Neutrophils Are Essential for Early Anti-*Listeria* Defense in the Liver, but Not in the Spleen or Peritoneal Cavity, as Revealed by a Granulocyte-depleting Monoclonal Antibody

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Summary

This study shows that in mice selectively depleted of neutrophils by treatment with a monoclonal antibody, RB6-8C5, listeriosis is severely exacerbated in the liver, but not in the spleen or peritoneal cavity during the crucial first day of infection. At sites of infection in the livers of neutrophil-depleted mice, *Listeria monocytogenes* grew to large numbers inside hepatocytes. By contrast, in the livers of normal mice neutrophils rapidly accumulated at infectious foci and this was associated with the lysis of infected hepatocytes that served to abort infection in these permissive cells. In the spleen the situation was different, in that depletion of neutrophils did not result in appreciable exacerbation of infection. In this organ intact infected cells, many of which appeared to be fibroblast-like stromal cells, were found at foci of infection in the presence or absence of large numbers of neutrophils. This suggests that neutrophils are less effective at destroying *L. monocytogenes*–infected target cells in the spleen than in the liver. Consequently, at least during the first day, the organism remained free to multiply intracellularly in the spleen in cells that are permissive for its growth. Presumably, the same situation exists in the peritoneal cavity, because depleting neutrophils did not severely exacerbate infection initiated at this site.

It has been generally believed that *Listeria monocytogenes* is a facultative intracellular parasite that owes its virulence to its ability to survive and grow inside macrophages (1, 2). However, it is now known that *L. monocytogenes* can invade and proliferate in cells other than macrophages (3–8), including liver parenchymal cells (3). Thus, although most of an intravenous inoculum of *L. monocytogenes* is rapidly ingested and destroyed by liver Kupffer cells (9), some organisms avoid this early defense mechanism and are able to parasitize underlying hepatocytes that appear to be devoid of any antilisterial defenses. Consequently, *L. monocytogenes* can grow inside these cells without killing them and go on to directly infect neighboring cells by inducing the formation of intercellular cytoplasmic bridges (4, 10). In this way *L. monocytogenes* has the potential to infect large numbers of hepatocytes without having to confront phagocytic cells. However, under normal circumstances this phase of the infectious process is brought under control when the permissive hepatocytes in which *L. monocytogenes* resides are lysed. This serves to release the organism into the extracellular space for ingestion and killing by professional phagocytes.

Evidence has been presented from this laboratory (9, 11, 12) indicating that the task of lysing infected hepatocytes is achieved early in infection by neutrophils that rapidly accumulate at infectious foci. It is shown more recently (13) that this same defense mechanism probably operates to destroy hepatocytes infected with other facultative intracellular bacteria. However, the interpretation that neutrophils mediate lysis of infected hepatocytes is based on histological evidence showing that they are the predominant cells that accumulate at foci of infection in the liver during the first day, and that many of them make contact with, or are in close physical proximity to, infected hepatocytes undergoing dissolution. When neutrophils were prevented from accumulating at infectious foci (9, 12) by treating mice with mAb directed against the CD11b chain of the CD11b/CD18 β-2 integrin molecule, hepatocytes were not lysed and *L. monocytogenes* continued to replicate within them. Although convincing, these findings do not represent unequivocal evidence that neutrophils are exclusively responsible for lysing infected hepatocytes because it remains possible that small numbers of other CD11b-bearing cells other than neutrophils, which are not obvious in histological preparations, might have contributed to hepatocyte lysis. More direct evidence for an exclusive role of neutrophils in destroying infected hepatocytes would be provided by showing that in mice selectively depleted of neutrophils, *Listeria*-infected hepatocytes are not lysed, and infection is exacerbated.

One purpose of the present study was to use this strategy to determine whether neutrophils are in fact required for lysing infected hepatocytes during early stages of infection. Its other purpose was to determine whether neutrophils play this early

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defensive role at sites of infection other than the liver. The use of anti-CD11b mAbs was considered unsuitable for answering this second question because of reports showing that these mAb do not prevent extravasation of neutrophils into all tissues (14). Therefore, the early course of listeriosis in the liver, spleen, and peritoneal cavity was examined in mice treated with a specific granulocyte-depleting mAb (15), RB6-8C5. The results show that, RB6-8C5, by selectively depleting neutrophils to the extent that these cells were not available for recruitment to infectious foci, caused severe exacerbation of infection in the liver, but not in the spleen or peritoneal cavity.

Materials and Methods

Mice. Male CB6/F_1 mice were obtained from the Trudeau Institute Animal Breeding Facility. They were used when they were 9–12 wk old.

Bacteria. A logarithmic-phase broth culture of *L. monocytogenes* (strain EDG) was prepared as described previously (13) and stored frozen at −70°C in 1-ml aliquots. For each experiment vials of frozen bacteria were thawed, pelleted by centrifugation (16,000 *g* for 5 min), resuspended, and diluted in sterile 0.9% (wt/vol) saline to the required inoculum concentration.

Experimental Infections. Liver and spleen infections were established by intravenous inoculation of 10^4 CFU *L. monocytogenes* in a volume of 0.2 ml. At 12 and 24 h of infection, mice were killed by cervical dislocation, their livers and spleens removed and homogenized separately, and 10-fold serial dilutions of organ homogenates were plated on Trypticase-soy agar. Bacterial colonies were counted after incubation for 24 h at 37°C. To establish infection in the peritoneal cavity, mice were inoculated intraperitoneally with 5×10^6 CFU of *L. monocytogenes* in a volume of 0.2 ml. At 12 and 24 h of infection mice were killed as above and peritoneal cells (PC)[1] were collected. PC were harvested by lavaging the peritoneal cavity twice with 3-ml volumes of ice-cold PBS containing 20 U/ml heparin, using a syringe fitted with a 20-gauge needle. The peritoneal cavity was gently massaged before withdrawing the wash. Once collected, PC were kept on ice. Total cell counts were determined using a hemocytometer and trypan blue exclusion staining. For differential counts, aliquots of PC were pelleted by low-speed centrifugation (300 *g* for 5 min), resuspended in a few drops of FCS, and smeared onto glass slides with a fine camel hair paint brush. Smears were dried in a stream of warm air and stained with Diff Quik (Baxter Corp., Miami, FL). Differential counts were determined by counting 200 consecutive cells in each smear. To determine the number of bacteria recovered in the peritoneal wash, 1 ml of PC was added to 4 ml sterile distilled water, vortex-mixed and sonicated to disrupt host cells, and the sonicate then plated as above. To determine the number of host cell-associated bacteria, PC were pelleted as above and the CFU of bacteria in the supernatant determined.

Treatment of Mice with mAb RB6-8C5. RB6-8C5 is a rat IgG 2b mAb that is said to cause specific depletion of mouse neutrophils and eosinophils (15). The RB6-8C5 hybridoma was a kind gift from Dr. R. Coffman, DNAX Research Institute (Palo Alto, CA). mAb RB6-8C5 for depletion was obtained from mouse ascites and partially purified by ammonium sulfate precipitation. Antibody concentration was estimated from the height of the IgG peak eluting from an HPLC-size exclusion column. mAb RB6-8C5 was given intravenously in a dose of 0.25 mg 2 d and 4 h before inoculation of bacteria. Control mice were left untreated. We felt that it was unnecessary to treat control mice with a control mAb because in numerous published studies from this and other laboratories the administration of similar amounts of isotype-matched antilymphocyte and anticytokine mAbs had no appreciable effect on the course of listeriosis during the first day.

Cytofluorometry. FITC was conjugated to RB6-8C5 (whole antibody) that had been purified to homogeneity by DEAE-sephacel ion exchange chromatography. A whole antibody FITC-conjugate of an isotype-matched mAb (labeled at a threefold higher FITC to immunoglobulin ratio than for RB6-8C5) directed against KLH served as a control for nonspecific staining via Fc receptor binding. This mAb was produced in this institute by Dr. L. Johnson. The $F(ab')_2$ fragment of rat IgG anti-Thy 1.2 (clone 30.H.12; American Type Culture Collection, Rockville, MD) was conjugated to biotin. FITC and biotin conjugation was carried out as previously described (16). These conjugates were used for one- or two-color cytofluorimetric analysis of single cell suspensions of spleen cells or lymph node cells as described elsewhere (16). For two-color analyses, cells were stained first with FITC-RB6 and then with biotinylated anti-Thy 1.2 and streptavidin-phycoerythrin (Molecular Probes Inc., Eugene, OR). Staining was carried out in buffer containing 100 μg/ml of normal rat IgG (Sigma Chemical Co., St. Louis, MO) to block nonspecific binding of RB6-8C5 to cells via their Fc receptors. This represents a 100-fold excess of normal IgG over the working concentration of FITC-RB6-8C5. To determine which cells are recognized by mAb RB6-8C5 in vivo, cardiac blood was collected from mice 1 h after intravenous administration of this mAb. Erythrocytes were lysed by osmotic shocking in distilled water and RB6-8C5-positive leukocytes were detected using $F(ab')_2$ FITC-conjugated polyclonal goat anti-rat antibody. The conjugate alone failed to bind to leukocytes prepared as above from control mice (data not shown). Stained cells were analyzed using a FACScan® cytofluorograph or they were sorted using a FACStar Plus® cytofluorograph (both from Becton Dickinson & Co., Mountain View, CA) both fitted with Lysis II software. Cytocentrifuge smears of sorted cells were stained with Diff-Quik and examined by microscopy.

Histology. Mice were inoculated intravenously with 10^4 or 10^6 CFU of *L. monocytogenes*, and killed at 24 h of infection. Their livers and spleens were removed whole, cut into small pieces, and fixed in 10% buffered formalin for 24 h. Fixed tissue was dehydrated in ethanol and embedded in plastic (JB-4 embedding kit; Polysciences Inc., Warrington, PA). Thin sections (1–2 μM thick) were cut with glass knives, stained with MacNeal's tetrachrome stain, and examined with a Nikon-microphot Fx light microscope.

Results

Specificity and Depleting Capacity of mAb RB6-8C5. It has been reported by others (15) that mAb RB6-8C5 specifically binds to and selectively depletes mature murine neutrophils and eosinophils. However, the published study does not present detailed cytofluorimetric data in support of this interpretation. Moreover, it was recently reported by another group (17) that RB6-8C5 can bind to a significant proportion of $CD8^+$ and $CD4^+$ T cells from naive mice and from mice infected with *L. monocytogenes*. Given this latter claim it was necessary to reexamine the specificity of RB6-8C5 for neutrophils and eosinophils before conducting any in vivo ex-

[1] *Abbreviation used in this paper:* PC, peritoneal cells.

periments with it. The specificity of RB6-8C5 was assessed by cytofluorimetric analysis of its binding to isolated cells from the spleens of naive mice and from mice that had been infected 1 d earlier with 10^4 CFU of *L. monocytogenes*. This allowed the identification of RB6-8C5–positive resident spleen cells and cells recruited to infectious foci in the spleen during the first day of infection. Cells that stained with a fluorescence intensity of >1.5 log were considered positive. In a preliminary experiment a single cell suspension of spleen cells (10% of which were determined by differential counting to be neutrophils) was directly stained with FITC–RB6-8C5 or with a control FITC–anti-KLH mAb. Whereas 10.8% of this spleen cell population was RB6-8C5–positive, only 0.23% of the same population was positively stained by the control mAb. The failure of control mAb to stain was expected, given the large excess of normal rat IgG used in the staining protocol. In view of this result, positive staining of cells with FITC–RB6-8C5 was considered to be specific in all subsequent experiments.

As shown in Fig. 1 almost all (>90%) RB6-8C5–positive spleen cells fell within a single brightly staining population. The remaining few cells stained with much lesser intensity. When the total (bright plus dull) positive cell population (Fig. 1, population *A*) was positively sorted, smeared, stained, and examined by microscopy it was found to consist almost entirely of neutrophils with a few eosinophils and mononuclear cells. By contrast, when only the intensely stained population of cells (Fig. 1, population *B*) was sorted and examined, eosinophils were conspicuously absent, indicating that they were stained less intensely by RB6-8C5. However, a small percentage of mononuclear cells remained which probably represented nonstaining cells that were bound to neutrophils via the Fc moiety of RB6-8C5 during sorting. This interpretation is based on the observation that when sorting of RB6-8C5 positive cells was carried out in the absence of nonspecific rat IgG, the number of mononuclear cells in the sorted population increased to ~50%, and cell agglutination was obvious (not shown). To verify that mAb RB6-8C5 selectively binds to neutrophils in vivo, as well as in vitro, blood leukocytes taken from mice given the mAb 1 h earlier were examined by cytofluorimetry as described in Materials and Methods. By 1 h, mAb RB6-8C5 had reduced the percentage of neutrophils in blood to 3% of the total leukocytes. Despite this, when RB6-8C5–positive leukocytes were positively sorted they were found by differential cell counting to be >95% neutrophils.

To test whether RB6-8C5 binds to some T cells, as indicated by a recent publication (17), spleen cells and lymph node cells (pooled inguinal, mesenteric, and brachial lymph nodes) from mice infected 1, 2, 5, and 8 d earlier with 10^4 or 10^3 CFU of *L. monocytogenes* were dual stained with FITC–RB6-8C5 and streptavidin-phycoerythrin after being treated first with biotinylated anti-Thy 1.2. The cytofluorimetric profile of dual stained spleen cells from mice infected 1 d earlier is displayed in Fig. 2. It shows unequivocally that T cells failed

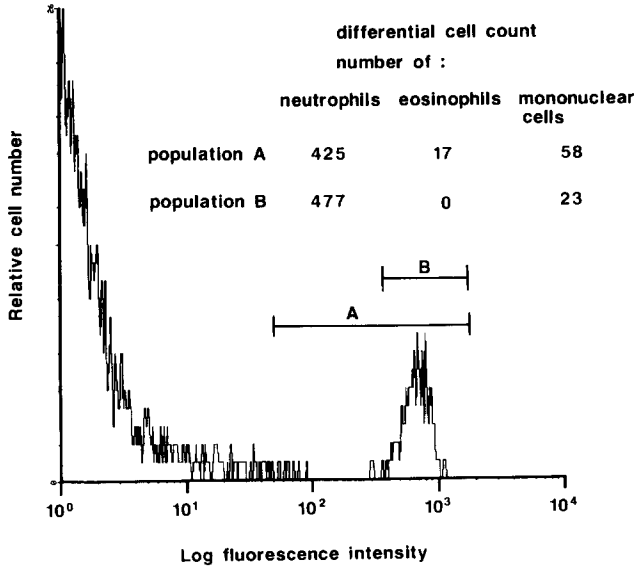


Figure 1. Identification of RB6-8C5–positive spleen cells. Cells were stained with FITC–RB6-8C5 and positively sorted by flow cytometry on the basis of their high (>1.5 log) fluorescence intensity. Cytofluorometric data is presented as a histogram plot of a cell suspension from which autofluorescent cells have been gated out. Most RB6-8C5–positive cells were contained in a single brightly staining cell population (*B*) with a few cells (*A* minus *B*) staining less intensely. After sorting, cytosmearing, and staining, differential counts (*inset*) were performed on RB6-8C5–positive cell populations *A* and *B*, 90–95% of which were found to be neutrophils and eosinophils.

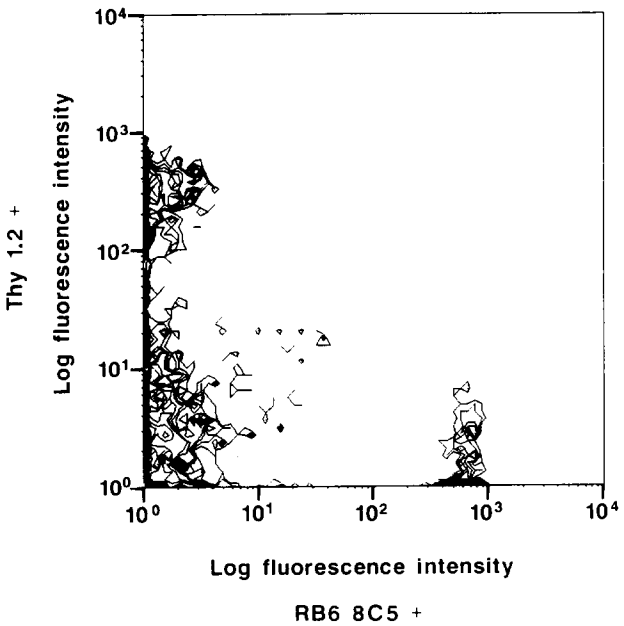


Figure 2. Two-color cytofluorimetric analysis of spleen cells stained with RB6-8C5 and anti-Thy 1.2. Spleen cells from a mouse inoculated 24 h earlier with *L. monocytogenes* were stained with FITC–RB6-8C5 (*x*-axis) and biotin–anti-Thy 1.2/streptavidin-phycoerythrin (*y*-axis). Data are presented as a contour plot of 80% logarithmic contours with a 1% threshold. No positive dual stained cells were detected, indicating that the antigen recognized by RB6-8C5 is not present on T cells.

to stain with RB6-8C5. Identical results were obtained with spleen and lymph node cells harvested and analyzed on all subsequent days of infection (data not shown).

The efficiency of mAb RB6-8C5 for depleting neutrophils was assessed by examining its effects on blood leukocyte numbers. The results presented in Table 1 show that RB6-8C5 treatment resulted in 95% depletion of neutrophils from blood. Thus, by the time RB6-8C5–treated mice were inoculated with *Listeria* they had >15-fold fewer blood neutrophils than control mice, but similar numbers of blood monocytes and lymphocytes.

Effect of Treating Mice with mAb RB6-8C5 on the Early Course of Listeriosis in the Liver vs. the Spleen. To determine the effects of depleting mice of neutrophils on the early course of listeriosis, the growth of *L. monocytogenes* was determined in the livers and spleens of control mice and RB6-8C5–treated mice at 12 and 24 h of infection. Table 2 shows that by 12 h after intravenous inoculation of 10^4 CFU of *L. monocytogenes* there were 10-fold more bacteria in the livers of RB6-8C5–treated mice than in the livers of control mice, and that by 24 h there were 140-fold more. In the spleen, in contrast, treating mice with mAb RB6-8C5 caused only a threefold exacerbation of infection during the same time period. These results indicate clearly, therefore, that in mice depleted of neutrophils, the liver, but not the spleen, is far more permissive for the growth of *L. monocytogenes*.

To determine why RB6-8C5 treatment severely exacerbates infection in the liver, but not in the spleen, these organs from control and mAb-treated mice were examined histologically 24 h after intravenous inoculation of 10^4 or 10^6 CFU of *L. monocytogenes*. As shown in Fig. 3, and described in detail below, foci of infection in the livers (Fig. 3 *a*) and spleens (Fig. 3 *b*) of control mice were populated by large numbers of neutrophils. By contrast, in RB6-8C5–treated mice, neutrophils were conspicuously absent from infectious foci in the liver and spleen, and from the blood circulating in these organs. Instead, foci of infection in the liver (Fig. 4 *a*) consisted of small groups of infected, morphologically intact hepatocytes some of which were very heavily infected, whereas others were less so. Presumably, *L. monocytogenes* grew to large numbers inside initially infected hepatocytes and then went on to directly infect neighboring hepatocytes at points of plasma membrane contact. This has been shown to be the case for intestinal epithelial cells in vivo (4) and a variety of other mammalian cells in vitro (10, 18, 19). In the spleen, by contrast, infectious foci in RB6-treated mice consisted of lightly infected cells in the marginal zone of the white pulp

Table 1. *Effect of Treating Mice with mAb RB6-8C5 on Blood Leukocyte Numbers*

Mice	Leukocytes		
	Lymphocytes	Monocytes	Neutrophils
	per mm^3 blood ± SD		
Controls (n = 5)	6620 ± 1520	328 ± 152	1100 ± 550
RB6-treated (n = 4)	4920 ± 492	402 ± 156	61 ± 42

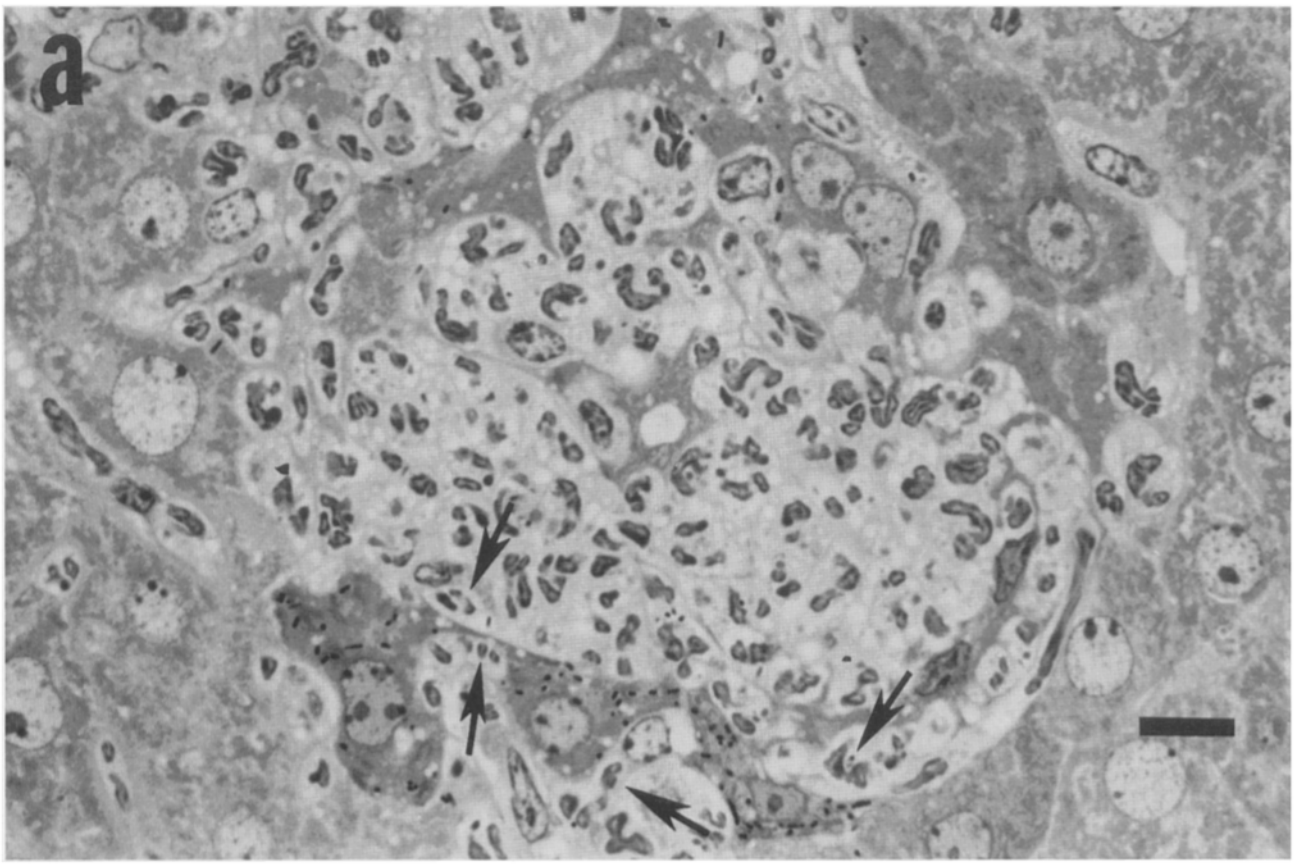
RB6-8C5–treated mice received 0.25 mg of mAb intravenously 2 d and 4 h earlier. Smears were made of peripheral blood collected from tail veins.

Table 2. *Recovery of Viable Bacteria from the Livers and Spleens of Control and RB6-8C5–treated Mice after Intravenous Inoculation of L. monocytogenes*

Mice	*L. monocytogenes*			
	12 h		*24 h*	
	Liver	*Spleen*	*Liver*	*Spleen*
	Log_{10} CFU ± SD			
Control	4.38 ± 0.16	4.75 ± 0.07	5.07 ± 0.15	5.78 ± 0.11
RB6-8C5–treated	5.42 ± 0.18	4.70 ± 0.15	7.22 ± 0.08	6.16 ± 0.11

RB6-8C5–treated mice received 0.25 mg of mAb intravenously 2 d and 4 h before intravenous inoculation of 10^4 CFU of *L. monocytogenes*. Control mice were untreated. Five mice per group were used.

Figure 3. Foci of *L. monocytogenes* infection in (*a*) the livers and (*b*) the spleens of infected control mice. By 24 h, foci of infection in the liver were indicated by large accumulations of neutrophils that occupied space originally occupied by parenchyma. At the peripheries of the microabscesses neutrophils are seen in close contact with infected hepatocytes (*arrows*) that are undergoing lysis. In the spleen, as in the liver, neutrophils were numerous at foci of infection. However, in contrast to the situation in the liver, intact infected cells (*arrows* and *insets*) are present in the centers of lesions in the spleen. This suggests that infected target cells in the spleen are less prone to destruction by neutrophils than are infected hepatocytes. Numbered insets show enlargements of cells indicated by arrows. Bar, 10 μm.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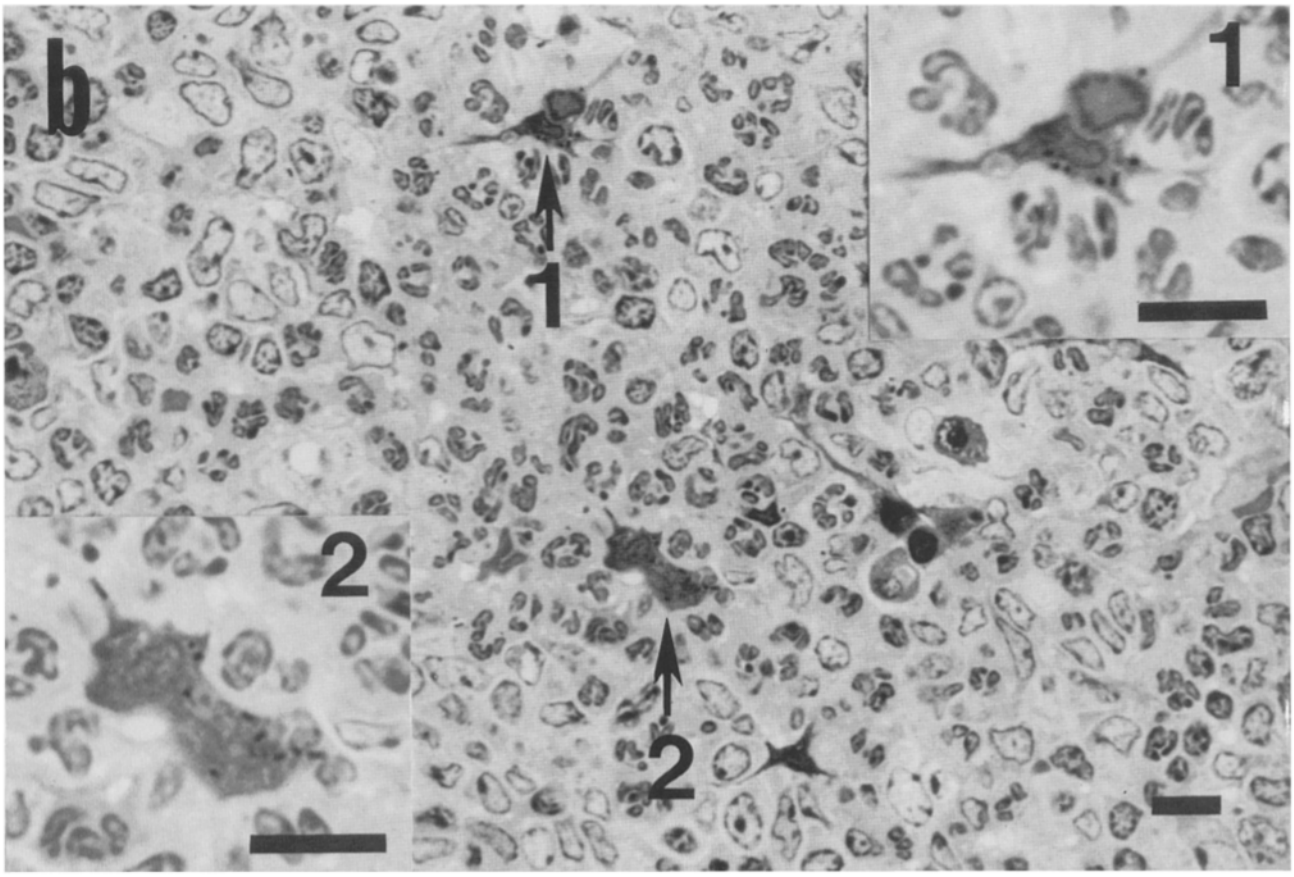
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(Fig. 4 *b*) and in the red pulp (not shown). The identity of the infected spleen cells was not determined by the present study, but many of them had the morphology and staining characteristics of recently described cells referred to as barrier cells (20), which are reticulum-like cells of uncertain function and origin, and usually present in the spleen in small numbers. Regardless of their identity, the histologic findings reported here suggest that target spleen cells in which *L. monocytogenes* resides are much less permissive for its growth than are hepatocytes. Thus, in the absence of neutrophils far more bacteria were found in individual hepatocytes than in individual spleen cells by 24 h of infection.

It is obvious from Fig. 3 *a* that in the livers of control mice, neutrophils that populate infectious loci occupy space once occupied by hepatocytes. This means that the hepatocytes that occupied the space must have been lysed. According to Fig. 4 *a* they would have been infected to one degree or another with *L. monocytogenes* before lysis occurred. Indeed, at the peripheries of liver lesions in control mice, neutrophils were found in close physical contact with infected hepatocytes that were in the process of being destroyed. In the spleens of control mice, as in the livers, infectious foci were also indicated by accumulations of neutrophils in the marginal zone and red pulp. Bacteria were found in these microabscesses inside intact fibroblast-like resident spleen cells (Fig. 4 *b*) and occasionally inside neutrophils. Although bacteria seen inside neutrophils might have come from initially infected spleen cells that had lysed, selective destruction of infected spleen cells in the vicinity of neutrophils was not obvious.

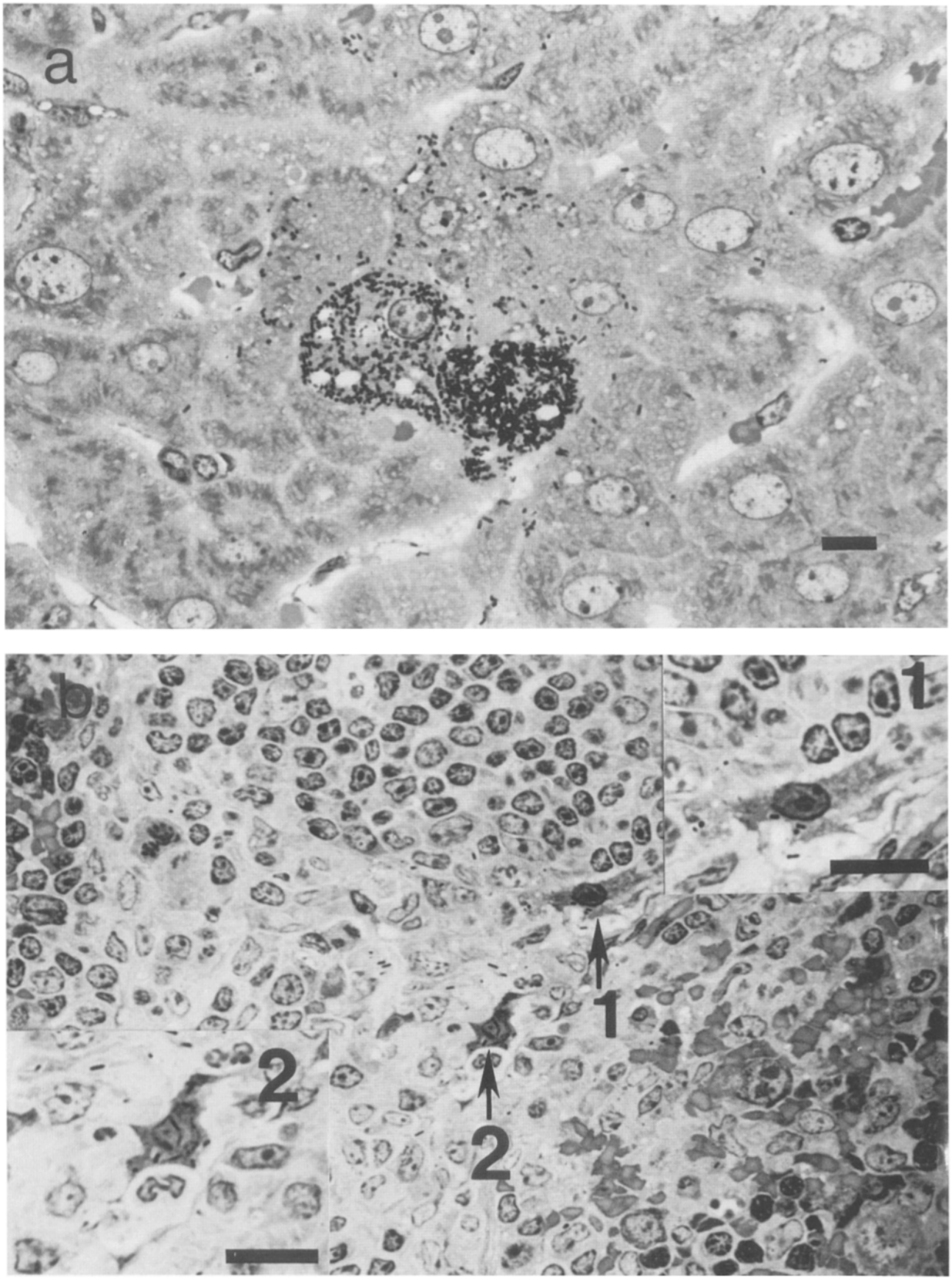
Effect of Treating Mice with mAb RB6-8C5 on Bacterial Growth in, and Neutrophil Recruitment to, the Peritoneal Cavity. Based on visual assessment of liver and spleen sections it seemed obvious that RB6-8C5 treatment depleted mice of neutrophils to the extent that few, if any, were available for recruitment to foci of infection. To demonstrate quantitatively the paucity of neutrophils available for recruitment to infectious foci an experiment was performed that measured the effects of RB6-8C5 pretreatment on the accumulation of neutrophils in the peritoneal cavities of mice inoculated intraperitoneally with *L. monocytogenes*. This experiment also examined the need for neutrophils to restrict early bacterial proliferation in the peritoneal cavity, as an example of infection in an extravascular compartment. To this end, the influx of inflammatory cells into the peritoneal cavities of control and RB6-8C5–treated mice was measured 12 and 24 h after intraperitoneal inoculation of 5×10^6 CFU of *L. monocytogenes*. Table 3 shows that in uninfected mice resident peritoneal leukocytes consisted mainly of macrophages and lym-

Table 3. *Effect of Treating Mice with mAb RB6-8C5 on Peritoneal Exudate Cell Composition and Bacterial Numbers after Intraperitoneal Inoculation of L. monocytogenes*

Mice	Time after inoculation	Peritoneal exudate cells				Log_{10} bacteria
		Total leukocytes	Lymphocytes	Macrophages	Neutrophils	
		$\times 10^4$				
Uninfected controls	–	337 ± 79	221 ± 55	107 ± 37	2 ± 0.24	–
Infected controls	12 h	586 ± 290	286 ± 117	96 ± 61	206 ± 108	4.21 ± 0.25
mAb-treated infected	12 h	389 ± 48	254 ± 43	113 ± 26	14 ± 67	4.58 ± 0.13
Infected controls	24 h	1,070 ± 230	304 ± 78	410 ± 89	344 ± 111	4.30 ± 0.14
mAb-treated infected	24 h	456 ± 240	264 ± 148	169 ± 75	12 ± 14.6	5.04 ± 0.37

RB6-8C5–treated mice received 0.25 mg of mAb intravenously 2 d and 4 h before intraperitoneal inoculation of 5×10^6 CFU *L. monocytogenes*. Control mice were untreated. Values shown are the mean ± SD using four mice per group.

Figure 4. Foci of *L. monocytogenes* infection in (*a*) the livers and (*b*) the spleens of mice depleted of neutrophils by treatment with mAb RB6-8C5. In the livers of neutrophil-depleted mice foci of infection consisted of groups of infected hepatocytes that appeared morphologically intact. Initially parasitized hepatocytes within infectious foci harbor large numbers of bacteria, whereas adjacent hepatocytes are less heavily infected. In the spleen, by contrast, *L. monocytogenes* did not grow to large numbers within individual infected cells. Many of the infected cells in the spleen had the morphological appearance of fibroblast-like barrier cells (*arrows* and *insets*). Numbered insets represent enlargements of cells indicated by arrows. Bar, 10 μm.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phocytes, and relatively few neutrophils. In infected control mice during the first 12 h after inoculation, there was a large influx of neutrophils into the peritoneal cavity, but no increase in the numbers of macrophages or lymphocytes. In the peritoneal cavities of RB6-8C5–treated mice, by contrast, by 12 h of infection there were 15-fold fewer recoverable neutrophils, representing an ~95% decrease in the number of these cells. However, at 12 h there was no decrease, in the number of macrophages or lymphocytes. Despite this selective decrease in the influx of neutrophils, there was little increase over control infected mice in the number of bacteria recovered from the peritoneal cavities of RB6-8C5–treated infected mice. This suggests that neutrophils were not necessary to restrict the growth of *L. monocytogenes* in the peritoneal cavity during the first 12 h.

At 24 h of infection large numbers of neutrophils were present in the peritoneal cavities of control infected mice, and there was a threefold increase in the numbers of macrophages. The number of lymphocytes, however, remained constant. By contrast, in RB6-8C5–treated mice at 24 h of infection, as at 12 h of infection, neutrophil numbers were low and there were approximately twofold fewer macrophages than in the peritoneal cavities of infected control mice. Because the antigen on neutrophils recognized by mAb RB6-8C5 reportedly is not expressed on macrophages, the observed decrease in the numbers of these cells in the peritoneal cavity in mAb-treated mice might indicate the abolition of a neutrophil-dependent macrophage recruitment mechanism.

As for the effect of RB6-8C5 treatment on infection in the peritoneal cavity, some fivefold more *Listeria* were recovered from the peritoneal cavities of treated mice than from control mice at 24 h of infection. Although substantial, this increase is small compared with the increase in bacterial numbers that occurs in the livers of RB6-8C5–treated mice. Indeed, there was little, if any, net bacterial growth between 12 and 24 h in the peritoneal cavities of infected control or infected mAb-treated mice. Moreover, in both cases >80% of the recoverable bacteria was pelleted by low speed (300 *g*) centrifugation suggesting that they were cell associated. In smears of peritoneal cells from RB6-8C5–treated mice <1% of macrophages were seen to contain *L. monocytogenes* bacteria, and then only in small numbers (<10 per macrophage). It is possible that these infected macrophages might be the natural reservoirs of *L. monocytogenes* infection in the peritoneal cavity, possibly because they are defective in their ability to kill this organism.

Discussion

According to the present study, treating mice with a granulocyte-specific mAb, RB6-8C5, abolishes a host defense mechanism that is critical for restricting the growth of *L. monocytogenes* in the liver during the first day of infection. According to the original description of mAb RB6-8C5 (15), it recognizes an antigen only present on mature eosinophils and neutrophils, and specifically depletes these granulocytes in vivo. The cytofluorimetric, hematological, and histological findings presented here support this claim by showing that FITC–RB6-8C5-stained neutrophils and eosinophils almost exclusively in a highly heterogeneous single cell suspension of spleen cells, and that RB6-8C5 treatment of mice selectively depleted them of blood neutrophils to numbers that were insufficient to subsequently populate foci of infection in the liver and spleen. That the surviving neutrophils in RB6-8C5–treated mice did not appear to focus to sites of liver or spleen infection might indicate a defect in their ability to do so, perhaps because of their having bound small amounts of mAb, or because they were RB6-8C5–negative immature cells. mAb RB6-8C5 was shown not to bind to T cells by the present study, a result not in agreement with the findings of others (17). The reason for this discrepancy is not obvious at this time, but might reflect differences in the reagents and staining methodologies used.

Although RB6-8C5 clearly binds to eosinophils, it is apparent that these cells are not found at foci of *L. monocytogenes* infection. It is reasonable to conclude, therefore, that the infection-enhancing action of RB6-8C5 in the liver is due to its ability to selectively deplete neutrophils thereby making them unavailable for recruitment to infectious foci. This strategy of specifically depleting mice of neutrophils shows in a more direct way than was previously shown with anti-CD11b–treated mice, the critical role that neutrophils play in defense against listeriosis of the liver. Although anti-CD11b mAbs prevented neutrophils from accumulating at infectious foci in the liver and caused exacerbation of infection in this organ, CD11b is not neutrophil-specific, but is also present on macrophages (3), certain T cells (21), and NK cells (22). However, given the evidence for the specificity of RB6-8C5 presented here, the results obtained with this mAb would appear to leave little doubt that neutrophils that accumulate at foci of infection in the liver, rather than other leukocytes, are responsible for early defense involving the lysis of infected hepatocytes. Moreover, it is clear from this and preceding studies (9, 11, 12) that neutrophils serve their protective function by destroying infected hepatocytes before *L. monocytogenes* has had the time to grow to large numbers within them.

According to other results presented here, most of the foregoing discussion does not apply to the function of neutrophils at other sites of *L. monocytogenes* infection. Thus, in contrast to the situation in the liver, treating mice with RB6-8C5 caused very little exacerbation of infection in the spleen, indicating that neutrophils are not as important in anti-listerial defense in this organ as in the liver during the first day of infection. This was surprising given the predominance of neutrophils at foci of infection in the spleen by 24 h after inoculation. Histological evidence indicates that neutrophils failed to substantially restrict *L. monocytogenes* multiplication in the spleen because the cells in which *Listeria* resides in this organ are less permissive for its growth than are hepatocytes. Even in the absence of neutrophils *L. monocytogenes* was not found in large numbers inside individual infected spleen cells. The evidence presented here is also consistent with the interpretation that infected target cells in the spleen are not lysed by neutrophils. The situation in the peritoneal cavity appears similar to that in the spleen, in that depleting mice of neutrophils did not result in severe exacerbation of infection ini-

tiated in this compartment. Whether infected peritoneal cells are normally lysed by neutrophils was not revealed by the present study. By contrast to these results, recent studies in our laboratory (unpublished data) showed that anti-CD11b mAbs failed to prevent recruitment of neutrophils to foci of *Listeria* infection in the spleen and peritoneal cavity, yet caused substantial exacerbation of infection in these two compartments during the first day. This failure of anti-CD11b mAbs to inhibit neutrophil recruitment into some tissues agrees with the findings of others (14, 23), and suggests that the infection-enhancing action of these mAbs in the spleen and peritoneal cavity is based on their ability to interfere with the antibacterial functions of phagocytes (24).

At the sites examined in the present study, neutrophil-mediated lysis of infected target cells as a strategy for controlling *L. monocytogenes* proliferation seemed to operate effectively only in the liver. However, this defense mechanism might be important at other sites not examined here. In this respect, the reported destruction of *Listeria*-infected enterocytes in areas of neutrophil accumulation in vivo after oral inoculation of bacteria into guinea pigs (4) is interesting given that enterocytes are very permissive for *L. monocytogenes* in vitro (6, 18).

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References

1. Hahn, H., and S.H.E. Kaufmann. 1981. The role of cell-mediated immunity in bacterial infections. *Rev. Infect. Dis.* 3:1221.
2. Mackaness, G.B. 1969. The influence of immunologically committed lymphoid cells on macrophage activity in vivo. *J. Exp. Med.* 129:973.
3. Rosen, H., S. Gordon, and R.J. North. 1989. Exacerbation of murine listeriosis by a monoclonal antibody specific for the type 3 complement receptor of myelomonocytic cells. *J. Exp. Med.* 170:27.
4. Racz, P., K. Tenner, and E. Mero. 1972. Experimental Listeria enteritis. I. An electron microscopic study of the epithelial phase in experimental listeria infection. *Lab. Invest.* 26:694.
5. Racz, P., K. Tenner, and K. Szivessy. 1970. Electron microscopic studies in experimental keratoconjunctivitis listeriosa. *Acta. Microbiol. Acad. Sci. Hung.* 17:221.
6. Gaillard, J.-L., P. Berche, J. Mournier, S. Richard, and P. Sansonetti. 1987. *In vitro* model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line Caco-2. *Infect. Immun.* 55:2822.
7. Havell, E.A. 1986. Synthesis and secretion of interferon by murine fibroblasts in response to intracellular *Listeria monocytogenes*. *Infect. Immun.* 54:787.
8. Portnoy, D.A., P.S. Jacks, and D.J. Hinrichs. 1988. Role of hemolysin for the intracellular growth of *Listeria monocytogenes*. *J. Exp. Med.* 167:1459.
9. Conlan, J.W., and R.J. North. 1991. Neutrophil-mediated dissolution of infected host cells as a defense strategy against a facultative intracellular bacterium. *J. Exp. Med.* 174:741.
10. Tilney, L.G., and D.A. Portnoy. 1989. Actin filaments and the growth, movement and spread of the intracellular bacterial parasite *Listeria monocytogenes*. *J. Cell Biol.* 109:1597.
11. Conlan, J.W., P.L. Dunn, and R.J. North. 1993. Leukocyte-mediated lysis of infected hepatocytes during listeriosis occurs in mice depleted of NK cells and $CD4^+$ $CD8^+$ $Thy1.2^+$ T cells. *Infect. Immun.* 61:2703.
12. Conlan, J.W., and R.J. North. 1992. Monoclonal antibody NIMP-R10 directed against the CD11b chain of the type three complement receptor can substitute for monoclonal antibody 5C6 to exacerbate listeriosis by preventing the focusing of myelomonocytic cells at infectious foci in the liver. *J. Leukocyte Biol.* 52:130.
13. Conlan, J.W., and R.J. North. 1992. 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. *Infect. Immun.* 60:5164.
14. Ferrante, A., R.F. Carter, A.F. Lopez, B. Rowan-Kelly, N.L. Hill, and M.A. Vadas. 1988. Depression of immunity to *Naegleria fowleri* in mice by selective depletion of neutrophils with a monoclonal antibody. *Infect. Immun.* 56:2286.
15. Tepper, R.I., R.L. Coffman, and P. Leder. 1992. An eosinophil-dependent mechanism for the anti-tumor effect of interleukin-4. *Science (Wash. DC).* 257:548.
16. Dunn, P.L., and R.J. North. 1991. Resolution of primary murine listeriosis and acquired resistance to lethal secondary infection can be mediated predominantly by $Thy1^+$ $CD4^-$ $CD8^-$ cells. *J. Infect. Dis.* 164:869.
17. Goossens, P.L., H. Jouin, and G. Milon. 1991. Dynamics of lymphocytes and inflammatory cells recruited in liver during murine listeriosis. *J. Immunol.* 147:3514.
18. Mournier, J., A. Ryter, M. Coquis-Rondon, and P. Sansonetti. 1990. Intracellular and cell-to-cell spread of *Listeria monocytogenes* involves interaction with F-actin in the enterocytelike cell line Caco-2. *Infect. Immun.* 58:1048.
19. Sanger, J.M., J.W. Sanger, and F.S. Southwick. 1992. Host

cell actin assembly is necessary and likely to provide the propulsive force for intracellular movement of *Listeria monocytogenes*. *Infect. Immun.* 60:3609.
20. Weiss, L. 1991. Barrier cells in the spleen. *Immunol. Today.* 12:24.
21. McFarland, H.I., S.R. Nahill, J.W. Maciaszek, and R.M. Welsh. 1992. CD11b (Mac-1): a marker for $CD8^+$ cytotoxic T cell activation and memory in virus infection. *J. Immunol.* 149:1326.
22. Holmberg, L.A., and K.A. Ault. 1984. Characterization of natural killer cells induced in peritoneal exudates of mice infected with *Listeria monocytogenes*. *Cell Immunol.* 89:151.
23. Winn, R.K., and J.M. Harlan. 1993. CD18-independent neutrophil and mononuclear leukocyte emigration into the peritoneum of rabbits. *J. Clin. Invest.* 92:1168.
24. Drevetts, D.A., B.P. Canono, and P.A. Campbell. 1992. Listericidal and nonlistericidal mouse macrophages differ in complement receptor type 3-mediated phagocytosis of *L. monocytogenes* and in preventing escape of the bacteria into the cytoplasm. *J. Leukocyte Biol.* 52:70.