ANTIBACTERIAL SYSTEMS OF SERUM IN RELATION TO NONSPECIFIC IMMUNITY TO INFECTION

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By nonspecific resistance to infection we presumably include those factors which determine why some animal species resist infections to which others readily succumb. These factors do not appear to be identifiable with specific antibody since there may be widely differing responses to two organisms which are antigenically almost identical. A case in point is the susceptibility of normal mice to *Salmonella typhimurium* and their native immunity to *Salmonella gallinarum* or *Salmonella schottmuelleri*, to be contrasted with the susceptibility of chickens to *S. gallinarum* and resistance to the other two bacterial strains. Because of the similarity in antigenic structure of these three *Salmonella* species it seems unlikely that the immunity of normal mice to *S. gallinarum* is due to a specific normal antibody which is directed successfully against this strain, but is ineffective against *S. typhimurium*. There are many other examples like this. The contribution, if any, serum factors may make in determining this natural species immunity will be discussed later.

When the experimental infections most commonly studied in the laboratory are considered it is apparent that the great majority of them are initiated through a hypodermic needle for convenience. This must not be forgotton when examining the problems of natural susceptibility. For example, the factors responsible for the native resistance of mice to *Diplococcus pneumoniae* are unlikely to be found in mouse serum, since although natural pneumococcal infection rarely if ever occurs in mice, these bacteria are extremely lethal when introduced artificially. Therefore, resistance is likely to be due to factors encountered before the organisms reach the blood stream. A few moments spent in clarifying terms may be helpful in getting these problems into focus. If the pathogenicity of an organism for a certain animals is defined as its ability to cause disease naturally in that animal, then this can be put into an equation as follows:

Pathogenicity = virulence $\times$

(invasiveness and communicability)

The terms "invasiveness and communicability" are used to include those ill-defined characteristics of bacteria which are involved before the organism gets established in the tissues or blood stream. In the laboratory we deliberately short-circuit these elements of this equation by introducing the organisms directly into the tissues. What we then measure is the artificial virulence of the organism for the animal species, and only if this measure is proportional to the pathogenicity of the organism can we hope to find the important natural resistance and susceptibility factors by examining serum. Conversely, where the virulence, as determined in the laboratory, is proportional to the pathogenicity in the field, it may fairly be expected that the reasons for these susceptibility differences could be found in the blood or tissues fluids, if only we knew how to look for them. This requirement is met by the two *Salmonella* strain in mice and chickens referred to earlier.

After this preamble let us examine the known antibacterial systems of serum to see what each may contribute to the over-all immunity of its host.

The most widely reported direct antibacterial systems of serum are:

(a) The complement-properdin complex, operative mainly against gram-negative bacteria.

(b) Lysozyme (1), of uncertain antibacterial spectrum *in vivo*.

(c) The β-lysins (2).

COMPLEMENT SYSTEM

Of these antibacterial systems of serum, by far the most impressive in its potentialities is the first. The complexities of this system make it a very good area for disagreements; for most reports about the activities of complement, another contradictory one can be found. In spite of this difficulty, it is now more or less agreed that for antibacterial activity to be manifested by serum all four components of complement are necessary, together with divalent cations and properdin (3). The function of so-called natural antibody

and its possible relation to properdin is in doubt (4). There is no doubt in anyone's mind, however, that fresh serum from most animals is a most effective agent in the test tube for killing many species of bacteria and it is surely reasonable to expect the system to be useful and operative *in vivo*. When attempting to assess its importance *in vivo* we might anticipate some correlation between serum resistance and virulence of organisms; indeed it has been reported that the virulence of *S. typhimurium* (5) and *Escherichia coli* (6) strains for mice is correlated with their resistance to sera containing complement. This correlation breaks down completely when the comparison is extended to other bacterial and animal species. *Proteus* strains, for example, are often much more resistant to killing by serum than are *S. typhimurium* strains and of course are rarely virulent (7). *Vibrio comma* is exquisitely sensitive to killing by serum and yet is pathogenic only for that animal species which appears to have one of the highest levels of serum complement activity, *i.e.*, man. It appears that the complement system cannot be implicated too deeply in the species-specific type of immunity but nevertheless the indication is strong that this system does participate in antibacterial defence *in vivo*, since animals which are deficient in complement activity are at a disadvantage and are generally susceptible to many infections (8). Another example suggesting the participation of complement in antibacterial defence *in vivo* may be provided by the study of kidney infections. Here we must account in particular for the ability of complement-sensitive coliform organisms to localize and multiply in the kidney while being incapable of multiplying in other areas of the body. Recent studies have shown that kidney tissue is peculiar in possessing high anticomplementary activity, apparently due to the production by the kidney of ammonia, which inactivates the fourth component of complement (9). The diminution of complement activity in the kidney may well provide a reason for the ability of gram-negative organisms to multiply there. One gets the impression that the serum bactericidal process provides a basic amount of immunity against many organisms but that it can by no means account for the variations in natural immunity between different animal species.

LYSOZYME

Lysozyme is another example of an antibacterial system highly effective *in vitro*, the significance of which *in vivo* is quite obscure. When first described by Fleming in 1922 the limited antibacterial spectrum of this enzyme militated against a broad field for this as a host defence mechanism. However, the fact that this was the only clear case of a pure enzyme having a specific action against some bacteria (10) has maintained interest and work in this area with two interesting recent results.

TABLE 1

Correlation between O-acetyl content and lysozyme sensitivity of Micrococcus lysodeikticus

Strain	Treatment	Cell Wall *O*-Acetyl Content	Reduction in OD by Lysozyme (1μg/ml)
		μmoles/g	
L2 (Sensitive)....	None	3	68
250 (Resistant)...	None	433	15
L2.....	Acetylation	305	26
250.....	Deacetylation	5	62

From Brumfitt *et al.* (13).

First, it has been shown by Repaske (11) that treatment of many gram-negative bacteria with metal binding agents such as Versene (ethylenediaminetetraacetic acid) renders these organisms sensitive to lysozyme. It is clear from this observation that the bacterial substrate on which lysozyme acts is of much wider distribution than is indicated by the *in vitro* bactericidal spectrum, and this may be of great significance in assessing the scope and importance of lysozyme. The second recent advance is of fundamental importance in providing a chemical explanation for the development of bacterial resistance to lysozyme. Several years ago it was suggested that this was a key question in microbiology and that methods were available for its solution (12). Brumfitt *et al.* (13) have now shown that resistance to lysozyme in strains of *Micrococcus lysodeikticus* can be correlated with the degree of *O*-acetylation in the *N*-acetyl muramic acid-glucosamine-polypeptide polymer which constitutes the substrate for the enzyme (table 1). It seems likely that these two advances will stimulate work so as to enable a more certain assessment to be made of the importance of lysozyme as a host defence factor.

β-LYSINS

In common with all the other reported bactericidal activities of serum *in vitro*, the role of

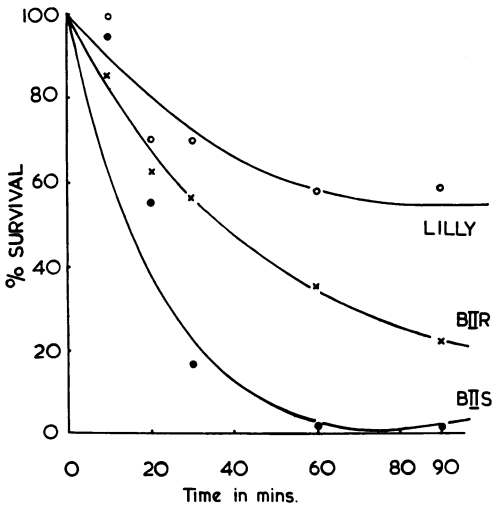

Figure 1. Sensitivity to normal saline of *Escherichia coli* LILLY and *Salmonella schottmuelleri* strains BIIS and BIIR.

the β-lysins in the intact animal is difficult to assess. It is often a matter for faith that an antibacterial action highly effective *in vitro* must have been provided by nature for a useful purpose *in vivo*. This understandable teleological tendency should be easier to avoid when the effective antibacterial activity which normal saline exhibits for many gram-negative bacteria (W. Ali and D. Rowley, *unpublished data*) is recalled (figure 1).

BACTERICIDAL ACTION OF MOUSE MONONUCLEAR PHAGOCYTES IN VIVO AND IN VITRO

Having discussed the more established work on serum bactericidal effects, a different approach will now be adopted, and an attempt will be made to analyze the sequence of events which follow the commonly used practice of intraperitoneal injection of bacteria. This will initially take us into cellular territory but it is possible to demonstrate that humoral factors play a decisive part even there.

For these studies, mice were used and various strains of gram-negative bacteria. Mice were chosen initially because of their convenience and also because they are almost unique in that the mouse peritoneum normally contains large numbers of macrophages and needs no previous stimulation with irritants (14). The principle of the method was to inject a series of normal mice with known numbers of viable organisms, then at timed intervals to sacrifice one mouse of the series and wash out the peritoneum with a tissue culture medium containing no antibiotics. At this point a portion of the washings was suitably diluted and plated to estimate the content of viable bacteria. One ml of the remainder of the washings was placed in a Porter flask containing a conventional "flying cover slip" and incubated for exactly 20 min, during which time the macrophages settled down and attached themselves to the bottom of the flask and to the cover slip. At the end of this time the supernatant fluid was removed and plated for viable count; the flying cover slip was picked out with sterile forceps, inverted on MacConkey's medium and pushed around vigorously to aid in disrupting the white cells.

The most striking initial finding arising from the use of this method was that the rate of disposal of the injected organisms could be greatly speeded by previously treating them with serum. The virulent strain of *S. typhimurium* was not removed appreciably from the mouse peritoneum unless the organisms had previously been sensitized by contact with horse serum or specific antiserum (15) (figure 2).

The second notable point which emerged was that if the difference between the total count per ml put into the flasks and the count per ml in the supernatant is accepted as a measure of the amount of phagocytosis, it can be seen that pretreatment with serum greatly promoted this activity, so that around 90 per cent of the bacteria were intracellular (table 2). Furthermore, the total viable organisms recovered from the cells and from the supernatant fluid were always far fewer than the total organisms known to have been put into the flask. It could easily be shown that the supernatant fluid was in no way antibacterial and in fact supported bacterial growth well. It may tentatively be assumed that the missing bacteria were killed by the cells. Evidence to be presented later supports this assumption.

It follows that if there has been considerable intracellular bacterial killing during the period of 30 min in the tissue culture flasks, the rapid reduction in viable count in the peritoneum which occurs with presensitized bacteria is likely to be due to the macrophages also.

This deduction is made more certain by the results of an experiment in which P^{32}-labeled

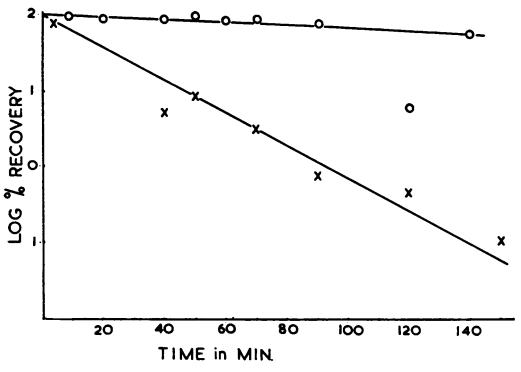

Figure 2. Recovery of *Salmonella typhimurium* strain C5 with or without previous serum treatment. X——X, organisms exposed to specific O-antiserum at a concentration of 5 agglutinating doses per ml for 5 min before dilution and injection into the mouse peritoneum; O——O, no serum treatment.

TABLE 2

Recovery of Escherichia coli 2206 from the mouse peritoneum

Series	Time	Total Bacteria Recovered/ml (T)	Total Bacteria in Supernatant/ml (S)	Total Bacteria in Cells (C)
	min			
A. Bacteria pretreated with dilute antiserum; inoculum, 380,000	0	276,000	112,000	7,500
	10	120,000	6,000	2,000
	30	18,000	300	2,000
B. Bacteria injected without previous serum treatment; inoculum 115,000	0	67,000	103,000	850
	15	67,000	79,000	2,250
	30	64,000	64,000	2,500

For simplicity this table shows only the results with three mice in each series of 10. Series *A* and *B* were carried out on the same day; dilutions of the same culture of *E. coli* were used.

bacteria, presensitized with dilute antiserum, were injected intraperitoneally into a series of mice; the number of viable bacteria was determined after various intervals, together with estimations of the total radioactivity. The results plotted in figure 3 show that 10 per cent of the P^{32} was still recoverable from the peritoneum at a time when the viable organisms had decreased to 0.05 per cent. The events leading to the remarkable decrease in bacterial numbers must have occurred in the peritoneum and could not be due to transport of the bacteria elsewhere (16).

Assuming that the observed bactericidal effect was due to the macrophages, there are various simple calculations which can be made with the data from this type of experiment. The time during which the bacteria can survive their intracellular environment is very similar irrespective of the situation *in vivo* or *in vitro* of the macrophages. The rather surprising finding was made that all the strains of bacteria examined, which included virulent and avirulent strains, were killed by the macrophages at similar rates (table 3). Viewed as a whole, the limiting factor in the interaction of bacteria and monocytes was the rate of phagocytosis, and this was dependent on the presence of opsonic factors. The virulent strains were not phagocytized at all unless they were presensitized, whereas avirulent strains were removed from the peritoneum quite rapidly even without presensitization, presumably because sufficient opsonic factors were available in the peritoneal fluids for this purpose (figure 4).

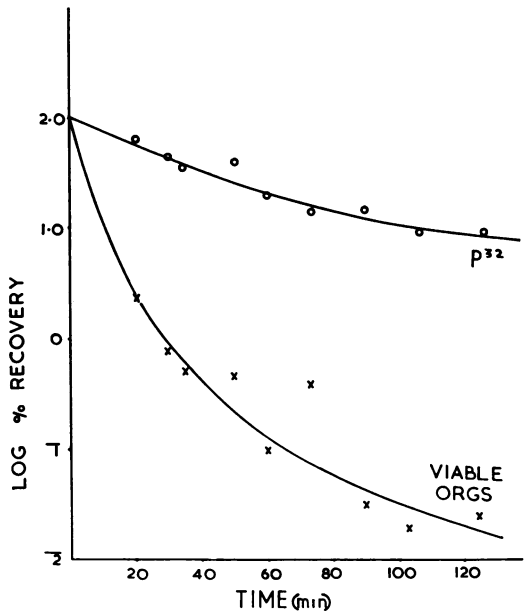

Figure 3. Recovery of P^{32} and viable bacteria from the peritoneum following injection of opsonized P^{32}-labeled *Escherichia coli* strain 2206.

TABLE 3

Bactericidal activity of mouse macrophages against various bacteria in the in vivo peritoneal environment or in tissue culture preparations

Organism	Avg 50 Per Cent Survival Time	
	In vivo	*In vitro*
	min	*min*
Pseudomonas aeruginosa	5	19
Escherichia coli 2206	9	19
E. coli 2380	7.5	25
E. coli 145	10	15
Salmonella typhimurium M206	10	20
Klebsiella pneumoniae	11.5	12
Salmonella typhimurium C5	12.5	17

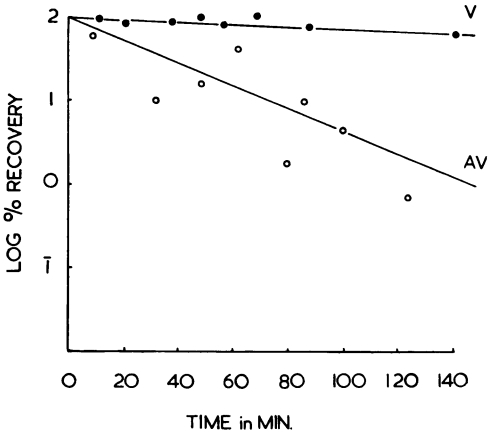

Figure 4. Removal of unsensitized, virulent (*V*) and avirulent (*AV*) *Salmonella typhimurium* strains from the mouse peritoneum.

The same kind of experiment may be used to test the ability of various sera to provide opsonic factors. Specific antiserum was found to be opsonic at a concentration which contained approximately $\frac{1}{50}$ of an agglutinating dose; the sera of various animals was in some cases highly effective, and this ability did not seem to be correlated with the content of specific antibody (17). In addition serum taken from mice which exhibited nonspecific immunity, due to the injection of lipopolysaccharide 48 hr previously, was found to be moderately effective, by comparison with normal mouse sera, in providing opsonins for antigenically quite unrelated bacteria (figure 5).

The interactions of macrophages with bacteria

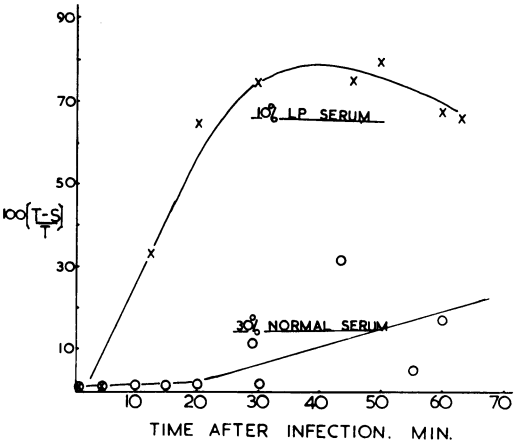

Figure 5. Phagocytosis of *Salmonella typhimurium* strain C5 *in vivo* pretreated with normal or LP serum. The fraction 100 (T − S)/(T) gives a measure of the percentage of organisms phagocytized.

can of course be followed entirely *in vitro* by allowing the peritoneal washings to settle down in the tissue culture flasks before infection. (It must be stressed that since antibiotics were avoided, it was possible to follow these interactions in the tissue culture flasks for only about 90 min. After this time multiplication of the extracellular bacteria began to swamp the system.) Under these conditions *in vitro* it is more nearly certain that the effects observed are due to mononuclear cells, since the small percentage of polymorphonuclear leucocytes which were originally present quickly degenerate and fall off the glass surface (figure 6). When 1-day-old cultures of macrophages are infected *in vitro*, the results are similar. In the absence of serum, little or no phagocytosis occurs and even in the presence of an adequate amount of opsonins the efficiency of phagocytosis is much reduced, probably due to the decreased chance of physical contact between cell and microbe. Nevertheless, once inside the cells, presensitized bacteria are rapidly killed. Figure 7 shows the intracellular bactericidal effect against *S. typhimurium* M206 (18).

One might expect that the most clear-cut results would be obtained from the type of experiment in which macrophage preparations were exposed to bacteria *in vitro* for a period, then washed to remove unphagocytized organisms; fresh medium was then added, and the survival of intracellular bacteria was followed. Unfortu-

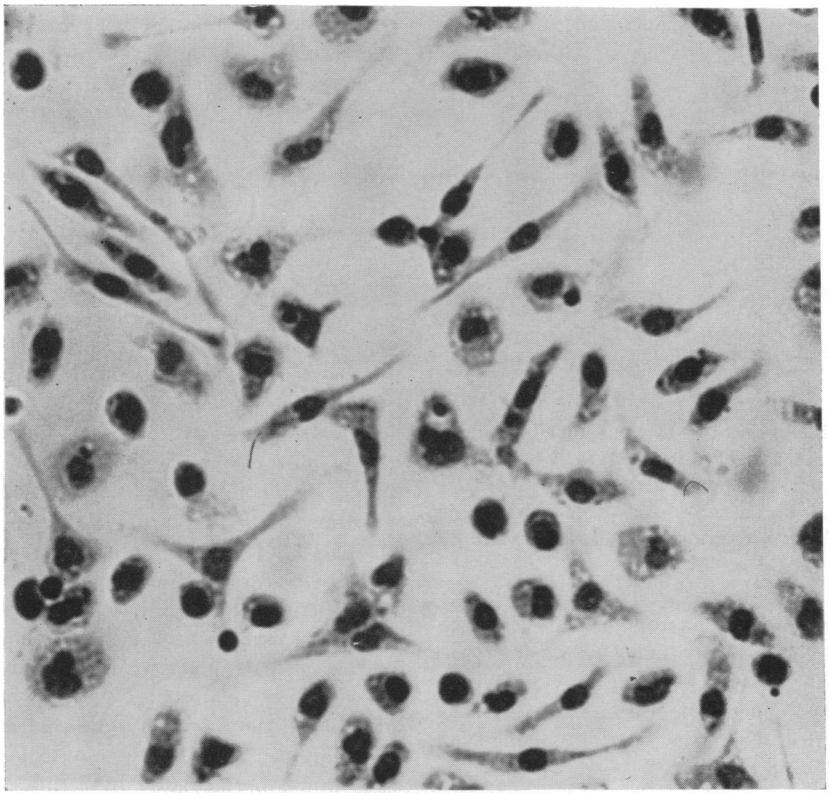

Figure 6. A 24-hr culture of mouse macrophages.

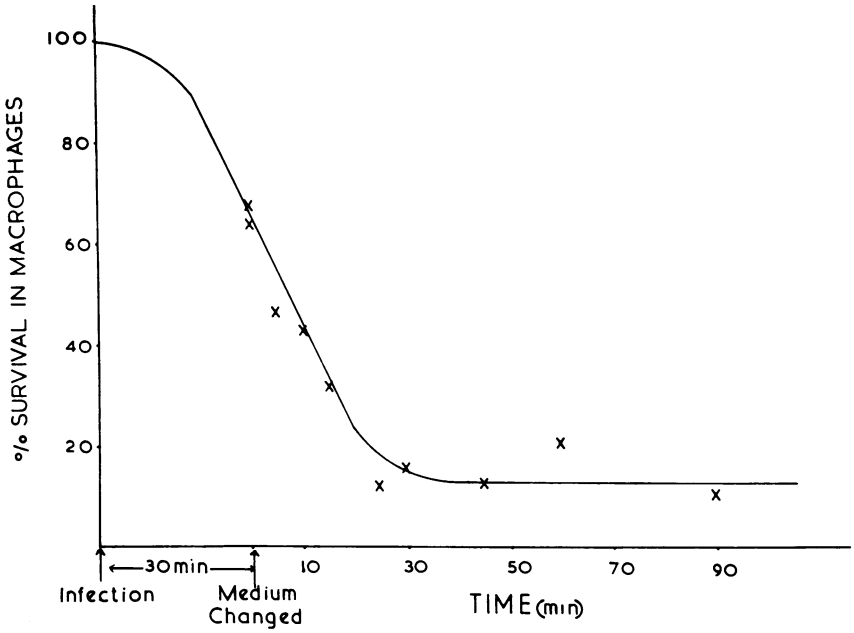

Figure 7. Bactericidal activity *in vitro* of mouse macrophages for *Salmonella typhimurium* strain M206.

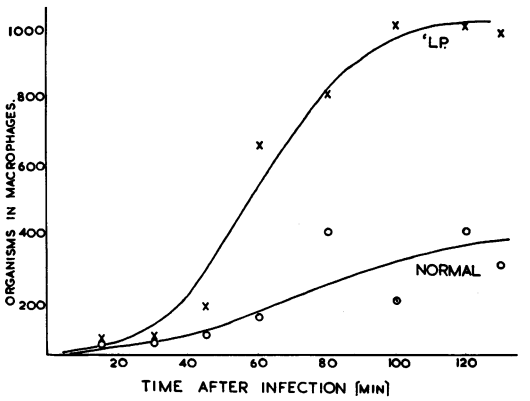

Figure 8. Uptake of weakly opsonized organisms (C5) by normal and LP macrophages. A comparison of the phagocytic activity for *Salmonella typhimurium* strain C5 by macrophages from normal mice and those from mice given 50 μg of *Escherichia coli* lipopolysaccharide 48 hr previously.

nately, the more the macrophages are subjected to shocks such as centrifuging or washing with fresh medium, the less effective they become, as indicated by reduction in the rate of their bactericidal effect. Nevertheless the same general results are found (table 3).

It has been mentioned earlier that serum taken from animals, in which nonspecific immunity had been induced by lipopolysaccharides, was more effective than normal serum in promoting phagocytosis. It is of course also possible to compare the macrophages from normal mice with those from specifically immunized or nonspecifically "immune" mice. We have found that such macrophages are more active in engulfing organisms, although the rates of the intracellular bactericidal processes are approximately the same. Furthermore, increased phagocytic activity can be produced *in vitro* by adding minute amounts of lipopolysaccharides to tissue culture preparations of macrophages; when examined 24 hr later their increased phagocytic ability can readily be observed by comparison with a similar control series (figure 8). This result is in keeping with reports in the literature that phagocytic cells are stimulated in several metabolic activities by contact with particulate materials of many kinds (19, 20).

Do these considerations throw any light on the mechanism of lipopolysaccharide-induced nonspecific immunity? So far, two important changes have been correlated with the onset of this immunity. First, it has been reported that the serum properdin levels of animals fall and later rise in parallel with the time course of decreased resistance to various infections, followed by an increase (21). Because of the wide range of bacteria involved it is clear that properdin, if it has any role, cannot act through a direct serum bactericidal action (22). On the other hand, it is possible that the increase in nonspecific opsonic activity in serum, which also accompanies the resistance to infection, may be due to properdin. This possibility is supported by the results of Howard and Wardlaw (23), who studied the opsonic factors in normal serum which are necessary for uptake of bacteria by the Kupffer cells of the perfused rat liver. They found that complement, natural antibody, and possibly properdin promote phagocytosis by these cells. The other important change which is correlated with this nonspecific immunity is the functional ability of the reticulo-endothelial system to remove intravenous colloids (24). If peritoneal macrophages can be regarded as part of the reticulo-endothelial system, then it seems likely from the present results that any increase in phagocytic ability of these reticulo-endothelial cells would be mediated by serum opsonic factors. This mechanism would explain the finding by Howard and Wardlaw that the liver of an animal stimulated by lipopolysaccharide, when perfused with bacteria suspended in a standard serum system, was no more actively phagocytic than a normal liver.

It is evidently impossible to discuss the antibacterial properties of serum out of the context of the cellular environment. One final example may serve to reinforce the thesis that these serum opsonins are real entities, which are not merely experimental artifacts but are of great practical importance to the intact animal. In this experiment portions of a culture of virulent *S. typhimurium* were incubated for 10 min with 30 per cent serum from various normal animals, or with five agglutinating doses of a specific O-antiserum. Each serum mixture was then diluted approximately 1:100,000 in normal saline and 0.2 ml of each injected intraperitoneally into several series of mice. The number of viable organisms in each serum mixture was determined immediately before injection. It can be seen from table 4 that although the pretreatment with sera did not affect the viability of the bacteria, yet in some cases the ability of the organisms to

TABLE 4

Effect of pretreating Salmonella typhimurium with sera of normal animals on its lethality for mice

Serum	Specific Hemagglutination Titer of Serum*	Mortality at 28 Days; Groups of 15 Mice
Horse	160	2
Human	80	10
Chick	80	12
Pig	8	1
Rat	2	4
Mouse	0	15
Guinea pig	0	15
Salmonella schottmuelleri-rabbit antiserum	80	8
Controls (no serum)	—	15

* Determined by coating sheep red cells with the specific lipopolysaccharide extracted from *Salmonella typhimurium* C5, and reacting them with the various sera. The results are expressed as the reciprocal of the agglutinating titer of the sera.

cause disease was greatly reduced, presumably due to rapid intraperitoneal phagocytosis and destruction of the opsonized organisms. Also, the protective ability of a particular serum was not necessarily correlated with its content of O-specific antibody, although of course the specific antiserum did protect (17).

The problem of defining the determinants of species immunity referred to earlier is a fascinating one and seems to defy explanation on the basis of O-specific antibody. Is it possible that the opsonic factors, which have been discussed at length in the present paper, may provide the explanation?

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DISCUSSION

In the "flying cover slip" preparations for study of phagocytosis and intracellular destruction of bacteria by peritoneal macrophages it is unlikely that the effects observed are attributable to aggregation of bacteria in a few of the phagocytes, as there were at least 1000 macrophages for each bacterium. It seems improbable, therefore, that reduction in number of bacterial colonies could be associated with phagocytosis of the microorganisms by only one or two macrophages (Rowley, London, England).

It is not known whether or not serum opsonin resulting from selective breeding and transmitted genetically could be responsible for the resistance of mice to *Salmonella typhimurium* infection (Synder, Baltimore).

Recent work by Amano and his colleagues (Amano, T., Inai, S., Seki, Y., Kashiba, S., Fujikawa, J., and Nishimura, S., 1954; Accelerating effect on the immune bacteriolysis by lysozyme-like substance of leucocytes and egg-white lysozyme. Med. J. Osaka Univ., **4,** 401–418) has shown that lysozyme may have a bactericidal effect upon ordinarily insusceptible microorganisms when in the presence of specific antibody to the bacterium. This may be an important observation in explaining the antibacterial effects of macrophages upon gram-negative bacilli in the presence of antibody or other opsonins (Watson, Minneapolis).