Brief Definitive Report

Neutrophil-mediated Dissolution of Infected Host Cells as a Defense Strategy against a Facultative Intracellular Bacterium

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Summary

The rate of growth of *Listeria monocytogenes* in the livers of mice infected intravenously with a lethal or sublethal inoculum of this facultative intracellular bacterium is greatly increased if neutrophils and other host cells are prevented from accumulating at foci of infection during the first 24 h by treatment with a monoclonal antibody (5C6) specific for the type 3 complement receptor of myelomonocytic cells. A histological examination of the livers of control mice showed that the accumulation of neutrophils at infectious foci resulted in the focal destruction of infected hepatocytes. In contrast, failure of neutrophils to accumulate at these sites in 5C6-treated mice allowed *Listeria* to multiply extensively in hepatocytes without destroying them. The results indicate that neutrophils play an important role in early defense against listeriosis in the liver by destroying infected hepatocytes, thereby reducing the opportunity for *Listeria* to multiply in permissive cells. In this way, neutrophils serve to break the chain of cell-to-cell spread of infection.

The facultative intracellular bacterium, *Listeria monocytogenes*, is a human pathogen of underestimated importance. Acquired immunity to listeriosis in mice is viewed as a convincing model of cellular immunity to infection in which *Listeria*-specific T cells activate the microbicidal mechanisms of macrophages that must ingest and destroy *Listeria* (1). It has been generally believed that *Listeria* is an intramacrophage parasite that can survive and multiply in normal, but not in microbicidally activated macrophages. It has been shown recently, however, that *Listeria* can parasitize and multiply in a variety of mammalian cells in vitro (reviewed in reference 2), and that the cell that it parasitizes in the livers of mice is the hepatocyte (3). It is now known that *Listeria* is a well adapted intracellular pathogen, in that it can multiply extensively intracellularly before killing its host cell (4), and can take advantage of host cell mechanisms to transport it directly into the cytoplasm of neighboring host cells (5, 6). Therefore, it would be of survival value to the host to possess a mechanism capable of destroying *Listeria*-infected host cells at the beginning of infection. It is shown here that neutrophils appear to perform this defense function in the livers of *Listeria*-infected mice by selectively lysing infected hepatocytes during the first 24 h of infection.

Materials and Methods

Mice. B6D2F$_1$ (C57BL/6 × DBA/2) adult female mice (11–14 wk old) were obtained from the Trudeau Institute Animal Breeding Facility (Saranac Lake, NY). Mice were reared under barrier-free conditions and were free of common viral pathogens according to tests performed by the diagnostic testing services of Charles River Professional Services (Wilmington, MA).

Bacteria. A log-phase culture of *L. monocytogenes*, strain EGD, serotype 1/2a, was grown in Trypticase-soy broth, aliquoted in 1-ml volumes, and stored at −70°C. For each experiment a vial was thawed, washed once in saline, and diluted appropriately in the same for intravenous inoculation in a volume of 0.2 ml. The organism was enumerated in the liver 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.

Monoclonal Antibody. The 5C6 mAb specific for an epitope on the type 3 complement receptor of mouse myelomonocytic cells (7) was obtained from the 5C6 hybridoma growing as an ascites in CD2F$_1$ mice. The mAb was purified from ascites by a one-step chromatographic procedure using an Avidal affinity column (Bioprobe International Inc., Tustin, CA). The antibody was injected intravenously in a dose of 0.5 mg 1 h before infection.

Histology. Mice were killed by cervical dislocation, their livers removed whole, cut into small pieces, and fixed in 10% buffered formalin solution. The tissue was then dehydrated in ethanol and embedded in glycol methacrylate (JB-4 embedding kit; Polysciences, Inc., Warrington, PA). Sections 1–2 μm in thickness were cut with glass knives and stained with McNeals tetrachrome stain. Microscopy was performed with a Nikon MicroPhot-Fx microscope.

Results and Discussion

It has been shown (8, 9) that in *Listeria*-infected mice, foci of infection in the liver and spleen are populated by neutrophils during the first 24 h of infection, and by mononuclear phagocytes thereafter. Because in sublethal infection *Listeria* multiplies progressively during the first 24 h, and is progres-

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Volume 174 September 1991 741–744

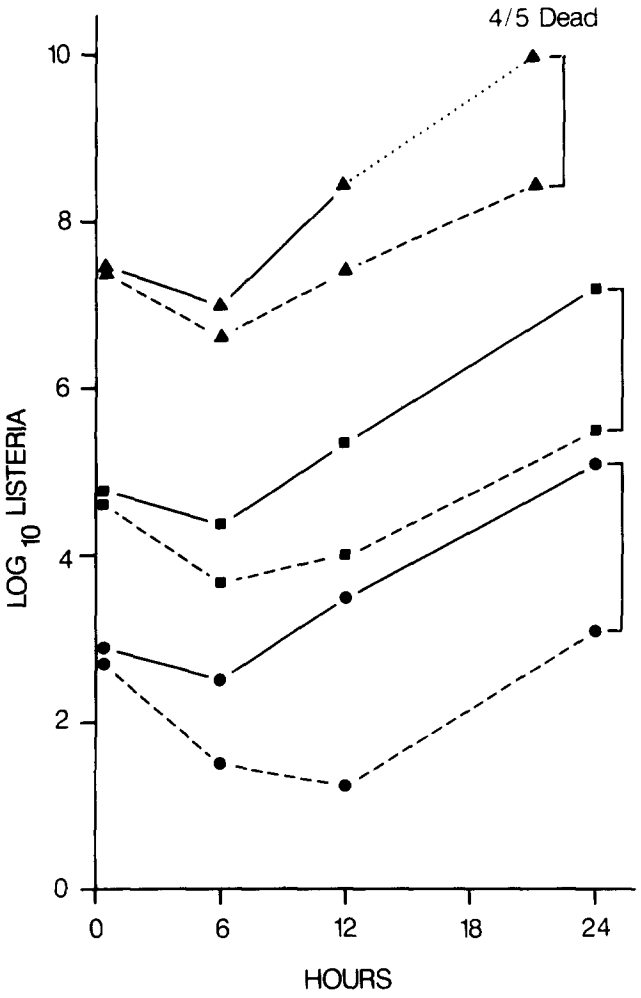

Figure 1. Evidence that intravenous injection of 0.5 mg of mAb 5C6 1 h before inoculation of 10^8 (▲), 10^5 (■), or 10^3 (●) *Listeria* resulted in each case in increased bacterial growth in the liver. (*Solid lines*) Growth in 5C6 treated animals; (*broken lines*) growth in control animals. Means of five mice per group. SEM < ± 0.3 $\log_{10}$.

sively destroyed after this time, it has been assumed that macrophages, but not neutrophils, are responsible for the control and resolution of infection. However, an important role for neutrophils in resistance to infection cannot be discounted until it is formally shown that bacterial growth does not increase in their absence. To test whether the absence of neutrophils at infectious foci in the liver at an early stage of infection results in increased bacterial growth in this organ, an experiment was performed that monitored *Listeria* growth in the livers of control mice and mice treated with a mAb (5C6) directed against the type 3 complement receptor of myelomonocytic cells (7). It was reasoned that, because treatment with 5C6 inhibits the accumulation of myelomonocytic cells at sites of inflammation (7), it should also prevent the accumulation of these cells at infectious foci. The results in Fig. 1 show that treating mice with 5C6 before inoculating them with *Listeria* resulted in 10 times more bacterial growth in their livers during the first 12 h of infection, and >50 times more bacterial growth at 24 h. This was the case, regardless of whether mice were inoculated with 10^8, 10^5 (lethal inocula), or 10^3 *Listeria*.

Evidence that the infection-enhancing effect of 5C6 treatment was caused by failure of neutrophils to accumulate at sites of bacterial multiplication was provided by a microscopic study of 1–2-μm sections of plastic-embedded livers of control and 5C6-treated mice. Selected photomicrographs are shown in Fig. 2, where it can be seen that foci of infection in the livers of control mice were heavily populated by neutrophils at 12 and 24 h, and that neutrophils occupied space that was originally occupied by hepatocytes. In other words, each focus of infection resulted in a focal accumulation of neutrophils that resulted, in turn, in focal destruction of hepatocytes. Each lesion was rimmed by intact noninfected and infected hepatocytes, and some of the latter cells appeared to be undergoing dissolution. It was apparent, moreover, that hepatocytes that were undergoing dissolution were being engaged by neutrophils, in that neutrophils were in close contact with them, and in some cases neutrophils had entered their cytoplasm. This focal dissolution of liver cells was evident as early as 6 h.

The situation in the livers of 5C6-treated mice was strikingly different, in that neutrophils did not accumulate at sites of infection. Instead, foci of infection were essentially devoid of inflammatory cells, and consisted of groups of infected hepatocytes that were morphologically intact, in spite of the fact that many of them contained a large number of *Listeria*, and that the liver as a whole contained 10–50-fold more organisms. Indeed, some hepatocytes, presumably those infected first, were literally packed with *Listeria*. This means that intracellular *Listeria* is not very toxic for hepatocytes, and that it can grow extensively in these cells and go on to infect neighboring cells without causing cell death. Indeed, according to this histological study, cell-to-cell spread in the livers of 5C6-treated mice was rapid and extensive during the first 24 h of infection, but morphological damage to the liver was not obvious.

This evidence for neutrophil-mediated lysis of infected hepatocytes is in keeping with evidence that neutrophils can lyse certain target cells in vitro and can be destructive of tissues in vivo (reviewed in reference 10). Neutrophils are enzymatically well equipped to perform these functions. Their plasma membrane possesses an NADPH oxidase system that generates a number of membrane-destructive oxygen metabolites. Moreover, the specific granules of neutrophils contain a variety of proteolytic enzymes capable of degrading dead cells and connective tissue components. Neutrophils can be activated to generate oxygen metabolites and to undergo degranulation in response to a variety of inflammatory stimuli, including IL-8 (11, 12) and other cytokines (13).

There is also evidence (14) that neutrophils can ingest and destroy *Listeria*. Therefore, their ability to destroy host cells harboring an organism that would otherwise remain inaccessible to ingestion by professional phagocytes, and remain free to multiply and directly infect adjacent host cells, is an important antibacterial defense strategy. Indeed, according to the results in Fig. 1, this neutrophil-mediated defense mech-

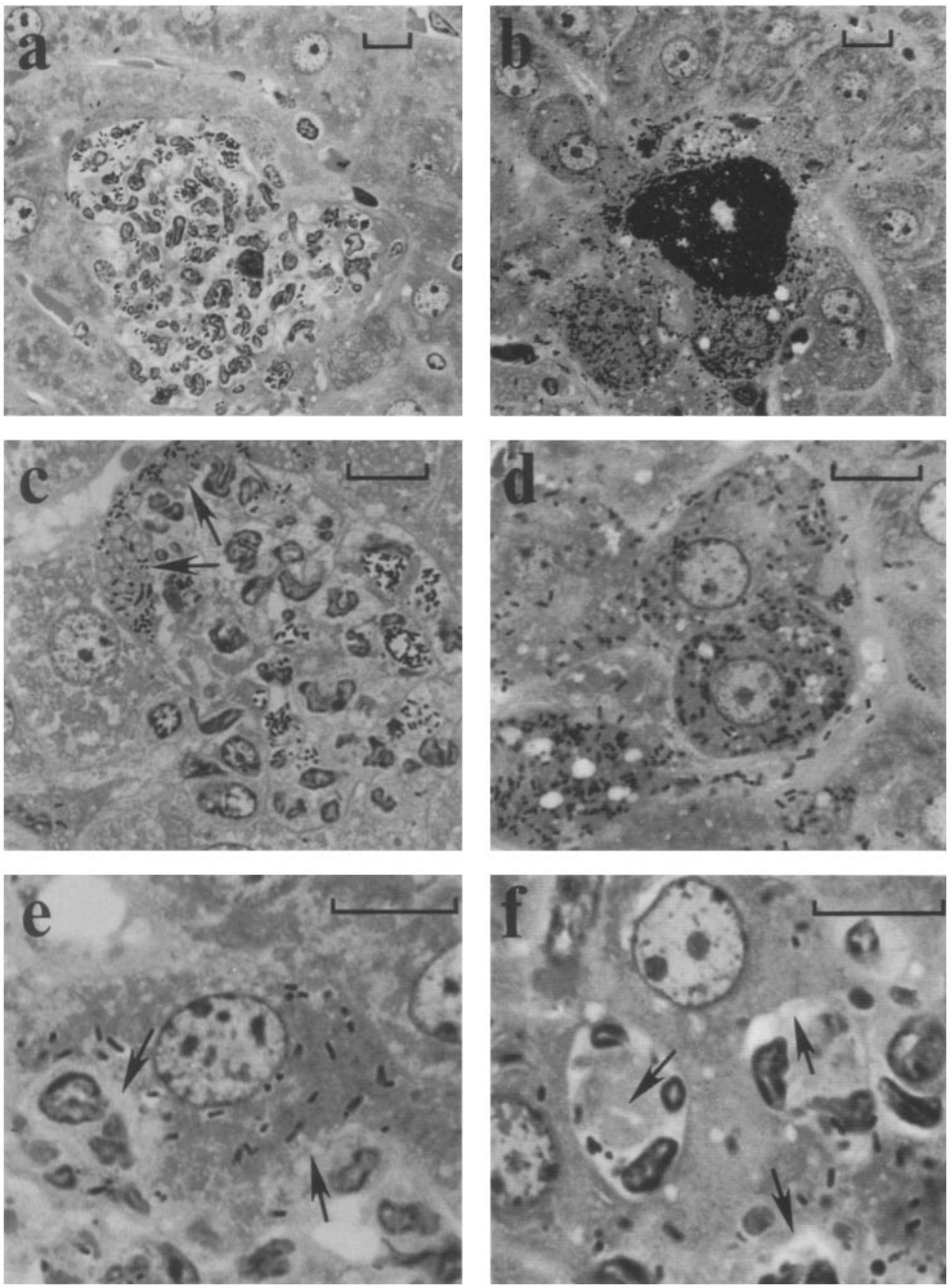

Figure 2. Photomicrographs of foci of infection in plastic-embedded sections of livers from control and 5C6-treated mice inoculated with 10^5 or 10^8 *Listeria* intravenously. (*a*) Infectious focus in the liver of a control mouse at 24 h of infection showing neutrophils occupying space originally occupied by hepatocytes and rimmed by intact hepatocytes. (*b*) Infectious focus in the liver of a 5C6-treated mouse at 24 h of infection showing heavily infected, but intact, hepatocytes with no involvement of neutrophils. (*c*) Focus of infection at 12 h showing hepatocytes undergoing dissolution (*arrows*). (*d*) Focus of infection at 12 h in 5C6-treated mice showing that infected hepatocytes appear not to be overtly damaged in the absence of neutrophils. (*e* and *f*) High-power micrographs of neutrophils apparently in the process of destroying hepatocytes (*arrows*) at the edge of a 12-h focus of infection. Bar, 10 μm.

anism is responsible for a >90% reduction in bacterial growth in the liver during the first 24 h of infection, regardless of the initial level of infection in this organ in the range of 10^3 to 10^8 organisms. One difference between infections resulting from inoculating 10^3 and 10^8 *Listeria* intravenously is that in the latter case there are 10^5 times more foci of infection. Therefore, the results suggest to us that within the first 24 h of infection the ability of neutrophils to reduce *Listeria* growth is the same at each focus of infection, regardless of the number of foci, and that failure to eliminate all *Listeria* at these sites is not the result of a shortage of neutrophils, but of limitations of this mechanism of defense. Presumably, it is the macrophage that makes up for this limitation from 24 h of infection on.

Lastly, the possibility that another cell, rather than the neutrophil, is responsible for destroying infected hepatocytes seems very unlikely for several reasons. First, neutrophils are enzymatically well equipped to perform this function, and appear to outnumber mononuclear cells at infectious foci by >100 to 1. Second, neutrophils were the only cells that were found within the cytoplasm of hepatocytes undergoing dissolution. Third, neutrophils are the only cells available in numbers large enough to heavily populate; in a matter of hours, as many as 5×10^7 foci of infection resulting from

inoculation of 10^8 organisms. Fourth, the destructive events under consideration were too early in infection to involve *Listeria*-specific T cells. NK cells may have been involved, although their cytolytic function remains essentially an in vitro phenomenon. It seems unlikely that neutrophil-mediated host cell lysis is a defense mechanism that exists exclusively to defend against listeriosis.

We thank Lynn Ryan, Ron LaCourse, Linda Schaefer, and Debra Duso for expert technical assistance, and Mary Durett for typing the manuscript.

This work was supported by an internal grant from the Trudeau Institute.

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Received for publication 25 May 1991.

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