INFECTION AND IMMUNITY, Mar. 1996, p. 1043–1047 Vol. 64, No. 3
0019-9567/96/$04.00+0

Neutrophils Prevent Extracellular Colonization of the Liver Microvasculature by *Salmonella typhimurium*

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Received 12 September 1995/Returned for modification 26 October 1995/Accepted 18 December 1995

The early course of hepatic infection with the facultative intracellular bacterial pathogen *Salmonella typhimurium* was examined in control mice and in mice selectively depleted of neutrophils by treatment with a granulocyte-specific monoclonal antibody. The results show that >200-fold more salmonellae were recovered in livers of the latter group of mice than in livers of the former group by 24 h of parenterally initiated infection. Comparative histological examination of the livers from both groups of mice indicated that neutrophils participate in early anti-*Salmonella* defense in the liver in part by aborting infection in permissive hepatocytes and by inhibiting extracellular bacterial colonization of the hepatic microvasculature. It is shown in addition that systemic salmonellosis was also severely exacerbated in neutropenic mice infected intragastrically with the pathogen.

Salmonella species can cause severe systemic infections, including typhoid, in humans (reviewed in references 1 and 7). Experimentally, parenteral inoculation of mice with sublethal doses of *Salmonella typhimurium* causes a systemic infection reminiscent of human typhoid (reviewed in references 6 and 9). Consequently, this murine model of salmonellosis has been extensively used to examine potentially clinically relevant mechanisms of anti-*Salmonella* host defense (3, 8, 9, 11–13). Much of a sublethal intravenous (i.v.) inoculum of *S. typhimurium* rapidly implants in the liver and goes on to cause an acute progressive infection in the parenchyma of this organ, lasting several days (8, 11). During this time, salmonellae invade and grow inside hepatocytes that express no obvious antibacterial activities, and leukocytes, almost exclusively neutrophils, extravasate and focally accumulate in large numbers at sites of hepatocyte infection (3, 8, 9, 11). Because *S. typhimurium* multiplies exponentially in the face of this extensive neutrophil buildup, these phagocytes have been considered to be only marginally important in anti-*Salmonella* defense. On the contrary, because control and resolution of murine salmonellosis coincides with the replacement of neutrophils by macrophages at infectious foci, the latter phagocytes stimulated by T cells, aided by antibodies, have been generally regarded as the principal expressors of anti-*Salmonella* immunity (2, 9). However, recent publications from this laboratory (3, 15) show that neutrophils also are critical for anti-*Salmonella* defense. In one of these studies (3) the need for neutrophils in anti-*Salmonella* resistance was assessed in mice treated with a monoclonal antibody (MAb) directed against the β2-integrin, Mac-1, a molecule expressed at the surface of neutrophils that independently acts as both a phagocytic receptor for bacteria (5) as well as an adhesion molecule that mediates neutrophil binding to the vasculature (16), the latter of which is a prerequisite for subsequent neutrophil extravasation. Early salmonellosis in mice treated with this MAb was severely exacerbated to the extent that >100-fold more salmonellae were recovered from the livers of these mice than from the livers of control mice by 24 h of infection. Histological examination of the livers showed that in anti-Mac-1 MAb-treated mice, neutrophils ingested but failed to destroy blood-borne extracellular salmonellae. In control mice, in contrast, salmonellae were seldom found inside intravascular neutrophils, suggesting that these phagocytes normally rapidly destroy the circulating salmonellae they ingest. Additionally, anti-Mac-1 MAb treatment prevented neutrophils from extravasating into foci of infected hepatocytes. Under these circumstances, *S. typhimurium* rapidly multiplied inside parasitized hepatocytes without destroying them. By contrast, in immunocompetent mice, this aspect of the infectious process is controlled by neutrophils that rapidly migrate into infectious foci where they engage and lyse infected hepatocytes. Neutrophil-mediated lysis of infected hepatocytes serves to deprive *S. typhimurium* of an otherwise highly permissive growth environment and to release this pathogen into the extracellular space for exposure to leukocytes that can kill it. The importance of neutrophils in anti-*Salmonella* defense was confirmed by a recent study (15) using mice selectively depleted of neutrophils as a result of treatment with a granulocyte-specific MAb. Early *Salmonella* infection in the livers of neutropenic mice, as in mice treated with anti-Mac-1 MAb, was severely exacerbated by 24 h. The present study examines the early fate of *S. typhimurium* in the livers of neutropenic mice. It shows that in the absence of neutrophils, *S. typhimurium*, in addition to multiplying uncontrollably inside hepatocytes, also rapidly proliferates extracellularly within the liver microvasculature, a finding not revealed by earlier studies using anti-Mac-1 MAb, which does not deplete neutrophils from the blood.

CB6/F1 male mice were obtained from the Trudeau Institute Animal Breeding Facility and were used when they were 10 weeks old. To deplete neutrophils, mice were inoculated intraperitoneally with 0.5 mg of a purified anti-granulocyte (17) MAb, RB6-8C5, 24 h prior to initiating infection. Control mice were similarly treated with an equal amount of isotype-matched, irrelevant anti-keyhole limpet hemocyanin MAb (10). To examine the need for neutrophils for controlling early hepatic *Salmonella* infection, mice treated with MAb RB6-8C5 or the control MAb were infected by i.v. inoculation of 10^4, or for histology with 10^6, CFU of *S. typhimurium* C5R organisms as previously described (3). At 24 h of infection, blood was collected from a tail vein for total and differential leukocyte counts performed as described elsewhere (4). Then, mice were

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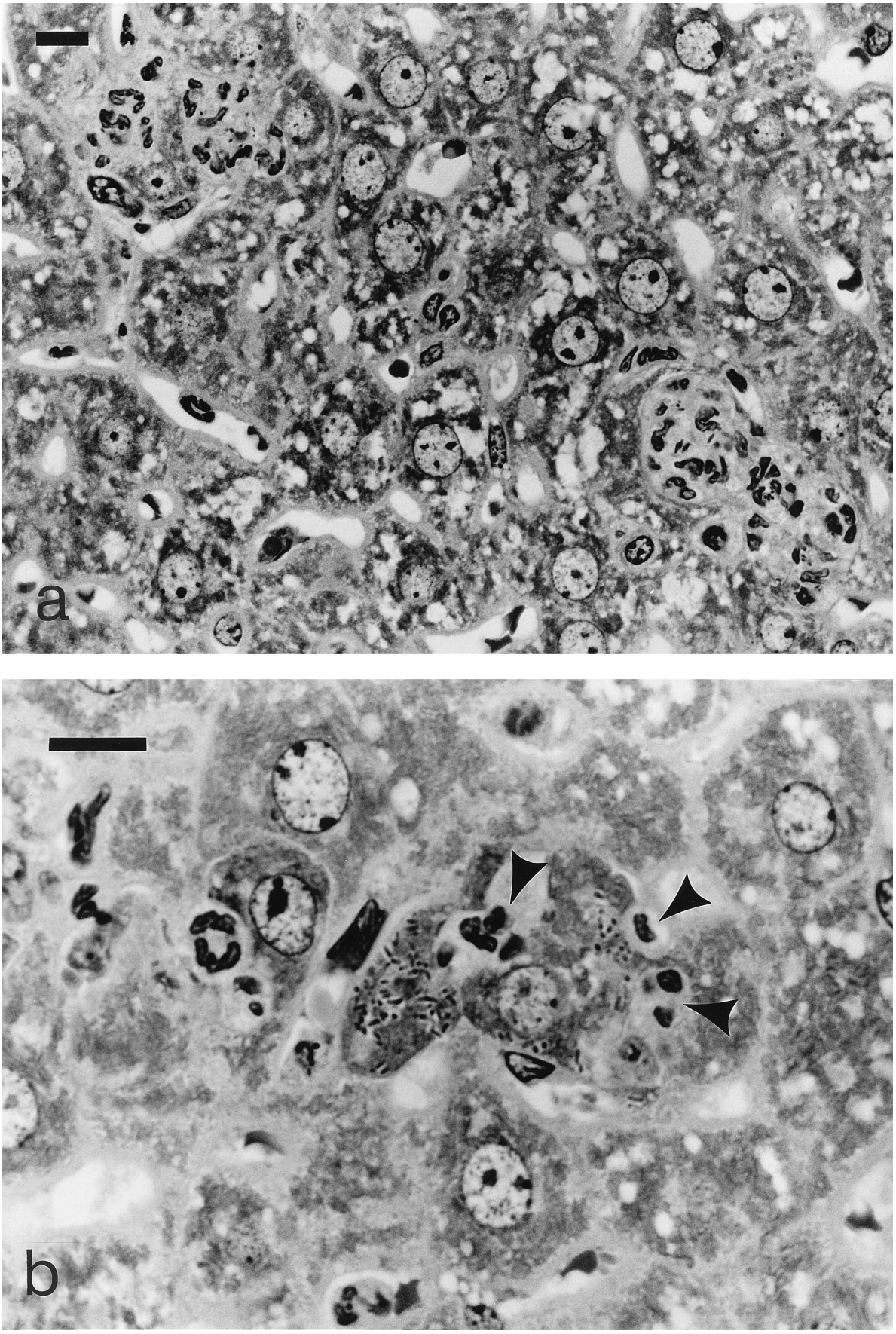

FIG. 1. Appearance of infectious foci in the livers of *Salmonella*-infected control mice. (a) By 24 h in control mice, infectious foci are occupied by small focal accumulations of neutrophils. (b) In some of these microabscesses, neutrophils (arrowheads) make intimate contact with infected hepatocytes that appear to be lysing. Bar = 10 μm.

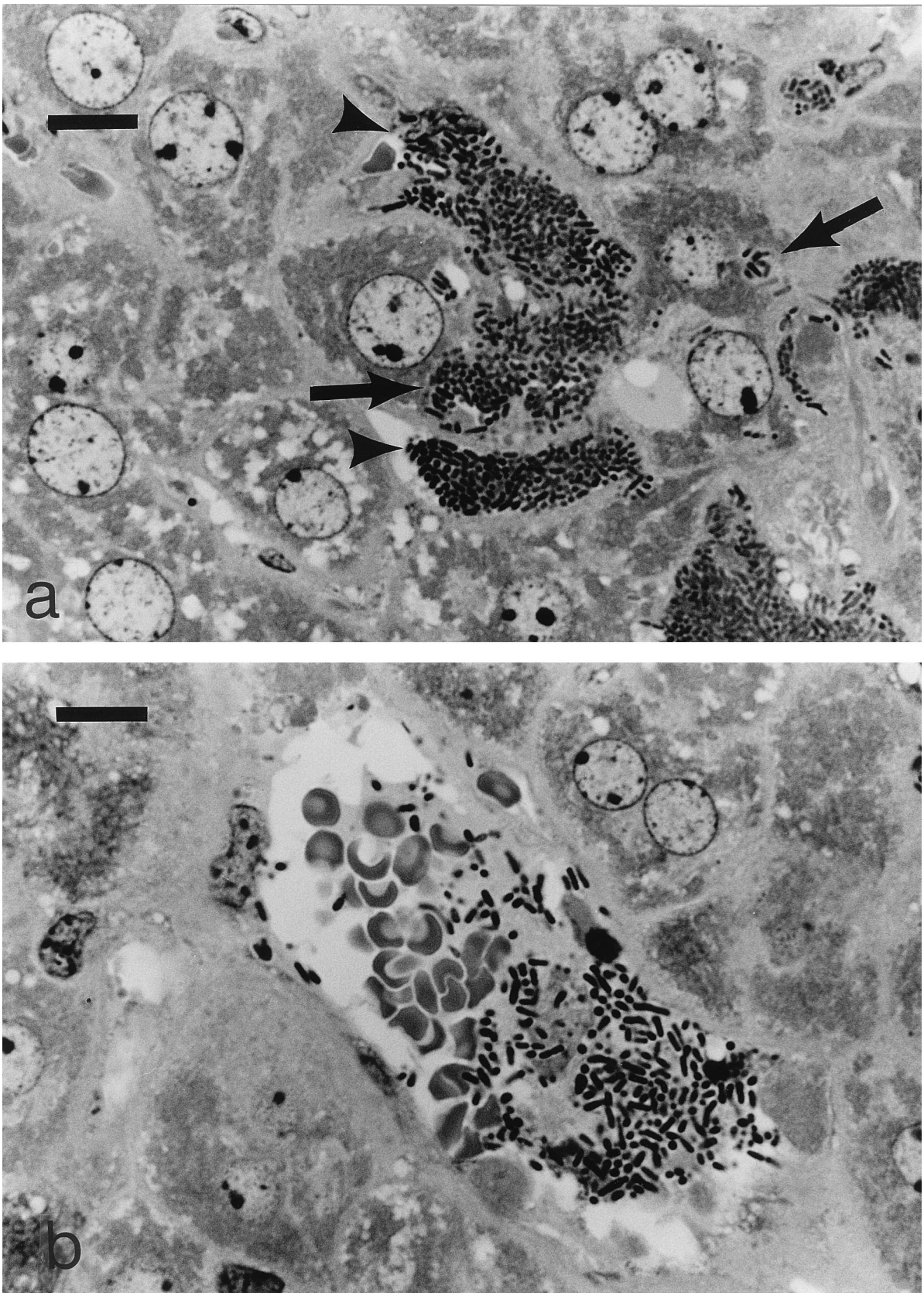

FIG. 2. Appearance of infectious foci in the livers of neutrophil-depleted mice by 24 h of *Salmonella* infection. By 24 h in neutrophil-depleted mice, salmonellae have parasitized hepatocytes (arrows) and colonized sinusoids (arrowheads) (a) and blood vessels (b). Bar = 10 μm.

killed by cervical dislocation, and their livers were taken for bacteriology or histology. *Salmonella* burdens were determined by plating 10-fold serial dilutions of infected liver homogenates on Trypticase-soy agar. Colonies were counted after 24 h of incubation at 37°C. For histology, livers were fixed, dehydrated, and embedded in plastic as previously detailed (3, 4). Liver sections were cut at 1 μm, stained with Macneal's tetrachrome, and examined with a Nikon microphot-FX light microscope.

Although MAb RB6-8C5 has been convincingly shown to selectively bind to and deplete only neutrophils and eosinophils (14, 17) from the circulating leukocyte pool, it remained possible that this MAb affects tissue macrophages, including Kupffer cells that appear to participate in anti-*Salmonella* defense (3). Therefore, in a preliminary experiment, the distribution of Kupffer cells was estimated histologically by examining the intracellular localization of i.v. injected fluorescent latex microspheres (1 μm in diameter) within liver sinusoids. By this criterion, MAb RB6-8C5 did not cause obvious depletion of Kupffer cells. Next, the phagocytic capacity of Kupffer cells was determined by measuring blood clearance of an i.v. inoculum of 10^8 CFU of *Listeria monocytogenes* bacilli by a published method (14). Because Kupffer cells normally rapidly ingest most blood-borne listeriae (14), this is a sensitive assay for monitoring whether MAb RB6-8C5 impairs phagocytosis by these tissue macrophages. By 15 min postinoculation, >98% of listeriae had been cleared from the blood of RB6-8C5-treated and control mice. Moreover, by this time, similar numbers of *L. monocytogenes* organisms were recovered from the livers of neutrophil-depleted and control mice (7.86 ± 0.06 $\log_{10}$ CFU and 7.82 ± 0.08 $\log_{10}$ CFU, respectively). These findings imply that MAb RB6-8C5 does not interfere with Kupffer cell phagocytosis.

On the basis of the foregoing results, it seems reasonable to propose that any infection-enhancing effect of MAb RB6-8C5 on hepatic salmonellosis will be due to its ability to deplete mice of neutrophils, because eosinophils do not appear to accumulate at foci of *Salmonella* infection (4, 9, 11). In this regard, mice treated with the control MAb or with the neutrophil-depleting MAb RB6-8C5 1 day earlier were inoculated i.v. with 10^4 CFU of *S. typhimurium*. At 24 h of infection, the numbers of neutrophils in the blood of control mice and RB6-8C5-treated mice were $160.6 \times 10^4 \pm 44.5 \times 10^4$ and $1.6 \times 10^4 \pm 0.51 \times 10^4$ per ml of blood, respectively (mean ± standard deviation; $n = 5$ mice per group). Also at 24 h of infection, the bacterial burdens in the livers of the control mice and RB6-8C5-treated mice were 3.16 ± 0.13 $\log_{10}$ CFU and 5.60 ± 0.16 $\log_{10}$ CFU per liver, respectively (mean ± standard deviation; $n = 5$ mice per group). These results show that parenterally initiated *Salmonella* infection in the livers of RB6-8C5-treated mice was severely exacerbated by 24 h. Similar results were obtained on three separate occasions.

Histological examination (Fig. 1) revealed that in the livers of control mice by 24 h, sites of infection were populated by small focal accumulations of neutrophils in sinusoids and parenchyma (Fig. 1a). Under these circumstances, neutrophils were sometimes found in intimate contact with infected hepatocytes that appeared to be lysing (Fig. 1b). Small numbers of poorly staining and apparently degenerating bacteria were found in some of these microabscesses. During systematic screening of random liver sections from three separate infected control mice, >100 granulocytic microabscesses, but no free-growing microcolonies of *S. typhimurium*, were encountered. This suggests that in control mice all foci of hepatic infection were occupied by neutrophils by 24 h. By contrast, neutrophils failed to accumulate at any of >100 infectious foci examined in liver sections from RB6-8C5-treated mice. Instead, by 24 h in the livers of these mice, salmonellae invaded and multiplied inside hepatocytes without destroying them (Fig. 2a). Additionally, by this time in the absence of neutrophils, there was extensive extracellular proliferation of the pathogen in liver sinusoids and elsewhere in the microvasculature (Fig. 2). Together, the bacteriological and histological findings indicate the critical importance of neutrophils for early anti-*Salmonella* defense in the liver.

Enteroinvasion is the normal route of systemic infection by salmonellae. Next, therefore, an experiment was performed to determine whether neutrophils impede systemic infection initiated by ingestion of *S. typhimurium*. This involved inoculating RB6-8C5-treated and control mice intragastrically with 5×10^8 CFU of *S. typhimurium*. Bacterial burdens in the livers of these mice were determined on day 4 of infection to allow time for salmonellae to disseminate to this organ from the gut. By this time an average of >100-fold more salmonellae were recovered from the livers of RB6-8C5-treated than from the livers of control mice (6.82 ± 1.09 $\log_{10}$ CFU and 4.67 ± 0.26 $\log_{10}$ CFU, respectively; $n = 5$ mice per group).

The findings of this and earlier publications (3, 15) from this laboratory leave no doubt that neutrophils are crucial for effective host defense against salmonellosis. Specifically, neutrophils act early in infection to restrict *Salmonella* growth to a level that can be adequately dealt with by other host defenses generated and mobilized later. In the liver, neutrophils restrict early infection by several means, including ingesting and killing blood-borne salmonellae that can otherwise colonize extracellular sites and aborting infection inside hepatocytes to thereby expose the otherwise sheltered pathogen to professional phagocytes. It remains unclear how *S. typhimurium* manages to colonize the hepatic microvasculature. Conceivably, *Salmonella* lipopolysaccharide induces intravascular blood clots to form locally at sites of bacterial implantation that are subsequently colonized by this pathogen. Finally, Kupffer cells are known to ingest some blood-borne salmonellae and are thought to contribute to anti-*Salmonella* defense in the liver (3). The present study does not refute this possibility. Clearly, however, Kupffer cells alone are unable to prevent uncontrolled proliferation of *S. typhimurium* in the livers of neutropenic mice.

I thank Louise Hartson for expert technical assistance, Pam Dunn and Robert North for their helpful advice and discussion, and Robert Coffmann (DNAX Research Institute, Palo Alto, Calif.) for providing the RB6-8C5 hybridoma.

This study was funded by a grant (IHP-42) from the Trudeau Institute.

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Editor: J. R. McGhee