56C
min								
20	0	3.9 × 10^3			0	7.4 × 10^3		
40			0	6.3 × 10^3			0	1.0 × 10^4

[a] See footnotes, Table 1.

RESULTS

Two representative experiments are shown in Table 1. The 50% fresh serum killed more than 10^4 organisms/ml within 5 min, whereas 20 and 10% fresh serum required at least 20 and 40 min, respectively, to kill a comparable number of test organisms. Identical results were obtained with L serum. Fresh serum was equally effective in all four fluid media used. Tables 2 and 3 show results obtained with L serum versus *E. coli* strains C and 0111 in TGCB and TSB. Virtually identical results were recorded with different amounts of T serum versus all test organisms in the same fluid media. In these experiments the organisms were pregrown in the same broth that later served as the diluent for serum in the bactericidal experiments. Only the results obtained with 20 and 10% serum are recorded; these are the final serum concentrations in human blood cultures. None of the four broths depressed the bactericidal activity of serum.

The experiments employing Liquoid at different final concentrations showed that 0.0125 to 0.025% Liquoid is sufficient to neutralize the bactericidal activity of 50% fresh human serum, whereas 0.006% of this substance was capable of inactivating this activity in both 20 and 10% fresh serum. This was true only when Liquoid was added to serum prior to the addition of the organisms (Table 4).

TABLE 4. *T serum and L serum versus E. coli in three media*[a]

Time after exposure of organisms to serum	Liquoid				C′ Co	56 C Co
	0.05%	0.025%	0.0125%	0.006%		
min						
5[b]	3.7×10^3	3.9×10^3	4.0×10^3	8.9×10^2	0	3.7×10^3
20[c]	5.1×10^3	4.8×10^3	5.8×10^3	5.4×10^3	0	5.3×10^3
40[d]	3.6×10^4	3.7×10^4	4.4×10^4	4.4×10^4	0	3.9×10^4

[a] C′ = fresh serum; Co-control; 56 C = heat-inactivated serum; 0 = less than 10 organisms/ml.
[b] T serum (50%) versus *E. coli* C in NB; 3.8×10^3 organisms/ml at 0 time.
[c] L serum (20%) versus *E. coli* C in TGCB; 4.7×10^3 organisms/ml at 0 time.
[d] L serum (10%) versus *E. coli* C in TSB; 3.2×10^4 organisms/ml at 0 time.

TABLE 5. *T serum (50%) versus E. coli C in NB; Liquoid added after inoculation of organisms*[a]

Time after exposure of organisms[b] to serum	Liquoid				C′ Co	56 C Co
	0.05%	0.025%	0.0125%	0.006%		
min						
3	4.3×10^3	4.8×10^3	9.0×10^2	0	0	7.7×10^3
5	4.7×10^3	4.7×10^3	6.0×10^2	0	0	6.6×10^3

[a] See footnotes, Tables 1 and 4.
[b] At zero time, 8.0×10^3 organisms/ml.

When Liquoid was added immediately after inoculation of the test organisms into broth containing 50% fresh serum, the serum was capable of killing half the number of organisms present before Liquoid stopped the reaction (Table 5). Also, 50% fresh serum rendered almost 10^4 organisms per ml nonviable within a period of less than 3 min. No such "pre-Liquoid" effect was noted in the case of 20 and 10% fresh serum.

In a final series of experiments, fresh human serum was exposed to Liquoid (0.05, 0.025, 0.0125, and 0.006% final concentration); the treated fresh serum, untreated fresh serum, and heat-inactivated serum samples were then centrifuged at 1,085 × *g* for 20 min in the cold. The supernatant fluids from these samples (diluted 1:2 in broth to yield 50% serum, respectively) were allowed to interact with very small inocula of test organisms. The bactericidal activity of 50 and 20% fresh serum was abolished by 0.05 and 0.025% Liquoid (Table 6).

Upon the addition of Liquoid (0.05 and 0.025% final concentration) to 50 and 20%, but not to 10%, fresh human serum, an instantaneous precipitation occurs, causing a pronounced diffuse turbidity in the serum solution. No such event takes place when heat-inactivated serum is exposed to Liquoid. This suggests that Liquoid is capable of interacting with and precipitating a heat-labile component(s) of fresh serum. This turbidity phenomenon has already been described by others who attributed it to the inactivation of one of the subcomponents of the third component of complement (7). Currently experiments in this laboratory are aimed at further characterizing the nature of the precipitate obtained from Liquoid-treated fresh serum.

TABLE 6. *Exposure of E. coli C to the supernatant fluids of Liquoid-treated fresh, untreated fresh, and untreated heat-inactivated T sera*[a]

Liquoid	20% Serum[b]	50% Serum[c]
%		
0.05	8.8×10^2	6.4×10^2
0.025	8.3×10^2	5.1×10^2
0.0125	1.5×10^1	0
0.006	0	0
C′ Co	1.0×10^1	0
56 C Co	1.0×10^3	7.1×10^2

[a] At 20 min after exposure of organisms to serum. See footnotes, Tables 1 and 4.
[b] At zero time, 9.0×10^2 organisms/ml.
[c] At zero time, 6.0×10^2 organisms/ml.

DISCUSSION

These findings serve to reemphasize the astonishing rapidity with which 50% or more fresh human serum is bactericidally active (2, 5). However, 20 and 10% fresh sera are less rapidly active in this respect. The clinical microbiology laboratory is confronted with these latter two serum concentrations after a patient's blood

specimen (10 to 20 ml) has been inoculated into bottles containing thioglycolate or Trypticase Soy Broth (70 to 100 ml). Yet, over a period of 20 to 40 min, fresh serum at a concentration of 10 to 20% can still kill an impressive number of coliform organisms. Approximately half of the strains of several species of *Enterobacteriaceae* isolated from bacteremic or septicemic patients have been found to be sensitive to the patient's own serum (8). Thus the clinical microbiologist has but two alternatives to ensure the recovery of serum-sensitive coliform pathogens present in exceedingly small numbers in a given blood specimen. One approach is to rapidly dilute and subsequently ultrafilter the specimen (10). However, because serum is so rapidly bactericidal, it is recommended that laboratories employ Liquoid at a final concentration of 0.025%, or better yet, 0.05%, to neutralize at once the bactericidal activity of serum. Once this has been achieved, the laboratory can employ any method for obtaining isolated colonies of the responsible coliform pathogen.

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