HOST-PARASITE RELATIONS IN MOUSE TYPHOID*

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Mouse typhoid is a naturally occurring infection which provides an excellent experimental model of the generalized *Samonella* infections of man. It has been used in many outstanding studies on topics such as herd immunity (4), the genetic (3) and nutritional (16) aspects of host resistance, and other important problems. But questions of fundamental importance still remain unanswered. Not the least of these concerns the mechanism of acquired resistance itself. In this matter two conflicting views are held: one school (5, 6, 11, 14, 15) believes that a cellular mechanism is involved; the other (7, 13) is equally persuaded that immunity is due to specific opsonic antibodies. Those subscribing to the latter view have set themselves the problem of identifying the antigens against which "protective" antibodies are directed (8). But if acquired resistance is not based on a mechanism of this sort, it is unlikely that this approach will lead to the development of effective procedures for immunization against *Salmonella* infections.

The present investigations were undertaken in the hope of obtaining a clearer picture of the relative importance of humoral and cellular factors in resistance to mouse typhoid. Although microbial enumeration has often been used to good effect in studying the evolution of the host response to *Salmonella typhimurium* infections in mice (12, 5), its use on this occasion has been more extensive, and the objectives somewhat different from those of previous workers in this field.

Materials and Methods

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

Organisms.—Two virulent strains of *Salmonella typhimurium* were used. One was streptomycin-sensitive (strain C_5S) and the other a streptomycin-resistant mutant derived from it (strain C_5R). They were of comparable virulence (intravenous $LD_{50} = 4 \times 10^3$; intraperitoneal $LD_{50} < 10$). Suspensions for injection were prepared from log-phase culture grown in tryptic soy broth (Difco Laboratories Inc., Detroit). Plate counts were performed on blood base agar (Difco) with or without added streptomycin (50 $\mu g/ml$.)

Bacterial Enumeration in Spleen and Liver.—Spleens and livers from individual mice were removed aseptically and homogenized in a final volume of 10 ml nutrient broth using a motor-

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ized teflon pestle at 2000 rpm. After serial dilution, aliquots were plated on well dried agar medium.

Immune Serum.—Anti-*Salmonella* serum was obtained from four sources: (*a*) from mice surviving on the 20th day following an intravenous injection of 0.1 LD_{50} of virulent *S. typhimurium* (C_5S). (*b*) from similar mice reinfected 1 and 4 wk later by the intravenous injection of 100 and 500 LD_{50}'s of the same organism. All animals survived and were bled 1 wk later (*c*) From mice injected intravenously with a single dose of 10^9 organisms heat-killed at 56°C for 90 min. They were bled 15 days later. (*d*) From mice immunized by three, weekly intraperitoneal injections of 10^8 heat-killed *S. typhimurium* and bled 7 days after the third inject tion.

All sera were sterilized by membrane filtration and were used immediately or stored in small volumes at −20°C. They were not heat-inactivated before use, and were never refrozen. Mice of a similar age were used as a source of normal serum.

Antibody Titers.—Antibody titers against somatic and flagellar antigens were performed by tube agglutination using monospecific antigen suspensions.
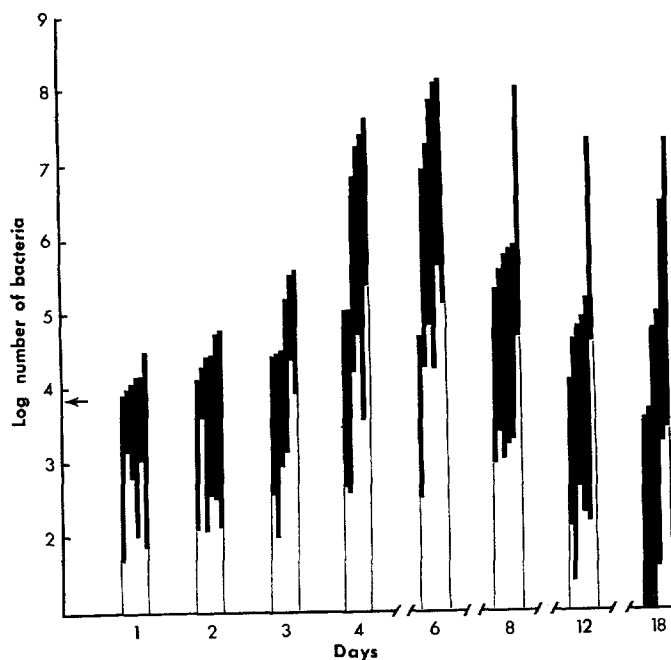

FIG. 1. Numbers of *S. typhimurium* (C_5S) in the spleen and liver (black) and in the blood (white) of individual mice at intervals during the course of a primary intravenous infection. Infecting dose is indicated by arrow.

RESULTS

Growth Pattern of S. typhimurium in Normal Mice.—

Normal female mice were injected intravenously with 7×10^3 living *S. typhimurium* (C_5S). Viable counts of organisms in the spleens and livers of 5 individual mice were made at inter-

vals following infection. At the same time 0.1 ml of heart blood was plated on agar to determine the numbers of bacteria present in the circulation.

The numbers of living *S. typhimurium* (C_5S) present in the spleen and liver and in the blood of individual mice at intervals during the course of a primary infection are recorded in Fig. 1. Since mice which died or appeared moribund were excluded from the experiment, the data do not accurately represent the behavior of *S. typhimurium* in normal mice. The fact that an increasing proportion of survivors showed fewer organisms in the blood and tissues on the 8th and subsequent days could be explained by the elimination of innately susceptible mice. Hobson (5) has shown, however, that mice of a given strain are relatively uniform in their susceptibility to this organism, and equally capable of developing acquired resistance. It is possible, therefore, that the discernible tendency for bacterial populations to decline from the 6th day onwards is an expression of developing resistance. The question was examined more objectively in the following experiments.

Resistance to Reinfection with S. typhimurium.—

Mice were infected intravenously with 5×10^3 *S. typhimurium* (C_5S). Groups of surviving mice (regardless of their condition) were reinjected on days 8, 11, 14, and 18 with the virulent streptomycin-resistant mutant, strain C_5R. On each occasion a corresponding number of normal mice were injected with the same bacterial suspensions. Viable counts were made at intervals on spleen and liver homogenates and on the blood of 5 mice from each group. The plate counts performed on doubly infected animals were made both in the presence and absence of streptomycin so that the two bacterial populations could be counted independently.

Fig. 2 shows a progressive increase in the efficiency with which infected mice deal with a super infecting population of *S. typhimurium*. By the 14th day most animals were able to eliminate the challenge organism completely. Since the same organism multiplied progressively in *all* normal controls, it is concluded that *every* mouse in the experiment was initially susceptible. It follows that animals which survive a primary infection do so by virtue of a highly effective form of acquired antibacterial immunity. It will be noted, however, that the organisms of the primary infection persisted in almost undiminished numbers (Fig. 2); they contributed exclusively to the bacteremia present in most mice on the 14th and 18th day of infection (the bacteremia arising from the primary bacterial population is not recorded in Fig. 2).

The data of Fig. 1 suggest that whenever a large increase occurs in the total bacterial population in liver and spleen it is always associated with a gross bacteremia. This might be thought to indicate that overwhelming infections are the result of inadequate blood clearance. The absence of streptomycin-resistant organisms from the blood (Fig. 2) indicates, however, that infected mice were already capable of eliminating a superinfecting population of bacteria from the circulation as early as the 8th day of the primary infection. This implies that defective clearance is not the reason for the persistence of the primary
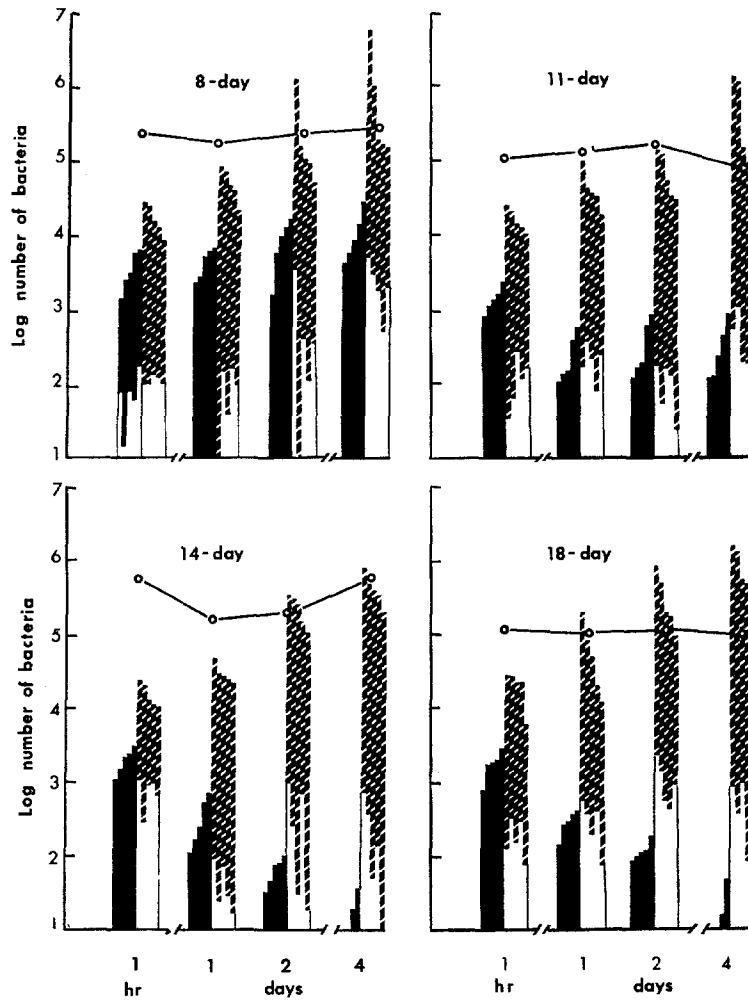
Fig. 2. Comparison of the numbers of *S. typhimurium* (C_5R) in the spleens and livers of normal mice (hatched) and reinfected mice (black) challenged at 4 stages of the primary infection with *S. typhimurium* (C_5S). The corresponding number of organisms in the blood of each mouse is recorded in white. The mean number of organisms (C_5S) of the primary infection is also shown (O——O).

infection, and suggests that the blood is being continuously reseeded. In this circumstance the ability of infected animals to suppress a second infecting dose of organisms could still be due to increased blood clearance rates. Tests were therefore made to determine whether passively transferred immune serum, with its known content of opsonic antibody, is capable of reproducing the pattern of antibacterial resistance found in actively infected mice.

The Influence of Immune Serum from Actively Infected Mice on the Host-parasite Relationship in Mouse Typhoid.—

A washed log-phase culture of *S. typhimurium* (C_5S) was used to prepare two suspensions of bacteria, one in whole fresh serum from normal mice and the other in fresh serum from mice surviving in a healthy state on the 20th day following the intravenous injection of approximately 0.1 LD_{50} virulent *S. typhimurium* (C_5S). The two suspensions contained approximately 5×10^5 organisms per ml. They were dispersed for 10 seconds with ultrasound (60 w
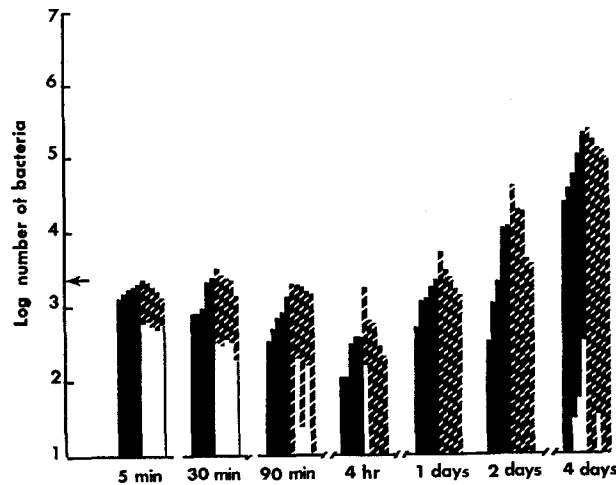

Fig. 3. Numbers of *S. typhimurium* (C_5R) in the spleens and livers of individual mice infected intravenously with a challenge inoculum suspended in normal serum (hatched) or serum taken from resistant donors on day 20 of an active infection (black). In both groups the corresponding number of organisms in the blood is indicated in white. The infecting dose of organisms is indicated by the arrow.

at 20 kc/sec), and a plate count was performed. The suspensions were stood for 90 min at 4°C, and were then redispersed before making a second plate count. The two suspensions, which contained no bacterial aggregates when examined by phase microscopy, were injected intravenously into two groups of normal mice. Each mouse received a volume of 0.2 ml of whole serum (equivalent to 20% of plasma volume). At intervals the spleens and livers of 5 mice of each group were removed for bacterial enumeration.

The rate of uptake, survival, and subsequent growth of *S. typhimurium* in mice treated with normal or immune serum is recorded in Fig. 3. The agglutinin titers of the sera are presented in Table I. The bacteria, which had been pretreated with serum, were injected intravenously in a volume of 0.2 ml of whole serum, each inoculum contained no aggregates and an identical number of viable organisms (2.0×10^3). It is apparent that the immune serum caused a more rapid clearance of organisms from the blood and a slightly more effective control over the bacterial population during the initial stages of the infection. It is quite certain, however, that it did not materially influence the subsequent

growth of organisms in the tissues. By the 4th day bacteria had begun to reappear in the blood, and the tissue populations had reached almost to the levels found in the recipients of normal serum. Since the donors of immune serum were the infected animals surviving from the experiment recorded in Fig. 2, they were known to be completely resistant to reinfection with the streptomycin-resistant strain of *S. typhimurium*. A similar result was obtained with serum from animals which had survived two additional injections of 100 and 500 LD_{50}'s of virulent organisms. It is clear, therefore, that a single injection of immune serum cannot reproduce the resistant state observed in the infected donor. It could be objected, of course, that the recipients were only briefly "protected" because of the short half-life of immunoglobulins in pas-

TABLE I

Reciprocals of the Agglutinin Titers of Normal Serum and Serum Obtained on Day 20 or a Primary S. typhimurium Infection in Mice

Antigens		Serum	
		Normal	Immune
Somatic	1	<10	<10
	4	<10	<10
	5	<10	80
	12	<10	<10
Flagellar	i	<10	320
	1, 2	<10	<10

sively immunized mice (17). In order to settle this point it was necessary to study the fate of *S. typhimurium* in actively immunized animals in which a shortage of opsonic factors could not be held to account for any breakdown of the clearance mechanism.

The Influence of Serum from Vaccinated Mice on the Host-Parasite Relationship in Mouse Typhoid.—It has been claimed that the antibodies produced during active infection promote the intracellular killing of *S. typhimurium*, but that those produced by immunization with dead organisms do not (9). It was therefore necessary to repeat the foregoing experiment with serum from animals immunized with a heat-killed vaccine before proceeding to an examination of the protective effects of active immunization with dead organisms. Two experiments were performed: one with serum obtained from mice injected intraperitoneally on three occasions at weekly intervals with 10^8 heat-killed *S. typhimurium* (C₅S), and the other with serum obtained 15 days after a single intravenous injection of the same vaccine containing 10^9 bacterial cells. The results of the latter experiment, and the agglutinin titers of the sera
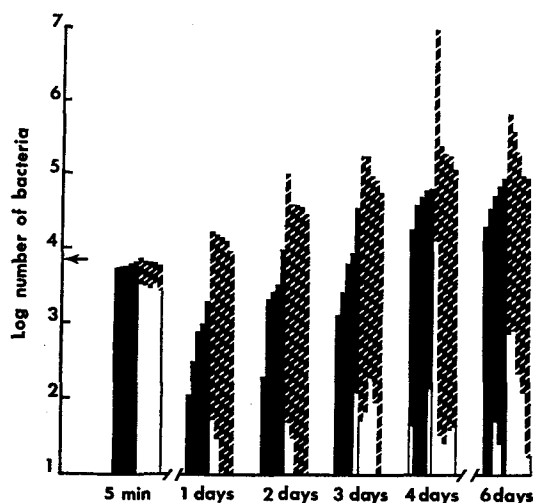
FIG. 4. Numbers of *S. typhimurium* (C₅R) in the spleens and livers of individual mice intravenously infected with organisms suspended in normal serum (hatched) or serum from mice immunized with heat-killed organisms (black). For each mouse the corresponding number of organisms in the blood is shown in white and the challenge inoculum by the arrow.

used, are recorded in Fig. 4 and Table II, respectively. It appears that serum from animals immunized in this way was as effective as that obtained from actively infected animals in promoting a rapid and complete clearance of the blood, and a little more effective in controlling the subsequent increase in bacterial numbers in spleen and liver. Although the antibody titer was higher than in the serum from actively infected mice, the same limitation on its availability after passive transfer still remains. This stricture should not apply, however, in the case of actively immunized animals.

TABLE II

Reciprocals of the Agglutinin Titers of Normal Serum and Serum from Mice Vaccinated with 10^9 Heat-Killed S. typhimurium

Antigens		Serum	
		Normal	Immune
Somatic	1	<10	<10
	4	<10	40
	5	<10	160
	12	<10	<10
Flagellar	i	<10	320
	1, 2	<10	<10

The Host-Parasite Relationship in Mice Immunized with a Heat-Killed Vaccine.—Fig. 5 shows a comparison of the growth pattern of *S. typhimurium* (C_5S) in normal and immunized mice challenged 15 days after the intravenous injection of 10^9 heat-killed *S. typhimurium* (C_5S). It is apparent that the parasite behaved in vaccinated mice exactly as it did in mice which had been passively immunized with serum from highly resistant animals with an active infection, or with serum from animals immunized with a dead vaccine. In all
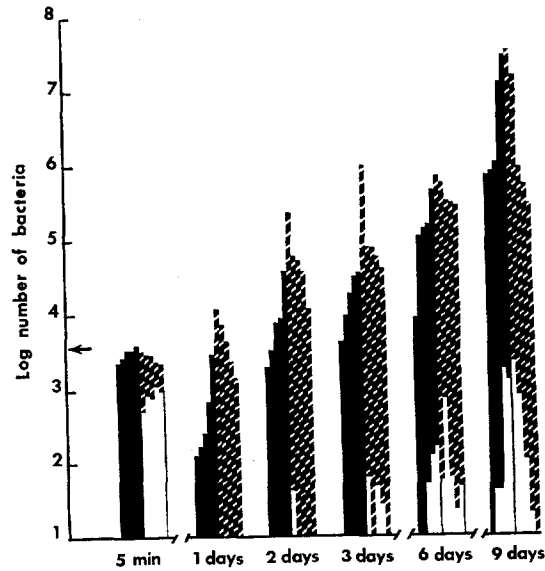

FIG. 5. Numbers of *S. typhimurium* (C_5R) in the spleens and livers of normal mice (hatched) or mice actively immunized with a heat-killed vaccine (black). For each mouse the corresponding number of organisms in the blood is shown in white and the challenge inoculum by the arrow.

cases the blood remained sterile for a period of 3 days, but the bacterial populations in spleen and liver increased progressively during this period, and did so at a rate comparable with that found in normal controls.

DISCUSSION

The foregoing studies describe the evolution of the host's response to a small intravenous inoculum of virulent *S. typhimurium*. Virtually all organisms could be recovered from the liver and spleen shortly after injection. A high proportion of them were subsequently killed (Fig. 3), but some survived and multiplied, presumably within cells which could not inactivate them (7). Although this initial multiplication of bacteria presumably takes place in cells, it should be remembered that living *S. typhimurium* are highly toxic for mouse macrophages.

Their inherent cytotoxicity is implicit in the early necrosis which occurs at infective foci in vivo (12). Gelzer and Suter (2) have shown in vitro that macrophages infected with virulent *S. typhimurium* are completely destroyed within a few hours; but when ingested in the presence of immune serum, the immediate cytotoxicity of the organism is abolished. This, and the prevention of early intravascular multiplication as seems to have occurred in one control mouse (Fig. 3), could account for the minor effect of antibody on the growth pattern of organisms in passively immunized or vaccinated mice. Any influence tending to prevent the creation of cyto-destructive foci at the site of implantation would slow the rate of progress of the infection.

Reference to Figs. 2 to 5 will show that organisms are seldom found in the blood 24 hr after the injection of a 50% lethal dose of *S. typhimurium*, but that a secondary bacteremia develops almost invariably at a later date. Large bacterial populations never exist in the absence of a substantial bacteremia. Histological observations made in the course of the present studies showed that primary lesions in the liver all developed a necrotic center within 48 hr. Some of them were located in the vicinity of large venules into which they subsequently eroded. The observations of Meynell and Stocker (10) are highly pertinent in this context. When mice were infected with equal numbers of two distinguishable variants of *Salmonellae* in doses of about 1.0 LD_{50} it was found that only one of the variants was represented in the terminal bacteremia which developed in a proportion of animals. These authors concluded that the development of a bacteremia is a chance event which can arise from a single bacterial cell. Although unable to deduce what circumstances permitted the multiplication and dissemination of only a proportion of the infecting inoculum, they suggested that it was not due to phenotypic or genotypic variation in the bacterial population. In their opinion it was determined by local conditions prevailing at the site of implantation. It would seem from the present observations that the most important determining factor is the anatomical distribution of primary lesions; only those located in the vicinity of veins being suitably placed to give rise to a secondary bacteremia at an early stage of infection. If vascular invasion occurs soon after implantation, before the development of acquired resistance, a proportion of the lesions derived from the reimplantation of organisms would also give origin to foci from which further dissemination could occur. This would account for the enormous variation between mice, some showing a rapid build-up of the infecting population, and others a much slower march of events.

In view of the behavior of reinfecting bacteria in animals already infected with the homologous organisms, it is clear that mice are capable of developing a mechanism which ensures the death of newly implanted organisms, thus preventing the development of a self-sustaining infection. When this stage of host resistance is reached there can be little doubt that bacteria released into the

circulation from primary foci are also subject to the same processes of removal from the blood and inactivation in the tissues. But the ultimate destruction of organisms within the primary foci from which they arise is obviously more difficult to accomplish. Since they persist in the face of a very efficient immune mechanism, conditions there must be most unfavorable to the effective operation of host defenses. This is not hard to imagine in view of the necrotic lesions that are present in the tissues of infected mice (12). Thus, the paradox of a persisting primary infection in the presence of complete resistance to reinfection is not difficult to comprehend.

It now remains to discuss the nature of the mechanism by which the infected animal so effectively suppresses freshly implanted organisms whether they arise endogenously or are deliberately introduced. It has been shown that neither the passive transfer of serum from highly resistant mice, nor active immunization with heat-killed organisms, is capable of reproducing this degree of resistance. The most that these immunizing procedures can accomplish is a more rapid uptake of organisms from the blood and a somewhat longer delay before any net increase occurs in the size of the infecting bacterial population. Apparently the infected animal develops an additional, and much more effective antibacterial mechanism. A similar conclusion has been reached by others on somewhat different grounds (5, 6, 11, 14, 15). The nature of this mechanism is dealt with in the ensuing paper (1).

SUMMARY

The development of acquired resistance to *Salmonella typhimurium* has been studied in mice infected intravenously with small numbers of streptomycin-sensitive or streptomycin-resistant organisms. By the 14th day of a primary infection the mouse develops a mechanism capable of destroying completely a super infecting dose of organisms, but is unable to eliminate organisms of the primary infection. The latter are constantly returned to the circulation from necrotic foci at the sites of implantation. Passive transfer of serum from actively infected or vaccinated animals, and immunization with heat-killed organisms, increase the capacity of the host to clear organisms from the blood, but do not interfere to any significant extent with their subsequent multiplication in the tissues. It is concluded that the resistance of actively infected animals depends on a nonhumoral mechanism capable of destroying organisms from endogenous or exogenous sources.

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