Comparison of the Bactericidal Activity of Different Vertebrate Sera

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ABSTRACT

SCHWAB, G. E. (University of Adelaide, Adelaide, South Australia), AND P. R. REEVES. Comparison of the bactericidal activity of different vertebrate sera. J. Bacteriol. 91:106–112. 1966.—The bactericidal activity for gram-negative bacteria of normal sera from eight species of vertebrates was investigated to compare complement-mediated killing in sera from animals representing various classes of vertebrates. Although all the sera were bactericidal, there was considerable variation in the range of bacterial species killed and in the bactericidal titers. The temperature dependence of the bactericidal and hemolytic complement activities was also studied. The curves relating activity to temperature were similar in shape for sera from a homeotherm and poikilotherms, but those for the homeotherm reached their maximum at temperatures of 5 to 10 C higher than the poikilotherms. The role of lysozyme and complement in killing rough gram-negative bacteria was examined, and the results suggest that, as for smooth organisms, killing is due to antibody plus complement. These natural antibodies, like those for smooth strains, were shown to be specific.

There is current interest in the question of antibody production by animals lower on the evolutionary scale than mammals. An easy screening test for this is to see whether the serum of such animals participates in the complement-mediated bactericidal reaction, since natural antibody has been found to be a necessary reactant. The present study was carried out in conjunction with work with invertebrates (Schwab, Reeves, and Turner, *unpublished data*) and was concerned with comparing the bactericidal activities of normal sera from various animals, all of which are known to produce specific acquired antibody on immunization.

MATERIALS AND METHODS

Media for bacterial culture. Nutrient broth (code no. CM67) and agar (code no. CM55) were prepared from Oxoid dehydrated culture media reconstituted as specified by the manufacturer.

Serum broth is the above medium containing 25% heat-inactivated sterile horse serum.

Maintenance of strains. Stock cultures of bacteria were maintained in a freeze-dried state in ampoules. From each freeze-dried ampoule, six agar slope cultures were prepared; each was used 10 times, then discarded, and a new culture was opened.

Bacterial strains. The bacterial strains used, to-

gether with their source and antigenic structure (where known), are listed in Table 1.

Collection of serum. Human blood was obtained by venipuncture from blood donors of group O. Each pool consisted of the serum from three persons.

Pig blood was collected aseptically from the jugular veins of animals slaughtered at the local abattoirs. A pool was made from about 15 animals. Sera from fetal pigs were also used; the embryos were obtained from slaughtered sows. After being detached from the placenta the embryos were bled by cardiac puncture. Each pool consisted of the serum from about 15 embryos. Kangaroos (*Macropus rufus*) were shot and then bled from the jugular vein. About 20 animals were bled for each serum pool. An inbred line of white leghorn fowls were bled from the wing vein. Each pool consisted of the serum from approximately 50 birds. "Sleepy" lizards (*Tiliqua rugosa*), 20 or more, were bled by cardiac puncture, and the sera were pooled. Toads (*Bufo marinus*) were pithed, a median incision was made, and blood was withdrawn from the exposed heart. Approximately 60 animals were bled for each serum pool. "Gummy" sharks (*Emissola antartica*) were bled by severing the tail. Blood was obtained from tench (*Tinca tinca*) and conger eel (*Conger conger*) by cardiac puncture.

In all cases, the blood was allowed to clot for 1 hr at 37 C, after which the serum was separated by centrifugation at 1,000 × *g* for 30 min. After dis-

TABLE 1. *Bacterial strains used for investigating serum bactericidal activity*

Bacterial strain	Antigenic structure	Reference	Remarks
Escherichia coli Lilly.............	—*	16	Rough strain
E. coli BV.......................	OIII; B4; H12	12	Referred to as strain E2206 (AV); smooth strain
E. coli CV.......................	OIII; B4; H12	12	Referred to as strain E2380; smooth strain
Salmonella typhimurium C5.......	I, IV, V, XII; i; 1, 2, 3	4	Mouse virulent
S. typhimurium M206.............	I, IV, V, XII; i; 1, 2, 3	7	Strain 206 Maaløe; avirulent for mice
S. gallinarum 9240...............	I, IX, XII	14	Referred to as strain 9.ex. NCTC 9240
S. paratyphi BIS.................	I, IV, V, XII; b; 1, 2	6	Smooth strain was derived from the rough
S. paratyphi BIR.................	—*	6	

* Antigenic structure not known.

pensing in 5-ml volumes, the sera were stored at −20 C.

Preparation of antibacterial immune sera. Rabbits were immunized by 13 successive injections at 3-day intervals. The first injection of 5 × 10⁷ steam-killed (2 hr) bacteria was given intraperitoneally, followed by four intravenous injections with doses progressively increasing to 5 × 10⁸ bacteria. These injections were followed successively by three intravenous injections of alcohol-killed bacteria and three injections of bacteria disrupted in an ultrasonic disintegrator. The animals were finally given three injections of living cells, the first (5 × 10⁷) intraperitoneally. The animals were bled by cardiac puncture 7 days after the last injection.

Preparation of inocula for bactericidal assays. The bacteria were incubated overnight in shaken serum broth at 37 C. A 1:100 dilution of this culture was made in fresh serum broth which was incubated for 4 hr. The inocula for the serum assays were prepared by diluting this culture in minimal medium.

Bactericidal assays. The sera were diluted in the minimal medium of Davis and Mingioli (3). Glucose was omitted for all strains except *Escherichia coli* Lilly. Conger eel serum was diluted in sterile seawater. The final volume of diluted serum was 1 ml. Serum dilutions were transferred to a water bath, and, after 5 min, 0.1 ml of a dilution of culture containing approximately 5 × 10³ bacteria was added. A control for each bacterial strain was included in the diluent alone. At time intervals, 0.02-ml samples from each tube were dropped onto quadrants of a nutrient agar plate. After overnight incubation at 30 C, the colonies were counted. The end point of the titration was the highest dilution of serum which killed 50% or more of the inoculum over a period of 90 min.

Hemolytic complement assay. A small-scale modification of the assay system of Kabat and Mayer (5) was used. The reagents were added in the following order: 0.4 ml of sensitized erythrocytes; sufficient buffer to make the final volume 1.2 ml; then 0.4, 0.3, 0.2, or 0.1 ml of an appropriate serum dilution. The tubes were incubated in a water bath for 30 min, after which the residual red cells were removed by low-speed centrifugation. The optical density of the clear lysate was measured against a diluent blank in a Coleman Junior spectrophotometer at 541 mμ. The 50% lytic end point was determined by interpolation from a standard curve prepared by lysing known concentrations of the sensitized sheep red blood cells.

Assay of lysozyme. Lysozyme was assayed according to the method of Wardlaw (16).

Serum absorptions. Bacteria were grown overnight at 37 C in shaken nutrient broth cultures. The centrifuged deposit was suspended in 70% alcohol for 16 hr at room temperature, and the killed bacteria were washed in saline. The dry weight of this suspension was estimated after making allowance for the salt present. For absorption, the required amount of bacterial suspension was centrifuged, resuspended in the undiluted serum, and stored overnight at 4 C, followed by removal of the bacteria by centrifugation.

Assay of serum lysozyme. A *Micrococcus lysodeikticus* suspension was prepared from lawn plates incubated 48 hr at 30 C. The bacteria were washed in saline, resuspended in test buffer, and killed by ultraviolet irradiation in thin layers for 15 min. This standard suspension was kept at −20 C. The assay procedure used was that of Wardlaw (16). All measurements of optical density were made on a Unicam SP 600 spectrophotometer at 540 mμ. A standard curve, relating percentage lysis to egg white lysozyme concentration, was prepared for each series of assays, by use of lysozyme levels from 0.25 to 2 μg/ml of reaction mixture. The unknown concentrations of lysozyme were calculated by interpolation from the standard curve. Egg white lysozyme used throughout these studies was Calbiochem grade B.

RESULTS

Bactericidal spectra of the sera. The bactericidal activities of the sera are shown in Tables 2 and 3. Although the bactericidal activities of different

TABLE 2. *Range of gram-negative bacteria killed by the various normal sera**

Bacterial strain	Normal animal sera†					
	Human	Pig	Kangaroo	Lizard	Fowl	Toad
Escherichia coli BV.....................	10	20	20	40	20	2
E. coli Lilly..........................	300	100	30	60	5	40
Salmonella typhimurium M206..........	50	40	20	100	40	40
S. typhimurium C5.....................	0	0	0	8	0	0
S. gallinarum 9240.....................	2	0	0	20	0	0
S. paratyphi BIS......................	2	10	0	10	0	2
S. paratyphi BIR......................	80	40	8	10	0	2

* Results expressed as the reciprocal of bactericidal titer. The bactericidal activity of human, pig, kangaroo, lizard, and fowl sera was estimated at 37 C; toad, at 25 C.

† 0 = no killing at a serum dilution of 1:2.

TABLE 3. *Range of gram-negative bacteria killed by the various normal fish sera*

Bacterial strain	Sera					
	Shark		Tench		Conger eel*	
	25 C	37 C	25 C	37 C	22 C	37 C
Escherichia coli BV......	0†	0	0	0	>16	8
E. coli Lilly.............	20	10	10	5	—	—
E. coli CV..............	—	—	—	—	>16	4

* These results were obtained by D. Rowley while working at the Laboratoire Arago, Banyuls, France, during the summer of 1956.

† Results expressed as reciprocal of bactericidal titer. Symbols: —, not tested; 0, no killing at a serum dilution of 1:2. The other five strains were not killed by either shark or tench sera at a dilution of 1:2.

serum pools from one animal species were not constant, the variation was less than that among pools of sera from different animal species. The ratio between the bactericidal titers for the smooth and rough strains of *E. coli* (BV and Lilly, respectively) varied considerably in different animal sera. Although the smooth strain was the more resistant to the lethal activities of human, pig, and toad sera, with fowl serum the sensitivities were reversed. Kangaroo and lizard sera occupied an intermediary position, both strains being equally sensitive to them. There were similar but less pronounced differences in the relative sensitivities of the smooth and rough strains of *Salmonella paratyphi* B.

Effect of temperature on the bactericidal and hemolytic activities. The effect of temperature on the level of bactericidal activity for *E. coli* Lilly in pig, lizard, and toad sera was studied by estimating the bactericidal titer at 5 C intervals from
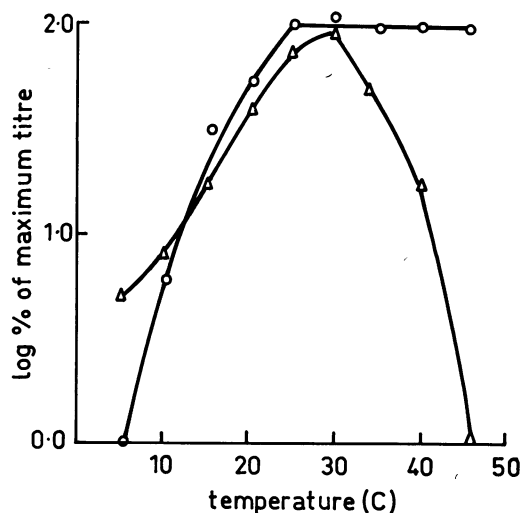

FIG. 1. *Effect of temperature on the level of bactericidal activity of pig and toad sera for Escherichia coli Lilly. Pig serum,* ○; *toad serum,* △. *The titers for each serum are expressed as the log percentage of the maximal titer for that serum.*

5 to 45 C. Curves for pig and toad sera are shown in Fig. 1; the lizard serum behaved similarly to toad. The bactericidal activities of fish sera were also greater at 25 C than at 37 C (Table 3).

The effect of temperature on the hemolytic activity of lizard and toad sera was similar to the effect on bactericidal activity, but, with pig serum, hemolytic activity was at a maximum between 35 and 40 C. The temperature versus hemolytic-activity curve for shark serum was similar to those for lizard and toad sera. Curves for pig and toad sera are shown in Fig. 2.

Heat lability of toad and lizard serum complement. All the hemolytic and bactericidal activity (as estimated at 25 C) in toad serum was lost

when the serum was previously heated at 45 C for 30 min. Both activities in lizard serum were reduced by 70% when treated similarly. Heating at 40 C for 30 min reduced the titers in both sera by 30%. Both the bactericidal and hemolytic titers of the heated sera estimated at 25 C were greater than those of untreated sera tested at 40 or 45 C.

Specificity of natural antibodies. The specificity of the natural antibodies for smooth strains of gram-negative bacteria were studied by absorbing

FIG. 2. *Effect of temperature on the hemolytic complement activities of pig and toad sera. Pig serum, ○; toad serum, △. The titers of each serum are expressed as a percentage of the maximal titer for that serum.*

sera with graded quantities of one bacterial serotype, then determining what effect these absorptions had on the bactericidal activity for homologous and heterologous strains.

The bactericidal titers of human serum for several strains of bacteria after absorption with either *E. coli* BV or *S. typhimurium* M206 are shown in Table 4. These studies suggested that the natural antibodies for these smooth strains were specific. Similar studies showed that the natural antibodies in lizard serum for *S. typhimurium* C5 were also specific. That antibody was removed during these absorptions was shown by the restoration of bactericidal activity to absorbed sera by suitable dilutions of homologous rabbit antibody. The bactericidal activity of human serum for *E. coli* Lilly was also reduced by absorption with the homologous strain without affecting the killing of *E. coli* BV. However, although 2 mg (dry weight) of bacteria per ml of serum produced a significant reduction in bactericidal titer for smooth strains, the quantity had to be increased 25-fold to give a similar decrease in titer for *E. coli* Lilly.

Study of normal pig serum for antibody to S. typhimurium C5. Since both antibody and complement are necessary for serum killing of gram-negative bacteria, the possibility was considered that lack of bactericidal activity could be due to the absence of complement-fixing antibodies. Pig serum, nonbactericidal for *S. typhimurium* C5, was absorbed for 30 min at 37 C with graded amounts of a living suspension of *S. typhimurium* C5. After the absorbing bacteria had been removed by centrifugation, the sera were assayed
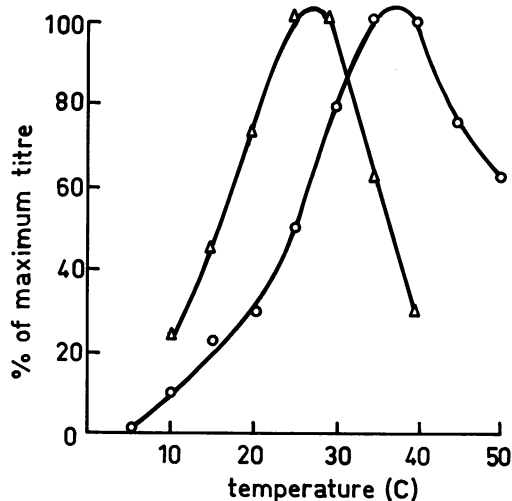
TABLE 4. *Specificity of natural sensitizers in human serum after absorption with Escherichia coli BV or Salmonella typhimurium M206*

Organism used for absorption	Bacterial strain	Dry weight of absorbing suspension per ml of serum			Unabsorbed control
		8 mg	4 mg	2 mg	
E. coli BV	*E. coli* BV	2*	2	2	10
	S. typhimurium M206	50	50	50	50
	S. paratyphi BIR	40	80	80	80
	E. coli Lilly	200	200	200	200
	C'H$_{50}$ per ml of serum	25	35	35	35
S. typhimurium M206	*E. coli* BV	10	10	10	10
	S. typhimurium M206	5	15	15	60
	S. paratyphi BIR	40	40	40	40
	E. coli Lilly	200	200	200	200
	C'H$_{50}$ per ml of serum	35	35	35	35

* Results expressed as reciprocal of bactericidal titer.

for residual hemolytic complement. When the dry weight of absorbing bacteria exceeded 1 mg/ml of serum, all hemolytic activity was lost (Table 5).

The resistance of *S. typhimurium* C5 to the bactericidal activity of pig serum would appear to be caused by the inability of complement fixed to the cell surface to initiate the reactions leading to the death of the cell, rather than by the absence of complement-fixing antibodies.

Serum factors participating in the killing of E. coli Lilly. Since it has been suggested (15, 16) that complement or complement and lysozyme are responsible for the killing of rough strains of gram-negative bacteria without the mediation of antibody, an effort was made to discover whether a correlation existed between lysozyme and hemolytic complement levels in individual serum pools and the strength of the bactericidal reaction for *E. coli* Lilly.

The lysozyme and hemolytic complement levels, together with the bactericidal titers for *E. coli* Lilly, of six sera are shown in Table 6. The only relationship found was that, in human, pig, and kangaroo sera, the product of the lysozyme con-

TABLE 5. *Effect of the quantity of absorbing suspension of Salmonella typhimurium C5 on the fixation of complement from normal pig serum*

Absorbing dose per ml of serum	Residual hemolytic complement ($C'H_{50}$ per ml of serum)
mg	
0	86
0.5	46
1.0	25
2.0	0
4.0	0
8.0	0

TABLE 6. *Relationship between the bactericidal titers of each of the sera for Escherichia coli Lilly and the levels of hemolytic complement and serum lysozyme**

Serum	Serum lysozyme per ml of serum	Reciprocal of bactericidal titer	$C'H_{50}$ per ml of serum
	μg		
Human	9	300	40
Pig	0.6	40	53
Kangaroo	2	30	32
Lizard	11	20	43
Toad	0.25	20	100
Fetal pig	0.25	10	ND†

* All figures refer to tests made at 37 C.
† Not detected.

TABLE 7. *Comparison of the bactericidal titers of fetal and adult pig sera for several species of gram-negative bacteria*

Bacterial strain	Serum	
	Fetal pig	Normal pig
Escherichia coli Lilly	10*	100
E. coli BV	2	20
Salmonella typhimurium M206	10	40
S. typhimurium C5	0	0
S. gallinarum 9240	0	0
S. paratyphi BIS	0	10
S. paratyphi BIR	5	40
$C'H_{50}$ per ml of serum	0 units	53 units

* Results expressed as reciprocal of bactericidal titer.

centration and $C'H_{50}$ levels approximated the reciprocal of the bactericidal titer for *E. coli* Lilly. This correlation was not found with lizard, toad, or fetal pig sera.

The addition of 10 μg of egg white lysozyme per ml to toad or pig serum did not increase the bactericidal titers for *E. coli* Lilly, although the levels of both lysozyme and hemolytic complement were then in excess of those in human serum, the serum having the highest bactericidal titer for *E. coli* Lilly.

Bactericidal activity of fetal pig serum. It has been suggested (15) that fetal pigs are unable to produce antibodies, and, hence, their serum does not kill smooth strains of gram-negative bacteria. Since the fetal pig serum killed rough strains it was postulated (15) that this killing was not mediated by antibody. This concept of the mechanism of serum killing conflicted with our own observations, so we investigated the bactericidal spectrum of fetal pig serum. Smooth and rough strains of gram-negative bacteria were killed by two pools of fetal pig serum. Except for *S. paratyphi* BIS the same strains were killed by fetal and adult sera, but the titers for the former were consistently lower (Table 7). Since no one doubts that antibody is necessary for the serum killing of smooth strains of gram-negative bacteria, we concluded that antibody was present.

DISCUSSION

Although the complement-mediated bactericidal activity of mammalian sera has evoked much attention since the first demonstration of this phenomenon in the latter part of the last century, little attention has been paid to a study of this phenomenon in other vertebrate sera. In the

present study, we have investigated killing by serum from poikilothermic, as well as homeothermic, vertebrates. The mechanism of the killing appears to be the same for all the sera studied.

The data presented in Table 2 show that the range of bacteria killed by sera from different animal species differs considerably. Though some sera appear to be generally more active than others and some bacteria more sensitive than others, it is not possible to put the various sera or the various bacterial strains in an unambiguous order with respect to these properties, and it is obvious that there is a considerable degree of specificity involved.

The concept that smooth Enterobacteriaceae are more resistant to serum killing than their rough variants has been generally accepted (8, 13). This concept may apply to the sera usually studied, but it does not hold true in all situations as the present study shows.

Both the lethal and hemolytic activities of the sera were influenced by temperature, but the effect was not constant. Although the shapes of the curves relating activity to temperature were similar for sera from both homeo- and poikilotherms, those for a homeotherm were, in general, transposed towards higher temperatures, presumably reflecting the higher body temperatures of these animals. The better killing by fish sera at 22 to 25 C than at 37 C might also be due to lability of complement at the higher temperature, although this was not tested. Cushing (1, 2), working with mammalian, amphibian, and fish sera, reported a similar effect of temperature on the lysis of red blood cells, and, in addition, he was unable to affect a mutual interchange of all the components of complement among the sera.

With toad and lizard sera, the hemolytic and bactericidal activities show a very similar temperature dependence; likewise, these two activities are similarly inactivated by heat, suggesting that the same agent, presumably complement, is involved both in hemolysis and killing. We have observed that other treatments, such as aging and absorption with antigen-antibody precipitates which inactivated hemolytic complement, also depleted the bactericidal activity of these sera, further pointing to the involvement of complement in the bactericidal reaction. Pig serum, however, gives an anomalous result, since there is a plateau in the bactericidal activity but a peak in the hemolytic activity. However, the difference may exist because in the bactericidal assay the overall reaction including antigen-antibody interaction is studied, whereas in hemolysis the reaction of complement with cells presensitized with antibody is measured, and, in the

reaction of pig serum with E. coli Lilly, antibody may be limiting with temperatures higher than 25 C.

It now seems firmly established that natural antibodies for smooth strains of gram-negative bacteria are specific (9), but the question has been posed whether antibodies are necessary for the serum killing of rough strains (15, 16). Muschel and Jackson (11) have, however, obtained results compatible with the classical concept that antibody is a mediator in the serum killing of rough as well as smooth strains. The strain specificity of natural antibodies for smooth strains has been corroborated in the present study, and, in addition, the bactericidal activity for rough strains has been specifically depleted by absorption.

Absorbing doses of a different order of magnitude were required to produce a similar percentage decrease in bactericidal activity for smooth compared with rough strains. This could be due either to serum containing more antibodies to rough strains than smooth, or rough strains containing less antigen than smooth.

The findings of Wardlaw (17) that, whereas lipopolysaccharide constitutes 9% of the cell walls of a smooth E. coli, only 1% of the walls of the rough strain E. coli Lilly is lipopolysaccharide, support the latter alternative.

Our inability to find a consistent correlation between the bactericidal titer for E. coli Lilly and the level of serum lysozyme suggests that lysozyme may not be responsible for the bactericidal titer of a serum, although it may lyse those bacteria which have been killed by complement and antibody (10).

During recent years, much effort has been directed towards determining the time during development at which animals become immunologically competent. Sterzl et al. (15) believe that with pigs this is a postnatal event, and, hence, killing by fetal serum cannot be antibody-mediated. Now that we have shown that fetal pig serum can kill smooth as well as rough strains, we must conclude that antibody is present, and we do not have to postulate a special mechanism against rough strains.

Our studies show that the antibody-complement bactericidal system is present in the sera of diverse species of vertebrates, and they support the hypothesis that all vertebrate serum killing of gram-negative bacteria is mediated by the antibody-complement system.

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