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Comparative histopathology in mouse typhoid among genetically diverse mice

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Summary. Genetically resistant CBA and A/J mice and susceptible BALB/c and C57BL/6 mice were challenged with either an identical infective dose or a minimal lethal dose of *Salmonella typhimurium*. The histopathological progression of the disease was examined in tissue sections prepared by the JB-4 Plus resin embedding method and compared between the resistant and susceptible mice. In a fatal disease, the lesions in both animal hosts began with focal abscesses within the first three days post infection. Mononuclear cell infiltration started by day 4 and transformed the lesions into granulomata. Well-formed granulomata were evident by day 7 and persisted in sublethally infected resistant mice. Massive bacterial proliferation and extensive tissue degeneration marked the terminal stage of a lethal challenge. There were no distinguishable features that would identify the tissue response to infection in a resistant host from a susceptible one, except that the lesions in the sublethally infected resistant mice advanced slower and were discrete and self-limiting.

Keywords: genetic diversity, histopathology, mouse typhoid

Inbred mouse strains have varying degrees of innate resistance to mouse typhoid. The minimal lethal doses of virulent *Salmonella typhimurium* SR-11 for the resistant A/J and CBA mice and the susceptible C57BL/6 and BALB/c mice are approximately 6×10^4 and $< 10^2$ bacteria, respectively, by the intraperitoneal (i.p.) challenge route (Xu & Hsu 1992). When 2×10^3 salmonellae are inoculated i.p., the susceptible mice will develop a progressive disease and die within 10 days, while the resistant ones will survive invariably without overt symptoms.

In previous publications, we have documented the distinguishing characteristics of primary lesions in mouse typhoid, as compared to lesions in mice immunized with attenuated (Nakoneczna & Hsu 1980) and inactivated (Nakoneczna & Hsu 1983) salmonellae, as well as their bacterial components (Hsu *et al*. 1985; Ding *et al*. 1990). The primary lesions in the susceptible mice begin with an early infiltration of polymorphs with ensuing necrosis and confluence. They gradually transform into granulomata with central necrosis and progressive tissue damage leading to a fatal disease. The secondary lesions, as seen in mice immunized with an attenuated vaccine, are noted for their immediate mononuclear cell response and early granuloma formation, followed by subsequent tissue recovery. In contrast, lesions of mice immunized with inactivated salmonellae or their components are reminiscent of those seen in primary lesions at the onset with an initial polymorph infiltration and subsequent

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granuloma formation. The differentiating feature is that the latter lesions are discrete, self-limiting and without progressive tissue injury. The animals usually recover from an infectious challenge.

The present investigation is intended to explore if there is any identifiable difference in the histopathological progression between the inbred resistant and susceptible mice. The developing lesions were compared after the mice were challenged with either an identical infective dose or with a minimal lethal dose appropriate to the individual mouse strains. Tissue specimens are embedded in a plastic resin, with which bacteria in tissues can be made visible because of its thin tissue sections.

Materials and methods

Mice

Four strains of inbred mice were purchased from the National Cancer Institute, Frederick, MD. The A/JCr and CBA/NCr mice are highly resistant to virulent *S. typhimurium*, while the C57BL/6NCr and BALB/CAnNCr mice are highly susceptible to the same pathogen. The mice were housed in the Central Animal Care Facility at the Medical College of Virginia campus and typically used in our experiments at 8–9 weeks of age.

Salmonella typhimurium

The virulent strain SR-11 was used in this study. The organism was maintained in Tryptic Soy Agar (Difco) and propagated in Tryptic Soy Broth (Difco). A 6 h broth culture, grown in a rotating drum at 37°C, was washed in saline in a procedure described previously (Hsu & Radcliffe 1968). An optically standardized saline suspension of bacteria contained approximately 2×10^9 viable organisms/ml. By i.p. inoculation into mice, the minimal lethal dose of this saline preparation was approximately 6×10^4 and $<10^2$ bacteria, respectively, in the resistant A/JCr and CBA/NCr mice and the susceptible C57BL/6NCr and BALB/CAnNCr mice.

Infection of mice and preparation of tissue sections

Mice were inoculated i.p. with 0.5 ml of a saline suspension containing the designated number of viable *S. typhimurium*. At daily intervals, randomly selected mice were sacrificed by ether inhalation. Tissue sections were removed from the liver and spleen and fixed overnight in 2% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2. The tissue samples were then kept in the buffer solution at 5 °C until embedding.

The tissue samples were dehydrated in increasing concentrations of ethanol and embedded in JB-4 Plus resin (Polysciences Inc., Warrington, PA). They were sectioned at $<2\,\mu$m thick in a Sorvall JB-4 microtome. The mounted sections were stained in Dip Quick Stain (J-322 A-2, Jorgensen Laboratories, Inc., Loveland, CO), containing Eosin Y and Methylene Blue. Bacteria were rendered visible in these thin tissue sections.

Differential leucocyte counts in lesions

Lesions were arbitrarily picked from tissue sections. Their compositions of inflammatory cells were differentiated by counting an entire lesion under a high dry microscopy (420× magnification). The cells were separated by percentage of polymorphs, macrophages and lymphocytes.

Results

Paired experiments were designed to compare the histopathological reactions to mouse typhoid between the resistant A/JCr and the susceptible C57BL/6NCr mice and between the resistant CBA/NCr and the susceptible BALB/CAnNCr mice. In each experiment, the two contrasting strains of mice were either infected i.p. with an identical dose of *S. typhimurium* or with a minimal lethal dose for that strain. Thus, the resistant and susceptible mice were either inoculated both with 2×10^3 bacteria or with 7×10^4 and 2×10^2 organisms, respectively. When 2×10^3 salmonellae were given, the susceptible mice began to die from the disease beginning from day 5, while the resistant mice would invariably survive the challenge.

Table 1 summarizes the number of mice infected and sacrificed for tissue sampling at various intervals. Thus,

Table 1. Record of total numbers of infected mice in each strain sacrificed for tissue sampling at daily intervals

	Number of mice sacrificed on each day post infection														
Dosage	2	3	4	5	6	7	8	9	10	11	12	13	19	21	22
BALB/CAnNCr															
2×10^2	1	2	2	2	2	2	1	2							
2×10^3	1	2	2	2	2	1									
CBA/NCr															
2×10^3	1	3	2	2	2	2	1	1	1		2				
7×10^4	1	2	2	2	2	3	1		1						
C57BL/6NCr															
2×10^2	1	4	3	3	3	1	1								
2×10^3	1	3	3	2	2	2	1								
A/JCr															
2×10^3	1	4	4	4	4	2	4		2	2	2	1	1	1	
7×10^4	1	4	4	4	4	2	4			2	1		1		1

tissue specimens were taken from the liver and spleen daily from each of the mouse strains after infection. In paired experiments, histological sections were normally prepared and examined by one of two authors. Typical lesions in these specimens were photographed on Ektachrome slides. The senior author, a pathologist, was then given both the tissue sections and the colour slides for review, knowing at this time only the intervals of the specimens but not the mouse strains or the infective doses.

The lesions developing in the livers provided a representative picture of the histopathological progression of the disease. They were not specifically diagnostic when viewed as individual, unidentified lesions. What is a lesion in a susceptible mice given a very low, but lethal dose of salmonellae may be indistinguishable from a lesion of the same age in a resistant animal challenged with an overwhelming, lethal dose. Similarly, when infected with an identical dose of pathogen, the pattern of developing lesions will proceed in a much more rapid and aggressive manner among the susceptible than the resistant mice. Hence, in the resistant mice infected with a sublethal dose of 10^3 bacteria, the lesions would become self-limiting with the regeneration of tissues. Nevertheless, the ensuing lesions do fit together to form a three dimensional spectrum which is defined by the parameters of the infective dose, the host susceptibility and the time after infection, as described in the following five phases.

Phase I

The first definable change was the focal infiltration of the hepatic sinusoids with polymorphs, as seen on day 3 in the susceptible C57BL/6NCr mice infected with 2×10^2 salmonellae and in the resistant CBA/NCr mice infected with 7×10^4 bacteria (Figure 1).

Phase II

The lesions progressed to discrete micronodules of polymorphs by days 3 and 4 in the susceptible C57BL/6NCr mice receiving a lethal dose of 2×10^3 organisms (Figure 2). Similar changes were observed in the resistant A/JCr mice receiving the sublethal dose of 2×10^3 on day 7 (Figure 3). Florid lesions might show the progression to microabscess formation with hepatocellular necrosis and more extensive fibrin in the susceptible C57BL/6NCr mice receiving 2×10^3 salmonellae on day 3 (Figure 4). It is apparent that the progression of lesions was delayed among the resistant mice given a sublethal dose.

Phase III

These polymorph lesions were followed by mononuclear cell infiltration in the susceptible C57BL/6NCr mice challenged with 2×10^2 bacteria on day 4 and the resistant CBA/NCr mice infected with 2×10^3 organisms up to day 11 (Figure 5).

Phase IV

Well-formed macrophage nodules or microgranulomata were found in the susceptible C57BL/6NCr mice (Figure 6), as well as in the resistant A/JCr mice (Figure 7), both receiving 2×10^3 bacteria after 5–7 days post infection.

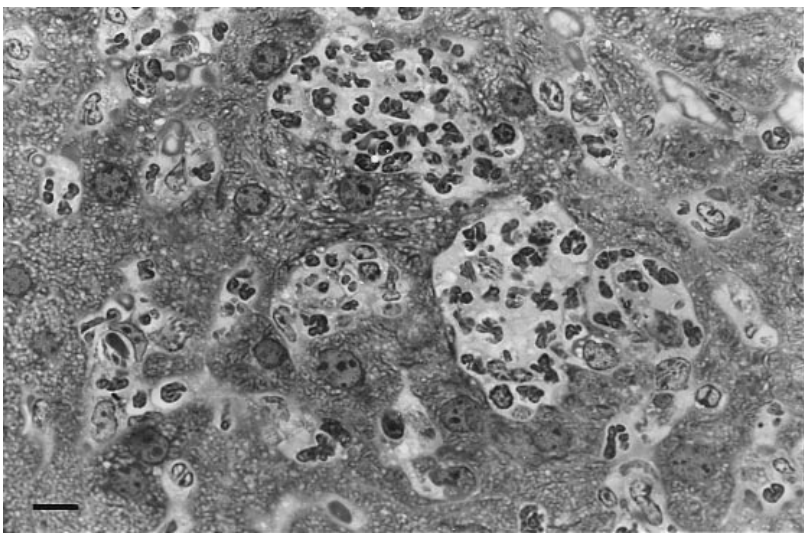

Figure 1. Liver of CBA/NCr mouse 3 days after infection with 7×10^4 *S. typhimurium*, showing well-preserved hepatocytes and distinct focal infiltration by polymorphs in sinusoids. Bar = 10 μm.

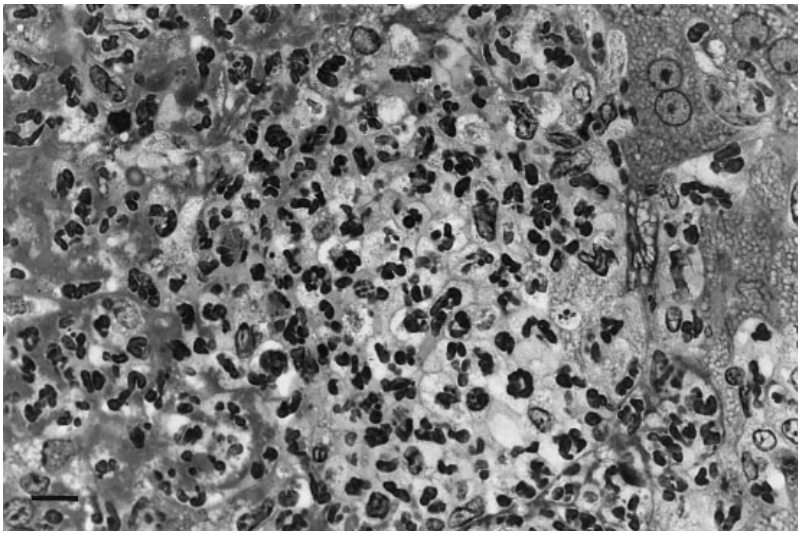

Figure 2. Liver of C57BL/6NCr mouse 3 days after infection with 2×10^3 *S. typhimurium*, showing individual hepatocytes with necrosis and a nodular accumulation of polymorphs. Bar = 10 μm.

The granulomata could persist among the resistant mice infected with this sublethal dose for at least 19 days.

Phase V

A major advantage of the thin plastic section technology employed in this study is the distinct preservation and revelation of bacterial morphology in relation to the tissues. The intracellular and extracellular localization is clear and the bacterial staining is intense. This allows us to identify areas of bacterial proliferation and accumulation. Overgrowth of organisms tended to occur in the degenerating tissues at the terminal phase of a fatal infection, as observed in mature lesions of susceptible C57BL/6NCr mice and of the resistant CBA/NCr mice (Figure 8a,b). But it was absent from resistant mice receiving a sublethal infective dose.

Our exhaustive reviews of tissue specimens did not suggest the likelihood of bacterial proliferation within macrophages. However, bacterial accumulation could be found within hepatocytes (Figure 9), sinusoids (Figure 8a,b) and areas of necrosis (Figure 10).

From the preceding tissue examinations, it is apparent that the transition from acute abscess to granuloma would proceed in a comparable time frame so long as both the resistant and susceptible mice were challenged with a minimal lethal dose. In an attempt to support this observation, differential counts were made on tissue

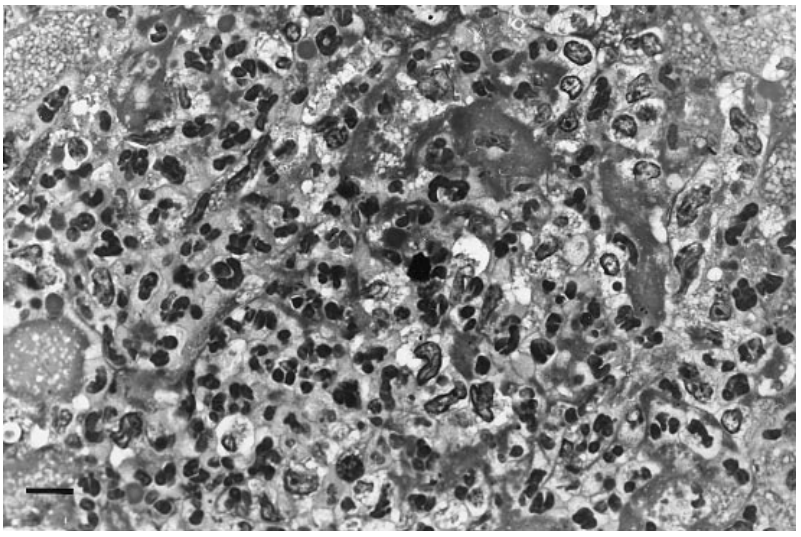

Figure 3. Liver of A/JCr mouse 7 days after infection with 2×10^3 *S. typhimurium*, showing individual hepatocyte necrosis and nodular infiltration of polymorphs, as lesions progressing toward microabscess formation. Bar = 10 μm.

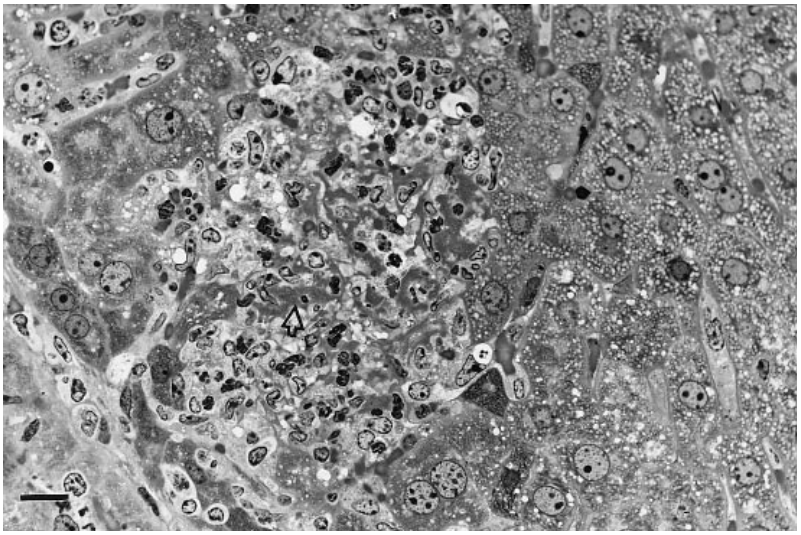

Figure 4. Liver of C57BL/6NCr mouse 3 days after infection with 2×10^3 *S. typhimurium*, showing nodular infiltration of polymorphs with more extensive fibrin exudation (arrow) and tissue necrosis. Bar = 10 μm.

lesions of the resistant and susceptible mice, both challenged with a minimal lethal dose and between days 3 and 6 post infection. During this period, the peak of polymorph response would decline, accompanied by a proportionate increase of macrophages. Even by day 6, the content of lymphocytes was typically < 5% among these fatally infected mice.

With each of the four strains of mice infected with their respective minimal lethal dose, at least 3 large lesions (each in the size of a high dry field or slightly larger) or up to 10 small neighbouring foci were counted at each time intervals. The total number of inflammatory cells counted ranged from 400 to 1100, from which the differential percentages were calculated in each specimen. Figure 11 plots the data of the decline of polymorph percentage in each mouse strain between days 3 and 6 of the infectious process. The corresponding balance of cell population at each interval would represent essentially that of macrophages. The data clearly reveal no overt difference in the rate of transition from polymorphs to macrophages between the resistant A/JCr and CBA/NCr mice and the susceptible C57BL/6NCr and BALB/CAnNCr mice, all challenged with their respective minimal lethal dose.

Discussion

The advantage of our present plastic thin-tissue sectioning technique is that it enables the visualization of bacteria in the tissues, which is not possible with the

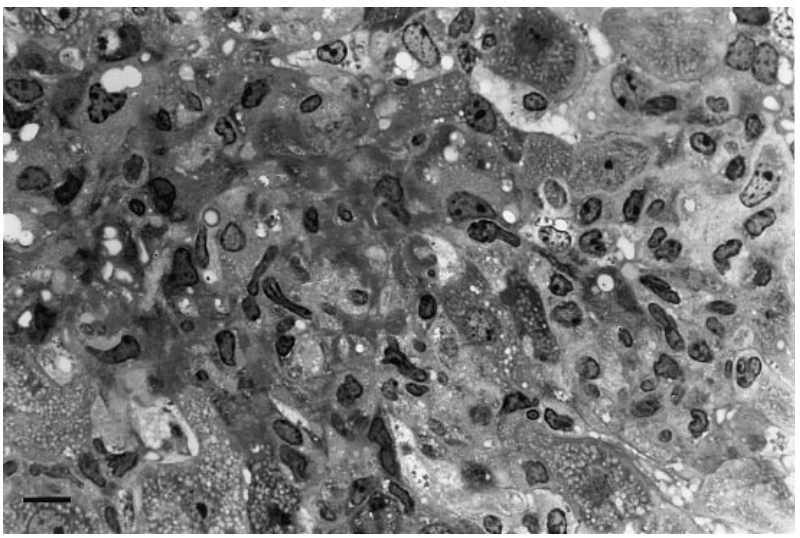

Figure 5. Liver of CBA/NCr mouse 11 days after infection with 2×10^3 *S. typhimurium*, showing lesion with infiltration of mononuclear cells and resolution of polymorphs. Bar = 10 μm.

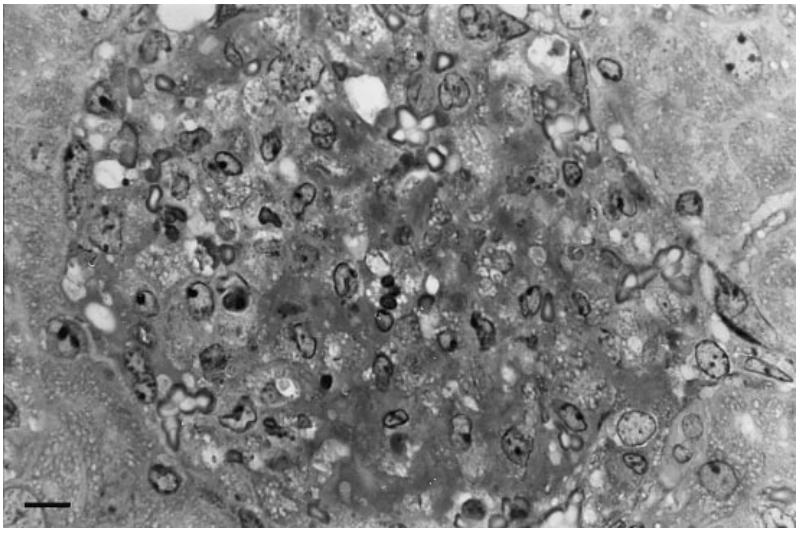

Figure 6. Liver of C57BL/6NCr mouse 6 days after infection with 2×10^3 *S. typhimurium*, showing a distinct macrophage nodule at this phase. Bar = 10 μm.

paraffin-embedding method used previously. It thereby provides a more definitive view of the interaction between host tissues and bacteria. We have reported some of our findings earlier using this procedure in international meetings on salmonellosis (Hsu 1992, 1993, 1995). Based on extensive examinations of four mouse strains as recorded in Table 1, we present here a comparative picture of the ensuing tissue pathology of mouse typhoid among genetically diverse mice.

The five phases in the histopathological progression of lesions in mice inherently resistant or susceptible to mouse typhoid show that they began as focal abscesses in response to the initial bacterial invasion (Figures 1–4) and are later transformed into granulomata (Figures 5–7). In mice infected with a lethal dose of pathogen, the lesions resulted in extensive bacterial proliferation, both extracellularly and within hepatocytes, along with tissue degeneration in the case of a fatal challenge (Figures 8–10). However, there were no identifiable features in their histopathological changes which could help to distinguish the resistant from the susceptible mice in a fatal infection. The susceptible BALB/CAnNCr and C57BL/6NCr mice were so sensitive to salmonella challenge that < 50 organisms were sufficient to initiate a fatal disease. In contrast, the resistant CBA/NCr and A/JCr mice required at least 6×10^4 virulent salmonellae as a minimal lethal dose. With a fatal infection, the ensuing lesions in both the resistant and susceptible mice described here shared the identical progressive characteristics with those previously described as

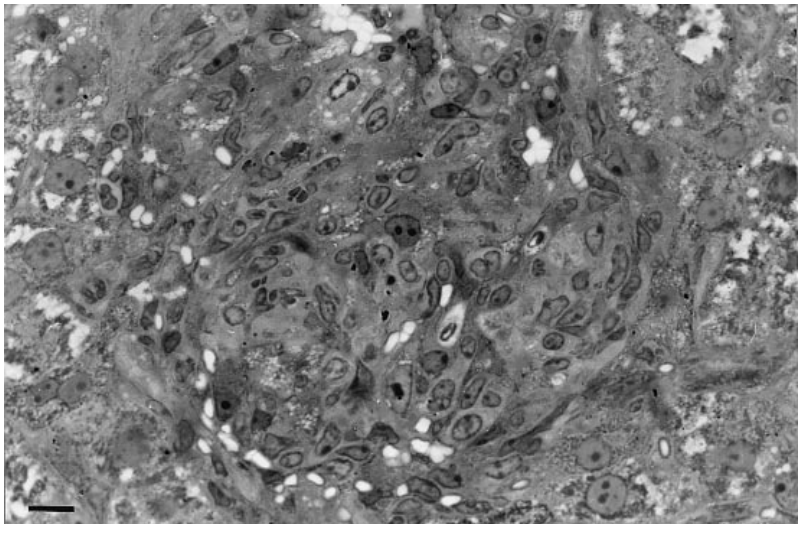

Figure 7. Liver of A/JCr mouse 5 days after infection with 2×10^3 *S. typhimurium*, showing a well-circumscribed microgranuloma. Bar = 10 μm.

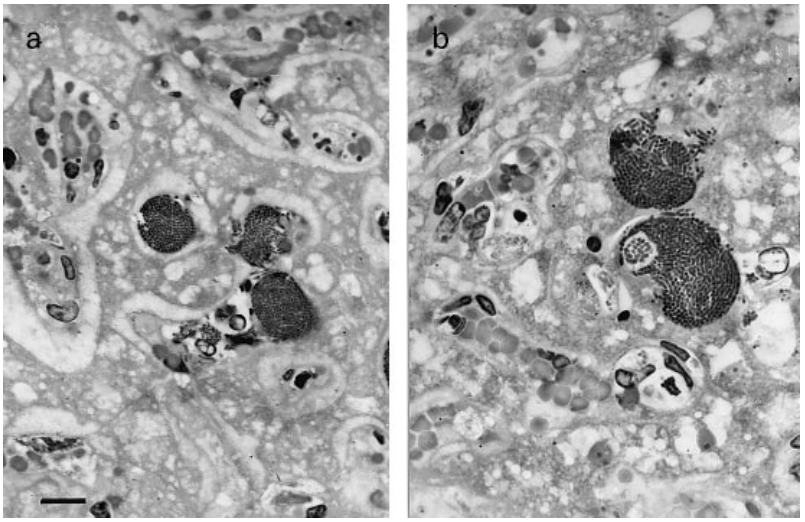

Figure 8. Massive bacterial proliferation in hepatic sinusoids of degenerating tissue lesions at terminal stage, as seen in susceptible C57BL/6NCr mouse receiving 2×10^3 bacteria (a) and resistant CBA/NCr mouse receiving 7×10^4 bacteria (b), both at 7 days post infection. Bar $=10\,\mu$m.

primary lesions in the outbred Swiss-Webster RFW mice (Nakoneczna & Hsu 1980).

The resistant CBA/NCr and A/JCr mice invariably survived a challenge of 2×10^3 virulent salmonellae without overt symptoms. With such a sublethal dose, the development of lesions was delayed and self-limiting (Figures 3 and 5), leading to the regeneration of tissues and the recovery from infection. This sequence of events is parallel to that seen in the histopathological development in mice protected by vaccination with killed salmonellae (Nakoneczna & Hsu 1983) or with their bacterial components (Hsu *et al.* 1985; Ding *et al.* 1990). In both cases, the lesions are characterized by the initial focal polymorph response, followed by the transformation into discrete granuloma. On the other hand, secondary lesions of mice vaccinated with attenuated salmonellae and subsequently re-infected with the pathogen are marked by the early formation of granulomata without any evidence of necrosis in the lesions (Nakoneczna & Hsu 1980).

Our present understanding of the pathogenesis of mouse typhoid is that an invasive disease is largely dependent on the early rapid proliferation of the pathogen in the extracellular locations of host tissues and within hepatocytes (Hsu 1989, 1993, 1995). Once the virulent salmonellae are ingested by inflammatory phagocytes, both polymorphs and macrophages, they are likely to be inactivated. With the re-infection type of

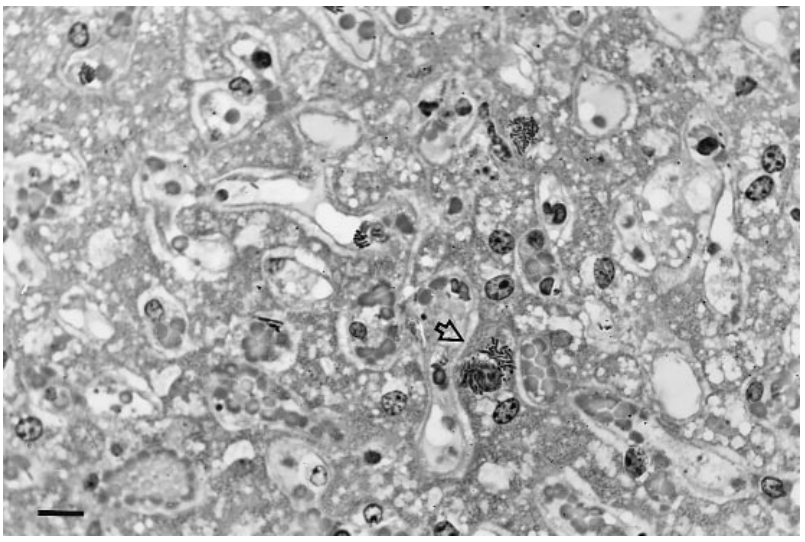

Figure 9. Accumulation of bacteria within hepatocytes (arrow) and extracellular locations of lesion in C57BL/6NCr mouse 5 days after infection with 2×10^2 *S. typhimurium*. Bar $=10\,\mu$m.

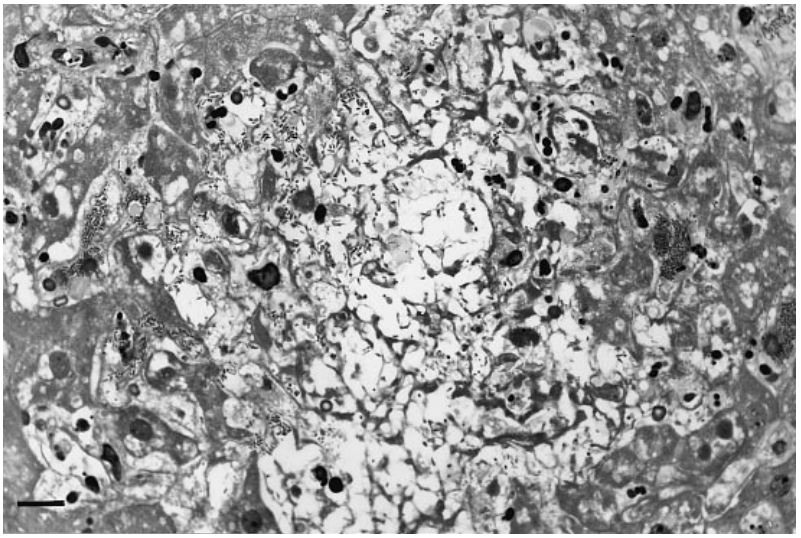

Figure 10. Diffused bacterial proliferation throughout necrotic lesion in liver of CBA/NCr mouse 6 days after infection with 7×10^4 *S. typhimurium*. Bar = 10 μm.

challenge, the secondary lesions are highly effective in terminating the infection by a synergistic action of acquired humoral immunity and delayed-type hypersensitivity. In contrast, the protective immunity offered by nonviable vaccines is related to the level of antibody at the time of challenge (Xu *et al*. 1993a). The opsonic function of antibody undoubtedly facilitates the effective elimination of the pathogen by the infiltrating inflammatory phagocytes.

These results suggest a logical explanation of the early events in the pathogenesis of mouse typhoid: Successful establishment of the pathogen in host tissues is attained by its predominantly extracellular proliferation at the early stage of the infection. This can be intercepted by an influx of a higher ratio of polymorphs (effector cells) among the acute inflammatory cells, as in the case of the genetically resistant mice, or by an influx of both antibody and acute inflammatory cells, as in the case of mice protected with nonviable vaccine. The survival of the host is therefore dependent on the early containment of the pathogen and its subsequent dissemination from the site of infection. So long as the host can retard the early bacterial proliferation, it would have time to overcome the infection with the sequential induction and elicitation of acquired immunity, both humoral and cellular. Thus, the minimal lethal dose is significantly higher in the resistant than in the susceptible mice because it takes more organisms to overwhelm the host defense mechanism in the resistant host. By the same analogy, mice protected by nonviable vaccine can also succumb to a sufficiently large infective dose (Nakoneczna & Hsu 1983; Hsu *et al*. 1985).

This is consistent with the observation that at the sublethal infective dose the rate of bacterial proliferation *in vivo* is suppressed among the resistant mice within the first two days of infection in comparison with their susceptible counterparts (Xu & Hsu 1992). Conversely, once the pathogen successfully establishes itself in host tissues, i.e. at the minimal lethal dose, the bacterial replication proceeds at a comparable rate regardless of the innate host resistance. The present investigation reveals no distinguishable characteristic between the resistant and susceptible mice on the basis of their

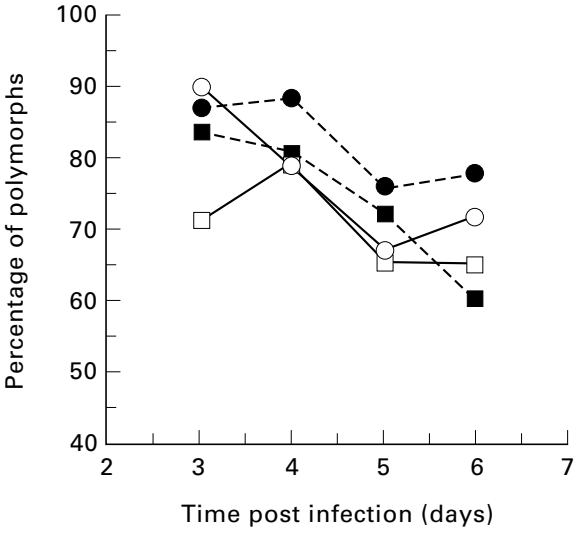

Figure 11. Comparative changes in percentages of polymorphs within lesions of resistant (open symbols) and susceptible (closed symbols) mice between 3 and 6 days post infection. □ A/JCr mice; ○ CBA/NCr mice; ■ C57BL/6NCr; ● BALB/CAnNCr mice.

histopathological progression. Figure 11 affirms quantitatively that the rate of transition from polymorphs to macrophages in lesions from day 3 to day 6 is comparable between the resistant and susceptible mice. Collectively, our observations also show that in a lethal infection the subsequent influx of lymphocytes into lesions is insignificant due to the extensive necrosis of tissues beyond day 5 of the infection (Figures 9 and 10). The absence of a difference in tissue response is consistent with our previous findings that there is no apparent difference between the diverse mouse strains in their antibody response to salmonella antigens (Xu *et al.* 1993b). Hence, the outcome of the infection is almost entirely determined by the host defense at the very early stage of infection.

Recently, other investigators have also used the thin plastic-embedded tissue sections and observed the significance of polymorphs in the early stage of several bacterial infections, including *S. typhimurium* (Conlan & North 1992). Other studies support our earlier assertion that polymorphs are effective in the early containment of the pathogen at the site of bacterial invasion (Conlan *et al.* 1993; Sjostedt *et al.* 1994; Conlan 1996). However, alternative to their interpretation, hepatocytes may provide a safe haven for the unrestricted proliferation of the pathogen and protect its attack by the infiltrating polymorphs, as we suggested previously (Lin *et al.* 1987; Hsu 1993). Similar findings in the contribution of neutrophils were observed in both primary and acquired immunity to listeria (Czuprynski *et al.* 1994).

Although the plastic tissue sections are uniquely useful in revealing the histopathological events in tissues and especially the relation between bacteria and host tissues, they do not provide a definitive observation on the fate of bacteria within host cells. One can only infer intracellular bacterial multiplication by the intracellular accumulation. There was no evidence in our study here to show the proliferation of salmonellae within phagocytic cells. However, our previous reports have produced ample documentation of bacterial replication within hepatocytes and their destruction within polymorphs and macrophages, based on their morphological integrity and division (Wang *et al.* 1988; Lin *et al.* 1987, 1989; Hsu 1989, 1993). By definition therefore *S. typhimurium* does not meet the traditional classification of a facultative intracellular pathogen (Hsu 1993).

In general, the lesions of mouse typhoid as presented here resemble human typhoid nodules both in their histopathology and in their distribution such that they bear no constant relation to the architecture of the hepatic lobule. Similar lesions are also noted in the spleen of the experimental animals as they are in humans. However, the histopathological progression is more easily traced in the liver. Resistant mice infected with a sublethal inoculum go on to develop lesions resembling typhoid nodules but lacking the necrosis and bacterial proliferation associated with a fatal human typhoid or with the fatal disease in susceptible mice. Thus, this study lends credence to the general consensus that mouse typhoid is an appropriate experimental model to study human typhoid.

Acknowledgements

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