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Early Pathogenesis of Infection in the Liver with the Facultative Intracellular Bacteria *Listeria monocytogenes*, *Francisella tularensis*, and *Salmonella typhimurium* Involves Lysis of Infected Hepatocytes by Leukocytes

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The results show that *Listeria monocytogenes*, *Francisella tularensis*, and *Salmonella typhimurium* are facultative intracellular bacteria with a capacity to invade and grow in nonphagocytic cells in vivo. In the liver, all of these pathogens were seen to invade and to multiply extensively in hepatocytes. In all three cases, inflammatory phagocytes were rapidly marshalled to foci of infection where they appeared to cause the destruction of infected hepatocytes, thereby releasing bacteria into the extracellular space, in which presumably they could be ingested and destroyed by the phagocytes. If phagocytic cells were prevented from accumulating at foci of liver infection by treatment of the mice with a monoclonal antibody (NIMP-R10) directed against the type 3 complement receptor of myelomonocytic cells, then lysis of hepatocytes failed to occur and bacteria proliferated unrestrictedly within them. Under these circumstances, otherwise sublethal infections became rapidly lethal. These findings strongly suggest that lysis of infected hepatocytes by phagocytic cells is an important general early-defense strategy against liver infection with at least three different intracellular bacteria.

Facultative intracellular bacteria are thought to be pathogenic, at least in part, because of their ability to survive and grow inside host macrophages (15, 28). Therefore, it is generally believed that T-cell-mediated immunity is protective against these pathogens because it activates the bactericidal mechanisms of macrophages, thereby making them nonpermissive for intracellular growth of bacteria (26). In the case of *Listeria monocytogenes* and *Francisella tularensis*, this view is supported by the results of in vitro studies (13, 33) demonstrating lymphokine-mediated augmentation of macrophage microbicidal and microbistatic activities against these bacteria. It is also supported by studies showing that treating mice infected with *L. monocytogenes* or *F. tularensis* with neutralizing antibodies directed against certain lymphokines can cause exacerbation of infection (5, 10, 17, 23). *Salmonella typhimurium* is also considered a facultative intracellular pathogen, although its ability to survive and grow in professional phagocytes in vivo is uncertain (19, 24). The role of cell-mediated immunity in antisalmonella resistance is also unclear in that there are conflicting reports (21, 29, 38) regarding the need for T-cell-derived cytokines for antisalmonella defense. Furthermore, specific antibody alone (for a review, see reference 19) has been shown to provide significant protection against salmonellosis under some circumstances.

Whatever the role of specific cellular immunity, it is becoming apparent that there is more to resistance to bacterial infection than such immunity alone. For example, because it takes several days for a T-cell-mediated response to develop and for it to mobilize macrophages to sites of bacterial implantation (31), an earlier mechanism of defense must exist to prevent intracellular pathogens with short generation times from multiplying to numbers capable of overwhelming any T-cell-mediated immunity that is generated. In murine listeriosis, this early defense in the liver is provided first by the innate microbicidal mechanisms of Kupffer cells (31), which ingest 90% of an intravenous (i.v.) inoculum within 15 min and inactivate greater than 95% of this bacterial load over the next 6 h or so. However, because bacteria that survive destruction by Kupffer cells rapidly go on to infect and grow in adjoining hepatocytes, an additional early mechanism of defense needs to be expressed to control this phase of the infectious process. It was shown in recent publications (7–9) that this task is performed predominantly by neutrophils that accumulate at infectious foci during the first 24 h of infection, where they function to cause dissolution of infected hepatocytes, thereby releasing *L. monocytogenes* into the extracellular environment for ingestion by neutrophils themselves and by macrophages. The importance of this antibacterial defense strategy was demonstrated by treating mice with monoclonal antibodies (MAb) that bind to epitopes on the CD11b polypeptide chain of the type 3 complement receptor (CR3) of neutrophils and prevent them from accumulating at foci of liver infection (7, 9). In mice so treated, lysis of infected hepatocytes fails to occur and *L. monocytogenes* is left free to grow unrestrictedly within these permissive cells. This results in an otherwise sublethal inoculum rapidly giving rise to lethal numbers of bacteria. Thus, this early neutrophil-mediated defense strategy serves to hold infection to a level that can be resolved by mechanisms of resistance that are subsequently acquired.

The present study was undertaken to determine whether this same early-defense strategy is used to defend against infections with two other facultative intracellular pathogens, namely, *F. tularensis* and *S. typhimurium*. To this end, the early pathogenesis of *L. monocytogenes*, *F. tularensis*, and *S. typhimurium* infections in the livers of normal mice and of mice treated with the anti-CR3 MAb, NIMP-R10, was studied. The results show that *F. tularensis* and *S. typhimurium*, like *L. monocytogenes*, can invade and grow inside hepato-

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cytes. They show, in addition, that infected hepatocytes are consequently lysed by host inflammatory cells.

MATERIALS AND METHODS

Mice. Male B6D2F$_1$ (C57BL/6 × DBA/2) mice were used when they were 8 to 12 weeks old for experiments with *L. monocytogenes* or *F. tularensis*. Male CD2F$_1$ (BALB/c × DBA/2) mice of similar ages were used for experiments with *S. typhimurium*. All mice were obtained from the Trudeau Institute Animal Breeding Facility and were known to be free of common viral pathogens according to the results of routine screening by the Research Animal Diagnostic Laboratory, University of Missouri, Columbia.

Bacteria. *L. monocytogenes* EGD was grown to log phase in Trypticase soy broth and aliquoted in 1-ml volumes (2 × 10^8 CFU/ml), and the aliquots were frozen at −70°C. A streptomycin-resistant variant of *S. typhimurium* C5 (20) was grown similarly and frozen in aliquots (2 × 10^8 CFU/ml) in the presence of 10% (vol/vol) dimethyl sulfoxide (Sigma Chemical Co., St. Louis, Mo.). *F. tularensis* LVS was obtained in freeze-dried form from the American Type Culture Collection, Rockville, Md. It was revived on cystine heart agar supplemented with 5% (vol/vol) fresh defibrinated rabbit blood. A single colony was used to grow a log-phase culture (2.5 × 10^8 CFU/ml) in modified Mueller-Hinton broth (3), which was then aliquoted in 2-ml volumes and frozen at −70°C in the presence of 10% (wt/vol) sucrose. The 50% lethal dose (LD_{50}) of *F. tularensis* determined over 28 days postinfection was approximately 1.5 × 10^5 CFU, the 28-day LD_{50} of *S. typhimurium* was approximately 1.5 × 10^3 CFU, and the 14-day LD_{50} of *L. monocytogenes* was approximately 7.8 × 10^3 CFU.

For each experiment, the required number of frozen vials was thawed, and the bacteria were washed once in 0.9% (wt/vol) saline and diluted appropriately in saline for intravenous inoculation in a volume of 0.2 ml. *L. monocytogenes* and *S. typhimurium* in the livers and spleens were enumerated by plating 10-fold serial dilutions of organ homogenates on Trypticase soy agar and counting bacterial colonies after incubation for 24 h at 37°C. Organ homogenates from mice infected with *F. tularensis* were plated on cystine heart agar supplemented with 1% (wt/vol) hemoglobin. Bacterial colonies were counted after incubation for 72 h at 37°C. All bacteriological media were obtained from Difco Laboratories, Detroit, Mich.

MAb. MAb NIMP-R10, directed against an epitope on the CD11b polypeptide chain of the CD11b/CD18 heterodimeric CR3 (25), was a kind gift from A. Lopez, Institute of Medical and Veterinary Science, Adelaide, South Australia. This antibody prevents the recruitment of neutrophils and macrophages to foci of *L. monocytogenes* infection in the liver (9). MAb was isolated from ascites fluid by a one-step chromatographic procedure with an Avidal affinity column (Bioprobe International Inc., Tustin, Calif.). The antibody was given i.v. in a dose of 0.2 mg (protein) 1 h before infection. In pilot experiments, an isotype-matched anti-CD4 MAb (GK 1.5) did not alter the pathogenesis of infection during the first 48 h. This is in keeping with previously published work from this laboratory with GK 1.5 (11). Accordingly, control antibody was not used further in this study.

Histology. Mice were killed by cervical dislocation, and their livers were removed whole, cut into small pieces, and fixed in 10% buffered formalin. The tissue was then dehydrated in ethanol and embedded in glycol methacrylate (JB-4 embedding kit; Polysciences Inc., Warrington, Pa.). Sections 1 to 2 μm in thickness were cut with glass knives and stained with aqueous McNeal's tetrachrome stain or with phenolic crystal violet (22). Light microscopy was performed with a Nikon Microphot-Fx microscope.

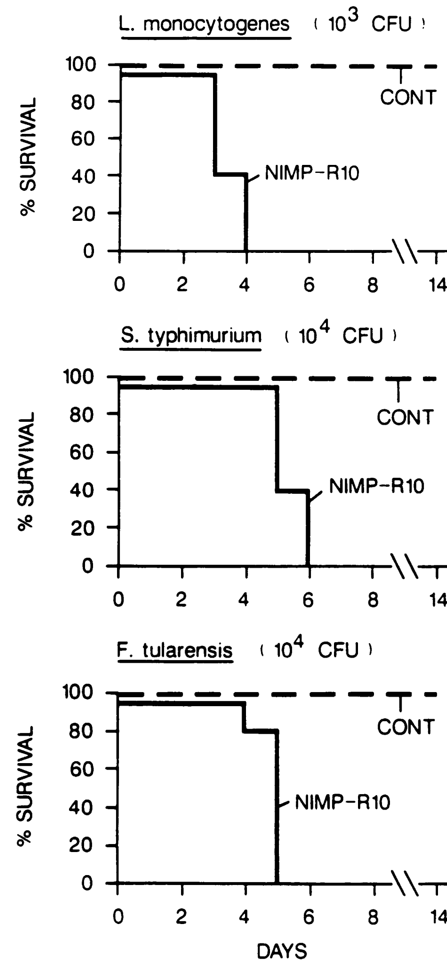

FIG. 1. Mice treated with MAb NIMP-R10 (solid lines) 1 h before infection were rapidly killed by i.v. inocula of *L. monocytogenes*, *S. typhimurium*, or *F. tularensis* that were sublethal for controls (cont; broken lines). Five mice per group were used, and deaths were recorded daily.

RESULTS

Treatment with MAb NIMP-R10 rendered mice more susceptible to infection with *L. monocytogenes*, *F. tularensis*, or *S. typhimurium*. Figure 1 illustrates the importance of CR3-dependent mechanisms of defense against infection with *L. monocytogenes*, *F. tularensis*, and *S. typhimurium*. It shows that inocula of these organisms that were sublethal for control mice for a period of 14 days were rapidly lethal for mice treated with anti-CR3 MAb NIMP-R10. The growth of these organisms in the livers of control and NIMP-R10-treated mice during the first 48 h of infection is shown in Fig. 2. It shows that NIMP-R10 treatment greatly exacerbated infections with *L. monocytogenes* or *S. typhimurium*, to the extent that by 24 h, in the livers of the treated mice there were up to 100-fold more bacteria than in those of the controls; there were up to 1,000-fold more by 48 h. This was

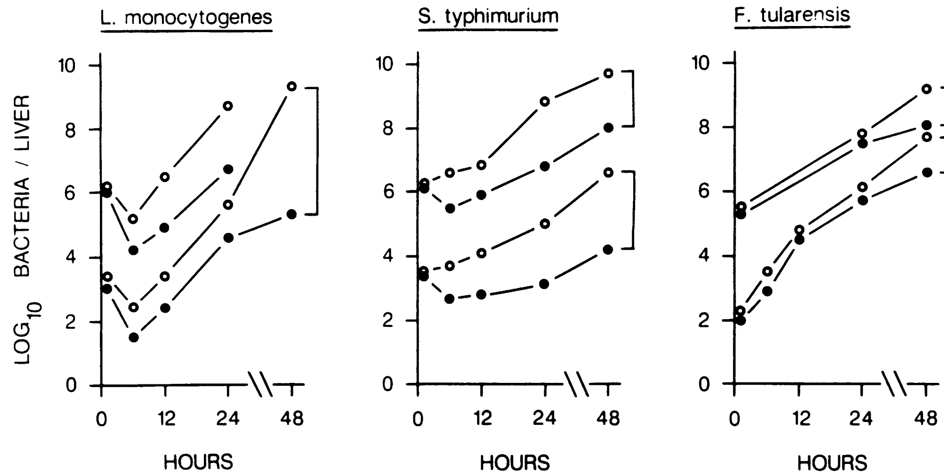

FIG. 2. Comparison of the growth of *L. monocytogenes*, *S. typhimurium*, and *F. tularensis* in the livers of mice treated with MAb NIMP-R10 (○) and in control mice (●). Mice were inoculated i.v. with 10^4 or 10^7 CFU of one of the organisms, and growth of bacteria in livers was monitored over 48 h. The means for five mice per group are shown. The standard errors of the means were <0.25 $\log_{10}$ unit.

the case with small (10^4 CFU) as well as large (10^7 CFU) inocula of these two organisms. In addition, the results show that NIMP-R10 treatment had only a marginal effect on the inactivation of *L. monocytogenes* that occurs during the first 6 h. By contrast, treatment with NIMP-R10 totally abolished the initial inactivation of *S. typhimurium* that occurs in the livers of control mice during the first 6 h. This suggests that *L. monocytogenes* and *S. typhimurium* are killed by different defense mechanisms during this early stage of liver infection. The situation with *F. tularensis* was different again, in that there was no apparent inactivation of this organism in the livers of control mice during the first 6 h. Therefore, NIMP-R10 had no influence on bacterial numbers during this stage of infection. In addition, it did not affect the growth of *F. tularensis* during the first 24 h of infection. However, by 48 h, NIMP-R10-treated mice had 10-fold more bacteria in their livers.

Early histopathogenesis of *L. monocytogenes*, *F. tularensis*, and *S. typhimurium* infections in the livers of NIMP-R10-treated and control mice. The cellular events during the first 48 h of infection in the livers of control and NIMP-R10-treated mice after inoculation of *L. monocytogenes*, *F. tularensis*, or *S. typhimurium* were monitored histologically. Large i.v. inocula (10^7 or 10^8 CFU) were used for this study to facilitate the detection of bacteria in thin (1- to 2-μm) sections. Because the early pathogenesis of listeriosis in the livers of mice treated with anti-CR3 MAb has already been described elsewhere (7–9, 35), it is dealt with here only briefly for comparative purposes. In NIMP-R10-treated mice, *L. monocytogenes* appeared to grow exclusively within the cytoplasm of infected hepatocytes. As shown in Fig. 3a, infection of hepatocytes by *L. monocytogenes* and its multiplication therein was evident by 24 h, by which time it had not only grown to large numbers inside the hepatocytes that it initially infected but had spread to infect neighboring hepatocytes. Retention of the morphological integrity of infected hepatocytes in mice treated with NIMP-R10 is noteworthy, as is the absence of inflammatory cells. In normal mice, in contrast, large numbers of neutrophils had accumulated at foci of infection, where they appeared to have caused extensive dissolution of infected hepatocytes (Fig. 3b). Under these circumstances, it was difficult to find any intact infected hepatocytes. As expected from the growth curves, far more bacteria were seen at infectious foci in the livers of NIMP-R10-treated mice than in those of controls. This suggests that in control mice, infected hepatocytes were destroyed before *L. monocytogenes* could grow to large numbers within them.

Because of its small size, *F. tularensis* was difficult to find in thin sections during the first several hours of infection. However, by 16 h in NIMP-R10-treated mice, this organism had multiplied to sufficient numbers to be recognized as aggregates inside Kupffer cells (Fig. 4a), as well as within vacuoles of hepatocytes (Fig. 4b). It was apparent that at 16 h some heavily infected Kupffer cells had lysed and released clumps of bacteria into sinusoids, in which they formed microcolonies that increased in size with time. From 16 to 48 h, *F. tularensis* bacteria increased in numbers both inside Kupffer cells and inside hepatocytes (Fig. 4c and d), and by 48 h some infected hepatocytes were showing various degrees of degeneration. Many bacteria were doublets at this time (Fig. 4d). In contrast to the situation with listeriosis, spread of infection to neighboring hepatocytes was not obvious by 48 h with *F. tularensis* (Fig. 4c). In terms of its ability to infect hepatocytes, the live vaccine strain of *F. tularensis* used in this study would appear to behave like environmental isolates of *F. tularensis* (27). After 16 h of infection in *F. tularensis*-infected control mice, accumulations of both neutrophils and mononuclear cells were seen at sites of infection and infected hepatocytes were obviously in the process of undergoing dissolution (Fig. 4e and f).

The situation with *S. typhimurium* was different from that seen with both other organisms, in that at 1 h postinoculation, *S. typhimurium* was found in the livers of NIMP-R10-treated mice in small numbers, not only inside Kupffer cells, but also inside neutrophils in sinusoids, as well as extracellularly in the sinusoids. Both Kupffer cells (Fig. 5a) and neutrophils (Fig. 5b) were heavily laden with *S. typhimurium* by 24 h of infection, although whether this occurred as a result of intracellular bacterial multiplication or of continuous phagocytosis is not known. In NIMP-R10-treated mice, neutrophils were found only in the sinusoids and did not migrate into the parenchyma. *S. typhimurium* also grew extracellularly in the liver sinusoids of these mice during this time. From 24 h of infection, *S. typhimurium* infected hepatocytes and multiplied extensively, apparently within

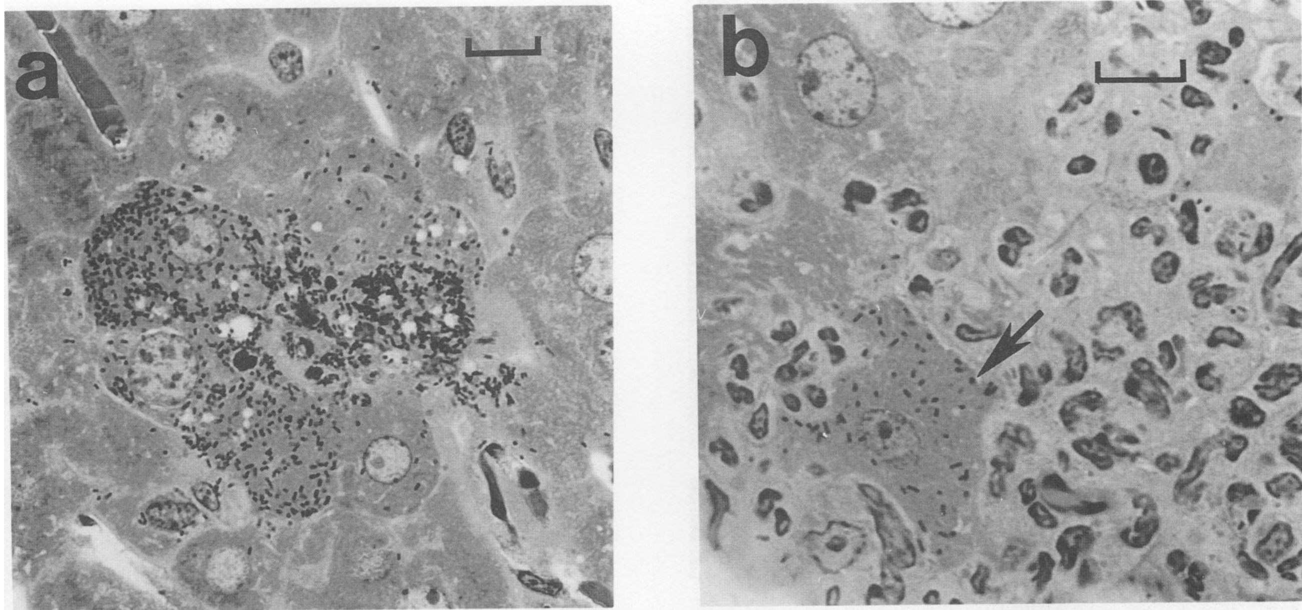

FIG. 3. Foci of *L. monocytogenes* infection in the livers of NIMP-R10-treated (a) and control (b) mice. By 24 h, foci of *L. monocytogenes* infection in mice treated with MAb NIMP-R10 consisted of clusters of intact, heavily infected hepatocytes, with inflammatory cells being conspicuously absent. In the livers of control mice, by contrast, large numbers of neutrophils had accumulated at foci of infection to occupy space previously occupied by infected hepatocytes. At the peripheries of infectious foci, neutrophils were found in close contact with infected hepatocytes (arrows) which were undergoing dissolution. Bar, 10 μm.

large vacuoles in these cells (Fig. 5c and d). By 36 h, the predominant liver lesions consisted of groups of hepatocytes that were uniformly infected with large numbers of *S. typhimurium* cells. By contrast, the situation in the livers of control mice infected with *S. typhimurium* was strikingly different, in that it was difficult to find any bacteria at all by 24 h of infection. Instead, foci of infection were indicated by the presence of focal accumulations of inflammatory cells that were mostly neutrophils. Between 24 and 48 h of infection, neutrophils were found in close physical contact with infected hepatocytes that were obviously undergoing dissolution (Fig. 5e and f). However, in contrast to the situation in NIMP-R10-treated mice, *S. typhimurium* was rarely found inside neutrophils in control mice. This suggests that in the latter mice, neutrophils were able to kill *S. typhimurium* bacteria that they ingested and that this ability is dependent on CR3 function. This finding is in keeping with the results of others who have shown (16) that NIMP-R10 can interfere with the ability of neutrophils to kill ingested *Proteus mirabilis* and *Staphylococcus aureus*. It is possible that neutrophils are responsible for the initial CR3-dependent inactivation of *S. typhimurium* (Fig. 2) in the livers of normal mice. As was the case with *L. monocytogenes* infection, far fewer *S. typhimurium* bacteria were observed at foci of infected hepatocytes in the livers of control mice than in those of mice treated with NIMP-R10.

DISCUSSION

It is generally considered that the pathogenicity of facultative intracellular bacteria depends on their ability to survive and grow inside host macrophages (15, 28). However, the potential for facultative intracellular bacteria to parasitize host cells other than macrophages in vivo and the consequences thereof have been largely overlooked. In this connection, it has been shown by others (24, 27) and confirmed by the present study that *F. tularensis* and *S. typhimurium*, in addition to being able to parasitize macrophages, can, like *L. monocytogenes*, infect liver hepatocytes. Indeed, it is obvious from the present study that hepatocytes are highly permissive for the growth of all three of these organisms. This means that in order to be accessible for ingestion and destruction by activated macrophages, bacteria growing in hepatocytes must be released into the extracellular space. According to the results presented herein, lysis of hepatocytes harboring any one of the three organisms under study during the first 48 h of infection is achieved by leukocytes that accumulate at sites of infection. This conclusion is based on the finding that extensive dissolution of infected hepatocytes occurred at foci of infection populated by neutrophils and macrophages in the livers of control mice but not in those of mice treated with the anti-CR3 MAb NIMP-R10, which prevents recruitment of inflammatory phagocytes to infectious foci. In the case of *L. monocytogenes* infection, neutrophils appear to be responsible for hepatocyte lysis during the early phase of infection, since they far outnumber other cells and they are the only cells that physically engage infected cells. Furthermore, depleting mice of NK cells or T cells (6) does not inhibit accumulation of neutrophils or lysis of infected hepatocytes at foci of *L. monocytogenes* infection. Neutrophils may also be involved in destroying hepatocytes infected with *S. typhimurium* or *F. tularensis*. In these two cases, however, mononuclear cells were also numerous at sites of infection by 48 h. Therefore, participation of monocytes, NK cells, or T cells in hepatocyte dissolution cannot be excluded, especially since NIMP-R10 clearly prevented the recruitment of mononuclear cells as well as neutrophils to foci of liver infection. It is unlikely, however, that T cells were responsible for lysing hepatocytes during early infection, because according to other studies (2, 11, 18, 29, 32), T-cell-mediated immunity does not contribute to resistance until after 48 h.

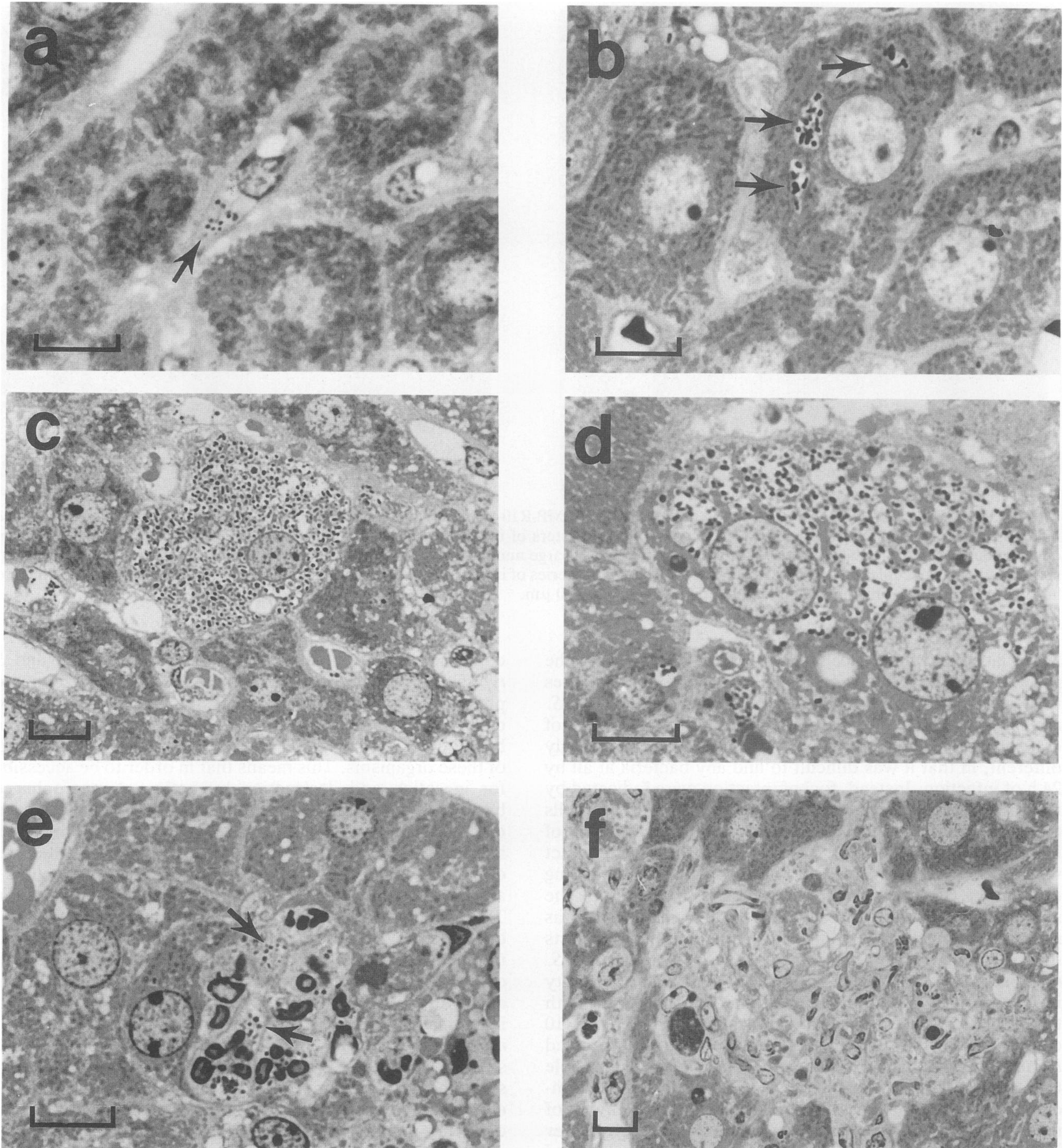

FIG. 4. *F. tularensis* infection in the livers of NIMP-R10-treated (a to d) and control (e and f) mice. By 16 h, *F. tularensis* was found in the livers of mice treated with NIMP-R10, inside Kupffer cells (a) and inside hepatocytes (b) apparently within vacuoles (arrows). By 36 to 48 h, *F. tularensis* multiplied to occupy most of the cytoplasm of infected hepatocytes (c and d); however, hepatocyte-to-hepatocyte spread of infection was not obvious (c), and many bacteria appeared as doublets in hepatocyte cytoplasm (d). By 16 h, small numbers of leukocytes had accumulated at foci of infection in the livers of control mice (e) and appeared to have been in the process of engaging and lysing infected hepatocytes. Small aggregates of bacteria (arrows) were present in these lesions. By 48 h (f), extensive dissolution of hepatocytes had occurred at foci of leukocyte accumulation and *F. tularensis* was difficult to find. Bar, 10 μm.

The ability of *S. typhimurium* to infect and grow in hepatocytes appears to contradict a recent study by others (30), who failed to recover infected hepatocytes from the livers of rats disrupted by collagenase perfusion. However, if, as we propose, infected hepatocytes are rapidly engaged and destroyed by mobile leukocytes in vivo, then they would not exist in cell preparations from infected livers.

Obviously, to infect hepatocytes, *L. monocytogenes*, *F.*

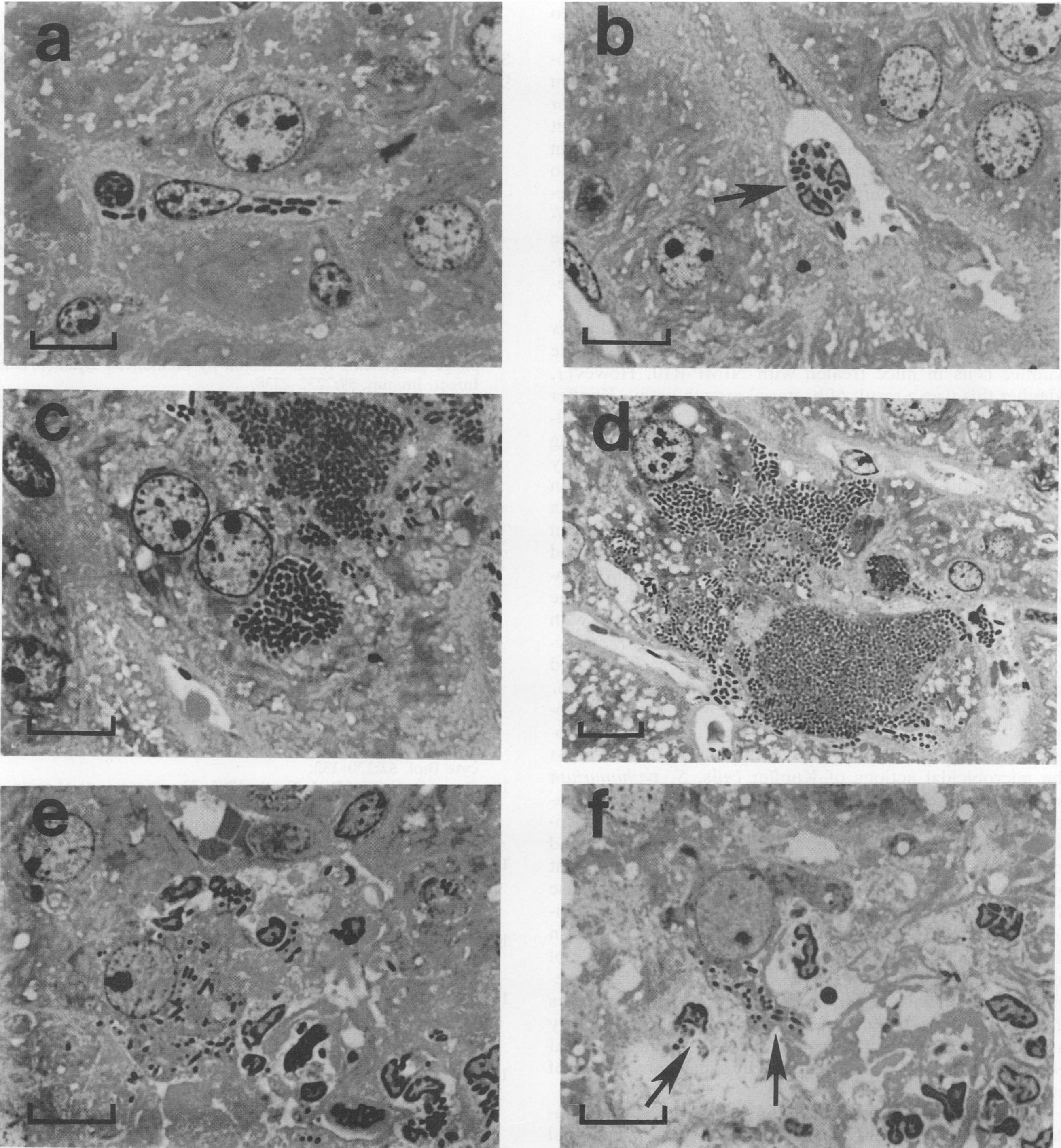

FIG. 5. *S. typhimurium* infection in the livers of mice treated with NIMP-R10 (a to d) and of control mice (e and f). In NIMP-R10-treated mice, *S. typhimurium* was found during the first 24 h in the livers inside Kupffer cells (a) and neutrophils (b) in sinusoids. Between 24 and 48 h, in NIMP-R10-treated mice, *S. typhimurium* cells had invaded and grown to large numbers inside hepatocytes (c and d). From 24 h, in control mice (e and f), leukocytes had moved out of the sinusoids and into the parenchyma to accumulate at the foci of infected hepatocytes. Hepatocytes apparently were in the process of being lysed (arrows) at sites of leukocyte engagement. Bar, 10 μm.

tularensis, and *S. typhimurium* must first cross the endothelium of liver sinusoids. *L. monocytogenes* presumably relies on its ability to produce a hemolysin (listeriolysin) to achieve this, since it is this molecule that allows it to escape from the phagosomes (14, 37) and to enter the cytoplasm of the Kupffer cells that removed it from the blood. Thenceforth, it can exploit a listeriolysin-dependent mechanism (34, 37) to infect neighboring hepatocytes via points of contact with infected Kupffer cells. Once inside hepatocyte vacuoles, *L. monocytogenes* cells presumably can use listerolysin to gain

access to the cytoplasm, where they multiply and go on to invade adjoining hepatocytes. By contrast, neither *F. tularensis* nor *S. typhimurium* is known to produce a listeriolysin-like hemolysin that would allow them to escape from the phagocytic vacuoles of the cells that ingest them. As for the ability of *S. typhimurium* to enter nonphagocytic cells, it has been shown elsewhere (36) that this organism can directly invade and penetrate epithelial cells to gain access to underlying tissues. Furthermore, it has been shown elsewhere (12) that to invade epithelial cells in vitro, *S. typhimurium* must first synthesize new membrane proteins. The same processes might occur in vivo to enable *S. typhimurium* to invade hepatocytes. It is not known how *F. tularensis* might invade hepatocytes.

In addition to growing inside hepatocytes, *F. tularensis* and *S. typhimurium* were also found in large numbers inside Kupffer cells in mice treated with NIMP-R10. However, whether this was a result of bacterial growth within Kupffer cells or of continuous phagocytosis of circulating extracellular organisms is not known. In either case, this finding suggests that *S. typhimurium* and *F. tularensis* are relatively resistant to the microbicidal mechanisms of Kupffer cells. In the case of *S. typhimurium*, this finding is in keeping with a recent report by others (30) demonstrating that this pathogen was intracellularly associated with Kupffer cells recovered from infected livers after collagenase perfusion and disruption. By contrast, *L. monocytogenes* is quickly killed by the Kupffer cells that ingest it, even in mice treated with NIMP-R10. Indeed, only a small percentage of *L. monocytogenes* cells avoid being killed by Kupffer cells and proceed to cause infection in hepatocytes. *F. tularensis* and *S. typhimurium*, however, can survive inside macrophage phagosomes possibly because they inhibit phagosome-lysosome fusion (1, 4). Although it was apparently able to resist the microbicidal actions of Kupffer cells, *S. typhimurium* was highly susceptible to a CR3-dependent killing mechanism that was apparently responsible for inactivating approximately 90% of the bacteria that were initially implanted in the liver. The results suggest that neutrophils might have been responsible for this initial inactivation, because *S. typhimurium* was found inside neutrophils in liver sinusoids by 1 h postinoculation and increased in numbers in these cells with time in mice treated with NIMP-R10 but not in controls. This suggestion is in keeping with the interpretation of an electron-microscopic study (24) that suggests that ingested *S. typhimurium* bacteria are destroyed by neutrophils. It is also supported by the finding (16) that NIMP-R10 interferes with the bactericidal capabilities of neutrophils.

In conclusion, the present study shows that CR3-dependent inflammatory cell accumulation at sites of bacterial implantation in the liver serves to drastically restrict the growth of *S. typhimurium* and *L. monocytogenes* during the early phase of infection by lysing hepatocytes infected with these bacteria, thereby releasing the bacteria for ingestion and destruction by phagocytic cells. *F. tularensis* was more able to resist the antibacterial actions of this defense, even though it caused the release of this organism from permissive hepatocytes and prevented (Fig. 1) *F. tularensis* from otherwise growing to lethal numbers. On the basis of the findings presented herein, it seems reasonable to suppose that phagocyte-mediated lysis of infected cells might be a general defense strategy against parasites of parenchymal cells.

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