# THE ROLE OF OPSONINS IN NON-SPECIFIC IMMUNITY

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It has recently been shown that in the peritoneal environment and also under tissue culture conditions (1), mouse peritoneal macrophages are able to kill many species of Gram-negative bacteria with great rapidity (2, 3). For any appreciable amount of phagocytosis to occur the bacteria must have previously been treated with serum, containing opsonic factors. Once inside the macrophages, however, the bacteria were killed with equal rapidity, irrespective of the virulence for mice of the bacterial strains. The *in vivo* experiments revealed that mice previously given bacterial lipopolysaccharides (LP) to provoke non-specific immunity, were able to clear unrelated bacteria much more rapidly from the peritoneum. This increased efficiency did not appear to be due to changes in the macrophages themselves, since these cells when recovered from LP-treated mice possessed similar bactericidal activity to that of normal cells. If this analysis of the elimination of bacteria from the mouse peritoneum is correct, it follows that the most probable reason for the increased clearance would be a greater supply of the necessary opsonic factors, thus enabling the rate-limiting step of phagocytosis to occur more rapidly. In this paper this possibility has been put to the test and in addition the direct effects of lipopolysaccharides on mouse macrophages are described.

## *Materials and Methods*

*Strains of Bacteria.*—The following were used. *Escherichia coli* 2206 and 2380 described by Rowley (4) and *Salmonella typhimurium* $C_5$ (5). The strains were kept on agar slopes in screw-capped bottles at 4°C. from which they were subcultured as required.

*Mice.*—These were all of the L.A.B. strain of grey mice bred in this Institute and weighed 20 to 25 gm.

*Bacterial Antisera.*—When used for opsonizing bacteria these were diluted to a strength 5 times greater than the final agglutinating titre. All sera were heated at 56°C. for 30 minutes before use.

*Tissue Culture Methods.*—Porter flasks (6) made of neutral glass and of 3.5 cm. internal diameter were used for all cultures; into each of these a "flying coverslip" was inserted. Glassware was cleaned with hot calgon and sodium metasilicate solution followed by prolonged

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rinsing in distilled water as described by Hanks (7). The tissue culture medium No. 199 was used throughout (8). This medium contained no antibiotic and was adjusted to pH 7.2 by adding fresh 1 per cent sodium bicarbonate solution. In any experiments which involved keeping the macrophages for several hours or more, 10 per cent calf serum was added to this medium.

*Bacterial Lipopolysaccharide.*—This was prepared from the cell walls of *E. coli* 2206 by the phenol water extraction method of Westphal and Lüderitz (9).

*$P^{32}$-Labelled E. coli* 2380.—Five millicurie of carrier-free $P^{32}$ was added to 20 ml. nutrient broth in a 100 ml. bottle and the medium inoculated with a loopful of *E. coli* 2380. After 6 hours of aerated growth, the culture was centrifuged and washed twice with saline by centrifuging. The washed bacteria were suspended in 199 medium so as to contain $5 \times 10^8$ organisms/ml.

## RESULTS

*Comparison between Elimination of $P^{32}$ and Viable Bacteria from the Peritoneum.*—It will be apparent from earlier work (see also Table I) that fully opsonised bacteria rapidly disappear from the peritoneum. Since macrophages have been shown to kill bacteria *in vitro*, it may be supposed that the reduction in viable count *in vivo* is due to the lethal effect of the peritoneal cells and that very little of this reduction in peritoneal bacteria is due to transport elsewhere in the body.

It seemed desirable to have direct evidence on this point.

For this purpose living $P^{32}$-labelled *E. coli* were presensitized with dilute antiserum before injecting 0.2 ml., containing approximately $10^6$ bacteria intraperitoneally (IP) in each of 10 mice. Immediately after injection and at timed intervals thereafter, mice were sacrificed and peritoneal lavage performed aseptically using 1.5 ml. of tissue culture medium 199 containing 5 u/ml. heparin. Viable counts and determinations of $P^{32}$ content were done on each washout.

The results plotted in Fig. 1 show that the reduction in viable numbers occurs much more rapidly than the decrease in $P^{32}$ content. In other words, the events leading to death of the bacteria are occurring in the peritoneum.

*Opsonic Effect of Serum from Lipopolysaccharide-Treated Mice.*—

Pooled serum was obtained from mice which had received an intravenous injection 2 days previously of 50 μg. lipopolysaccharide prepared from *E. coli* 2206. A suspension of *S. typhimurium* $C_5$ was diluted with this serum and with normal mouse serum so that the final suspensions contained $10^7$ organisms/ml. in 30 per cent *v/v* serum in 199 medium. Other similar mixtures were prepared to contain various concentrations of either serum. After incubating at 37°C. for 20 minutes the suspensions were diluted tenfold in 199 medium and 0.2 ml. containing approximately $2 \times 10^5$ organisms were injected intraperitoneally into each mouse of two groups, one group for lipopolysaccharide serum, the other for the normal mouse serum control. At various times mice from each group were sacrificed and washed out as above with 199 medium. After counting a portion of the washout, 1 ml. of the remainder was introduced into a Porter flask and incubated for 20 minutes to allow the macrophages to settle down and stick to the glass bottom of the flask. At this time a sample of the supernatant fluid was with-

drawn and plated for viable count. The difference between the total numbers introduced initially into the flask and the numbers surviving in the supernatant fluid was taken to indicate the numbers of phagocytosed organisms.

Table I gives the results of such an experiment in which the bacteria were pretreated with 30 per cent of serum from normal or lipopolysaccharide-treated mice. There is dual evidence of a greater opsonic effect by the LP serum, firstly the total recovery of organisms rapidly diminished and secondly the

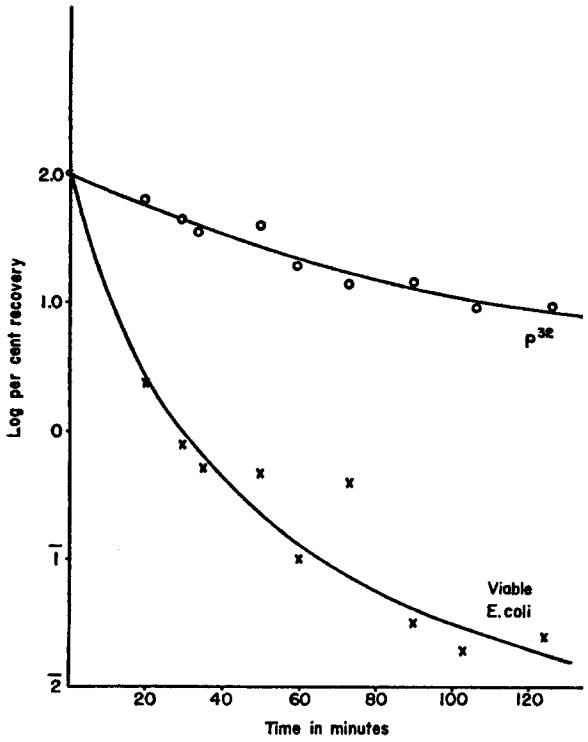


FIG. 1. The recovery of $P^{32}$ and of viable *E. coli* 2206 after intraperitoneal injection of the radioactive organisms.

amount of phagocytosis in each sample was much greater. It should be noted that this LP serum contained no detectable antibodies against the *S. typhimurium* used as a test organism—the opsonic factors appeared to be nonspecific. The opsonic effect of the LP serum was still apparent at the 5 per cent concentration by contrast to the normal mouse serum which showed no opsonic effect even at a concentration of 50 per cent.

*Comparison of Phagocytic Activity by Macrophages from Normal or LP-*

*Treated Mice.*—The interactions of bacteria and macrophages can, of course, be studied entirely *in vitro*, but this type of experiment is limited by the much decreased efficiency of phagocytosis in the tissue culture preparations compared to that occurring in the mouse peritoneum (3). In order to compare the phagocytic ability of macrophages it is, however, preferable to use the less active *in vitro* system.

TABLE I

*Comparison of the Opsonin Powers between Serum from Normal Mice and That from Lipopolysaccharide-Treated Mice*

| Time | Total organisms recovered | Organisms in supernatant | Phagocytosis by difference |
|---|---|---|---|
| *min.* | | | *per cent* |
| A. Inoculum 140,000 *S. typhimurium* $C_5$ pretreated with 30 per cent normal mouse serum | | | |
| 0 | 84,000 | 78,000 | 7 |
| 5 | 78,000 | 75,000 | 4 |
| 10 | 65,000 | 60,000 | 8 |
| 20 | 68,000 | 68,000 | 0 |
| 30 | 50,000 | 50,000 | 0 |
| 60 | 45,000 | 38,000 | 15 |
| B. Inoculum 250,000 *S. typhimurium* $C_5$ pretreated with 30 per cent serum from LP-treated mice | | | |
| 0 | 200,000 | 100,000 | 50 |
| 5 | 150,000 | 8,000 | 95 |
| 10 | 6,000 | 700 | 88 |
| 15 | 60,000 | 5,000 | 91 |
| 20 | 30,000 | 450 | 98 |
| 30 | 15,000 | 1,500 | 90 |
| 60 | 5,000 | 400 | 92 |

Tissue culture preparations, in Porter flasks, were made of macrophages from normal mice or from mice which 48 hours previously had been injected intravenously with 50 μg. lipopolysaccharide from *E. coli* 2206. Each flask contained a "flying coverslip" which occupied ⅕ of the floor area and which could be removed to provide a representative sample of the macrophages. After 24 hours in the flasks, the macrophages were showing active spreading (2). The supernatant medium was removed from each preparation and replaced by 1 ml. of fresh 199 medium containing 5,200 organisms/ml. of *S. typhimurium* $C_5$ which had been treated with 25 per cent LP-mouse serum before dilution. At intervals after infection flasks were removed from the incubator and an aliquot of the supernatant fluid removed for viable counting. The "flying coverslip" with its adhering macrophages was removed aseptically, turned upside-down on a plate of MacConkey's medium and rubbed around vigorously to aid in disrupting the macrophages.

The results of this experiment, shown in Table II, reveal that the macro-

phages from lipopolysaccharide-treated mice were more actively phagocytic than were those of normal mice.

TABLE II

*The Phagocytic Activity of Macrophages, from Normal and Lipopolysaccharide-Treated Mice, for Weakly Opsonised S. typhimurium $C_5$*

| Time | Organisms/ml. in supernatant | Organisms in cells | Phagocytosis |
|---|---|---|---|
| *min.* | | | *per cent* |
| | A. Macrophages from LP-treated mice | | |
| 0 | 5,000 | 110 | 2 |
| 0 | 5,000 | 90 | 2 |
| 17 | 5,000 | 260 | 5 |
| 32 | 4,800 | 250 | 5 |
| 45 | 5,000 | 750 | 13 |
| 65 | 4,500 | 1,000 | 18 |
| 80 | 4,500 | 3,500 | 44 |
| 100 | 5,500 | 5,000 | 47 |
| 120 | 5,000 | 5,000 | 50 |
| 130 | 5,800 | 7,500 | 56 |
| | B. Macrophages from normal mice | | |
| 0 | 4,800 | 190 | 4 |
| 0 | 4,500 | 100 | 2 |
| 15 | 4,800 | 200 | 4 |
| 30 | 5,000 | 175 | 4 |
| 45 | 4,800 | 100 | 2 |
| 62 | 5,800 | 400 | 6 |
| 80 | 6,200 | 2,000 | 24 |
| 100 | 6,000 | 200 | 3 |
| 122 | 6,800 | 2,000 | 23 |
| 130 | 7,500 | 1,500 | 17 |

Each flask in both series was infected with the same suspension, containing 5,200 *S. typhimurium*/ml., sensitized by 25 per cent LP-mouse serum before dilution.

*Effect of Lipopolysaccharides on Macrophages in Vitro.*—

Macrophages were washed out of normal mice and placed in flasks as above. After several hours the medium was sucked off all the flasks and replaced in half of them by fresh 199 and 10 per cent calf serum and in the other half by the same medium containing 10 μg. lipopolysaccharide/ml. All flasks were reincubated for 18 hours, then the medium was again removed and replaced in all the flasks by 1 ml. of 199 containing approximately 5,000 organisms/ml. of *S. typhimurium* $C_5$ which had been sensitized by 25 per cent LP-serum before dilution, as in the previous experiment.

The amounts of phagocytosis which occurred in each flask were estimated

from viable counts on the supernatant and cells as before. The intracellular counts are plotted in Fig. 2, from which it seems clear that the lipopolysaccharide treatment *in vitro* resulted in an increased phagocytic ability of the macrophages.

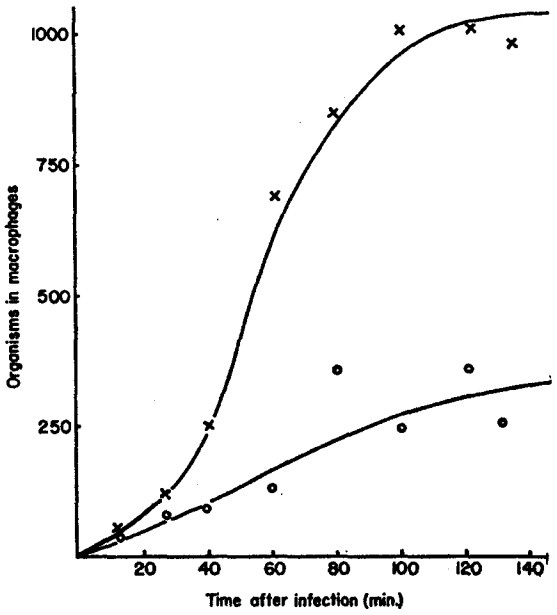


FIG. 2. The uptake of weakly opsonised *Salmonella typhimurium* $C_5$ by O—O normal 24 hour culture of mouse macrophages —X—X macrophages treated with 10 $\mu$g./ml. lipopolysaccharide 24 hours earlier.

## DISCUSSION

The phagocytosis-promoting effect of serum has been well established ever since Almroth Wright coined the term "opsonins" in 1904 to describe the hypothetical substances in serum responsible for this effect. Since that time there has been a sustained controversy about the nature of these substances in normal serum. This problem is still not resolved but many of the differences in opinion which have been expressed in the past are no doubt due to the experimental techniques on which they are based. In the present work it has been shown that under certain conditions serum factors are all-important in determining uptake of bacteria by macrophages. On the other hand under conditions when these serum factors are restricted, the phagocytic ability of cells derived from normal or from lipopolysaccharide-treated mice can be distinguished.

By studying the intraperitoneal fate of injected bacteria it can be seen that in the normal animal the supply of opsonic factors in the peritoneum is the

limiting factor which determines the outcome of the infection. The concentration of these opsonins needed to ensure phagocytosis varies according to the virulence of the microbe. For example, two strains of *S. typhimurium* $C_5$ and M206, virulent and avirulent for mice, when pretreated with a 1/1000 dilution of specific O-antiserum were phagocytosed to 16 and 96 per cent respectively by the peritoneal macrophages.

The mechanism by which immunity can be rapidly induced in animals by the intravenous injection of minute quantities of bacterial lipopolysaccharides is still unresolved. There appear to be several changes which may be correlated with the time course of the non-specific immunity (10). On the one hand there are variations in the serum bactericidal activity (11) which may be accompanied by alterations in the serum properdin level (12) and on the other hand there is a parallel change in the ability of the reticuloendothelial system to remove intravenous colloids (13). Howard and Wardlaw have shown that the phagocytic activity of the liver R.E.S. is dependent on serum opsonins, so that even in this instance the separation of humoral from cellular activities is obscured (14). In the present work it has been found that the serum from animals previously treated with lipopolysaccharides was more active than normal mouse serum when measured by its ability to prepare bacteria for phagocytosis by macrophages. Under somewhat different conditions the macrophages which had encountered lipopolysaccharide either *in vivo* or *in vitro* could be shown to possess increased phagocytic activity in their own right. This is in agreement with the observations (15, 16) that uptake of particulate materials increases a variety of enzymic activities of these cells. It is suggested from this work that the stimulation of non-specific immunity may be by a dual mechanism; an increase in non-specific opsonic factors of uncertain nature and a direct stimulation of some, at least, of the bodies phagocytic cells.

## SUMMARY

A study of the recoveries of radioactivity, and of viable bacteria following injection of $P^{32}$-labelled *E. coli* into the mouse peritoneum, has indicated that the rapid decrease in viable bacteria which occurs is largely due to peritoneal events, and not to the transport of bacteria elsewhere.

The serum from mice given lipopolysaccharides 48 hours previously, when used to pretreat bacteria before intraperitoneal injection, was found to stimulate phagocytosis to a greater extent than did pretreatment with normal serum. In addition, macrophages themselves were found to be affected by contact with lipopolysaccharides, either *in vivo* or *in vitro* in such a way as to promote their phagocytic abilities. It is suggested that the provocation of non-specific immunity by bacterial lipopolysaccharides involves two facets at least; firstly, an increase in the opsonic capacity of the serum, and secondly an increase in the inherent capacity of phagocytic cells to perform this function.

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