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Early Expression of Cytokine mRNA in Mice Infected with *Listeria monocytogenes*

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Protective immunity first becomes evident at 3 to 4 days after inoculation of mice with a sublethal dose of *Listeria monocytogenes*. Recent evidence suggests that production of gamma interferon (IFN-γ) occurs earlier (within the first 24 h of infection). The purpose of this study was to define better the sequence of cytokine mRNA expression during the early stages of *L. monocytogenes* infection. Cytokine mRNA expression was detected by polymerase chain reaction-assisted amplification of RNA extracted from the spleen cells of individual mice euthanized at 0.5 to 120 h after *L. monocytogenes* challenge. By using this method, mRNAs for tumor necrosis factor alpha, interleukin-1α (IL-1α), IL-2, IL-4, IL-5, and IFN-γ were detected in RNA from the spleen cells of uninfected mice. The intensity of the bands for IFN-γ, however, was increased greatly at 16 h after intravenous injection of 5×10^4 CFU (nearly 1 50% lethal dose) of *L. monocytogenes*. IL-6 and granulocyte-macrophage colony-stimulating factor mRNAs were not detected in spleen cell RNA from uninfected mice but were induced within 30 and 60 min, respectively, after inoculation with *L. monocytogenes*. Increased amounts of mRNAs for IFN-γ, IL-6, and granulocyte-macrophage colony-stimulating factor were detected after injection of viable, but not killed, *L. monocytogenes*. IL-3 mRNA was not detected at any time in RNA extracted from the spleen cells of uninfected or *L. monocytogenes*-infected mice. These results suggest that infection with *L. monocytogenes* elicits a detectable cytokine mRNA response within the first few hours of infection.

Murine listeriosis is a valuable model for investigating the role of leukocytes and cytokines in the development of cellular immunity (21, 23, 33). In mice undergoing a sublethal infection with *Listeria monocytogenes*, the bacterial burden typically reaches a peak in the spleen and liver at approximately 3 to 4 days after challenge and thereafter decreases (5, 6). Accordingly, it had been assumed that protective immunity against primary *L. monocytogenes* infection was established at or shortly before this time. Although it is assumed that the protective immune response is initiated considerably earlier, there are few published data on this subject. Recent reports provided evidence that gamma interferon (IFN-γ) production occurred by 24 h after *L. monocytogenes* challenge (26, 27, 29). The purpose of this study was to determine the time and sequence of mRNA expression for several cytokines in the host response to a primary *L. monocytogenes* infection.

It is difficult to detect the small amounts of cytokines secreted at sites of infection in vivo with bioassays or immunoassays (15). As a result, identifying the profile of cytokine mRNA in the tissues of *L. monocytogenes*-infected mice is an alternative strategy for identifying the cytokine response during infections. Advances in molecular biotechnology have made it possible to detect cytokine mRNA in various tissues (30). Although several investigators have used Northern (RNA) blot analysis to examine cytokine mRNA expression in tissues of mice infected with *L. monocytogenes* or other microbes (17–20, 29, 32), this method may not be sensitive enough to detect small amounts of mRNA transcripts. The polymerase chain reaction (PCR) amplification method facilitates detection of very small amounts of mRNA (9, 10). In addition, the commercial availability of cytokine primers allows the analysis of mRNA for an array of immunoregulatory peptides in individual mice. The primary purpose of this study was to identify changes in the profiles of cytokines that are produced during the first 24 h of the immune response to a primary *L. monocytogenes* infection by using PCR-assisted amplification of mRNA extracted from the spleens of mice at various times after challenge. Our data indicate that most of the changes in cytokine mRNA expression occur during the first 24 h after *L. monocytogenes* infection, with notable changes occurring within several hours after challenge.

MATERIALS AND METHODS

Mice. Male mice (C57BL/6 × DBA/2)F_1 (BDF_1) 5 to 6 weeks old were obtained from The Jackson Laboratory (Bar Harbor, Maine). These mice were housed under plastic microisolator caps (Lab Products, Frederick, Md.) at the Animal Care Facility of the University of Wisconsin School of Veterinary Medicine (a facility approved by the American Association for Laboratory Animal Care). Mice were given Purina Lab Chow (Ralston Purina, St. Louis, Mo.) and water ad libitum. The mice were stated by the supplier to be free of infection by adventitious agents such as Sendai virus and mouse hepatitis virus. Mice were allowed to acclimate to our animal care facility for at least 1 week before being used in an experiment.

Bacterial infection. *L. monocytogenes* EGD was maintained as described previously (7). Log-phase bacteria were suspended in tryptose phosphate broth containing 20% glycerol, and aliquots were stored at −70°C. Listeriae were freshly thawed and diluted in pyrogen-free phosphate-buffered saline (PBS) at appropriate working concentrations immediately prior to injection. Mice were infected intravenously (i.v.) via a lateral tail vein with approximately 5×10^4

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viable listeriae in a total volume of 0.2 ml. In some experiments, the bacteria were killed by heating at 70°C for 90 min before being injected i.v. into mice. At various times after infection, the mice were euthanized by cervical dislocation and their spleens were removed. About one-fourth of each spleen was reserved and processed further for RNA extraction as described below. The remainder of each spleen was homogenized thoroughly in distilled water, the homogenates were serially diluted in distilled water, and appropriate dilutions were plated on blood agar (Remel, Lenexa, Kansas). Plates were incubated for 24 h at 37°C, and the colonies were enumerated. Results were expressed as the mean $\log_{10}$ viable *L. monocytogenes* per spleen for three mice per time point, ± standard error of the mean.

RNA extraction and cDNA preparation. A portion of each spleen was teased apart in Hanks' balanced salt solution containing 0.02% azide. The spleen cells were washed once, suspended in 500 μl of lysis solution (4 M guanidine, 0.5% *N*-lauroylsarcosine, 25 mM sodium citrate, 100 mM 2-mercaptoethanol), and stored at −70°C until further processing. After lysates were thawed, 33 μl of 3 M sodium acetate (pH 4.0), 500 μl of water-saturated phenol, and 100 μl of chloroform were added to the lysates, with the mixture being thoroughly vortexed after each addition. The mixture was then chilled on ice for 15 min and centrifuged at 10,000 × *g* for 10 min at 4°C. The aqueous phase was recovered, and the RNA was precipitated with an equal volume of isopropanol (Sigma, St. Louis, Mo.) at −20°C for at least 90 min. The precipitates were centrifuged at 4°C (10,000 × *g* for 10 min), washed once with 75% ethanol in diethylpyrocarbonate-treated double-distilled water, and centrifuged again at 4°C at 10,000 × *g* for 10 min. The tubes were inverted to air dry the pellets. The pellets were then resuspended in 14.5 μl of diethylpyrocarbonate-treated double-distilled water. One microgram of oligo(dT) (Promega Biotec, Madison, Wis.) was added to the suspension, and the mixture was heated at 65°C for 5 min. After cooling on ice, the mixture was incubated for 2 h at 42°C with 14 μl of the following mixture: 20 mM dithiothreitol (Sigma); 1 mM (each) dATP, dGTP, dCTP, and dTTP; 35 U of RNasin (Promega); and 525 U of Moloney murine leukemia virus reverse transcriptase (GIBCO BRL Life Technologies, Inc., Gaithersburg, Md.) in reverse transcription buffer (Bethesda Research Laboratories, Inc.). Samples were stored at −20°C until subjected to PCR amplification.

PCR procedure. PCR primers for murine β-actin, tumor necrosis factor alpha (TNF-α), interleukin-1α (IL-1α), IL-2, IL-3, IL-4, IL-5, IL-6, IFN-γ, and granulocyte-macrophage colony-stimulating factor (GM-CSF) were purchased from Clontech (Palo Alto, Calif.). One microliter of cDNA prepared as described above was amplified in 0.5-ml microcentrifuge tubes in the presence of 500 nM (final concentration) 5′ and 3′ primers, 200 μM (each) dATP, dGTP, dCTP, and dTTP, and 1.25 U of *Taq* DNA polymerase (Promega) in a final volume of 50 μl of *Taq* DNA polymerase 10× buffer (Promega). The reaction mixture was overlaid with 45 μl of mineral oil, and PCR was performed in a Coy Tempcycler (Coy Laboratory Products Inc., Ann Arbor, Mich.) for 30 cycles, in which each cycle consisted of 1 min of denaturation at 94°C, 2 min of annealing at 60°C (except when using the primer for IL-5, for which the annealing temperature was 63°C), and 3 min of extension at 72°C. The reaction product was visualized by electrophoresis of 25 μl of the reaction mixture at 100 V for 60 min in a 1.5% agarose gel containing 1 μg of ethidium bromide per ml. The gels were then examined on a UV light box and photographed. One microgram of pGEM markers (Promega) was run in parallel as molecular weight markers (providing bands at 2,645, 1,605, 1,198, 676, 517, 460, 396, 350, and 222 bp). Specificities of the amplified bands were validated by their predicted sizes and by Southern blot procedures for IFN-γ, IL-2, IL-4, IL-6, GM-CSF, and β-actin.

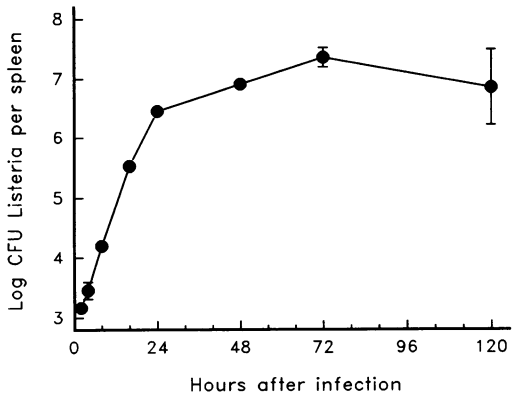

FIG. 1. Kinetics of bacterial multiplication in the spleens of mice infected with *L. monocytogenes* EGD. The data are expressed as the mean $\log_{10}$ CFU of *L. monocytogenes* per spleen ± standard error of the mean for three mice per datum point.

RESULTS

Patterns of cytokine mRNA expression in the spleens of *L. monocytogenes*-infected mice. Mice were challenged with 5 × 10^4 CFU of listeriae per mouse (nearly 1 50% lethal dose). This resulted in a characteristic primary infection in which the numbers of listeriae recovered from the spleens increased rapidly from 24 to 72 h and then plateaued and began to decrease slightly (Fig. 1).

The pattern of cytokine mRNA expression in spleen cells during the course of *L. monocytogenes* infection is summarized in Table 1. Figures 2 to 4 illustrate representative bands after PCR amplification with the various cytokine primers. Cytokine mRNA expression appeared to follow four distinct patterns. In the first, low levels of cytokine mRNA were constitutively present for uninfected mice, and these increased during *L. monocytogenes* infection. This pattern was exemplified by TNF-α, IL-1α, and IFN-γ. As illustrated in Fig. 2, mRNAs for TNF-α and IL-1α were detected in spleen cells of uninfected mice, although these bands were weak. The intensities of the mRNA bands for TNF-α and IL-1α increased rapidly during infection, although for some mice the bands remained similar in intensity to those for uninfected mice. IFN-γ mRNA bands were also detected in spleen cells from uninfected mice. The intensity of the IFN-γ mRNA band, however, markedly increased by 16 h after infection (Table 1; see also Fig. 4). A second pattern of cytokine mRNA expression was one in which cytokine mRNA was constitutively present in uninfected mice and did not increase appreciably, or even decreased somewhat, during *L. monocytogenes* infection. IL-2, IL-4, and IL-5 demonstrated this pattern. The intensities of the mRNA bands for these cytokines did not increase during *L. monocytogenes* infection (Fig. 2 and 3). On the contrary, the IL-4 mRNA bands disappeared entirely by 120 h after challenge. A third pattern of cytokine mRNA expression that was noted was the absence of detectable mRNA in cells from uninfected mice, followed by a rapid, substantial increase in

TABLE 1. Kinetics of expression of cytokine mRNA in spleen cells of mice infected with *L. monocytogenes* EGD[a]

Cytokine primer	Expression at time (h) after *L. monocytogenes* challenge										
	Prechallenge	0.5	1	2	4	8	16	24	48	72	120
TNF-α	+	++	++	++	++	++	++	++	++	++	++
	+	++	++	+	++	++	++	++	+	++	+
	−	+	+	+	+	+	+	+	+	−	−
IL-1α	+	++	++	++	++	++	++	++	++	++	++
	+	++	++	++	++	++	+	++	+	++	++
	−	−	++	+	−	+	+	+	+	+	+
IFN-γ	+	+	+	+	+	+	++	++	++	++	++
	+	+	+	+	+	+	++	++	++	++	++
	+	+	+	+	+	+	++	++	+	++	++
IL-2	+	+	+	+	+	+	+	+	+	+	+
	+	−	−	+	+	+	+	+	−	+	+
	−	−	−	−	−	−	+	+	−	−	+
IL-4	+	+	+	+	+	+	+	+	+	+	−
	+	+	+	+	+	+	+	+	+	+	−
	+	+	+	−	+	+	+	+	−	−	−
IL-5	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+
IL-6	−	+	+	+	+	+	+	++	++	++	+
	−	+	+	+	+	+	+	++	+	++	+
	−	−	+	+	+	+	+	+	+	+	−
GM-CSF	−	−	+	+	+	+	+	++	+	+	+
	−	−	−	−	−	+	+	+	−	+	−
	−	−	−	−	−	−	+	+	−	−	−
IL-3	−	−	−	−	−	−	−	−	−	−	−
	−	−	−	−	−	−	−	−	−	−	−
	−	−	−	−	−	−	−	−	−	−	−
β-actin	++	++	++	++	++	++	++	++	++	++	++
	++	++	++	++	++	++	++	++	++	++	++
	++	++	++	++	++	++	++	++	++	++	++

[a] BDF_1 male mice were injected i.v. with 5×10^4 CFU of *L. monocytogenes* EGD. At the indicated times, three mice were killed and their spleens were removed. Total RNA was extracted from the spleen cells and transcribed into cDNA by reverse transcriptase. cDNA was subjected to 30 cycles of PCR with the indicated primers. The reaction products were visualized by electrophoresis and were scored by the intensity of bands as follows: −, negative; +, weak band; ++, strong band. Each symbol represents the result obtained with a single mouse. These semiquantitative scores can be used only for comparing the amounts of products from samples obtained with the same primer.

signal within hours after challenge. This pattern was illustrated by IL-6 and GM-CSF, for which mRNA signals were visible within 0.5 and 1 h, respectively, after *L. monocytogenes* challenge (Table 1 and Fig. 4). The intensity of the IL-6 mRNA bands continued to increase at 24 to 72 h postchallenge, whereas the intensity of the GM-CSF mRNA bands decreased at later time points. A fourth pattern, characterized by no detectable cytokine mRNA at any of the time points examined, was demonstrated by IL-3 (Table 1 and Fig. 2).

Increased cytokine mRNA expression requires injection of viable *L. monocytogenes*. Next, we examined whether the observed increases in IFN-γ, IL-6, and GM-CSF mRNAs required actual infection with *L. monocytogenes* or were merely a nonspecific response to the stress of restraint and injection. Mice were injected i.v. with viable listeriae, heat-killed listeriae, or PBS alone. RNA was extracted from their spleen cells, and the expression of IL-6, IFN-γ, and GM-CSF mRNAs was determined at 1 and 24 h after injection

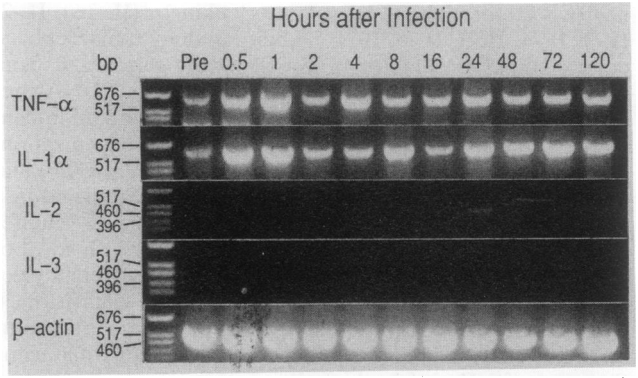

FIG. 2. PCR-assisted amplification of TNF-α, IL-1α, IL-2, IL-3, and β-actin mRNAs from spleen cells of representative individual mice at various times after *L. monocytogenes* challenge. On the original gels and photographs, bands for TNF-α, IL-1α, and β-actin were observed at all time points. Faint bands were observed for IL-2 at all time points except for 48 h after infection. Pre, prechallenge.

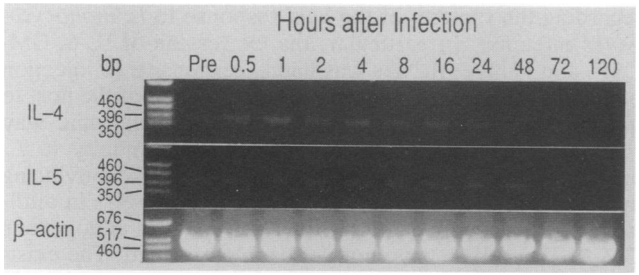

FIG. 3. PCR-assisted amplification of IL-4, IL-5, and β-actin mRNAs from spleen cells of representative individual mice at various times after *L. monocytogenes* challenge. On the original gels and photographs, faint bands were observed for IL-5 at all time points and for IL-4 at all time points except for 120 h. Pre, prechallenge.

(Fig. 5). Strong signals for IFN-γ and GM-CSF mRNAs were induced only by the injection of viable listeriae. As indicated in Fig. 4, a weak signal for IFN-γ was observed for uninfected mice. A similar signal was seen for mice injected with killed listeriae or PBS (Fig. 5). Although some IL-6 mRNA was induced at 1 h after injection of killed listeriae or PBS, a strong signal at 24 h after injection was observed only for mice that had been injected with viable listeriae.

DISCUSSION

The present study used a PCR amplification technique to demonstrate that the pattern of expression of cytokine mRNA by spleen cells during *L. monocytogenes* infection can be divided into four groups: (i) cytokines with constitutive expression of mRNA in uninfected mice and an increase in cytokine mRNA during infection, a group which includes TNF-α, IL-1α, and IFN-γ; (ii) cytokines with constitutive mRNA expression in uninfected mice that is largely unaffected by *L. monocytogenes* infection, a group which includes IL-2, IL-4, and IL-5; (iii) cytokines without constitutive mRNA expression in uninfected mice whose mRNA expression was induced by *L. monocytogenes* infection, a group which includes IL-6 and GM-CSF; and (iv) a fourth group, consisting of IL-3 alone, whose mRNA was not detected at any time before or during the course of the infection.

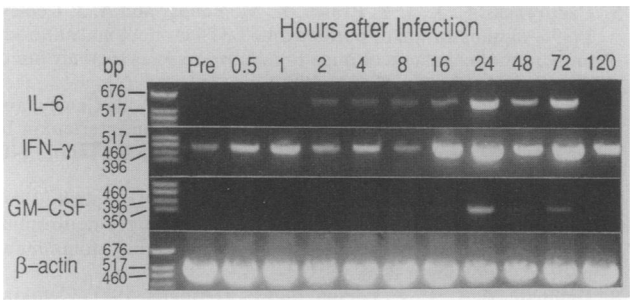

FIG. 4. PCR-assisted amplification of IL-6, IFN-γ, GM-CSF, and β-actin mRNAs from spleen cells of representative individual mice at various times after *L. monocytogenes* challenge. On the original gels and photographs, bands for IFN-γ were observed at all time points, for IL-6 at all time points except for preinjection and at 120 h, and for GM-CSF at all time points 2 h or later after infection. Pre, prechallenge.

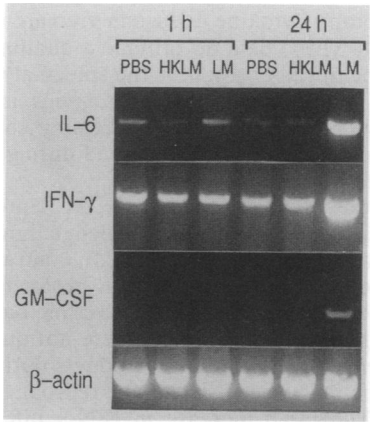

FIG. 5. Influence of injection with PBS or heat-killed or viable *L. monocytogenes* on the expression of cytokine mRNA in spleen cells of mice. Mice were injected i.v. with viable listeriae (LM; 5×10^4 CFU) and an equal number of heat-killed listeriae (HKLM) or with the vehicle alone (0.2 ml of PBS). PCR-assisted amplification of cytokine mRNA from the spleen cells of individual mice was performed at 1 and 24 h after injection. A representative band from a single mouse is shown for each cytokine. On the original gels and photographs, bands for IL-6, IFN-γ, and β-actin were observed at all time points, whereas bands for GM-CSF were observed only at 24 h after injection of viable listeriae.

We assume that the low levels of mRNA for the cytokines in group 1 (TNF-α, IL-1α, and IFN-γ) and group 2 (IL-2, IL-4, and IL-5) in the spleen cells of uninfected mice reflect normal homeostasis. The mice used in this study were confirmed to be free of infection by adventitious agents as described in Materials and Methods, thus suggesting that these cytokine mRNA signals reflect the normal state rather than a response to an underlying infectious agent. In addition, we have confirmed that no PCR products were observed when cDNA templates were omitted from the PCR mixture (data not shown). Therefore, the possibility that DNA contamination occurred in the PCR mixture can be excluded. Although some investigators did not detect IFN-γ mRNA in uninfected spleen cells by Northern blot analysis (17, 29, 32), our observations are consistent with those of Dallman et al. (8), who used a PCR amplification technique to demonstrate IFN-γ mRNA expression in cardiac tissue from normal mice. It is likely that the presence in uninfected mice of low levels of mRNA for cytokines produced by both TH1 cells (IL-2 and IFN-γ) and TH2 cells (IL-4 and IL-5) reflects the dynamic nature of immune regulation even in the absence of microbial invasion (24). Expression of TNF-α and IL-1α mRNAs by uninfected mice may reflect continual stimulation of the mononuclear phagocyte system by small numbers of microbes and microbial products in the bloodstream.

It should be noted that the PCR results presented in this study are principally qualitative. Differences in band intensity may reflect changes in gene transcription, cell number, or cell types present in the spleen at various times during *L. monocytogenes* infection. We recently presented evidence, however, that band intensity correlates with binding of radiolabeled cytokine probes (13). We recognize that the results presented do not prove that the respective cytokine peptides were released from spleen cells during *L. monocytogenes* infection in vivo. Such determinations are difficult to perform, although efforts to develop procedures that will

allow us to identify cytokine release in vivo are underway in our laboratory. These data do provide a qualitative evaluation of cytokine mRNA present in the spleen at various time points during a primary *L. monocytogenes* infection. As such, they give an indication of how rapidly changes in the regulation of cytokines can be observed during *L. monocytogenes* infection in mice.

The intensities of TNF-α and IL-1α PCR products were increased after *L. monocytogenes* challenge; however, these responses were not uniform (Table 1). In contrast to TNF-α and IL-1α, the intensity of IFN-γ PCR products was markedly and uniformly increased by 16 h after bacterial challenge. Although the PCR products were not quantified, the increased intensity of IFN-γ PCR products during *L. monocytogenes* infection was consistent and reproducible (Table 1 and Fig. 5). The rapid increase in IFN-γ mRNA that we observed is consistent with a previous report that IFN-γ was secreted from spleen cells within 1 day after *L. monocytogenes* challenge (26, 29). The same workers demonstrated that splenic IFN-γ mRNA, as detected by Northern blot analysis, was prominent at 1 to 3 days after *L. monocytogenes* challenge.

The kinetics of IL-6 mRNA expression observed in the present study indicated that peak expression of IL-6 mRNA occurred at 24 to 72 h after infection and declined to background levels by 120 h after infection. This is consistent with the work of Havell and Sehgal (16), who observed that *L. monocytogenes* infection induced peak concentrations of IL-6 in the sera and spleens of mice at 2 days after challenge. Overall, both studies suggest that IL-6 mRNA expression and serum IL-6 levels reflect the bacterial burden in the spleens of *L. monocytogenes*-infected mice. Although a weak IL-6 mRNA signal was induced by injection of killed listeriae or PBS, this probably reflects the response of the mononuclear phagocyte system to the nonspecific stress of restraint and injection (28, 34). Increased expression of IL-6 mRNA, however, required injection of viable listeriae capable of multiplying in vivo (Fig. 5).

The peak mRNA signal for GM-CSF was observed at 24 h after *L. monocytogenes* challenge. Cheers et al. (4) detected small amounts of GM-CSF in sera of mice after *L. monocytogenes* infection. They also demonstrated that the amounts of GM-CSF increased in accordance with the severity of infection. In our study, GM-CSF mRNA expression was induced by injection of viable listeriae but not by injection of heat-killed listeriae or PBS. These results suggest that bacterial proliferation is needed to stimulate the expression of GM-CSF mRNA. Cheers et al. (4) did not detect IL-3 in the sera of *L. monocytogenes*-infected mice, an observation that is consistent with the absence of IL-3 mRNA detectable by the methods used in our study.

Although IL-4 mRNA was expressed in uninfected mice, it disappeared entirely at 72 to 120 h after *L. monocytogenes* infection. This is in contrast with IFN-γ mRNA expression, for which intense mRNA signals were observed for at least 120 h after bacterial challenge. Although the mechanism remains to be established, down-regulation of IL-4 mRNA may be beneficial in the establishment of protective immunity against *L. monocytogenes* infection. IL-4 is known to have anti-inflammatory properties (1, 2, 11, 12, 14, 22) that can inhibit the effects of IFN-γ in the development of protective immunity against infectious agents (31). Previous work from this laboratory demonstrated that administration of a neutralizing anti-IL-4 monoclonal antibody increased the resistance of mice to *L. monocytogenes* infection (13).

The results obtained in this study provide new information regarding the rapidity of the host response to *L. monocytogenes* infection. In particular, the expression of IL-6, GM-CSF, and IFN-γ mRNAs was highly responsive to injection of viable listeriae. It is assumed that the ability of the host to quickly initiate these cytokine responses is in some way related to the development of protective immunity to *L. monocytogenes* infection. Certainly, there is convincing evidence for the importance of endogenous IFN-γ in antilisteria resistance (3, 25, 26). Similar analyses of endogenous GM-CSF and IL-6 have not yet been performed. The existing evidence suggests that levels of these two cytokines in plasma parallel the bacterial burden in the spleens and livers of *L. monocytogenes*-infected mice, rather than necessarily being an indicator of bacterial clearance (4, 16). At this point, it would be premature to propose a detailed scheme for the series of immunoregulatory events that occur during *L. monocytogenes* infection. The results of this study do provide evidence, however, that our thinking about the regulation of protective immunity must recognize that the host is beginning to make a detectable response within hours after challenge. This occurs well before clinical signs of disease or obvious manifestations of cellular immunity (e.g., delayed-type hypersensitivity) become apparent.

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