THE EFFECT OF ANTIBODY ON INTRACELLULAR PARASITISM OF SALMONELLA TYPHIMURIUM IN MONONUCLEAR PHAGOCYTES IN VITRO

Prolonged Survival of Infected Monocytes in Presence of Antibody*

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During *Salmonella* infections the phagocytic cells within lymphoid tissue of the intestine and in other parts of the RES[1] harbor the infectious agents during incubation and septicemia (1). *In vitro* studies have demonstrated that such *intracellular* bacilli are protected from the action of circulating antibody (2). Contrariwise, the effect of antibody upon *extracellular Salmonella* remains debatable in view of evidence obtained from *in vivo* or *in vitro* experiments (3–7). Therefore, the relationship between circulating antibody and acquired immunity still requires test, although more recent observations on specific tolerance to the somatic *Salmonella* antigen have suggested a protective role of antibody (8).

In this paper experiments are reported that demonstrated an effect of humoral antibody upon the interaction between *Salmonella typhimurium* and mononuclear phagocytes *in vitro*. It has been found recently that successful facultative intracellular parasitism of *Salmonella* depends on the virulence of the strain used for the experiment (9). Thus, by choosing an organism which is able to survive and multiply within phagocytes *in vitro*, one can specifically study effect and site of action of antibody on both extra- and intracellular *Salmonellae in vitro* while controlling other antibacterial mechanisms that may complicate animal experimentation (10, 11). Evidence is presented suggesting a synergism between humoral antibody and intracellular environment resulting in a more stabilized interaction between MN[1] and *S. typhimurium*. A popu-

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[1] The following abbreviations are used throughout the paper:—RES = reticulo-endothelial system; MN = mononuclear phagocytes; SM = standard medium; RGG = rabbit gamma globulin.

lation of mononuclear phagocytes from normal rabbits is rapidly destroyed *in vitro* when infected with a virulent strain of *S. typhimurium*. In presence of antibody the phagocytized organisms lose much of their destructive effect upon the cells while retaining their ability to multiply intra- and extracellularly.

Materials and Methods

Animals.—Male and female New Zealand albino rabbits, weighing between 2 and 3 kg., were obtained from a local rabbit farm and fed with pellets (Brownlee Feed and Seed Co., Gainesville) and water *ad lib.*

Mononuclear Phagocytes.—Suspensions rich in MN were obtained according to the technique described in an earlier paper (12). Rabbits were injected intraperitoneally with 30 ml. of a 12 per cent solution of sodium caseinate in saline at pH 7 (soluble purified casein from the Fisher Scientific Company, New York). Five days later the animals were killed by bleeding from the heart; the peritoneal cavity was opened aseptically and washed with 50 ml. physiological saline containing 0.005 per cent heparin (heparin sodium, U.S.P. 1 gm = 100,000 units, Fisher Scientific Company). The diluted exudate was then collected through a stainless steel tube with a sieve-like end. This cell suspension was centrifuged at 900 to 1000 R.P.M. for 9 to 10 minutes and resuspended in the standard medium described below. The number of cells was determined in the hemocytometer (AO Spencer chamber). The viability of the cells was estimated by use of the trypan blue test (12).

Bacteria.—The following strains of *Salmonella* were used: A mouse virulent strain of *Salmonella typhimurium* (2,4,5,12: i-1,2,3,5), No. 176 (13), obtained from the stock collection of the Department of Microbiology, Emory University School of Medicine, Atlanta; a strain of *Salmonella aberdeen* (11: i-1,2) and *Salmonella abony* (1,4,5,12: b-e,n,x) (both received through the courtesy of Dr. P. R. Edwards, Enteric Bacteriologic Unit of the Communicable Disease Center, Public Health Service, Chamblee, Georgia). The organisms were maintained by weekly transfers on blood-agar-base (Difco) plates and were cultivated for each experiment at 37°C. for 8 hours in brain-heart-infusion (Difco) with an initial pH of 7.8. The cultures were then diluted in SM[1] to give the desired inoculum for the experiment. If heat-killed bacilli were used, the suspended organisms were placed in a 66°–70°C. waterbath for 20 minutes.

The number of bacteria in the culture was determined by plating a standard amount of serial 10-fold saline dilutions into nutrient agar (BBL) and was estimated by reading the optical density in a "spectronic 20" at $\lambda = 625$ mμ.

Experimental Media.—If not stated otherwise, the experiments were performed in SM consisting of 4 parts Krebs-Ringer carbonate buffer (14) and 1 part heat-inactivated homologous serum. Glucose was added to give a final concentration of 0.1 per cent. The initial pH was approximately 7.8 with phenol red 1:50,000 as indicator.

Sera from several rabbits were pooled, passed through a Seitz filter and inactivated at 56°C. for 30 minutes. They were stored at −5°C. If complement was required, fresh rabbit serum or commercially available guinea pig complement (Hyland Laboratories, Los Angeles) was added.

Unless stated otherwise, pooled rabbit immune serum with an agglutination titer of 1:2560 was used for all experiments with antibody. The serum was added to the SM in a final concentration of 1:10 or 1:30. An equivalent amount of normal serum was present in all control tubes.

Immunization of Rabbits.—Washed saline suspensions of *S. typhimurium* derived from 8 hour broth cultures were injected intravenously three times weekly on alternate days over a period of 3 to 4 weeks. During the first week 50 to 100 million heat-killed organisms were injected three times, whereas for all subsequent injections similar numbers of living bacilli

were used. The immunized animals were bled 5 days after the last injection. The immune sera were inactivated at 56°C. for 30 minutes and stored at −5°C.

Fractionation and Characterization of the Immune Sera.—Gamma globulin was prepared according to Kendall's method by fractionation of immune serum with ammonium sulfate (15). The protein concentration of sera and globulin fractions was estimated colorimetrically by the method of Folin-Ciocalteu (15). Agglutinin titrations were performed on whole serum and globulin fractions using a standard macroscopic method. The antisomatic antibody was removed by precipitating the antiserum with purified lipopolysaccharide derived from *S. typhimurium* according to the technique described by Westphal *et al.* (16).

Infection of MN and Intracellular Multiplication.—Since these studies were concerned with the outcome of the interaction between the pathogen and the MN after phagocytosis and not with the process of phagocytosis, it was important to obtain uniformly infected MN regardless of whether antibody was present or not during phagocytosis. The experiments were done in the following two steps.

1. Approximately equal numbers of MN and *S. typhimurium* suspended in SM with or without antibody were mixed in screw-capped tubes (16 × 125 mm.) which were incubated at 37°C. for 45 minutes in a horizontal position. The cells were then scraped off the glass with wooden applicators, centrifuged (900 R.P.M. for 10 minutes) and washed twice in SM to remove most free bacteria and residual antibody. The cells were finally resuspended in SM and either incubated in the same tubes or transferred to special flasks.

2. The infected MN were incubated at 37°C. for a period from 3 to 8 hours. The fate of *S. typhimurium* and MN was followed during the period of incubation.

(*a*) *Determination of viable units:* The increase in the total number of viable units of intra- and extracellular bacteria was determined by removing replicate tubes, resuspending the cells in the culture fluid, and plating out serial dilutions. Smears were also prepared from each suspension.

(*b*) *Indirect measurement of extracellular multiplication:* Release of bacteria from the cells and subsequent multiplication was followed indirectly by measuring the rate of CO_2 fixation by the multiplying bacteria. It has been shown that the amount of $C^{14}O_2$ fixed was a direct function of extracellular multiplication even if the bacteria were clumped by agglutinins (17). The suspension of infected MN was placed in specially designed flasks which allowed generating an atmosphere of 5 volume per cent CO_2 containing 5 to 6 μc. $C^{14}O_2$ and sampling of aliquots of the suspension at intervals of time for determination of radioactivity.

(*c*) *Morphologic determination* of the fate of intracellular *S. typhimurium* was done using coverslip cultures of infected MN. The technique described for the study of intracellular multiplication of tubercle bacilli was employed (18). The infected MN were allowed to settle on small coverslips and placed in screw-capped tubes. Individual coverslips were removed at intervals of time, fixed in methanol, and stained with Giemsa. The intracellular population was determined by counting 100 MN containing bacteria and classifying them into groups of cells containing 1 to 3, 3 to 10, or more than 10 bacteria (18).

Calculation.—The average generation time of *S. typhimurium* was derived from the slope of the curves obtained when the logarithm to the base 2 of the amounts of $C^{14}O_2$ fixed was plotted against time of incubation. When initial and final viable counts were available a "multiplication factor" was determined by dividing the final by the initial count. A ratio was obtained dividing the experimental values by the control value.

Fluorescent Staining for S. typhimurium Antibody.—The fluorescent antibody technique of Coons was used for the visualization of *S. typhimurium* antibody (19, 20). Coverslip cultures prepared as described above were washed in phosphate-buffered saline (19), fixed in cooled acetone, and stored in the deep freeze at −20°C. (21). Globulin from anti-rabbit sheep serum (obtained through the courtesy of Dr. J. Vazquez, Department of Pathology, University of

Pittsburgh School of Medicine) was precipitated with ammonium sulfate. The globulin fraction (anti-RGG) was then conjugated with either fluorescein isocyanate (Sylvana Chemical Company, Orange, New Jersey) using Coons original method (19) or with lissamine rhodamine B 200 (obtained through the courtesy of Dr. F. Adler, Public Health Research Institute of New York, New York) according to the technique described by Chadwick *et al.* (22). For staining, the dry coverslip cultures were washed in buffered saline, overlayed with the conjugate, and kept in a moist chamber at 37°C. for 30 minutes. A companion set of coverslips was stained with Giemsa. After careful washing with buffered saline the coverslips were mounted in carbonate-buffered glycerol (pH 8.2) and examined with the fluorescent microscope. The specificity of the fluorescent labelled anti-RGG for the rabbit globulin was established by performing the following biological (*a*) and technical (*b*) controls:

(*a*) 1. Uninfected MN cultures treated with anti-*S. typhimurium* globulin.
2. MN cultures infected with *S. typhimurium* in *absence* of anti *S. typhimurium globulin.*

(*b*) 1. MN cultures infected with *S. typhimurium* in *presence* of anti-*S. typhimurium* globulin, but previously overlayed with *non*-fluorescent anti-RGG (blocking technique, 20).

None of the above controls showed specific fluorescence. For direct staining of the organisms anti-*Salmonella typhimurium* globulin was conjugated by the same procedure with fluorescein isocyanate.

A Wild M 20 research microscope with dark field and a Wild universal lamp (HBO 200, mercury vapor) was used. Two UG 1 and one BG 23 served as exciter filters, and a GG 4 as barrier filter, Wild Heerbrugg Ltd., Port Washington, New York.

RESULTS

Effect of Antibody on Salmonella typhimurium.—As determined by turbidity readings, the presence of antibody did not interfere with the multiplication of *S. typhimurium* in either nutrient broth or SM even though the antiserum caused clumps containing 50 to 100 organisms. When these organisms were grown in an atmosphere of 5 per cent CO_2 containing 5 μc. $C^{14}O_2$, the rate of CO_2 fixation was proportional to the growth rate irrespective of whether the organisms were finely dispersed or heavily clumped (17). The average generation times for *S. typhimurium* in SM in presence or absence of antibody are given in Table I.

The generation time was found to be similar in both cases indicating that antibody did not have any effect upon the rate of multiplication of *S. typhimurium.* The addition of complement as fresh rabbit or guinea pig serum did not appear to alter the activity of the antiserum.

Effect of Antiserum on Phagocytosis.—Uniform conditions were required for the study of intracellular multiplication of *S. typhimurium* within MN. Therefore, it was necessary to determine the effect of antibody upon rate and extent of phagocytosis of *S. typhimurium* by MN.

When mixtures of about 20 million MN and an equal number of *S. typhimurium* were incubated approximately 30 to 40 per cent of the cells contained bacteria after 45 minutes of incubation. Only very few organisms were seen extracellularly after the cells had been washed twice.

TABLE I

Average Generation Time in Minutes of S. typhimurium Growing in SM with or without Specific Rabbit Antiserum

Condition	Generation time
Control	43 ± 3.6*
Antiserum 1:10	42 ± 2.8

* Standard deviation.

TABLE II

Phagocytosis of S. typhimurium by Rabbit MN in Presence and Absence of Specific Antiserum

Condition	Phagocytosis* Per cent MN	Phagocytosis* No. of bacteria per 100 cells
Control	32 ± 8.2‡	232 ± 34
Antiserum 1:10	37 ± 8.9	230 ± 29

* MN and *S. typhimurium* were incubated for 45 minutes at 37°C.
‡ Standard deviation.

TABLE III

*Effect of Antibody on Intracellular S. typhimurium within Rabbit MN**

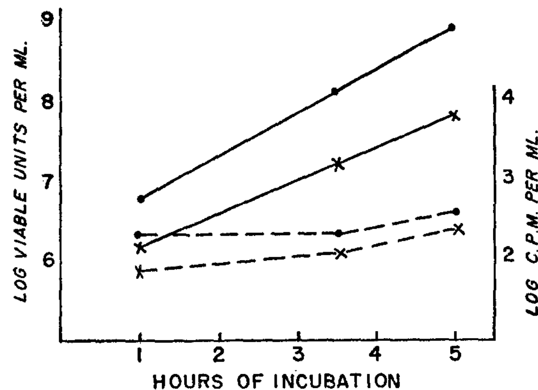
Condition	Viable Counts		CO_2 fixation		Morphology
	Multiplication factor	Ratio	C.P.M. × 10^2	Ratio	
Control	733 ± 492	—	246 ± 95	—	Early destruction of MN and extracellular growth
Antiserum	3.4 ± 3.3	0.0046	6.6 ± 2.5	0.027	MN containing large numbers of bacteria

* MN which had phagocytized *S. tymphimurium* were incubated for 5 to 6 hours in SM in an atmosphere of 5 per cent CO_2 containing 5 μc. $C^{14}O_2$. Determinations of viable units were done at the beginning and end of incubation period and radioactivity counts in the culture medium at the end of the experiment.

Table II gives the results of counts done on smears prepared with suspensions of cells used for the phase of intracellular multiplication. It can be seen that antibody had little or no effect on the percentage of cells ingesting bacteria or on the number of organisms per cell. This is also shown in Figs. 1 and 2.

Fate of Intracellular S. typhimurium within MN after phagocytosis.—During the phase of intracellular multiplication the infected MN were kept in SM

containing heat-inactivated, normal rabbit serum. The experimental sets were identical with the exception that antibody was present during the period of phagocytosis in one and not in the other. These experiments gave the following results:



TEXT-FIG. 1. The effect of presence of antibody during phagocytosis on viable counts and CO_2 fixation during intracellular multiplication of *S. typhimurium* within MN. Legend: Viable units in presence ●— — —● and absence of antibody ●——●; C.P.M. in presence X— — —X and absence of antibody X——X.

TABLE IV

Effect of Different Concentrations of Antiserum and of Globulin Fraction Present during Phagocytosis on Intracellular Multiplication of S. typhimurium in MN

Experiment No.	Dilution of antibody or protein fraction	Multiplication factor	Ratio	Extent of cell destruction
37	No antiserum (control)	180	—	+++
	1:10	3	0.02	−
	1:100	20	0.11	±
	1:1000	17	0.09	±
	1:10,000	56	0.31	+
	1:100,000	150	0.83	++
42	Albumin fraction (control)	32	—	+++
	Antiserum	3	0.09	−
	Globulin fraction	3	0.09	−

In absence of antibody the number of viable units rose steadily during the period of incubation (3 to 5 hours). Similarly, the rate of CO_2 fixation was constant over the same period of time (Table III and Text-Fig. 1).

In stained preparation an increasing number of intracellular organisms was observed. However, after a short period of incubation the cells containing large

numbers of organisms appeared injured. Many cells were lysed and released bacteria into the extracellular environment, resulting in progressive destruction of the cell population and in overwhelming numbers of extracellular *S. typhirium* (Fig. 3). In contrast, in cultures of MN which had phagocytized *S. typhimurium* in presence of antibody, the number of viable units increased only very slowly and the amount of CO_2 fixed was almost negligible. Evaluation of stained preparations revealed that the number of intracellular organisms increased and that the cells contained very large numbers of bacteria without lysis occurring at a time when the majority of the control cells had lysed (Figs. 2 and 4). However, upon continued incubation (8 to 16 hours), these cells lysed and released large numbers of organisms into the culture medium.

TABLE V

Effect of Complement Added either during the Phagocytosis or during Intracellular Multiplication in Presence or Absence of Antibody during Phagocytosis

Complement present		Antibody 1:10	Multiplication factor	Ratio
Phagocytosis	Intracellular multiplication			
—	—	—	64	—
+	—	—	60	0.94
—	+	—	48	0.75
—	—	+	3.4	0.05
+	—	+	3.2	0.05
—	+	+	4.2	0.06

The antiserum used had an agglutination titer of 1:2560. When increasing dilutions of the antiserum were added to the medium during phagocytosis the results presented in Table IV were obtained. As can be seen, the effectiveness of the antiserum was ten times weaker at a dilution 1:100 than it was at 1:10. Further dilution caused a gradual decrease of activity. However, the effect was still detectable at dilutions greater than 1:2560 at which agglutination was no longer measurable.

The total protein content of the antiserum was 40 mg./ml., of which 9.2 mg./ml. was found in the globulin fraction. The agglutinin titer of the globulin was the same as the titer of the original antiserum. As shown in Table IV, intracellular activity of the antiserum was recovered with the globulin fraction whereas the albumin fraction was without any effect.

Role of Complement—As shown in Table V, the addition of complement as guinea pig serum to the medium either during phagocytosis or intracellular multiplication did not alter the effect of antibody. Fresh rabbit serum was similarly inactive in this respect.

Affinity of Antiserum.—In all experiments antiserum was added to a mixture

of *S. typhimurium* and MN. If either bacteria or MN were exposed individually to antiserum and washed before combining them for phagocytosis, the results represented in Table VI were obtained.

It can be seen that pretreatment of the bacteria with antiserum had a similar effect on the intracellular fate of *S. typhimurium* as did the presence of antiserum during phagocytosis. This was not the case when MN were exposed to antiserum.

TABLE VI

Effect of Sensitization of either MN or S. typhimurium with Antibody on Intracellular Multiplication within MN

Sensitization*	Antibody during phagocytosis	Multiplication factor	Ratio
None	None	265	—
MN	None	325	1.23
S. typhimurium	None	20	0.07
None	1:10	6	0.02
MN	1:10	2.7	0.01

* Either MN or *S. typhimurium* were incubated for 30 minutes at 37°C. in SM containing 1:10 antiserum. The cells were then washed and resuspended in SM.

TABLE VII

Effect of Anti-S. typhimurium Antibody on Intracellular Multiplication of Salmonella aberdeen and Salmonella abony

Salmonella	Antigens		Antiserum during phagocytosis	Multiplication factor	Ratio
	O	H			
typhimurium	2,4,5,12	i-1,2,3(5)	none	143	—
			1:10	13.6	0.09
aberdeen	11	i-1,2	none	97	—
			1:10	41	0.45
abony	1,4,5,12	b-e,n,x	none	87	—
			1:10	5.3	0.061

Specificity of the Effect of Antibody.—The antiserum used contained a mixture of somatic and flagellar antibody. An attempt was made to determine the responsible antigen.

(*a*) *Precipitation with Endotoxin.*—Small amounts of homologous lipopolysaccharide endotoxin were added in succession to antiserum until no further precipitation was observed. The removal of antisomatic antibody was further verified by the results of agar diffusion experiments. When this antiserum was used for the experiments, no effect on intracellular multiplication could be observed.

(*b*) *The Effect of Anti-S typhimurium Antibody upon Intracellular Fate of Other Types of Salmonella within MN.*—*Salmonella abony* and *Salmonella aberdeen* which share somatic and flagellar antigens respectively with *S. typhimurium* appeared to multiply within MN of normal rabbits. Suspension of both organisms were agglutinated by the antiserum against *S. typhimurium.* The results given in Table VII show that the intracellular parasitism of *S. abony* was altered by the presence of antibody during phagocytosis, whereas *S. aberdeen* remained unaffected by the antibody.

Visualization of Antibody within MN and Its Entry into the Cells.—The results obtained indicate that the antibody exerted its influence on the cell-parasite relationship within the MN. Therefore, an attempt was made to localize the antibody within the cells using the fluorescent antibody technique. Acetone-fixed, infected monocytes on coverslips were stained for rabbit gamma globulin with anti-RGG sheep serum previously labelled with fluorescein or lissamine rhodamine. The organisms phagocytized in presence of rabbit antibody reacted with anti-RGG, which was not the case with organisms engulfed in absence of antibody. In both cases large numbers of bacteria were seen within the MN in Giemsa-stained preparations.

To determine whether antibody entered the cell after combining with the surface of the bacteria, the following experiment was done. MN were allowed to phagocytize *S. typhimurium,* and were washed. They were then overlayed with anti-*S. typhimurium* antibody labelled with fluorescein, and incubated at 37°C. for 30 minutes. Another batch of cells was incubated for phagocytosis in presence of the same labelled anti-*S. typhimurium* antibody. Fluorescent intracellular bacteria were observed only in the case when phagocytosis occurred in presence of the labelled antibody. These experiments would demonstrate that the antibody is carried into the cells in combination with the bacteria and does not enter the cell once the bacteria have been phagocytized.

DISCUSSION

The experiments here reported dealt with the dynamics of intracellular parasitism of *Salmonella typhimurium* within rabbit monocytes and concerned the effect and site of action of somatic antiserum altering the interaction between cell and parasite.

There is sufficient evidence to conclude that *S. typhimurium* multiplied within MN of normal rabbits. The inevitable destruction of cells shortly after ingestion of bacteria and upon their proliferation has complicated the analysis of events. Increase of the intracellular population, as evidenced by counts of stainable and visible bacilli, preceded extracellular growth. During the first few hours of cultivation the per cent of infected cells remained constant while the number of bacilli per infected cell increased rapidly. This indicated that population changes were limited to an intracellular process. Continuous observation of infected cells on a warm stage confirmed this. Destruction of cells

apparently occurred as a consequence of an increase of the intracellular population. Phagocytosis of large numbers of heat-killed *S. typhimurium*, or exposure of the cells to the endotoxin, did not cause destruction of the cells even after prolonged cultivation. It has to be assumed that some product of bacterial metabolism was involved, but no attempts were made to identify such products. Further evidence for intracellular multiplication of *S. typhimurium* was obtained by adding bacteriophage to the infected and washed MN. Bacteriophage attacked the extracellular bacteria while the number of intracellular organism steadily increased (23). Viable counts represented the sum of extra- and intracellular organisms, although complete dispersion of intracellular bacteria was unlikely. As far as could be determined, CO_2 fixation occurred only in the extracellular phase. Therefore the rate of CO_2 fixation, as measured by the appearance of organic C^{14} in the culture, was directly proportional to the rate of destruction of MN and subsequent extracellular multiplication.

Rabbit antiserum did not inhibit extracellular growth of *S. typhimurium* even in presence of complement. On the contrary, CO_2 fixation by bacteria alone was frequently found to be somewhat higher in flasks in which antibody was present (17). Stimulation of bacterial respiration had been observed with other bacteria (24, 25). In our experiments it was noticed that the drop of pH in cultures with antibody occurred more slowly and to a less extent than in absence of antibody. Stimulation of oxidative processes over fermentative ones had to be assumed.

Phagocytosis of *S. typhimurium* by MN in presence of antibody resulted in considerable postponement of cell destruction, although large numbers of bacteria accumulated within the cells. Evidence is presented that the active part of the antibody reacted specifically with the somatic antigen of the bacteria, *e.g.* activity of the antiserum was removed by precipitation with the endotoxin, and the intracellular parasitism of *Salmonella abony* containing a common O-antigen with *S. typhimurium* was similarly altered by antiserum as was that of *S. typhimurium*. The fact that the antibody had no effect on the intracellular fate of *Salmonella aberdeen*, which shares flagellar antigens with *S. typhimurium*, allowed the exclusion of the possibility that agglutination alone was responsible for the observed phenomenon.

It was difficult to rule out experimentally the participation of complement in the effect of antibody, because phagocytes may contain complement even after repeated washing (26). However, addition of an excess of complement to the system did not influence the effect of antibody in either direction.

The mode of action of the antibody could not be elucidated. Neutralization of endotoxin as mechanism is unlikely, but this possibility should not be ruled out completely. The lipid moiety of the lipopolysaccharide, which is made responsible for most of the biological manifestations of endotoxin, was not available for these experiments. Furthermore, it has been shown that the polysaccharide moiety of the endotoxin combines with the antibody (27).

It has recently been reported that somatic antibody against *Salmonella* prepared in guinea pigs induces formation of spheroplasts in *Salmonella typhosa* in presence of complement and lysozyme (28). In view of the fact that complement was not required in the phagocytic system such a mechanism must be excluded, although lysozyme is supposedly present in monocytes. In addition, spheroblasts were not observed in any of the preparations.

The results obtained when fluorescein-labelled antibody was added before and after phagocytosis strongly supported the view that the site of activity of antibody was within the cellular cytoplasm. The results also confirmed in a more direct way earlier findings by Rous and Jones (2), according to which antibody has no access to intracellularly located bacteria or red blood cells. It is quite interesting that antibody did not penetrate into the phagocyte when added to the cells after they had ingested the respective antigen. This limitation to the extracellular phase of accessibility of a particulate antigen for antibody is almost identical with that encountered with viruses (29). However, the quantitative limitations of the technique employed have to be kept in mind. Traces of antigen cannot be demonstrated, inasmuch as the minimum quantity of antigen present has to be not less than 0.1 μg. per ml. (30).

In a few experiments MN from rabbits which had been actively immunized were used. If the cells were washed repeatedly before infecting them with *S. typhimurium*, the results of the interaction were identical with those obtained with normal MN. Incomplete washing, however, allowed enough antibody to remain in the cell suspension to alter the conditions of the experiment. There was, therefore, no evidence of participation of a cellular or a cytophylic antibody.

The protective effect of an immune globulin described in this paper appears to be different from the factor found to protect MN when infected *in vitro* with virulent tubercle bacilli. In this system the cultivation of MN derived from immunized animals in a medium containing immune serum resulted in increased resistance of the cells to the destructive effect of intracellular, virulent tubercle bacilli. Neither "immune" cells nor immune serum alone were fully effective. Furthermore the effect was non-specific, for MN derived from rabbits vaccinated with BCG were protected against tubercle bacilli when cultivated in media containing immune sera derived from animals immunized with antigenic materials from *Salmonella*, *Brucella*, or *Mycobacteria* (31). The protective factor was not in the globulin fraction and appeared in the serum 5 days after intradermal injection into rabbits of either live or heat-killed BCG (32).

The findings reported suggest that there exist more ways than hitherto recognized by which the immune response may influence and alter interactions between phagocytic cells and pathogens. Some of the more important mechanisms are as follows:

(*a*) Enhancement of phagocytosis by an antibody reacting with a surface

component of the pathogen as is the case in infections with pneumococci and beta hemolytic streptococci (33, 34).

(*b*) Neutralization of an exotoxin, such as leucocidin of virulent staphylococci (35, reviewed in reference 36).

(*c*) Immune adherence of pathogens to erythrocytes and subsequent increased phagocytosis of the pathogen (37).

(*d*) Increased capacity of the mononuclear phagocytes to restrict intracellular multiplication of the pathogen as described in the case of *Mycobacteria* and *Brucella* (38–41).

(*e*) The results reported in this paper support the view that circulating antibody against the somatic antigen of *S. typhimurium* can enter the cells upon phagocytosis of the bacteria. The presence of the antibody within the phagocyte appears to stabilize the interplay between cell and parasite in favor of the former without inhibiting intracellular multiplication. Prolonged intracellular residence of the parasite presumably results in restricting the spread of the pathogen.

SUMMARY

The effect of antibody on the fate of *Salmonella typhimurium* within mononuclear phagocytes (MN) of rabbits was studied *in vitro*. Monocytes and bacteria were incubated either in absence or presence of antibody. After 45 minutes during which phagocytosis occurred infected cells were washed to remove extracellular bacilli and free antibody. The cells were then reincubated in a medium without addition of antibody, and the interaction between the MN and bacteria was followed, correlating bacterial viability and the morphology of the mixture. The following results were obtained.

The anti-*Salmonella* antibody was not bactericidal even in presence of complement and did not enhance phagocytosis.

Regardless of whether antibody was present or absent during phagocytosis, the bacteria appeared to multiply within the cells. When no antibody was present during phagocytosis the infected cells were severely damaged within a few hours of incubation, and extensive extracellular multiplication was dominating. When antibody was present during phagocytosis MN packed with bacteria persisted for a long time. Little or no extracellular growth occurred.

It was possible to demonstrate the presence of the antibody within the infected MN, using the fluorescent antibody technique. The antibody appeared as a coat around the bacteria. Antibody entered the cells only during phagocytosis, presumably attached to the bacteria.

The active factor of the immune serum was found in the gamma globulin fraction and reacted specifically with the somatic antigen of *Salmonella typhimurium*. The antiflagellar portion of the antiserum was not involved in the phenomenon described.

It is concluded that this antibody protects monocytes against the effect of intracellularly located *Salmonella*.

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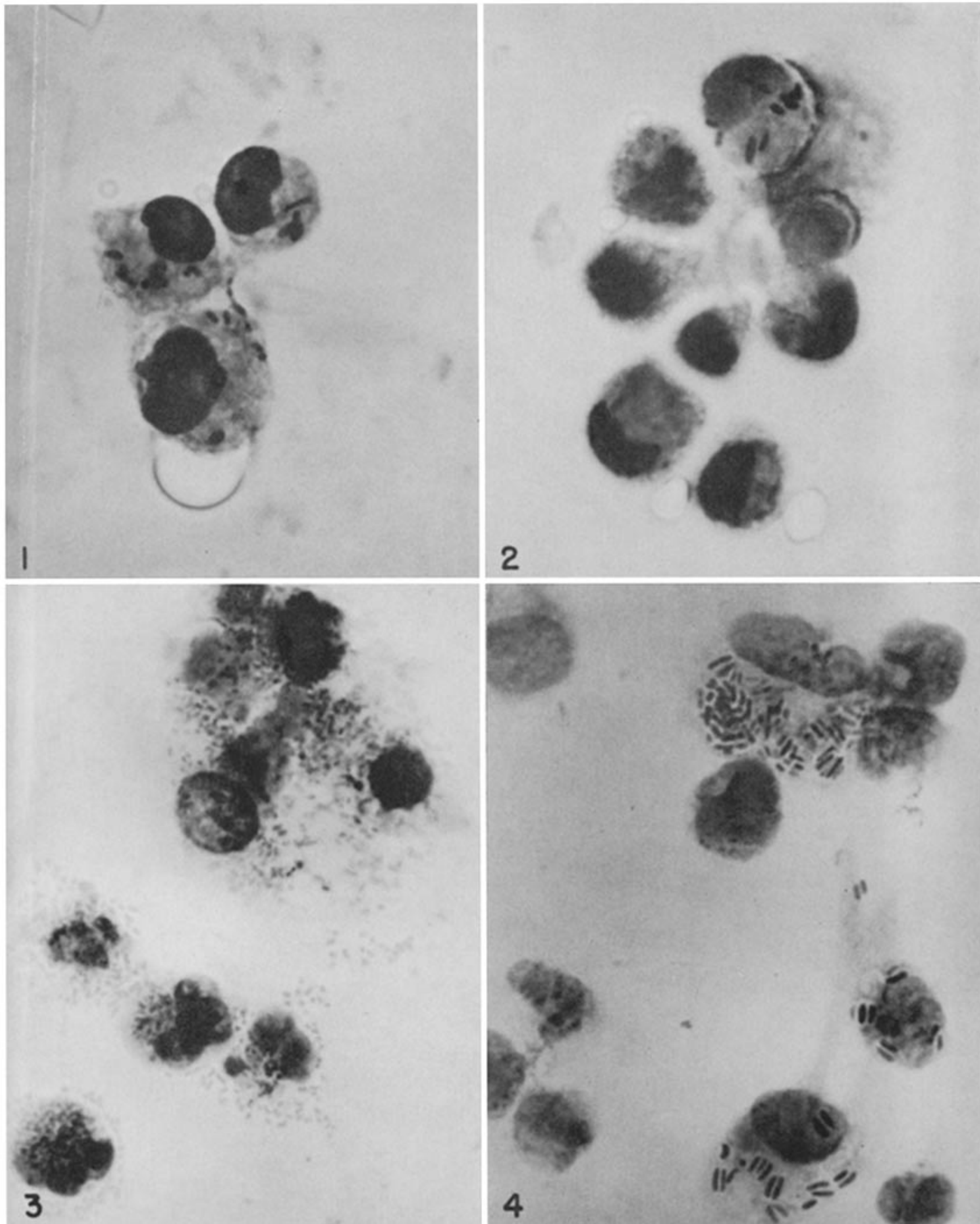
EXPLANATION OF PLATE 62

The effect of antibody on the interaction between *Salmonella typhimurium* and MN. A mixture of MN and *S. typhimurium* were allowed to settle on coverslips in the presence (Figs. 1 and 3) or absence (Figs. 2 and 4) of antiserum. After 45 minutes the slides were washed and reincubated in SM. Giemsa stain. × 1250.

FIGS. 1 and 2. Beginning of incubation period. MN in both sets contain similar numbers of intracellular organisms.

FIGS. 3 and 4. After 5 hours of incubation in SM. In Fig. 3 (phagocytosis in absence of antibody) large numbers of intra- and extracellular organisms are visible. The cells appear overwhelmed by the rapidly increasing bacterial population now present within and without them. The MN in Fig. 4 appear healthy although some contain a large number of organisms. There are few extracellular organisms visible. The organisms are large and are surrounded by a halo indicative either of an intracellular vacuole or of a capsule-like structure.



(Gelzer and Suter: Effect of antibody on *Salmonella* within monocytes)