INFECTION AND IMMUNITY, July 1993, p. 3068–3072
0019-9567/93/073068-05$02.00/0

Vol. 61, No. 7

Multiplication of *Listeria monocytogenes* in a Murine Hepatocyte Cell Line

STEVE WOOD, NANCY MAROUSHEK, AND CHARLES J. CZUPRYNSKI*

Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, Madison, Wisconsin 53706

Received 2 November 1992/Accepted 26 April 1993

***Listeria monocytogenes* was shown to invade and multiply in a murine hepatocyte cell line (ATCC TIB73). Hemolytic and nonhemolytic *L. monocytogenes* strains exhibited similar abilities to invade hepatocytes, but only hemolytic *L. monocytogenes* multiplied within this cell line. Microscopic evaluation of monolayers stained with Wright stain demonstrated focal necrosis (plaques) in the hepatocyte monolayers, with large numbers of intracellular listeriae visible within the hepatocytes that lined the margins of these plaques. Murine recombinant interleukin-1α, human recombinant tumor necrosis factor alpha, and murine recombinant gamma interferon did not affect the multiplication of *L. monocytogenes* in the hepatocytes. These data confirm in vivo observations of the intracellular multiplication of *L. monocytogenes* in hepatic lesions in infected mice.**

Murine listeriosis has been widely used as a model infection for studying cellular immunity. When injected intravenously into mice, the majority of the listeriae rapidly accumulate in the liver and to a lesser extent in the spleen (2, 24). Following an initial decline in the numbers of viable listeriae recovered from these organs, the surviving listeriae increase in number for 2 to 4 days and then decline as the host mobilizes a protective cellular immune response. Until recently, most had considered *Listeria monocytogenes* to be a pathogen of tissue macrophages, such as the Kupffer cells in the liver (17). Recently, several lines of investigation have indicated that considerable multiplication of *L. monocytogenes* occurs in the hepatocytes of experimentally infected mice (2, 10, 15, 25).

In the present study, we describe the ability of hemolytic *L. monocytogenes* to multiply in a murine hepatocyte cell line. To do this, we used a murine embryonic hepatocyte cell line obtained from the American Type Culture Collection (ATCC TIB73). The cells were cultured in Dulbecco modified Eagle tissue culture medium (DMEM) (Sigma) supplemented with 4 mM glutamine, 2 mM pyruvate, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 25 mM D-glucose, 26.7 mM sodium bicarbonate, 1 mg of gentamicin sulfate per liter, and 10% heat-inactivated fetal bovine serum (FBS) (Hyclone lot 11111026 and GIBCO lot 46K7319). Hepatocytes were added to wells at a density of 3×10^5 cells per ml in 24-well tissue culture plates and were incubated for 24 h at 37°C with 5% CO_2.

Two hemolytic strains of *L. monocytogenes* (EGD and 1WK91), a nonhemolytic mutant of 1WK91 (DPL-215), and a hemolytic revertant of DPL-215 (DPL 291) were generously provided by D. Portnoy, Philadelphia, Pa. (23), and stored as aliquots at −70°C. Before each experiment, the bacteria were thawed, washed 3 times in phosphate-buffered saline (PBS), and adjusted to a concentration of 10^5 bacteria per ml. Each hepatocyte monolayer was incubated with 1 ml of the bacterial suspension for 2 h at 37°C with CO_2. Following infection, the extracellular listeriae were removed by three washes with warm DMEM. The infected hepatocyte monolayers were then incubated for 24 h in DMEM with 10% FBS and 1 μg of gentamicin per ml. Preliminary data demonstrated that this concentration of gentamicin inhibited extracellular multiplication of the listeriae while allowing an approximately 100-fold increase in intracellular *L. monocytogenes* to occur in a 24-h incubation period (data not shown). Similar results were obtained with a gentamicin concentration of 5 μg/ml, whereas gentamicin concentrations of 25 and 50 μg/ml reduced the number of intracellular listeriae recovered by 0.78 $\log_{10}$ CFU and 1.17 $\log_{10}$ CFU, respectively. The latter observations probably reflect the fact that gentamicin is not totally excluded from entering cells in vitro (13) and that high extracellular concentrations of gentamicin may have resulted in an intracellular gentamicin concentration that was somewhat inhibitory to *L. monocytogenes* multiplication in the hepatocyte cytoplasm. When the effects of cytokines were to be evaluated, they were added to the hepatocyte monolayers before addition of the listeriae and remained present throughout the subsequent 24-h incubation period. Cytokines used included recombinant murine interleukin-1α (IL-1α) (Hoffmann-LaRoche, Nutley, N.J.), human tumor necrosis factor alpha (TNF-α) (Cetus, Emeryville, Calif.), gamma interferon (IFN-γ) (American Cancer Society, New York, N.Y.), and human IL-6 (Genetics Institute, Cambridge, Mass.). At appropriate time points, the hepatocytes were lysed with sterile deionized water, and the lysates were serially diluted in PBS. The dilutions were plated in duplicate on blood agar (BBL Microbiology Systems, Cockeyville, Md.) and incubated for 24 h at 37°C. Results were expressed as the mean ± standard error of the mean (SEM) $\log_{10}$ CFU of *L. monocytogenes* per well. Some hepatocyte monolayers were grown on sterile glass coverslips, infected with *L. monocytogenes*, and incubated as described above. At the end of the incubation period, the coverslips were removed, washed, fixed with methanol, and stained with a Wright stain. The coverslips were inverted, mounted on a clean glass slide with Permount (Fisher), examined microscopically, and photographed.

We first examined the ability of *L. monocytogenes* EGD to invade and multiply within the TIB73 murine hepatocyte cell line. Preliminary experiments indicated that a 2-h incubation of *L. monocytogenes* with hepatocyte monolayers resulted in approximately 15 to 20% of the bacterial inoculum becoming cell associated, as determined by lysis of the monolayers

* Corresponding author.

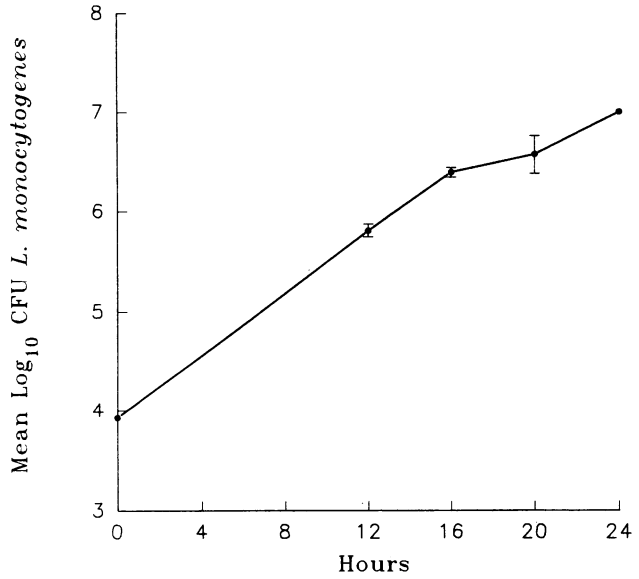

FIG. 1. Intracellular multiplication of *L. monocytogenes* EGD in mouse hepatocyte cell line ATCC TIB73. Hepatocyte monolayers were grown and infected as described in Materials and Methods. At the indicated time points, triplicate monolayers were washed and lysed with distilled H_2O, and the lysates were diluted in PBS and plated on blood agar. Results are expressed as the mean ± SEM $\log_{10}$ *L. monocytogenes* CFU from a representative experiment.

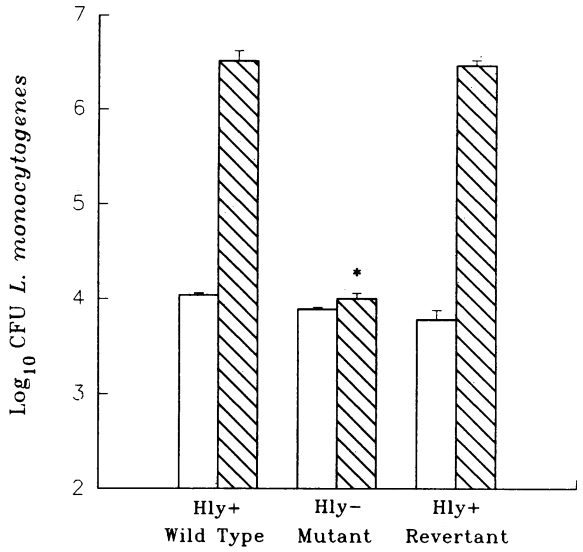

FIG. 2. Hemolytic *L. monocytogenes* multiply within a murine hepatocyte cell line, whereas nonhemolytic *L. monocytogenes* do not. Hepatocyte monolayers were infected with Hly^+ strain 1WK91, Hly^- strain DPL-215, and Hly^+ strain DPL-291 (a revertant of DPL-215) as indicated in Materials and Methods. Monolayers were lysed, and the lysates were plated on blood agar immediately after infection (□) and after the infected monolayers were incubated for 24 h at 37°C (▨). Results are the mean ± SEM $\log_{10}$ *L. monocytogenes* CFU per monolayer as determined in four separate experiments.

and plating on blood agar (data not shown). Because 1 μg of gentamicin per ml is microbiostatic rather microbicidal, it is likely that only a portion of these were internalized and that the remainder were adherent to the surface of the hepatocytes. The intracellular listeriae multiplied more than 100-fold in the infected monolayers when they were incubated for 24 h in DMEM that contained 1 μg of gentamicin per ml to inhibit extracellular listerial growth (Fig. 1). Similar results were observed at a gentamicin concentration of 5 μg/ml, which was microbicidal for *L. monocytogenes*.

Intracellular multiplication, but not invasion, required that the *L. monocytogenes* be hemolytic. Both strain EGD and a second hemolytic strain (1WK91) exhibited significant growth within hepatocytes compared with a nonhemolytic mutant (DPL-215) that invaded hepatocytes but failed to multiply ($P < 0.001$). (see Fig. 2). A hemolytic revertant (DPL-291) of the nonhemolytic mutant multiplied in hepatocytes to an extent similar to that of the parental hemolytic strain (Fig. 2). Infection of hepatocytes with hemolytic *L. monocytogenes* resulted in focal necrosis (plaques) (Fig. 3A). Cells adjacent to plaques contained large numbers of intracellular listeriae. In contrast, plaques did not form in monolayers infected with nonhemolytic *L. monocytogenes* (Fig. 3B) and far fewer intracellular listeriae were visible within hepatocytes. Hemolytic and nonhemolytic strains of *L. monocytogenes* grew equally well in DMEM and were inhibited by similar concentrations of gentamicin (data not shown), providing further evidence that this system detected intracellular growth of *L. monocytogenes*.

Various cytokines, including IL-1α (5, 14), IL-2 (11), TNF-α (15, 19, 24), and IFN-γ (1, 19), have been reported to increase the resistance of mice to experimental *L. monocytogenes* infection. We were interested in determining whether cytokines might exert their effects in part by restricting the intracellular multiplication of *L. monocytogenes* in hepatocytes. This was tested in vitro by incubating hepatocytes with graded amounts of cytokines before and during infection with *L. monocytogenes*. We observed no obvious difference in the initial number of intracellular listeriae, or in their ability to multiply, in hepatocytes incubated with murine recombinant IL-1α, human recombinant TNF-α, or murine recombinant IFN-γ compared with control hepatocytes in the same experiments (Table 1). Treatment of hepatocytes with recombinant human IL-6 decreased the intracellular multiplication of *L. monocytogenes*, but this effect was very modest (Table 1). This difference was not present at 12 to 16 h of incubation, suggesting that IL-6 caused a slight decrease (approximately twofold) in the total number of listeriae rather than a significant decrease in the rate of listerial multiplication.

The results of this study indicate that *L. monocytogenes* can invade and multiply within a murine hepatocyte cell line. This finding is consistent with recent reports that considerable multiplication of *L. monocytogenes* occurs in the hepatocytes of experimentally infected mice rather than in hepatic macrophages, as had been surmised previously (2, 10, 15, 25). Hepatic involvement in human listeriosis has also been reported (9). The in vitro study presented here confirms these in vivo observations and provides additional evidence that our thinking about the regulation of antilisteria resistance in the liver must recognize that listeriae are multiplying in hepatocytes. The bacterial multiplication and plaque formation that we observed in vitro are consistent with the histopathologic picture presented by the liver during the early stages of *L. monocytogenes* infection. One of the earliest lesions detected in experimentally infected mice is the formation of small zones of necrotic hepatocytes that soon become infiltrated with polymorphonuclear and mono-

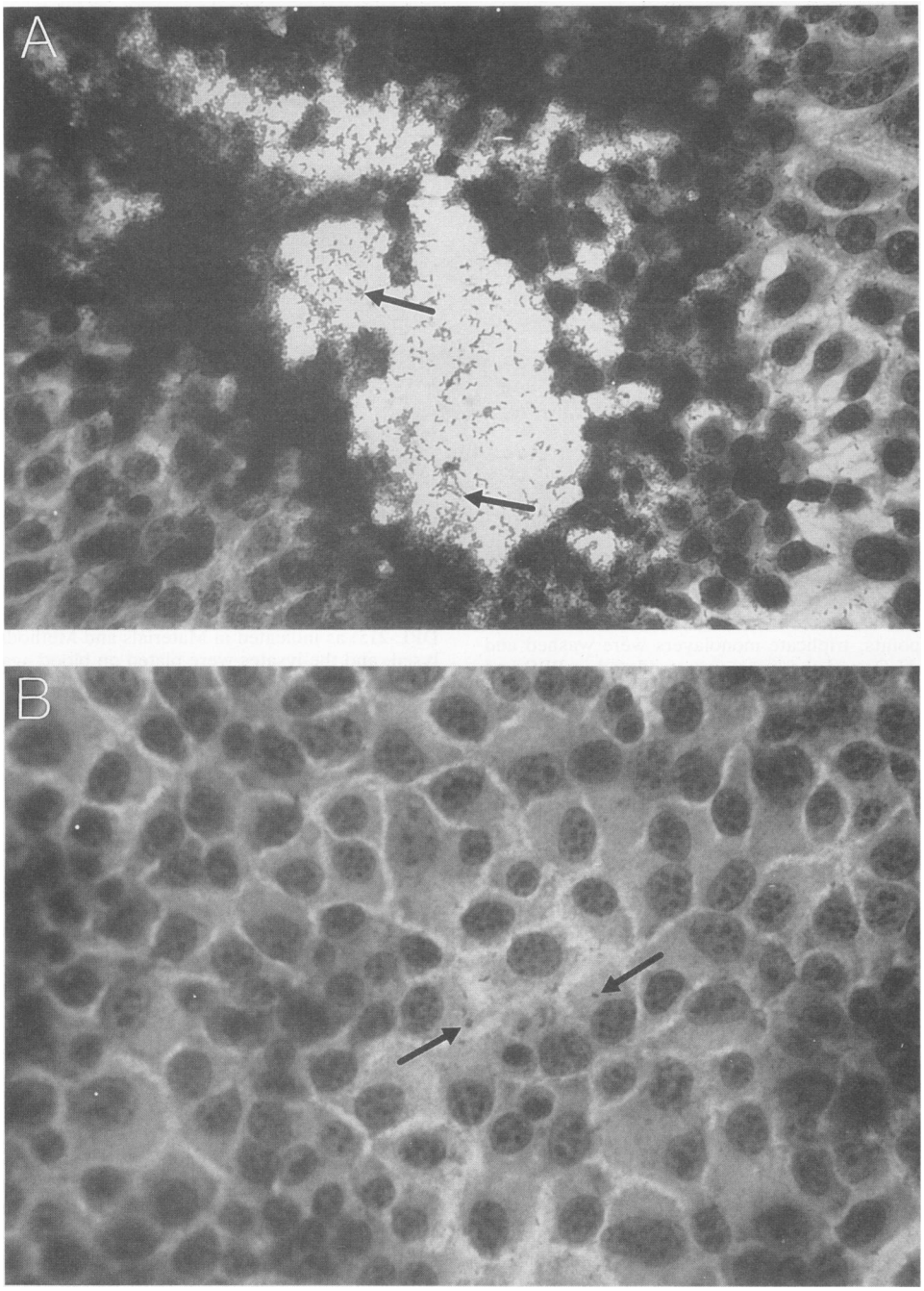

FIG. 3. Microscopic examination of hepatocyte monolayers infected with Hly^+ (A) and Hly^- (B) *L. monocytogenes*. The Hly^+ strain caused formation of plaques. The intensely staining cells adjacent to the plaques are packed with intracellular listeriae that are released as the hepatocytes lyse (arrows). In contrast, plaques did not form in monolayers infected with Hly^- *L. monocytogenes* and intracellular listeriae were infrequent (arrows). Wright stain; magnification, ×400.

nuclear cells (2, 15, 25). Both light and electron microscopic evaluation of these liver lesions has provided evidence of invasion and multiplication of *L. monocytogenes* within hepatocytes (2, 15, 25).

Using strains of *L. monocytogenes* provided by D. Portnoy (23), we noted that both hemolytic and nonhemolytic strains were taken up by hepatocytes but only hemolytic *L. monocytogenes* multiplied and caused plaques in the hepatocyte monolayer. This observation is consistent with recent information about the molecular pathogenesis of *L. monocytogenes* (reviewed in reference 22), which indicates that bacterial invasion involves a surface protein, internalin (7), escape from the membrane-bound vacuole and subsequent intracellular multiplication require the hemolysin (23), and movement through the cytoplasm and into adjacent cells require a protein (ActA) on the bacterial surface that activates formation of actin filaments (6). Although a previous report indicated that *L. monocytogenes* can bind to hepato-

TABLE 1. Effects of continuous treatment with recombinant human IL-6, human IL-1α, human TNF-α, and murine IFN-γ on the intracellular growth of *L. monocytogenes* in hepatocytes[a]

Cytokine (100 ng/ml)	No. of expts	Log_{10} CFU (mean ± SEM)	
		Control	With cytokine
IL-6	7	6.95 ± 0.14	6.41 ± 0.16[b]
IL-1α	3	6.75 ± 0.21	6.46 ± 0.15
TNF-α	2	6.58 ± 0.03	6.57 ± 0.02
IFN-γ	3	7.27 ± 0.18	7.23 ± 0.22

[a] All cytokines were added pre- and postinfection. Hepatocyte monolayers were incubated with 10^5 CFU of *L. monocytogenes* for 2 h at 37°C, washed 3 times with warm DMEM, and incubated in DMEM with 10% FCS and gentamicin (1 μg/ml) for 24 h at 37°C with 5% CO_2. Monolayers were washed 3 times with DMEM and lysed with distilled water, and the lysates were serially diluted and plated in duplicate on blood agar.

[b] Statistically significant decrease from control cultures as calculated by the Bonferroni modification of the Student *t* test ($P < 0.0065$).

carcinoma cells in vitro (3), the present study is the first to demonstrate invasion and intracellular multiplication in hepatocytes in vitro.

Several cytokines have been shown to increase the resistance of mice to *L. monocytogenes* in vivo (4, 11, 16, 24). The mechanisms by which these cytokines exert their beneficial effects have not yet been identified. We examined the possibility that several of these cytokines might directly reduce the multiplication of *L. monocytogenes* in hepatocytes. This proved not to be the case for IL-1α, TNF-α, and IFN-γ, all of which are known to increase antilisteria resistance in vivo (1, 4, 11, 14, 15, 19, 24). Other investigators have reported that IL-6 is a potent hepatocyte-activating agent in vivo and in vitro (8, 12, 21, 26). We therefore investigated what effect IL-6 might have on multiplication of *L. monocytogenes* in hepatocytes. Treatment of hepatocytes with IL-6 reduced the intracellular multiplication of *L. monocytogenes*; however, this effect was far too modest (approximately a twofold reduction) to account for the dramatic decrease in numbers of *L. monocytogenes* in the liver during the latter stage of infection. This conclusion is consistent with observations that high levels of IL-6 in plasma occur in mice unable to resist *L. monocytogenes* infection (2, 15, 20) and that administration of human recombinant IL-6 does not increase antilisteria resistance in vivo (5). Liu et al. recently reported, however, that high concentrations (20 to 50 μg per mouse) of murine recombinant IL-6 increased antilisteria resistance in vivo if given 4 h before experimental challenge (18).

In conclusion, the results of this study provide new evidence for the ability of *L. monocytogenes* to invade and multiply within hepatocytes. This confirms recent in vivo observations and provides a new model system for investigating the regulation of *L. monocytogenes* growth in a nonphagocytic cell line that is relevant to the pathogenesis of listeriosis.

This work was supported by funds from the U.S. Public Health Service (grants AI-21343 and BRSG RPO 5912) and the University of Wisconsin Graduate School.

We thank the School of Veterinary Medicine word processing personnel for preparing the manuscript.

REFERENCES

1. **Buchmeier, N. A., and R. D. Schreiber.** 1985. Requirement of endogenous interferon-γ production for resolution of *Listeria monocytogenes* infection. Proc. Natl. Acad. Sci. USA **82**:7404–7408.
2. **Conlan, J. C., 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**:741–744.
3. **Cowart, R. E., J. Lashmet, M. E. McIntosh, and T. J. Adams.** 1990. Adherence of a virulent strain of *Listeria monocytogenes* to the surface of a hepatocarcinoma cell line via lectin-substrate interaction. Arch. Microbiol. **153**:282–286.
4. **Czuprynski, C. J., and J. F. Brown.** 1987. Recombinant murine interleukin-1α enhancement of nonspecific antibacterial resistance. Infect. Immun. **55**:2061–2065.
5. **Czuprynski, C. J., M. Haak-Frendscho, N. Maroushek, and J. F. Brown.** 1992. Effects of recombinant human interleukin-6 alone, and in combination with recombinant interleukin-1α and tumor necrosis factor alpha, on antibacterial resistance in mice. Antimicrob. Agents Chemother. **36**:68–70.
6. **Dabiri, G. A., J. M. Sanger, D. A. Portnoy, and F. S. Southwick.** 1990. *Listeria monocytogenes* moves rapidly through the host-cell cytoplasm by inducing directional actin assembly. Proc. Natl. Acad. Sci. USA **87**:6068–6072.
7. **Gaillard, J. L., P. Berche, C. Frehel, E. Goolin, and P. Cossart.** 1991. Entry of *L. monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-positive cocci. Cell **65**:1127–1141.
8. **Gauldie, J., W. Northemann, and G. H. Fey.** 1990. Hepatocytes undergoing acute phase responses require exogenous IL-6. J. Immunol. **144**:3804–3808.
9. **Gebauer, K., J. C. Hall, J. B. Donlon, R. Herrmann, S. Rofe, and C. Platell.** 1989. Hepatic involvement in listeriosis. Aust. N.Z. J. Med. **19**:486–487.
10. **Gregory, S. H., L. K. Barczynski, and E. J. Wing.** 1992. Effector function of hepatocytes and Kupffer cells in the resolution of systemic bacterial infections. J. Leuk. Biol. **51**:421–424.
11. **Haak-Frendscho, M., K. M. Young, and C. J. Czuprynski.** 1989. Treatment of mice with human recombinant interleukin-2 augments resistance to the facultative intracellular pathogen *Listeria monocytogenes*. Infect. Immun. **57**:3014–3021.
12. **Hagiwara, T., H. Suzuki, I. Kono, H. Kashiwagi, Y. Akiyama, and K. Onozaki.** 1990. Regulation of fibronectin synthesis by interleukin-1 and interleukin-6 in rat hepatocytes. Am. J. Pathol. **136**:39–47.
13. **Hand, W. L., and N. L. King-Thompson.** 1986. Contrasts between phagocyte antibiotic uptake and subsequent intracellular bactericidal activity. Antimicrob. Agents Chemother. **29**:135–140.
14. **Havell, E. A., L. L. Moldawer, D. Helfgott, P. L. Kilian, and P. B. Sehgal.** 1992. Type I IL-1 receptor blockade exacerbates murine listeriosis. J. Immunol. **148**:1486–1492.
15. **Havell, E. A., and P. B. Sehgal.** 1991. Tumor necrosis factor-independent IL-6 production during murine listeriosis. J. Immunol. **146**:756–761.
16. **Kiderlen, A. F., S. H. E. Kaufman, and M. L. Lohmann-Mathes.** 1984. Protection of mice against the intracellular bacterium *Listeria monocytogenes* by recombinant immune interferon. Eur. J. Immunol. **14**:964–967.
17. **Lepay, D. A., R. M. Steinman, C. F. Nathan, H. W. Murray, and Z. A. Cohn.** 1985. Cell-mediated immunity is correlated with an influx of macrophages capable of generating reactive oxygen intermediates. J. Exp. Med. **161**:1503–1512.
18. **Liu, Z., R. J. Simpson, and C. Cheers.** 1992. Recombinant interleukin-6 protects mice against experimental bacterial infection. Infect. Immun. **60**:4402–4406.
19. **Nakane, A., T. Minagawa, M. Kohanawa, Y. Chen, H. Sato, M. Moriyama, and N. Tsuruoka.** 1989. Interactions between endogenous gamma interferon and tumor necrosis factor in host resistance against primary and secondary *Listeria monocytogenes* infections. Infect. Immun. **57**:3331–3337.
20. **Nakane, A., A. Numata, and T. Minagawa.** 1992. Endogenous tumor necrosis factor, interleukin-6, and gamma interferon levels during *Listeria monocytogenes* infection in mice. Infect. Immun. **60**:523–528.
21. **Nussler, A., J. C. Drapier, L. Remin, S. Pied, F. Miltgen, M. Gentilini, and D. Mazier.** 1991. Arginine-dependent destruction of intrahepatic malaria parasites in response to tumor necrosis factor and/or interleukin-6 stimulation. Eur. J. Immunol. **21**:227.

22. **Portnoy, D. A., T. Chakraborty, W. Goebel, and P. Cossart.** 1992. Molecular determinants of *Listeria monocytogenes* pathogenesis. Infect. Immun. **60:**1263–1267.
23. **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:**1459–1471.
24. **Roll, J. T., K. M. Young, R. S. Kurtz, and C. J. Czuprynski.** 1990. Human rTNFα augments anti-bacterial resistance in mice: potentiation of its effects by recombinant human rIL-1α. Immunology **69:**316–322.
25. **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. Absence of monocytes at infective foci allows *Listeria* to multiply in non-phagocytic cells. J. Exp. Med. **170:**27–37.
26. **Zuraw, B. L., and M. Lotz.** 1990. Regulation of the hepatic synthesis of C1 inhibitor by the hepatocyte stimulating factors interleukin 6 and interferon γ. J. Biol. Chem. **265:**12664–12670.