IN VITRO STUDIES ON THE INTERACTION BETWEEN MOUSE PERITONEAL MACROPHAGES AND STRAINS OF SALMONELLA AND ESCHERICHIA COLI*

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(Received for publication, April 13, 1960)

Studies on *Salmonella* infections in mice showed that the injected bacteria are cleared rapidly from the circulation, and may be found mainly in the liver, and spleen, the principal organs of the reticuloendothelial system (RES) (1). Investigations of the fate of the bacteria in these organs revealed that whereas the population of a virulent strain of *Salmonella* increases, that of an avirulent strain slowly declines (2, 3). Since mouse serum appears to lack a bactericidal mechanism *in vitro*, it has been assumed that the destruction of bacteria by the mouse is dependent on its cellular defenses (4, 5). Thus it has been suggested that the virulence of strains of *Salmonella* for the mouse reflects the ability of the strain to multiply within the phagocytic cells, presumably those of the RES.

The technical difficulties involved in a study of the fate of *Salmonella* strains, within the fixed phagocytic cells of the liver and spleen, has led workers to investigate the behaviour of bacteria of known virulence within peritoneal macrophages, or other animal cells easily maintained in tissue culture (6–8). The results of these experiments in different laboratories have been contradictory. Furness (6) found that mouse peritoneal macrophages phagocytosed virulent and avirulent strains of *Salmonella* to the same extent in the presence of 10 per cent heated calf serum. After an initial period of killing, the virulent strain multiplied within the mononuclear cells, whilst the avirulent strain maintained itself only in small numbers. These results are contrary to the findings of Rowley and Whitby (7) who studied the fate of the same strains of *Salmonella typhimurium* within mouse macrophages. These workers observed that the virulent strain required a greater amount of serum opsonins for phagocytosis than the avirulent strains, but once within the phagocyte both strains were killed at the

* This work was supported by grants from the Department of the Army (DA-49-007-MD-590) and the United States Public Health Service (E-2094).

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same rate, irrespective of their virulence. In all these experiments specific antiserum was used as a source of opsonic factors.

The interaction between host and bacterial parasite is a complex one, the survival of the one or the other being favoured by such factors as (*a*) the titre of specific and non-specific opsonins, (*b*) the ability of the phagocytic cells to ingest and kill bacteria, and (*c*) the capacity of the bacteria to multiply within the host environment. At best one may study, at one time, only a few of these parameters.

It seemed desirable to investigate systematically, the influence of some of these factors on the ability of peritoneal macrophages to phagocytose and kill bacteria. Conditions were chosen such that peritoneal macrophages were exposed to bacteria *in vitro* in a ratio of about 100:1, assuming then that the events studied would be the result of infection of macrophages by a single organism. The observations on the ability of peritoneal macrophages to kill phagocytosed bacteria were carried out both in the presence and absence of bactericidal concentrations of streptomycin, as it was felt that a more meaningful interpretation could be made of the fate of the intracellular bacteria from such a comparative study.

In a first series of experiments the ability of normal mouse peritoneal macrophages to phagocytose and kill bacteria of varied virulence for mice, after opsonization with fresh mouse serum, was investigated. Significant differences were observed in the way avirulent *Escherichia coli* and *Salmonella* strains, and virulent *Salmonella* strains were dealt with. In other experiments a study was made of the effect of using specific immune serum, or of increasing natural resistance by non-specific methods, such as infection with *Mycobacterium tuberculosis* (BCG) (9, 10) or endotoxin treatment (11), on the reaction between bacteria and peritoneal macrophages. Previous opsonization with immune serum improved the ability of normal macrophages to kill phagocytosed bacteria. Animals whose natural resistance had been enhanced by BCG infection, or bacterial lipopolysaccharide treatment, were observed to have both an increase in the level of serum opsonins, and peritoneal macrophages with improved ability to destroy intracellular, virulent *Salmonella*. Thus both humoral and cellular factors contribute to natural resistance, the outcome of infections with *Salmonella* being conditioned by the ability of the macrophages to kill phagocytosed bacteria.

Material and Methods

Bacterial Strains.—Two strains of *Salmonella typhimurium* were used, a virulent strain C3 (LD_{50}, 200 organisms) and an avirulent strain M206 (LD_{50}, 10^6 organisms) (6, 7). Also studied was a strain of *Salmonella enteritidis* (Danysz) S1 obtained from Dr. Biozzi, Hopital Broussais, Paris, (10) (LD_{50}, 10^8 organisms) and two strains of *Escherichia coli*, E 2380 (12) and one (0111 B4) previously studied in this laboratory (13).

Mice.—Swiss-Webster, male and female weighing 20 to 24 gm. were used.

Endotoxin-Treated Mice.—Mice were injected intravenously or intraperitoneally with 50 μg. of lipopolysaccharide, prepared from *E. coli* 2206 (12) by the phenol/water method of Westphal, Luderitz, and Bister (14), and purified by alcohol precipitation and high speed centrifugation. Plasma and peritoneal macrophages were obtained from these mice 48 hours after the injection of endotoxin.

Specific Antiserum.—Specific antiserum was prepared against *Salmonella enteritidis* in rabbits and the scheme adopted for immunization was that outlined in Wadsworth, Standard Methods (15). The rabbit serum used had an agglutinin titer of 1:1000.

Opsonization of Bacteria.—Three to five mice were bled from the retroorbital venous plexus with a sterile Pasteur pipette, the blood pooled, defibrinated, and centrifuged at 1500 R.P.M. for 10 minutes. The clear plasma was carefully withdrawn and placed in a tube standing in ice. Previous experiments had shown that there was no difference in the opsonizing capacity of mouse serum or mouse plasma. For opsonization 0.2 ml. of the plasma was mixed with 0.2 ml. of a 24 hour broth culture of the bacteria containing 5×10^8 bacteria/ml. The mixture was incubated at 37°C. for 20 minutes and then placed in ice. In experiments using specific antiserum, final dilutions of the antiserum were made in normal mouse plasma.

Studies with Mouse Peritoneal Macrophages in Antibiotic-Free Medium.—The experimental techniques used here were similar to those previously described by Whitby and Rowley (16). Mice were injected intraperitoneally with 1.5 ml. of 199 medium (17) (obtained from Microbiological Associates, Bethesda), containing 5 units/ml. of heparin, and killed by breaking the neck. The abdomen was gently massaged and the skin reflected after swabbing with alcohol. One ml. of the injected 199 medium was carefully withdrawn through a No. 19 needle, and slowly introduced into a Porter flask (18) containing a flying coverslip, known to cover ¼ of the floor area. Counts on the cell population showed that individual peritoneal wash-outs contained from 1×10^6 to 5×10^6 cells per ml. Fifteen flasks were thus inoculated, the macrophage suspension from one mouse being used for one flask. The flasks were incubated at 37°C. for ½ hour to allow the macrophages to settle and adhere to the glass. Following this the supernatants were withdrawn and 1 ml. of 199 medium containing between 10^4 and 10^5 opsonized bacteria was added. Thus the ratio of bacteria to cells was of the order of 1:100. With each series of flasks containing macrophages, five flasks containing no macrophages were similarly inoculated, as controls for bacterial growth, during the period of phagocytosis. All the flasks were then reincubated for a further 60 minutes. At the end of this time, the flasks were removed from the incubator, and the control flasks plus three flasks containing macrophages placed on ice. The supernatants from the remaining twelve flasks were withdrawn, and the number of bacteria determined by plating 0.1 ml. of a suitable dilution made in normal saline, onto a nutrient agar plate. After washing the macrophages by addition of 199 medium and gently rocking the flask, 1 ml. of fresh 199 medium was added, and the flasks replaced in the incubator. This procedure took on the average 10 minutes. Supernatant counts were then made on the flasks which had been ice-cooled. The flying coverslips from each of the three flasks containing macrophages were removed and washed gently in a sterile Petri dish containing saline. Each coverslip was then rubbed vigorously over the surface of a nutrient agar plate, disrupting the macrophages and releasing the viable bacteria. At 30 minute intervals three more flasks were removed from the incubator and the number of viable bacteria in the macrophages determined as above.

Calculation of the Percentage Phagocytosis and Survival.—It was assumed that the difference (D) between the supernatant counts of the experimental flasks (E) and the control flasks (C) at the end of the 60 minute period of incubation represented the number bacteria phagocytosed ($C-E = D$). The percentage phagocytosis would therefore be $D/C \times 100$. The number of viable bacteria (N) within the total population of macrophages could be determined by multiplying the flying coverslip count by 4. The percentage survival at any time during

the experiment could therefore be calculated from $N/D \times 100$. These results were plotted as log per cent survival against time.

It is realized that the percentage phagocytosis and percentage survival, which are calculated by inference, may not represent the true values, but since experiments had shown that the bacteria were in the lag phase during the greater part of the time allowed for phagocytosis, it is felt that these inferred results are close to the true values.

Macrophage Studies in the Presence of Antibiotics.—The sensitivity of the various strains to streptomycin was determined in 199 medium. (Table I) In experiments with macrophages various concentrations were used. Phagocytosis took place in antibiotic-free medium, and at the end of this period all flying coverslips were removed and placed carefully in Porter flasks containing 1 ml. of 199 medium plus streptomycin. In order to allow the antibiotic time to kill the extracellular bacteria, coverslips taken from the first three flasks to determine the amount of bacteria viable at the end of the phagocytic period, were placed in ice cold anti-

TABLE I

Effect of Various Concentrations of Streptomycin on the Survival of Various Salmonella Strains

Bacterial strain	Per cent survival after 60 minutes				
	Streptomycin μg./ml. 199 medium				
	0.5	1	2	5	10
M 206	60	40	5	0	0
S1	50	40	10	0	0
C5	80	50	30	5	0

biotic medium; they stood in ice for 30 minutes before plating. The other twelve flasks containing coverslips were incubated for various periods of time and treated as previously described.

In Vivo Infection Studies.—Mice were injected intravenously or intraperitoneally with 0.2 ml. of 199 medium containing 3×10^8 bacteria S1. The mice were followed for a period of 28 days and deaths recorded.

Infection of Mice with BCG.—Bacillus Calmette-Guérin was grown for 10 days at 37°C. in Dubos and Middlebrook medium (19). The bacteria were harvested by centrifuging at 2000 R.P.M. for 5 minutes and resuspended in the same medium to give a concentration of 5 mg. wet weight of bacteria/ml. Mice were injected intravenously with 0.2 ml. of this suspension. Peritoneal macrophages and plasma were obtained from the infected mice 14 to 21 days after injection.

RESULTS

Phagocytosis and Survival of Bacteria after Opsonization with Normal Mouse Plasma.—Peritoneal macrophages obtained from normal mice were infected with strains of *Salmonella* and *E. coli*. The results of such experiments are illustrated in Fig. 1. Each curve is the result of two or more experiments, and thus each point represents the average percentage survival of bacteria within macrophages from at least six flasks. The two strains of *E. coli* behaved similarly and the results from each were summated. These experiments show that under the

above conditions, virulent strains of *Salmonella* are not phagocytosed to the same extent as the avirulent strains. Differences in the percentage survival of bacteria within the macrophages at the end of the phagocytic period were also apparent; a higher percentage of the ingested virulent bacteria could be recovered on plating. The behavior of the bacteria within the macrophages suggested that the virulent strains of *Salmonella* could multiply intracellularly, whereas continued killing occurred with the avirulent strains, (M206, *E. coli*) some multiplication appearing to take place toward the end of the time period studied.

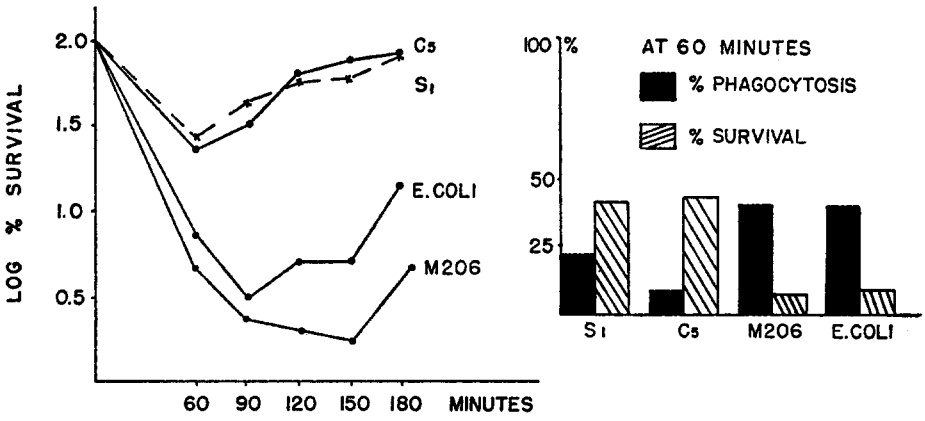

FIG. 1. Phagocytosis by normal mouse peritoneal macrophages of various strains of *Salmonella* and *E. coli* and their intracellular fate, after opsonization with normal mouse plasma. C5, virulent strain, *Salmonella typhimurium*; M206, avirulent strain, *Salmonella typhimurium*; S1, virulent strain, *Salmonella enteritidis* (Danysz).

It is difficult to say from these experiments where extracellular growth is not excluded, that the late apparent increase in the intracellular population of bacteria, is not due to multiplication of bacteria that are stuck to the surface of the macrophages.

The experiments were therefore repeated using several concentrations of streptomycin. The amount of antibiotic in the medium was found to be critical. At concentrations which were bacteriostatic for the strains under investigation, or bactericidal to the extent that 60 per cent of the bacteria were killed within 60 minutes, intracellular growth of the virulent strains could be demonstrated at a time when the numbers of extracellular bacteria fell rapdily (Fig. 2). However with higher concentrations of streptomycin no multiplication took place, and the bacteria were killed within the phagocytic cells. Even with the smallest concentrations of streptomycin that allowed intracellular growth whilst killing extracellular bacteria, a sudden fall in numbers of intracellular bacteria was

frequently observed towards the end of the time period studied (Fig. 3). This suggests that the macrophages were damaged by the multiplying intracellular bacteria allowing streptomycin to enter the cell. The cytotoxic effect of multiplying intracellular bacteria which may depend on the rate of multiplication has been reported by others (8, 20).

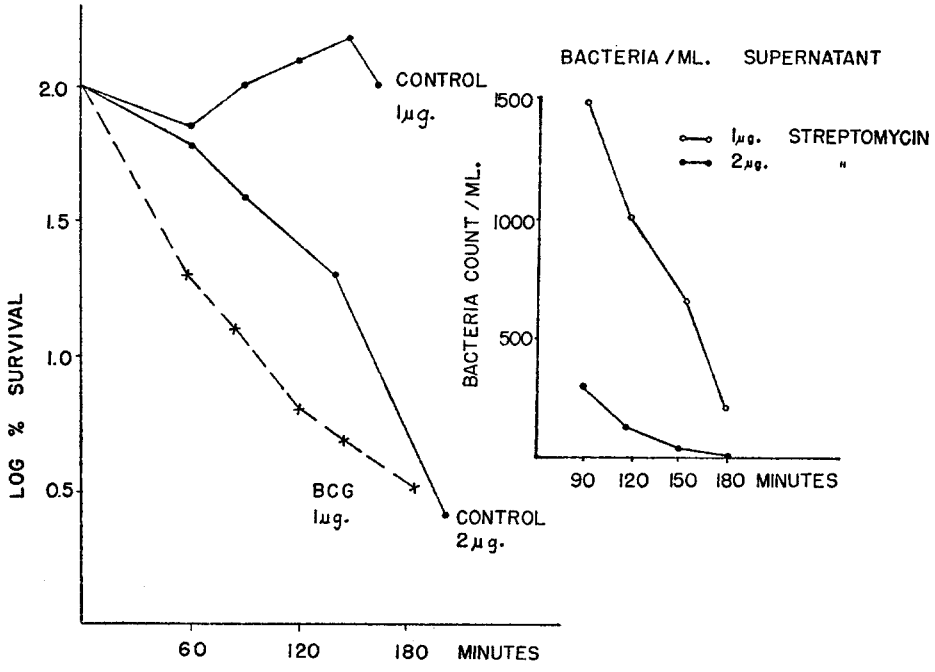

FIG. 2 *a* FIG. 2 *b*

FIG. 2 *a*. Effect of various doses of streptomycin on the survival of *Salmonella enteritidis* (Danysz) S1 in the supernatant from Porter flasks.

FIG. 2 *b*. Effect of optimum concentrations of streptomycin on the survival of *Salmonella enteritidis* (Danysz) within peritoneal macrophages from normal mice and BCG-infected mice, after opsonization with normal and BCG plasma.

Control: Normal macrophages and normal plasma.

Comparison of the results obtained with and without antibiotics, suggests that the amount of extracellular growth taking place on the surface of the cells in antibiotic-free medium was small, and not sufficient to mask events taking place within the cell. From these findings it is possible to conclude that the virulent strains are able to multiply within the mononuclear cells, whilst the avirulent strains are slowly killed. It should be noted that after the first 60 minutes the rate of killing decreased for each of the strains investigated.

Phagocytosis and Survival of Salmonella enteritidis (Danysz) after Opsonization with Specific Antiserum.—*Salmonella enteritidis*, a strain virulent for mice, was

opsonized with various dilutions of a rabbit-specific antiserum which had been heated at 56°C. for 30 minutes to destroy complement activity. From the results presented in Fig. 4 it may be seen that over quite a wide range of dilution the opsonizing capacity of the antiserum appears to be optimal. When compared with the results obtained using normal mouse serum as a source of opsonins, it is apparent that opsonizing bacteria with a specific antiserum not only increases the amount of bacteria phagocytosed but also increases the amount killed, as indicated by the smaller numbers of intracellular bacteria recovered at the end of the phagocytic period. Peritoneal macrophages obtained from normal mice treat a virulent strain of *Salmonella* opsonized with specific

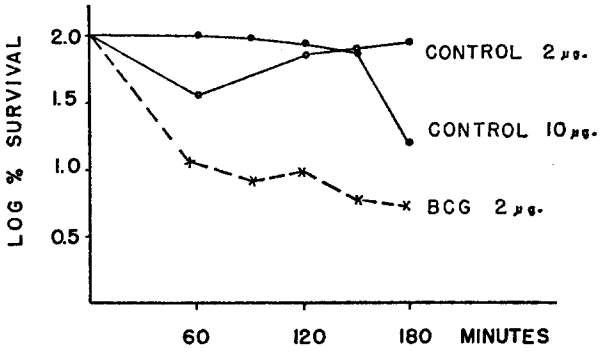

FIG. 3. Effect of optimum concentrations of streptomycin on the survival of *Salmonella typhimurium* (C5) within peritoneal macrophages from normal mice and BCG-infected mice, after opsonization with normal and BCG plasma.

Control: Normal macrophages and normal plasma.

rabbit antiserum, as they would an avirulent strain opsonized with normal mouse plasma.

Phagocytosis and Survival in BCG Macrophages of Salmonella Strains after Opsonization with Plasma from BCG-Infected Mice.—For the sake of clarity plasma and peritoneal macrophages obtained from BCG-infected mice will be referred to as BCG plasma and BCG macrophages.

Investigations on the phagocytosis of virulent strains of *Salmonella* opsonized with BCG plasma, by BCG macrophages, and their survival within the phagocytes, show that under these conditions virulent strains are treated as if they were avirulent, phagocytosis being increased and intracellular survival decreased (Fig. 5). Similar results were obtained using macrophages and plasma from endotoxin-treated mice (Fig. 6).

In order to determine whether these results were due to cellular and/or humoral factors, the interaction between normal mouse serum and bacteria opsonized with BCG plasma was studied. From these investigations (Fig. 7) it is apparent that BCG plasma has a higher opsonic titer than normal mouse

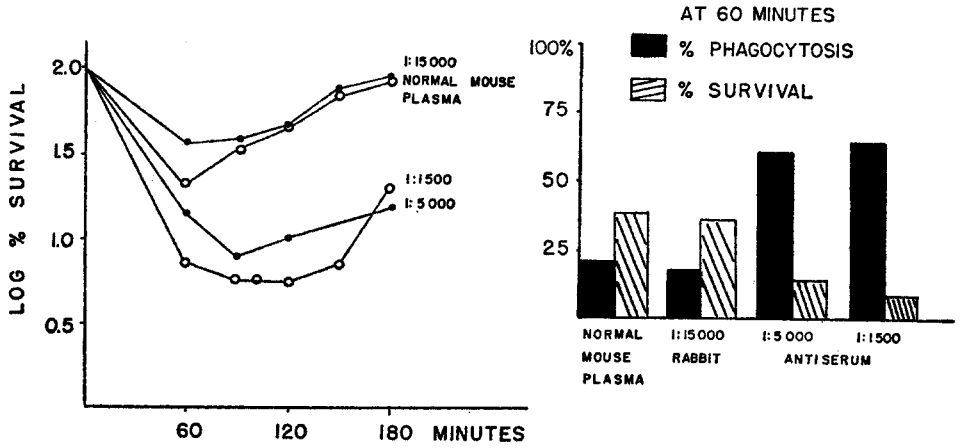

FIG. 4. Effect of various dilutions of rabbit anti-*Salmonella* S1 serum on the opsonization of *Salmonella enteritidis* S1, and intracellular survival after phagocytosis by normal mouse peritoneal macrophages.

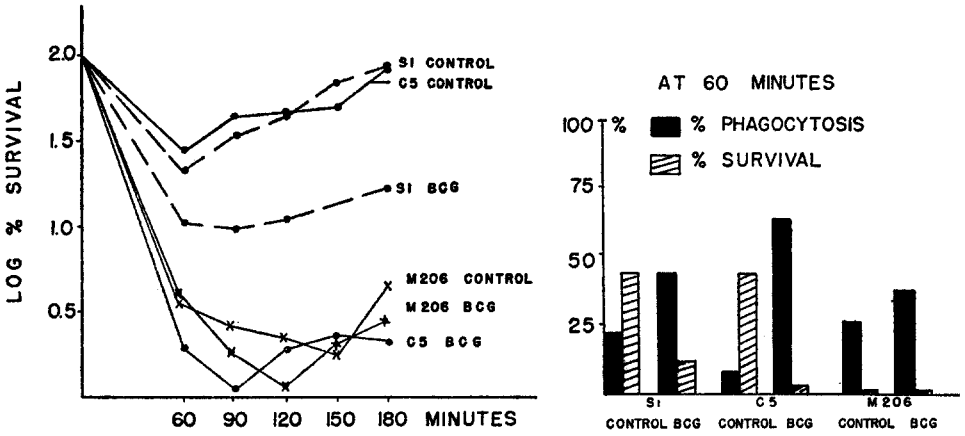

FIG. 5. Phagocytosis by peritoneal macrophages from normal and BCG-infected mice, of virulent and avirulent strains of *Salmonella* and their survival within the phagocytes. Opsonization with normal mouse plasma in the case of normal macrophages (control) and with BCG mouse plasma with respect to BCG macrophages (BCG).

C5, virulent strain of *Salmonella typhimurium*; M206, avirulent strain of *Salmonella typhimurium*; S1, virulent strain of *Salmonella enteritidis* (Danysz).

plasma, and that a combination of BCG macrophages and BCG plasma is a more efficient phagocytic and bactericidal system than one of peritoneal macrophages from normal mice and BCG plasma.

It is interesting that little difference could be detected in the behaviour of

the avirulent strain (M206) within BCG macrophages after opsonization with BCG plasma, compared with its survival within macrophages from normal mice

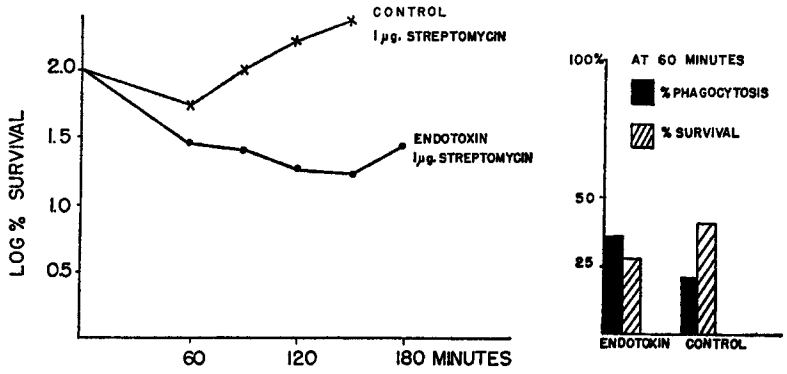

FIG. 6. Effect of treatment of mice with 50 μg. of *E. coli* lipopolysaccharide on the opsonic properties of the serum and on the capacity of peritoneal macrophages to suppress intracellular growth of *Salmonealla enteritidis* S1.

Controls: Normal macrophages infected with S1 opsonized with normal mouse plasma.

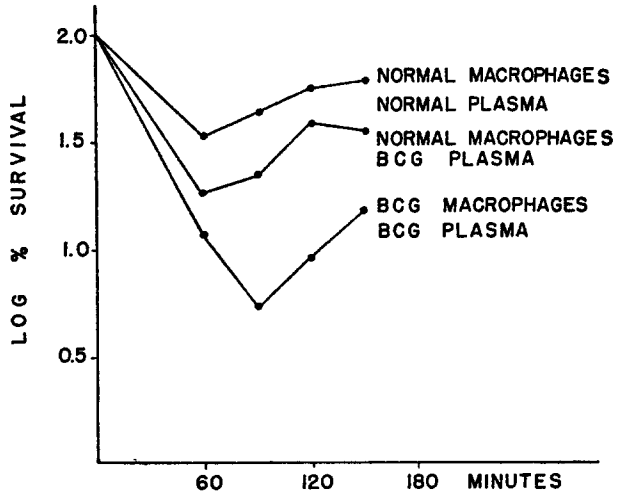

FIG. 7. Comparative studies on the phagocytic and bactericidal properties of macrophages from normal and BCG-infected mice, against *Salmonella enteritidis* (Danysz) after opsonization with normal and BCG plasma.

after opsonization with normal mouse plasma. This suggests that normal mouse plasma contains sufficient opsonins to effect efficient phagocytosis and killing of the avirulent strain.

TABLE II

Effect of Previous Opsonization of the Bacteria with Normal Mouse Plasma, BCG Plasma, and Rabbit-Specific Antiserum on the Mortality of Mice Infected with Salmonella enteritidis (Danysz) S1

Route of challenge and treatment of bacteria	Cumulative mortality, days after challenge													
	2	4	6	8	10	12	14	16	18	20	22	24	26	28
Intravenous challenge														
Opsonized normal plasma	0/20*	6/20	10/20	10/20	17/20	19/20	20/20							
Opsonized BCG plasma	0/20	4/20	13/20	14/20	17/20	18/20	18/20	18/20	18/20	18/20	18/20	18/20	18/20	18/20
Opsonized 1:1500 antiserum	0/20	2/20	7/20	11/20	14/20	14/20	15/20	15/20	15/20	15/20	15/20	15/20	15/20	15/20
Opsonized 1:15000 antiserum	0/20	1/20	12/20	17/20	20/20									
Intraperitoneal challenge														
Opsonized normal plasma (Experiment I)	1/20	2/20	5/20	7/20	14/20	15/20	15/20	16/20	16/20	16/20	16/20	16/20	16/20	16/20
Opsonized normal plasma (Experiment II)	2/20	3/20	6/20	14/20	17/20	20/20								
Opsonized BCG plasma	0/20	0/20	3/20	9/20	17/20	18/20	20/20							
Opsonized 1:1500 antiserum (Experiment I)	0/20	0/20	3/20	6/20	9/20	10/20	10/20	10/20	12/20	12/20	12/20	12/20	12/20	12/20
Opsonized 1:1500 antiserum (Experiment II)	0/20	1/20	2/20	5/20	9/20	10/20	10/20	10/20	10/20	10/20	10/20	10/20	10/20	10/20
Opsonized 1:15000 antiserum	0/20	0/20	1/20	4/20	4/20	10/20	13/20	13/20	13/20	13/20	13/20	13/20	13/20	13/20
BCG—infected mice														
Intravenous challenge	0/20	0/20	0/20	5/20	6/20	13/20	18/20	18/20	20/20					
Intraperitoneal challenge	0/20	0/20	0/20	4/20	9/20	9/20	15/20	15/20	19/20	19/20	19/20	19/20	19/20	19/20

* Number of animals dead / Number of animals challenged.

These experiments were repeated with streptomycin and it was found that using a system of BCG macrophages and bacteria opsonized with BCG plasma, killing of the virulent strains extended over the time period studied, but not to as marked degree as in the first 60 minutes (Fig. 2 and 3). Heating BCG plasma to 56°C. for 30 minutes completely destroyed the opsonizing capacity of the plasma.

The amount of bacteria phagocytosed by a culture of cells is limited to a great extent by the degree of opsonization. It became apparent in the present study that opsonization also affected the survival of the bacteria within the cell.

In Vivo Studies on Salmonella Infections in Mice.—The *in vitro* studies presented here, have suggested a reason for the increased resistance to *Salmonella* infections displayed by mice infected with BCG. Some of the *in vitro* findings were applied to *in vivo* studies and the fate of normal mice followed after they had been injected intravenously and intraperitoneally, with *Salmonella enteritidis* opsonized with normal mouse plasma, BCG plasma, and specific antiserum. (Table II). Mice that had been injected with bacteria opsonized with BCG plasma, displayed no increased resistance to infection. However the group of mice challenged with bacteria opsonized with specific antiserum, displayed some resistance in terms of survival, but this was more apparent when the challenge was given intraperitoneally, and kept small.

DISCUSSION

The present studies have shown that unless Gram-negative bacteria have been opsonized they are not phagocytosed by mouse peritoneal macrophages. These observations are in agreement with the findings of Whitby and Rowley (16). Contrary to the observations reported by Furness (6) it was found that virulent strains of *Salmonella* were never phagocytosed as well as avirulent strains, unles they had been previously well opsonized with specific antiserum, or plasma from BCG-infected mice. The observations of Whitby and Rowley (7) that intracellular bacteria are killed at the same rate irrespective of their virulence, was found to be limited to conditions under which the virulent bacteria had been well opsonized. The results presented in this paper show that virulent strains of *Salmonella* opsonized with normal mouse plasma are not phagocytosed as well as avirulent strains, and that a much greater percentage of the virulent bacteria survive and multiply within the macrophages. The differences between the results of Whitby and Rowley and of our own could be explained by the fact that these investigators used a specific antiserum as a source of opsonic factors. Under these conditions virulent strains of *Salmonella* are treated by mouse peritoneal macrophages as if they were avirulent. Treating these bacteria with plasma or serum of different opsonic properties indicated a relationship between the extent of opsonization and intracellular survival. Phagocytosis of poorly opsonized virulent bacteria is never great and

of the phagocytosed bacteria, large numbers survive to multiply intracellularly. However if these same strains are well opsonized with BCG plasma or specific antiserum, phagocytosis is greatly increased, and then only small numbers of the ingested bacteria survive within the cells.

These findings may in part explain the observations that mononuclear cells from an animal specifically immunized against the bacteria under study, destroy these bacteria more efficiently than those from a normal animal (21–24). In many of the studies reported to have shown these differences, ingestion of bacteria has taken place in the serum or peritoneal exudate from the immune animal. In such cases the macrophages from the immune animal would be phagocytosing well opsonized bacteria. Further it is very difficult to be sure that washing immune cells removes all traces of antibody, minute amounts being sufficient to greatly alter phagocytosis and intracellular killing. By comparing the percentage degeneration of monocytes in tissue culture after phagocytosing bacteria in normal and immune serum, several workers have suggested that immune serum protects the cell against the cytotoxic effect of the bacteria. From the results discussed in this paper this "protective" effect could well be a measure of the efficiency of killing, and not as has been suggested protection of the phagocyte *per se* (25, 26).

Plasma from BCG-infected, or endotoxin-treated mice had an elevated opsonic titer, and peritoneal macrophages from these mice were able to kill phagocytosed virulent *Salmonella* which would grow within normal macrophages. These results were particularly striking in the presence of optimum levels of streptomycin, that destroyed extracellular bacteria. While both humoral and cellular factors are likely to contribute to the resistance of mice when challenged with virulent *Salmonella*, the lack of protection observed *in vivo* after opsonization of the bacteria with BCG plasma indicates that cellular factors are essentially responsible for the favourable outcome of the infection. The mechanism responsible for the improved cellular resistance is not understood, but certain conclusions may be drawn from an analysis of the curves of bacterial survival within macrophages, bearing in mind that the experimental procedure favoured the infection of individual macrophages by a single bacterium. In the case of normal macrophages and virulent bacteria, significant killing was observed in the first 60 minutes, followed by growth. These findings indicate that the macrophage population is heterogeneous with respect to its ability to destroy the ingested bacteria. The experiments with BCG macrophages and virulent bacteria showed continuous killing for 2 or 3 hours, suggesting that the population of macrophages was more homogeneous with respect to its ability to kill intracellular bacteria. These differences may be a reflection of the number of mature and immature cells in the macrophage population, the mature cells possessing phagocytic and bactericidal properties,

whilst the immature cells are capable of ingesting bacteria but not killing them, growth taking place in the latter.

The RES of the spleen and liver is a system of phagocytic cells in various stages of maturation, and there is likely to be some proportion of immature cells. Under the stimulus of lipopolysaccharide or BCG-infection (both of which produce a hyperplasia of the RES) a speeding up of the maturation process may occur such that a greater number of mature macrophages are present in these organs than would be present in normal animals.

One could thus postulate that the resistance displayed by BCG mice to *Salmonella* infections is due to two factors; a greater proportion of mature macrophages, and a rise in serum opsonic factors increasing phagocytosis and intracellular killing.

SUMMARY

Virulent strains of *Salmonella* opsonized with normal mouse plasma are never phagocytosed as well as avirulent strains.

The virulent strains of *Salmonella* phagocytosed after opsonization with normal mouse plasma are able to multiply within normal mouse peritoneal macrophages, whereas under similar experimental conditions the avirulent strains are killed.

When virulent strains of *Salmonella* are opsonized with specific antiserum or plasma from BCG-infected mice, they are treated by normal mouse macrophages as if they were avirulent.

Virulent bacteria opsonized with BCG plasma are phagocytosed and killed better by peritoneal macrophages from BCG-infected mice, than peritoneal macrophages from normal mice.

We wish to thank D. L. Palmer for his assistance with some of these experiments.

BIBLIOGRAPHY

1. Øistov, J., Jensen, K. A., and Kobayshi, K., Studien über Breslauinfektion. der mause speziel mit Rucksicht auf die bedentung des Retikuloendothelial gewebes, *Z. Immunitätsforsch.*, 1928, **55**, 34.
2. Schneider, H. A., and Zinder, N. D., Nutrition of the host and natural resistance to infection. V. An improved assay employing genetic markers in the double strain inoculation test, *J. Exp. Med.*, 1956, **103**, 207.
3. Pike, R. M., and Mackenzie, G. M., Virulence of *Salmonella typhimurium*. I. Analysis of experimental infection in mice with strains of high and low virulence, *J. Bact.*, 1940, **40**, 171.
4. Marcus, S., Esplin, D. W., and Donaldson, D. H., Lack of bactericidal effect of mouse serum on a number of common micro-organisms, *Science*, 1954, **119**, 877.
5. Muschel, L. H., and Muto, T., Bactericidal reaction of mouse serum, *Science*, 1956, **123**, 62.

6. Furness, G., Interaction between *Salmonella typhimurium* and phagocytic cells in tissue culture, *J. Infect. Dis.*, 1958, **103,** 272.
7. Rowley, D., and Whitby, J. L., The bactericidal activity of mouse macrophages *in vitro*, *Brit. J. Exp. Path.*, 1959, **40,** 570.
8. Shepard, C. C., Phagocytosis by Hela cells and their susceptibility to infection by human tubercle bacilli, *Proc. Soc. Exp. Biol. and Med.*, 1955, **90,** 392.
9. Dubos, R. J., and Schaedler, R. W., Effects of cellular constitutents of mycobacteria on the resistance of mice to heterologous infections. I. Protective effects, *J. Exp. Med.*, 1957, **106,** 703.
10. Howard, J. G., Biozzi, G., Halpern, B., Stiffel, C., and Mouton, D., The effect of *Mycobacterium tuberculosis* (BCG) on the resistance of mice to bacterial endotoxin and *Salmonella enteritidis* infection, *Brit. J. Exp. Path.*, 1959, **40,** 281.
11. Howard, J. G., Rowley, D., and Wardlaw, A. C., Investigations on the mechanism of stimulation of non specific immunity by bacterial lipopolysaccharide, *Immunology*, 1958, **1,** 181.
12. Rowley, D., Rapidly induced changes in the levels of non-specific immunity in laboratory animals, *Brit. J. Exp. Path.*, 1956, **37,** 223.
13. Benacerraf, B., Sebestyan, M. M., and Schlossman, S., A quantitative study of the kinetics of blood clearance of P^{32}-labelled *Escherichia coli*, and staphylococcus by the reticulo-endothelial system, *J. Exp. Med.*, 1959, **110,** 27.
14. Westphal, Ö., Luderitz, Ö., and Bister, F., Uber der extraktion von bakterien mit phenol/wassel, *Z. Naturforsch.*, 1952, **73,** 148.
15. Wadsworth, A. B., Standard Methods, The Williams & Wilkins Co., Baltimore, 3rd edition, 1927.
16. Whitby, J. L., and Rowley, D., The role of macrophages in the elimination of bacteria from the mouse peritoneum, *Brit. J. Exp. Path.*, 1959, **40,** 358.
17. Morgan, J. F., Morton, H. J., and Parker, R. C., Nutrition of animal cells in tissue culture; initial studies on synthetic medium, *Proc. Soc. Exp. Biol. and Med.*, 1950, **73,** 1.
18. Porter, K. R., Claude, A., and Fulham, E. F., A study of tissue culture cells by electron microscopy. Methods of preliminary observations, *J. Exp. Med.*, 1945, **81,** 233.
19. Dubos, R. J., and Middlebrook, G., Media for tubercle bacilli, *Am. Rev. Tuberc.*, 1947, **46,** 334.
20. Mackaness, G. B., Smith, N., and Wells, A. Q., Growth of intracellular tubercle bacilli in relation to their virulence, *Am. Rev. Tuberc.*, 1954, **69,** 479.
21. Lurie, M., Studies on the mechanism of immunity to tuberculosis. The fate of tubercle bacilli ingested by mononuclear phagocytes derived from normal and immunized animals, *J. Exp. Med.*, 1942, **74,** 247.
22. Suter, E., The multiplication of tubercle bacillus within mononuclear phagocytes in tissue cultures derived from normal animals and animals vaccinated with BCG, *J. Exp. Med.*, 1953, **97,** 235.
23. Mackaness, G. B., Growth of tubercle bacilli in monocytes from normal and vaccinated rabbits, *Am. Rev. Tuberc.*, 1954, **69,** 495.
24. Pomales-Lebrón, A., and Stinebring, W. R., Intracellular multiplication of

Brucella abortus in normal and immune mononuclear phagocytes, *Proc. Soc. Exp. Biol. and Med.*, 1957, **94**, 78.

25. Fong, J., Schneider, P., and Elberg, S. S., Studies on tubercle bacillus-monocyte relationship. I. Quantitative analysis of the effect of serum of animals vaccinated with BCG upon bacterium-monocyte system, *J. Exp. Med.*, 1956, **104**, 455.
26. Gelzer, J., and Suter, E., The effect of antibody on intracellular parasitism of *Salmonella typhimurium* in mononuclear phagocytes *in vitro*. Prolonged survival of infected monocytes in presence of antibody, *J. Exp. Med.*, 1959, **110**, 715.