STUDIES RELATING TO THE SERUM RESISTANCE OF CERTAIN GRAM-NEGATIVE BACTERIA

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Bactericidal effects that require the participation of antibody and complement were observed first with the cholera vibrios (2) and have been shown subsequently to affect a great many Gram-negative species including the *Escherichia*, *Salmonella*, *Proteus*, *Shigella*, *Chromobacter*, *Hemophilus*, and *Brucella* (3). Notable exceptions exist, however, and some strains of *Paracolobactrum ballerup*,[1] *Salmonella typhimurium*, and *Salmonella paratyphosa C*, under usual test conditions, are resistant to serum bactericidal action with "normal" or immune serum as an antibody source (4, 5). The properties associated with this resistance are not readily apparent and may vary in different organisms. For example, although O-inagglutinability has been correlated with serum resistance among strains of *Escherichia coli*, *S. paratyphosa C* is O-agglutinable (1).

This work was undertaken to study further the resistance of certain strains of the Enterobacteriaceae to serum bactericidal action. Most of the experiments were performed with *P. ballerup*, an organism which has been used often in studies of typhoid fever immunology because of its Vi antigen. The Vi antigen is an effective bactericidal target in *Salmonella typhosa*, and antisera against *P. ballerup*, and *S. paratyphosa C*, also a Vi containing organism, are highly bactericidal against Vi containing strains of *S. typhosa* (4). Yet these same antisera have been ineffective against their homologous organisms.

Materials and Methods

Bacterial Cultures.—*S. typhosa* strain Ty2, *P. ballerup* strain 7851/39, *S. paratyphosa C* strain 32 V, and *S. typhimurium* strain 37 were obtained from Mr. Arthur Abrams of WRAIR and maintained on meat extract agar.

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[1]This organism is a member of the *Ballerup* group, which has been reclassified in the *Citrobacter* group. However, *P. ballerup* has been used widely in typhoid fever serology, and the designation, familiar to many workers, has been retained in this publication.

Bactericidal Assays.— (*a*) *Photometric growth assay:* The basic method used has been described previously (6). The organism to be tested was streaked on meat extract agar and kept at 37°C or other experimental temperatures for about 16 hours prior to its use in the bactericidal tests. The bacterial growth was suspended in broth, a measured amount of the suspension containing about 2×10^7 organisms was added to tubes containing appropriate amounts of serum substances, modified according to the experimental purpose, and the tubes incubated at 37°C for 60 minutes. Assay of the surviving organisms was made then by addition of 2½ volumes of broth to each tube. Upon reaching a suitable reading range with the control tubes still in the log-growth phase, the optical densities of the tubes were determined, and, from these data, the percentage of organisms killed was calculated. When the percentages killed, converted to probits, were plotted against the logarithm of the serum amounts, a linear representation of data resulted. The 50 per cent serum dosage was read off by interpolation; the reciprocal of this amount represented the serum titer. (*b*) *Plate count method:* The test organism was an 18 hour meat extract agar subculture washed off with saline and diluted with saline according to test requirements. Equal volumes of serum substances and the bacterial suspension were mixed and incubated at 37°C for different periods of time. Assay of surviving organisms was performed by spreading 0.1 ml samples of the test mixtures on meat extract agar plates with sterile glass beads. The plates were incubated for about 16 hours and the colonies counted (7). (*c*) *Antibiotic inhibition:* The procedure used was similar to the photometric growth assay technique for serum bactericidal action except that graded amounts of antibiotic were used in place of serum substances (8).

Vi antigen determination: Acidic extracts of acetone-dried organisms of *P. ballerup* cultured at different temperatures were prepared. The smallest amount of the neutralized extract required to inhibit the agglutination of maximally sensitized red blood cells coated with purified Vi antigen by an anti-Vi serum was determined (9). This value provided a basis, therefore, for determination of the relative Vi content of the different organisms tested.

RESULTS

Experimental Variations Designed to Overcome the Resistance of P. ballerup to Serum Bactericidal Action.—

1. Changes in the photometric growth assay *procedure*: (*a*) the reaction time, usually 1 hour, was varied between ½ and 4 hours; (*b*) serums, from different animals including the guinea pig, rabbit, pig, and human were used as a complement source alone and with rabbit anti-*P. ballerup*. None of these attempts resulted in detectable bactericidal action with the growth assay procedure.

2. The possible inhibitory effect of a "secondary" antibody was suggested by Adler's finding that an "antibody" in normal serum capable of adsorption by R-state cells inhibited the bactericidal action of the "primary" antibody against *S. typhosa* strain 0901 (10). Normal rabbit serum and rabbit anti-*P. ballerup* serum were both absorbed twice with heat-killed saline washed *S. typhosa* strain Ty6S, a rough organism possessing Vi antigen, small amount of H antigen, and no O antigen. The absorbed sera, like the unabsorbed samples, were lacking in bactericidal activity. Probably a rough variant of *P. ballerup* would have been a better choice as an adsorbing organism but it was unavailable.

3. The bacterial cells were treated with various enzymes in order to expose possible target antigens. Lysozyme, protease, papain, chymotrypsin, and trypsin were used, but none of these altered the organism's serum resistance.

Complement Fixation.—It seemed important to determine whether the sensitized cells of *P. ballerup*, apparently insensitive to the bactericidal action of complement, were capable of complement fixation. In a standard hemolytic system (11), with heat (30 minutes at 56°C) killed cells of *P. ballerup* as antigen, a normal rabbit preimmunization serum specimen gave a titer of 8 whereas the titer of the postimmunization serum was 128. Thus, the inability of antiserum, conjunction with complement, to kill *P. ballerup* could not be correlated with the lack of complement fixation in this system.

TABLE I

Bactericidal Titers of Preimmunization and Postimmunization Sera of Rabbits against the Homologous Organisms Cultured at Different Temperatures

Organism	Serum	Cultural temperature						
		17°C	23°C	30°C	37°C	39°C	41°C	43°C
P. ballerup 7851/39	Preimmunization....	<5	<5	<5	<5	<5	286	264
	Postimmunization....	<5	<5	<5	<5	<5	16,700	12,500
S. typhosa Ty2	Preimmunization....	105	83	<5	10	N.D.	N.D.	227
	Postimmunization....	15,000	4200	1930	1930	N.D.	N.D.	29,000
S. paratyphosa C 32V	Preimmunization....	<5	<5	<5	<5	N.D.	N.D.	<5
	Postimmunization....	<5	<5	<5	<5	N.D.	N.D.	<5

N.D., not done.

Effect of Different Temperatures of Cultivation on the Susceptibility of P. ballerup.—Previous work (9) indicated that when *S. typhosa* strain Ty 2 was cultured either at temperatures of 18° or 41.5°C, rather than 37°C, it lost most of its Vi antigen and became increasingly susceptible to serum bactericidal action. Since *P. ballerup* is a Vi-containing organism, it appeared reasonable to determine the effect of different temperatures of cultivation prior to the performance of the bactericidal reaction at 37°C. Accordingly, *P. ballerup*, as well as *S. typhosa*, and *S. paratyphosa C*, was cultured on meat extract agar at different temperatures for 16 hours prior to their use. The results with the photometric growth assay method in Table I indicate that *P. ballerup* grown at 41°C became susceptible to normal rabbit serum and extremely susceptible to antiserum, but that, unlike *S. typhosa* Ty 2, cultivation at 17°C did not alter its serum resistance. Growth of *P. ballerup* at 43°C, compared to 41°C, resulted in

a slight loss of its susceptibility. Recultivation of the 41°C-grown *P. ballerup* at 37°C resulted in a return to serum resistance. The serum susceptibility of the organism when grown at higher temperatures represents, therefore, a phenotypic change which is not transmissible. *S. paratyphosa* C maintained its resistance despite the change in temperature of cultivation.

It may be noted, finally, that temperature differences from 37–41°C during the 1 hour period of the bactericidal reaction has no appreciable effect on the test results with the organisms cultivated at different temperatures prior to their testing in the bactericidal reaction.

Properties of P. ballerup after Cultivation at Different Temperatures.— Tests for the presence of Vi antigen were made in *P. ballerup* cultures grown at different temperatures. At higher temperatures of cultivation, *P. ballerup* lost its Vi antigen as indicated by the loss in the ability of dried extracts of its cells to inhibit the agglutination of Vi-sensitized red cells by anti-Vi. The Vi hemagglutination inhibition titer after cultivation at 43°, 41°, and 39°C was, respectively, less than 2, 2, and 16, whereas at temperatures of 37° and 17°C, the titers were 64. The retention of Vi antigen by *P. ballerup* cultured at 17°C is associated with its serum resistance when cultured at that temperature (Table I). Furthermore, the immunization of rabbits with cells of *P. ballerup* cultured at 37°C and treated with 0.5 per cent formalin elicited a mean Vi hemagglutinin response of 64 in the sera from 3 rabbits in contrast to a lack of detectable Vi antibody with similarly treated cells from 41°C cultures. These results suggested that the Vi antigen was not the bactericidal target with normal or anti-*P. ballerup* sera in these experiments. Finally, the presence of 2 mg of purified Vi antigen derived from *E. coli* 5396/38 (12) did not inhibit the bactericidal activity of anti-*P. ballerup* against the homologous organism grown at 41°C.

When *P. ballerup* was grown in brain heart infusion broth at temperatures from 37–43°C for 16 hours, a progressive tendency toward granular growth was noted as the temperature of incubation increased. Such growth is probably associated with the change from bactericidal resistance to susceptibility. Granular growth in broth and serum susceptibility are two of the criteria used in defining the rough state of an organism (13).

Antibiotic Sensitivity.— It was of interest to determine whether the susceptibility of *P. ballerup* to serum as a result of its cultivation at elevated temperatures was paralleled by an enhanced sensitivity to other destructive agents. As the results indicate (Table II), the sensitivity of the organism to penicillin was increased at least 4-fold as a result of growth at elevated temperatures, but with relatively slight changes in chloramphenicol sensitivity. In addition, although its serum sensitivity was increased by growth at elevated temperatures, *S. typhosa* Ty 2 become less sensitive to the action of both antibiotics when grown at temperatures above 37°C.

Demonstrated Susceptibility of P. ballerup Cultured at 37°C to Serum Bac-

tericidal Action.— By using a small inoculum containing about 10^3 organisms and determination of the surviving organisms by plate count after extended contact between *P. ballerup* and serum, bactericidal effects of serum were easily accomplished (Table III). The lack of a detectable bactericidal effect noted previously after a similar reaction period of 4 hours is related to the large

TABLE II

The Sensitivity of P. ballerup, Cultured at Different Temperatures, to Pencillin and Chloramphenicol

Cultural temperature	Penicillin	Chloramphenicol
°*C*	*mg*	*μg*
37	>0.3*	1.6
39	>0.3	2.5
41	0.27	2.6
43	0.07	1.4

* Represents the amount of antibiotic required for 50 per cent growth inhibition of a standardized inoculum.

TABLE III

The Bactericidal Action of Normal and Immune Rabbit Serum Plus Complement (C′) against P. ballerup

Reaction time	Normal serum plus C′	Immune serum plus C′	C′alone	Inactivated (56°C for 1 hr.) C′	Saline alone
hrs.					
0	88*	117	99	101	96
1	139	118	138	136	117
2	304	278	224	230	105
3	172	260	213	336	104
4	10	62	45	>400	96
5	0	5	30	>400	75
6	0	2	14	>400	41
7	0	0	12	>400	17

* Represents number of surviving *P. ballerup* determined by plate count.

inoculum required when using the photometric growth assay method. In contrast to methods using smaller inocula an approximate inverse proportion has been observed between the inoculum density and serum titer with the photometric growth assay method (6). After the first 2 hours of reaction between a relatively small number of organisms of *P. ballerup* and serum substances, there is an increase in the number of organisms and it is only after 4 hours of contact that an appreciable bactericidal effect occurs (Table III). The complement source in these experiments was guinea pig serum absorbed once with heat-

killed saline-washed cells of *P. ballerup*. Its bactericidal activity is a reflection probably of inadequate absorption of naturally occurring "antibody." Also noteworthy is the comparable sensitizing activity of normal serum and immune serum under these experimental conditions. Similar experiments performed with Gram-negative organisms such as *S. typhosa* 0901 or Ty 2, which are readily susceptible to detectable serum bactericidal action even with inocula of over 10^7 organisms, have indicated that bactericidal action is readily detectable after only 30 minutes contact between the cells with antiserum and complement.

TABLE IV

Bactericidal Action of Normal Guinea Pig Serum Resulting from an Extended Reaction Period

Reaction time	*S. paratyphosa C*		*S. typhimurium*	
	Active normal serum	Inactivated (56°C for 30 min.) normal serum	Active normal serum	Inactivated (56°C for 30 min.) normal serum
hrs.				
0	85*	68	69	79
1	51	69	91	76
2	99	101	109	68
3	130	96	89	174
4	20	124	83	468
5	10	129	85	>500
6	7	203	44	>500
7	2	199	73	>500

* Represents number of surviving organisms determined by plate count.

Further experiments have shown that the extended reaction period between serum substances and *P. ballerup*, prior to killing of the organism, was not related to prolonged complement action. The lengthy reaction period apparently allows the organism to achieve a proper physiological state, presently undefined, for their reactivity with complement. Sensitized cells in such a state may be destroyed relatively rapidly. For example, after sensitization of *P. ballerup* by inactivated rabbit antiserum, the cells were washed and suspended in heat inactivated guinea pig serum, diluted 1:3, which served as a source of nutrient. After 4 hours at 37°C, specifically absorbed guinea pig serum as a complement source was added resulting in over 90 per cent killing of the *P. ballerup* in less than 30 minutes. Similar results were obtained without prior sensitization of the cells by addition of untreated guinea pig serum, serving as a source of both normal "antibody" and complement, or antiserum plus absorbed guinea pig serum after the 4 hour incubation period.

Serum Bactericidal Action against S. typhimurium and S. paratyphosa C.— Strains of these organisms which have been regarded as resistant to bactericidal

action may be destroyed even by normal guinea pig serum provided that at least 4 hours of contact were allowed between the organisms and the serum (Table IV). Moreover, the lack of effect of inactivated guinea pig, which served as a control, may be seen.

DISCUSSION

The results of this study have indicated primarily that certain Gram-negative bacteria, considered insusceptible to the bactericidal action of the antibody-complement system, are merely less susceptible than other bacteria and that special experimental conditions are required for their destruction by serum substances. Instead of an absolute distinction between susceptible and insusceptible organisms, probably a normal distribution of serum susceptibility occurs among the Gram-negative bacteria.

Cultured at temperatures of 41°C or higher, *P. ballerup*, an organism insusceptible to serum substances when cultured at normal body temperature, was markedly susceptible not only to immune, but also to normal serum acting in conjunction with complement. Low temperature cultivation, however, produced no detectable effect. It was found also that *S. typhosa* lost its Vi antigen and became increasingly serum sensitive when cultured at temperatures below as well as above 37°C. This difference between the two organisms was related to the Vi antigen content of the organism cultured at different temperatures.

The loss of serum resistance by these and other organisms (14) when cultured at elevated temperatures is significant obviously in considerations of the role of fever in host resistance. Fever has been considered to influence resistance to infection either in its effect upon the microorganism, or indirectly by thermal alteration of one or more of the various cellular or humoral mechanisms responsible for resistance to infection (15). The increased serum susceptibility of some microorganisms cultured at elevated temperatures resulted from changes to the microorganisms themselves, which conceivably might enhance a host's humoral defenses. Whether or not a temperature change would influence the bactericidal action of plasma *in vivo* without a concomitant change in the organism was not determined with our experimental model. Nonetheless this model is one of the few which satisfies nicely conditions that must be met if the adage that fever may enhance host resistance to infection is verified experimentally. These conditions involve first an agent that is pathogenic at normal body temperature. Secondly, the disease produced must elicit a febrile response, and lastly, the fever must injure the parasite or activate defensive mechanisms within the host (15).

Antigenic changes induced by temperature variation are not unique with the Gram-negative bacteria. The capacity for expression of alternative antigens on *Paramecium aurelia* for example, is determined by several environmental and nutritional factors including temperature (16). In treponemal infections, it is not known whether the therapeutic effect of fever results from the treponemicidal action of the higher temperatures or possible antigenic changes induced by the fever which render the treponemes more susceptible to the antibody-complement system (17). Unlike the induction of

fever by the Gram-negative bacteria, treponemal infections do not elicit a marked febrile response and fever should not be invoked, therefore, as a host defense mechanism (15).

The results of the tests for antibiotic sensitivity indicated no simple relationship between serum and penicillin sensitivity. This was not unexpected in view of the probable mechanisms of action of the two agents. Penicillin acts by interference with the synthesis of the "mucocomplex" of the bacterial cell wall (18), whereas serum bactericidal action results probably from an enzymatic attack by complement on the cell wall (19). When *P. ballerup* was cultivated at increased temperatures, it became increasingly sensitive to penicillin, but *S. typhosa* showed decreased sensitivity. Whereas the increased sensitivity of these Vi antigen–containing organisms to serum has been correlated with a loss of that antigen after cultivation at different temperatures, the underlying physiological changes associated with changes in penicillin sensitivity are not known. Obviously, susceptibility to penicillin depends on the amount of mucocomplex in the bacterial cell wall, and it would be pertinent to determine quantitatively the effect of temperature differences on mucocomplex formation. Serum or penicillin susceptibility, as might be suspected, was not associated with inhibition by chloramphenicol, which is a specific inhibitor of protein synthesis. In any event, the results of the antibiotic sensitivity experiments indicated that the increased susceptibility of *P. ballerup* to serum as a result of its cultivation at elevated temperatures was not paralleled by an indiscriminate increase in susceptibility to other destructive agents.

The finding that certain Gram-negative bacteria may be destroyed by the antibody-complement system only after they have been allowed to incubate for extended periods of time was of considerable interest. Cells of *P. ballerup* after growth overnight on meat extract agar and suspension in a small volume of inactivated absorbed guinea pig serum used as nutrient and diluted with saline became susceptible to serum bactericidal action only after an incubation period of at least 4 hours. In this experimental situation, the associated antigens or other changes in the organism rendering it serum sensitive have not been investigated, but warrant further study. Similarly the immobilization of *Treponema pallidum* by the antibody-complement system increases with time and 16 hours' incubation is used generally for the immobilization test procedure (20). The puzzling observation made by Kornfeld *et al.* (7) with a strain of *Escherichia coli* that required an extended incubation period with serum substances before demonstration of their bactericidal effect is also comparable. That strain too was resistant to serum with larger inocula and the usual reaction period of 60 to 90 minutes. No experimental evidence is available to explain the need for a long period of incubation for serum bactericidal action against certain organisms. It is likely that an organism must reach a certain stage in its growth cycle prior to its reactivity with the antibody-complement system. Even with Gram-negative organisms killed maximally after 60 minutes of contact with serum substances, diminished bactericidal activity has been observed with starved cells or with the surviving cells in a week-old culture (6).

Although these studies have indicated that probably all Gram-negative enteric bacteria may be destroyed by the antibody-complement system, it is

likely that *in vivo* this bactericidal reaction may not be sufficiently potent against those strains that require special conditions for their destruction. Considerable evidence is available which suggests that relatively high serum resistance is one of the requisites for virulence among the Gram-negative enteric bacteria (14, 21). For example, active immunization with Vi antigen protected mice against challenge with serum-sensitive *S. typhosa*, but did not provide protection against Vi-containing, ordinarily serum-resistant *S. paratyphosa C* (22). Similarly, antisera against *S. paratyphosa C* which did not give protection against challenge with the homologous organism readily protected mice against challenge with *S. typhosa* and, in conjunction with complement, was bactericidal only against the latter organism under standard laboratory conditions. Obviously, in addition to the bactericidal reaction, other immune mechanisms may play a part in mouse protection tests, but other comparable experiments have shown also a close correlation between mouse protection and serum bactericidal activity (23). Other *in vitro* studies to supplement the data provided by the model systems of this study should be undertaken.

SUMMARY

Paracolobactrum ballerup, an organism considered completely insusceptible to the bactericidal action of the antibody-complement system, became extremely sensitive to immune serum and even to normal serum, in conjunction with complement, when cultivated at temperatures above 37°C. This conversion to serum sensitivity was associated with the loss of the organism's Vi antigen and its tendency to assume a rough state. It did not result in a genetic change in the organism, however, since reincubation of serum-sensitive *P. ballerup* at 37°C resulted in a restoration of its resistance. The loss of serum resistance as a result of cultivation temperatures above 37°C may be of significance as an example of the enhancement of host defense mechanisms induced by fever.

Special conditions of testing indicated that *P. ballerup* cultured at 37°C and *S. paratyphosa C*, organisms considered insusceptible to serum bactericidal action, were not entirely refractory to serum. These conditions included simply a relatively low ratio of the number of test organisms to serum volume and an extended incubation period of the organisms with serum bactericidal substances, or even without these substances, prior to the initiation of bactericidal action. It is likely, therefore, that an absolute distinction between serum-sensitive and serum-resistant Gram-negative organisms does not exist, but rather that there is a very broad distribution of serum sensitivity among these organisms.

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