Growth of Typhoid and Paratyphoid Bacilli in Intravenously Infected Mice

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The in vivo growth of Salmonella paratyphi A, S. paratyphi B, S. paratyphi C, and S. typhi, as well as of an S. typhi-typhimurium hybrid, was studied in three different strains of mice. S. paratyphi A and B and S. typhi demonstrated very little growth potential in any of the intravenously infected mice, even after as many as 20 serial mouse passages. It was noted, however, that small numbers of viable S. paratyphi B and S. typhi persisted in the spleens of infected mice for up to 28 days. Salmonella paratyphi C and the S. typhi-typhimurium hybrid gave rise to progressive systemic infections beginning from very small intravenous inocula. The median lethal doses for the C57Bl strain of mouse were about five organisms. The relevance of these findings with regard to the development of an animal model for studying human typhoid fever vaccines is discussed.

Enteric fever is a severe human disease usually caused by Salmonella typhi or one of the paratyphoid bacilli (28). Although the etiology of this important infectious disease has been understood for nearly a century, it has been only relatively recently that the host-parasite relationships involved in the expression of acquired resistance to enteric disease has become clearer (2). One of the main limiting factors in any study of immunity to typhoid fever is the virtual absence of a suitable experimental model of the human disease. Neither the mouse nor the guinea pig is naturally susceptible to infection by S. typhi and, although a number of elegant protection studies have been carried out in the chimpanzee (8), and in human volunteers (13), progress in this whole area has been understandably slow. The only model which has been used for the routine assessment of human typhoid vaccines involves intraperitoneally or intracerebrally infected mice (15, 24). Protection is usually assessed from the percentage survival by mice exposed 7 or 14 days previously to a single dose of killed vaccine and then subjected to a lethal intraperitoneal challenge with S. typhi strain Ty2 (9). In order to kill 100% of the control mice, large numbers of S. typhi must be injected, and in many cases hog gastric mucin has also been included to inhibit the early phagocytosis of the inoculum (18). Under such conditions, death occurs within 48 h in most of the control animals due to an overwhelming peritonitis and endotoxemia. On the other hand, the vaccinated animals control the extracellular growth of the challenge population within the peritoneal cavity by increasing the rate of phagocytosis of the S. typhi in the presence of the specific opsonin. Most of the intracellular bacilli are then removed from the peritoneal cavity and are rapidly inactivated in the liver and spleen. Despite this accelerated rate of inactivation of the opsonized bacilli, viable organisms can persist in vivo for considerable periods of time (11, 12). Infection of mice with large oral doses of S. typhi induces an early septicemia in many of the animals. However, the systemic infection usually remains quite low and the disease is usually nonfatal. The use of massive infecting doses renders these mouse protection tests open to the criticism that the experimental model is not biologically relevant, so that the data have little value so far as the naturally acquired human disease is concerned (2). Some workers have gone so far as to question the relevance of the mouse model in any study of human typhoid fever (25). Much the same type of criticism also holds for tests employing S. paratyphi A or B infections in mice (11). Only in the case of S. paratyphi C do we see a progressive systemic infection in mice exposed to intravenous or intraperitoneal challenges (14). Unfortunately, detailed quantitative growth data is still not available for this organism.

Recent studies carried out in this laboratory (3) and elsewhere (22) indicate that specific pathogen-free C57Bl mice (as well as several of its hybrids) are more susceptible to systemic
and oral *Salmonella* challenges than has been the case for the widely used outbred CD-1 strain of mouse (5). However, most of these studies have been carried out using *S. typhimurium* or *Salmonella enteritidis* as the infectious agent. In the present study, growth of a number of enteric fever bacilli has been followed serially in the livers and spleens of C57Bl mice or one of its F1 hybrids (B6D2). The resulting growth curves indicate that *S. typhi* does persist in the livers and spleens of C57Bl and B6D2 mice, offering the hope that an improved animal model for studying *S. typhi* infections may now be available.

**MATERIALS AND METHODS**

**Organisms.** *S. typhi* strains Ty-2 and 0-901 were obtained from Richard Hornick, University of Maryland. *S. typhi* strains 9-53, 9-57, 9-67, 9-83, and 10-36 were recent human isolates supplied by Joseph Winter of the Beth Israel Hospital, New York. E. M. Johnson of the Walter Reed Army Institute of Research kindly supplied us with cultures LCDC #1 (9,12;i-1), LCDC #34 (9,12,Vi:i-1-2), and LCDC #42 (9,12,Vi:d-1,2) from their collection of *S. typhityphimurium* hybrids (7). *S. typhimurium* C5 was described in an earlier paper (17). *S. paratyphi A* was a long established laboratory strain obtained from Morris Wagner, University of Notre Dame. *S. paratyphi* B was a recent human isolate received from Dr. Winter. *S. paratyphi* C (NCTC 5735) was obtained from the National Collection of Type Cultures, Colindale, England. Seed lots of all cultures were preserved by lyophilization, but working cultures were maintained at room temperature on Trypticase soy agar (TSA; BBL, Cockeysville, Md.) slants subcultured at 3-month intervals. The cultures were discarded after 12 months, and fresh ones were prepared from lyophilized stocks.

**Animals.** Specific pathogen-free CD-1 mice (Charles River Farms, Wilmington, Mass.), C57Bl/6J (Jackson Laboratories, Bar Harbor, Me.), and C57Bl/6xDBA/2F1 hybrid mice (B6D2) were maintained 10 to a cage under isocaps (Carworth, New City, N.Y.) on sterile bedding with free access to sterile food and water (3). Young adult males, 5 to 7 weeks of age, were used throughout. Fifty animals were used in each experiment, with five mice taken for each time point.

**Intravenous challenge.** The inoculum was grown in 5 ml of tryptose soy broth, shaken at 37 C for 6 h. The logarithmic-phase culture was diluted suitably in saline, and the inoculum was injected intravenously into the left lateral tail vein. The number of viable organisms in each inoculum was determined by plating suitable saline dilutions onto TSA. The plates were incubated overnight at 37 C and the colonies were counted.

**Bacterial enumeration technique.** Livers and spleens were aseptically removed from five randomly selected animals at each time point. The organs were homogenized separately in sterile saline as described elsewhere (6). Bacterial counts of the organ homogenates were made on TSA, with a standard error similar to that reported earlier (1, 6).

**Serial passage of enteric fever organisms in mice.** Mice were infected intravenously with approximately 10⁴ viable *S. typhi* or *S. paratyphi* A, B, or C. The spleen was removed 2 or 3 days later, and the infecting organism was recovered by inoculation of 0.1 ml of the homogenate into digest broth. Purity of the culture was checked by plating the homogenate onto TSA. After overnight incubation at 37 C, the growth was inspected for purity and the organism was reinoculated into a fresh animal. This procedure was repeated 10 to 20 times, after which the mouse virulence of the organism was checked by median lethal dose (LD₅₀) determination (3) and by following its growth in the livers and spleens of B6D2 mice.

**LD₅₀ determinations.** These were estimated by a method described earlier (3) using the 30-day mortality data for groups of 10 mice inoculated intravenously with increasing numbers of viable organisms. LD₅₀ determinations were also made from time to time on the mouse-passaged organisms, which were injected either in the form of suitably diluted spleen homogenates or as a broth-grown suspension prepared from this homogenate.

**RESULTS**

**Growth of paratyphoid bacilli in normal mice.** Thirty B6D2 mice were infected intravenously with 2 × 10⁴*S. paratyphi* A. The number of viable bacteria recovered from the liver and spleen fell steeply over the first 24 h, and by the second day very few bacilli were recovered from either organ (Fig. 1). Despite this extensive inactivation, some mice still contained as many as 100 viable *S. paratyphi* A in their livers on day 14. Variations between replicate counts in these animals were understandably high, however.

Mice receiving the corresponding inoculum of *S. paratyphi* B only inactivated 50% of the inoculum during the first 24 h (Fig. 2). This was followed by a slow period of growth in both the liver and spleen and, although none of the infected mice appeared to be ill at any stage of the infection, a few viable bacilli could usually be recovered from the liver for up to 28 days. There were no deaths at this challenge dose.

A third group of mice were infected with about 10⁴ viable *S. paratyphi* C (Fig. 3). Again, there was an early extensive inactivation of the inoculum and only 1% of the challenge population survived the first 24 h. There was a substantial regrowth by both the liver and spleen populations, so that as many as 10⁴ viable bacilli were recovered in vivo on day 6. Several of the infected mice died as a result of this infection. The growth patterns for the three
organisms are consistent with the LD₅₀ values recorded in Table 1.

**Growth of S. typhi in normal mice.** S. typhi has little virulence for normal mice unless introduced into the peritoneal cavity in large numbers (11). When 5 × 10⁴ viable S. typhi strain Ty2 were injected intravenously into B6D2 mice, 95% of the inoculum was inactivated during the first 24 h (Fig. 4). However, viable salmonellae were still recoverable from both the liver and spleen some 14 days later, and a few residual bacilli were isolated from the spleens of two out of five mice examined after 28 days.

The original culture of S. typhi Ty2 contained two colony types. The major colony type had a typical smooth surface morphology (26), and on inoculation of this organism into B6D2 mice the growth curve shown in Fig. 5 was recorded. The spleen counts were 10 times as high as that observed with the original (mixed) culture (Fig. 4). The second colony type was slightly larger, with a less convex surface and a margin which tended to be undulate. The growth curve for this variant obtained in B6D2 mice was similar to that for the smooth colony, but the liver and spleen populations were numerically 100-fold lower than those observed with the fully smooth strain (Fig. 5). Thus, it seems likely that the growth curve shown in Fig. 4 is actually a composite of the growth curves for the two substrains. The purified smooth strain was

**Table 1.** LD₅₀ determinations for S. typhi Ty2 and S. paratyphi A, B, and C injected intravenously into specific pathogen-free CD-1, B6D2, and C57Bl mice

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<thead>
<tr>
<th>Organism</th>
<th>Mice</th>
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<tr>
<td></td>
<td>CD-1</td>
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<tr>
<td><strong>S. typhi Ty2</strong></td>
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<tr>
<td>Broth grown</td>
<td>8 x 10⁷</td>
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<tr>
<td>Mouse passaged</td>
<td>8 x 10⁷</td>
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<tr>
<td>Spleen adapted</td>
<td>&gt;10⁴</td>
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<tr>
<td><strong>S. typhi O-901</strong></td>
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<tr>
<td>Broth grown</td>
<td>&gt;10⁴</td>
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<tr>
<td><strong>S. paratyphi A</strong></td>
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<tr>
<td>Broth grown</td>
<td>7 x 10⁷</td>
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<tr>
<td>Mouse passaged</td>
<td>&gt;10⁷</td>
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<td>Spleen adapted</td>
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<td><strong>S. paratyphi B</strong></td>
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<td>Broth grown</td>
<td>4 x 10⁸</td>
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<td>Mouse passaged</td>
<td>3 x 10⁸</td>
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<tr>
<td>Spleen adapted</td>
<td>&gt;10⁴</td>
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<tr>
<td><strong>S. paratyphi C</strong></td>
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<tr>
<td>Broth grown</td>
<td>3 x 10⁹</td>
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<tr>
<td>Mouse passaged</td>
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<tr>
<td>Spleen adapted</td>
<td>2 x 10⁸</td>
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<tr>
<td><strong>S. typhi-typhimurium 42</strong></td>
<td></td>
</tr>
<tr>
<td>Broth grown</td>
<td>3 x 10⁹</td>
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* - , Not done.
stable on subculture and was used in all of the subsequent studies.

The effect of the presence or absence of Vi antigen on the growth of S. typhi in vivo was examined by comparing the above growth curves with those obtained in B6D2 mice infected with the Vi-antigen-deficient strain, S. typhi 0-901. The resulting liver and spleen growth curves are shown in Fig. 6. The absence of Vi antigen seemed to have no effect on the persistence of the typhoid bacilli in vivo. In fact, the number of viable S. typhi 0-901 actually increased in the spleen some 20-fold over a 3-day period, and this bacterial population then remained constant for several days before declining again slowly (Fig. 6). No deaths were observed in the mice challenged with $10^4$ viable O-901, and this is consistent with the intravenous LD$_{50}$ value of more than $10^4$ viable S. typhi O-901 in all three strains of mice (Table 1).

S. typhi Ty2 has been maintained on laboratory media for many years and may not be typical of the wild-type strain of S. typhi. Groups of mice were therefore infected intravenously with five strains of S. typhi recently isolated from patients suffering from clinically severe typhoid fever. The liver and spleen growth curves shown in Fig. 7 for strain 9-83 are typical of the results obtained with all five strains. It can be seen that these curves closely resembled those obtained earlier with S. typhi Ty2.

**Effect of repeated mouse passage on virulence.** Serial passage of S. typhi Ty2 through normal mice failed to increase the virulence of the organism, as judged from the intravenous LD$_{50}$ values shown in Table 1. Little change was observed in the virulence of S. paratyphi A or B subjected to the same treatment, but there was a 10-fold decrease in the intravenous LD$_{50}$ for the passed S. paratyphi C, both in CD-1 and B6D2 mice (Table 1). Even higher levels of mouse virulence could be observed when infected spleen homogenates were injected intravenously into fresh animals. As expected, the virulence of the spleen-adapted organisms was somewhat higher in the B6D2 than in the CD-1 mice (Table 1); intravenous LD$_{50}$ values as low as $10^4$ viable S. paratyphi C were recorded using B6D2 mice, a value which approaches that noted earlier for unpassed cultures of S. enteritidis 5694 (3). The 3- or 4-day spleen counts for S. paratyphi A and B and S. typhi Ty2 did not increase sufficiently to permit serial mouse-to-mouse transfer by means of spleen homogenates, and so LD$_{50}$ determinations for such preparations could not be made (Table 1).

**Growth of Salmonella typhi-typhimurium hybrids in normal mice.** The virulence of hybrid strains of S. typhi-typhimurium was first checked in C57Bl/6 mice. All three strains of bacilli recorded intravenous LD$_{50}$ values of less than 40 organisms for this host. S. typhi-typhimurium hybrid LCDC #42 most closely resembled S. typhi Ty2 so far as its somatic antigenic structure was concerned, and this organism was thus selected for the subsequent

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**Fig. 4. Growth of unpassed S. typhi Ty2 in intravenously infected B6D2 mice.**

**Fig. 5. Growth curves of the smooth and rough substrains of S. typhi Ty2 in B6D2 mice after intravenous challenge.** (Sp = spleen; Lr = liver; Bl = blood.)
growth studies. *S. typhi-typhimurium* 42 was considerably more mouse virulent than either *S. typhi* Ty2 or O-901 when tested in the three strains of mice (Table 1). However, it was the C57Bl mouse which appeared to be most susceptible to intravenous infection with the hybrid; the reason for the greater resistance of the B6D2 and CD-1 strains of mice is not known. The LD₅₀ values for *S. typhimurium* C5 (7 x 10⁴) were significantly higher than for *S. typhi-typhimurium* hybrid 42 (4 x 10⁴). Furthermore, deaths in the *S. typhimurium*-infected mice began on day 6 and essentially ended by the 11th day of the infection. On the other hand, deaths in the *S. typhi-typhimurium* 42-infected mice began on day 8 with a peak about day 12, but a few deaths continued to be noted even after 30 days. Those mice which died about this time showed large liver abscesses at necropsy, and large numbers of salmonellae, antigenically identical to hybrid #42, were isolated from the lesions.

C57Bl mice infected intravenously with 8 x 10⁸ *S. typhi-typhimurium* 42 developed severe, progressive systemic infections (Fig. 8). Viable bacilli were first recovered from the liver and spleen homogenates on day 2, but then the bacterial counts increased progressively and mice began to die from the seventh day. Identical growth curves were obtained both in the B6D2 and CD-1 mice up to day 7 (Fig. 8). From day 6 onwards, the viable numbers seen in the B6D2 and CD-1 mice remained constant, whereas those in the C57Bl mice continued to increase steadily with a considerable mortality beginning on day 7.

**DISCUSSION**

Most studies of *S. typhi* infections in mice have been limited by the fact that this organism has low pathogenicity for this animal species. When a challenge population of 10 million viable *S. typhi* Ty2 is introduced into the normal mouse peritoneal cavity, extensive growth occurs over the first 24 h, and the number of viable bacilli within the cavity itself and in the draining mediastinal lymph nodes may increase to lethal proportions within a few hours. When the same dose of organisms is injected directly into the blood stream, the bacilli are removed by the phagocytic cells of the liver and spleen, and there is less opportunity for the bacilli to multiply freely within the tissues (11, 12). As a result, the intravenously introduced inoculum is unable to multiply extracellularly and fails to develop into a lethal infection. None of such mice die as a result of this infection, and therefore some other means besides death or survival must be used to assess the protective qualities of a given typhoid vaccine. Serial enumeration of the number of *S. typhi* in the livers and spleens of intravenously infected mice enables the demonstration of an antibacterial type of response by the vaccinated, not by the unvaccinated, control animals. At present, very little quantitative data
exist on the growth of S. typhi within the reticuloendothelial organs of normal mice. Ge-Richter (12) reported that S. typhi multiplied in vivo when introduced in very large numbers into the peritoneal cavities of white mice. Most of his mice died within 24 to 48 h after challenge, due to overwhelming endotoxemia. A few mice usually survived, and these were found to harbor significant numbers of viable S. typhi within their spleens for many weeks. The present study shows that even moderate doses of S. typhi can establish persistent systemic infection in the majority of the challenged mice and that small numbers of bacilli may still be recovered from the tissues for at least 3 weeks. The low degree of mouse virulence shown by S. typhi Ty2 is due to the extensive early inactivation of the infecting population during the first 24 h following challenge. The survivors seem incapable of multiplying in vivo to any great extent, and so none of the animals actually succumbs to the challenge. The growth curves shown in Fig. 4 for the S. typhi Ty2 in B6D2 mice resemble those observed earlier for S. enteritidis infections in LBN rats (26) or for S. gallinarum in normal CD-1 mice (6). Contrary to our earlier expectations, the S. typhi population did not decline progressively to zero, as had been seen for the mouse-avirulent strain of Salmonella pullorum (3, 6). The ability of S. typhi to establish a resident persisting infection in the intravenously infected mice makes it possible to devise an experimental mouse model which can be used to study antityphoid immunity in appropriately vaccinated mice.

Attempts to increase the mouse virulence of S. typhi by means of serial mouse passage were unsuccessful. This failure was somewhat unexpected, since this experimental device has been used successfully in maintaining maximal levels of mouse virulence for Listeria monocytogenes (16) and for Diplococcus pneumoniae and other human pathogens (27). Animal passage has also been routinely used to maintain fully smooth bacterial suspensions for agglutination tests (23). Mouse passage of S. typhi was thought to be worth attempting, since both quantitative and qualitative antigenic differences have been shown to exist between in vivo and in vitro growth preparations of S. typhi (9, 21). Repeated mouse passage reduced the extent of the early inactivation phase seen in the intravenously infected mice, but there was little increase in the rate of growth by the survivors in vivo (P. B. Carter, unpublished data). Much the same picture emerged for the passaged cultures of S. paratyphi A and B. However, when the mouse-passaged S. paratyphi C was tested, a substantial increase in mouse virulence was observed. This was partly due to a reduced early inactivation of the inoculum, as well as to a more extensive growth period in vivo. Whereas it was possible that the mouse-adapted S. paratyphi C also produced new surface antigens which somehow rendered it more resistant to the bactericidal action of the normal mouse phagocytes (analogous to those changes described recently for other in vivo-grown bacteria [10, 19]), it was unlikely that the change involved the Vi antigen component. Even in vitro-grown S. paratyphi C produced sufficient quantities of this antigen to render the organism inagglutinable to group C sera. The growth curves obtained with the passaged strain suggested that the increase in virulence correlated best with the increase in rate of growth shown by this organism in vivo.

The growth curves shown in Fig. 8 confirm and extend the recent report by Dierna et al. (7) that S. typhi-typhimurium hybrid 42 is capable of inducing a progressively fatal, systemic infection in mice. The hybrid strain had an almost identical somatic antigenic structure to S. typhi but somehow retained the higher mouse virulence of the S. typhimurium parent. There was a 100- to 1,000-fold increase in viable counts seen with the hybrid strain over the first 6-day period of the challenge, after which further growth seemed to be prevented by an emerging immune response. The unexpected finding that the hybrid strain multiplied in the livers and spleens of the C57Bl mice long after the other two mouse strains had effectively controlled the infection was observed on a number of occasions and appears to be a real phenomenon. The growth curve shown in Fig. 8 is therefore consistent with the lowered LD50 values for this
organism in the C57Bl mice (Table 1). Other workers have suggested that C57Bl mice are less able to develop an effective immunity to salmonellosis than other inbred strains of mice (22). This is confirmed by the progressive increase in the liver and spleen counts in the C57Bl but not the C57Bl × DBA F1, hybrid mice from the seventh day of the infection. The latter strain was able to develop a fully effective resistance to the further growth of the S. typhi-
typhimurium in vivo (7). It is not known whether strain 42 is virulent for man or whether any resulting intestinal infection would conform to the pathogenic characteristics of the S. typhi or the S. typhimurium parent. Thus, we do not know whether the infection induced by the hybrid strain 42 would be more analogous to human typhoid fever or to gastroenteritis. Certainly the disease caused by the hybrid strain in mice bears considerable resemblance to the progressive type of systemic S. typhi infection which develops in naturally infected humans. It is hoped that cross-protection tests carried out in B6D2 mice infected with S. typhi Ty2 and the hybrid 42 will provide useful data on the relevance of this mouse model to the human disease.

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