

## MECHANISMS OF ACQUIRED RESISTANCE IN MOUSE TYPHOID\*

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A previous paper (1) showed that when the serum of mice infected with *Salmonella typhimurium* was injected intravenously into normal mice, it did not materially influence the course of an intravenous infection in the recipients, even though the donors of the serum were completely resistant to reinfection. This implies that factors other than circulating antibody are necessary for the full expression of acquired resistance in this disease. The same conclusion has been reached by other workers (2, 3) who have postulated the existence of a cellular form of immunity.

Acquired cellular resistance has been shown to occur in tuberculosis (4), brucellosis (5, 6), and listeriosis (7, 8). The increased microbicidal capacity of macrophages from animals infected with these bacteria is not specifically directed against the organisms which induced it (6), with the result that cross-resistance has been found to exist between these infections (2, 6, 9, 10). Resistance disappears with the resolution of the infection, but can be rapidly recalled by reinfection with the homologous organism (6). Heterologous organisms do not have this effect, suggesting an immunological basis for acquired cellular resistance (6). It seems, therefore, that a *specific* immunological reaction is involved in the production of macrophages with enhanced, but *nonspecific*, microbicidal ability.

However, in the case of mouse typhoid an alternative mechanism has been proposed (11) to explain the cellular resistance which appears to develop in this disease. Specific macroglobulin antibody can be eluted from the peritoneal cells of mice infected with *S. typhimurium* (12). This antibody produced rapid clearance of virulent *S. typhimurium* from the peritoneal cavities of normal mice. From this and other evidence (13) it was inferred that the enhanced microbicidal activity of the macrophages of infected animals is a property conferred on them by adsorbed antibody and is not due to an intrinsic change in cell function. An essential corollary would then be that the increased bacteri-

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cidal ability of such macrophages is wholly specific, a feature which would make them readily distinguishable from cells with nonspecific microbicidal activity.

The present investigation assesses the relative contributions of humoral and cellular factors in acquired resistance to *S. typhimurium*, and examines the specificity of the bactericidal mechanisms involved.

#### *Materials and Methods*

*Animals.*—Mice of the outbred Swiss-Webster strain were used at 6 to 10 wk of age.

*Organisms.*—The strains of *Salmonella typhimurium* (C<sub>5</sub>S and C<sub>5</sub>R) were the same as those used in the preceding paper (1). The strain of *Listeria monocytogenes* was a recent human isolate supplied by Dr. E. G. D. Murray, and was virulent for mice (intravenous LD<sub>50</sub> = 1.0 × 10<sup>5</sup>). It was grown in tryptic soy broth (Difco Laboratories, Inc., Detroit). Plate counts were performed on well dried tryptic soy agar (Difco).

*Serum.*—Antiserum to *S. typhimurium* was obtained from two sources: (a) Serum of actively infected mice. A small group of animals surviving on the 21st day after intraperitoneal injection of 10<sup>5</sup> living *S. typhimurium* were bled from the tail under vacuum. (b) Serum of mice immunized with heat-killed *S. typhimurium*. The vaccine was prepared by heating a dense saline suspension of the organisms at 56°C for 1 hr. Plating revealed no viable bacteria. Five doses each of 10<sup>8</sup> organisms were given alternately by the intravenous and intraperitoneal routes over a period of 2 wk. The sterility of the vaccine was confirmed by plating homogenates of the livers and spleens of five randomly selected mice 2 wk after the last injection when all other mice were bled.

Antiserum to *Listeria monocytogenes* was obtained from mice infected intraperitoneally with 5 × 10<sup>2</sup>, and then reinfected 1 month later with 5 × 10<sup>5</sup> viable organisms. The animals were bled 9 days after the second injection.

Normal serum was obtained from unimmunized mice of similar age. All sera were sterilized by membrane filtration, stored at -20°C in small volumes, and used immediately after thawing.

*Experiments in Vitro.*—Log-phase cultures of *S. typhimurium* were dispersed for 10 sec by ultrasound (Bronwill Biosonik). Direct counts were made in a Petroff-Hausser chamber. The cultures were diluted and suspended in 20% serum in balanced salt solution (BSS) at a concentration of 10<sup>7</sup> per ml. The suspensions were stood for 30 min at 4°C and then diluted 1:10 with BSS containing 1% foetal calf serum (FCS). After redispersing with ultrasound duplicate viable counts were made immediately prior to injection. Approximately 10<sup>5</sup> bacteria were injected intraperitoneally into test mice in a volume of 0.1 ml. After precisely timed intervals the animals were killed. 1 min later their peritoneal cavities were washed out with 2.5 ml of BSS containing 10 IU heparin per ml at a temperature of 38°C. Aliquots (0.1 ml) of the peritoneal washings from each mouse were dispensed into a series of paraffin-lined tubes containing 1.9 ml of 1% FCS in BSS at 38°C. At accurately timed intervals a tube from each series was rapidly cooled to 2°C. Preliminary experiments had shown that chilling prevented any further bacterial inactivation. Duplicate platings of 0.1 ml each were made from individual tubes to give the total number of viable bacteria present at each time point. All tubes were then centrifuged for 5 min (420 g) to deposit cells (14). The numbers of extracellular bacteria were determined from duplicate platings of the supernatant. The number of cell-associated viable bacteria at each point was calculated by subtracting this number from the total viable counts. Automatic-zero pipettes (error ± 0.001 ml) were used in all the foregoing procedures. Streptomycin agar was used to differentiate between streptomycin-sensitive and streptomycin-resistant strains where necessary (1).

*Bacterial Enumeration in the Spleen and Liver.*—The method used has been described previously (1).

## RESULTS

The failure of immune serum to influence bacterial growth in liver and spleen suggests that cellular factors may play an essential part in acquired resistance to *S. typhimurium* (1). Experiments were therefore conducted *in vitro* to determine whether enhancement of the microbicidal ability of macrophages occurs in infected animals.

*Explanation of the Technique used to Study Bacterial Survival in Macrophages.*—To compare the microbicidal properties of normal and “immune” macrophages it was essential to ensure a rapid uptake of organisms by each cell population.

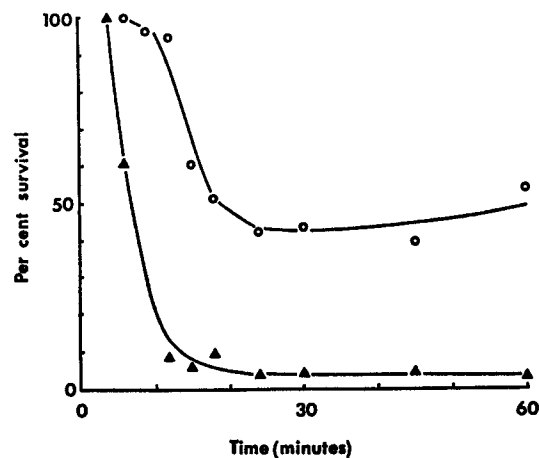


FIG. 1. Curves showing the intracellular inactivation of *S. typhimurium* (C<sub>6</sub>R) in macrophages from normal (O—O) and *S. typhimurium*-infected (▲—▲) mice.

For technical reasons this cannot be done *in vitro*, but can be achieved in the peritoneal cavity of the mouse. However, *S. typhimurium* is not phagocytized readily in the peritoneal cavities of normal mice unless it has been pretreated with specific antibody. Moreover, the peritoneal cavities of actively immunized mice would be expected to contain specific antibody, which has been said to influence bacterial survival after ingestion (15–17). Thus it was important for three reasons that it be made available so that comparable conditions would prevail during phagocytosis by both normal and immune cell populations. It was also essential to limit the period of phagocytosis to the shortest possible interval of time so that the duration of intracellular residence of organisms could be defined within narrow limits, thus allowing accurate comparison of the rates of inactivation in the two cell populations. Preliminary experiments showed that adequate phagocytosis of opsonized bacteria could not be achieved in normal animals in less than 5 min of intraperitoneal residence. But as bacterial inactivation in normal cells could not be detected within this period

it was possible to allow sufficient time for phagocytosis and still observe the whole of the bacterial inactivation curve in vitro. A shorter period for phagocytosis (2.5 min) was possible in immune animals because of more rapid ingestion.

*The Inactivation of S. typhimurium (C<sub>5</sub>R) by Macrophages of Normal and Specifically Immunized Mice.*—Experiments were performed in vitro to compare the behavior of *S. typhimurium* (C<sub>5</sub>R) within isolated macrophages from normal and immune mice. The latter were animals surviving 21 to 26 days after infec-

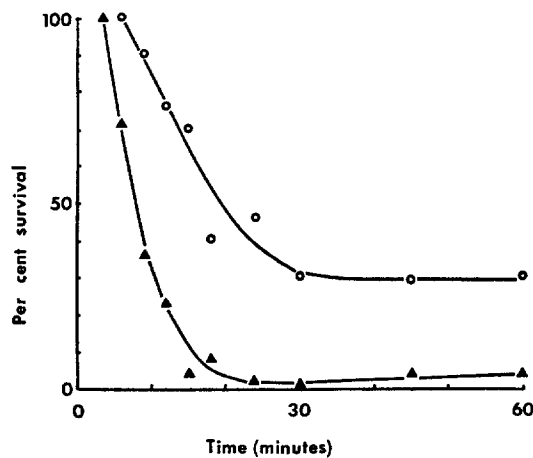


FIG. 2. Curves showing the intracellular inactivation of *S. typhimurium* (C<sub>5</sub>S) in macrophages from normal (O—O) and *Listeria*-infected (▲—▲) mice.

tion with *S. typhimurium* (C<sub>5</sub>S). Bacterial suspensions were treated with serum from mice actively infected with *S. typhimurium* (C<sub>5</sub>S).

The bacterial survival curves shown in Fig. 1 are typical of five identical experiments. In "immune" macrophages, bacterial inactivation was already in progress 3.5 min after introducing organisms into the peritoneal cavity and continued rapidly until 95% of the initial viable intracellular population had been killed. In normal macrophages little killing was observed before 9 min and only 50 to 60% of intracellular bacteria were killed. In all five experiments bacterial inactivation had virtually ceased by 30 min in normal macrophages (mean percentage kill  $57.7 \pm 7.4$ ) and by 15 min in "immune" macrophages (mean percentage kill  $97.0 \pm 2.5$ ).

These findings do not deny the existence of a specific cell-bound antibody which confers on "immune" cells their obviously enhanced bactericidal activity (12). To examine this question experiments were performed with cells from heterospecifically immunized mice.

*The Inactivation of S. typhimurium (C<sub>5</sub>S) by Macrophages of Normal and*

*Listeria-Immunized Mice.*—*Listeria monocytogenes* is known to produce in mice an acquired cellular resistance which cross-protects against unrelated organisms (6).

Mice were injected intraperitoneally with a sublethal dose of *L. monocytogenes* ( $5 \times 10^4$ ). 7 days later these mice were used in an experiment similar to that described in the previous section.

Fig. 2 shows that macrophages from *Listeria*-infected mice are as active against *S. typhimurium* (C<sub>6</sub>S) as those from animals specifically immunized with *S. typhimurium* itself. If this result were due to the presence of cross-reacting antibody bound to the macrophages of *Listeria*-immunized animals its production should be reflected in the serum or cells of animals repeatedly

TABLE I  
Mean\* Percentage Phagocytosis of *S. typhimurium* (C<sub>6</sub>S) in the Peritoneal Cavity of Normal and *Listeria*-Infected Mice

| Test mice                      | Serum       |                         |                           |
|--------------------------------|-------------|-------------------------|---------------------------|
|                                | Normal      | <i>Listeria</i> -immune | <i>Salmonella</i> -immune |
| Normal.....                    | 12.9 ± 5.6  | 11.8 ± 5.7              | 93.6 ± 3.7                |
| <i>Listeria</i> -infected..... | 27.6 ± 10.6 | 30.5 ± 0.6              | 97.6 ± 0.5                |

\* Means and standard deviations were calculated on data obtained from groups of 5 mice.

infected with *L. monocytogenes*; the peritoneal macrophages of *Listeria*-infected animals should all be capable of phagocytosing *S. typhimurium* in the absence of specific opsonic antiserum. Attempts were therefore made to detect cross-reacting antibody in *Listeria*-immunized mice.

*Phagocytic and Opsonic Activity in Cells and Serum of Listeria-Infected Mice.*—The sera used to treat *S. typhimurium* (C<sub>6</sub>S) were obtained from normal mice and from mice which had survived infection with large doses of *L. monocytogenes* or *S. typhimurium*. The experimental procedure was similar to that used to study intracellular survival, but in this case the bacteria were left for 5 min in the peritoneal cavity and from the total and extracellular bacterial counts a percentage phagocytosis was computed for each serum. The results set out in Table I show that serum from *Listeria*-infected animals did not promote phagocytosis of *S. typhimurium*. However, the macrophages of *Listeria*-infected animals were more actively phagocytic than those of normal mice regardless of the serum used. This is analogous to the finding (4) that macrophages of tuberculous animals show, both in vivo and in vitro, an increased phagocytic activity towards a variety of particles (carbon, collodion, staphylococci). It is possible that some of the increased uptake of organisms was due to the larger

cell populations in the *Listeria*-infected animals; they contained approximately 50% more cells than normal. However, phagocytosis of bacteria treated with specific antiserum was almost complete in the peritoneal cavities of normal mice (Table I), indicating that virtually all bacteria made contact with macrophages during 5 min of intraperitoneal residence. Hence the influence of cell numbers could only be of minor significance. If all bacteria injected into the peritoneal cavities of *Listeria*-immune animals also made contact with macrophages, it is significant that in the absence of specific antibody 70% of them did not become permanently cell-associated.

TABLE II  
*Agglutinin Titers against the Somatic Antigens of S. typhimurium in the Sera of 5 Individual Mice which had been Vaccinated with Heat-Killed S. typhimurium*

| Mouse | Somatic antigen |      |       |       |
|-------|-----------------|------|-------|-------|
|       | 1               | 4    | 5     | 12    |
| 1     | I.S.*           | I.S. | 1/80  | <1/10 |
| 2     | <1/10           | 1/20 | 1/40  | <1/10 |
| 3     | I.S.            | 1/80 | 1/80  | <1/10 |
| 4     | <1/10           | 1/40 | 1/160 | <1/10 |
| 5     | I.S.            | I.S. | 1/80  | <1/10 |

\* I.S., insufficient serum for test.

In the previous experiment (Fig. 2), in which opsonized bacteria were used, virtually all macrophages of *Listeria*-infected mice were shown to be capable of the rapid inactivation of *S. typhimurium*. If the enhanced and nonspecific microbicidal ability of all the cells of *Listeria*-infected mice were due to cross-reacting cell-bound antibody, it follows that this antibody does not promote phagocytosis, since only 30% of unopsonized bacteria were phagocytized (Table I). It could also be argued that the hypothetical antibody was bound to only a proportion of cells. As neither of these propositions seems at all likely, it is reasonable to conclude that no cross-reacting antibody exists in *Listeria*-infected mice.

*The Resistance of Listeria-Infected and Salmonella-Vaccinated Mice to Challenge with S. typhimurium.*—Since the foregoing studies on intracellular survival of *S. typhimurium* in vitro could not be performed without specific antiserum it was necessary to use an in vivo technique to compare the relative contributions of humoral and cellular factors in resistance to this organism. For this purpose

mice of the same age and sex were divided into four groups. They were treated as follows: Groups 1 and 2 were given 2 intravenous injections of  $10^8$  heat-killed *S. typhimurium* (C<sub>6</sub>S) a week apart. 1 wk later groups 2 and 3 were given 1.0 LD<sub>50</sub> of living *L. monocytogenes* intravenously. Group 4 was untreated. After 1 further wk all surviving animals were challenged intravenously with  $2.6 \times 10^8$  *S. typhimurium* (C<sub>6</sub>S). Liver and spleen counts were performed on 5 animals of each group at 90 min and 1, 2, and 4 days after challenge. Table II shows the

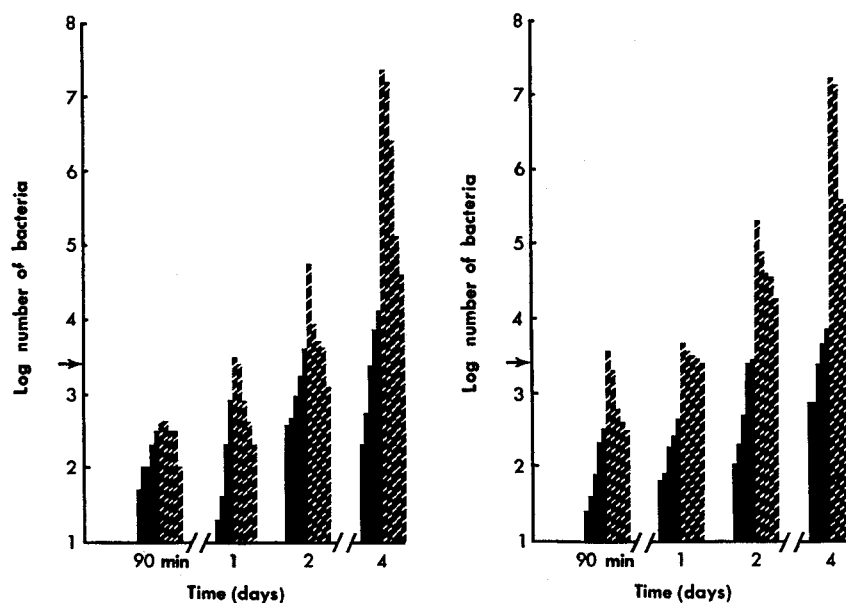


FIG. 3. Behavior of *S. typhimurium* (C<sub>6</sub>S) in the livers and spleens of mice vaccinated as follows: *Left*, heat-killed *S. typhimurium* (hatched) and heat-killed *S. typhimurium* followed by living *L. monocytogenes* (black). *Right*, living *L. monocytogenes* (black) and unvaccinated controls (hatched). Inocula indicated by arrow.

agglutinin titers against the 4 somatic antigens of *S. typhimurium* in the individual sera of 5 mice from the *Salmonella*-vaccinated groups at the time of challenge. Similar tests on sera from 5 *Listeria*-infected mice gave negative results at a dilution of 1:10. Intraperitoneal phagocytosis tests were also performed on the above sera; while those from *Salmonella*-vaccinated mice were highly active, sera from *Listeria*-infected animals were totally inactive.

The behavior of *S. typhimurium* (C<sub>6</sub>S) in the four groups of animals described above is depicted in Fig. 3. The presence of specific circulating antibody had little influence on the course of infection. On the other hand the numbers of viable *Salmonellae* in the livers and spleens of *Listeria*-infected animals were

much lower than in normal mice; the presence of specific antibody had no additional antibacterial effect in these animals. The enhancement of macrophage activity known to be produced by the *Listeria* infection clearly contributed more to host resistance than the specific opsonic antibody produced by the heat-killed vaccine.

The repeated observation that heat-killed vaccines do not afford significant protection against *Salmonella* infections has been attributed to the lability of antigens that determine virulence. In the case of *S. typhimurium*, it has been suggested that the heat-lability of somatic antigen 5 (18) accounts for the in-

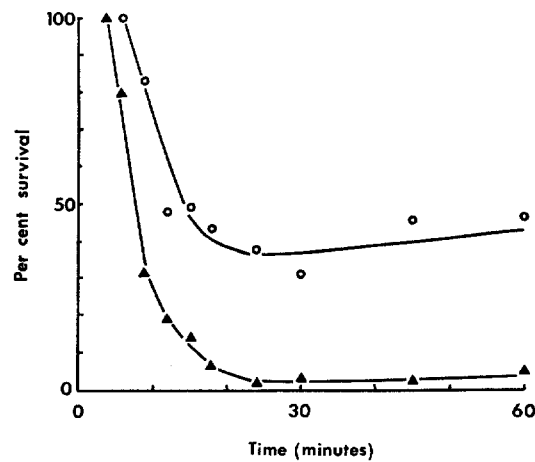


FIG. 4. Curves showing the intracellular inactivation of *S. typhimurium* (C<sub>6</sub>S) in macrophages from normal (○—○) and *Listeria*-infected (▲—▲) mice. The organisms were pretreated with serum from animals immunized with heat-killed *S. typhimurium*.

effectiveness of heat-killed vaccines (17, 19). Failure of the vaccine used in the present experiment to change the course of infection cannot be due to an absence of antibody to factor 5 (Table II). However, it has been stated (17) that "only certain antibodies are effective in promoting killing by phagocytic cells," and that those produced with heat-killed vaccines are lacking in this quality. Experiments were therefore performed *in vitro* to examine from this viewpoint the properties of antibodies produced by a heat-killed vaccine.

*Intracellular Survival of S. typhimurium Treated with Antibody Resulting from a Heat-Killed Vaccine.*—Five experiments *in vitro*, similar to those described previously, were performed using macrophages from normal and *Listeria*-infected mice, except that the serum used for pretreatment of bacteria was produced with a heat-killed vaccine. The results of a representative experiment are shown in Fig. 4. The intracellular survival curves in normal and "immune" macrophages are similar to those obtained with serum from actively infected



and highly resistant mice (Figs. 1 and 2), indicating that the inadequacy of heat-killed vaccines is not due to production of antibody which does not promote intracellular killing of bacteria.

*Acquired Cellular Resistance in Salmonella-Infected Mice.*—Since specific antibody seems to afford mice little protection to intravenous challenge with *S. typhimurium*, it seems likely that the enhanced microbicidal activity of

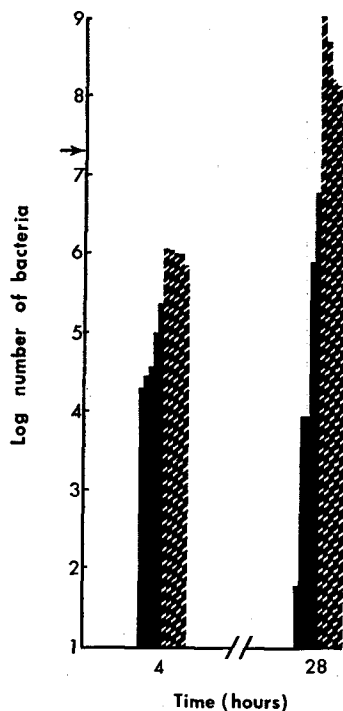


FIG. 5 Behavior of *L. monocytogenes* in the livers and spleens of normal (hatched) and *S. typhimurium*-infected mice (black). Inoculum indicated by arrow.

macrophages is of prime importance in determining the level of resistance to infection by this route. As described in the introduction, cellular resistance is nonspecific in its microbicidal effects. *Salmonella*-infected mice should therefore be resistant to challenge by other facultative intracellular parasites.

Mice preinjected intravenously with approximately  $2 \times 10^2$  *S. typhimurium* (C<sub>6</sub>S) were challenged intravenously on the 18th day of infection, together with a group of normal controls, with  $2 \times 10^7$  (200 LD<sub>50</sub>'s) *L. monocytogenes*. Spleen and liver counts of both infecting organisms were performed on 5 animals from each group 4 and 28 hr after challenge. The two bacterial populations were distinguishable by colonial characteristics. The remaining 10 animals of each group were used to observe mortality rates.

Fig. 5 shows that the majority of *Salmonella*-infected mice progressively inactivated a challenge inoculum of *L. monocytogenes*; whereas, after 4 hr, rapid multiplication occurred in normal controls. The mortality rates confirmed this trend: all remaining control mice died 36 to 72 hr after challenge; all *Salmonella*-infected mice survived.

In the foregoing experiment an interesting association was observed between the numbers of residual *Salmonellae* in the tissues and the level of resistance to the *Listeria* infection. This is shown by the regression line in Fig. 6, which re-

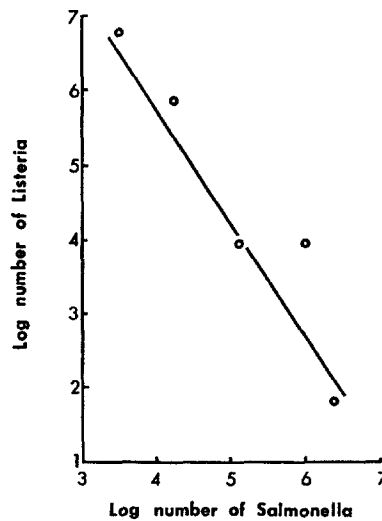


FIG. 6. Regression line showing the relationship between numbers of *S. typhimurium* and *L. monocytogenes* present in the livers and spleens of individual mice 28 hr after intravenous challenge of *S. typhimurium*-infected mice with  $2 \times 10^7$  *L. monocytogenes*.

veals an inverse relationship ( $P < 0.05$ ) between the two bacterial populations 28 hr after challenge. This phenomenon is more fully documented in the ensuing paper (20).

The results described so far in this and the previous paper (1) are at variance with the view that specific antibody makes any substantial direct contributions to host resistance in mouse typhoid. However, the studies conducted in vivo were all performed on intravenously infected animals, while those reporting a significant protective effect of immune serum have all made use of the intraperitoneal route of challenge with a relatively small dose of organisms (11, 13, 15, 21). It was necessary, therefore, to investigate the outcome of infections initiated by these two routes.

*Susceptibility of Mice to Intravenous and Intraperitoneal Infection with S. typhimurium.*—Four hundred normal mice were divided into groups of 20. Each

**TABLE III**  
*Mortality Rates in Mice Infected with Graded Doses of S. typhimurium (C<sub>6</sub>S) Injected by the Intravenous or Intraperitoneal Routes after Treatment with Normal Mouse Serum or Serum from Mice Vaccinated with Heat-Killed C<sub>6</sub>S*

| Serum  | Route of inoculation | Challenge dose      | Percentage death on day: |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     | Total (day 28) | LD <sub>50</sub> |
|--------|----------------------|---------------------|--------------------------|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|-----|----------------|------------------|
|        |                      |                     | 2                        | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |     |                |                  |
| Normal | Intravenous          | 5                   |                          |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | 5   |                |                  |
|        |                      | 50                  |                          |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     | 20             |                  |
|        |                      | 500                 |                          |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     | 35             |                  |
|        |                      | 5 × 10 <sup>3</sup> |                          |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     | 35             |                  |
|        | Intraperitoneal      | 5 × 10 <sup>4</sup> |                          |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     | 85             |                  |
|        |                      | 5                   |                          |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     | 60             |                  |
|        |                      | 50                  |                          |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     | 100            |                  |
|        |                      | 500                 |                          |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     | 100            |                  |
| Immune | Intravenous          | 5 × 10 <sup>3</sup> |                          |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | 90  |                |                  |
|        |                      | 5 × 10 <sup>4</sup> |                          |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | 100 |                |                  |
|        |                      | 5                   |                          |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | 0   |                |                  |
|        |                      | 50                  |                          |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | 10  |                |                  |
|        | Intraperitoneal      | 500                 |                          |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     | 20             |                  |
|        |                      | 5 × 10 <sup>3</sup> |                          |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     | 70             |                  |
|        |                      | 5 × 10 <sup>4</sup> |                          |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     | 45             |                  |
|        |                      | 5                   |                          |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     | 5              |                  |
| Normal | Intravenous          | 50                  |                          |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | 20  |                |                  |
|        |                      | 500                 |                          |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | 45  |                |                  |
|        |                      | 5 × 10 <sup>3</sup> |                          |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | 60  |                |                  |
|        |                      | 5 × 10 <sup>4</sup> |                          |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    | 45  |                |                  |
|        | Intraperitoneal      | 5                   |                          |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     | 5              |                  |
|        |                      | 50                  |                          |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     | 20             |                  |
|        |                      | 500                 |                          |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     | 45             |                  |
|        |                      | 5 × 10 <sup>3</sup> |                          |   |   |   |   |   |   |   |    |    |    |    |    |    |    |    |    |    |    |    |    |    |    |     | 35             |                  |

group was injected intraperitoneally or intravenously with one of 5 graded doses of *S. typhimurium* (C<sub>5</sub>S) which had been treated with normal mouse serum or serum from mice vaccinated with heat-killed organisms. The progressive mortality rates in these groups are recorded in Table III along with LD<sub>50</sub>'s as calculated by the method of Reed and Muench (22). Mortality rates appeared to be dose dependent except in groups receiving normal serum-treated organisms intraperitoneally. In these groups mortality was high, and time to death short. If the growth curve of *S. typhimurium* in the livers and spleens of normal mice is consulted (Fig. 3) it is apparent that in order to reach a lethal level by the 4th day, a population of 5 organisms introduced into the peritoneal cavity in the absence of specific antibody must multiply much more rapidly than a similar population injected intravenously. Since virulent *Salmonellae* are phagocytized inefficiently in the peritoneal cavities of normal mice in the absence of specific antibody (Table I), rapid extracellular multiplication in the peritoneum could account for the results recorded in Table III. In effect, specific antibody converts a peritoneal infection from a rapidly lethal disease to one resembling an intravenous infection.

#### DISCUSSION

The present studies have shown that the macrophages from *Salmonella*-infected mice possess conspicuously enhanced microbicidal properties. The improvement in the immune macrophage population involves not only an increase in the percentage of cells able to inactivate ingested organisms but also a marked acceleration of the inactivation process itself. One group of workers have implied that macrophages may become altered by virtue of adsorbed antibody, and that no intrinsic change is necessarily present in the cells themselves (12). Since this antibody was reported to cause not only phagocytosis but also the subsequent killing of ingested *Salmonellae* (15, 16), their hypothesis demands that the enhanced microbicidal ability of "immune" macrophages exhibit specificity (11). However, the present studies have demonstrated an absence of specificity in the antibacterial mechanisms developed during infection with *S. typhimurium*. Infected animals inactivated *Listeria monocytogenes* in their spleens and livers and were completely resistant to challenge by 200 LD<sub>50</sub>'s of this organism, resistance to which depends upon enhancement of the microbicidal ability of host macrophages (7, 8, 23). Furthermore, *Listeria*-infected animals were resistant to *S. typhimurium*, and their macrophages possessed enhanced microbicidal activity for that organism *in vitro*; cross-resistance occurred in the absence of antibody capable of opsonizing or agglutinating *S. typhimurium*.

By way of contrast, specific immunization with heat-killed vaccines did not limit the growth of *S. typhimurium* *in vivo* after intravenous challenge (reference 1 and Fig. 3). This conforms to the general experience that killed vaccines

are not protective (1, 3, 24). Japanese investigators have emphasized the fact that increased antibacterial activity in macrophages, readily found when living vaccines of *S. enteritidis* are used to immunize, is absent from the cells of animals immunized with dead organisms (25). They conclude, therefore, that the lack of protective effect of heat-killed vaccines is due to their failure to induce a significant degree of cellular resistance. Tests performed in vitro on peritoneal cells from vaccinated mice by the methods used in the present investigation confirmed this point with respect to heat-killed *S. typhimurium*.

There remains the alternative explanation of the inefficacy of heat-killed vaccines proposed by Jenkin and Rowley (13, 17). They contend that antibody produced in response to heat-killed *S. typhimurium* is capable of promoting phagocytosis but not intracellular killing of virulent organisms. However, it is clear from Figs. 1, 2, and 3 that intracellular inactivation of *S. typhimurium* treated with serum from mice vaccinated with heat-killed organisms is in no way different from that resulting from treatment with serum from mice immunized with live organisms. Thus, the type of circulating antibody present in the vaccinated animals is not the reason for their inability to resist an intravenous infection; their deficiency in comparison with actively infected mice lies in the functional normality of their macrophages.

Nonetheless, heat-killed antiserum was highly protective when used to treat an intraperitoneal inoculum of virulent organisms. Its effect in this situation seems to depend upon its ability to promote rapid phagocytosis in the peritoneal cavity, thus preventing extracellular multiplication which would occur in its absence. This idea is supported by the findings of Ushiba et al. (26) who have described the behavior of *Salmonella enteritidis* in the peritoneal cavities of normal and vaccinated mice. In the normal mouse rapid growth occurred, but in the vaccinated animals bacterial numbers in the peritoneal cavity declined during the first 24 hr after injection. It is interesting in this connection to review the findings of MacLeod (27) and of Greenwood, Topley, and Wilson (28) who showed that mice immunized with a heat-killed vaccine of *S. typhimurium*, though partially resistant to intraperitoneal challenge (27, 29), were not protected against an experimental infection by the oral route (27) or a naturally acquired infection under epidemic conditions (28). These results, and the finding of Ørskov et al. (30), and Seiffert et al. (31) that peritonitis is not a feature of the early stages of mouse typhoid acquired by natural means, suggest that protection tests based upon intraperitoneal challenge have limited significance.

It is quite certain from the present studies that *S. typhimurium*-infected mice develop a mechanism which can eliminate a super infecting dose of organisms from the liver and spleen (1). This mechanism has the characteristics of the acquired cellular resistance which develops in tuberculosis, brucellosis, and listeriosis. In these diseases, the level of resistance at any time is determined by the number of the initiating bacterial species present in the tissues, whether

they are persisting from the primary infection or have been reintroduced (6, 20). A similar phenomenon was observed in the present studies. The higher the numbers of *Salmonellae* present in the tissues the greater was the resistance to challenge with *L. monocytogenes* (Fig. 6). If circulating or cell-bound antibody were responsible for resistance to the *S. typhimurium* infection, and if cross-reaction with this antibody produced protection against *L. monocytogenes*, one would expect the direct reverse; mice which had combatted the *Salmonella* infection more successfully should have been more resistant to *L. monocytogenes*.

#### SUMMARY

Experiments in vitro comparing normal mouse peritoneal macrophages with cells from *Salmonella typhimurium*-infected mice have shown that the "immune" macrophages have conspicuously enhanced microbicidal properties. Whereas normal macrophages could inactivate only 50 to 60% of intracellular *S. typhimurium* pretreated with immune serum, cells from infected animals killed virtually all ingested organisms and did so at an accelerated rate. Macrophages from *Listeria monocytogenes*-infected mice were shown to possess similarly enhanced microbicidal activity against *S. typhimurium*. Furthermore, the growth of *S. typhimurium* in the liver and spleen was more effectively restricted in *Listeria*-infected mice than in animals vaccinated with heat-killed *S. typhimurium*, even though the *Listeria*-infected animals possessed no demonstrable cross-reacting antibody to *S. typhimurium*. The lack of resistance in the mice vaccinated with heat-killed organisms could not be attributed to any deficiency of humoral factors, since the serum from these animals was as effective at promoting phagocytosis and killing by macrophages as serum from actively infected (and demonstrably resistant) mice. Conversely, *Salmonella*-infected mice were totally resistant to intravenous challenge with *L. monocytogenes*. The level of resistance in individual animals was related to the numbers of residual *Salmonellae* remaining in the tissues; mice with heavier residual infections being the more resistant.

Specific antiserum from mice vaccinated with heat-killed *S. typhimurium* was found to be significantly protective only when the intraperitoneal route of challenge was employed.

The foregoing studies have been interpreted to mean that enhancement of the microbicidal ability of macrophages is the mechanism of major importance in acquired resistance to *S. typhimurium* infection in mice.

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