THE EFFECT OF OPSONINS ON THE INTRACELLULAR SURVIVAL OF BACTERIA*

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THE importance of serum opsonins in promoting phagocytosis of bacteria by macrophages needs no longer be stressed. We have suggested from such studies concerned with the survival of the ingested bacteria, that opsonins not only enhance phagocytosis but play some role in determining the fate of the ingested bacteria (Rowley, 1958; Jenkin and Benacerraf, 1960; Rowley and Jenkin, 1962). This latter conclusion has been arrived at by following the survival of bacteria within mouse peritoneal macrophages under different conditions of opsonization, and hence under conditions which lead to differences in the rates of phagocytosis. It has been impossible therefore to decide conclusively that opsonins do effect the survival of ingested bacteria, since the differences observed between the intracellular behaviour of well opsonized, and poorly opsonized bacteria, may to some extent be a reflection of the differences in the rates of phagocytosis.

Ideally, in order to arrive at a more definite answer, one must arrange two experimental systems in which phagocytosis of a constant inoculum of bacteria takes place at the same rate, but in one case opsonins specific for the bacteria should be absent or in low titre, whilst in the other instance the opsonins should be non-limiting. Under these conditions with similar rates of phagocytosis, the influence of opsonins on intracellular events would be reflected by differences in the survival of the phagocytosed bacteria.

This paper describes a method by means of which bacteria may be phagocytosed in the absence of specific serum opsonins, at rates comparable to those observed if the bacteria had been well opsonized. A comparison has been made of the intracellular survival of bacteria under such differing conditions, and it has been found that serum opsonins play a definite, but as yet unclear role, in determining the fate of the intracellular bacteria.

MATERIAL AND METHODS

Bacterial strains.—The strains of *Salmonella typhimurium* used in these studies were C5 and S63 (virulent) and M206 (avirulent) (2).

Development of strains of Salm. typhimurium *lysogenic for phage* P22.—One ml. of a bacterial suspension containing approximately 5×10^8 organisms/ml. was poured over the surface of a nutrient agar plate and the superfluous fluid removed. A series of plates was thus prepared and on to these was poured 1 ml. samples of various dilutions of phage, one plate being used for one phage dilution. After removal of excess fluid the plates were incubated at 37° overnight. Following incubation, a plate was selected containing 100–200 plaques. From the centres of such plaques, bacterial colonies were selected apparently resistant to lysis by the phage. Such colonies were tested against a specific antiserum, for their lysogenicity, and for their ability to absorb phage P22. These strains were designated C5R,

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S63R and M206R, depending on their origin. These derived strains were similar to the parent strains except for the fact that they were lysogenic for phage P22.

Preparation of phage.—Phage was prepared and assayed according to standard techniques (Adams, 1959). The phage P22 for all these experiments was propagated in strain C5.

In vivo *clearance studies.*—Isotopically labelled bacteria were prepared and stored according to the methods previously published (Jenkin and Rowley, 1961). Swiss white mice, males and females, weighing from 18–20 g. were used in all these experiments. The method for the study of the clearance of the bacteria from the blood stream was that described by Biozzi, Benacerraf and Halpern (1953). The rate of clearance for a dose of bacteria 10^9/100 g. weight was expressed as the phagocytic index K, where

$$K = \frac{\text{Log . C1} - \text{Log . C2}}{\text{T2} - \text{T1}}$$

Each clearance curve is the summation of results obtained from 6 mice. The radioactivity of each blood sample, giving a measure of the concentration of bacteria, was assayed as previously reported (Jenkin and Rowley, 1961).

Source of serum.—Pig serum was obtained from adult pigs at slaughter. Mouse blood was collected by bleeding from the retro-orbital plexus. The blood was heparinised with 10 units heparin/ml., and the cellular constituents of the blood removed by centrifuging 1500 rpm for 10 min.

Opsonization of bacteria.—Bacteria were opsonized by mixing 1 ml. of a bacterial suspension containing 10^9 bacteria/ml. with 1 ml. of the serum. The mixture was kept at 4° for 20 min. Following this the mixture was centrifuged at 3000 rpm for 20 min. and finally resuspended in saline for injection.

In vitro *phagocytic studies with mouse peritoneal macrophages.*—The technique was similar to that previously described with one or two modifications (Jenkin and Benacerraf, 1960). Mouse peritoneal macrophages were harvested in Hanks' tissue culture medium (Hanks, 1955) containing 0·5 per cent bovine serum albumin fraction V and 5 units/ml. of heparin. The macrophages were allowed to settle in Leighton tubes for at least one hour before experimental use. Each tube was gassed at the start with a mixture of 5 per cent CO_2 and air. At the end of the settling period, the macrophages were carefully examined microscopically for signs of degeneration and only those monolayers in which the macrophages had begun to spread were kept for experimental purposes. In general, less than 5 per cent of the tubes were discarded. After examination of the monolayers, opsonized bacteria in the ratio of about one bacterium to 500 macrophages was added to each tube in 0·7 ml. of the tissue culture medium, and the tubes incubated for a further 60 min. With each series of tubes containing macrophages, four or five tubes containing no macrophages were similarly inoculated, as controls for bacterial growth during this period. At the end of this time (phagocytic period) the tubes were removed and counts made on the bacterial population of the supernatants. Three tubes were then immediately placed on ice. The monolayers of macrophages in the remaining tubes were washed with ice-cold Hanks' medium containing bovine serum albumin, and finally returned to the incubator after addition of 0·7 ml. of tissue culture fluid. Three tubes were then removed at time intervals indicated in the various figures presented in the text. The three tubes placed in ice were washed as above and 0·5 ml. of saline added. The monolayer of macrophages in each tube was removed by scraping with a small knife blade mounted on a handle. This proved an extremely efficient way of removing the macrophages from the glass surface, as evidenced by microscopic examination of the tubes. Clumps of macrophages were broken up by vigorous pipetting and finally 0·1 ml. of the macrophage suspension plated onto a nutrient agar plate and briskly spread with a metal spreader. In the experiments that were duplicated with antibiotics in the medium, 1 μg. of streptomycin/ml. of medium was added after the 60 min. period allowed for phagocytosis. In order to allow the streptomycin time to react with extracellular bacteria in the tubes taken at the end of the phagocytic period, ice-cold antibiotic medium was added to the monolayers, and the tubes were kept at 4° for 30 min. before removal of the macrophages for plating. In experiments using phage P22 the bacteria were contacted with phage in a ratio of 10 phage particles to 1 bacterium for a period of 15 min. at 37°. Following this, the mixture was centrifuged at 3000 rpm and washed with saline. After re-centrifuging, the washed suspension of bacteria was resuspended in 1 ml. of tissue culture medium and added to the serum for opsonization. Bacteria so treated were designed C5RP, S63RP and M206RP.

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 min. period of incubation represented the number of 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 the macrophages could be determined by multiplying the plated macrophage sample by 5. The percentage survival at any time during the experiment could be calculated from $N/D \times 100$. These results were plotted as log per cent survival against time.

Absorption of mouse serum with bacteria.—Mouse serum was absorbed with a suspension of bacteria that had been boiled for 2 hr. One ml. of mouse serum was absorbed with 10^9 bacteria overnight at 4°. Bacteria were removed from the serum by centrifuging at 3000 rpm for 20 min.

RESULTS

Rates of clearance of virulent strains of Salm. typhimurium *S63R and C5R from the circulation of mice before and after contact with phage P22*

Both strains of P32 labelled Salmonellae were mixed with phage P22 in a ratio of 10 phage particles to one bacterium. After incubation at 37° for 15 min.,

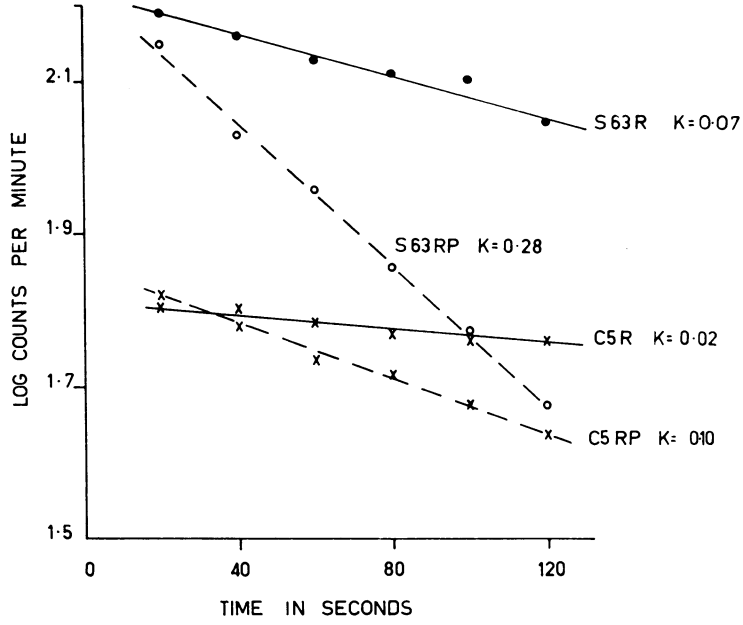

FIG. 1.—Rates of clearance (expressed as the phagocytic index K) of virulent strains of *Salmonella typhimurium* S63R and C5R before and after contact with phage P22.

the bacteria were centrifuged and washed and 0·2 ml. of the washed suspension containing 2×10^8 bacteria injected intravenously. The elimination of the bacteria from the circulation was followed, and the rates of clearance compared with those observed when untreated bacteria were injected (Fig. 1). It is apparent from these results that bacteria after they have been in contact with phage are removed from the blood stream at faster rates than are the untreated bacteria. This type of experiment was repeated using different ratios of phage particles to

TABLE I.—*Rates of Clearance of Virulent Strains of* Salm. typhimurium *S63R and C5R Before and After Contact with Phage P22. Phage and Bacteria Incubated together at 37° for 15 min.*

Strain of *Salm. typhimurium*	Ratio of phage to bacteria	Phagocytic Index K
C5R	0	0·02
	10 : 1	0·09
	25 : 1	0·09
	50 : 1	0·08
S63R	0	0·07
	10 : 1	0·28
	25 : 1	0·27
	50 : 1	0·28

bacterial cells (Table I). The effect of incubating a constant number of phage particles with bacteria for various periods of time on the subsequent clearance of these bacteria from the circulation was also measured (Table II). The results of these experiments show that the rates of elimination of the bacteria are not affected by the ratio of phage particles to bacterial cells used in these tests, neither does prolonged incubation of the bacteria with the phage increase the rate of clearance. As a result of these experiments the ratio of phage particles to bacterial cells was kept in the order of 10 : 1 and the time of incubation at 37° 15 min.

TABLE II.—*Rates of Clearance of Virulent Strains of* Salm. typhimurium *S63R and C5R After Contact with Phage* P22 *for varying Periods of Time at* 37°. *Constant Ratio of* 10 *Phage Particles to One Bacterium*

Strain of *Salm. typhimurium*	Time/min. at 37°	Phagocytic Index K
C5R	15	0·09
	30	0·09
	60	0·09
	120	0·10
	Control no phage	0·02
S63R	15	0·27
	30	0·28
	60	0·27
	120	0·30
	Control no phage	0·07

Virulence of Salm. typhimurium *strains before and after contact with phage P22*

Both strains after contact with phage reacted to full titre with a specific antiserum. The bacteria were treated with phage as above, washed three times, and finally resuspended in saline. Mice were challenged intraperitoneally and followed for a period of 28 days. The results given in Table III show that contact of bacteria with phage does not alter their virulence for mice.

In vitro *studies on the phagocytosis of phage treated bacteria*

The phagocytosis of *Salm. typhimurium* C5R before and after treatment with phage was followed *in vitro* using mouse peritoneal macrophages. As a source of opsonins mouse serum was used, since this has previously been shown to contain a low titre of these factors reacting against this particular highly virulent strain (Jenkin and Benacerraf, 1960). From the results illustrated in Fig. 2 and 3 it

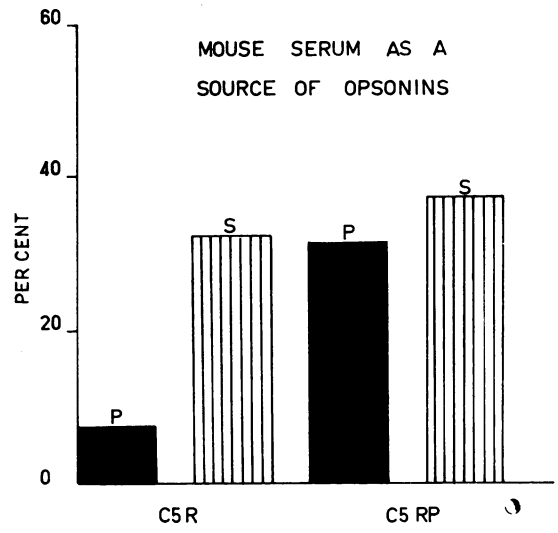

FIG. 2.—Phagocytosis and intracellular survival of C5R before and after 60 min. contact with phage P22. P = phagocytosis. S = survival

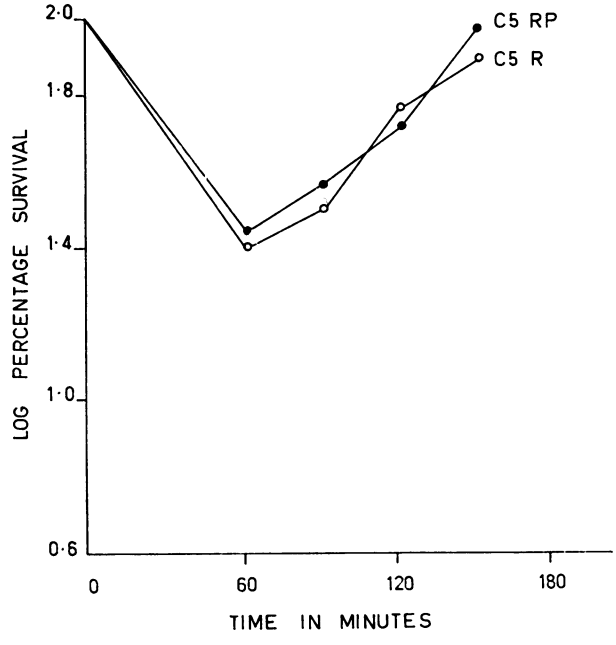

FIG. 3.—Intracellular behaviour of C5R before and after contact with phage P22. Mouse serum used as source of opsonins.

TABLE III

Strain	Challenge dose	Cumulative deaths, days after challenge 2	4	6	8	10	12	14	16	18	20	28
(1) C5	800	0	2	4	9	15	19	19	19	19	19	19
(2) C5R	1000	0	1	5	10	12	15	16	16	16	17	17
(3) C5RP	1200	0	1	3	12	14	17	18	18	18	19	19
(1) S 63	10^4	0	6	10	14	18	18	19	19	20	20	20
(2) S 63R	10^4	0	2	8	9	16	20	20	20	20	20	20
(3) S 63RP	10^4	1	2	7	11	17	19	19	19	19	19	19

(1) Original parent strain from which the (2) lysogenic strain was derived.
(3) Lysogenic strain contacted with phage in a ratio of 10 phage particles to one bacterial cell and incubated at 37° for 15 min. Twenty mice challenged by intraperitoneal injection in each group.

may be seen that despite differences in the rates of phagocytosis, the intracellular survival of the bacteria was similar in the two experiments, a large proportion of the bacteria surviving and multiplying intracellularly. These results suggest that in the particular system under investigation the fate of the ingested bacteria is not determined by differences in the rates of phagocytosis. As a further illustration of this point the phagocytosis and intracellular survival of C5R was studied under the following conditions. Bacteria were first treated with phage and then divided into 2 samples. To one sample mouse serum was added, whilst to the other pig serum which had previously been shown to contain a high titre of serum opsonins directed against this virulent strain (Jenkin, 1962 ; Rowley and Jenkin, 1962). Under these experimental conditions it was found that though the rates of phagocytosis were comparable between the two systems, the fates of the ingested bacteria were quite different. Where mouse serum was used as the source of opsonins the bacteria survived and multiplied within the cells, whereas when pig serum was used the bacteria continued to be killed over the time period studied (Figs. 4 and 5). The survival of the bacteria within the cells was followed in the presence and in the absence of streptomycin, to eliminate the possibility that growth observed in some of the experiments was due to multiplication of bacteria stuck to the surface of the cells. The results of these two sets of experiments were essentially the same, and therefore it is reasonable to assume that the observed increase in bacterial numbers was due to actual intracellular multiplication.

Phagocytic studies using mouse serum and absorbed mouse serum as a source of opsonins

In the following experiments *Salm. typhimurium* C5R (virulent) and M206R (avirulent), were used before and after contact with phage. Mouse serum as a source of opsonins was absorbed with the above strains before and after contact with phage P22. Using this serum the phagocytosis and survival of the ingested bacteria was followed and compared with results obtained using unabsorbed mouse serum. As a further extension of these experiments the phagocytosis of both strains before and after treatment with phage was studied in the absence of serum opsonins. The results of these experiments are presented in Tables IV and V and in Figs. 6 and 7. They show that in the absence of specific bacterial opsonic factors little or no killing of the ingested bacteria takes place. This is shown

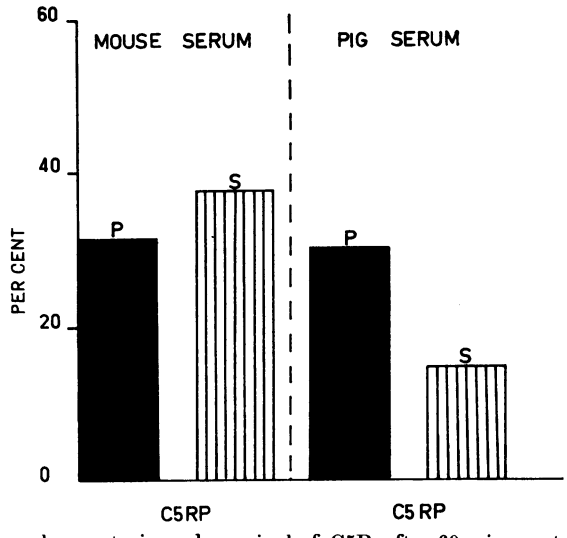

FIG. 4.—Percentage phagocytosis and survival of C5R after 60 min. contact with phage P22. Mouse serum and pig serum used as a source of opsonins. P = phagocytosis. S = survival.

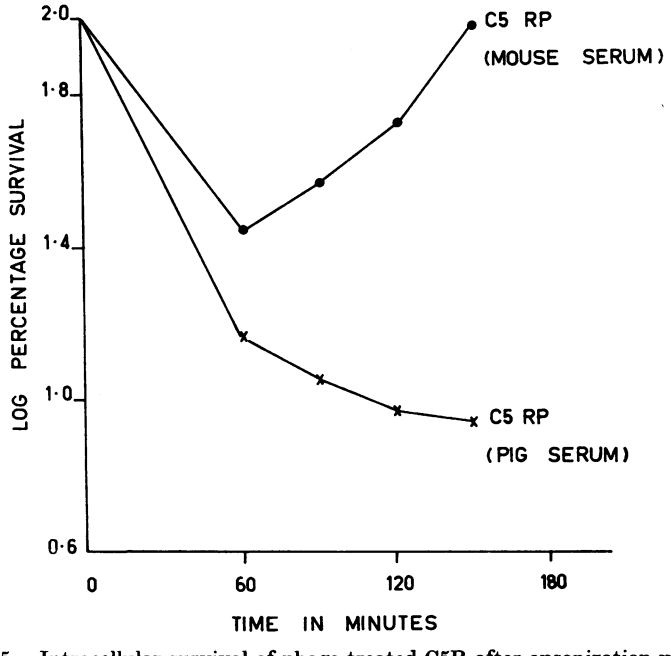

FIG. 5.—Intracellular survival of phage treated C5R after opsonization with mouse serum and pig serum.

TABLE IV.—*The Phagocytosis of C5R (Before and After Contact with Phage P22) by Mouse Peritoneal Macrophages in Tissue Culture, and the Intracellular Survival of the Bacteria Calculated at the end of the 60 min. Period allowed for Phagocytosis to take place. Mouse Serum as a Source of Opsonins was Subjected to Various Treatments as Indicated*

	C5R no phage		C5R + phage (C5RP)	
	Per cent P	Per cent S	Per cent P	Per cent S
Mouse serum	12	59	30	40
Mouse serum absorbed C5R	4	100	16	100
*Mouse serum absorbed C5RP	3	100	4	100

P = Phagocytosis. S = Survival.
* Similar results obtained in the absence of serum opsonins.

TABLE V.—*The Phagocytosis of M206R (Before and After Contact with Phage P22) by Mouse Peritoneal Macrophages in Tissue Culture, and the Intracellular Survival of the Bacteria Calculated at the end of the 60 min. Period allowed for Phagocytosis to take place. Mouse Serum as a source of Opsonins was Subjected to Various Treatments as indicated*

	M206R no phage		M206R + phage (M206RP)	
	Per cent P	Per cent S	Per cent P	Per cent S
Mouse serum	33	10	30	6
Mouse serum absorbed M206R	3	100	29	100
*Mouse serum absorbed M206RP	4	100	5	100

P = Phagocytosis. S = Survival.
* Similar results obtained in the absence of serum opsonins.

particularly well in experiments with the avirulent strain of *Salm. typhimurium* M206R, which if opsonized with normal mouse serum is killed once ingested. However, if the same bacteria treated with phage P22 are phagocytosed in the absence of serum factors for the bacteria they survive and persist intracellularly without any apparent reduction in number. This occurs even though the rates of phagocytosis between the two experiments are comparable.

DISCUSSION

Shortly after d'Herelle discovered bacteriophage, reports appeared regarding the influence of phage on the phagocytosis of bacteria by polymorphonuclear cells. In general, these papers are difficult to interpret since good quantitative data are lacking. However, one paper in particular is of interest (Smith, 1928). This report described the phagocytosis of staphylococci before and after contact with bacteriophage. Using Wright's method of quantitating the reaction between cell and bacterium the observed differences in the rates of phagocytosis were so striking that they seemed worthy of further experimentation, since they suggested that phage might prove a useful tool to explore certain problems concerned with the intracellular survival of bacteria under different conditions of opsonization. In general, this phenomenon has been confirmed using strains of *Salm. typhimurium* though our results are at variance in some other aspects since no increase

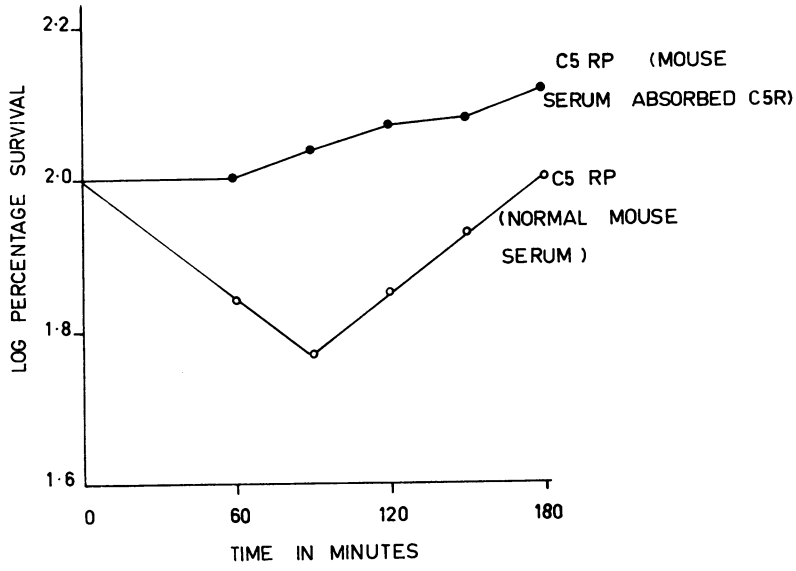

FIG. 6.—Intracellular survival of phage treated C5R after opsonization with mouse serum, and mouse serum that has been absorbed with C5R.

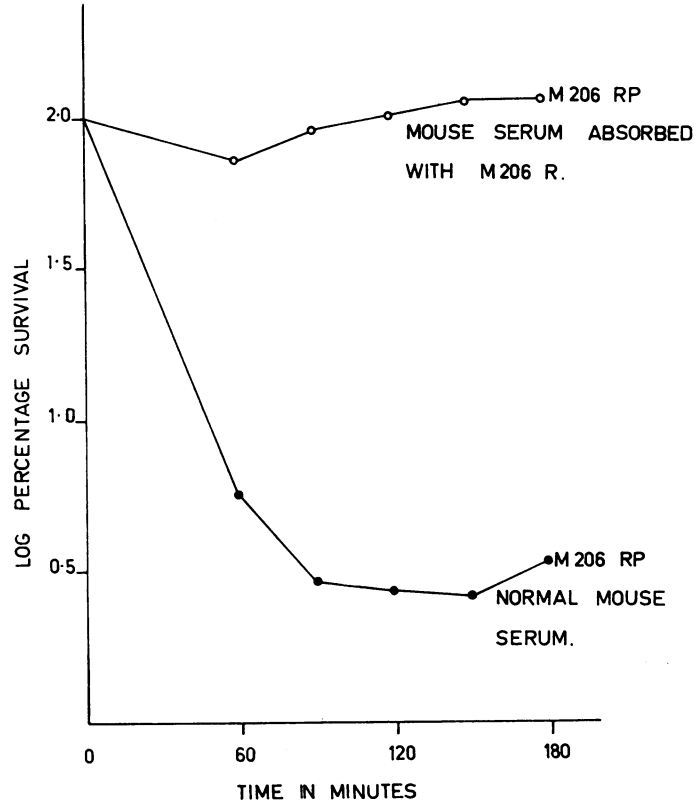

FIG. 7.—Intracellular survival of phage treated M206R after opsonization with normal mouse serum and mouse serum that has been absorbed with M206R.

in the rates of phagocytosis following prolonged incubation of the cells with phage was observed. It is visualised that phage plays an entirely passive role in the reaction between the bacterium and the phagocytic cell. That this is probably so is suggested by the findings that mouse serum that has been absorbed with bacteria is still capable of promoting phagocytosis providing the bacterium has first been contacted with phage. If, however, the serum is absorbed with phage-coated bacteria, then its phagocytic promoting properties are lost. In the absence of any serum factors few bacteria are actually associated with the phagocytic cells. The number of bacteria associated with the cells is not increased in the presence of phage. Thus the increased rates of phagocytosis observed cannot be due to phage particles by themselves, increasing the probability of the bacteria adhering to the phagocytic cells once contact between cell and bacterium has been made. The reaction is illustrated schematically as follows (Fig. 8).

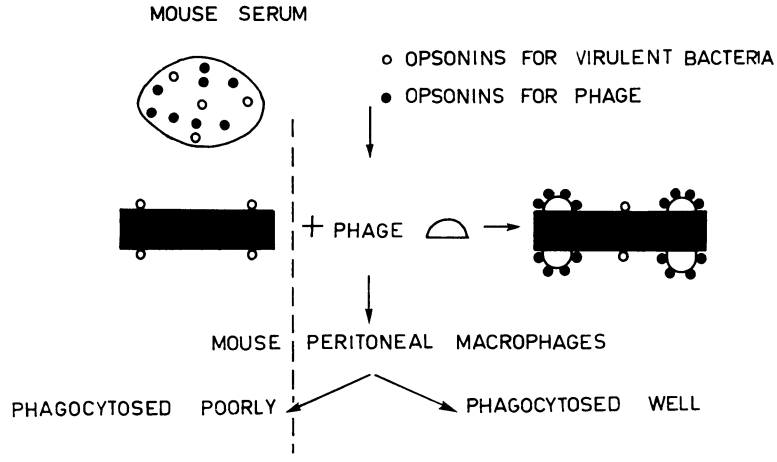

FIG. 8.—Diagram of the reaction between phage, opsonins and bacteria.

It should be emphasised that the increased rates of phagocytosis were observed only when using a serum in which opsonins specific for the strain were lacking or in low titre.

By using phage it has been possible to compare in some quantitative manner the effect of serum opsonins on the intracellular survival of strains of *Salm. typhimurium*. These results confirm our earlier conclusions and show that the intracellular fate of the bacteria is not wholly determined by differences in the rates of phagocytosis. It is apparent that opsonins affect in some, as yet undetermined manner, the survival of the ingested bacteria. This point is strikingly revealed if one considers the results obtained using the avirulent strain of *Salm. typhimurium* M206R. Earlier work had shown that mouse serum is capable of promoting rapid phagocytosis of these bacteria, the greater proportion of the ingested bacteria being killed (Jenkin and Benacerraf, 1960). If these opsonins are removed by absorption the serum will still promote phagocytosis of phage-coated bacteria to a degree comparable with unabsorbed serum, but in this instance the ingested bacteria survive. It is of interest to note that even in the

absence of opsonins, a small percentage of the added bacteria are found associated with the macrophages. Since these cell-associated bacteria multiply without evidence of any killing having taken place, even in the presence of streptomycin in the extracellular medium, it is reasonable to assume that the bacteria are in fact intracellular.

These observations are similar to the early findings of Rowley (1958) who showed that mouse peritoneal macrophages which had taken up small numbers of *Escherichia coli* in the absence of serum factors were unable to kill the ingested bacteria which multiplied intracellularly. However, if the same bacteria were treated with horse serum, rapid phagocytosis took place and the ingested bacteria were killed.

The above findings emphasise the importance of studying host-parasite relationship with cells and serum obtained from the susceptible animal. Many previous studies concerned with the relationship have used cells and serum from species of animals which may be more resistant to infection by the parasite than the normal host. In some cases the cells have been obtained from one species of animal whilst the serum from another. Under these conditions whilst the results obtained may be a true reflection of what is happening in that particular system, completely fallacious conclusions may be reached in attempting to relate these observations to what occurs in the susceptible host.

SUMMARY

Using phage it has been possible to study the phagocytosis of bacteria by mouse peritoneal macrophages in the absence of specific serum opsonins. A quantitative comparison has been made of the intracellular survival of bacteria phagocytosed in the presence and absence of these serum factors. In the absence of serum opsonins bacteria that are ingested by mouse peritoneal macrophages survive and multiply intracellularly.

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