Dissemination and proliferation of *Salmonella typhimurium* in genetically resistant and susceptible mice

H. R. XU and H. S. HSU*

Department of Microbiology and Immunology, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298, USA

Summary. Genetically resistant A/J and CBA mice were inoculated intraperitoneally with either 10^3 or 10^4 organisms of a virulent strain of *Salmonella typhimurium*; susceptible C57BL/6J and BALB/c mice were inoculated with either 10^2 or 10^3 organisms. Except with the smaller dose in resistant mice, fatal infection ensued. Bacteraemia occurred within 1 h after inoculation, except that it was not detectable during the first 6 h in the susceptible mice inoculated with 10^2 organisms. From day 2, the circulating bacterial population continued to increase in all infected mice, except that it remained under control in the resistant mice inoculated with the lower dose (10^3 organisms). The pathogen proliferated logarithmically in the liver from day 2, and a bacterial count of $c. 10^8$ cfu/g of tissue was reached when the animals died at 5–7 days; again, the resistant mice inoculated with 10^3 organisms were an exception in which the hepatic bacterial population was kept under control and the mice survived.

Introduction

Host resistance in murine salmonellosis is under genetic control. Inbred mouse strains have been derived with varying degrees of native resistance to the disease, some highly susceptible, others highly resistant.¹ The variation can in part be demonstrated by the difference in the net bacterial growth in the liver and spleen during the infection. It has been suggested that the difference was attributable to the relative capacity of the host's macrophages to control the rate of bacterial proliferation.^{2,3} Histopathological evidence throws doubt on this contention, because the early lesions in the liver and spleen contain predominantly inflammatory polymorphs rather than macrophages, and also the pathogen replicates extracellularly in lesions and sinusoids as well as within hepatocytes.⁴

The present study was designed to detect dissemination of the pathogen at early stages of the infection and its subsequent proliferation in the liver, in an attempt to ascertain the ability or inability of the host to contain the bacteria. We believe this to be the first step in a systematic analysis of the basic difference between resistant and susceptible hosts.

Materials and methods

Strain of Salmonella typhimurium

The virulent strain SR-11⁵ was maintained on Tryptic Soy Agar (TSA; Difco) and propagated in Tryptic Soy Broth (TSB; Difco) for 6 h at 37 °C in a rotating drum. The organisms were washed in saline and the suspension was standardised optically by the previously described procedure⁵ to contain $c. 2 \times 10^9$ viable bacteria/ml.

Mice

Inbred, male mice weighing $c. 20$ – 25 g were used. A/J mice (Jackson Laboratories, Bar Harbour, ME) and CBA mice (National Cancer Institute, Frederick, MD) are genetically typed *Ity^r*, being resistant to infection with *S. typhimurium* by the intraperitoneal (i.p.) route, with an LD50 of $c. 10^4$ bacteria. C57BL/6J mice (Jackson Laboratories) and BALB/c mice (National Cancer Institute) are typed *Ity^s*, being hypersusceptible to a similar infection, with an LD50 of $< 10^2$ organisms. The animals were housed in the Central Animal Care Facility at the Medical College of Virginia for at least 1 week before they were used.

Infection of mice and sampling of bacteria in blood and liver

Mice were inoculated i.p. with a designated number of bacteria. Heparinised Natelson capillary tubes

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* Correspondence should be sent to Dr H. S. Hsu.

(Baxter Corp., McGaw Park, IL) were used to obtain 12.5- μ l samples of blood from the retro-orbital plexus of individual infected mice selected by rotation at designated intervals. Each blood sample was placed in 1 ml of saline and sonicated for 15 s in a Biosonik II apparatus (Bronwill Scientific, Rochester, NY), at a "needle" probe intensity setting of 30, to disrupt the leucocytes and to disperse the bacteria. The sample was diluted in saline and plated on TSA to enumerate the bacteria.

Randomly selected mice were killed with ether at daily intervals from day 2 after inoculation. The liver was removed, and weighed after a small section had been taken for histopathological examination. It was then ground in 12 ml of chilled saline in a 20-ml sterilised stainless steel cup with a Waring tissue blender (model 34BL21) at no. 2 setting for 2–3 min. The homogenised tissue was centrifuged at 60 *g* for 10 min at 5°C to sediment debris. The absence of intact tissue cells in the supernate was confirmed by microscopy. A sample of it, containing disrupted cells and released bacteria, was diluted for bacterial quantitation on TSA plates.

Results

Bacterial dissemination and proliferation in resistant (A/J) and susceptible (C57BL/6J) mice

Three experiments were performed as follows. The resistant mice were divided into two groups of 6–8 animals each, and inoculated i.p. with either 2×10^3 or 5×10^4 bacteria. Likewise, two groups of 5–6 susceptible mice were inoculated with either 1×10^2 or 2×10^3 bacteria. To assay the level of bacteraemia, peripheral blood samples were taken from one infected mouse in each of these four groups, selected by rotation, at 1, 2, 4, 6, 8, 10 and 24 h after inoculation. At daily intervals from day 2, one mouse from each group was bled for bacterial count, and killed; its liver also was removed for bacterial count.

A dose of 2×10^3 bacteria did not cause a fatal infection in the A/J mice, whereas a dose of 5×10^4 bacteria was invariably lethal. In contrast, both 1×10^2 and 2×10^3 bacteria induced a rapidly fatal infection in the C57BL/6J mice. Figs. 1 A and B show the changing bacterial population in the blood and liver, respectively, in the two types of mouse. Each point represents the average bacterial count from three mice in these three experiments.

As seen in fig. 1 A, the pathogen was not isolated from the peripheral blood of the C57BL/6J mice inoculated with 1×10^2 bacteria within the first 6 h of the infection. On the other hand, bacteraemia was detected within 1 h in both the C57BL/6J and A/J mice inoculated with 2×10^3 bacteria and in the A/J mice inoculated with 5×10^4 organisms. From day 2, the bacterial population in the circulation continued to increase until the mice died (from day 5 onwards),

Fig. 1. A, Bacteraemia, and B, bacterial proliferation in the liver, in naturally resistant A/J mice and susceptible C57BL/6J mice. The A/J mice were inoculated i.p. with either 2×10^3 (▽) or 5×10^4 (○) bacteria, and the C57BL/6J mice were inoculated with either 1×10^2 (▲) or 2×10^3 (●) bacteria.

except in the A/J mice inoculated with 2×10^3 bacteria, which were able to maintain a low level of circulating bacteria and to survive the infection.

Fig. 1 B shows that the number of bacteria increased logarithmically in the liver from day 2. With the exception of the A/J mice inoculated with 2×10^3 bacteria, a bacterial count of *c.* 10^8 /g of tissue was reached when mice died—from day 5 among the susceptible C57BL/6J mice and from day 7 among the resistant A/J mice. In contrast, the A/J mice inoculated with the lower dose of 2×10^3 bacteria were able to contain the bacterial population below *c.* 10^6 /g and to retard its subsequent growth.

Bacterial dissemination and proliferation in resistant (CBA) and susceptible (BALB/c) mice

The above experiments were repeated with different strains of resistant and susceptible mice. Two groups of 18 BALB/c mice were inoculated with either 1×10^2 or 2×10^3 bacteria; 21 CBA mice were inoculated with 2×10^3 bacteria and 26 CBA mice with 6×10^4 bacteria. At each designated interval, bacterial counts in the blood and liver were determined from either two or three mice in each of these four groups and the

Fig. 2. A, Bacteraemia, and B, bacterial proliferation in the liver, in natively resistant CBA mice and susceptible BALB/c mice. The CBA mice were inoculated i.p. with either 2×10^3 (▽) or 6×10^4 (○) bacteria, and the BALB/c mice were inoculated with either 1×10^3 (▲) or 2×10^3 (●) bacteria.

averages were plotted in figs. 2A and B, respectively.

Consistent with the preceding observations, fig. 2A shows that the pathogen entered the circulation within 1 h after the i.p. inoculation of 2×10^3 and 6×10^4 bacteria, whereas bacteraemia was not detected until 6 h after inoculation with 1×10^3 bacteria in BALB/c mice. There was also an increase of bacteria in the circulation from day 2 until the animals succumbed to the disease, except in CBA mice inoculated with 2×10^3 organisms. The latter group also controlled the initial bacterial invasion in the liver on day 2 and suppressed the subsequent bacterial growth after day 4 (fig. 2B). Again, only this group survived the disease. In the three other groups, the bacterial count reached c. 10^8 organisms/g of liver; the susceptible BALB/c mice began to die on day 5 and the resistant CBA mice from day 7.

Discussion

The *It*y gene on chromosome 1 is commonly cited as the primary controlling factor in murine host resistance to salmonellosis.⁶ It appears to affect the net growth rate of virulent *S. typhimurium* in the liver and spleen of inbred mouse strains during infection.^{1,7-9} A/J and CBA mice are both genetically typed *It*y^r and are consistently resistant to an i.p. inoculation of 10^3 viable units of *S. typhimurium* strain SR-11. In

contrast, C57BL/6J and BALB/c mice are genetically typed *It*y^s and invariably succumb to a similar inoculation with 10^2 bacteria. The purpose of the present investigation was to compare the early dissemination and the subsequent proliferation of the pathogen in resistant and susceptible hosts. The organisms were inoculated into the peritoneal cavity to simulate a local primary infection and initiate a systemic infection. The changing bacterial population in the liver was enumerated to represent their proliferation *in vivo*, because this organ is in the reticuloendothelial system (RES) but not in the lymphoid system.

Treatment of blood by dilution and sonication is likely to disperse the intracellular⁴ as well as extracellular organisms into single bacteria. In contrast, the vigorous grinding of the liver in a Waring blender is unlikely to disintegrate the tissue such that its total viable bacterial population can be released and recovered as individual organisms. But the procedure seems adequate and is suitable for comparative purposes. It is important to reiterate that the increase of bacterial population in an organ, such as the liver or spleen, does not represent the proliferation of the pathogen within host macrophages, nor does its suppression necessarily reflect the expression of antibacterial cellular immunity.⁴

The bacteria disseminated rapidly to establish a systemic infection, as evidenced by the appearance of bacteraemia within 1 h, when $\geq 10^3$ organisms were inoculated into either the genetically resistant or susceptible mice (figs. 1A and 2A). Even when 1×10^2 bacteria were injected into a susceptible host, they became detectable in the blood by 6 h. This affirms the i.p. route as an efficient way of introducing a systemic inoculum. There appeared to be no complete clearance of the bacteria from the circulation during the early infection as one would normally expect. This observation may well be unique to this experimental design, in which the initial growth of the organisms in the peritoneal cavity would continue to dispatch them into the blood. The pathogen undoubtedly gained a foothold in the organs of the RES at an early stage, and its proliferation could be assessed by the net increase in the bacterial population in the liver. In these experiments, the infective doses were low, so that the rapid initial bacterial growth would not be influenced by the effect of endotoxin. Also, consistent with earlier reports,^{4,10-12} the histopathological reactions in these infected livers showed the early accumulation of polymorphs and the absence of macrophages (unpublished observations). Hence, the suggestion that macrophage function may be hindered by endotoxin¹³ is irrelevant at the early stage of the infection.

In the present experiments, the fate of virulent bacteria within resistant and susceptible hosts appeared almost identical in two strains of each type of host. The circulating bacterial population (figs. 1A and 2A) and the hepatic bacterial population (figs. 1B and 2B) went into rapid growth from day 2 to day 5, except in the resistant mice inoculated with 2×10^3

bacteria in which both the hepatic and the circulating populations failed to increase significantly. The similarity between the changes in bacterial population in the blood and in the liver suggests that the peripheral bacteria may represent predominantly the release of the propagating organisms from the organs and, probably, the peritoneal cavity into the circulation, rather than bacterial multiplication in the blood, as in septicaemia, except perhaps at the terminal phase of a fatal disease. The bacterial growth rate appeared faster in the susceptible host than in the resistant host (figs. 1B and 2B). This is consistent with observations by other investigators.^{1,7,8} As the pathogen attained a level of 10^8 bacteria/g of liver, the mice began to die. Thus, susceptible mice died sooner than the resistant ones because their bacterial population reached its peak sooner: it appears that the susceptible host provides a better milieu for bacterial proliferation than its resistant counterpart.

The data here show that there was no difference in the ability of the two types of host to localise the pathogen at the site of entry in the i.p. infection. The organisms rapidly entered the circulation for systemic dissemination (figs. 1A and 2A); but once they reached an organ of the RES, they were less able to establish themselves and replicate in the resistant host (figs. 1B and 2B), which appeared better able to contain the pathogen, rendering it less invasive. Thus, the resistant A/J and CBA mice were able to withstand an i.p. inoculum of 2×10^3 virulent bacteria, whereas the susceptible C57BL/6J and BALB/c mice rapidly succumbed to a dose of 1×10^2 bacteria. However, there is clearly a threshold of tolerance beyond which the resistant host can no longer suppress the initial challenge. Hence, an i.p. inoculum of 5×10^4 and 6×10^4 bacteria into the A/J and CBA mice, respectively, led to an invariably fatal disease. Therefore, the degree of native resistance in the host was relative.

We made no attempt to differentiate between the relative abilities of the resistant and susceptible hosts to kill and to permit growth of the pathogen, as others reported.^{2,9,14,15} A review has been presented⁴ of the possible genetic control of host macrophages on salmonellae, either by inference from in-vivo experiments or by cell-culture studies. While some investigators speculated on the concept of subsets of macrophages as the safe site for bacterial proliferation, the relevance of macrophages as effector cells, especially in the early stage of murine salmonellosis, remains contrary to histopathological evidence.^{4,10-12} Indeed, the present observations reveal that the ability or inability of the host to contain the systemic bacterial multiplication was shown within the first 4-5 days after inoculation, when macrophages are absent. Moreover, there is now ample in-vivo evidence^{4,16-19} of the destruction of virulent *S. typhimurium* within inflammatory phagocytes, both polymorphs and macrophages, and of its multiplication in the extracellular space of sinusoids and lesions and within hepatocytes. The genetic control on host resistance may well reside in tissue reactions and the early acquired immune response to infection. If the host tissues are viewed as the milieu of bacterial propagation, its net growth rate will be determined by a dynamic balance of the relative ability of the host tissues to interact with the invading bacteria against that of the pathogen to survive and reproduce therein. The contributing factors to this balance are clearly not limited to the macrophage alone. Hence, the genetic basis of host resistance to murine salmonellosis needs to be radically reassessed. Our comparative study on the bacterial dissemination and proliferation in host tissues provides the first step toward that goal.

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