Bacteriostatic Activity of Serum Against Staphylococci

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ABSTRACT

CYBULSKA, JANINA (State Institute of Hygiene, Warsaw, Poland), AND J. JELJASZEWICZ. Bacteriostatic activity of serum against staphylococci. *J. Bacteriol.* 91:953-962. 1966.—Antistaphylococcal activity of normal serum against strains exhibiting various patterns of coagulase, clumping-factor, and staphylokinase production is not connected with the presence of these factors. Purified coagulase does not influence this property of serum. Coagulase-negative strains with clumping-factor activity grow in normal serum as typical pathogenic staphylococci. Serum bacteriostatic activity against staphylococci may be reversed by several nonspecific factors, such as sterile broth, supernatant fluids of coagulase-negative strains, and ammonium sulfate precipitates of culture supernatant fluids of various staphylococci. Immune sera with a high agglutinating titer for staphylococcal cells do not prevent growth of serum-resistant strains; serum-susceptible strains are inhibited as in normal serum control. Activation or blocking of the serum fibrinolytic system does not influence serum bacteriostatic activity. The growth rate of serum-resistant strains is identical in serum and in Todd-Hewitt broth; serum-susceptible strains are inhibited to the inoculum level, but decreases and increases in viable count are noted during a 24-hr observation period. Observations made with sera of 10 animal species clearly demonstrated differences in serum bacteriostatic activity, mouse serum being completely noninhibitory and cat serum only weakly inhibitory. The technique of quantitative determination of serum susceptibility of staphylococci is described, and the importance of serum antistaphylococcal activity in vitro is discussed. Experimental staphylococcal infection produced in rabbits by intravenous injection of different *Staphylococcus aureus* strains did not result in significant changes in serum antistaphylococcal activity. The technique of experimental infection used caused chronic infection, with a peak on the 14th day; this was proved by means of a newly developed 5'-nucleotidase test. At the same time, sera of infected animals exhibited slight inhibitory properties, which returned to initial values 1 week later. Infection was produced by strains recognized as nonpathogenic and was inhibited in vitro by sera from both normal and infected rabbits. It is concluded that antistaphylococcal activity of serum should be considered as an "in vitro" phenomenon, which seems to have no importance in defense mechanisms of rabbits infected intravenously with staphylococci.

Coagulase-positive strains of *Staphylococcus aureus* isolated from pathological material multiply readily in normal human serum, whereas nonpathogenic strains are inhibited (19, 20, 21). This phenomenon was found to be independent of complement and lysozyme and to be produced by a thermostable factor present in fresh serum (8, 18). Resistance of pathogenic staphylococci to the action of normal sera has aroused interest in the mechanism of nonspecific resistance to infection by these strains, especially after presentation

of a hypothesis on reversing of serum antistaphylococcal activity by coagulase (5). According to Ekstedt (3, 4), this activity depends on the presence of a thermostable serum factor which is neutralized by coagulase. Coagulase is supposed to activate respiration of staphylococci, and when adsorbed on coagulase-negative cells, prevents their destruction by the serum (6). The active factor was found in the globulin fraction of normal serum, and is not active toward *S. aureus* or *Bacillus subtilis* pretreated with coagulase (25).

This role of coagulase was, however, not confirmed (23, 24) and was also more or less doubtful to other investigators (9, 15, 18). It is not known whether serum of an animal infected with staphylococci exhibits bacteriostatic activity in vitro.

This communication is concerned with anti-staphylococcal activity of normal sera derived from 10 animal species and the influence of various factors including coagulase on this phenomenon in vitro. Bacteriostatic activities of sera derived from rabbits infected with eight strains of *S. aureus* and with single strains of *Streptococcus pyogenes* and *Escherichia coli* were observed and compared.

MATERIALS AND METHODS

Strains. Characteristics of the 12 strains of *S. aureus* used are given in Tables 2 and 3. The sources of the strains were as follows: strain Smith (diffuse and compact variants) was received from D. E. Rogers; 8447-B from D. D. Smith; Newman D₂C, A-224, and E-7 from E. S. Duthie; Jacherts from D. Jacherts; Mendita from S. I. Morse; strain Czerszer was isolated by us from the nose of a child during an epidemic of staphylococcal enterocolitis in Katowice hospital; strain Zak was isolated from a case of conjunctivitis occurring in this laboratory; Kulba was from the blood of a patient with staphylococcal bacteremia in Warsaw University Dermatological Clinic; and Bekasiak was isolated from the nose of a child with scarlet fever in Poznan Children's Hospital for Infectious Diseases.

The following strains of *S. aureus* were used for animal experiments: Smith compact and diffuse, E-7, Bekasiak, 8447-B, Jacherts, Mendita, and Newman D₂C. Infections with *Streptococcus pyogenes* (type 15) and *E. coli* O:26 were made for control and comparative purposes.

Production of toxins. Coagulase was determined at 20 and 37 C with 10 plasmas of the various species used in these experiments; fresh citrated plasma diluted 1:5 with saline was used. Results were read after 1, 3, 6, and 24 hr. Clumping factor (or bound coagulase) was tested with undiluted plasma. The details have been described elsewhere (13). Staphylokinase was detected by the plate method devised by Glanville (*personal communication*). Human, rabbit, dog, cat, and guinea pig plasmas were used. Hemolysins were differentiated according to Elek and Levy (7) by use of anti- α -hemolysin received from the National Institute for Medical Research, London, England, and anti- β -hemolysin donated by H. Proom (Burroughs Wellcome & Co., Beckenham, Kent, England). Erythrocytes of all tested genera were used.

Antibiotic sensitivity. The disc method described by Gawenda-Dzierzynska and Wasiewicz (10) was used. Commercially available discs were utilized (Sera and Vaccines Laboratory, Warsaw, Poland).

Bacteriophage typing. An international set of phages and the method described by Blair and Williams (1) were applied.

Growth of staphylococci in serum and broth. Growth

curves of typical staphylococcal strains in normal rabbit serum and Todd-Hewitt broth were prepared. Smith compact variant, multiplying readily in serum, and strain Mendita, which is inhibited in its growth, were chosen. Measurements were made in duplicate serum and broth samples. Viable counts were calculated in samples taken every 2 hr during a 24-hr period. Viable counts and determination of bacteriostatic activity of serum were performed as described below.

Plasma samples. Heart blood was drawn into sodium citrate solution from rabbits, mice, guinea pigs, rats, and cats, and venous blood from humans, sheep, horses, dogs, and roosters. Pools of samples derived from several individuals were used on the same day.

Serum samples. Blood samples were kept for 1 hr at 37 C and then 1 hr at 4 C. Serum was removed, centrifuged if necessary, and then pooled with similar sera from several animals. It should be stressed here that sera were always used within a few hours, i.e., the same day that they were collected. All tests were started at physiological pH.

Collection of blood from infected rabbits for determination of serum antistaphylococcal activity. Samples of blood were taken from the heart before infection, and then on the 7th, 14th, and 21st day after the first infection. Every sample of serum was tested quantitatively for its bacteriostatic activity against the Mendita (inhibited in normal sera) and Smith compact (multiplying in normal sera) strains.

Determination of bacteriostatic activity of serum. Measurements were carried out with 0.5-ml volumes of sera in Pyrex serological test tubes closed with rubber stoppers. The inoculum was prepared as follows. An 18-hr Todd-Hewitt broth culture was centrifuged, the sediment was thoroughly suspended in 5 ml of saline, and was then diluted 1,000-fold with saline. Volumes of 0.1 ml of the diluted suspensions were added to each test tube containing 0.5 ml of serum; 0.2 ml of suspension was added to the tube containing 1 ml of saline. Serum samples were incubated at 37 C in a water bath for 24 hr. The test tubes with saline only served for the initial viable-count calculation. For this purpose, the suspension was diluted 10^{-1} , 10^{-2} , and 10^{-3} , and 0.5-ml portions were transferred onto sheep blood-agar plates and spread with a sterile glass rod. The blood-agar plates had been dried at 37 C for 2 hr. Inoculated plates were incubated at 37 C overnight, and the viable count was calculated. The inocula prepared in this manner are reproducible. After incubation, serum samples inoculated with staphylococci were thoroughly shaken, since some strains grow in serum as a dense granular sediment. Samples (0.5 ml) were withdrawn from each tube. Readily visible bacterial growth suggested dilution of 10^6 to 10^8 , and absence of turbidity, 10^1 to 10^5 . The samples were then inoculated onto blood-agar as described for inoculum, with the use of four plates for abundantly growing strains and five plates for strains which did not produce serum turbidity. It should be noted here that strains producing β -hemolysin may cause opalescence of serum; this is, however, easily distinguishable from growth. The

inoculated plates are then incubated at 37 C for 24 hr, and the number of colonies is detected by use of an electric counter. Bacteriostatic activity of serum was evaluated on the basis of serum antistaphylococcal index (growth index), calculated from the following equation: number of colonies after 24 hr of incubation/number of colonies at 0 hr $\times$ 100. Each serum pool was checked for sterility. The results obtained by this method are comparable, although the same strain tested with different sera may result in varying values of the growth index. However, strains inhibited in their growth in serum regularly resulted in an index below 1.0, whereas strains multiplying in serum gave indexes higher than 1.0.

Purified coagulase. Two samples of purified coagulase were used. One was supplied by Morris Tager and was exhausted during preliminary experiments. Another was prepared as described previously (14).

ϵ -Aminocaproic acid (EACA). Crystalline EACA, received from S. Niewiarowski, was dissolved in saline to a concentration of 100 mg/ml, sterilized by boiling, and added to serum to a final concentration of 10 mg/ml. Serum plus EACA was incubated for 30 min at 37 C prior to inoculation.

Ethylenediaminetetraacetic acid (EDTA). The sodium salt of EDTA (Nutritional Biochemicals Corp., Cleveland, Ohio) was dissolved in saline to a concentration of 10 mg/ml, sterilized by boiling, and added to serum in a final amount of 1 mg/ml. Serum plus EDTA samples were preincubated at 37 C for 30 min.

Streptokinase. A sample of Distreptase (Sera and Vaccines Laboratory, Warsaw, Poland), containing 23,000 Christensen units of streptokinase and 17,000 units of streptodornase per ampoule, was diluted to achieve a concentration of 500 units of streptokinase per ml of serum. Samples were then preincubated for 5 min at 37 C.

Staphylokinase. Purified and concentrated staphylokinase was prepared as described by Glanville (11). Solutions of initial concentration of 5 mg/ml were sterilized by filtration through Schott G-5 filters and used in a final concentration of 500 μ g/ml of serum. A 1-mg amount of the staphylokinase sample contained 1,200 caseinolytic units. Serum with added staphylokinase was preincubated for 30 min at 37 C.

Complement. A commercial product (Lublin, Sera and Vaccines Laboratory) was used. The contents of the ampoule, corresponding to 2 ml of guinea pig serum, were dissolved in 1 ml of distilled water; 10% of complement (v/v) was added to the fresh mouse serum. Samples were then preincubated for 30 min at 37 C.

Supernatant fluids of staphylococcal cultures. Newman D₂C and Smith compact strains were grown in Todd-Hewitt broth for 18 hr. After centrifugation, the supernatant fluids were sterilized by filtration through Schott G-5 filters; the first portion was discarded, and the remainder was used for experiments. To the sera, 10 or 30% (v/v) of the supernatant fluids were added. The controls consisted of the same volume of sterile broth and saline. All samples were incubated for 40 min at 37 C prior to inoculation.

Todd-Hewitt broth. The broth was prepared in the conventional manner. Broth (10 or 30%) was added to serum, incubated for 30 min at 37 C, and used for control determinations.

Reversal of serum bacteriostatic activity by coagulase. Sterile coagulase, purified as described above, was added directly to the serum to achieve a final concentration of 2 mg/ml of serum. After the coagulase dissolved, serum samples were preincubated for 1 hr at 37 C.

Immune sera. Rabbits were immunized with formalinized and heat-killed suspensions of staphylococci by intravenous injections, as described for preparation of streptococcal grouping sera (Rockefeller Institute Hospital procedure). Sera agglutinating with homologous suspensions in dilutions higher than 1:600 were obtained.

Experimental infection. Rabbits of both sexes, received from a commercial source and weighing 2,500 to 3,000 g, were used. It was established that intravenous injection of rabbits with a dose of about 6×10^5 to 10×10^6 staphylococcal cells, followed by injection of a 10-fold higher dose after 6 days, results in chronic staphylococcal infection with final localization in kidneys, and pyelonephritis (Piekacz, Jeljaszewicz, and Zak, in preparation). Groups of six rabbits were infected by injection into the marginal ear vein of suspensions of viable staphylococci at the concentrations indicated in Table 1.

Histochemical determination of 5'-nucleotidase activity in leukocytes. This was performed by the technique described by Wachstein and Meisel (23). Results were expressed as percentages of cells with enzyme activity.

RESULTS

Strains with different properties were selected for the purposes of this study. They differed in phage patterns, antibiotic sensitivity, and production of coagulase, clumping factor, and staphylokinase (Tables 2 and 3).

In the preliminary experiments, strains differing in regard to coagulase production were selected.

TABLE 1. *Suspensions used for infection of rabbits*

Strain	No. of cells	
	First infection	Second infection (6 days later)
Smith compact . . .	6.3×10^5	11.4×10^7
Smith diffuse	8.3×10^5	6.2×10^7
E-7	1.6×10^6	8.2×10^7
Bekasiak	8.4×10^5	10.8×10^7
8447-B	2.8×10^6	6.2×10^7
Jacherts	1.6×10^6	11.8×10^7
Mendita	2.4×10^6	7.3×10^7
Newman D ₂ C	8.5×10^5	5.0×10^7
<i>Streptococcus</i>		
<i>pyogenes</i>	6.5×10^5	14.0×10^7
<i>Escherichia coli</i> . .	2.5×10^6	8.0×10^7

TABLE 2. *Phage types and antibiotic susceptibility of strains used for testing of serum antistaphylococcal activity*

Strain	Phage type	Resistant to
Smith compact . . .	RTD: NT; 1,000 × RTD: 29+*	—
Smith diffuse	RTD: NT; 1,000 × RTD: 29+*	—
Jacherts	83A	Streptomycin, penicillin
Mendita	NT	—
8447-B	3A/3B/3C/6/7/42E/47/54/75	—
Bekasiak	RTD: NT; 1,000 × RTD: 29	Penicillin
E-7	NT	—
Newman D ₂ C	7/47/53/54/75/77	Penicillin
Zak	NT	Penicillin
Kulba	NT	Penicillin, erythromycin, tetracyclines, chloramphenicol
A-224	7/47/53/54/75/77/83A	Penicillin
Czerszer	75/77	Penicillin, streptomycin, tetracyclines

* Plus indicates weak reaction.

Of 12 strains chosen for the study of antistaphylococcal activity of normal sera, six were coagulase-positive and six coagulase-negative. Two coagulase-positive strains were clumping factor-negative, and two coagulase-negative strains were clumping factor-positive. Thus, all the different possibilities of appearance of coagulase and clumping factor, were represented. These activities were determined on the basis of results obtained with human and rabbit plasma. It is well known, however, that with other plasmas, coagulase clotting activity may appear negative. For instance, Smith compact strain was coagulase-positive with human, rabbit, cat, mouse, horse, and rat plasma, positive with dog plasma at 20 C only, positive with undiluted sheep plasma, and negative in guinea pig plasma. The same strain exhibited clumping factor activity with human, rabbit, dog, cat, mouse, sheep and rat plasma, but no clumping occurred in guinea pig and rooster plasma. Other coagulase-positive strains failed to clot mouse plasma. Guinea pig plasma was clotted only by the E-7 strain.

Observation of growth curves in serum and broth of typical staphylococcal strains revealed that some strains are only inhibited, and others grow in serum as well as in broth. Strains susceptible to the serum action decreased in number of cells, when exposed to the serum; starting with the 6th hour, viable counts increased, reaching the initial value after 24 hr of incubation (Fig. 1). Therefore, the overall effect of the inhibitory activity of serum against tested strains of *S. aureus*, after 24 hr of incubation, should be considered as bacteriostatic rather than bactericidal.

Species differences in serum bacteriostatic activity were noted (Table 4). Mouse serum showed complete lack of antistaphylococcal activity against all studied strains. Although the growth

indexes of strains Mendita and 8447-B were lower than with the remaining strains, growth was nevertheless very distinct. Opposite results were achieved with the rooster serum. Growth of staphylococci was strongly inhibited even in the case of Bekasiak and Jacherts strains, which multiplied abundantly in other sera. It should be mentioned here that coagulase-negative strains exhibiting the clumping factor activity grew in serum as did typical coagulase-positive staphylococci.

The Kulba strain, coagulase- and clumping factor-negative with all 10 plasmas used, was the only strain of such type which exhibited moderate growth in human and rabbit serum. The E-7 strain, of animal origin, was strongly inhibited by human and rabbit serum, but grew in several other sera. Cat serum was found to be weakly bacteriostatic for staphylococci, inhibiting only the Zak strain. Very similar results were obtained with human, rabbit, guinea pig, and dog plasma.

No correlation was found between excretion of coagulase, clumping factor, or staphylokinase, and the ability of a given strain to multiply in serum (Table 5).

Purified coagulase did not change the growth index of serum-susceptible strains of staphylococci (Table 6). Reversal of serum antistaphylococcal activity could be achieved, however, by several simple procedures. It was shown that supernatant fluids of staphylococcal cultures, whether coagulase-positive or -negative, could reverse the serum antistaphylococcal activity. Examples of such data are presented in Fig. 2 and 3 with four different serum-susceptible strains. Saline and sterile broth had similar effects. Also, a precipitate obtained from a cell-free supernatant fluid of a coagulase-positive *Staphylococcus* resulted in this nonspecific reversal.

TABLE 3. Coagulase, clumping factor, staphylokinase, and hemolysins in strains of *Staphylococcus aureus* used for testing of antistaphylococcal activity of serum

Strain	Hemolysin pattern	Plasma*															
		Rabbit			Dog			Human			Cat			Guinea pig†			Rooster‡
		SC	CF	S	SC	CF	S	SC	CF	S	SC	CF	S	SC	CF	S	
Smith compact . . .	α, β	3+	3+	+	2+	2+	+	3+	+	+	2+	3+	+	+	3+	+	—
Smith diffuse . . .	α, β	3+	—	+	2+	—	+	3+	—	+	2+	3+	—	—	3+	—	—
Jacherts . . .	α, β	3+	3+	+	—	—	+	3+	+	+	+	3+	+	+	3+	+	3+
Mendita . . .	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
8447-B . . .	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Bekasiak . . .	β	3+	—	+	3+	—	+	3+	—	+	3+	—	+	—	—	—	—
E-7 . . .	β	—	3+	—	2+	3+	—	—	3+	—	3+	—	—	—	—	—	—
Newman D ₂ C . . .	Nonspecific	—	3+	+	—	—	+	—	3+	+	—	—	—	—	—	—	2+
Zak . . .	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Kulba . . .	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
A-224 . . .	α, β	3+	3+	+	+	+	+	3+	2+	+	2+	—	—	—	—	—	—
Czerszer . . .	α, β	3+	3+	+	±	3+	+	3+	3+	+	2+	—	—	—	3+	+	—

* SC, staphylocoagulase; CF, clumping factor; S, staphylokinase. All positive results with rooster plasma were obtained at 20 C. Intensity of SC and CF positiveness determined as described previously (13).
† Positive results of coagulase test obtained at 20 C.
‡ Doubtful-positive results obtained at 20 C.

FIG. 1. Growth curves of serum-resistant strain *Smith compact* and serum-susceptible strain *Mendita*. Normal rabbit serum and Todd-Hewitt broth.

The observed phenomenon of serum anti-staphylococcal activity does not seem to be a simple immunological mechanism. Serum-susceptible strains were still inhibited in immune sera, whereas serum-resistant strains grew as easily as in normal sera (Table 7). Addition of complement to mouse serum does not change its activity toward staphylococci (Table 8). It is also evident that antistaphylococcal activity of serum is not dependent of the fibrinolytic system. Plasminogen activators, such as streptokinase or staphylokinase, added in high concentrations, failed to exert any influence; equally ineffective was EACA, a potent inhibitor of serum fibrino-

TABLE 5. Production of coagulase, clumping factor, and staphylokinase by 99 strains of *Staphylococcus aureus* and their ability to multiply in normal rabbit serum

Total no. of strains	Growth in serum*		Production of		
	+	-	Coagulase	Clumping factor	Staphylokinase
67	67	0	+	+	+
5	5	0	-	+	+
7	7	0	+	-	+
1	1	0	-	-	+
2	2	0	+	+	-
3	1	2	-	+	-
2	2	0	+	-	-
12	1	11	-	-	-

* See Materials and Methods section. Growth index higher than 1.0 was accepted as positive result.

TABLE 4. Growth of 12 different strains of *Staphylococcus aureus* in sera of 10 species*

Strain	Coagulase and clumping factor	Serum									
		Horse	Sheep	Rabbit	Human	Mouse	Guinea pig	Rooster	Cat	Dog	Rat
Smith compact..	SC+, CF+	4.48	4.42	840.00	34.30	109.80	57.60	3.70	134.00	38.80	268.00
Smith diffuse....	SC+, CF-	11.90	4.18	139.10	6.38	252.00	90.00	0.01	41.10	111.60	0.59
Jacherts.....	SC+, CF+	40.20	11.30	227.50	34.70	87.90	182.00	0.44	108.00	117.80	2.42
Mendita.....	SC-, CF-	0.01	0.05	0.01	0.01	12.20	0.12	0.01	3.45	0.07	0.01
8447-B.....	SC-, CF-	0.01	0.05	0.01	0.08	13.70	0.69	0.01	10.00	1.00	0.01
Bekasiak.....	SC+, CF-	44.30	5.20	178.50	12.50	154.00	116.80	0.02	121.60	109.00	164.00
E-7.....	SC-, CF+	0.05	28.00	0.01	0.25	162.20	93.90	7.60	39.40	33.50	0.02
Newman D ₂ C.....	SC-, CF+	12.30	4.60	942.00	76.30	74.00	38.00	0.01	214.00	160.00	284.00
Zak.....	SC-, CF-	0.03	0.07	0.01	0.07	108.00	0.09	0.01	0.39	0.01	0.02
Kulba.....	SC-, CF-	0.84	0.14	0.09	1.31	164.30	1.00	0.01	9.16	0.01	8.80
A-224.....	SC+, CF+	83.90	3.30	88.00	73.20	80.00	49.40	55.00	52.50	161.50	0.40
Czerszer.....	SC+, CF+	57.50	2.24	550.00	85.80	65.30	41.00	72.00	125.00	273.00	9.75

* Results are expressed in growth index values (see Materials and Methods). Staphylocoagulase (SC) and clumping factor (CF) activities determined with rabbit plasma.

TABLE 6. Influence of coagulase on multiplication of serum-susceptible strains of *Staphylococcus aureus* in human and rabbit serum^a

Test strain	Growth index in			
	Normal rabbit serum ^b	Normal rabbit serum + coagulase ^c	Normal human serum ^b	Normal human serum + coagulase ^c
Mendita	0.01	0.03	0.01	0.07
Zak	0.01	0.01	0.10	0.12
Kulba	0.15	0.19	1.60	1.98
8447-B	0.01	0.02	0.12	0.09
1 ^d	0.11	0.07	0.17	0.21
2 ^d	0.01	0.03	0.01	0.03
3 ^d	0.01	0.01	0.01	0.05
4 ^d	0.08	0.03	0.11	0.17
5 ^d	0.07	0.11	0.09	0.09
6 ^d	0.03	0.07	0.07	0.09
Control (Smith compact)	820.00	818.00	35.10	38.20

^a Results are given as growth index values (see Materials and Methods).

^b Sera from 10 healthy individuals.

^c Final concentration of coagulase in serum: 2 mg/ml. (Coagulase samples used in this study clotted rabbit citrate plasma in 15 to 20 sec when used at a concentration of 100 μ g/ml and did not exhibit activity of any staphylococcal toxins.)

^d Staphylococcal strains freshly isolated from clinical material (coagulase and clumping factor-negative).

FIG. 2. Reversal of serum antistaphylococcal activity toward serum-susceptible strains Mendita and Zak. Normal rabbit serum. Todd-Hewitt broth. Symbols: O, viable count at 0 hr; ●, viable count after 24 hr of incubation at 37°C. Supernatant 1: cell-free supernatant of Newman D₂C strain. Supernatant 2: cell-free supernatant of Smith compact strain.

FIG. 3. Reversal of serum antistaphylococcal activity toward serum-susceptible strains Kulba and E-7. For explanation see Fig. 2.

TABLE 7. Comparison of growth in normal and immune serum of *Staphylococcus aureus* strains Smith compact and Mendita*

Test strain	Rabbit immune serum against Mendita	Rabbit immune serum against Smith compact	Normal rabbit serum
Smith compact	940.00	960.00	860.00
Mendita	0.02	0.02	0.01

* Results expressed as growth index values (see Materials and Methods).

TABLE 8. Activation and blocking of serum fibrinolytic system, presence of EDTA and addition of complement to mouse serum, and antistaphylococcal activity of serum*

Substance added	Serum used	Growth†	Control: growth in normal serum
Streptokinase	Human	—	— (human)
Staphylokinase	Rabbit	—	— (rabbit)
Staphylokinase	Human	—	— (human)
EACA	Human	—	— (human)
EACA	Rabbit	—	— (rabbit)
EDTA	Rabbit	—	— (rabbit)
Complement	Mouse	+	+

* Test strains were Mendita, Kulba, and Zak.

† Growth index higher than 1.0 was accepted as positive result.

lytic activity. The antistaphylococcal serum system was also unaffected by the presence of EDTA.

Rabbits were infected with different strains of staphylococci: three of the strains used (E-7,

Mendita, and 8447-B) were inhibited in vitro by sera of healthy animals, whereas five others grew abundantly.

Staphylococcal infection in rabbits does not significantly influence serum antistaphylococcal activity, which is the same as in sera of normal animals. Regardless of whether infection was with a growing strain or with one inhibited in normal serum in vitro, the test strain Smith compact grew easily, whereas strain Mendita was still susceptible to the action of serum derived from the infected rabbit. Virtually identical results were obtained with all strains used for infection and all serum samples. A typical example is given in Fig. 4. Intravenous infection with *Streptococcus pyogenes* and *Escherichia coli* also produced no changes in the studied phenomenon (Fig. 5).

Near the 14th day of infection, sera of infected rabbits exhibited transitory inhibitory properties, returning to initial values on approximately the 21st day. This inhibition, was, however, slight, and should not be considered as bacteriostatic activity: growth indexes were only slightly lower. A correlation was noted with the results of determinations of 5'-nucleotidase activity in peripheral blood polymorphonuclear leukocytes of infected rabbits (Table 9). The percentage of cells with activity of this enzyme was also highest on the 14th day of infection and returned to approximately the initial value 1 week later. At 3 weeks after the first injection of staphylococci, agglutinating titers of sera were tested against the Smith compact strain. Titers were generally low (the

highest reaching 1:32 in only five sera); seven sera did not agglutinate at all.

All rabbits were killed and tested bacteriologically. The presence of infection, very frequently detectable in kidneys only, was established.

DISCUSSION

Sera derived from various species differ in their antistaphylococcal activity. Rooster serum is inhibitory for almost all staphylococcal strains. Horse and sheep sera were more inhibitory than the others observed in this series. On the other hand, cat serum was only weakly bacteriostatic, and mouse serum failed to inhibit multiplication of any of the staphylococcal strains used. Mouse serum was found to be inactive against gram-negative bacilli also (16), and this was attributed

FIG. 4. Growth of *Staphylococcus aureus* Smith compact and Mendita in sera of rabbits infected with strains Mendita and Newman D₂C. On ordinate, logarithm values of the growth index are given. Symbols: ○, growth of Smith compact strain in Mendita serum; ×, growth of Smith compact strain in Newman D₂C serum; △, growth of Mendita strain in Mendita serum; ●, growth of Mendita strain in Newman D₂C serum.

FIG. 5. Growth of *Staphylococcus aureus* Smith compact and Mendita in sera of rabbits infected with *Streptococcus pyogenes* and *Escherichia coli*. Symbols: ○, growth of Smith compact strain in *S. pyogenes* serum; ×, growth of Smith compact strain in *E. coli* serum; △, growth of Mendita strain in *S. pyogenes* serum; ●, growth of Mendita strain in *E. coli* serum.

TABLE 9. 5'-Nucleotidase activity in peripheral blood leukocytes of rabbits infected with various strains of *Staphylococcus aureus* and single strains of *Streptococcus pyogenes* and *Escherichia coli*

Microorganism used for infection	Day of infection			
	0	7	14	21
<i>Staphylococcus aureus</i>	19.6	48.2	76.3	32.6
<i>Streptococcus pyogenes</i>	21.2	64.2	75.1	43.1
<i>Escherichia coli</i>	18.2	29.5	31.3	27.4

* Results are expressed as the percentage of leukocytes with 5'-nucleotidase activity. Mean values of determinations from all rabbits in each group. Individual differences were slight.

to the low level or absence of C'2 and C'3 complement components (17). When, however, guinea pig serum rich in complement is added to the mouse serum, this results in the appearance of its bactericidal activity against gram-negative bacilli. We did not observe changes in mouse serum antistaphylococcal activity after addition of complement. Species differences in bacteriostatic activity of serum against staphylococci are at present difficult to explain and need further study.

Coagulase exerts no influence on the antistaphylococcal activity of serum toward serum-susceptible staphylococci. This is clearly evident from experiments with coagulase-containing sera, and by isolation of serum-resistant strains not producing detectable traces of coagulase; such strains are usually clumping factor-positive and grow in normal sera as typical coagulase-positive staphylococci. This is in accordance with previous observations from this laboratory (23, 24). It was shown, however, that several very simple factors remove serum antistaphylococcal activity. This may be done by pure broth precipitate obtained by full saturation of staphylococcal culture supernatant fluids regardless of their ability to produce coagulase. This reversal of inhibitor contained in serum seems nonspecific, and should not be attributed to coagulase, which, used in a purified form and in controlled conditions, does not cause any significant changes in serum bacteriostatic activity for staphylococci. Similar results were reported by Fletcher (9), who found that the inhibitor is active at an alkaline pH and is resistant to heating at 100 C. This author was unable to confirm the findings of Ekstedt cited in the introduction to this paper. Fletcher (1962) found that many proteinaceous materials, such as peptone or even a pool of amino acids, remove the inhibitor from serum.

Serum antistaphylococcal activity is also not dependent on the presence of agglutinating antibodies against staphylococcal cells. On the assumption that staphylococci may destroy the inhibitor in serum by activation of the serum fibrinolytic system, experiments with streptokinase and staphylokinase were performed. However, neither activation of this system nor its blocking by EACA resulted in any changes of the serum antistaphylococcal activity.

The mechanism of this phenomenon remains unknown, and further research is needed. It seems very interesting, in view of observations made by Hirsch (12) and Des Prez et al. (2), to study the release of bactericidins from platelets after their damage, which occurs also during physiological clotting.

Experimental staphylococcal infection evoked

in rabbits by the intravenous route does not result in significant changes in serum antistaphylococcal activity of these animals. The same applies to infection caused by *Streptococcus pyogenes* and *Escherichia coli*. Sera from infected animals, like sera from healthy rabbits, are inhibitory for serum-susceptible staphylococci, whereas pathogenic strains multiply rapidly.

The slower multiplication rate of staphylococci in sera of rabbits taken 2 weeks after infection, together with the increase in the number of polymorphonuclear leukocytes with 5'-nucleotidase activity, may suggest that this moment presents the peak of mobilization of defense mechanisms and infection progress. After the 14th day of experimental infection, both growth index and leukocyte 5'-nucleotidase activity return to the initial value. It has been recently proposed that number of polymorphonuclear leukocytes with 5'-nucleotidase activity may be considered as a measure of staphylococcal and streptococcal infection (Szmigielski, Jeljaszewicz, and Wilczynski, *Pathol. Microbiol.*, *in press*).

Bacteriostatic activity of serum against some strains of staphylococci seems to present one of the nonspecific resistance factors. Its importance, however, is doubtful, at least in experimental staphylococcal infection of the rabbit. It should be noted also that, by the method of infection used, strains of *S. aureus* recognized as nonpathogenic on the basis of all bacteriological criteria and inhibited in serum *in vitro* (Mendita and 8447-B) caused pyelonephritis in rabbits.

Bacteriostatic activity of serum against staphylococci should be rather considered as an "in vitro" phenomenon, which may be helpful for indirect laboratory recognition of "pathogenic" and "nonpathogenic" staphylococci. This may be done by a recently described plate test (Cybulska and Jeljaszewicz, *J. Clin. Pathol.*, *in press*), by means of which classification of pathogenic strains may be as easy as by the coagulase test. It should be mentioned here that some coagulase-negative strains are responsible for infections and, as shown in this communication with a Kulba strain, only detection of its moderate resistance to the action of serum enabled the differentiation of this strain from other coagulase-negative staphylococci. Antistaphylococcal activity of serum seems to have no importance in defense mechanisms of rabbits infected intravenously with staphylococci. Its real role in pathogenesis of staphylococcal infection and nonspecific defense mechanisms of the host is not known. It does not seem, however, that this phenomenon is of primary importance.

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