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CELLULAR AND HUMORAL ASPECTS OF HOST RESISTANCE IN MURINE SALMONELLOSIS*

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Summary.—Mice were challenged with a highly virulent strain of *Salmonella typhimurium* by intraperitoneal injections. At relatively low infecting doses, immunizations with either viable attenuated or heat killed *Salm. typhimurium* were found to be equally protective against otherwise fatal infections. Pre-opsonization of virulent salmonellae significantly increased the survival rate of mice infected with small numbers of the pathogen. By a cell culture method, peritoneal macrophages of mice were shown to be innately capable of destroying the ingested virulent *Salm. typhimurium*. Macrophages from previously infected mice did not appear to have any significant increase in their bactericidal activity against salmonellae, but they possessed cytophilic antibodies specific against the H and the O antigens of *Salm. typhimurium*. It is believed that humoral elements play an important role in acquired immunity in murine salmonellosis by opsonization of the pathogen.

IN EXPERIMENTAL murine salmonellosis, pathogenic salmonellae are considered by some investigators to be facultative intracellular parasites capable of proliferating freely within host cells (Blanden, 1968; Collins, 1969*a*, *b*; Mitsuhashi, Sato and Tanaka, 1961). Acquired immunity of the host depends largely on the ability of the activated macrophages to eliminate the ingested pathogens. In this context, specific antibodies against salmonellae, either produced by the host resulting from vaccination with killed bacteria or acquired by passive transfer of immune serum, promote phagocytosis of the pathogen but do not offer significant protection against a challenging infection (Collins, 1969*a*, *b*, 1973).

Contrary to this view, there is an increasing number of reports recently which provide convincing data to show the protective value of killed vaccines or of passive transfer of antiserum against otherwise fatal challenges with virulent salmonellae (Cronly-Dillon, 1972; Germanier, 1972; Herzberg, Nash and Hino, 1972; Ornellas, Roantree and Steward, 1970; Venneman and Berry, 1971). Studies using guinea-pigs as the experimental model in this laboratory have shown that: (a) the intracellular environment of macrophages is unfavourable for the survival of virulent *Salm. typhimurium* or *Salm. enteritidis* (Hsu and Mayo, 1973; Rhodes and Hsu, 1974); (b) macrophages from guinea-pigs previously infected with *Salm. typhimurium* are not endowed with any significant increase in their ability to destroy ingested virulent *Salm. typhimurium* (Hsu and Mayo, 1973); (c) these immune macrophages possess cytophilic antibodies specific against the H and the O antigens of *Salm. typhimurium* (Hsu and Mayo, 1973); (d) immune serum promotes phagocytosis of salmonellae by macrophages and, conversely, the virulence of different strains of *Salm. typhimurium* is directly related to their resis-

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tance to phagocytosis by host cells (Wells and Hsu, 1970); and (e) virulent *Salm. typhimurium* pre-opsonized with immune serum generates a less severe infection as assayed by cutaneous lesions in guinea-pigs (Hsu and Piper, 1972). Results presented in this paper confirm these principal observations using mice as the experimental model of salmonellosis.

MATERIALS AND METHODS

Salm. typhimurium.—The virulent strain SR-11 and the avirulent strain RIA were used in this study (Hsu and Radcliffe, 1968). By intraperitoneal (i.p.) injection into mice, the virulent strain had an LD_{50} of approximately 10 bacteria, while the avirulent strain had an LD_{50} of $> 10^6$ bacteria. The bacteria were grown in Tryptic Soy Broth (Difco) for 5–6 h and washed in saline by the procedure described previously (Hsu and Radcliffe, 1968). The optically standardized saline suspensions of bacteria contained approximately $2{\cdot}5 \times 10^9$ viable organisms/ml of strain SR-11 or 10^9 viable organisms/ml of strain RIA.

Preparations of bacterial antigens.—The preparation of the H or the O antigens of salmonellae or *Esch. coli* was described previously (Hsu and Mayo, 1973). The heat killed vaccines of *Salm. typhimurium* were prepared with strains SR-11 and RIA. The bacteria were grown in Tryptic Soy Broth for 6 h, harvested by centrifugation at 1100 *g* for 20 min and washed twice with saline. The suspensions were adjusted to a proper optical density representing approximately 10^9 viable bacteria/ml and then heated in a water bath at 70° for 30 min. After the suspension was checked for sterility, it was used as vaccine without further washing.

Animals.—Mice of the Swiss–Webster strain were originally purchased from Rockland Farm (Gilbertsville, Pennsylvania) and inbred in the animal facilities of the Department of Microbiology, Medical College of Virginia. Male mice weighing between 20 and 30 g each were used for infection with *Salm. typhimurium* and as macrophage donors. Male albino guinea-pigs were purchased from commercial sources and used as serum donors. They weighed between 600 and 800 g each.

Infection and immunization of mice.—Mice were infected or immunized by the i.p. route. An appropriate number of viable or killed bacteria in a volume of 0·5 ml was injected into mice according to the schedule specified in each experiment.

Collection of serum.—Mice were anaesthetized with chloroform and exsanguinated directly from the exposed heart with needle and syringe. Serum was pooled from several mice after the blood was allowed to clot overnight at 4°. It was checked for sterility and frozen until use. Serum was also pooled from guinea-pigs bled by cardiac puncture. Serum derived from animals previously infected with *Salm. typhimurium* is hereafter referred to as immune serum.

Cultivation of infected macrophages.—The basic procedure was previously developed for the cultivation of infected macrophages derived from guinea-pigs (Hsu and Radcliffe, 1968; Hsu and Mayo, 1973). Mice were injected i.p. with 1 ml of mineral oil (Marcol no. 90, Humble Oil and Refining Co.) and killed by cervical dislocation 2–3 days later. The peritoneal cavity was washed with approximately 7 ml of Hanks' solution containing 1% homologous serum and 6 u/ml of heparin (Liquaemin sodium " 10 ", Organon Inc.). After separation from the oil, the leucocytes were sedimented by centrifugation at 220 *g* for 10 min at 5°. Only peritoneal washings containing primarily leucocytes were pooled from 4–6 mice.

The pooled leucocyteswere suspended in 10 ml of Hanks' solution containing 0·25% trypsin (Nutritional Biochemicals Corp.) and incubated in an Eberbach waterbath at 37° with horizontal shaking at approximately 72 cycles/min for 10 min. The trypsinized cells were collected by centrifugation at 200 *g* for 10 min at 5° and resuspended in 2–4 ml of Hanks' solution containing heparin and 1% homologous serum. The cellular population was determined in a haemacytometer. Such a leucocyte suspension usually contained over 80% monocytes.

For the ingestion of virulent *Salm. typhimurium* by macrophages, a volume containing approximately 3×10^7 leucocytes was mixed with a volume containing $8{\cdot}75 \times 10^8$ viable bacteria in a test tube. Six-tenths of 1 ml of homologous normal serum was added and the final volume was brought to 4·5 ml with Hanks' solution. After the mixed suspension was rotated in a drum at 25 rev/min for 15 min at 37°, it was transferred into 2 40-ml silicone coated centrifuge tubes, each containing 30 ml of chilled Hanks' solution with 6 u/ml of heparin and 25 μg/ml of kanamycin (Kantrex, Bristol Lab.). The infected cells were separated from the uningested bacteria by centrifugation at 150 *g* for 10 min at 5°, pooled and resuspended in 4 ml of cell culture medium composed of 80% Medium 199 (Microbiological Associates, Inc.), 10% homologous serum, 10% isotonic 1·4% sodium bicarbonate (CO_2-saturated) and 25 μg/ml of kanamycin. The cell culture was maintained in a 25-ml silicone-coated Erlenmeyer flask which was closed with a rubber stopper and incubated in the Eberbach shaking water bath. At the beginning of cell culture and after each sampling, the flasks were flushed

gently with 5% CO_2 in air to maintain the cell suspension in a high CO_2 tension.

Determination of viable intracellular bacteria at intervals.—The principle of assessing the fate of *Salm. typhimurium* within infected macrophages by quantitative recovery of viable cell associated bacteria has been described previously (Hsu and Radcliffe, 1968; Hsu and Mayo, 1973). Before the incubation of the infected cell culture and at designated intervals thereafter, 0·25 ml of the cell suspension was withdrawn with a 1-ml pipette and treated as follows: (i) 0·1 ml of the sample was put into 1·9 ml of chilled Hanks' solution and centrifuged at 100 *g* for 5 min at 5° to sediment the infected cells. The supernatant fluid was removed and diluted with 2 ml saline. The population of viable bacteria in this fluid was determined after serial dilution and by the pour-plate method using Tryptic Soy Agar. The sedimented infected cells were resuspended in 2 ml of saline and sonicated in a polyethylene test tube in a Biosonik II apparatus (Bronwill Scientific) at a "needle" probe intensity setting of 30 for 15 sec. The released viable bacteria were enumerated by pour-plate method after ten-fold serial dilutions. (ii) 0·1 ml of the sample was mixed with equal volume of 0·25% eosin-Y (Fisher Scientific Co.). From this, the total and viable cellular populations were determined in a haemacytomoter. In all the experiments reported here, over 90% of the leucocytes retained their ability to reject the dye throughout the period of observation.

The ratio of bacteria to cells for each interval was computed by dividing the number of viable bacteria recovered from the infected cell sediment in 0·1 ml sample by the total number of leucocytes present in 0·1 ml sample. The phagocytic index was previously defined as the ratio of bacteria/cell determined at 0 h (Wells and Hsu, 1970). The fate of intracellular bacteria was presented by plotting the ratios calculated by dividing the number of bacteria/cell recovered at intervals by the phagocytic index.

Detection of cytophilic antibodies on macrophages.—Mice were inoculated with 10^5 viable bacteria of the avirulent strain, followed by a second inoculum of 10^4 viable bacteria of the virulent strain 2–3 weeks later. Peritoneal macrophages were pooled from 2 or 3 normal or infected mice after stimulation with mineral oil. The cellular suspensions were adjusted to a concentration of approximately 7×10^6 leucocytes/ml and inactivated with Merthiolate in Hanks' solution at a final concentration of 1 : 5000. Using Pasteur pipettes, one drop of the cellular suspension and one drop of the bacterial antigens were mixed on an agglutination slide by manual rotation. Specific agglutination of macrophages with bacterial antigens was usually seen within 30 min.

RESULTS

Acquired resistance to reinfection with virulent Salm. typhimurium

Groups of mice were inoculated i.p. with 10^5 or 10^6 viable bacteria of the avirulent *Salm. typhimurium* RIA. Three weeks later, the majority of these mice survived. They were challenged with either 10^2 or 10^3 bacteria of the virulent *Salm. typhimurium*, along with groups of normal mice. Figure 1 shows that the infecting dose of either 10^2 or 10^3 virulent salmonellae was fatal to at least 80% of the normal mice within 10 days after the infection. By contrast, over 90% of the previously infected animals were protected from such a fatal infection. The only exception appeared in the group where the mice were inoculated with 10^5 avirulent bacteria followed by 10^2 virulent bacteria. The survival rate here was 77% at the end of 3 weeks post infection. Seven control mice inoculated with 10^6 viable bacteria of the avirulent strain at the beginning of this experiment survived through the 6-week period of the experiment, indicating that the primary infection did not incur further mortality after the the first 3 weeks of the experiment.

Protective effect of heat killed vaccine against salmonella infection

Bacterial suspensions inactivated by heating at 70° for 30 min were used as vaccine. The equivalence of 10^6 or 10^7 viable bacteria of either the avirulent or the virulent *Salm. typhimurium* were injected i.p. into groups of mice. Three weeks after immunization, the mice were challenged with 10, 10^2 or 10^3 viable virulent *Salm. typhimurium.* Figure 2 shows that in this series of experiments, the LD_{50} of the challenging salmonellae was approximately 10 viable bacteria. The heat killed vaccine inoculated 3 weeks before infection protected over 80% of the mice from a fatal infection even at the challenging dose of 10^3 bacteria. The vaccine prepared from the avirulent strain was as effective as that prepared from the

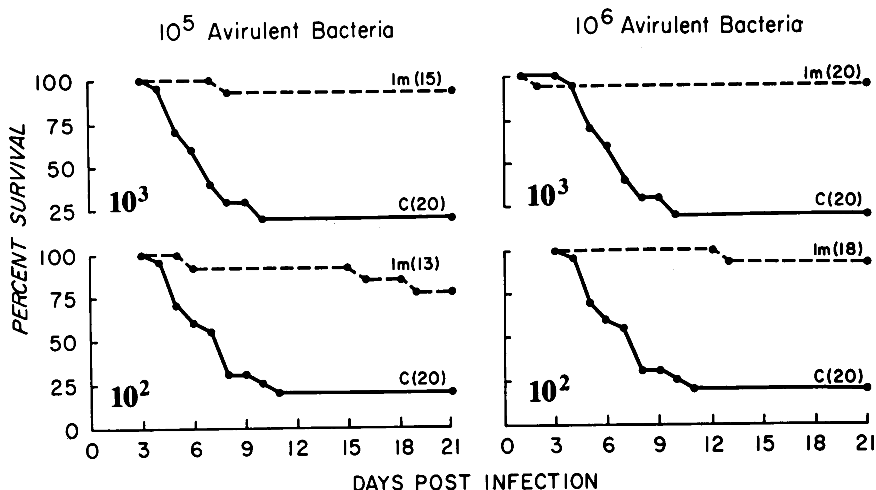

FIG. 1.—Acquired resistance to reinfection with virulent *Salm. typhimurium.* Mice were inoculated with a primary infection of 10^5 or 10^6 avirulent *Salm. typhimurium* (Im) and then challenged with 10^2 or 10^3 virulent *Salm. typhimurium,* along with normal mice (C). The number on the lower left corner of each section indicates the challenge dose of virulent bacteria. The number in parentheses indicates the number of mice in each group. The same groups of normal mice (C) are shown in the left and the right columns.

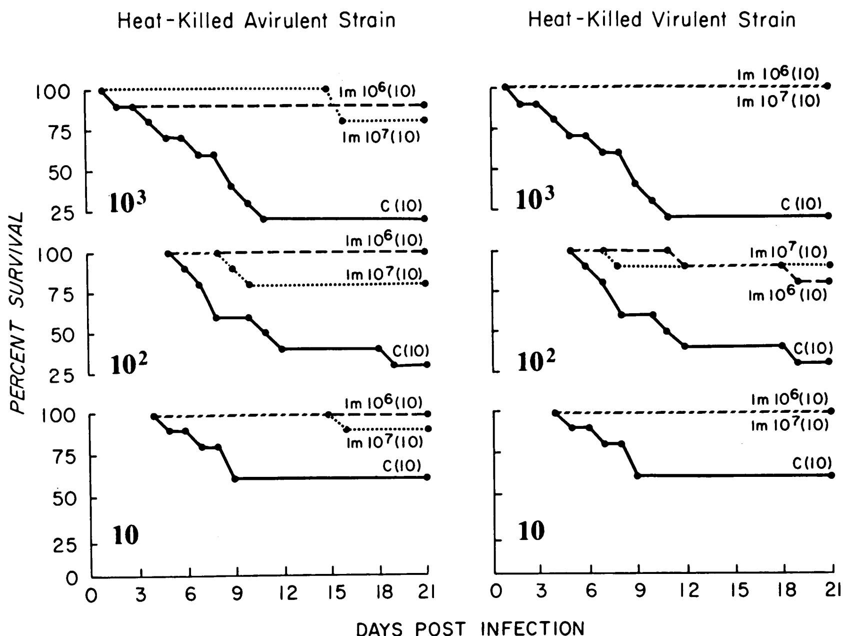

FIG. 2.—Protective effect of heat killed vaccine against salmonella infection. Mice were immunized with 10^6 or 10^7 heat killed *Salm. typhimurium* (Im) and then challenged with 10, 10^2 or 10^3 virulent *Salm. typhimurium,* along with normal mice (C). The number following Im indicates the dose of vaccine. The number on the lower left corner of each section indicates the challenge dose of virulent bacteria. The number in parentheses indicates the number of mice in each group. The same groups of normal mice (C) are shown in the left and the right columns.

challenging virulent strain. Furthermore, vaccines containing 10^7 killed bacteria did not appear to be any more effective against the levels of challenging doses than those containing 10^6 killed bacteria.

Protection against salmonella infection by passive transfer of serum

Immune serum was collected from guinea-pigs infected with virulent *Salm. typhimurium* as described previously (Hsu and Mayo, 1973). Mice were inoculated i.p. with 10^2 viable virulent *Salm. typhimurium*. Eight days thereafter the apparently healthy survivors were bled. Pools of immune serum were tested for sterility before use for passive transfer. The immune serum from guinea-pigs had an agglutination titre against the O Ag of *Salm. typhimurium* SR-11 of approximately 1 : 160, while the immune serum from mice had a titre of $< 1 : 10$. These immune sera did not have any demonstrable bactericidal activity against *Salm. typhimurium* SR-11.

Saline suspensions of the virulent strain SR-11 were adjusted to a concentration of approximately 8×10^4 viable bacteria/ml and mixed in equal volume with saline, normal or immune serum. The mixtures were left standing at room temperature for 15 min and serial dilutions were made in saline, so that each inoculum of 0·5 ml contained 2×10^3, 2×10^2 or 2×10 bacteria. Vigorous pipetting was done throughout to ensure dispersion of the bacteria. Samples were taken from each dilution for viable bacterial counts to verify the concentration of the inocula.

Several sets of experiments were performed according to this protocol. The results are compiled in Fig. 3. The LD_{50}

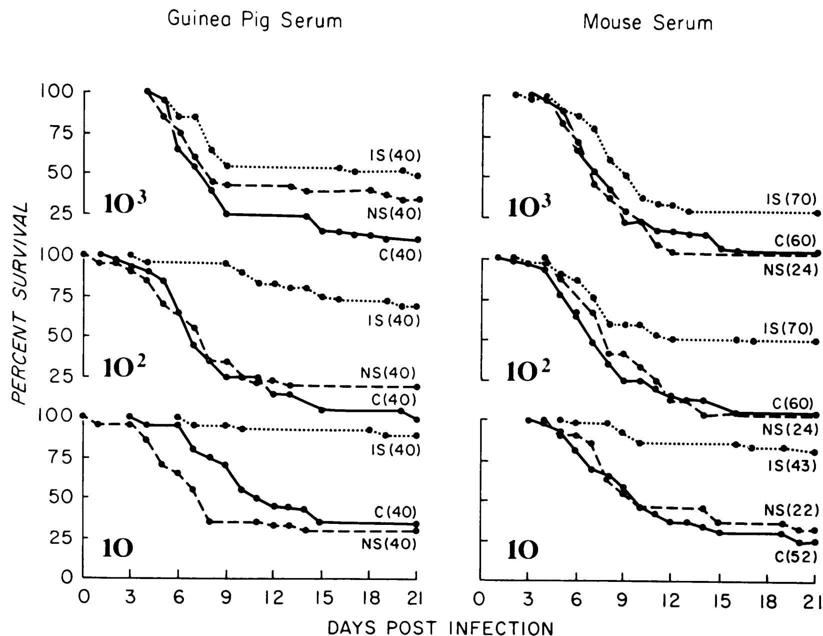

FIG. 3.—Passive protection against salmonella infection by immune serum. Mice were challenged with 10, 10^2 or 10^3 virulent *Salm. typhimurium* pre-opsonized with immune serum (IS) from guinea-pigs or mice. Their survival rate was compared with mice challenged with same doses of bacteria suspended in saline (C), or in normal serum (NS) from guinea-pigs or mice. The number on the lower left corner of each section indicates the challenge dose of virulent bacteria. The number in parentheses indicates the number of mice in each group.

of the virulent *Salm. typhimurium* as determined in these experiments was <20 bacteria. The addition of normal serum to the infecting inocula did not alter the survival rate of the infected mice, except in the case of normal guinea-pig serum, where it appeared to have slightly improved the survival rate at the infectious dose of 10^3 bacteria. On the other hand, the presence of immune serum, either from guinea-pigs or mice, had clearly provided a significant protective effect on mice against infectious doses of 10 or 10^2 bacteria. Even at the infectious dose of 10^3 bacteria, there was noticeable improvement in the survival of the infected mice.

Fate of virulent Salm. typhimurium *within macrophages of normal and previously infected mice*

Peritoneal macrophages were pooled from normal mice and, without prior trypsinization, allowed to ingest virulent *Salm. typhimurium*. The infected cells were washed and cultured in medium containing normal serum. The fate of the intracellular bacteria was determined. Figure 4A depicts the average of 2 sets of 3 experiments, one at the average phagocytic index of $0{\cdot}3 \pm 0{\cdot}01$ (s.d.) and the other at $0{\cdot}5 \pm 0{\cdot}1$. The macrophages of normal mice were highly efficient in eliminating the ingested salmonellae. The initial intracellular destruction of bacteria within the first 2 h was significantly greater at the lower phagocytic index of 0·3 ($P < 0{\cdot}05$); as determined by the Student's *t*-test. Simple regression analysis showed that there was no significant difference in the rate of the subsequent destruction of the intracellular bacteria ($P > 0{\cdot}1$).

When pooled peritoneal macrophages from previously infected mice were ex-

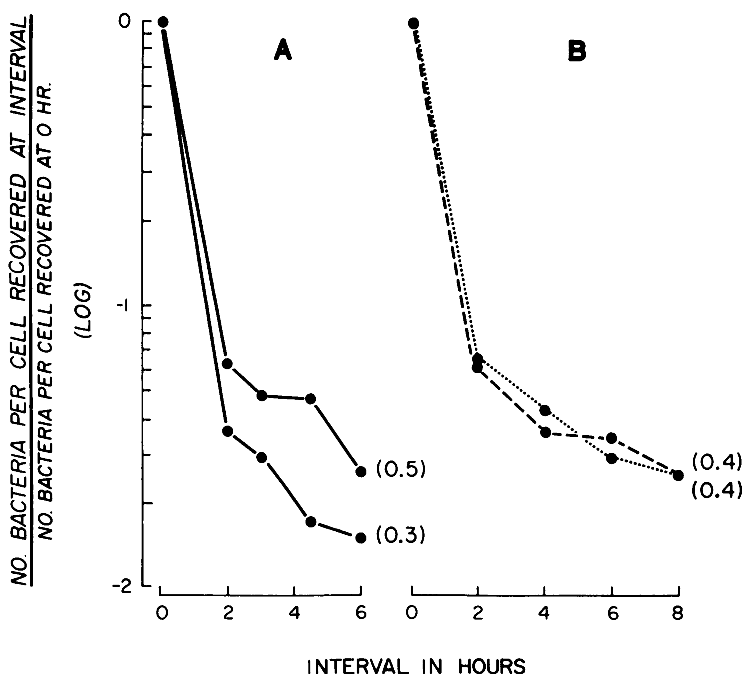

FIG. 4.—A. Destruction of virulent *Salm. typhimurium* within macrophages of normal mice. The number in parentheses represents the phagocytic index of each set of experiments. B. Comparative rate of intracellular destruction of *Salm. typhimurium* within the trypsinized (dotted line) and the untreated (broken line) normal macrophages. The number in parentheses represents the phagocytic index of each set of experiments.

posed to virulent *Salm. typhimurium*, they clumped together tightly. This interfered with the enumeration of total cellular population in sampling. If these macrophages were first trypsinized at 37° for 15 min, they would not clump together after infection. The following experiments were made to check the effect of trypsin treatment of macrophages on their capacity to destroy *Salm. typhimurium*.

Peritoneal macrophages were divided into 2 portions; one was treated with trypsin as described above and the other was incubated in Hanks' solution as control. The cells were then washed and allowed to ingest virulent *Salm. typhimurium* and cultured in the presence of normal serum. Figure 4B presents the average of 3 experiments which had an average phagocytic index of 0·4 ± 0·01 (s.d.) for the trypsinized cells, and 0·4 ± 0·03 for the untreated cells. Trypsinization of macrophages before infection appeared to have no significant effect on their ability to ingest or to destroy salmonellae.

Mice were immunized with 10^5 viable bacteria of the avirulent *Salm. typhimurium* and then used as donors of macrophages and immune serum 2–3 weeks thereafter. Pooled suspensions of normal or immune macrophages were sampled on Tryptic Soy Agar plates. The suspensions containing normal cells were consistently sterile whereas that of immune cells usually produced a few colonies of *Salm. typhimurium* residual from the primary immunization. The latter was not considered sufficient to interfere with the quantitation of intracellular salmonellae in cell culture. The cells were trypsinized, infected with virulent *Salm. typhimurium* in the presence of normal serum and cultured.

Figure 5A shows the average of 3

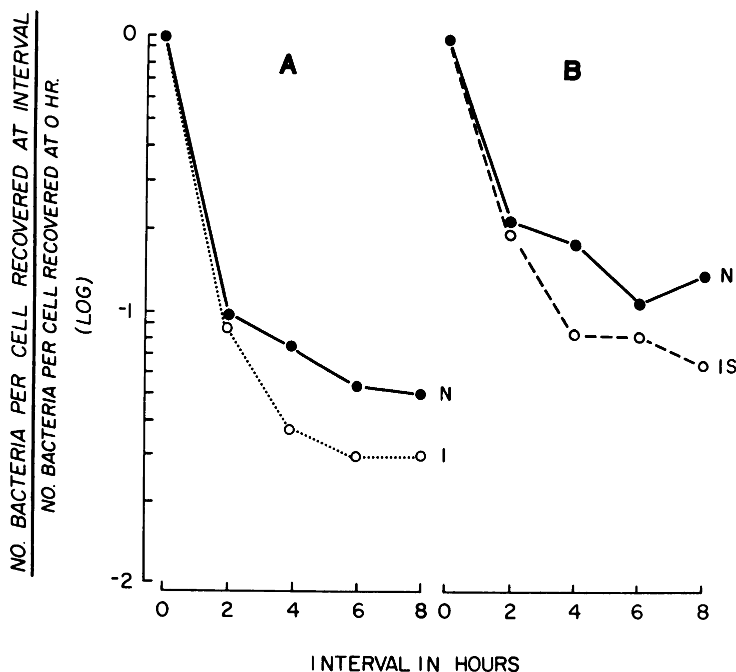

FIG. 5.—A. Comparative rate of destruction of virulent *Salm. typhimurium* within normal (N) and immune (I) macrophages, both cultured in normal serum. B. Comparative rate of destruction of virulent *Salm. typhimurium* within normal serum (N) and immune macrophages cultured in immune serum (IS).

experiments in which both the infected normal and immune macrophages were cultured in medium containing normal serum. The average phagocytic indices in these experiments were $0 \cdot 3 \pm 0 \cdot 05$ (s.d.) for the normal cells and $0 \cdot 3 \pm 0 \cdot 07$ for the immune cells. It appeared that the immune cells possessed a slightly greater capacity to destroy the ingested salmonellae. However, the difference was not shown to be significant by analysis of variance ($P > 0 \cdot 1$).

An additional set of 3 experiments were performed in which both the normal cells and the immune cells were allowed to ingest virulent *Salm. typhimurium* in the presence of normal serum, but the infected normal cells were then cultured in medium containing normal serum, whereas the infected immune cells were cultured in medium containing immune serum. The results are presented in Fig. 5B. The phagocytic indices in these experiments were $0 \cdot 5 \pm 0 \cdot 1$ (s.d.) for the normal cells and $0 \cdot 4 \pm 0 \cdot 09$ for the immune cells. Again, there appeared to be a trend for the immune cells to have a somewhat greater capacity to eliminate the ingested salmonellae than the normal cells. However, t-test analysis of the initial destruction ($P > 0 \cdot 1$) and regression analysis of the continued destruction ($P > 0 \cdot 1$) failed to show any significant difference in the bactericidal activities of these 2 populations of macrophages.

Opsonic effect of immune serum on Salm. typhimurium

A comparison was made of the opsonic effect of immune serum of mice on the virulent strain of *Salm. typhimurium.* In the procedure for phagocytosis described above, 3×10^7 normal peritoneal leucocytes were mixed with $8 \cdot 75 \times 10^8$ bacteria (an approximate ratio of 1 : 30) in a final volume of 4·5 ml containing 0·6 ml normal serum. In order to achieve a closely identical phagocytic index when phagocytosis was to occur in the presence of immune serum, 3×10^7 normal leucocytes were mixed with $6 \cdot 75 \times 10^8$ bacteria (an approximate ratio of 1 : 22) in a final volume of 4·5 ml containing 0·4 ml immune serum. In 4 experiments set up in this manner, the average phagocytic index was $0 \cdot 4 \pm 0 \cdot 02$ (s.d.) for both the normal and the immune serum. This implies that the normal peritoneal macrophages have an enhanced phagocytic activity in the presence of immune serum.

Specific cytophilic antibodies on macrophages of mice previously infected with Salm. typhimurium

When macrophages from previously infected mice were allowed to ingest virulent *Salm. typhimurium* and cultured *in vitro*, the cells clumped together and could not be dispersed even with vigorous pipetting. It was presumed that this clumping was due to the presence of cytophilic antibodies on the cellular surface. Freshly harvested peritoneal macrophages from normal and previously infected mice were tested for their ability to agglutinate with various bacterial antigens as described above. The results are shown in the Table. In the present

TABLE.—*Agglutination of Macrophages with Bacterial Antigens*

		*Salm. typhimurium**			*Salm. enteritidis*	*Salm. typhi*	*Esch. coli*
	Saline	Live	O Ag	H Ag	O Ag	O Ag	O Ag
NC	−	−	−	−	−	−	−
IC	−	+	+	+	−	−	−

Ag = antigen,
NC = normal cells,
IC = immune cells,
* Both strains RIA and SR-11 of *Salm. typhimurium* were used.

conditions of infecting the mice with the avirulent, followed by the virulent *Salm. typhimurium*, the immune macrophages agglutinated with the live, the H and the O antigens of both strains of *Salm. typhimurium*. However, at the level of sensitivity of the present test, there was no demonstrable cross-agglutination of immune macrophages with the O antigens of different species of *Salmonella*, nor with

the O antigens of another genus of gram-negative bacilli.

DISCUSSION

In experimental salmonellosis, it is generally held that a previous infection with viable attenuated vaccine provides a more effective immunity to subsequent challenge with virulent salmonellae than does killed vaccine (Collins, 1969*b*, 1973; Germanier, 1972; Herzberg *et al.*, 1972). Nevertheless, killed vaccines were shown in many studies to be effective in reducing the mortality significantly against an otherwise fatal infection (Cronly-Dillon, 1972; Germanier, 1972; Herzberg *et al.*, 1972; Ornellas *et al.*, 1970). There appear to be two major differences in these two procedures of immunization. Animals protected by killed vaccines failed to suppress the proliferation of challenge inocula in the reticuloendothelial system despite an initial reduction of the inocula, as did those immunized with attentuated bacteria (Collins, 1969*b*; Mackaness, Blanden and Collins, 1966). In addition to the development of protective immunity viable vaccines induced in the host the development of delayed-type hypersensitivity to a protein containing fraction of the salmonella culture, which was not attained by killed vaccines (Collins and Mackaness, 1968).

Despite the criticism that peritonitis is not a feature of the early stages of mouse typhoid (Blanden, Mackaness and Collins, 1966), one must concede that the peritoneal cavity does provide certain advantages in analysing the relative role of cellular and humoral factors in the infection such that the pathogen may freely multiply intracellularly or extracellularly, or both, without being rapidly removed at the early phase of the infection by the reticuloendothelial system as in the case of intravenous inoculation. In the present study, the virulent *Salm. typhimurium* has an LD_{50} of approximately 10 bacteria by i.p. injection (Fig. 1, 2, 3). When protection against infection was evaluated by mortality resulting from i.p. infection, small challenge inocula were used in this study in order to avoid the complication of an early effect of bacterial endotoxins. Figure 1 shows that mice given a primary i.p. infection of 10^5 or 10^6 avirulent *Salm. typhimurium* became immune to a challenge infection of 10^2 or 10^3 virulent *Salm. typhimurium.* Figure 2 shows that an essentially identical degree of protection against similar infection could be achieved by immunization with 10^6 or 10^7 heat-killed bacteria of either the avirulent or the virulent strain of *Salm. typhimurium.* Whether killed vaccine could provide an equally effective protection as viable vaccine against much higher challenge doses was not assessed in this study. In another study, virulent *Salm. typhimurium* were premixed with saline, normal or immune serum from mice or guinea-pigs, diluted to appropriate quantities and injected i.p. into mice. Figure 3 shows that the presence of normal serum in the inoculum had no effect on the challenged animals. By contrast, the presence of immune serum protected the mice from an otherwise fatal infection with 10 organisms and offered substantial protection against an infection with 10^2 bacteria. Even at the challenge dose of 10^3 bacteria, there was consistent evidence of increased survival of the infected mice. Similarly, immune serum was also shown to be effective in reducing the size of cutaneous lesions generated by virulent *Salm. typhimurium* in guinea-pigs (Hsu and Piper, 1972).

It has long been asserted that salmonellae are facultative intracellular parasites (Collins, 1969*b*) and that the antibacterial immunity in murine salmonellosis is cellular in nature with humoral elements playing a relatively minor role (Collins, 1969*a*). There appears to be the misleading premise that the proliferation of salmonellae in the organs of the reticuloendothelial system is necessarily confined to the intracellular location but not the extracellular tissues (Blanden *et al.*, 1966; Collins, 1969*b*) and that passive protection against salmonellosis by the transfer of

immune lymphoid cells necessarily excludes the ability of the transferred cells to produce protective antibodies in the recipients (Smith and Bigley, 1972). Otherwise, there is little authenticated experimental evidence of proliferation of salmonellae within host leucocytes. Investigations were undertaken in this laboratory on the fate of virulent salmonellae within macrophages of guinea-pigs and mice by a cell culture method. The present study shows that the intracellular environment in mice is also unfavourable for the intracellular survival of virulent *Salm. typhimurium* (Fig. 4, 5), as has been shown previously in the case of guinea-pigs (Hsu and Mayo, 1973; Rhodes and Hsu, 1974). It was argued that since macrophages of normal animals possessed an innate capacity to inactivate the virulent salmonellae, the expression of antibacterial cellular immunity as an enhanced capacity of the immune macrophages to destroy the ingested pathogen could no longer be considered a crucial issue in the acquired resistance of the host (Hsu and Mayo, 1973). Figure 5 confirms that the macrophages from previously infected mice were not endowed with a significant increase in their capacity to eliminate the ingested salmonellae, regardless of whether the host cells were maintained in culture medium containing normal or immune serum. This observation is therefore contrary to the claims made by other investigators (Blanden, 1968; Blanden *et al.*, 1966; Mitsuhashi *et al.*, 1961). It should be noted parenthetically that the immune macrophages used here were derived from infected mice at a "carrier state", since salmonellae were isolated from their peritoneal washings. On the other hand, the presence of specific cytophilic antibodies on the surface of immune macrophages was regarded as a manifestation of acquired cellular immunity by enhanced clumping and phagocytic activities against the invading pathogen (Hsu and Mayo, 1973). The Table depicts the species specific nature of the cytophilic antibodies on the macrophages of previously infected mice against the H and the O antigens of both the avirulent and the virulent strains of *Salm. typhimurium*. At the level of sensitivity of the present test, there appeared to be no cross-agglutination of the Merthiolate-inactivated immune macrophages with the O antigens of other species of *Salmonella* or of *Esch. coli*. Previous studies on the cytophilic antibodies in guinea-pigs further showed that they could be eluted from immune macrophages by incubation in the absence of immune serum and, conversely, they could be passively transferred on to normal macrophages by incubation in the presence of immune serum (Hsu and Mayo, 1973) Furthermore, there appeared to be cytophilic antibodies of at least 2 specificities (the H and the O antigens) on the surface of the immune macrophages. It appears likely that the cytophilic antibodies described here are comparable with the cell bound antibodies described by Rowley, Turner and Jenkin (1964) and by Ushiba *et al.* (1966). On the other hand, these cytophilic antibodies are not identical to those seen on lymphoid cells of immunized mice (Margolis and Bigley, 1972). The latter were found to be a macroglobulin reacting specifically against protein-rich components of the whole cells of *Salm. typhimurium*.

The use of cell culture methods in the analysis of host–parasite interactions offers the distinct advantage of identifying the relative role of cellular and humoral elements in host resistance to infectious agents. However, certain technical pitfalls inherent with the experimental procedure must be recognized. First, the multiplication of *Salm. typhimurium* in the cell culture medium must be effectively controlled. Otherwise, the determination of the fate of intracellular salmonellae would be complicated by a continuous phagocytosis of the extracellular bacteria by the host cells. Kanamycin was incorporated into the cell culture medium to suppress the extracellular bacterial multiplication. It was shown previously (Rhodes and Hsu,

1974) that the destruction of salmonellae by macrophages of guinea-pigs was not likely to be due to the interference of kanamycin entering into the host cells. Second, the quantitative recovery of viable intracellular bacterial population in a sample of infected cell culture necessitated a complete disintegration of the host cells without significant damage to the intracellular parasite. The failure to achieve this objective, together with the clumping of the infected immune macrophages due to cytophilic antibodies, was most likely to be responsible for the apparently enhanced intracellular killing by immune macrophages as reported by Blanden *et al.* (1966). In the present experimental design, it was also necessary to maintain a dispersed infected cell suspension in the culture in order to quantitate the leucocytic population when sampling. Pretreatment of macrophages with trypsin before infection *in vitro* prevented the clumping of the infected immune cells in culture. Figure 4B shows that trypsinization of macrophages had no effect on the bactericidal action of the macrophages on *Salm. typhimurium*. Trypsinization apparently destroyed the antigen binding activities of the cytophilic antibodies in the immune macrophages, but it probably did not remove the immunoglobulin from the cellular surface (Hsu and Mayo, 1973). Reconstitution of the *in vitro* environment by incorporating immune serum into the culture medium did not confer on the immune macrophages any altered ability to cope with the ingested salmonellae (Fig. 5B). Collectively, the cell culture experiments reported here demonstrated that the macrophages of normal mice were innately capable of eliminating the ingested virulent *Salm. typhimurium*, provided that the extracellular bacterial population was effectively controlled. The efficiency of this intracellular killing was dependent on the phagocytic index (Fig. 4A) but independent of the presence of immune serum (Fig. 5B). As consistent with the previous report (Wells and Hsu, 1970), immune serum of mice facilitated the ingestion of salmonellae by macrophages of mice.

It is clear from the data presented here that the host–parasite relationship in experimental salmonellosis in mice is basically analogous to that previously observed in guinea pigs. With the information derived from the present investigation, the following composite analysis of the host–parasite interactions in murine salmonellosis may be proposed: In view of the innate capacity of the host macrophages to destroy intracellular salmonellae, the pathogenicity of virulent *Salm. typhimurium* depends largely on its resistance to phagocytosis and its ability to propagate extracellularly in the host tissue. Conversely, host resistance to the pathogen depends greatly on the efficiency with which the extracellular bacteria are eliminated by phagocytosis, which is enhanced by immune serum. On this basis, when a population of virulent *Salm. typhimurium* is inoculated into the peritoneal cavity of a mouse, the majority of the pathogens avoid being phagocytized, multiply extracellularly and disseminate to the organs of the reticuloendothelial system until a lethal bacterial population is attained. On the other hand, when 10 or 10^2 bacteria are pre-opsonized before inoculation (Fig. 3), they would be effectively eliminated by the macrophages in the peritoneal cavity. However, when a larger number of pre-opsonized bacteria is introduced, some of the organisms successfully escape ingestion by the limited population of phagocytes initially present. These organisms multiply and their progeny would no longer be opsonized due to the low level of antibodies transferred into the site of infection. A fatal infection would then ensue. The major advantage of immunization with heat-killed bacteria (Fig. 2) might be the active production of protective antibodies in the host so that the inflammatory response to the peritoneal infection would infuse the site with specific opsonins and phagocytes to cope with the proliferating extracellular patho-

gen. It would therefore be possible for the host to protect itself against a much larger initial challenge. The induction of delayed-type hypersensitivity by immunization with viable attenuated vaccines or by a previous infection might be considered highly beneficial to the acquired resistance of the host, in that its elicitation by a subsequent infection would accelerate the influx of cellular and humoral elements into the foci of infection by its enhanced inflammatory response. Such an additional advantage might in part account for the ability of the immunized host to inhibit the growth of the parasite *in vivo* (Collins, 1969*b*; Mackaness *et al.*, 1966).

The above proposal is in agreement with the general thesis of the cellular nature of host immunity against salmonellosis in that it is the innate bactericidal capacity of the macrophages which is ultimately responsible for the elimination of the pathogen. There is no evidence of bacteriolytic effect by the immune serum in the present experimental model since the strain of *Salm. typhimurium* used was not sensitive to the antiserum. Rather, protective antibodies play a crucial role in acquired immunity by facilitating the removal of the proliferating salmonellae from their extracellular location into a hostile intracellular environment. Immune macrophages armed with specific cytophilic antibodies may represent a manifestation of antibacterial cellular immunity by enhanced clumping and phagocytic activities against the pathogen. Hence, antibacterial cellular immunity in salmonellosis is not analogous to that expressed in tuberculosis, in which the immune macrophages were shown to have an enhanced capacity to suppress the intracellular multiplication of tubercle bacilli (Hsu, 1965).

In essence, therefore, the innate capacity of macrophages to destroy ingested salmonellae, the enhanced cellular activities of macrophages mediated by cytophilic antibodies, the opsonic and agglutinating actions of immune serum and the accelerated inflammation due to hypersensitivities constitute the integral components of acquired immunity of the host working in synergism against salmonellosis.

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