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Restricted Replication of *Listeria monocytogenes* in a Gamma Interferon-Activated Murine Hepatocyte Line

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The intracellular pathogen *Listeria monocytogenes* replicates mainly in resting macrophages and hepatocytes residing in infected tissues. Both innate and acquired resistance strongly depend on activation of listericidal capacities of macrophages by gamma interferon (IFN-γ) produced by natural killer cells and T lymphocytes. In contrast to macrophages, hepatocytes have been considered to serve purely as a cellular habitat, prolonging survival of the pathogen in the host. By using an immortalized murine hepatocyte line, the relationship between *L. monocytogenes* and this cell type has been analyzed in more detail. Our data reveal that hepatocytes are able to eradicate listeriolysin-deficient (avirulent) *L. monocytogenes* but fail to control growth of listeriolysin-expressing (virulent) *L. monocytogenes* organisms. Following stimulation with IFN-γ, hepatocytes gained the capacity to restrict growth of virulent *L. monocytogenes*, although less efficiently than the highly listericidal IFN-γ-activated macrophages. Hepatocytes costimulated with a combination of IFN-γ, interleukin 6 (IL-6), and tumor necrosis factor alpha (TNF-α) expressed the highest antilisterial activities. Although IFN-γ-stimulated hepatocytes produced demonstrable levels of reactive nitrogen intermediates (RNI), the results of inhibition studies do not support a major role for these molecules in antilisterial hepatocyte activities. In contrast, inhibition of RNI produced by macrophages neutralized their antilisterial effects. IFN-γ-stimulated, *L. monocytogenes*-infected hepatocytes expressed TNF-α mRNA, suggesting that they are a source of this cytokine during listeriosis. These studies suggest a novel function for hepatocytes in listeriosis: first, IFN-γ-stimulated hepatocytes could contribute to listerial growth restriction in the liver, and second, through secretion of proinflammatory cytokines, they could promote phagocyte influx to the site of listerial growth.

Facultative intracellular bacteria including *Listeria monocytogenes* exploit resting mononuclear phagocytes as a cellular habitat to promote their survival and replication in the infected host (34). The sulfhydryl-activated cytolysin, listeriolysin, which renders *L. monocytogenes* hemolytic, has been identified as a major virulence factor which promotes egression from the phagosome into the cytoplasm (18, 33, 46). Aquired resistance to *L. monocytogenes* is mediated by T lymphocytes (34, 41). Cytokines are central to host immunity against listeriosis (34). Although the macrophage-activating cytokine (gamma interferon [IFN-γ]) is essential for protection, other cytokines such as tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), and IL-1 also participate (5, 9, 27, 37, 39). Activation of murine macrophages by IFN-γ causes production of reactive nitrogen intermediates (RNI), which are highly toxic to various intracellular pathogens, including *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Leishmania major*, and *Toxoplasma gondii*, in vitro (1, 6, 11, 16, 22). The relative contribution of RNI to antilisterial defense, however, remains unclear.

Intracellular pathogens exploit not only resting macrophages but also nonprofessional phagocytes as an important niche to elude host defense mechanisms (48). For example, *L. monocytogenes* infects both Kupffer cells and hepatocytes of the liver (26). It was first assumed that antimicrobial activities cannot be activated in hepatocytes. Therefore, hepatocytes were viewed as host cells exclusively serving as shelters which protect listeriae from host attack, thereby promoting their survival. More recent observations that hepatocytes produce RNI after appropriate stimulation have challenged the assumption that they are not purely pathogen-permissive host cells and indicate that they could also participate in antilisterial resistance in the liver (8, 44).

To investigate the potential contribution of hepatocytes to antilisterial defense, we studied the survival of *L. monocytogenes* strains of differing virulence in a murine hepatocyte line in vitro. It was found that (i) only virulent *L. monocytogenes* strains grow inside resting hepatocytes, whereas avirulent strains are eliminated effectively; (ii) cytokine stimulation induces antilisterial activities in hepatocytes, with IFN-γ being the most potent cytokine; (iii) antilisterial capacities of cytokine-stimulated hepatocytes are less pronounced than those of activated macrophages; (iv) although hepatocytes produce RNI after cytokine stimulation, the contribution of their listerial growth inhibition properties appears minimal. We conclude that hepatocytes are not merely a cellular shelter for *L. monocytogenes* but contribute to antilisterial defense in the liver once they are properly activated by cytokines.

MATERIALS AND METHODS

Microorganisms. The following *L. monocytogenes* strains were used: (i) the hemolytic strain EGD; (ii) the ahemolytic mutant M3, with the transposon *916* (Tn*916*) insert in the promoter region of the listeriolysin gene; and (iii) the ahemolytic strain SLCC53 (ATCC 43250) with a deletion in the regulatory gene *prfA* (38). Bacteria were grown in tryptic soy broth (Difco), and samples were frozen at −70°C until used.

Cell lines and cell culture. The mouse liver cell line ATCC TIB 75 (*H-2*^{d}) was characterized as a hepatocyte line by morphology and microfluorometric analysis. The TIB 75 cell line was propagated in Dulbecco's modified Eagle medium (DMEM) (GIBCO) with 10% fetal calf serum (FCS). Bone marrow-derived macrophages (BMMϕ) from the femora of 8- to 12-week-old C57BL/6 mice (*H-2*^{b}) and BALB/c mice (*H-2*^{d}) were cultured in DMEM supplemented with 10% FCS, 5% horse serum, and 30% conditioned medium from L929 cells at 37°C in 10% CO_2 for 9 days as described previously (17). For microfluorometric analysis, BMMϕ and TIB 75 cells were harvested and stained with anti-mouse macrophage monoclonal antibody (MAb) F4/80 (American Type Culture Collection, Rockville, Md.) conjugated with fluorescein isothiocyanate for 30 min at

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4°C in phosphate-buffered saline (PBS) containing 0.1% sodium azide. Fc receptors of cells were blocked with an anti-mouse Fc-receptor MAb (2.4G2; American Type Culture Collection). Control cells remained unstained. Cells were analyzed with a FACScan (Becton Dickinson) at 10,000 cells per sample.

Cytokines and reagents. Murine recombinant IFN-γ (rIFN-γ) was kindly provided by G. Adolf, Ernst Boehringer Institut für Arzneimittel-Forschung, Vienna, Austria; murine rTNF-α was a gift from F. H. Hillen, BASF, Ludwigshafen, Germany; human rIL-6 was kindly provided by L. Aarden, CLB, Amsterdam, The Netherlands. L-Arginine-depleted RPMI 1640 medium was prepared by using an RPMI 1640 select amin kit (GIBCO). N^G-monomethyl-L-arginine (NMLA) was purchased from Calbiochem (La Jolla, Calif.). Catalase, superoxide dismutase, mannitol, L-histidine, and lucigenin were obtained from Sigma Chemical Co., St. Louis, Mo.

Intracellular growth in vitro. Hepatocytes (2×10^4 per well) or BMMϕ (5×10^4 per well) were seeded into flat-bottom microdilution plates in DMEM supplemented with 10% FCS, without antibiotics. The cells were infected with different *L. monocytogenes* strains at an infection rate of 20:1. At 60 min after infection of hepatocytes or 30 min after infection of BMMϕ, gentamicin sulfate (10 μg/ml) was added to kill extracellular bacteria. Cells were lysed by treatment with 0.1% saponin dissolved in PBS at the time points indicated in the figures. Bacteria from six wells were pooled, serial dilutions were plated onto tryptic soy plates (Difco), and the numbers of CFU were determined. The intracellular growth of *L. monocytogenes* was not affected by 5 or 10 μg of gentamicin per ml. But at 5 μg/ml extracellular growth of listeriae was incompletely inhibited. Therefore, 10 μg/ml was used in all experiments discussed here.

Inhibition of listerial growth by hepatocytes. Listerial growth inhibition was assessed by seeding hepatocytes (2×10^4 cells per well) into flat-bottom microdilution plates in DMEM supplemented with 10% FCS, without antibiotics; this was followed by stimulation with rIL-6, rTNF-α, rIFN-γ, and all possible combinations. After 24 h, the medium was removed and hepatocytes were infected with 10^6 live *L. monocytogenes* EGD cells per well. After 1 h of incubation, gentamicin sulfate was added. At 2 and 24 h after infection, supernatants were removed, cells were lysed as described above, and the numbers of CFU were determined. NMLA as an RNI scavenger or catalase, superoxide dismutase, mannitol, and L-histidine as scavengers for reactive oxygen intermediates (ROI) were added 3 h before infection.

Nitrite determination. The NO_2^- concentrations in cell cultures were measured with the Griess reagent (13, 24). Briefly, 50 μl of supernatant was mixed with 50 μl of Griess reagent [1% sulfanilamide, 0.1% (naphthyl)ethylenediamine dihydrochloride, 2.5% H_3PO_4] and incubated for 10 min at room temperature. As a standard for NO_2^- production, $NaNO_2$ was dissolved in DMEM and the A_{541} was measured in an Immunoreader NJ 2000 (Intermed). Griess reagent detects accumulated NO_2^- in a range of 1 to 300 μM. Production is presented as nanomoles per 10^5 cells.

Determination of superoxide anion production. Production of superoxide anions was measured by lucigenin-dependent chemiluminiscence (CL) with a MicroLumat (Berthold, Wildbad, Germany) as described previously (15). Briefly, hepatocytes were cultured and stimulated as described above. Before infection, medium was removed and replaced by HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered RPMI 1640 medium (Biochrom, Berlin, Germany) containing 250 μM lucigenin, and background CL was recorded. After 2 min, *L. monocytogenes* (infection ratio, 20 bacteria per cell) was added, and counts per minute of CL was determined up to 1 h at 1-min intervals at a constant temperature of 37°C. For positive controls, BMMϕ were treated with zymosan and CL was measured.

PCR analysis. The following primer sequences for IL-6, TNF-α, inducible NO synthase (iNOS), and β-actin were used in the PCR: IL-6, 456–4GACAAAGC CAGAGTCCTTCAGAG (sense); IL-6, 684–6CTAGGTTTGCCGAGTAGGG GATCTC (antisense); TNF-α, 820–8GGCAGGTCTACTTTGGAGTCATTGC (sense); TNF-α, 1127–11ACATTCGAGGCTCCAGTGAATTCGG (antisense); iNOS, 256–2ATGGCTTGCCCCTGGAAGGGGGTTTC (sense); iNOS, 595–5GGACTTGCAAGTGAAATCCGATG (antisense); β-actin, 886–9 TGGAAT CCTGTGGCATCCATGAAAC (sense); β-actin, 1234–12TAAAACGCAGCT CAGTAACAGTCCG (antisense).

Total cellular RNAs from hepatocytes were extracted by a rapid protocol. Cells were resuspended in 500 μl of lysis buffer (4 M guanidine thiocyanate, 0.5% *N*-lauroylsarcosine, 25 mM trisodium citrate-2-hydrate, 100 mM 2-mercaptoethanol). Subsequently, 33 μl of 3 M sodium acetate (pH 4.8), 500 μl of phenol, and 100 μl of chloroform were added. The aqueous phase was isopropanol precipitated at −20°C overnight. The RNA pellet was totally reverse transcribed (4 μl of 5× reaction buffer, 2 μl of 0.1 M dithiothreitol, 0.5 μl of 25 mM deoxynucleotide triphosphates, 1 μl of Superscript-RT [GIBCO/BRL], and water were added to a final volume of 20 μl) using 1 μl of oligo(dT)12–18 (500 ng/μl; Pharmacia). A standard PCR reaction mixture (50 μl) contained 5 μl of 10× GIBCO reaction buffer, 0.5 μl of 25 mM deoxynucleotide triphosphates, 2 μl of cDNA, 1 μl of sense and antisense primer (500 ng/μl), 1 μl of 250 mM $MgCl_2$ (final concentration, 5 mM), and 0.5 μl of *Taq* DNA polymerase (5 U/μl; GIBCO/BRL). PCR was carried out in a Perkin-Elmer Cetus DNA thermal cycler for 34 cycles (96°C for 2 min, 54°C for 2 min, and 72°C for 2.5 min). Subsequently, 20 μl of the reaction mixture was fractionated by electrophoresis on a 1.5% agarose gel (0.5× Tris–boric acid–EDTA buffer [pH 8.3]), and the amplified DNA fragments were visualized by ethidium bromide staining and UV transillumination of gels.

RESULTS

Characterization of cell line TIB 75. The hepatocyte line (ATCC TIB 75) used originated from cell line TIB 73, which has been described as a hepatocyte line by others (49). The hepatocyte origin was verified by biochemical markers, morphology, and phenotype. TIB 75 cells produced urea and transferases as typical biochemical liver markers and showed hepatocyte morphology (Fig. 1A). Since two predominant populations in the liver are macrophages and hepatocytes, we stained TIB 75 cells and control BMMϕ with anti-mouse macrophage MAb F4/80. For cell line TIB 75, staining was negative, whereas control BMMϕ expressed this marker (Fig. 1B). On the basis of these findings we consider TIB 75 a hepatocyte line.

Invasion of and intracellular growth in hepatocytes by *L. monocytogenes*. In a first attempt to analyze the relationship between *L. monocytogenes* and hepatocytes in vitro, cells of the hepatocyte line TIB 75 were infected with hemolytic and ahemolytic strains of *L. monocytogenes*. Consistent with findings by Wood et al. (49) using the hepatocyte line TIB 73, these hepatocytes were parasitized by both strains of *L. monocytogenes*. Yet, only hemolytic, and not ahemolytic, *L. monocytogenes* strains were capable of replicating in hepatocytes (Fig. 2) (49). With respect to uptake and intracellular growth restriction of *L. monocytogenes*, hepatocytes were less efficient than BMMϕ of either the C57BL/6 or BALB/c mouse strain. The virulent *L. monocytogenes* strain grew in an almost unrestricted fashion in hepatocytes (2-log increase of CFU during 24 h), whereas in C57BL/6 or BALB/c BMMϕ bacterial numbers were only slightly increased. These data show that (i) *L. monocytogenes* is capable of parasitizing hepatocytes in vitro and (ii) its intracellular survival and replication in hepatocytes depend on listeriolysin. All subsequent experiments were performed with the listeriolysin-expressing virulent *L. monocytogenes* strain EGD.

Hepatocyte stimulation by cytokines. IL-6, TNF-α, and IFN-γ have been detected in livers of mice after *L. monocytogenes* infection, and all three cytokines are known to contribute to resistance of mice to listeriosis (5, 15, 27, 40, 43). We, therefore, were interested in the possible effects of these cytokines on the fate of *L. monocytogenes* within hepatocytes. To this end, hepatocytes were preincubated with 500 U of IL-6, TNF-α, IFN-γ, or a combination thereof per ml. The cytokine-containing medium was removed after 24 h, and hepatocytes were infected with *L. monocytogenes*. IL-6 and TNF-α alone had no or only marginal effects on intracellular bacterial growth, whereas the combination of both cytokines caused small but definite growth inhibition of *L. monocytogenes* (Table 1). In contrast, IFN-γ alone or combinations of the other cytokines with IFN-γ induced marked growth inhibition of *L. monocytogenes*. Comparison of IFN-γ-stimulated hepatocytes and BMMϕ revealed that listeriae failed to grow in stimulated BALB/c BMMϕ and were killed in C57BL/6 BMMϕ, whereas hepatocytes only reduced bacterial growth (Fig. 3). It could be argued that restriction of bacterial growth was caused by increased gentamicin pinocytosis of cytokine-stimulated hepatocytes (14). We consider such effects unlikely, in particular because we used at least fivefold-lower gentamicin concentration than was used in the studies in which such effects were observed (14).

NO_2^- production by hepatocytes. Previous studies have provided compelling evidence for a decisive role of NO_2^- in growth inhibition of numerous intracellular pathogens by mu-

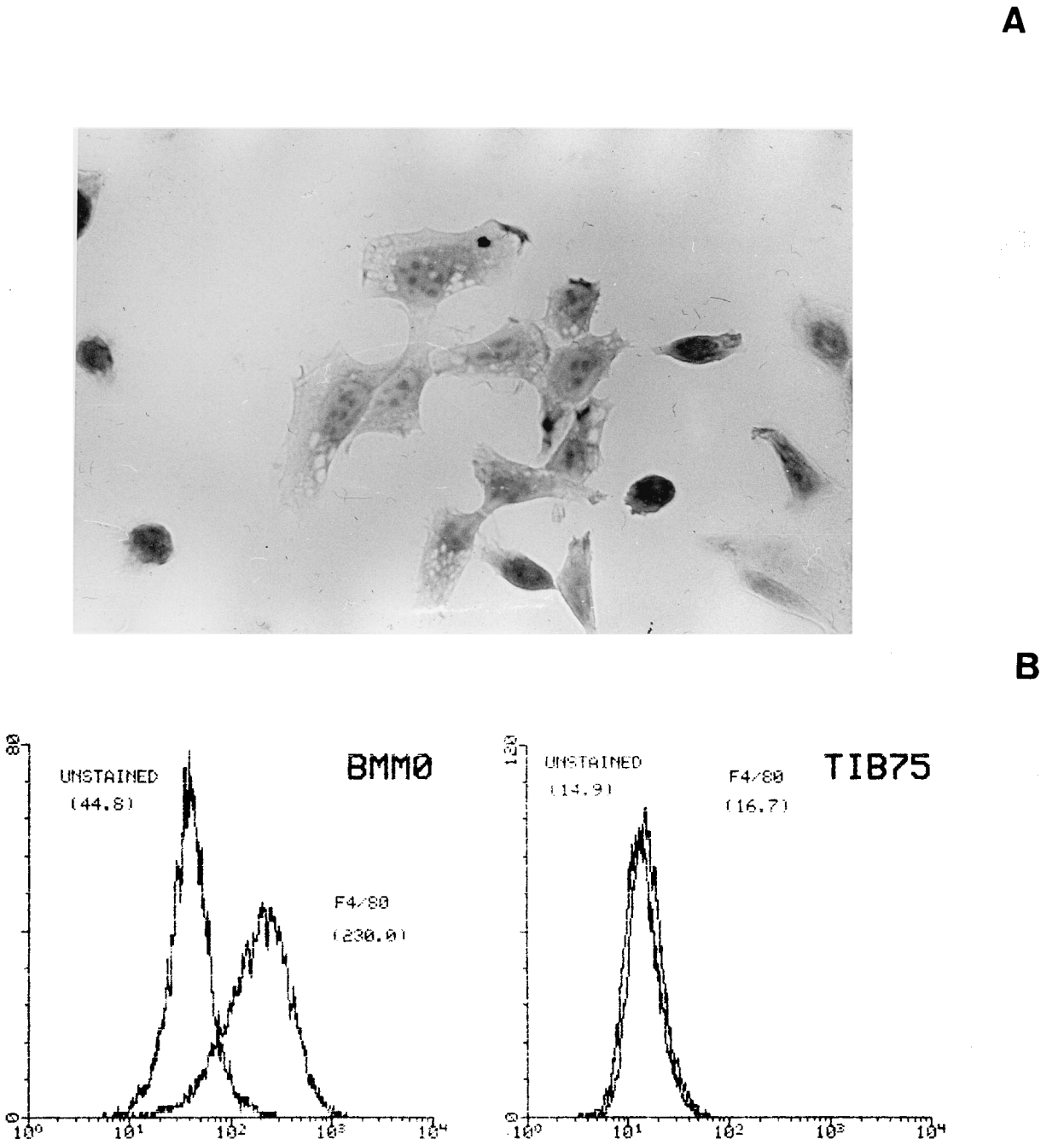

FIG. 1. Characterization of hepatocyte line TIB 75. (A) Morphological examination. Cells were stained with hematoxylin-eosin, and photomicrographs were taken with an Olympus photomicroscope. (B) Staining with anti-mouse macrophage MAb. BMMϕ and TIB 75 cells were stained with anti-mouse macrophage MAb F4/80 conjugated with fluorescein isothiocyanate or left unstained. Data are presented as cell numbers versus log fluorescence intensity.

rine macrophages (1, 6, 11, 14, 22). It was therefore decided to analyze NO_2^- production by hepatocytes after cytokine stimulation and infection with *L. monocytogenes*. As shown in Fig. 4, hepatocytes produced NO_2^- after stimulation with IFN-γ and *L. monocytogenes* infection. NO_2^- production was marginally increased by the combination of IFN-γ with either IL-6 or TNF-α or by costimulation with all three cytokines. Cytokine-stimulated C57BL/6 or BALB/c BMMϕ produced NO_2^- in a similar way but at higher concentrations. Importantly, concomitant IFN-γ stimulation and *L. monocytogenes* infection were required for NO_2^- production to occur, either stimulus alone being insufficient. To verify the dual requirement for cytokine stimulation and listerial infection in RNI production by hepatocytes, expression of iNOS mRNA was examined by PCR. Even with this highly sensitive system, iNOS mRNA expression was detected only in hepatocytes after both IFN-γ stimulation and *L. monocytogenes* infection (Fig. 5). We conclude that hepatocytes are capable of producing RNI and that both cytokine stimulation and intracellular infection are required.

Attempts to identify antilisterial effector mechanisms. RNI

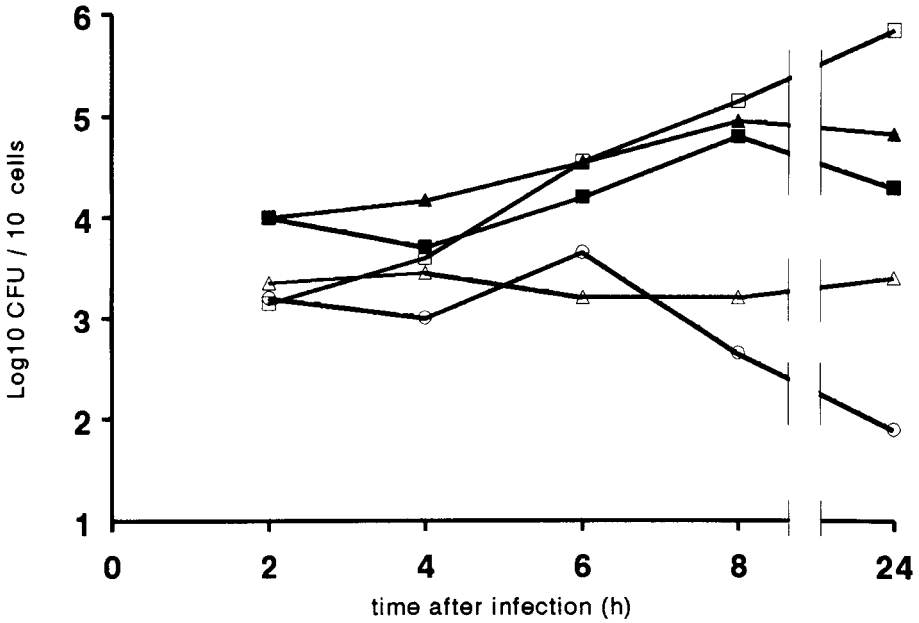

FIG. 2. Growth of hemolytic and ahemolytic *L. monocytogenes* strains in hepatocytes. Hepatocytes were infected with hemolytic strain EGD (□), ahemolytic Tn*916* mutant M3 (△), or ahemolytic deletion mutant SLCC53 (○) and compared with C57BL/6 BMMϕ (■) or BALB/c BMMϕ (▲) infected with EGD. Cells were lysed at the indicated time points, and numbers of CFU were determined by serial dilution. Experiments were performed three times with similar results.

are exclusively produced from L-arginine, and lack of arginine or addition of inhibitory L-arginine analogs interferes with NO_2^- production (8, 31). To assess the possible contribution of RNI to listerial growth inhibition by hepatocytes and BMMϕ, cells were stimulated with IFN-γ in medium containing L-arginine to guarantee normal protein synthesis during activation. The medium was then removed, and cells were infected with *L. monocytogenes* in L-arginine-deficient medium. Alternatively, hepatocytes or BMMϕ were infected with *L. monocytogenes* in L-arginine-containing medium in the presence of graded concentrations of the L-arginine inhibitor NMLA. NO_2^- production by hepatocytes as well as by BMMϕ was abolished under both conditions. While inhibition of listerial growth in hepatocytes was only partially reversed in the presence of NMLA and not at all affected by the lack of L-arginine, the absence of arginine or presence of NMLA in BMMϕ cultures facilitated growth of *L. monocytogenes* (Fig. 6; Table 2). In arginine-free C57BL/6 BMMϕ cultures, the remaining low level of nitrite could result from an endogeneous pool of arginine. Apparently this had no influence on listerial growth. The possible participation of ROI in antilisterial defense in hepatocytes was assessed by measuring O_2^- production with CL. We failed to detect any O_2^- production in stimulated, infected hepatocytes (data not shown). Furthermore, addition of catalase, superoxide dismutase, mannitol, or histidine, employed as ROI scavengers, did not affect growth inhibition of *L. monocytogenes* by cytokine-stimulated hepatocytes (data not shown). These findings indicate that different antibacterial effector mechanisms are operative in BMMϕ and hepatocytes. While BMMϕ inhibit listerial growth primarily by producing RNI, hepatocytes seem to use RNI- and ROI-independent mechanisms.

TABLE 1. Growth inhibition of hemolytic *L. monocytogenes* EGD[a]

Cytokine	CFU/10^5 hepatocytes	% Growth inhibition
None	1.7×10^6	
IL-6	2.0×10^6	−17.6
TNF-α	1.6×10^6	11.7
IFN-γ	6.0×10^5	64.7
IL-6 + TNF	9.2×10^5	45.2
IL-6 + IFN-γ	6.5×10^5	61.7
TNF-α + IFN-γ	3.3×10^5	77.0
IL-6 + TNF-α + IFN-γ	2.2×10^5	87.0

[a] Hepatocytes were preincubated with cytokines (500 U/ml) for 24 h and infected at an infection rate of 20:1, and numbers of CFU were determined after 2 and 24 h. After 2 h, numbers of CFU were similar in all hepatocytes (between log 10 3.5 and 3.8). Experiments were performed three times with similar results.

Cytokine mRNA induction in hepatocytes. Previous findings have revealed expression of IL-1, IL-6, and TNF-α mRNA in livers of *L. monocytogenes*-infected mice, and these cytokines have been implicated in antilisterial defense (9, 15, 27, 40). We, therefore assessed whether expression of these cytokines is restricted to macrophages or also occurs in hepatocytes. To this end, hepatocytes were stimulated with IFN-γ and infected with *L. monocytogenes*. Subsequently mRNA transcripts of IL-6 and TNF-α were analyzed by PCR. Although none of these mRNAs were detected in resting hepatocytes, transcripts of IL-6 mRNA were inconsistently seen, whereas those of TNF-α were clearly demonstrable after IFN-γ stimulation plus *L. monocytogenes* infection. Thus, IFN-γ pretreatment and infection were required for upregulation of either iNOS mRNA or TNF-α mRNA. These findings suggest autocrine production of TNF-α by IFN-γ-stimulated and *L. monocytogenes*-infected hepatocytes. TNF-α could act (i) as a proinflammatory cytokine promoting phagocyte attraction and (ii) as a macrophage and hepatocyte costimulator at the site of listerial growth in the liver (Fig. 7).

DISCUSSION

L. monocytogenes is an intracellular bacterium capable of replicating in resting mononuclear phagocytes (34). Acquired resistance against *L. monocytogenes* is mediated by specific T

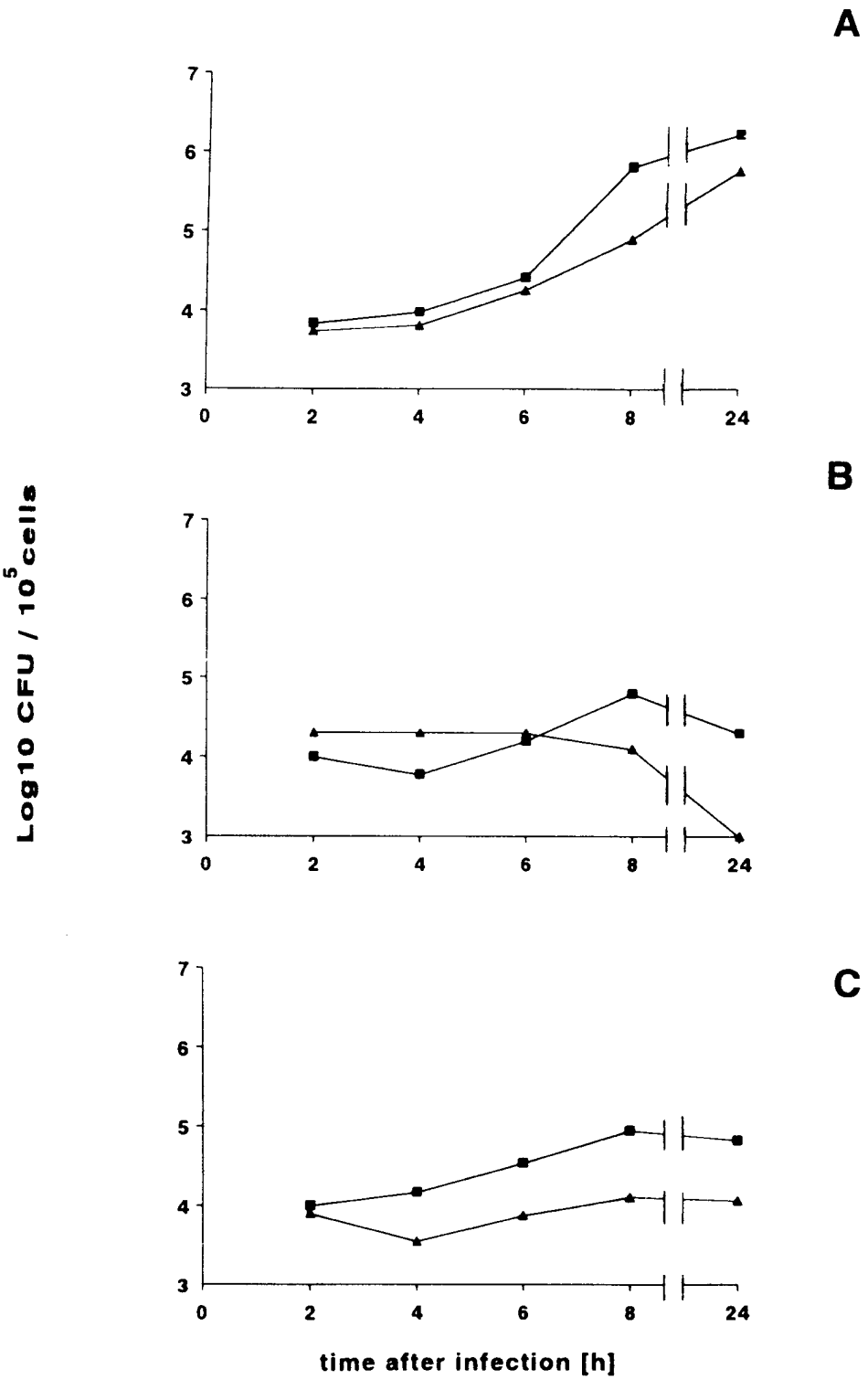

FIG. 3. Listerial growth inhibition by IFN-γ-stimulated hepatocytes. Hepatocytes (A), C57BL/6 BMMϕ (B), and BALB/c BMMϕ (C) were stimulated with IFN-γ (500 U/ml) (▲) or remained unstimulated (■). At 24 h after stimulation, cells were infected with *L. monocytogenes* EGD, and numbers of CFU were determined at the indicated time points. Experiments were repeated three times with comparable results.

cells and afforded by macrophages (10, 36, 40). The major requirement for the acquisition of antilisterial resistance is the activation of antibacterial capacities in macrophages by IFN-γ from T lymphocytes and natural killer cells with support by other cytokines including IL-1, IL-6, and TNF-α from macrophages (5, 12, 27, 28, 37, 40). Compelling evidence exists that *L. monocytogenes* not only replicates in resting macrophages but also invades certain nonprofessional phagocytes, in partic-

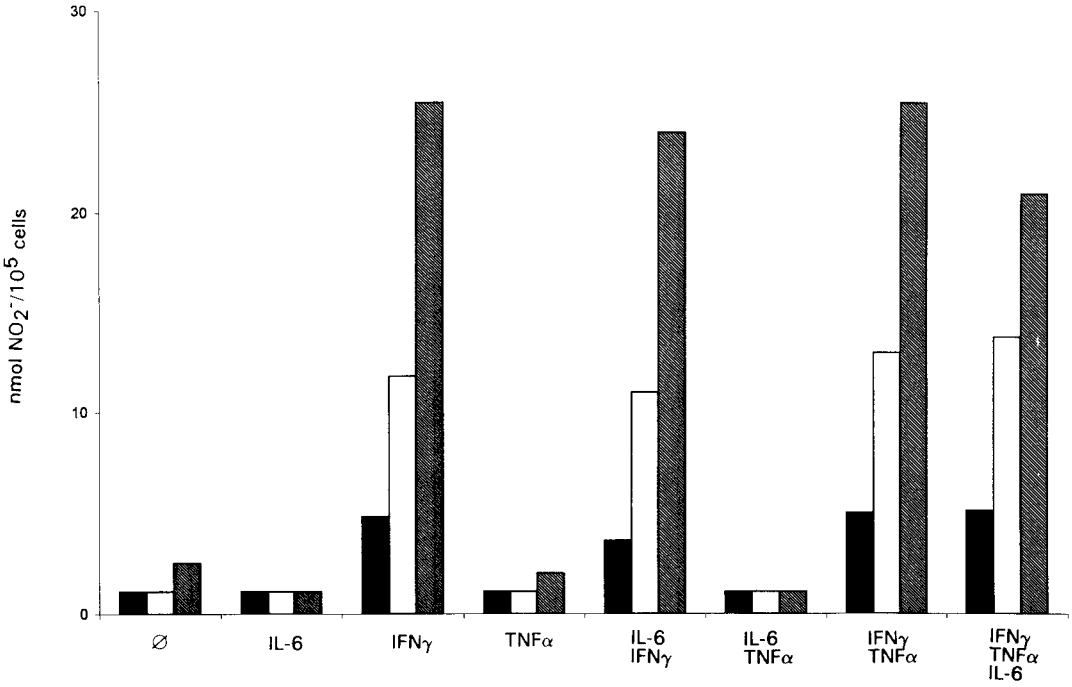

FIG. 4. NO_2^- production by cytokine-stimulated and *L. monocytogenes*-infected hepatocytes or BMMϕ. Hepatocytes (■), C57BL/6 BMMϕ (□), or BALB/c BMMϕ (▨) were stimulated with cytokines (500 U/ml) for 24 h, washed, and infected with *L. monocytogenes* for another 24 h. Subsequently, supernatants were collected and NO_2^- production was determined by Griess reaction. Experiments were repeated twice with comparable results.

ular, hepatocytes (25, 48). These cells are considered a major cellular reservoir for *L. monocytogenes* in the infected mouse. Using an immortalized murine hepatocyte line as a model system, we show here that *L. monocytogenes* exploits hepatocytes as a habitat in vitro, provided the bacterium expresses the hemolysis-inducing listeriolysin. Intracellular growth of listeriolysin-deficient *L. monocytogenes* strains was markedly restricted by resting hepatocytes, indicating an innate antibacterial potential of these nonprofessional phagocytes or restricted availability of nutritional factors in these cells. In contrast, listeriolysin-expressing wild-type *L. monocytogenes* organisms multiplied in an uncontrolled way, emphasizing the central role of listeriolysin in listerial survival within hepatocytes. IFN-γ stimulation rendered hepatocytes capable of restricting growth of hemolytic *L. monocytogenes*, though to a lesser degree than the restriction seen in IFN-γ-activated macrophages.

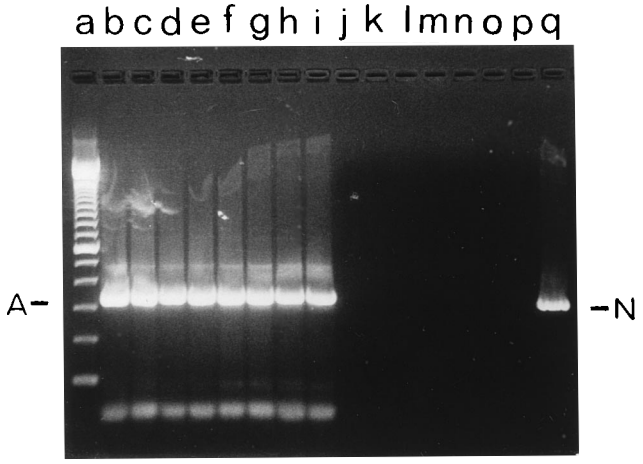

FIG. 5. Induction of iNOS mRNA in cytokine-stimulated and *L. monocytogenes*-infected hepatocytes. Lane a, marker. Lanes b to i, probed for β-actin: b, hepatocytes; c, EGD-infected hepatocytes; d, TNF-α-stimulated hepatocytes; e, TNF-α-stimulated and EGD-infected hepatocytes; f, IL-6-stimulated hepatocytes; g, IL-6-stimulated and EGD-infected hepatocytes; h, IFN-γ-stimulated hepatocytes; i, IFN-γ-stimulated and EGD-infected hepatocytes. Lanes j to q, probed for iNOS: j, hepatocytes; k, EGD-infected hepatocytes; l, TNF-α-stimulated hepatocytes; m, TNF-α-stimulated and EGD-infected hepatocytes; n, IL-6-stimulated hepatocytes; o, IL-6-stimulated and EGD-infected hepatocytes; p, IFN-γ-stimulated hepatocytes; q, IFN-γ-stimulated and EGD-infected hepatocytes. A, β-actin probe (348 bp); N, iNOS probe (339 bp).

Our findings that hepatocytes are invaded by *L. monocytogenes* in vitro are consistent with results of recent experiments by others using an immortalized embryonic hepatocyte line or freshly isolated hepatocytes from *L. monocytogenes*-infected mice (7, 49). We are aware that immortalized cell lines may not fully reflect the functional spectrum of hepatocytes in vivo. On the other hand, primary cultures of hepatocytes are often contaminated with Kupffer cells, thus complicating interpretation of data regarding antilisterial capacities of the two cell populations.

Numerous cytokines including IFN-γ, TNF-α, IL-1, and IL-6 contribute to acquired resistance to *L. monocytogenes* (5, 9, 27, 37, 39). These cytokines exert their protective effects primarily by stimulating antimicrobial activities in infected macrophages. Our study showing that IFN-γ also activates antilisterial capacities in hepatocytes points to the potential contribution of activated hepatocytes to defense against this pathogen. Although IL-6 and TNF-α alone failed to stimulate antilisterial activities in hepatocytes, a combination of these cytokines inhibited *L. monocytogenes* growth to a limited degree. However, costimulation by IFN-γ, IL-6, and TNF-α caused the most pronounced growth inhibition in hepatocytes, though still lower than in BMMϕ.

Since IFN-γ stimulation of *L. monocytogenes*-infected hepatocytes induced TNF-α mRNA expression, we assume that

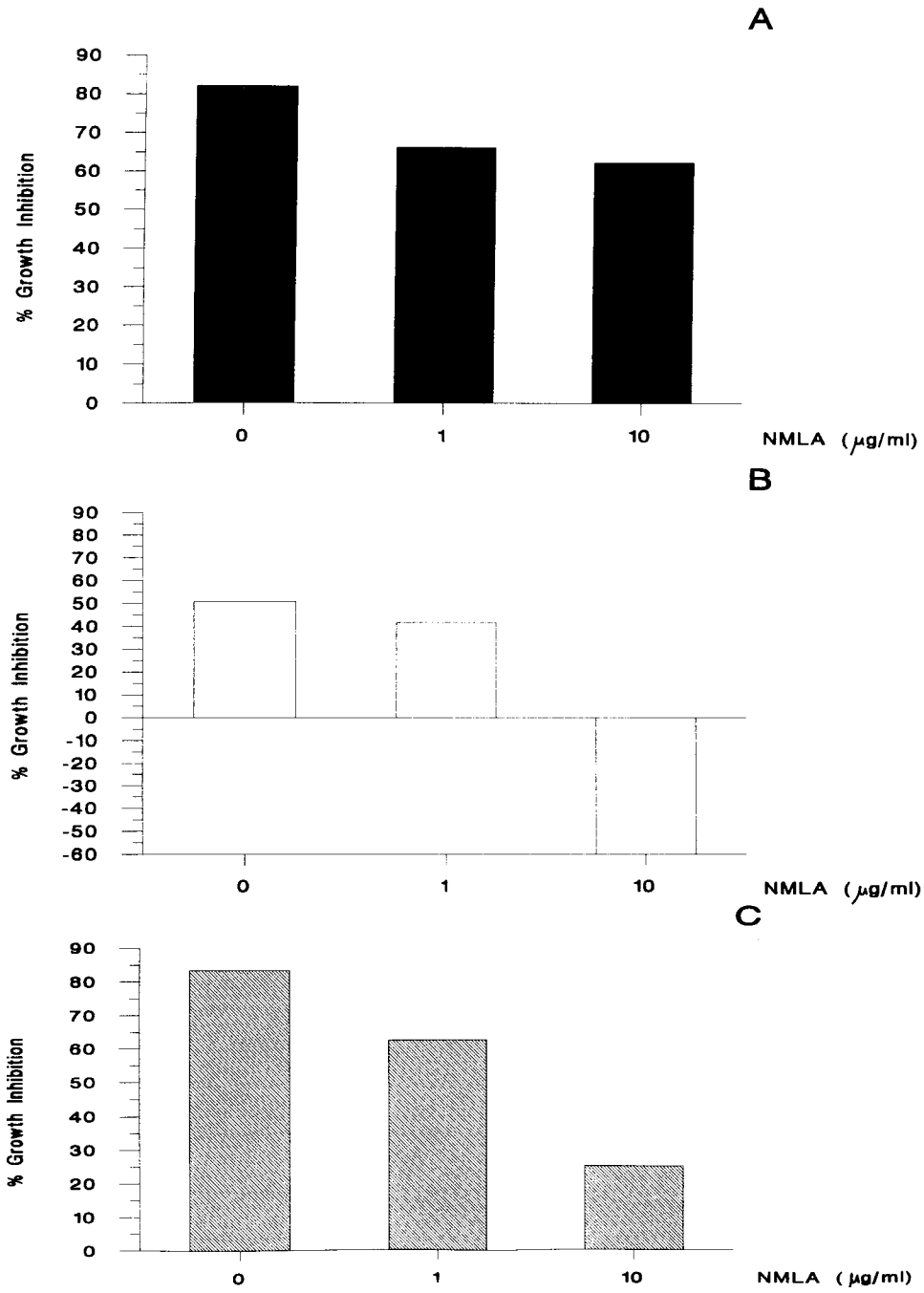

FIG. 6. Failure to inhibit *L. monocytogenes* growth in hepatocytes by NMLA. Hepatocytes (A), C57BL/6 BMMϕ (B), or BALB/c BMMϕ (C) were stimulated with IFN-γ for 24 h, washed, and infected with *L. monocytogenes* EGD in the presence of increasing amounts of NMLA. Percent listerial growth was determined 24 h later. Control without stimulation after 24 h: 1.3×10^6 CFU/10^5 hepatocytes, 5.5×10^4 CFU/10^5 C57BL/6 BMMϕ, and 4.8×10^4 CFU/10^5 BALB/c BMMϕ. Data represent a typical experiment of two performed.

autocrine TNF-α production participates in hepatocyte activation. In addition, TNF-α possesses proinflammatory activities and, hence, could promote attraction of inflammatory phagocytes to the site of listerial growth (21). Studies by Conlan et al. (7) have shown that immigrant granulocytes cause destruction of *L. monocytogenes*-infected hepatocytes.

Recent interest in antibacterial effector mechanisms in mice has focused on RNI (32). Although there is convincing evidence that RNI are central to the defense against numerous intracellular pathogens, the relevance of RNI in antilisterial protection remains equivocal (1, 4, 6, 11, 15, 22, 25, 29, 30, 38). In our experiments, IFN-γ stimulation plus *L. monocytogenes* infection caused pronounced NO_2^- production by hepatocytes. Production of RNI by hepatocytes in other systems has also been described (20, 23, 42, 44, 45). Yet, the results of our experiments with L-arginine-deficient medium and the L-arginine inhibitor NMLA argue against a major role for RNI in listerial growth inhibition by hepatocytes. Further, our data do

TABLE 2. Effect of arginine-free medium on growth inhibition of *L. monocytogenes*[a]

Cells and IFN-γ (U/ml)	With arginine			Without arginine		
	CFU	% Inhibition	nmol of NO_2^-[b]	CFU	% Inhibition	nmol of NO_2^-
Hepatocytes						
0	8.5×10^5		<0.5	1.6×10^6		<0.5
250	3.2×10^5	62.3	2.3	3.0×10^5	70.0	<0.5
500	2.6×10^5	69.4	1.9	3.8×10^5	62.0	<0.5
C57BL/6 BMMϕ						
0	1.3×10^4		<0.5	1.4×10^4		<0.5
250	4.1×10^3	68.4	11.7	1.1×10^4	21.4	4.5
500	2.6×10^3	69.2	10.0	1.2×10^4	14.2	3.5
BALB/c BMMϕ						
0	3.0×10^3		<0.5	4.8×10^3		<0.5
250	5.0×10^2	83.3	17.0	7.5×10^3	−56.2	<0.5
500	2.3×10^2	92.3	13.0	7.3×10^3	−52.0	<0.5

[a] Cells were stimulated with IFN-γ and afterwards infected with *L. monocytogenes* (infection rate, 20:1). At 24 h after infection, CFU and NO_2^- were determined and calculated for 10^5 cells. Experiments were repeated twice with similar results.
[b] Threshold value, 0.5 nmol of NO_2^- per 10^5 cells.

not support a contribution of ROI to antilisterial hepatocyte functions. Additional studies are therefore required to elucidate the RNI- and ROI-independent mechanisms responsible for listerial growth inhibition by IFN-γ-stimulated hepatocytes. One possibility is an IFN-γ-mediated, delayed egression into the cytosol similar to that seen in macrophages (47). Another possibility is nutrient deprivation mechanisms. For example, listerial growth is dependent on acquisition of iron and iron deficiency is partly responsible for RNI- and ROI-independent growth restriction of *Legionella pneumophila* (2, 19).

Our data, together with those of Gregory et al. (26), suggest a novel function for hepatocytes in murine listeriosis. First, IFN-γ stimulation of *L. monocytogenes*-infected hepatocytes induced transcription of mRNA for the proinflammatory cytokine TNF-α, which in vivo promotes influx of inflammatory phagocytes to the site of listerial growth. Second, IFN-γ stimulation activated moderate antilisterial capacities in hepatocytes, which resulted in growth inhibition but failed to cause sterile eradication of *L. monocytogenes*. We assume that hepatocytes contribute to containment, yet permit persistence of *L. monocytogenes* in the liver. Expression of both functions was uniquely depended on IFN-γ stimulation. Therefore, any antilisterial activities of hepatocytes in vivo would require production of IFN-γ in the near vicinity. At early stages of infection, IFN-γ could be provided by natural killer cells and in later phases by T lymphocytes (3, 35). IFN-γ production by T lymphocytes would become more rapid and more pronounced during secondary listeriosis. In conclusion, our findings suggest a shift in the conception that hepatocytes serve as mere shelter for *L. monocytogenes* to their function as an active constituent of the protective host response in listeriosis.

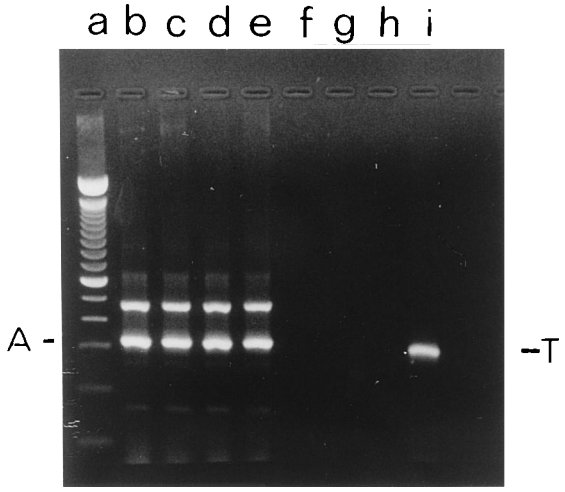

FIG. 7. Expression of TNF-α mRNA in cytokine-stimulated and *L. monocytogenes*-infected hepatocytes. Lane a, marker. Lanes b to e, probed for β-actin: b, hepatocytes; c, EGD-infected hepatocytes; d, IFN-γ-stimulated hepatocytes; e, IFN-γ-stimulated, EGD-infected hepatocytes. Lanes f to i, probed for TNF-α: f, hepatocytes; g, EGD-infected hepatocytes; h, IFN-γ-stimulated hepatocytes; i, IFN-γ-stimulated, EGD-infected hepatocytes. A, β-actin probe (348 bp); T, TNF-α probe (307 bp).

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