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Serum Bacteriostasis of *Staphylococcus aureus*

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Serum bacteriostasis of *Staphylococcus aureus* was characterized quantitatively and qualitatively. Bacteriostasis was proportional to the concentration of serum. Reproducibility was good; freezing and thawing did not materially affect the end point. Four of six different strains, including the propagating *S. aureus* strain for phage 73 which does not produce coagulase, were susceptible to serum bacteriostasis in similar titers; two were not susceptible at all. All six strains were effective inhibitors of bacteriostasis. Active and inactive coagulase were also inhibitors. In contrast to sensitive *S. aureus*, *S. epidermidis* and *Streptococcus salivarius* were not uniformly susceptible to bacteriostasis by different serums. *Escherichia coli*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Salmonella montevideo*, *S. zymogenes*, and *Diplococcus pneumoniae* were not susceptible. Among gram-positive bacteria, only *D. pneumoniae* inhibited *S. aureus* bacteriostasis. Agglutinins of *S. aureus* and nonspecific substances such as lysozyme, β-lysin, C-reactive protein, and transferrin were not responsible for *S. aureus* serum bacteriostasis. After diethylaminoethyl column fractionation of serum, the bacteriostatic principle was eluted in proximity to blood group antibody; immunoglobulins A, G, and M appeared to be present in bacteriostatic fractions. It is suggested that *S. aureus* bacteriostasis by serum is due to natural antibody and that inhibitory reactions with pneumococci and coagulase are due to common antigens.

Inhibition of *Staphylococcus aureus* growth by serum has been described by several investigators (8, 11, 18, 21, 25), but the mechanism of action is not clear. The effect is generally attributed to bactericidins (19), lysozyme (17), C-reactive protein (22), iron-binding protein (24), substances released from white blood cells during clotting (15), or progesterone (32), or a combination of these. Although human globulins are known to be active inhibitors of *S. aureus* growth (9, 11, 27), this action is generally not attributed to antibody because of lack of specificity, reversal of inhibitory action by coagulase, and failure to utilize complement (9, 11, 18). The biological significance of serum bacteriostasis is obscure. Protection against *S. aureus* infection in experimental animals has been related to serum bacteriostasis by some but not all investigators (5, 20, 31).

In human serum, bacteriostatic activities persist despite dilution (11). This permits quantitation. Thus, the present study was undertaken to characterize human serum bacteriostasis of *S. aureus* quantitatively and qualitatively and to determine its relation to nonspecific serum inhibitors.

MATERIALS AND METHODS

The following bacteria were used: *Diplococcus pneumoniae* ATCC 11733 (rough strain); *Enterobacter aerogenes*, human isolate; *Escherichia coli*, human isolate; *Gaffkya tetragena* ATCC 10875; *Klebsiella pneumoniae*, human isolate; *Sarcina lutea* ATCC 9341; *Salmonella montevideo*, human isolate; *S. aureus* HG, phage type 52/52A/80/81 [previously described (7)], serotype abcphkm 263-1; *S. aureus* 3A, propagating strain for phage 3A of the International Phage Typing set obtained from P. Byrd Smith of the Center for Disease Control, Atlanta, Ga., serotype bc_1m; *S. aureus* Wood 46 (lacking in Jensen antigen A), obtained from Arthur White (13); *S. aureus* 73, human isolate, phage type 81, serotype k 263-1; *S. aureus* 74, human isolate, phage type 29/52/80/6/7/42/47/53/54/75/77/81, serotype AB; *S. aureus* Tager, obtained from Morris Tager, phage type 77; *S. epidermidis* R, human isolate previously described (7); *S. epidermidis* CIIA, human isolate; *Streptococcus salivarius* ATCC 9759; and *S. zymogenes*, human isolate. (Serotyping of all *S. aureus* strains except Wood 46 and Tager was performed by Jay Cohen.)

Coagulase. Partially purified coagulase, a gift of Morris Tager, had no red cell lytic activity when tested on 5% rabbit red blood cell-agar; 10 μg/ml clotted heparinized human plasma in 2 hr. Inactivation of coagulase was carried out by boiling for 2 hr in

0.03 N NaOH (*p*H 9.0) after which *p*H was adjusted to 7.0 with 2 N HCl. After such treatment, 100 μg of coagulase per ml did not clot human plasma in 24 hr.

Procedures. Serum was obtained aseptically from healthy volunteers not receiving antibacterial medication. Blood was allowed to clot at room temperature and refrigerated for 2 to 6 hr before separation. Sera were stored at −20 C until used.

Colostrum was collected from postpartum women by means of a sterile breast pump. To remove contaminating skin bacteria, specimens were centrifuged at 20,000 rev/min for 30 min at 4 C in a refrigerated centrifuge.

Fresh guinea pig complement was prepared and stored at −70 C.

Bacteriostasis. To measure bacteriostasis, serial twofold dilutions in Trypticase soy broth (TSB; BBL) were added to a mixture of Tween 80, TSB, and bacteria from an overnight culture. The final volume was 1.5 to 2.0 ml and contained 0.1% Tween and approximately 100 bacteria. All tubes were kept on ice until the start of 4 hr of incubation at 37 C with shaking. One tube remained at 4 C and was used to determine the initial inoculum. After 4 hr, pour plates of 0.5 ml of each reaction mixture were made in duplicate, employing undiluted samples and samples diluted 10^{-1} and 10^{-2} in TSB. Control tubes were identical except that 0.85% saline replaced serum.

Two plates containing 50 to 300 colonies were counted, and the mean of duplicate counts was taken as the result. Plates showing contaminating bacterial colonies were discarded and the determination was repeated. Only tests in which controls had at least a 30-fold increase in numbers were considered valid. The end point was taken as the highest serum dilution showing 10-fold fewer colonies than the incubated control. A sample protocol is shown in Table 1.

Inhibition of serum bacteriostasis with dead bacteria was done with an overnight culture of organisms killed at 65 to 70 C for 2 hr or at 121 C for 10 min. The organisms were washed, and their density was adjusted in TSB to 2×10^8 to 8×10^8 bacteria per ml. The bacterial suspension was added to serum in a ratio of approximately 3 volumes to 1 and was reacted at 37 C for 4 to 8 hr and at 4 C for 1 to 3 days. (Shorter periods were found less effective in preliminary studies.) Additional dilutions were then made and the bacteriostatic system was tested as above. For control purposes, a specimen of serum diluted with plain TSB was carried through the procedure.

Inhibition with coagulase was carried out by adding various concentrations of active or inactive coagulase to serum and incubating as above; 0.85% saline served as the control. Testing for bacteriostasis was then done in the usual fashion.

Mercaptoethanol treatment was carried out with 0.2 M mercaptoethanol made up in 0.1 M NaCl and 0.2 M phosphate buffer (*p*H 7.2) to which an equal volume of serum was added. The mixture was reacted for 48 hr at 0 C and then diluted 1:10 in 0.85% NaCl; the serum proteins were then twice precipitated with 12 volumes of cold acetone to remove the mercaptoethanol. The sediment was reconstituted in solution in TSB for testing. A control was treated identically except that 0.85% saline replaced mercaptoethanol. Quantitative determinations of immunoglobulins and other proteins were carried out by accepted methods (10); immunodiffusion plates from Hyland Laboratories (Travenol Lab., Inc.) were used; anti-transferrin, anti-C-reactive protein, and anti-immunoglobulin antibodies were purchased from Hyland Laboratories or Hoechst Chemical Co. Diethylaminoethyl (DEAE) column chromatography was carried out by standard methods (23).

TABLE 1. *Sample protocol for Staphylococcus aureus HG*

Determination	Time (hr)	Serum dilution	Dilution of reaction mixture	Mean colony count
Control	0		10	182
	4		10^{-1}	TNTC[a]
			10^{-2}	185
Serum 12	0		10	182
	4	1:10	10^{-1}	24
		1:20	10^{-1}	35
		1:40	10^{-1}	94
		1:80[b]	10^{-1}	164
		1:160	10^{-1}	270
		1:320	10^{-1}	TNTC

[a] TNTC, >300 colonies.
[b] Serum bacteriostatic titer.

RESULTS

Reproducibility and quantitative relations of serum bacteriostasis. Changes in titer after refreezing and thawing were measured in 34 sera from 15 persons. After initial titration against *S. aureus* HG, sera were refrozen at −20 C, stored for 1 to 8 weeks, and then retitered. A total of fifty retitrations was done, and only two tests differed by as much as two dilutions; none differed by more. Hence, the 95% confidence interval for reproducibility of titer after refreezing is ±1 dilution. The amount of bacterial growth after inoculation of 10^2 organisms in various concentrations of 30 different sera was found to be inversely proportional to the amount of serum (Fig. 1).

The distribution of end points in sera from 21 infants, age 1 to 7 months, and 36 adults were compared and were found to be similar (Table 2). Colostrum from four women had no bacteriostatic activity at 1:20, although sera from these women were active, as were three of four umbilical cord sera from their infants (Table 3). *S. aureus* agglutinins were present in all four colostrum samples.

Bacteriostasis of different bacteria. Four of six strains of *S. aureus* (HG, 3A, 73, and 74) were susceptible to different sera in similar bacterio-

static titer. In 15 sera in which strain HG titered 1:20 to 1:320, the maximum difference in end point between strains HG, 3A, 73, and 74 was a two-dilution (fourfold) disparity; one dilution or no difference occurred in 38 of 45 (84%) tests. Against strains Tager and Wood 46, however, serum bacteriostasis could not be demonstrated. Growth of these two staphylococci was unaffected by a 1:10 dilution of serum inhibiting *S. aureus* HG in titer of 1:80. All *S. aureus* strains produced coagulase except strain 73.

Sera bacteriostatic for *S. aureus* HG at 1:80 or higher were tested against *S. epidermidis*, *S. salivarius*, *S. zymogenes*, *D. pneumoniae*, *E. coli*, *E. aerogenes*, *K. pneumoniae*, and *S. montevideo* (Table 4). Bacteriostasis of one strain of *S. epidermidis* was caused by 7 of 21 sera in titer of 1:20 to 1:80, and another strain by 6 of 20 sera in titers of 1:20 and 1:40. Sera bacteriostatic for one *S. epidermidis* strain were not necessarily bacteriostatic for the other. Among streptococci, 11 of 20 sera were inhibitory for *S. salivarius*, but none of 16 affected *S. zymogenes* at a titer of 1:20. The other bacteria tested were not susceptible to bacteriostasis in serum diluted 1:10 or 1:20.

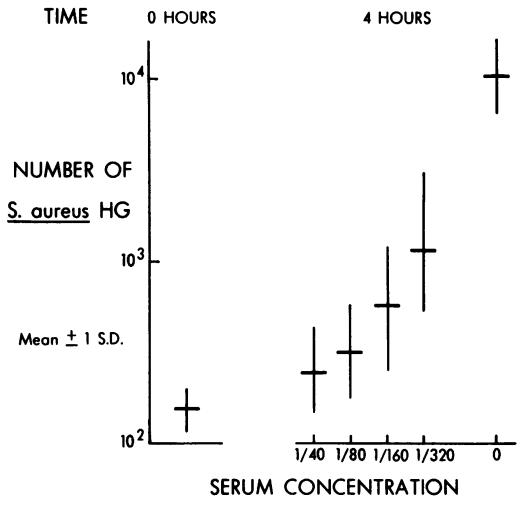

FIG. 1. *Summary of bacteriostatic activity of 30 sera. The mean number of Staphylococcus aureus HG found at 4 hr is inversely related to the serum concentration.*

Inhibition of serum bacteriostasis. Serum bacteriostatic action was inhibited by the addition of 10^8 autoclaved *S. aureus* HG cells; bacteriostatic titers were decreased two dilutions or more. Similar inhibition was brought about by strains Wood 46 and Tager. *S. epidermidis* R had no such effect (Table 5) nor did *G. tetragena*, *S. lutea*, *S. salivarius*, and *S. zymogenes*. However, *D. pneumoniae* was a particularly potent inhibitor of bacteriostasis, at times more inhibitory than *S. aureus* HG (Table 5).

Partially purified coagulase also inhibited serum bacteriostasis, the degree of inhibition being proportional to the concentration of coagulase. Inactivated coagulase, no longer able to clot serum, was also an effective inhibitor of bacteriostasis.

Effect of nonspecific factors on bacteriostasis: heat, dialysis, complement, Tween 80, kaolin, white blood cells, and mercaptoethanol. Bacteriostatic activity was not altered by 30-min heating of serum in a 56 C water bath, but activity was lost at 60 C. There was no loss of bacteriostasis after overnight dialysis against 0.02 M NaCl. Addition of five 50% units of fresh guinea pig complement per ml did not alter titers. End points were similar whether Tween 80 was present or absent. Absorption with 7 mg (dry weight) of kaolin per ml for 30 min was done to remove lysozyme (30). This did not affect the end point.

Sera prepared by clotting of plasma with white blood cells present to provide β-lysin, and without white blood cells, were made from two blood specimens taken simultaneously from the same individual. One blood sample collected in ordinary glassware was allowed to clot with white cell elements present, and the serum was removed in the usual way. The other was collected in siliconized glassware containing heparin. After blood cells settled, plasma free of white cells and platelets was aspirated and then allowed to clot. Titers in both serum preparations from the same person were similar.

Bacteriostatic activity was greatly decreased by mercaptoethanol. Agglutinins were not affected (Table 6).

TABLE 2. *Distribution of Staphylococcus aureus HG bacteriostatic titers in infants and adults*

Population	No. of sera	Distribution of bacteriostatic end points (%)						
		1:20	1:40	1:80	1:160	1:320	1:640	Total
Infants (1 to 7 months)	21	4.8	14.3	28.5	47.6	4.8	0	100
Adults	36	8.3	13.9	25.0	36.1	13.9	2.8	100

TABLE 3. *Staphylococcus aureus HG bacteriostatic levels in maternal serum, colostrum, and umbilical cord serum*

Subject	Serum	Colostrum	Umbilical cord serum
GA	1:640	<1:20	1:80
GR	1:40	<1:20	1:40
TH	1:80	<1:20	<1:20
AL	1:160	<1:20	1:40

TABLE 4. *Distribution of bacteriostatic titers in different sera against various bacteria*

Bacteria	No. of sera	Amt with Bacteriostatic titer of[a]				
		<1:20	20	40	80	>80
Staphylococcus epidermidis R	21	14	5	1	1	0
S. epidermidis CIIA	20	14	3	3	0	0
Streptococcus salivarius	20	9	6	5	0	0
S. zymogenes	16	16	0	0	0	0
Diplococcus pneumoniae 11733	3	3[b]	0	0	0	0
Escherichia coli	10	10[b]				
Enterobacter aerogenes	6	6[b]				
Klebsiella pneumonia	12	12[b]				
Salmonella montevideo	3	3[b]				

[a] All sera bacteriostatic for *S. aureus* HG in titer of 1:80 or >.

[b] Tested at 1:10 without bacteriostasis.

DEAE column fractionation of whole serum. Approximately 35 ml of serum from individual volunteers was dialyzed against 5 liters of 0.02 M NaCl in 0.005 M PO_4 buffer at *p*H 8 for 4 hr. The serum was centrifuged at 10,000 rev/min at 0 C for 30 min, and the supernatant fluid, adjusted to *p*H 8 and 0.02 M relative salt concentration, was applied to a DEAE cellulose column, 2.8 by 120 cm, which had been washed with PO_4 buffer at *p*H 8 and 0.02 M relative salt concentration. Eighty 15-ml effluent fractions were collected in the cold. After the effluent was collected, an increasing continuous saline gradient starting at 0.02 M and ending at 0.3 M NaCl was added and an additional 160 eluate fractions were collected.

In the effluent, no bacteriostatic activity was detected although *S. aureus* agglutinins were found here. In the eluate, bacteriostatic activity in a single peak was present in close proximity to blood group antibody (Fig. 2).

Fractions showing bacteriostatic activity were tested by immunoelectrophoresis; tests with anti-human C-reactive protein and anti-transferrin antibodies showed no reaction. Reactions with anti-immunoglobulin IgA, anti-IgM, and anti-IgG did occur.

Activity of fractions bacteriostatic to *S. aureus* HG was inhibited by dead *S. aureus* HG cells, pneumococci, and coagulase as before, but not by *S. epidermidis* R.

One active fraction was tested for complement fixation in a standard sheep cell hemolysin system

TABLE 5. *Inhibition of Staphylococcus aureus serum bacteriostasis by dead S. aureus, S. epidermidis, Diplococcus pneumoniae, or active or inactivated coagulase*

Serum no.	Bacteriostatic titer for *S. aureus* HG					
	Control	Inhibited with *S. aureus* HG	Inhibited with *S. epidermidis* R	Inhibited with *D. pneumoniae*	Inhibited with coagulase	
					40 μg/ml	4 μg/ml
12	1:160	1:40	1:160	—	—	—
105	1:160	<1:20	1:160	<1:10	—	—
MJC	1:80	1:20	1:80	<1:10	—	—
124	1:160	<1:20	1:160	—	—	—
101	1:320	1:40	—	<1:10	—	—
124A	1:320	—[a]	—	—	1:20[b]	1:80[b]
12A	1:320	—	—	—	1:20[b]	1:80[b]
DFE	1:320	—	—	—	1:20[b]	—
					1:40[c]	—
NJE	1:80	—	—	—	<1:20[b]	—
					1:20[c]	—

[a] Not tested.

[b] Inhibition with active coagulase.

[c] Inhibition with inactivated coagulase, done at the same time as the inhibition with active coagulase.

TABLE 6. *Effect of mercaptoethanol treatment on Staphylococcus aureus serum bacteriostasis and agglutination*

Serum no.	Bacteriostatic titer after mercaptoethanol treatment		Agglutinin titer after mercaptoethanol treatment	
	Control	Treated	Control	Treated
12	1:320	<1:20	1:160	1:320
111	1:160	<1:20	1:320	1:320
124	1:320	<1:50	1:160	1:160

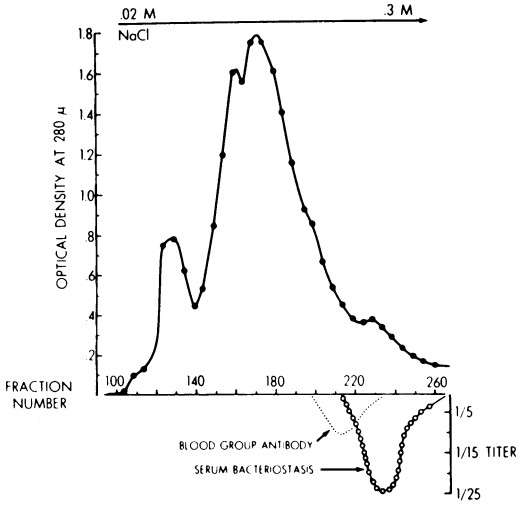

FIG. 2. *Plasma protein fractionation. Fractions 100 to 260 were eluted by an increasing saline gradient. Note proximity of Staphylococcus aureus bacteriostatic activity to blood group antibody.*

with guinea pig complement. Complement was not fixed.

DISCUSSION

A detailed investigation of the effect of human serum on growth of *S. aureus* revealed that bacteriostatic end points can be expressed as a simple serum dilution. The end points were reproducible. The 95% confidence interval after refreezing and thawing was ± one dilution of the first test. The bacteriostatic principle was present in umbilical cord serum and newborn infants in quantities similar to those in adults. It was absent from colostrum.

There are a number of studies demonstrating serum bacteriostasis of *S. aureus* (5, 9, 16), and methods of different investigators vary considerably. The system described by Cybulska and Jeljaszewicz (5) was inhibited by broth and saline and was clearly different from that employed here, in which both broth and saline were utilized. Ekstedt's results (9) and ours are similar, including inhibition by coagulase and pneumococci. However, some differences do exist. In Ekstedt's studies greater serum activity was found against *S. epidermidis* than *S. aureus*, and activity was lost after dialysis; in the present study neither of these conditions existed. The refinements of the present study, in which an end point is taken after 4 hr of incubation, may account for these differences.

The nature of the serum factor responsible for bacteriostasis was explored. Although such nonspecific factors as C-reactive protein, transferrin, β-lysins from white blood cells or lysozyme were ruled out, two important findings against specificity were observed. (i) Not all strains of *S. aureus* were susceptible to serum bacteriostasis, yet some streptococci were. (ii) Inhibition of serum bacteriostasis was brought about by pneumococci as well as *S. aureus*.

These two arguments against specificity merit closer inspection. The lack of susceptibility of *S. aureus* strains Tager and Wood 46 may simply be due to a deficiency in some cellular attribute as a result of long-term laboratory propagation. The Wood 46 strain is lacking in Jensen's antigen A although it contains abundant polysaccharide A (13); it may lack other substances which are necessary for susceptibility to bacteriostasis. Thus, early growth of strains Tager and 46 in serum is not necessarily an argument against specificity. Moreover, separate mechanisms of bacteriostasis for *S. aureus*, *S. epidermidis*, and *S. salivarius* appear to exist. Different sera were similar in their bacteriostatic effects against sensitive strains of *S. aureus*, but this was not the case for *S. epidermidis* or *S. salivarius* (Table 5). Finally, all six strains of *S. aureus*, including Wood 46 and Tager, were effective inhibitors of *S. aureus* bacteriostasis, whereas *S. epidermidis* and *S. salivarius* were not.

In inhibition tests, only active or boiled coagulase, pneumococci, and *S. aureus* were effective inhibitors of *S. aureus* bacteriostasis. Other gram-positive bacteria, including a variety of micrococcaceae, were not. The inhibitory effect of pneumococci may be due to the presence of a substance also found in *S. aureus*. Heterogenetic characteristics shared by different genera of gram-positive cocci have been described (3, 30). That strongly heated coagulase, pneumococci, and staphylococci were all inhibitory suggests that a polysaccharide-containing substance is responsible. In this regard, it may be noteworthy that cross-reactions with pneumococci and other

microorganisms appear to be due to similar polysaccharide end-groupings (14).

The bacteriostatic principle was sensitive to mercaptoethanol, and active fractions appear to contain immune globulins. In view of the proximity of blood group antibody and the bacteriostatic substance on DEAE fractionation (Fig. 2), it may be that serum bacteriostasis is an action of a natural antibody to *S. aureus* (4, 26). If serum bacteriostasis is due to an immune system, it would appear to be mediated by IgA, IgM, or both, in view of the sensitivity to mercaptoethanol. The absence of bacteriostatic activity in colostrum may indicate local production of IgA (28) without *S. aureus* antigenic exposure.

The biological significance of human globulins causing *S. aureus* bacteriostasis is not clear, despite the demonstration of their efficacy in preventing experimental infection (27, 31). Cybulska and Jeljaszewicz regard the phenomenon as a laboratory curiosity because of variation in animal species (5), whereas MacLeod and coworkers relate this variation to the variation in resistance of different animal species to *S. aureus* infection (20). In this regard, the absence of bacteriostatic activity in colostrum could be a factor in *S. aureus* breast abscesses of postpartum women.

Serum bacteriostasis may play an important role in preventing surgical wound infection. The first few hours after incision appears to be the important time for determining the occurrence of *S. aureus* surgical wound infections (1, 6). The number of *S. aureus* cells appears to reach a steady state between 2 and 4 hr (2). Thus, an important consideration in the initiation of *S. aureus* infection is whether a rapid increase in bacterial numbers in tissues occurs. Serum bacteriostasis may prevent wound infections by limiting the growth of *S. aureus* for a short time until other host defenses are mobilized. In the absence of host bacteriostasis, *S. aureus* could immediately start on its exponential growth phase and become invasive. The presence of staphylococcal products in tissues renders an animal more susceptible to local staphylococcal infection (12). This, too, may be due to inhibition of serum bacteriostasis at the site of infection.

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