THE INFLUENCE OF ERYTHROPHAGOCYTOSIS ON THE INTERACTION OF MACROPHAGES AND SALMONELLA IN VITRO*

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Previous studies have shown that mice with hemolysis are more susceptible to salmonella infection than normal mice. Administration of anti-mouse-erythrocyte serum, phenylhydrazine hydrochloride, antibody-coated mouse erythrocytes, or heterologous erythrocytes results in a marked increase in susceptibility of mice to infection with *Salmonella typhimurium* (1). Susceptibility is not altered by injection of normal mouse erythrocytes, hemoglobin solution, or stroma from homologous or heterologous erythrocytes. NZB/Bl mice with naturally occurring autoimmune hemolytic anemia were also shown to be more susceptible to *S. typhimurium* infection than NZB/Bl mice without hemolysis (2). Following clearance of salmonella from the blood, organisms multiply more rapidly and to a higher concentration in the livers and spleens of mice with hemolysis than in control mice (3). It was postulated that phagocytosis of erythrocytes by cells of the reticuloendothelial system alters the capacity of these cells to kill salmonella (3).

The present studies were undertaken to evaluate the effect of erythrophagocytosis by macrophages on the interaction of macrophages and salmonella in vitro.

Materials and Methods

Macrophages and Erythrocytes.—Male Swiss mice (CF 1 strain, Carworth Farms, New City, New York) served as the source of peritoneal macrophages and mouse erythrocytes. Macrophages were obtained by washing out the peritoneal cavities of 15 g mice with Eagle's basal medium (EM) as previously described (4). *Petri dish cultures* were prepared from 100 ml of pooled peritoneal aspirates. The cells were sedimented by centrifugation at 500 *g* for 5 min at 4°C and resuspended in 10 ml of EM at a concentration of about 1×10^6 cells per ml; 0.9 ml aliquots of the suspension were placed in 35 × 10 mm plastic Petri dishes (Falcon

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Plastics, Los Angeles) containing 0.1 ml of heat-inactivated normal rabbit serum (NRS). *Cover slip cultures* were prepared by layering 0.4 ml aliquots of the peritoneal aspirate from one mouse on sterile glass cover slips in plastic Petri dishes. Each 0.4 ml aliquot contained about 2×10^4 cells. The cover slips had been washed in Microsolv detergent (Microbiological Associates, Inc., Bethesda, Maryland) and rinsed 12 times in tap water and 4 times in distilled water before being autoclaved. Petri dish and cover slip cultures were incubated at 37°C in humid 5% CO_2 in air for 60 min to allow sedimentation and surface adherence of cells.

Mouse erythrocytes were obtained from 20 to 24 g etherized mice by aspirating blood directly from the heart into a heparinized syringe. The blood was centrifuged at 1500 *g* for 15 min at 4°C and the sedimented erythrocytes were washed twice in saline and resuspended in saline to give a final concentration of 50%.

Bacteria.—*S. typhimurium*, strain KK (5), was used in all studies. Stock cultures were maintained by storing at −20°C 1 ml aliquots of an 18 hr culture in trypticase soy broth (TSB). Bacterial inocula were prepared by adding 1 ml of stock to 9 ml of TSB and incubating for 18 hr at 37°C.

Anti-Mouse-Erythrocyte Serum (AES).—Rabbits were injected intraperitoneally once a week for 4 successive wk with a 50% suspension of thrice-washed mouse erythrocytes in saline. Serum obtained during the 5th wk agglutinated a 1% suspension of mouse erythrocytes at a dilution of 1:5120. *Antibody-coated erythrocytes* were prepared by incubating 2 ml of packed mouse erythrocytes with 0.5 ml of AES for 30 min. The erythrocytes were then washed twice in saline and once in EM prior to use. There was no clumping of erythrocytes in the final suspension.

Antisalmonella Serum (*AKK*).—Confluent colonies of strain KK on the surfaces of trypticase soy agar plates were washed off with saline. The bacteria were then washed three times in saline and resuspended in 10 ml of 70% ethyl alcohol at a final concentration of 7×10^{10} bacteria per ml. After 1 hr at room temperature and 18 hr at 4°C, the organisms were again washed three times and suspended in 10 ml of saline (salmonella vaccine). Broth cultures prepared from this suspension were sterile. Rabbits were inoculated intravenously with 0.001 ml of the salmonella vaccine on day 1, 0.01 ml on day 3, and 0.1 ml on days 5, 8, 12, 15, and 18. Serum obtained from heart blood 14 days after the last injection agglutinated a 5% suspension of the salmonella vaccine at a titer of 1:2560.

Phagocytosis and Determination of Surviving Bacteria in Petri Dish Cultures.—The *S. typhimurium* in 10 ml of an 18 hr TSB culture were washed in saline and diluted to 10^{-6} in EM. The final dilution contained 5% heat-inactivated AKK to opsonize the bacterial inoculum. There was no agglutination or killing of bacteria at these concentrations. The number of viable units in the inoculum was determined by spreading 0.1 ml aliquots on trypticase soy agar plates and counting the number of colonies appearing after incubation for 18 hr. The supernates were removed from Petri dish cultures containing 10^6 macrophages and 0.5 ml aliquots of the bacterial inoculum were added to these dishes and to control dishes without macrophages. The number of viable organisms used in each experiment was constant but in different experiments varied from 100 to 500. The dishes were then incubated at 37°C in humid 5% CO_2 in air. The supernates were removed from the dishes at varying times after adding the bacteria, and 0.1 ml aliquots were spread on trypticase soy agar plates. 2 ml of melted agar was then added to the dishes from which the supernates had been removed. The dishes and the agar plates inoculated with supernates were incubated at 37°C for 18 hr. The number of surviving extracellular bacteria was determined by counting the number of colonies appearing on the plates prepared from the supernates. The number of surviving bacteria associated with cells was determined by counting the number of colonies appearing in dishes

from which the supernates had been removed and trypticase soy agar added. Total bacterial counts in each dish were determined by adding the extracellular and cell-associated values.

Fig. 1 shows the total, extracellular, and cell-associated bacterial counts expressed as per cent of inoculum in preliminary experiments designed to demonstrate phagocytosis and killing of salmonella by macrophages. About 100% of the inoculum was recovered immediately after adding the bacteria (Fig. 1 A), demonstrating that all the viable bacteria in the inoculum could be recovered by this method. In controls without macrophages the total bacterial count remained stable for 60 min before multiplication occurred. In preparations containing macrophages the total bacterial count decreased for 90 min and then increased. Supernate counts decreased in both control and macrophage preparations (Fig. 1 B). However, there was a greater and more prolonged decrease in counts in the supernates of the macrophage preparations, presumably reflecting phagocytosis of bacteria. In control preparations the loss of bacteria from the supernates was reflected by a progressive increase in the number of organisms on the surfaces of control dishes (Fig. 1 C). Killing of bacteria by macrophages was indicated by the persistently small number of viable bacteria on the surfaces of dishes containing macrophages.

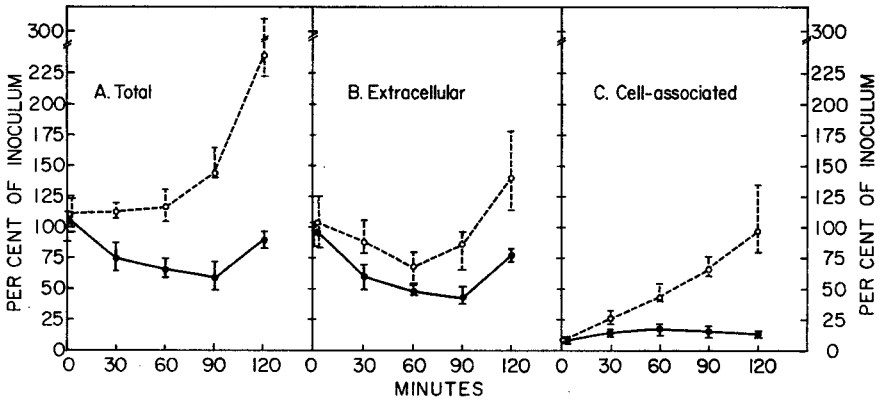

FIG. 1. Total (A), extracellular (B), and cell-associated (C) bacterial counts from Petri dish cultures of macrophages (●——●), and control dishes without macrophages (O-----O). The average and range of four experiments are shown.

In experiments testing the effect of erythrophagocytosis, extracellular and cell-associated counts were performed 30 and 60 min after the addition of bacteria since during this time phagocytosis and killing of bacteria could be measured in the absence of extracellular multiplication. The results of these experiments are expressed as *per cent phagocytosis* and *per cent of phagocytized bacteria killed*. The methods utilized in calculating these values are demonstrated in Table I which shows data from a representative experiment.

Although the assumptions behind these calculations do not account for possible adherence of bacteria to macrophages without phagocytosis, the fact that most of the bacteria removed from the supernate were not recovered by dish counts indicates that the bacteria were killed and therefore were almost certainly phagocytized. The extremely low bacteria to cell ratios (usually less than 1:1000) makes it highly unlikely that more than one colony forming unit was associated with one macrophage. There was less than a 5% difference in counts obtained

at 30 and 60 min in all experiments. The values shown in the tables in the section on Results represent averages of the 30 and 60 min counts.

Degradation of Salmonella by Macrophages on Cover Slips.—AKK with an agglutinin titer of 1:320 was conjugated with fluorescein isothiocyanate by Microbiological Associates, Inc. The bacterial sediment from 5 ml of an 18 hr KK culture was washed with saline and incubated with 0.5 ml of a 1:2 dilution of fluorescein-conjugated AKK (FAKK) for 30 min at 37°C. The coated bacteria were then washed three times in cold EM and resuspended in 15 ml of EM. There was no agglutination of bacteria in the final suspension. 3 ml of the suspension of FAKK-coated bacteria (about 1×10^7 viable KK) were added to each cover slip culture of macrophages. After incubation at 37°C in humid 5% CO_2 in air for 60 min, the cover slips were washed with EM to remove extracellular bacteria, placed in fresh EM and reincubated. At varying times after washing, cover slips were exposed for 15 min to 1%

TABLE I

*Method of Calculating Per Cent Phagocytosis and Per Cent of Phagocytized Bacteria Killed**

	Time, min		
	0	30	60
No. of bacteria in inoculum (I) 455			
No. of bacteria in supernate (S)	385	190	185
No. of bacteria in dish (D)	45	57	65
No. of bacteria phagocytized (P):			
$P = I - S$			
$P = 455 - 190$‡	70	265	270
Per cent phagocytosis:			
$P/I \times 100$			
$265/455 \times 100$‡	15	58	59
Per cent of phagocytized bacteria killed:			
$(P - D)/P \times 100$			
$(265 - 57)/265 \times 100$‡	36	78	76

* Each value is the average of duplicate determinations.

‡ Calculations shown are those for 30-min values.

trypan blue in saline, washed in saline, fixed in acetone for 7 min, and air dried. The cover slips were then rinsed in increasingly dilute concentrations of acetone in water, air dried, and mounted in buffered glycerol (pH 8.4) for phase-contrast and ultraviolet microscopy.

The rate of intracellular degradation of KK was measured by determining the rate of loss of fluorescence from intracellular bacteria by a method previously described (4). One hundred macrophages which contained bacteria and which were not stained with trypan blue were counted on each cover slip. The per cent of these macrophages which contained fluorescent bacteria and the per cent containing nonfluorescent bacteria was determined. Results were expressed as per cent of macrophages containing fluorescent bacteria at the time intervals studied. The large bacteria to cell ratio (500:1) resulted in phagocytosis of multiple bacteria by most macrophages.

RESULTS

Influence of Antibody-Coated Homologous Erythrocytes on Phagocytosis and Killing of S. typhimurium by Macrophages.—

Three techniques were used to determine whether phagocytosis of antibody-coated erythrocytes by macrophages altered the capacity of these cells to phagocytize and kill *S. typhimurium*.

Method 1 (*RBC before bacteria*): Petri dish cultures of macrophages were exposed to 1 ml of a 25% suspension of antibody-coated mouse erythrocytes for 45 min and then washed before the addition of bacteria. This resulted in phagocytosis of 1 to 10 erythrocytes by 85% of the macrophages.

Method 2 (*RBC with bacteria*): The bacterial inoculum was mixed with antibody-coated mouse erythrocytes and added to Petri dish cultures of macrophages which had not been previously exposed to erythrocytes. The final concentration of erythrocytes in the mixture was 25%. This technique resulted in the simultaneous exposure of bacteria and opsonized erythrocytes to normal macrophages.

Method 3 (*RBC before and with bacteria*): Petri dish cultures of macrophages were exposed to erythrocytes as in method 1. A mixture of bacteria and erythrocytes was then added to the dishes as in method 2. This technique resulted in the simultaneous exposure of opsonized erythrocytes and bacteria to macrophages which had previously ingested erythrocytes.

Controls in each experiment included identical macrophage cultures which were exposed to bacteria in the absence of erythrocytes and dishes with bacteria and erythrocytes but without macrophages. Nuclear exclusion of trypan blue was used as an indication of viability of macrophages.

TABLE II

*Influence of Antibody-Coated Homologous Erythrocytes on Phagocytosis and Killing of S. typhimurium by Macrophages**

Experiment No.	Phagocytosis of salmonella by macrophages exposed to				Phagocytized bacteria killed by macrophages exposed to			
	No RBC	RBC before bacteria	RBC with bacteria	RBC before and with bacteria	No RBC	RBC before bacteria	RBC with bacteria	RBC before and with bacteria
	%	%	%	%	%	%	%	%
1	58	59	—	—	70	75	—	—
2	91	91	13	—	99	92	1	—
3	50	—	4	17	69	—	0	0
4	72	—	—	28	92	—	—	48

* Each value is the average calculated from duplicate bacterial counts at 30 and 60 min after the addition of bacteria. The effect of erythrocytes in each experiment can be evaluated by comparison with values obtained in the absence of erythrocytes.

Table II summarizes the results of these experiments. There was marked inhibition of phagocytosis and killing of bacteria when bacteria and opsonized erythrocytes were simultaneously exposed to macrophages (experiments 2, 3,

and 4). Phagocytosis of erythrocytes by macrophages prior to exposure to bacteria did not influence subsequent phagocytosis and killing of the bacteria (experiments 1 and 2). The viability of macrophages was not affected by the presence of erythrocytes and erythrocytes had no effect on the multiplication of bacteria in the absence of macrophages.

Influence of Antibody-Coated and Uncoated Intact or Disrupted Erythrocytes.—

TABLE III

*Comparison of the Influence of Antibody-Coated or Uncoated Intact and Disrupted Erythrocytes on Phagocytosis and Killing of S. typhimurium by Macrophages**

Macrophages exposed to	Phagocytosis of salmonella by macrophages	Phagocytized bacteria killed by macrophages
	%	%
No RBC	67	80
Intact, coated RBC	26	40
Disrupted, coated RBC	54	74
Intact, uncoated RBC	59	73
Disrupted, uncoated RBC	52	70

* Each value is the average calculated from duplicate bacterial counts at 30 and 60 min after the addition of bacteria.

TABLE IV

*Influence of Heterologous Erythrocytes on Phagocytosis and Killing of S. typhimurium by Macrophages**

Experiment No.	Phagocytosis of salmonella by macrophages exposed to				Phagocytized bacteria killed by macrophages exposed to			
	No RBC	RBC before bacteria	RBC with bacteria	RBC before and with bacteria	No RBC	RBC before bacteria	RBC with bacteria	RBC before and with bacteria
	%	%	%	%	%	%	%	%
1	57	42	11	—	79	61	2	—
2	37	21	—	2	66	57	—	0
3	49	—	10	7	87	—	37	27

* Each value is the average calculated from duplicate bacterial counts at 30 and 60 min after the addition of bacteria.

The following suspensions were prepared from mouse erythrocytes: (*a*) intact antibody-coated erythrocytes in EM; (*b*) intact uncoated erythrocytes in EM; (*c*) disrupted antibody-coated erythrocytes prepared by freezing and thawing a 25% suspension of coated erythrocytes ten times; (*d*) disrupted uncoated erythrocytes prepared by freezing and thawing a 25% suspension of uncoated erythrocytes ten times. Macrophage Petri dish cultures were exposed to one of the suspensions for 45 min, washed, and then exposed to a mixture of the same suspension and bacteria. The final concentration of intact or disrupted erythrocytes in each mixture was 25%. Control cultures were exposed to bacteria in the absence of erythrocytes.

Table III summarizes the results of these experiments. There was no appreciable inhibition of phagocytosis or killing of bacteria in the presence of intact or disrupted uncoated or disrupted antibody-coated erythrocytes as compared to the marked inhibitory effect of intact antibody-coated erythrocytes.

Influence of Heterologous Erythrocytes.—The effect of washed human erythrocytes on the phagocytosis and killing of bacteria by mouse macrophage cultures was tested by methods identical to those utilized for measuring the effect of antibody-coated mouse erythrocytes. The results of these experiments are shown in Table IV. Phagocytosis and killing of bacteria was markedly inhibited when the bacteria and human erythrocytes were simultaneously exposed to

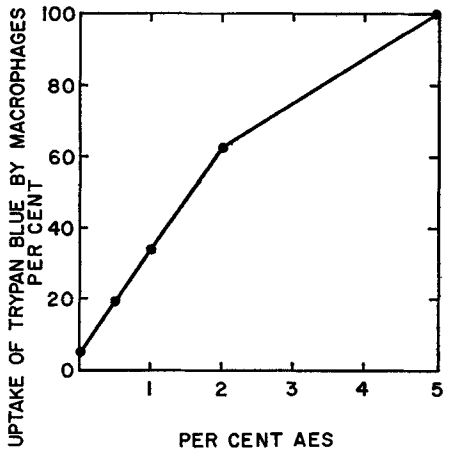

FIG. 2. Effect of varying concentrations of anti-mouse-erythrocyte serum (AES) on uptake of trypan blue by mouse macrophages.

macrophages. Human erythrocytes had no effect on the viability of macrophages or the multiplication of bacteria.

Effect of Anti-Mouse-Erythrocyte Serum on Macrophages.—The possibility that anti-mouse-erythrocyte serum might be toxic for mouse macrophages was suggested by the observation of Quie and Hirsch (6) that anti-rabbit-erythrocyte serum is toxic for rabbit polymorphonuclear leukocytes. To test the effect of AES on the viability of macrophages, cover slip cultures of macrophages were incubated for 90 min in varying concentrations of AES in NRS. The cover slips were then washed with saline, exposed to 1% trypan blue for 15 min at 37°C, again washed in saline, and examined by phase microscopy for nuclear exclusion of trypan blue.

The results of this experiment indicate that AES does exert a deleterious effect

on mouse macrophages (Fig. 2). The per cent of macrophages killed was directly proportional to the amount of AES present in the medium.

Intracellular Degradation of S. typhimurium by Macrophages.—

In order to evaluate the influence of erythrophagocytosis on degradation of bacteria by macrophages, cover slip cultures of macrophages which contained intracellular *S. typhimurium* coated with fluorescein-labeled antibody were prepared and observed as described in Materials and Methods. The rate of loss of fluorescence from the salmonella in normal macrophages

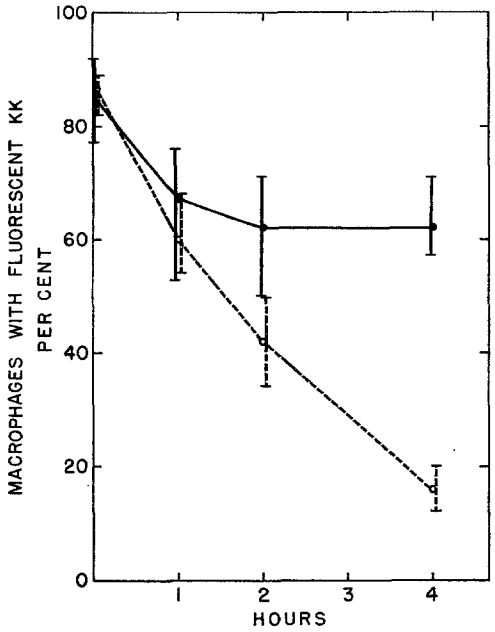

FIG. 3. Rate of loss of fluorescence from fluorescent-antibody–coated salmonella in normal macrophages (O——O), and macrophages which have previously phagocytized erythrocytes (●——●). The average and range of three experiments are shown.

was determined in three experiments. A progressive decrease in fluorescence occurred; after 4 hr of observation less than 20% of the macrophages contained fluorescent intracellular bacteria.

In other experiments cover slip cultures were exposed to a 25% suspension of antibody-coated mouse erythrocytes for 45 min, and then washed with EM before the addition of fluorescent-antibody–coated *S. typhimurium*. In each of these experiments the fluorescent-antibody–coated *S. typhimurium* were also added to control cover slip cultures not exposed to erythrocytes. In each experiment control and experimental cultures contained the same number of macrophages obtained from one mouse. The rate of loss of fluorescence from intracellular bacteria in these experiments is shown in Fig. 3.

Immediately after washing off extracellular bacteria 85% of erythrocyte-treated macrophages and 85% of control macrophages contained fluorescent

bacteria. 1 hr later about 65% of both types of macrophages contained fluorescent bacteria. However, at subsequent intervals the proportion of control macrophages containing fluorescent bacteria progressively decreased while there was no further decrease in the proportion of erythrocyte-treated macrophages containing fluorescent bacteria. By 4 hr, less than 20% of control macrophages contained fluorescent bacteria compared to more than 60% of erythrocyte-treated macrophages.

DISCUSSION

Kaye and Hook (3) observed that mice with hemolytic anemia are more susceptible to salmonella infection than normal mice and suggested that phagocytosis of erythrocytes by cells of the reticuloendothelial system alters the capacity of these cells to kill salmonella. The present studies support and extend this hypothesis by demonstrating that erythrophagocytosis in vitro inhibits phagocytic, bactericidal, and degradative activities of macrophages.

Inhibition of phagocytosis could be demonstrated during simultaneous exposure of bacteria and intact heterologous or opsonized homologous erythrocytes to macrophages. However, no inhibition of phagocytosis was observed when bacteria were exposed to macrophages which had ingested erythrocytes prior to exposure to bacteria. One explanation of these findings is that phagocytosis of bacteria is inhibited by competition between erythrocytes and bacteria for phagocytic sites on macrophages. Competition between two particles for phagocytic sites has also been suggested by the recent studies of Normann and Benditt (7). These investigators showed that simultaneous intravenous administration of carbon and aggregated albumin or heterologous erythrocytes decreased the rate of clearance of carbon from the blood of rats.

Inhibition of killing of bacteria was also demonstrated during simultaneous exposure of bacteria and heterologous or antibody-coated homologous erythrocytes to macrophages. However, macrophages which had previously ingested erythrocytes demonstrated normal bactericidal activity on subsequent challenge with bacteria. These observations suggest that the mechanisms responsible for the bactericidal action of macrophages may be temporarily overloaded or depleted by ingestion of erythrocytes, but that recovery is rapid. It is of interest to note that increased susceptibility of mice to salmonella infection occurs when bacteria and opsonized erythrocytes are simultaneously exposed to cells of the reticuloendothelial system (3).

The observation that phagocytic and bactericidal activity of macrophages is normal *after* phagocytosis of erythrocytes is consistent with studies of polymorphonuclear leukocytes (8, 9) in which prior particle ingestion did not inhibit subsequent phagocytosis and killing of bacteria. Louria and Brayton (8) observed no difference in phagocytosis of staphylococci by normal human polymorphonuclear leukocytes and leukocytes which had previously ingested *Candida* unless the leukocytes showed evidence of damage by pseudomycelia.

Cohn and Morse (9) showed that rabbit polymorphonuclear leukocytes which have phagocytized heat-killed bacteria have normal or enhanced phagocytic and bactericidal activity on subsequent exposure to live bacteria.

Gill and Cole (4) have shown that there is a progressive loss of fluorescence from fluorescent-antibody–coated streptococci after phagocytosis by macrophages. This observation has been interpreted as an indication of intracellular degradation of the bacterial cell wall. In the present study the rate of loss of fluorescence from fluorescent-antibody–coated *S. typhimurium* in normal macrophages was similar to the rate of loss of fluorescence previously reported for streptococci. However, the rate of loss of fluorescence from *S. typhimurium* in macrophages which had previously ingested erythrocytes was decreased. These results suggest that intracellular mechanisms responsible for degradation of bacteria are inhibited by prior phagocytosis of erythrocytes.

Macrophages which had phagocytized erythrocytes prior to exposure to bacteria had normal bactericidal activity but decreased ability to degrade labeled bacteria. Although different mechanisms may be responsible for degradation and killing of bacteria, the differences observed are more likely related to the techniques that were used to study the two functions. Studies of bactericidal activity of macrophages were continued for only 1 hr whereas observations on degradative capacity were extended over a period of 4 hr. In addition, a much higher ratio of bacteria to macrophages was used for studies of bacterial degradation than for studies of bacterial killing.

Quie and Hirsch (6) reported that anti-rabbit–erythrocyte serum is toxic for rabbit polymorphonuclear leukocytes. In the present study anti-mouse–erythrocyte serum was shown to be lethal for mouse macrophages. However, the observed effects of erythrophagocytosis on macrophage function cannot be explained by the lethal effect of anti-mouse–erythrocyte serum. Although phagocytic, bactericidal, and degradative activities of macrophages were inhibited by phagocytosis of antibody-coated mouse erythrocytes, no lethal effect on macrophages could be detected. Ingestion of heterologous erythrocytes by macrophages in the absence of anti-mouse–erythrocyte serum also inhibited phagocytosis and killing of bacteria.

SUMMARY

Phagocytosis and killing of *Salmonella typhimurium* by mouse peritoneal macrophages was inhibited when the bacteria and antibody-coated homologous erythrocytes or heterologous erythrocytes were simultaneously exposed to macrophages in vitro. No inhibition of phagocytosis or killing was observed in experiments employing uncoated or disrupted antibody-coated homologous erythrocytes.

Degradation of *S. typhimurium* as measured by the loss of fluorescence from intracellular salmonella coated with fluorescein-labeled antibody was inhibited

in macrophages which had previously ingested antibody-coated homologous erythrocytes.

Anti-mouse-erythrocyte serum was found to have a cytotoxic action on mouse macrophages. However, the viability of macrophages was not altered by phagocytosis of antibody-coated homologous erythrocytes or uncoated heterologous erythrocytes.

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