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Interactions Between Macrophages of Guinea Pigs and Salmonellae

III. Bactericidal Action and Cytophilic Antibodies of Macrophages of Infected Guinea Pigs

H. S. HSU AND DONALD R. MAYO

Department of Microbiology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298

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The fate of virulent *Salmonella typhimurium* within macrophages of guinea pigs was assessed by a suspended cell culture procedure. The present study confirmed that macrophages of normal guinea pigs were capable of inactivating the ingested salmonellae. Macrophages of previously infected guinea pigs were not endowed with any significant increase in their ability to eliminate the ingested pathogen. However, the immune macrophages were observed to clump together tightly when they were exposed to salmonellae. This phenomenon was attributed to the presence of specific cytophilic antibodies on the immune macrophages. When immune macrophages were inactivated with Merthiolate, they agglutinated with both the H and the O antigens of *S. typhimurium*, but not with the O antigens of other species of *Salmonella* nor with the O antigens of *Escherichia coli*. Cytophilic antibodies could be eluted from immune macrophages by incubation in the absence of immune serum. Conversely, cytophilic antibodies could be passively transferred onto normal macrophages by incubation in the presence of immune serum. Furthermore, using immune serum previously adsorbed with the O antigens of *S. typhimurium*, cytophilic antibodies against the H antigens alone could be transferred onto normal macrophages, or those against the O antigens alone could be eluted from immune macrophages.These data suggest that immune macrophages possess specific cytophilic antibodies against both the H and the O antigens of *S. typhimurium*. It is proposed that the presence of cytophilic antibodies on immune macrophages represents an expression of antibacterial cellular immunity by enhanced clumping and phagocytic activities of the macrophages.

Despite the classification of salmonellae as facultative intracellular bacteria (1, 18), there is little authenticated experimental evidence that *Salmonella typhimurium* or *S. enteritidis* can survive or multiply within macrophages of experimental animals. On the contrary, the intracellular environment of macrophages of guinea pigs was shown to be unfavorable for the proliferation of either one of these two species of salmonellae (9; M. W. Rhodes and H. S. Hsu, J. Reticuloendothel. Soc., accepted for publication). It was also shown that the rate of phagocytosis of *S. typhimurium* by macrophages of guinea pigs was significantly greater in the presence of specific antiserum and was inversely proportional to the relative virulence of the strains of the bacteria (24). Hence the virulence of salmonellae is greatly dependent on their ability to propagate in the extracellular location of the host tissue. Conversely, antibacterial cellular immunity of an immune host may be manifested as enhanced capacity of the macrophages to eliminate the ingested salmonellae or as greater clumping and phagocytic activities of the macrophages against the extracellular pathogen, or both. The present investigation was undertaken to examine these possibilities by using the cell culture method as previously described (9).

MATERIALS AND METHODS

Salmonella typhimurium The stock virulent strain SR-11 was used in this study. The mean lethal dose (LD_{50}) of this strain was approximately 10 bacteria upon intraperitoneal (i.p.) injection into mice. The preparation of bacterial suspensions in

saline was described previously (9). The optically standardized bacterial suspension contained approximately 2.5×10^9 viable bacteria/ml.

Preparation of bacterial antigens. For the preparation of the H or the O antigens, the following cultures were used: *S. typhimurium*, SR-11 and RIA; *S. enteritidis*, I-203; *S. typhi*, ATCC 6539; *Escherichia coli*, ATCC 10536. The bacteria were grown in Tryptic Soy Broth (Difco) for 6 h and harvested by centrifugation at $1,400 \times g$ for 20 min. The H anigens were prepared by suspending the bacteria in saline containing 0.5% Formalin at room temperature overnight. The O antigens were prepared by heating the bacterial suspension in saline in a boiling water bath for 30 min. The sterilized antigenic preparations were washed twice in saline and standardized to an optical density of 125 in a Klett-Summerson photoelectric colorimeter with a no. 66 filter (approximate spectral range of 640 to 700 nm).

Guinea pigs. Male albino guinea pigs were purchased from commercial sources and fed with guinea pig pellets, supplemented with multiple vitamins (Poly-Vi-Sol, Mead Johnson) in their drinking water. Each animal weighed between 600 and 800 g.

Immunization of guinea pigs with S. typhimurium. Guinea pigs were injected intracutaneously in two sites with 10^5 virulent *S. typhimurium*. After 2 to 4 weeks, they were given an i.p. injection of 2×10^4 bacteria of the same strain. These guinea pigs were used 3 to 5 weeks later as donors of peritoneal macrophages, which are hereafter referred to as immune macrophages. At the time of sacrifice, these infected guinea pigs were bled from the heart. The sera collected from three to five animals were pooled, checked for sterility, and frozen until use. This serum is hereafter referred to as immune serum.

Adsorption of immune serum with antigens. Immune serum derived from guinea pigs immunized with *S. typhimurium* was adsorbed with the O antigens of *S. typhimurium* SR-11 by the following protocol: the heat-killed bacteria were sedimented from 1 ml of the standardized suspension by centrifugation at $1,400 \times g$ for 30 min, resuspended with 4 ml of immune serum, and left standing at 5 C overnight. The antigen-antibody complexes were removed by centrifugation at $1,400 \times g$ for 30 min. The adsorbing procedure was repeated twice. The final supernatant serum was passed through a membrane filter (0.22-μm pore size, Millipore Corp.) and stored at −20 C.

Procedure for suspended culture of macrophages infected with S. typhimurium. Peritoneal exudates containing over 70% monocytes were harvested from normal or immunized guinea pigs injected 3 to 5 days previously with mineral oil. The procedures for the preparation of leukocyte suspensions and for their infection were described in detail in a previous publication (9). The initial infection of the leukocyte suspension was achieved by mixing 4.5×10^7 cells in 7 ml of Hanks solution containing 20% normal homologous serum with *S. typhimurium* at the ratio of 10 bacteria/cell. After the mixed suspension was rotated in a drum at 25 rpm at 37 C for 15 min, the infected cells were diluted 10-fold in chilled Hanks solution containing 6 U of heparin per ml (Liquaemin Sodium "10", Organon, Inc.). and 40 μg of kanamycin per ml (Kantrex, Bristol Lab.), using 40-ml silicone-coated centrifuge tubes. They were collected by centrifugation at $150 \times g$ for 10 min at 5 C and resuspended in culture medium composed of 70% medium 199 (Microbiological Associates, Inc.), 20% homologous serum, 10% isotonic 1.4% sodium bicarbonate (CO_2-saturated), and 40 μg of kanamycin per ml. The cell suspension was transferred to a 25-ml silicone-coated Erlenmeyer flask which was then closed with a rubber stopper and placed in an Eberbach water bath with horizontal shaking at approximately 72 cycles/min. At the beginning of cell culture and after each sampling thereafter, the flasks were flushed gently with 5% CO_2 in air to maintain the cell suspension in a high CO_2 tension.

Procedure for sampling the infected macrophage culture at intervals. The detailed procedure for removing samples from the infected cell cultures and for the quantitative recovery of cell-associated salmonellae was described previously (9). Instead of using a 0.5% solution of sodium deoxycholate to release the viable cell-associated bacteria, a modified procedure using sonic treatment was devised as follows. After the removal of supernatant fluid from the sedimented, infected cells from 0.2 ml of sample, the infected cells were resuspended in 2 ml of saline, transferred to a polyethylene tube, and sonically treated in a Biosonik II apparatus at a "needle" probe intensity setting of 30 for 15 s. This treatment resulted in the disintegration of over 95% of the leukocytes without any appreciable effect on the viability of *S. typhimurium*. One milliliter of this suspension was then mixed with 4 ml of saline, from which 0.5 ml was removed for making serial 10-fold dilutions up to 10^{-4}. A 0.5-ml amount of each dilution was placed in a petri dish for bacterial colony counting by the pour-plate method using Tryptic Soy Agar (Difco). At the same time, the total and viable leukocyte populations in the 0.2-ml sample were determined with eosin-Y staining in a hemocytometer.

The ratio of bacteria to cells was computed for each interval by correlating the number of viable bacteria recovered from the infected cell sediment from a 0.2-ml sample with the total number of leukocytes present in a 0.2-ml sample. The phagocytic index was previously defined as the ratio of bacteria per cell determined from the 0-h sample (24). The fate of intracellular bacteria was presented by plotting the ratio calculated by dividing the number of bacteria per cell recovered at intervals by the phagocytic index.

For the purpose of comparison, the procedure for the quantitative recovery of cell-associated salmonellae using 0.5% solution of sodium deoxycholate as described previously (9) was also used in one set of experiments reported herewith.

Procedure for the detection of cytophilic antibodies. To detect the presence of gamma globulin on the surface of macrophages, washed suspensions of peritoneal macrophages were collected by centrifugation at $220 \times g$ for 5 min and resuspended in 1 ml of 1:50 dilution of fluorescein-labeled, anti-guinea pig globulin antibodies from rabbit (Difco) in Hanks solution. The cell suspension was incubated in a water

bath at 37 C for 30 min. The cells were then sedimented by centrifugation and washed twice with 5 ml of chilled Hanks solution. The final cell sediment was resuspended in a small amount of Hanks solution, smeared, and fixed with methanol on glass slides for fluorescent microscopy.

To show agglutination of macrophages with bacterial antigens, the cell suspension was adjusted to a concentration of approximately 7×10^6 cells/ml and inactivated by exposure to Merthiolate in Hanks solution (1:5,000) for 20 min to prevent phagocytosis of the antigens. By using Pasteur pipettes, one drop of the cell suspension and one drop of the bacterial antigens were mixed by manual rotation on an agglutination slide. Specific agglutination was seen within 30 min.

RESULTS

Quantitative recovery of intracellular salmonellae by sonication. In previous studies (9) a 0.5% solution of sodium deoxycholate was used for the disintegration of leukocytes and the recovery of cell-associated *S. typhimurium*. It was suspected that this treatment was detrimental to the intracellular *S. typhimurium* which were already exposed to the injurious actions of the host macrophages. Preliminary experiments showed that when viable *S. typhimurium* was exposed to 0.5% sodium deoxycholate, 30 to 50% of the organisms were recovered on Tryptic Soy Agar plates. By contrast, when the same organism in saline suspension was sonically treated at the intensity described above, over 95% of the bacteria were recovered by colony counts.

The following set of experiments was performed to compare the fate of *S. typhimurium* SR-11 within macrophages of guinea pigs by using sodium deoxycholate or sonic treatment for the quantitative recovery of intracellular bacteria. Peritoneal macrophages were infected with *S. typhimurium* and maintained in culture flasks containing approximately 5×10^7 infected leukocytes in 7 ml of culture medium. At designated intervals, 0.7 ml of the cell culture was removed. From two samples of 0.2 ml each, the infected cells were sedimented by centrifugation. A third sample of 0.2 ml was mixed with eosin-Y for the determination of total and viable leukocyte population. One of the sedimented cell samples was treated with 1 ml of 0.5% sodium deoxycholate as described previously (9). The other was resuspended with 2 ml of saline and sonically treated. From these, viable bacterial populations were determined. Figure 1 shows the results compiled from four experiments, in which the infected cell cultures were maintained for 28 h. The fate of intracellular bacteria as determined by sodium deoxycholate treatment (SDC-A) was identical to that previously reported (9). There was a rapid destruction of the organism within the first 3 h, followed by a phase of increase in intracellular bacterial population between 3 and 22 h and by a stationary phase. By contrast, the fate of intracellular bacteria as determined by sonic treatment (SON-A) on samples of the same infected cell cultures showed a far lower initial destruction of the bacteria as compared to that determined by sodium deoxycholate treatment. Over a 28-h period, there was a slow but continuous decline in the intracellular bacterial population. Despite the fact that, throughout the subsequent intervals, consistently more bacteria were recovered from equivalent samples of the same cell cultures by sonic treatment than by sodium deoxycholate, the phagocytic indices were identical by these two methods of quantitation. In this series of experiments, the average phagocytic index was 0.5 ± 0.07 (standard deviation [SD]) as determined by sonic treatment and 0.5 ± 0.05 (SD) as determined by sodium deoxycholate treatment.

The result of three additional experiments (SON-B) also shows continuing destruction of intracellular *S. typhimurium* as determined by the method of sonic treatment. In these experiments, the average phagocytic index was 0.3 ± 0.04 (SD). This further illustrates that the host macrophages were more efficient in destroying the intracellular *S. typhimurium* when they were less heavily infected initially.

Fate of S. typhimurium within macrophages of normal and immunized guinea pigs. Peritoneal macrophages were harvested

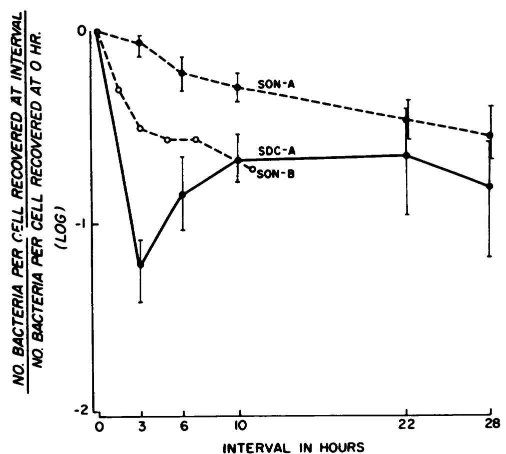

FIG. 1. *Fate of S. typhimurium within macrophages of normal guinea pigs as determined by the quantitative recovery of intracellular bacteria using sodium deoxycholate (SDC) or sonic treatment (SON). Crossbars at intervals represent range of data from which the average was taken.*

from normal or immunized guinea pigs. The cell suspensions were washed and adjusted to 7 × 10^6 cells/ml in culture medium containing 10% normal homologous serum and 20 μg of kanamycin per ml. They were maintained overnight in separate culture flasks in the shaking water bath at 37 C. In the meantime, samples of peritoneal washings from immunized animals were inoculated into Tryptic Soy Broth to assure sterility. On the following morning, the cells were collected by centrifugation at 150 × *g* for 10 min and resuspended in Hanks washing solution. The total and viable cell counts were obtained. Cell suspensions showing over 90% viability were infected with *S. typhimurium*. The infected normal macrophages were cultured in medium containing 20% normal serum, whereas the infected immune macrophages were cultured in medium containing 20% of either normal or immune serum. Samples were taken at designated intervals. The fate of intracellular bacteria under these conditions was determined from the average of three sets of experiments (Fig. 2). The phagocytic indices in these experiments varied between 0.3 to 0.5 in each condition.

Over a period of 8 h, there was a continuous destruction of intracellular *S. typhimurium*. The rate of bactericidal activities was essentially comparable within macrophages of normal or immunized guinea pigs. The reconstitution of immune macrophages into medium containing immune serum did not significantly

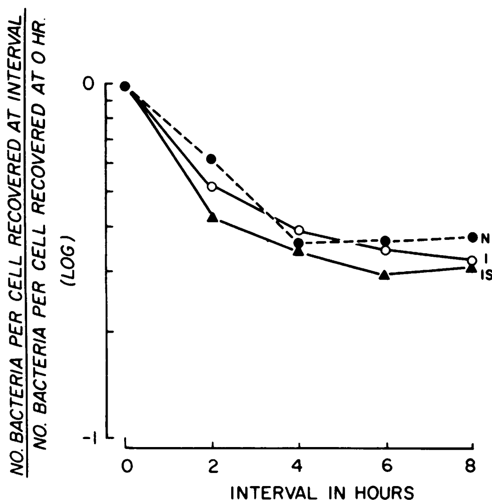

FIG. 2. *Comparative rate of destruction of S. typhimurium within normal macrophages cultured in normal serum (N), immune macrophages cultured in normal serum (I), or immune macrophages cultured in immune serum (IS).*

enhance the bactericidal activities of the immune cells. It may therefore be concluded that the intracellular environment is unfavorable for the intracellular survival of *S. typhimurium* and that macrophages derived from immunized guinea pigs are not endowed with any significant increase in their bactericidal action.

Detection of specific cytophilic antibodies on macrophages. Freshly harvested peritoneal macrophages from normal or immunized guinea pigs were tested for their ability to agglutinate with various bacterial antigens (Table 1). The H and the O antigens prepared from both the avirulent strain RIA and the virulent strain SR-11 of *S. typhimurium* were used in these tests. It is apparent that macrophages derived from animals previously infected with virulent *S. typhimurium* agglutinated with the live, the H, or the O antigens of organisms of the same species. However, at the level of sensitivity of the present test, there was no apparent cross-agglutination of the immune macrophages with the O antigens of different species of *Salmonella* nor with the O antigens of another genus of gram-negative bacilli. The agglutination of immune macrophages with salmonellae antigens is presumed to be due to the presence of specific cytophilic antibodies on the cellular surface which are cross-linked with bacteria to form large clumps. However, microscope examination of stained preparations of agglutinated cells failed to reveal bacteria between cells due to dense cell clumping.

Elution and passive transfer of cytophilic antibodies on macrophages. Preliminary studies showed that, when immune macrophages were incubated in medium containing 10% normal serum at 5 C or 37 C overnight, they lost the ability to agglutinate with specific salmonellae antigens. Peritoneal macrophages from normal or immunized guinea pigs were adjusted to a concentration of 7 × 10^6 cells/ml and suspended in culture medium containing one of the following variations of serum content: 30% normal serum, 10% normal serum plus 20% immune serum, or 10% normal serum plus 20% immune serum previously adsorbed with the O antigens. The addition of normal serum to the adsorbed immune serum was intended to replenish some of the complement depleted by the adsorption with the O antigens. The cell suspensions were placed in separate silicone-coated tubes (Vacutainer, Becton, Dickinson & Co.) which were then flushed with 5% CO_2 in air, stoppered tightly, and placed in a shaking water-bath at 37 C overnight. The following morning samples of the cell cultures were checked in eosin-Y to assure viability of at least 90% of the total cell

population. The cells were then collected by centrifugation at 150 × g for 10 min and inactivated with Merthiolate in Hanks solution at the final cell concentration of approximately 7 × 10^6 cells/ml. Slide agglutination tests were done by using the cultured macrophages and various bacterial antigens (Table 2).

When either normal or immune cells were cultured overnight in the presence of normal serum they did not agglutinate with either the H or the O antigens of *S. typhimurium*. This possibly indicates that the cytophilic antibodies on the immune cells were eluted in the process. On the other hand, after normal or immune cells were maintained overnight in the presence of immune serum, they were able to agglutinate with both the H and the O antigens of *S. typhimurium*. This indicates that circulating antibodies could be passively transferred onto normal macrophages and that the presence of circulating antibodies prevented the escape of cytophilic antibodies from immune cells. When immune serum were previously adsorbed with the O antigens of *S. typhimurium* and incorporated into the culture medium, both the normal and the immune cells agglutinated with only the H antigen of *S. typhimurium*. This suggests that the cytophilic antibodies had at least two specificities. Table 2 also confirms that there was no cross-agglutination of cytophilic antibodies with the O antigens of *S. enteritidis*, *S. typhi*, and *E. coli*.

Fluorescent antibodies staining of macrophages. By using fluorescent antibodies prepared from rabbit against guinea pig globulin, it was observed that the surface of washed normal and immune macrophages were coated with globulin. When immune macrophages were first incubated at 37 C for 20 min in Hanks solution containing 0.25% trypsin, the cells could no longer agglutinate with antigens of *S. typhimurium*. But this treatment did not remove their affinity for fluorescent antibodies.

TABLE 1. *Agglutination of macrophages with bacterial antigens*

Guinea pigs[a]	Saline	*S. typhimurium*[b]			*S. enteritidis* O Ag	*S. typhi* O Ag	*E. coli* O Ag
		Live	O Ag	H Ag			
NC	–	–	–	–	–	–	–
IC	–	+	+	+	–	–	–

[a] NC, normal cells; IC, immunized cells.
[b] Both strains RIA and SR-11 of *S. typhimurium* were used. Ag, antigen.

TABLE 2. *Agglutination of macrophages with bacterial antigens*[a]

Guinea pigs	Saline	*S. typhimurium*[b]		*S. enteritidis* O Ag	*S. typhi* O Ag	*E. coli* O Ag
		O Ag	H Ag			
NC + NS	–	–	–	–	–	–
NC + IS	–	+	+	–	–	–
NC + adIS	–	–	+	–	–	–
IC + NS	–	–	–	–	–	–
IC + IS	–	+	+	–	–	–
IC + adIS	–	–	+	–	–	–

[a] Slide agglutination test using peritoneal macrophages of normal (NC) or immunized (IC) guinea pigs and various bacterial antigens. The macrophages were first cultured overnight at 37 C in medium containing normal serum (NS), immune serum (IS), or immune serum previously adsorbed with the O antigens of *S. typhimurium* (adIS).
[b] Both strains RIA and SR-11 of *S. typhimurium* were used. Ag, antigen.

DISCUSSION

The basis for acquired immunity in salmonellosis has been a controversial issue. The concept of antibacterial cellular immunity as a basic mechanism for acquired resistance to salmonellosis was claimed by several investigators, including Mitsuhashi, Sato and Tanaka (14), Sato et al. (17) and Blanden (1). These workers showed that macrophages from an immune host have an enhanced capacity to suppress the growth of or to destroy intracellular salmonellae. The cellular basis for acquired immunity was supported by the evidence of the failure to passively transfer immunity with immune serum and to actively immunize animals with killed vaccines (6). Passive transfer of immune serum facilitated the removal of salmonellae from the circulation of the host but it did not interfere with the subsequent bacterial multiplication in the tissue (12).

Other workers subscribed to the view that acquired immunity in salmonellosis is due to protective antibodies. Jenkin et al. (10) showed that the rate of killing of virulent *S. typhimurium* within mouse peritoneal macrophages was determined by the presence of antibodies. Rowley et al. (16) demonstrated that immunity could be passively transferred by either macrophages or sera obtained from mice previously immunized with living attenuated *S. typhimurium*. In these studies, the expression of antibacterial cellular immunity was attributed to cell-bound antibodies. A cell-bound antibody against salmonellae was extracted with urea from immunized mice and described as a macroglobulin (19).

Contrary to previous reports (6, 12), Ornellas et al. (15) showed that heat-killed vaccines

provided significant protection against subsequent challenges with virulent salmonellae and that bacteria opsonized in vitro with species-specific antiserum were rendered less virulent. Other reports (3-5, 21-23) also supported the protective value of killed salmonella vaccines, although it was generally held that living attenuated salmonellae produce a more effective immunity in the host than did killed virulent organisms. Venneman and Berry (21, 22) reported that either peritoneal cells or sera, obtained from donor mice immunized with an attenuated *S. typhimurium*, heat-killed salmonellae, or immunogenic ribosomal preparations of a virulent *S. typhimurium* were effective in passively transferring resistance to normal recipient mice. When mice were immunized with immunogenic preparations of ribonucleic acid (RNA) from a virulent *S. typhimurium*, only peritoneal cells were capable of passive transfer of resistance (22). The immunogenic RNA was recently purified (20), but its mechanism of immunogenicity is unknown.

The conflicting conclusions on the basis of acquired immunity to salmonellosis may largely be attributed to the pitfalls in experimental designs, particularly when cell culture methods were used to compare the fate of salmonellae within normal and immune macrophages. It was previously emphasized (9; M. W. Rhodes and H. S. Hsu, J. Reticuloendothel. Soc., accepted for publication) that the validity of infected cell culture experiments depended on the quantitative recovery of viable intracellular bacteria by the total disintegration of the host cells and on the effective control of extracellular bacterial population.

In the previous publication (9), a 0.5% solution of sodium deoxycholate was used for the quantitative recovery of intracellular *S. typhimurium*. It was observed that between 1 and 7 h after the infection of macrophages, more cell-associated salmonellae were recovered when using water (which did not disintegrate the leukocytes) than when using sodium deoxycholate. It was proposed that, when the intracellular bacteria were exposed to digestive enzymes of the host cells, the bacterial cell surface was damaged in such a way that the cell membranes became susceptible to the adverse effect of sodium deoxycholate in a manner similar to mammalian cell membranes. In the present study, sonic treatment completely disintegrated the host leukocytes, but it apparently did not interfere with the viability of the released intracellular salmonellae. The increase in the intracellular bacterial population between 3 and 22 h as seen by the determination with sodium deoxycholate was not borne out by the determination with sonic treatment (Fig. 1). Whether there was an intracellular multiplication of a portion of the sodium deoxycholate-resistant salmonellae was unknown. It might also represent a regeneration of the structural integrity of the bacterial cell wall by a certain portion of the intracellular bacteria so that when they were released by sodium deoxycholate treatment, they were no longer subject to the adverse effect of the chemical. That a prolonged exposure of *S. typhimurium* to the intracellular environment rendered the bacteria sensitive to the injurious effect of sodium deoxycholate was supported by the observation that phagocytic indices determined by sodium deoxycholate treatment or by sonic treatment were identical. It is therefore evident that the fate of intracellular salmonellae is more dependably determined using sonic treatment for the quantitative recovery of viable cell-associated bacteria.

The present study clearly shows that macrophages of normal guinea pigs possessed an innate capacity to inactivate the ingested virulent *S. typhimurium*. Hence the widely accepted classification of salmonellae as a facultative intracellular parasite is rendered untenable. The expression of antibacterial cellular immunity as an enhanced capacity of the immune macrophages to destroy the ingested pathogen can no longer be considered a crucial issue in the acquired resistance of the host. The present study in fact fails to show any significant increase in the capacity of the immune macrophages to eliminate the ingested *S. typhimurium* (Fig. 2). Contrary to this observation, several investigators provided experimental data to show the existence of antibacterial cellular immunity in salmonellosis. Unfortunately, the decreased rate of bacterial multiplication within immune macrophages was assessed by the highly unreliable method of microscope counting of infected cell cultures (14, 17). Blanden et al. (2) presented data suggesting an enhanced intracellular destruction of salmonellae by immune cells. But in their experimental procedure, no attempt was made to disintegrate the infected cells for the quantitative recovery of cell-associated bacteria. The lower bacterial counts from a sample of infected immune cells might simply reflect clumping of these cells, as compared to the dispersed suspension of infected normal macrophages.

When freshly harvested macrophages derived from actively immunized guinea pigs were infected with *S. typhimurium* and cultured in vitro, the leukocytes clumped together tightly. Technically, the clumping of infected cells hampered the determination of the total leuko-

cyte population in the sampling of the cell culture. This problem was alleviated by overnight incubation of immune macrophages in the absence of immune serum prior to infection. The clumping of immune macrophages when infected with salmonellae was attributed to the presence of cytophilic antibodies on the cellular surface. This was confirmed by the agglutination of Merthiolate-inactivated immune macrophages with the H and the O antigens of *S. typhimurium* (Table 1). The cytophilic antibodies were species specific against *S. typhimurium*, since at this level of sensitivity there appeared to be no cross-agglutination with the O antigens of other species of *Salmonella* or the O antigens of *E. coli*. The failure of immune macrophages to clump together when infected with *S. typhimurium* after overnight culture in the absence of immune serum was evidently a result of the elution of cytophilic antibodies from the immune macrophages (Table 2). It is of particular interest to note that cytophilic antibodies could be passively transferred onto normal macrophages by overnight incubation with immune serum, which may in part explain the protective effect of passive transfer of immune serum (8, 13). The incubation of normal or immune macrophages in the presence of immune serum previously adsorbed with the O antigens of *S. typhimurium* further indicated that the cytophilic antibodies on the surface of macrophages possessed at least two specificities against the H and the O antigens of *S. typhimurium*. It is therefore apparent that the surface of macrophages were coated with immunoglobulins of various immunologic specificities, which may be freely exchanged with the circulating immunoglobulins as governed by their relative concentrations in the surrounding medium. The observation of cytophilic antibodies against salmonellae is therefore in agreement with the demonstration of cell-bound antibodies on immune macrophages (16).

Incubation of immune macrophages with trypsin prior to infection in vitro inhibited the clumping of cells, but it apparently did not remove the immunoglobulins from the cellular surface, as ascertained by fluorescent antibody staining of the treated cells. This is in agreement with the observation that cytophilic antibodies were bound to macrophages by the Fc fragment of the immunoglobulins (11). After the elution of cytophilic antibodies from immune macrophages, the reconstitution of the culture medium with immune serum did not endow the immune macrophages with any significant increase in their bactericidal activities against the ingested *S. typhimurium* (Fig. 2).

In view of the bactericidal action of macrophages against virulent S. typhimurium, the efficiency with which extracellular salmonellae are engulfed by the phagocytes can logically be considered as an essential factor in the acquired resistance of the host, or conversely, in the relative virulence of the pathogen. The demonstrations of opsonic function of immune serum and a greater resistance of the virulent than the avirulent *S. typhimurium* to phagocytosis (24) support this contention. On the other hand, immune bacteriolysis is not necessarily a mechanism of acquired resistance of the host, since the strain of *S. typhimurium* used in this study is not sensitive to immune serum (8).

Unlike acquired immunity to tuberculosis, in which antibacterial cellular immunity is expressed as an enhanced capacity of the immune macrophages to suppress the intracellular multiplication of *Mycobacterium tuberculosis* (7), this study shows that antibacterial cellular immunity against salmonellosis is apparently mediated by cytophilic antibodies which provide enhanced specific attraction and opsonic action for the pathogen. Passive transfer of immune serum exerts its protective effect on the recipient by its opsonic action on salmonellae (24) and by its transfer of cytophilic antibodies onto the macrophages of the recipient. On the basis of the present experimental data, the innate capacity of macrophages to destroy ingested salmonellae, the enhanced clumping and phagocytic activities of immune macrophages mediated by cytophilic antibodies, and the opsonic effect of immune serum constitute the integral components of acquired immunity of the host working in synergism at a cellular level against salmonellosis. The development of hypersensitive reactions (8) in the infectious process may be beneficial to host resistance by accelerating the inflammatory response in the tissue which in turn generates a greater influx of cellular and humoral elements into the site of infection.

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