

Effects of in vitro Growth Phase on the Pathogenesis of Salmonella typhimurium in Mice

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The growth phase of a bacterial (Salmonella typhimurium) culture was shown to have pronounced effects on the pathogenic properties of the harvested bacteria. Salmonellae obtained from a culture in primary (exponential) growth phase (PP) were more readily cleared from the blood and more readily killed by phagocytes than were salmonellae obtained from a more slowly growing secondary growth phase (SP) culture. PP salmonellae were observed to cause death of mice sooner than SP salmonellae. This appeared to be because the more rapid growth of PP, as compared to SP, salmonellae continued in the liver and spleen for several hours following intravenous injection, and more than compensated for their high in vivo death rate. As a result, within 4 h there were approximately 10-fold more live salmonellae in the spleens and livers of mice that had received PP, as compared to SP, salmonellae. This 10-fold difference was maintained until the death of the mice, indicating that after the first 4 h post-inoculation, the net in vivo growth of the salmonellae was the same regardless of their growth phase in the inoculating culture. This transition between PP and SP salmonellae occurred long before a dense stationary phase culture was obtained. Salmonellae grown in minimal media exhibited the biological properties of SP salmonellae and never entered as rapid a growth phase as did salmonellae in complete media.

INTRODUCTION

There have been many reports over the last 70 years indicating that rapidly growing bacteria are more susceptible to serum, to chemical, osmotic, lysosomal and certain antibiotic agents, as well as to temperature shock than are slower growing bacteria (Sherman & Albus, 1923; Sherman & Cameron, 1934; Rest et al., 1977; Taylor et al., 1981; Wilson & Miles, 1964). It has also been reported that the blood clearance of pneumococci can be affected by bacterial growth phase (Brown et al., 1981). Based on this type of observation there is an appreciation among most individuals working in microbial pathogenesis that bacterial growth conditions may have a pronounced effect on bacterial virulence.

In the present paper we report the results of a detailed investigation of the effects of bacterial growth phase on the pathogenesis of a bacterial species in mice. We have used mouse virulent Salmonella typhimurium for these studies since it has been increasingly studied as a model for infection by facultative intracellular parasites, such as S. typhi in man. A major topic of study of S. typhimurium pathogenesis in recent years has been the study of both mouse and Salmonella genes which affect the net growth and killing of salmonellae in the liver and spleen (Plant et al., 1982; Briles et al., 1981a; Hormaeche et al., 1981; Weinstein et al., 1984). In the course of these studies inoculations have frequently been given intravenously in an effort to carefully control the

Abbreviations: FCS, foetal calf serum; LR, lactate-Ringer's solution; PP, primary phase; SP, secondary phase; TH + Y, Todd-Hewitt broth plus 0.5% yeast extract.
size of the inoculum getting to the reticulo-endothelial system, and to avoid the effects of resistance genes that might control the penetration of salmonellae from the gut into the lymph and blood. In an effort to maximize the reproducibility of such studies and to facilitate the comparison of results between different studies we have examined in detail the relationship between the growth phase of this organism under standard culture conditions and its fate in vivo.

As with cultures of other bacteria, following a lag phase, salmonellae initially grow at a constant rate that results in an exponential increase in bacterial numbers. As growth proceeds, the rate of bacterial division declines, in part because of nutritional deficiencies, and although there may still be a net increase in bacterial numbers growth is no longer exponential.

Since the transition in biological properties occurs at the end of exponential growth, long before net growth of the cultures ceases, we have avoided the term 'stationary phase', and have used instead the term 'secondary phase' (SP). For the sake of continuity we have referred to the exponential phase of growth as the 'primary phase' (PP).

METHODS

Mice. BALB/cJN and C3H/HeN mice, 6-10 weeks old, were obtained from a specific pathogen free colony maintained by Dr Jerry McGhee in the Core Facility for Immunocompromised Mice, The Comprehensive Cancer Center, University of Alabama, Birmingham, Ala. USA. The BALB/cJN mice were used in all experiments except as indicated. C57BL/6J, LAFl/J and CB6Fl/J mice were purchased from Jackson Laboratories, Bar Harbor, Me., USA. BSVS mice were raised in our animal facility. Mice were maintained on sawdust bedding with a 12 h light-12 h dark cycle and provided with water and Purina Lab Chow (Ralston Purina, St Louis, Mo., USA) ad lib.

Salmonellae. S. typhimurium SR-11 (Schneider & Zinder, 1956) was obtained from L. J. Berry, University of Texas at Austin, Tx., USA; its virulence has been maintained by intravenous passage through mice. S. typhimurium SL1344 was obtained from S. Hoiseth and B. A. D. Stocker, Stanford (Hoiseth & Stocker, 1981) and S. typhimurium LT2-Z from C. Turnbough, University of Alabama at Birmingham. Both PP and SP cultures were grown in Todd-Hewitt broth (Difco) supplemented with 0.5% yeast extract (TH + Y). Inocula for SP cultures were obtained from stock cultures maintained at -70 °C in media. SP cultures were grown overnight (12-16 h) at 37 °C in a shaking water bath, harvested by centrifugation and suspended in lactate-Ringer's solution (LR) at 4 °C. PP cultures were inoculated from resuspended SP cultures to yield a starting density of 1 x 10^6 colony-forming units (c.f.u.) ml^-1 of fresh media and grown for approximately 2-5 to 3 h at 37 °C in a shaking water bath. After harvesting by centrifugation bacteria were washed once in LR, centrifuged again, and suspended in a known volume of LR before determination of OD_{420} using a Beckman model 25 spectrophotometer. Dilutions of the harvested bacteria, appropriate for the experiments, were made by assuming that an OD_{420} of 1 = 5 x 10^8 SP or 1 x 10^9 PP salmonellae ml^-1. Numbers of viable bacteria were always confirmed by plating. Cultures designated PP were always harvested before an OD_{420} of 0.2 was reached. Cultures designated SP were always harvested after an OD_{420} of 3.0 was reached. Initiation of PP and SP cultures was planned so that the SP and PP S. typhimurium injected into mice could be harvested at the same time. The pH of the media used was 7.5 at the initiation of the cultures and not less than 6.5 at the harvest of the SP salmonellae.

Control experiments, run with the various assay procedures described below, indicated that plating on either bismuth sulphite or nutrient agar gave essentially the same numbers of colonies. For most experiments, bismuth sulphite agar was used since visualization of S. typhimurium colonies was easier and strict sterility was not required. Bacteria were enumerated in pour plates, made by vortexing 0.7 ml of a serial dilution of bacteria into 8 ml bismuth sulphite agar at 50 °C, and immediately pouring the mixture into a 100 mm plastic Petri dish.

Growth kinetics of SR-11. Twelve identical tubes, each containing 7 ml of medium, were inoculated with 10^6 c.f.u. (OD_{420} 0.002) of a 16 h culture of SR-11 salmonellae. The tubes were incubated with shaking at 37 °C. From two of these tubes 0.1 ml of medium was removed at inoculation and at 1, 2, 3, 4, 5, 6, 8, 12, 17 and 25 h post-inoculation. These samples were appropriately diluted and plated. At 3, 4, 6, 12 and 25 h post-inoculation all the salmonellae in two additional tubes were harvested by centrifugation. The numbers of salmonellae in each tube were determined by plating samples taken before harvesting. OD_{420} values and the number of c.f.u. determined for harvested bacteria and pre-harvest bacteria were the same within counting error.

Recovery of viable salmonellae from blood, liver and spleen. Mice were injected intravenously with 0.2 ml LR containing 10^6 c.f.u. of PP or SP salmonellae. Blood samples (75 μl), taken at given times, were serially diluted in LR and plated to determine the number of c.f.u. per ml of blood. The total volume of blood per mouse was calculated from the weight, assuming a constant of 0.08 ml g^-1. The number of c.f.u. in the total blood volume was calculated and expressed as a percentage of the dose given.
Spleens, livers and other tissues were homogenized in 10 ml phosphate buffer (0.01 M, pH 7.2) at 4 °C using a stomacher (Tekmar, Cincinnati, Ohio, USA). Samples of the homogenate were serially diluted in buffered water and plated to determine the number of c.f.u. per organ. Data were expressed as a percentage of the dose given.

Growth kinetics in media and mouse serum. PP or SP cultures were diluted to 10^6 c.f.u. ml⁻¹ in fresh media (or serum) at 4 °C and then incubated in a shaking water bath at 37 °C. At given times after transfer to the water bath, measured volumes were removed, diluted in LR, and plated. Serum obtained fresh from retired breeder BALB/cJN mice was filter-sterilized before use. A portion of this serum was heat-inactivated at 56 °C for 45 min. Media used for culture of salmonellae were TH + Y broth and minimal media (73 mM-K₂HPO₄, 17 mM-NaH₂PO₄, 0.8 mM-MgSO₄, 10 mM-citric acid) (Vogel & Bonner, 1956).

In vitro and in vitro macrophage studies. Mice were given intraperitoneal injections of 1 ml Brewer-thioglycollate (Difco) 4 d before inoculation with salmonellae (Briels et al., 1981 b). PP and SP salmonellae resuspended in LR at 10⁷ c.f.u. ml⁻¹ were opsonized at 4 °C for 30 min with 20 μg ml⁻¹ of the Salmonella-specific hybridoma antibody, ST-1 (Briels et al., 1981 b), with periodic vortexing. Mice were injected intraperitoneally with 0-1 ml opsonized PP or SP salmonellae. The peritoneal cavity was washed out 10 min after infection with 3 ml LR at 4 °C containing 0-01% BSA and 15 units heparin ml⁻¹. As described previously (Briels et al., 1981 b), the peritoneal exudate cells were layered on filter sterilized (0-45 μm, Millipore) foetal calf serum (FCS) and centrifuged at 250 g for 10 min. The cells were resuspended in LR and recentrifuged through FCS. The pelleted cells were resuspended in RPMI 1640 containing 5% FCS (RPMI-FCS) at 4 °C. By plating the FCS supernatants and the cell pellets it was possible to determine what fraction of the salmonellae recovered from the peritoneal cavity were cell-associated. Virtually 100% of PP and SP salmonellae recovered from the peritoneal cavity after 10 min were cell-associated as reported previously (Briels et al., 1981 b). To determine the number of c.f.u. at zero time a sample of the cell suspension was removed, diluted in phosphate buffer containing 0-1% BSA and plated. The cell suspensions were then incubated at 37 °C in a shaking water bath and samples removed at given times for plating, after lysis as above. The numbers of c.f.u. recovered at the various times were expressed as a percentage of those recovered at zero time.

Statistical comparisons. The two-sample rank test (Goldstein, 1964) was used to provide statistical treatment groups.

RESULTS

Growth kinetics of S. typhimurium SR-11

The initial growth of S. typhimurium SR-11 in TH + Y was quite fast, with a doubling time of 25 min (Fig. 1a). The exponential PP was followed about 6 to 7 h post-inoculation by a SP at which time the growth rate slowed to a doubling time of 23 h.

Although there was almost a 25% reduction in c.f.u. from 17 to 25 h post-inoculation, bacterial death was probably not significant before 17 h since actual bacterial counts in a Peteroff-Hauser chamber indicated that the viability of the salmonellae was virtually 100% in both the 17 h SP culture and a 3 h PP culture. During the culture period the OD₄₂₀ of the culture increased less than did the numbers of c.f.u. (Fig. 1a, compared with Fig. 1b). This was due to a reduction in the optical density per c.f.u. of the bacteria as they passed from PP to SP. This is reflected in Fig. 1(c), where the number of c.f.u. per OD₄₂₀ unit is depicted for salmonellae harvested 3, 4, 6, 12 and 25 h post-inoculation. The light scattered by the individual bacteria, as measured by OD₄₂₀, actually decreased as they passed from PP to SP.

The higher light scattering power of the PP as compared to the SP salmonellae was consistent with the fact that PP salmonellae are much larger than SP salmonellae (Clark & Ruehl, 1919; Henrici, 1926). In our samples the difference in size of PP, as compared to SP, salmonellae was readily apparent by light microscopy of unstained bacteria.

Effects of the growth phase of salmonellae on subsequent recovery from livers and spleens of infected mice

We next determined the number of salmonellae recoverable from the liver and spleen of mice 4 h after inoculation with SR-11 cultures that had been grown to various optical densities before use (Fig. 2). Salmonellae were grown to OD₄₂₀ of 0-16, 0-65, 1-6 and 8-2, and injected intravenously into mice at a dose of 10⁸ c.f.u. per mouse. The two low density cultures were in PP while the culture with OD₄₂₀ of 1-6 was just at the end of PP, and the culture with an OD₄₂₀ of 8-2 was well within SP (Fig. 1).
Fig. 1. Growth kinetics of a culture of *S. typhimurium* SR-11 in 5 ml TH + Y. Cultures were inoculated with 10^6 salmonellae from an overnight SP culture. (a) ○, •, c.f.u. ml^-1 from each of two cultures, which were sampled at the times indicated; ★, means of duplicate platings of salmonellae from the tubes harvested to yield the OD_{420} values shown in (b). (b) OD_{420} values of cultures in five separate tubes; each tube was harvested at the time indicated. (c), Number of harvested c.f.u. per OD_{420} unit for each of the five cultures shown in (b).

Fig. 2. Recovery of viable salmonellae from the spleens (□) and livers (■) of mice inoculated intravenously 4 h earlier with salmonellae harvested at various culture densities (OD_{420}). The geometric mean number of bacteria recovered from the five mice in each group is plotted as a percentage of the inoculation dose. Bars indicate SE. All the data from cultures with OD_{420} < 1 are significantly different (P < 0.05) from the data from cultures with OD_{420} > 1, except for the comparison of the spleen data from the cultures with OD_{420} values of 0.65 and 1.6.
The results of the experiment (Fig. 2) indicated that as the concentration of viable bacteria in the cultures used for mouse inoculation increased, the number of live salmonellae recovered from the spleens and livers, expressed as a percentage of the inoculum, decreased. Thus, per cell, the salmonellae in the rapidly growing low density culture have a greater ability to infect the liver and spleen than salmonellae in slower growing, high density cultures. In particular, it was apparent that the greatest difference in *in vivo* recovery observed between the cultures occurred with the transition between PP and SP growth. In this experiment that transition occurred between the cultures with OD$_{420}$ values of 0.65 and 1.6.

**Kinetic analysis of the numbers of PP and SP salmonellae in livers and spleens following infection**

In these and subsequent studies of the differences in biological properties of PP and SP salmonellae, we used cultures with an OD$_{420}$ < 0.2 for PP salmonellae and cultures with an OD$_{420}$ > 3 for SP salmonellae. The numbers of PP and SP salmonellae in the livers and spleens of mice were determined 2, 3, 4 and 7 h after intravenous inoculation (Fig. 3a). These data make it clear that the differences observed 4 h post-inoculation are also apparent at the other times.

Since most studies of the pathogenesis of salmonellae have used much smaller inocula of salmonellae than we used in the short-term experiments above, we infected mice with 100 PP or SP salmonellae, and determined the numbers of salmonellae in their livers and spleens after 1, 2, 3 or 4 d: roughly 10 times more salmonellae were recovered from the livers and spleens of mice receiving PP, as compared to SP, salmonellae (Fig. 3b). The difference in numbers of salmonellae in mice inoculated with PP, as compared to SP, salmonellae was apparently established before the 24 h point, since the subsequent rate of accumulation of salmonellae in both groups of mice was the same. All remaining mice in both groups died between 5 and 7 d post-infection.

Because of mouse to mouse variations in time of death 1 week after infection, much larger numbers of mice would have been required to determine if there was a significant difference in the time of death of mice infected with PP, as compared to SP, salmonellae.

**Time of death of mice infected with PP and SP salmonellae**

To determine if there was a difference in the ability of SP and PP salmonellae to cause death, we infected mice with doses of salmonellae greater than 10$^5$. By using such high doses we expected death to occur sooner and hoped that the amount of variation in the time of death within each group would be minimized. From Fig. 4 it is apparent that from a dose range of from 2 x 10$^5$ to 2 x 10$^6$ there was a 12 to 18 h lag in deaths of mice infected with SP as compared to PP salmonellae.

**Effect of salmonellae growth phase on subsequent blood clearance**

The difference in numbers of salmonellae recovered from the spleens and livers of mice after intravenous injection with PP, as compared to SP, salmonellae did not appear to be due to differential blood clearance of PP and SP salmonellae. Although PP salmonellae were cleared from the blood slightly faster (statistically significant at $P < 0.002$, by rank order test) than SP salmonellae, more than 98% of both were cleared from the circulation 2 h after intravenous injection (data not shown). Thus the lower recovery of SP, as compared to PP, salmonellae from the liver and spleen at the 4 h point could not have been due to incomplete clearance of SP salmonellae.

**Localization of salmonellae in various tissues and organs**

One possible explanation for the differences in recovery from mouse livers and spleens of PP, as compared to SP, salmonellae is that PP and SP salmonellae may localize in different sites in the mouse. This did not appear to be the case in our experiments. When the numbers of PP and SP salmonellae in the spleens, livers, lungs, kidneys and mesenteric lymph nodes of five individual mice injected with PP or SP salmonellae were determined, about 80% of the recovered salmonellae were present in the liver and spleen, and the PP and SP salmonellae showed the same relative distribution among the different organs assayed (Table 1).
Fig. 3. (a) Recovery of viable salmonellae from the livers and spleens of mice 2 to 7 h after intravenous inoculation with about 10^5 PP or SP salmonellae; (b) recovery of salmonellae from livers and spleens of mice 1-4 d after intravenous inoculation with about 100 PP or SP salmonellae. Data are plotted as geometric mean number of salmonellae recovered from the liver (●) or spleen (○) of each mouse and are given as a percentage of the inoculation dose to normalize for slight differences in the numbers of PP and SP salmonellae given. Bars indicate SE. Each point represents the data from five mice. All data with PP salmonellae (---) are significantly different from the data with SP salmonellae (-----) from the same organ (P < 0.05) except for the comparison of spleen data at 3 h and 3 d post-inoculation.

Fig. 4. Time of death of mice infected with PP and SP salmonellae. Groups of six mice were infected with either 1.5 x 10^5, 5.2 x 10^5 or 7.7 x 10^6 PP salmonellae (●), or 2.2 x 10^5, 7.3 x 10^5 or 2.2 x 10^6 SP salmonellae (○). Deaths were recorded at 6 h intervals for a period of 90 h by which time all the mice had died. Mice infected with the lowest doses of PP salmonellae died significantly (P < 0.01) earlier than mice infected with the lowest doses of SP salmonellae. Similarly, mice infected with the intermediate dose of PP salmonellae died significantly (P < 0.01) sooner than mice infected with the intermediate dose of SP salmonellae. The significance of these comparisons is made even greater by the fact that for each of the two comparisons mice were injected with slightly fewer PP than SP salmonellae.

Growth kinetics of PP and SP salmonellae in vitro

Another possible explanation for the greater recovery of PP, as compared to SP, salmonellae from the spleens and livers of mice is that since they are growing exponentially they might be expected to show faster initial growth in vitro. The greater growth potential of PP as compared to SP salmonellae is shown in Fig. 5 where PP and SP salmonellae were inoculated into TH + Y media, fresh normal mouse serum, or heat-inactivated normal mouse serum. In all three instances, PP salmonellae continued growing exponentially, whereas the SP salmonellae had a lag phase of almost 1 h.
Bacterial growth phase and pathogenesis

Fig. 5. Growth of PP (-----) and SP (----) salmonellae in TH + Y (a), and in normal (○) and heat-
inactivated (▲) mouse serum (b). Cultures were inoculated with 10^6 c.f.u. ml⁻¹.

Table 1. Relative recovery of PP and SP salmonellae in various body tissues

Groups of three to five mice were inoculated intravenously with 10⁶ PP or SP salmonellae; after 4 h the mice were sacrificed. SE values were never >30% of the listed values.

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<tr>
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<th>PP salmonellae</th>
<th>SP salmonellae</th>
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<tr>
<td></td>
<td>Percentage of inoculum received</td>
<td>Percentage of inoculum recovered</td>
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<tr>
<td>Liver</td>
<td>31·1</td>
<td>31·5</td>
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<tr>
<td>Spleen</td>
<td>48·0</td>
<td>48·7</td>
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<tr>
<td>Kidney</td>
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<td>Mesenteric lymph node</td>
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<td>Total</td>
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The results obtained with fresh serum are particularly important since fresh serum is one of the environments in which salmonellae could grow in in vivo. Since heat-inactivation of the normal serum did not alter the growth kinetics of either PP or SP salmonellae (Fig. 5b), it seems unlikely that the difference in in vivo survival of PP and SP salmonellae is dependent on complement-mediated killing in normal serum.

Growth kinetics of PP and SP salmonellae associated with peritoneal cells

In an attempt to examine the growth rates of PP and SP salmonellae under conditions that more closely reflect those in vivo, we examined in vitro growth of salmonellae associated with thioglycollate elicited peritoneal exudate cells. Thioglycollate-elicited macrophages were chosen for these studies since they are highly phagocytic for salmonellae opsonized with ST-1, but much less bactericidal than resident macrophages (Briles et al., 1981b). These properties facilitated our studies on the growth of cell-associated bacteria, since almost 30% of the injected PP salmonellae and virtually all injected SP salmonellae were recovered from the peritoneal cavities (Fig. 6), and over 99% of the recovered salmonellae in both growth phases were found to be cell-associated.
Fig. 6. Growth of salmonellae in vitro in thioglycollate elicited peritoneal exudate cells from CB6 mice injected intraperitoneally with PP (■) or SP (□) salmonellae. Peritoneal cells were harvested at 10 min, washed free of non-attached salmonellae at 4 °C, and placed in culture at 37 °C. Time post-harvest refers to the time for which the cells were incubated at 37 °C. Each bar represents the data from triplicate cultures. At each time, the values for SP salmonellae were significantly different from those for PP salmonellae ($P < 0.05$). The 3 h values for SP and PP salmonellae were different from the earlier SP and PP time points ($P < 0.05$).

Fig. 7. Killing of macrophage-associated salmonellae by penicillin. Opsonized salmonellae were injected intraperitoneally into mice that had been previously injected with thioglycollate. After 10 min the mice were killed and the peritoneal exudate cells were removed and washed free of unattached salmonellae. The peritoneal exudate cells were then incubated in tissue culture media containing either 0 (a), 1 (b), 10 (c), 100 (d) or 1000 μg (e) penicillin. At various intervals samples of cells were removed, lysed, and plated to enumerate salmonellae. The zero time value represents the number of salmonellae associated with the cells when they were placed in culture. Each curve represents means of triplicate cultures.

The lower recovery of PP salmonellae was probably the result of a greater susceptibility of these bacteria to cell-associated killing. This conclusion is supported by the fact that in vitro incubation of the recovered macrophages with their associated salmonellae for 1 h resulted in a further decrease in the numbers of salmonellae in the cell suspensions from mice receiving PP, as compared to SP, salmonellae (Fig. 6).

However, between the 1 h and 3 h points the situation changed markedly. While the numbers of salmonellae in cells infected with SP salmonellae increased slightly, the numbers of salmonellae in the cells infected with PP salmonellae increased 10-fold to nearly twice the infecting dose. It seems likely that the cell growth we observed was intracellular since repassage
of the peritoneal cells through FCS after 3 h showed that over 90% of the salmonellae were still cell-associated. These results suggested that the greater recovery of salmonellae from mice infected with PP, as compared to SP, salmonellae is the result of more rapid growth of the PP salmonellae.

Examination of relative growth rates of salmonellae by use of penicillin

Since thioglycollate elicited macrophages are probably not typical of reticulo-endothelial macrophages, we examined the relative in vivo growth rates of PP and SP salmonellae by making use of the ability of penicillin to preferentially kill growing, rather than resting, bacteria (Lowrie et al., 1979). We measured the effects of penicillin on the growth and killing of PP and SP salmonellae in media, in association with peritoneal exudate cells, and in mice.

As expected, a greater penicillin-dependent reduction in c.f.u. occurred in cultures inoculated with PP, as compared to SP, salmonellae: at a concentration of 1000 μg penicillin ml⁻¹, only 0-3% of the PP salmonellae remained alive after 30 min as compared to 36% of the SP salmonellae. The best separation between the killing curves of PP and SP salmonellae occurred at a concentration of 100 μg ml⁻¹, where the killing of the SP salmonellae lagged slightly more than an hour behind that of PP salmonellae.

When the same concentrations of penicillin were added to salmonellae associated with macrophages the drug had only a slight effect at concentrations in the medium below 1000 μg ml⁻¹. This is consistent with the work of Lowrie et al. (1979) and with their finding that intracellular penicillin concentrations are less than extracellular penicillin concentrations. At 1000 μg penicillin ml⁻¹, a rapid decrease in numbers of PP salmonellae occurred between 1 h and 2 h post-infection (Fig. 7). This corresponds to the time when a rapid net increase of these salmonellae was first seen in peritoneal cells (Fig. 6). The SP salmonellae treated with the same concentration of penicillin showed only a modest decrease in numbers during the same time.

To obtain information about the in vivo growth rates of SP and PP salmonellae, mice were injected intraperitoneally with either 1 or 10 mg penicillin before intravenous injection of salmonellae (Fig. 8). In mice receiving either dose there was a marked penicillin-dependent decrease in recovery of salmonellae after 4 h from both the livers and spleens of mice receiving PP salmonellae (Fig. 8b). A much smaller penicillin-dependent decrease in numbers of SP salmonellae in livers and spleens was observed. As expected, in the control group, which was given no penicillin, five times as many salmonellae were recovered after 4 h from the livers of mice given PP, as compared to those given SP, salmonellae. These findings indicate that PP salmonellae divide more rapidly in vivo during the 4 h post-inoculation period than do SP salmonellae.

The 2 h data, like the 4 h data, indicate that PP salmonellae grow faster in vivo than do SP salmonellae (Fig. 8a). However, they also suggest that there is much more efficient cell-mediated killing of PP, than of SP, salmonellae. This conclusion is based on the fact that in control mice, receiving no penicillin, the number of salmonellae recovered after 2 h was the same regardless of whether they received PP or SP salmonellae. Thus the more rapid growth of PP salmonellae must be compensated for in these mice by a higher rate of killing of PP, as compared to SP, salmonellae. This finding supports the in vitro results presented in Fig. 6, indicating that PP, as compared to SP, salmonellae are killed more readily by thioglycollate elicited macrophages.

Generalization of results to other mouse strains, other bacterial strains and other media

The results described above were obtained with a single bacterial strain, SR-11, and a single bacteriological medium, TH + Y. Most of the experiments also made use of BALB/c mice. The results obtained, however, do not appear to be restricted to this set of conditions. We compared the numbers of PP and SP salmonellae recovered from the livers and spleens of mice infected with the highly virulent strain SL1344 (Hoiseth & Stocker, 1981), and the relatively avirulent strain LT2-Z, of *S. typhimurium*. Although a larger percentage of SL1344 than of LT2-Z was recovered from spleens and livers, the effect of using PP, as compared to SP, salmonellae of both of these strains was similar to that observed with SR-11 (data not shown). Bacteria grown in
Fig. 8. In vivo killing of salmonellae by penicillin. Mice were injected with the indicated amounts of penicillin 10 min before intravenous inoculation with PP (■) or SP (□) salmonellae. After 2 h (a) and 4 h (b) the mice were killed and the numbers of salmonellae in their livers and spleens were determined. Each bar represents the data from three mice. In all cases where penicillin was used, significantly fewer (P < 0·05) PP than SP salmonellae were recovered except for the 4 h data with bacteria from the liver. In the case of PP salmonellae penicillin significantly (P < 0·05) reduced the recovery of the bacteria from the liver and spleen. Although penicillin had a much smaller effect on the recovery of SP salmonellae the effect was significant (P < 0·05) in all cases except for the spleen data at 2 and 4 h with 1 mg penicillin.

Penassay broth, another complex medium, gave the same general result that was obtained when the bacteria were grown in TH + Y. When bacteria were grown in minimal medium, however, where even the PP growth rate was quite slow, (124 min doubling time as compared to 17 to 23 min for TH + Y, Penassay broth and brain heart infusion broth), there was no difference in liver accumulation of PP and SP salmonellae.

In other studies (data not shown), we have determined that the differential recovery of PP, as compared to SP, salmonellae from livers and spleens can also be seen with C3H/HeN, C57BL/6J, BSVS, CB6F1/j and LAFl/J mice.

DISCUSSION

Our studies have shown that in the mouse PP and SP salmonellae differ from each other in three very important ways. The PP bacteria are more readily cleared from the blood, and more easily killed by phagocytes, than SP bacteria, but can more than compensate for these relative
frailties by their more rapid initial in vivo division rate. The greater virulence of PP, as compared to SP, salmonellae was evident from the earlier death of mice infected with PP salmonellae. Also, larger numbers of salmonellae were recovered from the spleens and livers of mice injected intravenously with PP salmonellae harvested from various standard bacteriological media than were recovered from mice receiving SP salmonellae harvested from the same media. The lower virulence of SP, as compared to PP, salmonellae was not because SP salmonellae had a lower viability than PP salmonellae since (1) the SP cultures had a higher number of c.f.u. per OD$_{420}$ unit than the PP cultures, and (2) the inoculation doses of PP and SP salmonellae were based on the actual numbers of c.f.u. injected. The more rapid in vivo growth rates of PP, as compared to SP, salmonellae might be due to larger stores of metabolites in PP salmonellae, to a higher state of metabolic activity and the presence of more of the necessary enzymes to operate metabolic pathways in PP salmonellae, or to other differences between PP and SP salmonellae.

The differences in in vivo growth rates of PP, as compared to SP, salmonellae appears to be only temporary, since by several hours after inoculation the net in vivo increase in bacterial numbers (but not the total number of bacteria) was the same regardless of whether mice were inoculated with PP or SP salmonellae. The effect of growth phase on pathogenesis appeared to be dependent on rapid growth in vivo, since salmonellae grown in minimal media never entered a rapid growth phase and always exhibited the biological properties associated with SP salmonellae.

Our observation that in vivo killing of PP salmonellae was more rapid than killing of SP salmonellae was anticipated by Rest et al. (1977) who showed that exponential phase salmonellae are more readily killed in vitro by phagocytes than are stationary phase salmonellae; they also cited studies that showed exponential phase salmonellae to be more susceptible to chemical or physical killing. Most of our present study has focused on growth conditions used for preparing salmonellae for inoculation into mice (Briles et al., 1981a,b; Eisenstein et al., 1982; Hormaeche, 1979; O'Brien et al., 1981; Robson & Vas, 1972; Wilson et al., 1982). Two parameters affected by salmonellae growth phase, namely blood clearance and killing by phagocytic cells, are known to be influenced by surface structure (Biozzi et al., 1960; Krishnapillai, 1971; Lyman et al., 1977; Nakano & Saito, 1969; Valtonen, 1977; Valtonen et al., 1976).

It is well known that the smooth or complete O-antigen of S. typhimurium is an important virulence determinant in the mouse system. Smooth organisms are not removed from the blood by the reticuloendothelial system as rapidly as various rough mutants, and are much less susceptible to the bactericidal mechanism of phagocytes (Krishnapillai, 1971; Liang-Takasaki et al., 1983; Nakano & Saito, 1969; Tagesson & Stendahl, 1973; Valtonen, 1977). It is interesting that these are two parameters in which PP salmonellae differ from SP salmonellae. The PP salmonellae behave as though they are rougher than SP salmonellae for a short time after inoculation. This may be at least partially explained by a report that chemostat-grown salmonellae with a high growth rate showed decreased agglutination by anti-O sera and a concomitant 5 to 10 fold decrease in expression of O-antigen monosaccharides (Collins, 1964). The outer membrane composition of S. typhimurium, Klebsiella aerogenes and E. coli is also influenced by the growth of the culture (Robinson & Tempest, 1973; Schnaitman, 1974). However, S. typhimurium mutants which are deficient in some outer membrane proteins, and have altered expression of others, are still virulent (Valtonen et al., 1977).

In most studies of the pathogenesis of salmonellae, what is measured is not simply growth or killing but the net effects of these two activities. The results in this paper indicate that whether PP or SP salmonellae show more net growth in a particular experiment would depend on the relative importance of killing, as compared to growth, in the assay used. Important points, familiar to experienced workers in the field, but not obvious from the growth curves such as the one shown in Fig. 1 are that (1) the transition between PP and SP growth phase occurs long before the optical density of the culture stops increasing and (2) most of the PP growth occurs before the culture becomes visibly turbid. In fact, by the time the bacterial density is high enough to yield an easily recoverable cell pellet by centrifugation, the culture is too near to the transition between PP and SP bacteria for harvest of PP bacteria to be made without paying careful attention to the growth kinetics of the culture.
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