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Growth of Salmonellae in Orally Infected Germfree Mice

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Germfree mice were infected intragastrically, intravenously, or intraperitoneally with 10^3 to 10^9 viable *Salmonella typhi* Ty2, *S. gallinarum* 9240, or *S. enteritidis* 5694. The 50% lethal doses were compared with those for conventionally raised mice. Substantial growth of the salmonellae occurred in the intestinal tract of the germfree mice but, despite the presence of more than 10^9 viable *S. typhi* or *S. gallinarum* in the lumen, the liver and spleen cultures remained *Salmonella*-free, and all of the mice survived the oral challenge. The ileal and cecal Peyer's patches and the mesenteric lymph nodes of these mice contained 10^3 to 10^4 viable salmonellae within 24 h of introduction of the inoculum into the stomach. Despite this local involvement, the infection did not spread systemically even when host resistance was reduced by means of sublethal, whole-body gamma irradiation before oral challenge. Germfree mice infected orally with as few as 10 mouse-virulent *S. enteritidis* quickly developed severe diarrhea and died within 5 to 8 days as a result of a spreading systemic disease.

Salmonella typhi is an obligate human pathogen incapable of inducing progressive disease in normal mice (3, 5). A fatal infection can be induced after massive intraperitoneal challenge, especially if hog gastric mucin is added to the inoculum (17). In this system, the *S. typhi* inoculum multiplies extracellularly to induce early peritonitis, causing death due to acute endotoxic shock within 48 h. Quantitative growth studies of the behavior of the challenge population within the peritoneal cavity (5) make it clear that this infection does not resemble the progressive type of systemic disease seen in human enteric fever (10) or even that observed in mice infected orally with *S. enteritidis* (2).

Attempts to develop an oral assay system for salmonella vaccines have been severely limited by our inability to establish a reproducible infection in conventionally raised mice (8, 9). Small numbers of *S. typhi* can be recovered from the livers and spleens of the highly susceptible B6D2 strain of mouse after oral challenge (3), but the model is still not biologically relevant so far as the human disease is concerned. Resistance to oral *Salmonella* challenge has been ascribed to the inhibitory effects of the established intestinal flora on the growth of the introduced inoculum within the intestinal lumen (16). Elimination of the normal intestinal flora by massive oral antibiotic treatment increases host susceptibility to oral *Salmonella* challenge (12). Considering this fact, it was reasoned that susceptibility to oral challenge by *S. typhi* might be enhanced if the inoculum were introduced orally into germfree mice. Ruitenberg et al. (14) have previously reported growth of *S. panama* in orally challenged germfree mice and explained the extensive systemic growth of the organism in terms of the overgrowth of the intestinal tract of the germfree host by the salmonellae. The present study was carried out to examine the behavior of several strains of *Salmonella* of increasing mouse virulence after their intragastric inoculation into germfree mice.

MATERIALS AND METHODS

Animals. Germfree TRU:ICR mice were randomly bred at the Trudeau Animal Breeding Facility, Saranac Lake, N.Y., and maintained in plastic isolators on sterile commercial diet and acidified water ad lib (4). Male mice, 4 to 5 weeks of age (20 to 25 g each). were used throughout. Feces samples were routinely cultured on Trypticase soy agar and MacConkey agar plates as well as examined microscopically for indications of contamination before challenge. Conventionally raised, specific-pathogen-free mice were maintained under conditions described earlier (2).

Organisms. *S. typhi* Ty2, *S. enteritidis* 5694, and *S. gallinarum* 9240 were grown under conditions described earlier (2, 7). The broth culture was diluted suitably in sterile saline and used immediately, and the viability of the inoculum was checked by plate counts on Trypticase soy agar.

Virulence testing methods. A shaken Trypticase broth culture was concentrated approximately 10-fold by being centrifuged at 10,000 rpm for 20 min and suspended in fresh broth. Viable counts indicated populations of up to 5×10^{10} organisms per ml. Groups of five mice were infected intragastrically with 0.2 ml of the undiluted suspension or with serial 10-fold broth dilutions by using a feeding needle as described earlier (2). Intravenous and intraperitoneal challenges were

carried out by using the same suspensions, as described elsewhere (3, 5). The 50% lethal dose (LD_{50}) determinations were made by the method of Reed and Muench (13) from the 28-day cumulative mortality data.

Bacterial enumeration in vivo. The lungs, spleen, mesenteric lymph nodes, and duodenal, ileal, and cecal Peyer's patches were carefully dissected out aseptically from each of five randomly selected mice and homogenized separately in cold saline as described earlier (2). Counts were also carried out on the small intestine, cecum, and large intestinal contents suspended in 10 ml of sterile saline after brief homogenization and plating on Trypticase soy agar and MacConkey agar. Plates were incubated overnight at 37°C before counting.

Irradiation of germfree mice. Mice were placed in sterile double paper bags, removed from the isolator, and exposed to 400 rads of whole-body gamma irradiation in a small-animal irradiator (18). The animals were returned to the air lock of the isolator after the removal of the outer (contaminated) bag, the inner bag was sprayed with peracetic acid, and the mice were then removed from the bag and returned to their cages within the isolator. Exposure to the peracetic acid vapors was very brief (less than 1 min). The irradiated mice behaved almost identically to the unexposed controls (see Fig. 4), making it unlikely that the peracetic acid treatment had an additional deleterious effect on the irradiated germfree mice. There were four groups of mice: (i) irradiated germfree; (ii) irradiated conventional; (iii) non-irradiated germfree; and (iv) non-irradiated conventional mice. The four groups of mice were infected orally with 10^6 viable *S. typhi* Ty2 24 h after irradiation. The germfree mice were checked for non-*Salmonella* contaminants 1 and 4 days after irradiation, by both microscopic and cultural methods. Only salmonellae were recovered from the gut contents in all except one experiment, in which accidental contamination occurred at some time during irradiation. Several different morphological forms were observed in the feces 24 h after the mice were returned to the isolator. The growth of the *S. typhi* inoculum within the gut contents was minimal, and the entire experiment was abandoned and repeated after sterilization of the isolator.

RESULTS

Growth of *S. typhi* in orally challenged mice. The oral LD_{50} for *S. typhi* Ty2 in conventional and in germfee mice was in excess of 5×10^9 viable bacilli and could not be determined precisely (Table 1). When injected intraperitoneally, the LD_{50} for both germfree and conventional mice was between 5×10^6 and 10×10^6. Death occurred only during the first 48 h. The intravenous LD_{50}'s were approximately 10 times those for the intraperitoneal route but 50 to 100 times lower than the figure for the oral challenge (Table 1). *S. typhi* was virtually avirulent for both conventionally raised and germfree mice. Similar studies with another attenuated strain (*S. gallinarum*) and the mouse-virulent *S. enteriditis* indicated that *S. gallinarum* behaved in much the same way as *S. typhi* but that *S. enteritidis* was nearly 10^6 times more virulent for orally challenged germfree mice than the conventional host (Table 1). Interestingly enough, the LD_{50}'s for all three organisms tested by the three routes of challenge (with one important exception, which will be discussed in greater detail in a later section) were much the same in conventionally raised and in germfree mice. If anything, the germfree host was slightly more resistant than its conventionally raised counterpart to the oral *Salmonella* challenge (Table 1).

Thirty germfree mice were infected orally with about 10^7 viable *S. typhi* Ty2. Growth curves for the intestinal contents and the gut-associated lymphoid tissues are recorded in Fig. 1. The *S. typhi* multiplied extensively within the cecum and large intestine to reach 5×10^8 to 50×10^8 viable bacilli within 24 h. The count for the small intestine was about 10^7 viable organisms, and these counts did not change appreciably over the 42-day period of the study. The cecal Peyer's patch was heavily infected within 24 h (10^4 viable bacilli per patch) and remained at this level for most of the test period. The ileal Peyer's patches contained 500 to 1,000 viable *S. typhi*, whereas duodenal Peyer's patches gave counts of less than 100 viable bacilli throughout most of the 20-day period (Fig. 1). The mesenteric lymph node counts increased sharply from about 50 bacilli at 24 h to 10^4 viable salmonellae by day 10. These counts slowly declined to about 100 bacilli on day 20. Few viable salmonellae were detected in the spleen, liver, or lung ho-

TABLE 1. *LD_{50} determinations for orally challenged germfree and conventionally raised mice*

Organism	LD_{50} for:[a]					
	Germfree mice			Conventional mice		
	Oral	i.v.	i.p.	Oral	i.v.	i.p.
S. typhi	$>5 \times 10^9$	1.5×10^8	1.0×10^7	$>5 \times 10^9$	8×10^7	5×10^6
S. enteritidis	3–5	2×10^3	4×10^3	5×10^6	1×10^4	2.5×10^3
S. gallinarum	3×10^9	—[b]	—[b]	1×10^9	5×10^6	5×10^4

[a] Calculated from 28-day survival data.
[b] —, Not determined.

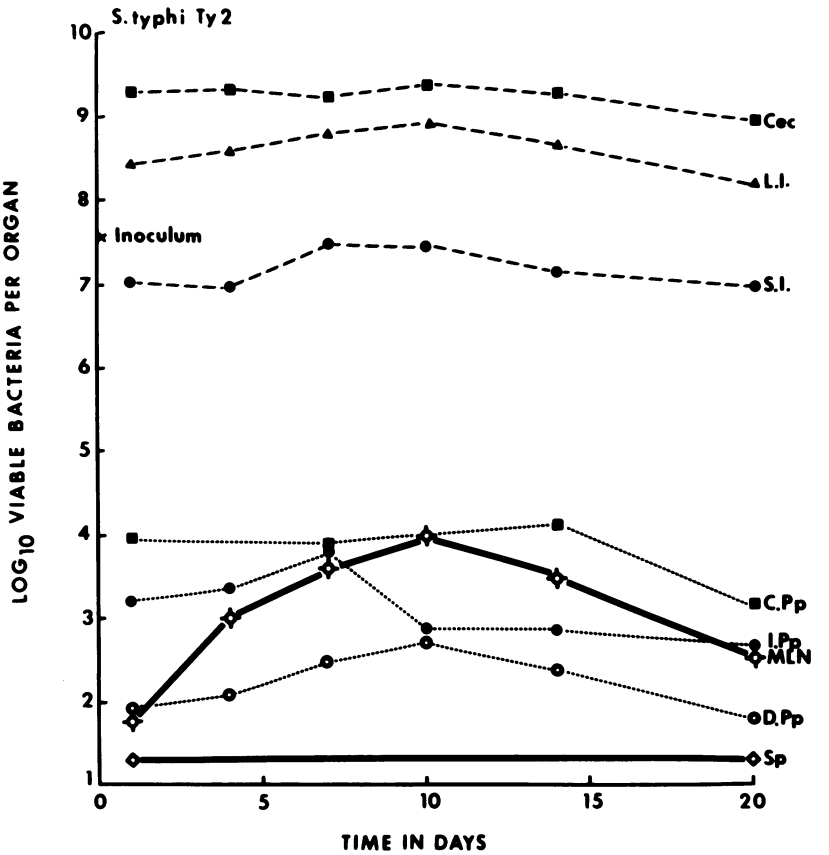

FIG. 1. *Growth of S. typhi Ty2 in germfree mice after oral challenge with 4 × 10⁷ viable organisms. S.I., Small intestine; Cec, cecum; L.I., large intestine; D.Pp, duodenal Peyer's patch; C.Pp, cecal Peyer's patch; I.Pp, ileal Peyer's patch; MLN, mesenteric lymph node; Sp, spleen.*

mogenates. The occasional colonies observed on these plates seemed to occur at random, and it was not possible to make an accurate determination of the size of the liver or spleen bacterial populations at any time. Lung homogenates prepared 24 h after the oral challenge were free of viable *S. typhi*, indicating that aerogenic contamination did not occur during the intragastric inoculation procedure (8).

***S. gallinarum* infection in orally challenged germfree mice.** A group of thirty germfree mice were infected orally with 2×10^7 viable *S. gallinarum*. The growth curves shown in Fig. 2 are essentially the same as the *S. typhi* curves. The *S. gallinarum* multiplied extensively in the cecum and large intestine, as well as infecting the ileal and cecal Peyer's patches and the mesenteric lymph nodes (Fig. 2). Few salmonellae were recovered from the lungs, liver, or spleen at any time during the experiment (Fig. 2), and none of the animals appeared to be ill or developed diarrhea over the 20-day period of the study.

***S. enteritidis* infection in orally challenged germfree mice.** Oral inocula ranging from 10 to 10^7 viable *S. enteritidis* resulted in a uniformly fatal infection in germfree mice. Typical growth curves for such mice infected orally with about 10^4 *S. enteritidis* are shown in Fig. 3. All of the mice developed progressive systemic infections involving the lungs, liver, spleen, and lymph nodes. The mice appeared to be ill (ruffled fur, hunched appearance) by day 4 of the experiment, with severe diarrhea developing a day or so later. This extensive diarrhea may account for the relatively low recoveries of viable salmonellae from the large intestines of these mice (Fig. 3).

Counts carried out on homogenates prepared from spleen increased steadily and reached lethal proportions in most mice by day 10. Presumably, it was the unrestricted growth by the liver and spleen populations that resulted in the death of most of the animals. The *S. enteritidis* was toxic for the germfree host, judging from the extensive diarrhea, ruffled fur, and hunched ap-

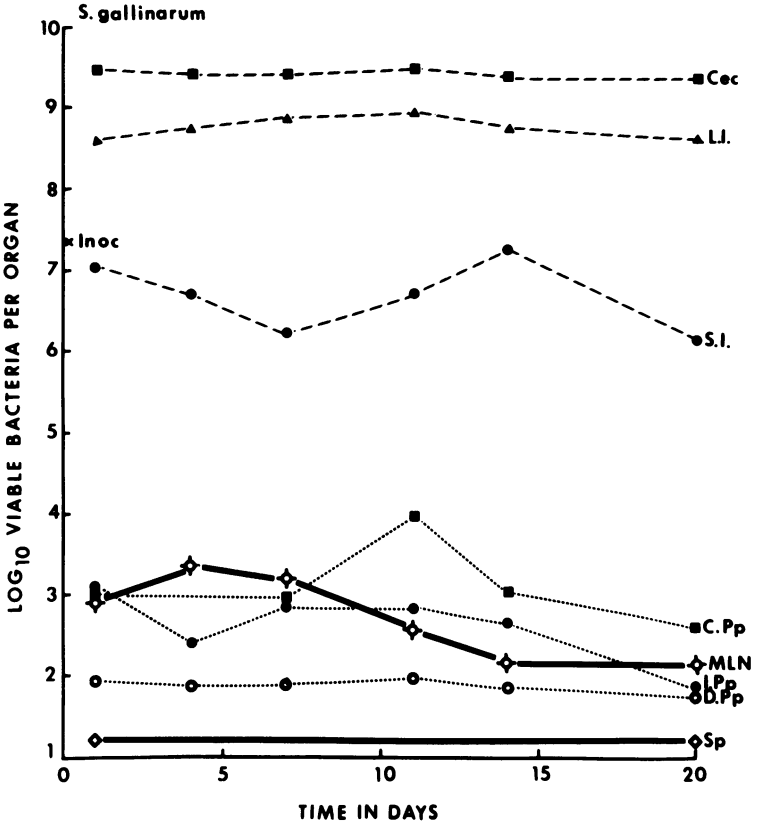

FIG. 2. *Growth of S. gallinarum in germfree mice challenged orally with 2 × 10^7 viable organisms. (See legend to Fig. 1 for further details.)*

pearance of the animals. The diarrhea observed in the *S. enteritidis*-infected mice could indicate the presence of an enterotoxin of the type reported earlier by Sandefur and Peterson for *S. typhimurium* (15). Presumably, the *S. enteritidis* population in the conventionally raised host never reached a point where it could express a detectable enterotoxic effect.

Effect of whole-body irradiation on the *S. typhi* infection of germfree mice. *S. typhi* failed to induce progressive systemic disease in mono-associated mice, despite the fact that substantial numbers of organisms reached the mesenteric lymph nodes (Fig. 1). The organism did not seem capable of infecting the lungs, liver, or spleen to any extent. Presumably, the normal cellular defenses were capable of inactivating the *S. typhi* which reach these central organs before they can establish an ongoing infection. Sublethal, whole-body irradiation is known to interfere with normal cellular defenses in mice (1). This temporary depression in host CMI has been ascribed to the inactivation of a radiosensitive monocyte precursor population in the bone marrow (18). Due to this circumstance, the host is unable to mobilize its normal phagocytic defenses and so may be more susceptible to the *S. typhi* challenge. Two groups of thirty germfree mice (together with the corresponding conventionally raised controls) were placed in isolators. One group of each type was later exposed to 400 rads of whole-body irradiation 24 h before oral challenge with nearly 10^6 viable *S. typhi*. The growth curves for the germfree mice are shown in Fig. 4. The conventionally raised mice (whether irradiated or not) failed to develop detectable counts in any of the test organs studied 24 h after challenge. In the irradiated germfree mice, the *S. typhi* multiplied in the intestinal contents to the same extent as in the non-irradiated controls (Fig. 4). The spleen and lung remained free of detectable salmonellae, despite a 100-fold increase in the ileal Peyer's patch counts over the first 9-day period. By day 14, the mesenteric lymph node counts in the irradiated host were 10 times higher than those in the corresponding controls. However, none of the mono-associated irradiated mice succumbed to

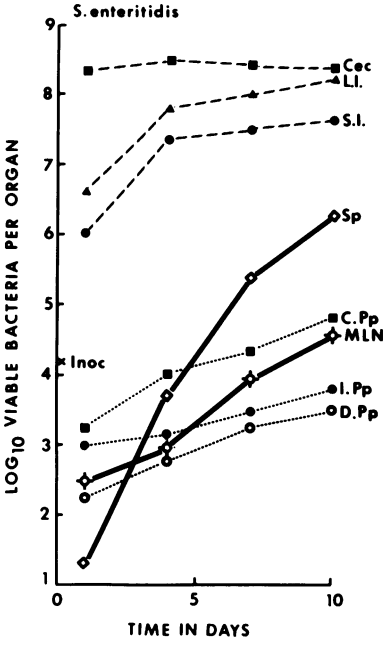

FIG. 3. *Growth of S. enteritidis in germfree mice challenged orally with 1.08 × 10⁴ viable organisms. (See legend to Fig. 1 for further details.)*

the intestinal challenge with *S. typhi*, even when the incubation period was extended to 30 days.

DISCUSSION

Salmonellosis in conventionally raised mice primarily involves the ileal and cecal Peyer's patches (2). The intestinal contents are normally free of residual salmonellae within a few hours, provided that coprophagy is somehow prevented (4, 6). When the host is exposed to a highly invasive intestinal pathogen, even this short exposure time is sufficient for some of the organisms to enter the ileal and cecal Peyer's patches. From there they eventually drain through the bloodstream to the liver and spleen, where they begin to multiply extensively. If the host is exposed to a strain of more modest virulence (such as *S. gallinarum*), the bacilli still reach the mesenteric lymph nodes but seem unable to go on to produce a generalized systemic infection (Fig. 2). One might argue that *S. gallinarum* was less capable of competing with the resident intestinal flora than was *S. enteritidis*, so that fewer attentuated organisms actually entered the tissues. However, this explanation can hardly be true for the germ-free host. Furthermore, equivalent numbers of *S. typhi*, *S. gallinarum*, and *S. enteritidis* appear in the Peyer's patch and mesenteric lymph node cultures at 24 h (cf. Fig. 1 and 3). The two former strains did not go on to produce progressive systemic disease. Counts as high as 10^{10} viable *S. typhi* per g of feces were found in many of the mono-associated mice within hours of challenge (Fig. 1). Careful removal of individual Peyer's patches (to avoid contamination with gut contents) indicated the presence of up to 10^4 bacilli in some of these organs. Presumably, viable *S. typhi* also reached the liver and spleen in substantial numbers but were inactivated immediately by the normal host defenses. Such a process can also be inferred from the immediate decline seen in viable counts for the liver and spleen after an intravenous challenge of normal mice with *S. typhi* Ty2 (3). If the normal liver and spleen cells are so effective, one might ask why the established infection within the Peyer's patches and mesenteric lymph nodes are not also eliminated rapidly. However, this apparent continued local persistence by the *S. typhi* may actually be the result of a continuous reinfection of the gut-associated lymphoid tissues by *S. typhi* crossing the intestinal mucosa.

S. typhi seems as capable of entering the ileal and cecal Peyer's patches as its more virulent relatives (cf. Fig. 1 and 3). The lack of mouse virulence exhibited by *S. typhi* seems to be due to the extreme sensitivity of this organism to the bactericidal action of normal mouse macrophages. Irradiation is known to depress the ability of the host to express an effective cell-mediated immune response against salmonella challenge, and so it was possible that the irradiated germfree mouse might be susceptible to a *S. typhi* challenge to the extent that a detectable systemic involvement would occur. However, the data in Fig. 4 make it clear that irradiation with 400 rads before oral challenge with *S. typhi* did not result in any substantial growth of the organisms in the liver and spleen. Apparently, the normal resident macrophages in the liver and spleen (which would be expected to be resistant to such a dose of irradiation) were capable of inactivating any typhoid bacilli reaching the liver and spleen from the mesenteric lymph nodes. This would imply that the mouse macrophage did not require immunological activation to kill all of the *S. typhi*. On the other hand, virulent *S. enteritidis* can only be inactivated by mouse macrophages after they have been activated by a cell-mediated immune response (11). It is interesting to note that the opposite seems to be true for these organisms when they are introduced into the human intestinal tract (10). The reason for this striking difference in inherent susceptibility observed in two antigenically related salmonellae when introduced by the oral route into two different host species remains a complete mystery.

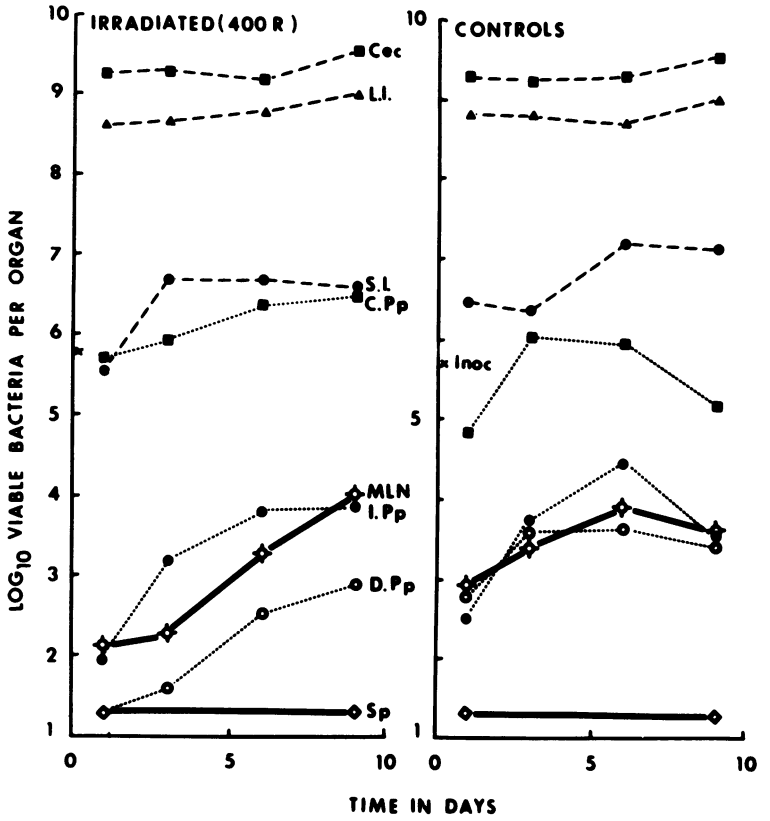

FIG. 4. *Growth of S. typhi in sublethally irradiated (400 rads, whole body) and non-irradiated germfree mice after challenge 24 h later with 8 × 10⁵ viable organisms. The conventional mice (both irradiated and control) failed to develop detectable infections and have been omitted from the figure. (See legend to Fig. 1 for further details.)*

The introduction of less than 10 viable *S. enteritidis* into the germfree intestinal tract represents a 100% lethal dose, whereas the corresponding value for the conventionally raised specific-pathogen-free host is almost 10^9 viable organisms. This difference is in sharp contrast to the relatively small difference observed in the LD_{50} determinations when the organisms was introduced intravenously or intraperitoneally (Table 1). An explanation for this apparent paradox lies in the unrestricted growth by the *Salmonellae* in the intestinal contents of the germfree host. The massive intestinal infection quickly overwhelmed the systemic defenses, whether the initial inoculum was 10 or 10^6 organisms. However, when the inoculum was introduced systemically, the organisms were taken up by the liver and spleen, where growth was relatively slower than in the intestinal tract. Eventually, the infection spread to the intestine, with a period of rapid and extensive local growth. However, by this time, most of the mice had developed some systemic immunity to the salmonellae, and this limited the infection to sublethal proportions. Thus, the parenterally infected animals were able to survive, whereas animals receiving a much smaller initial inoculum of the same organism by the oral route were rapidly killed.

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