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Induction of Cytokines in Phagocytic Mammalian Cells Infected with Virulent and Avirulent *Listeria* Strains

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The present paper analyzes the cytokine response of mouse macrophages during infection by *Listeria monocytogenes*. The use of different mutants of *L. monocytogenes* impaired in various steps of the infection process allowed us to dissect the cytokine response. Cytokine mRNA expression was detected by PCR-assisted amplification of RNA extracted from macrophages after infection with different *Listeria* strains. An increase in the amount of mRNA for tumor necrosis factor alpha (TNF-α), interleukin-1α (IL-1α), IL-1β, and IL-6 was detected in P388D$_1$ macrophages infected with *L. monocytogenes* at 4 h postinfection. Interestingly, only hemolytic strains of *L. monocytogenes* were able to induce IL-1α, IL-6, and TNF-α mRNA. This indicated that the induction of these cytokine mRNAs requires entry of the listeriae into the host cell cytoplasm. In contrast, IL-1β was also induced by infection with nonhemolytic mutants of *L. monocytogenes* which remain entrapped within the phagosome. The levels of TNF, IL-1α, and IL-6 found in the supernatants of *Listeria*-infected P388D$_1$ macrophages generally correlated well with the induction of the respective mRNAs, but it became obvious that cytokine activity is also regulated through posttranscriptional mechanisms. In vitro induction of the cytokines IL-1α, IL-1β, IL-6, and TNF-α was also observed by infection of bone-marrow-derived macrophages with *L. monocytogenes*.

Listeria monocytogenes, a pathogen responsible for opportunistic infections such as meningitis and septicemia in humans and animals (40, 41), is the best-characterized member of the genus *Listeria*, which comprises five additional species, i.e., *L. ivanovii*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, and *L. grayi* (16).

L. monocytogenes is a facultative intracellular bacterial species which can invade, survive, and replicate within nonprofessional phagocytic cells like enterocytes (10) and fibroblasts (23, 35) as well as in professional phagocytes such as macrophages (23, 35). It has been conclusively shown by a number of investigations that the extracellular protein listeriolysin O is absolutely required for intracellular survival and replication (10, 23, 35). The gene encoding listeriolysin O (30) is part of a gene cluster which includes genes encoding a phosphatidylinositol-specific phospholipase C (*plcA*) (24, 28), a metalloprotease (*mpl*) (6, 29), a protein necessary for induction of actin polymerization (*actA*) (7, 19), and a lecithinase (*plcB*) (45). These genes and several others which have been identified only through their gene products and which are not part of the virulence gene cluster (43) are under the control of a transcriptional activator PrfA (3, 25, 26).

The mechanism of T-cell-mediated protective immunity against *L. monocytogenes* has been studied extensively (18, 27, 38), and it is generally accepted that protective immunity can be induced only by immunization with viable, intracellularly growing *L. monocytogenes*. There is still little known, however, about the host cell response upon infection with *L. monocytogenes*.

The molecular characterization of a growing number of cytokines and their genes in recent years (42) now offers an additional approach to study the host response mechanisms in detail. The infection of cultured eucaryotic cells by *L. monocytogenes* has been shown to induce the release of different cytokines. Havell first showed that *L. monocytogenes*-infected primary cultures of mouse fibroblasts secrete alpha/beta interferon (IFN-α/β) (12), tumor necrosis factor alpha (TNF-α), and interleukin-6 (IL-6) (13). Infected mouse alveolar macrophages produced IL-6 and TNF-α in variable amounts after infection (13). Mouse peritoneal macrophages infected with viable but not heat-killed *L. monocytogenes* produced significant amounts of IL-1 (31).

Mice were infected with *L. monocytogenes*, and the levels of different cytokine mRNAs in the spleen and liver were analyzed at different time points postinfection (8, 15, 21, 33, 36). These studies showed that the mRNA levels for IL-1α, IL-1β, IL-6, IL-10, TNF-α, IFN-γ, the granulocyte-macrophage colony-stimulating factor, and the macrophage colony-stimulating factor were all elevated in various tissues.

In this study, we wanted to analyze the induction of cytokine mRNAs and the location of the bacteria in the host cell necessary for such induction. For this purpose, we infected the macrophage-like cell line P388D$_1$ and mouse bone-marrow-derived macrophages with *L. monocytogenes*, *L. monocytogenes* mutants, and *L. ivanovii*. *L. ivanovii* is a strongly hemolytic species, but nevertheless, it has only low virulence for humans.

Because of their well-characterized importance in inflammation processes, we decided to study initially the induction of the cytokines IL-1α, IL-1β, IL-6, and TNF-α at the transcriptional level. The data presented in this study show that hemolytic wild-type strains which are able to reach the cytoplasm of the P388D$_1$ host cell can induce IL-1α, IL-1β, IL-6, and TNF-α mRNA, whereas mutants remaining in the phagosome can only induce IL-1β. Furthermore, we show that the levels of cytokines found in the supernatant of *Listeria*-infected P388D$_1$ macrophages generally correlate with the mRNA levels. IL-1α,

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TABLE 1. *Listeria* strains used in this study

Strain	Hemolysis[a]	Comments	Source (reference)
L. monocytogenes Sv 1/2a EGD	+		S. H. E. Kaufmann
L. ivanovii Sv 5	++		SLCC[b]
L. monocytogenes prfA	−	*prfA* gene inactivated	T. Chakraborty (3)
L. monocytogenes plcA	+	*plcA* gene inactivated	E. Domann (5)
L. monocytogenes mpl	+	*mpl* gene inactivated	E. Domann (5)
L. monocytogenes actA	+	*actA* gene inactivated	E. Domann (5)
L. monocytogenes M3 *hly*	−	Tn*916* insertion into *hly* gene	S. Kathariou (17)

[a] Hemolysis zones on brain heart infusion agar plates with 5% sheep erythrocytes after 24 h at 37°C and 48 h at 4°C. Symbols: −, no hemolysis; +, hemolysis zones around colonies present; ++, extremely large hemolysis zones around colonies.
[b] SLCC, Special Listeria Culture Collection of the Institut for Medical Microbiology and Hygiene.

IL-1β, IL-6, and TNF-α were also induced in *L. monocytogenes*-infected bone-marrow-derived macrophages.

MATERIALS AND METHODS

Bacterial strains. All *Listeria* strains used in this study and their origins are listed in Table 1. The *prfA*, *plcA*, *mpl*, and *actA* mutants of *L. monocytogenes* were obtained through integration by homologous recombination of suicide vectors containing the respective cloned genes (3, 5). The nonhemolytic transposon mutant *L. monocytogenes* M3 *hly* was described earlier (17).

For all infections of cell cultures, overnight cultures of the *Listeria* strains were diluted in brain heart infusion broth (Difco) and incubated at 37°C until they reached the mid-log phase of growth. The bacteria were then harvested, washed once, and resuspended in phosphate-buffered-saline (PBS) with 15% glycerol and stored in aliquots at −80°C until used for infection. *Listeria* strains were heat killed by treatment at 75°C for 30 min.

Culture and infection of bone-marrow-derived and P388D$_1$ macrophages (23, 35). Female BALB/c mice were killed by cervical dislocation, and the femurs were isolated and flushed with RPMI-Click medium (Biochrom). Macrophage precursor cells were seeded in 60-mm-diameter tissue culture plates (Greiner; 5×10^5 cells per plate) in RPMI-Click medium supplemented with 10% heat-inactivated fetal calf serum (GIBCO), 2 mM L-glutamine (GIBCO), 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 0.05 mM 2-mercaptoethanol, penicillin (100 U/ml), streptomycin (100 μg/ml), and 20% L929 conditioned medium as a source for granulocyte-macrophage colony-stimulating factor in a humified 5% CO_2 atmosphere. The bone-marrow-derived macrophages were infected on days 6 to 10 after isolation.

The mouse macrophage-like cell line P388D$_1$ (ATCC CLL 46) was cultured in RPMI medium (Gibco) supplemented with 10% fetal calf serum and 2 mM L-glutamine in a humified 5% CO_2 atmosphere at 37°C. Twenty-four hours prior to infection, macrophages were seeded in either 60-mm-diameter tissue culture plates (2×10^6 macrophages per plate) for RNA extraction or in 12-well tissue culture plates (5×10^5 cells per well) for TNF activity detection.

After two washings with PBS, the macrophages were infected with a multiplicity of infection of 50 listeriae (in RPMI medium) per eucaryotic cell. The plates were then incubated for 40 min to allow the macrophages to phagocytose the bacteria. The monolayers were then washed three times with PBS to remove extracellular bacteria and further incubated in medium containing 50 μg of gentamicin per ml to kill extracellular bacteria and prevent reinfection. At the indicated time points, either aliquots of 0.1 ml of the supernatants were removed, centrifuged once, and stored at −20°C until used for determination of TNF activity or the macrophages were used for RNA extraction.

RNA isolation and cDNA synthesis. At 4 h postinfection, the total RNA was isolated from the macrophages of individual 60-mm plates by the single-step guanidinium thiocyanate procedure essentially as described previously (4).

Complementary DNA was synthesized with the Stratagene first-strand synthesis kit (San Diego, Calif.) by using the protocol of the manufacturer. Briefly, 5 μg of total RNA in 32 μl of diethyl-pyrocarbonate-treated water was mixed with 3 μl of oligo(dT)$_{15}$ primer (100 ng/ml) and heated at 65°C for 5 min. After being cooled to room temperature, the following reagents were added: 5 μl of 10× first-strand buffer (containing 500 mM Tris-Cl [pH 8.3], 750 mM KCl, and 30 mM $MgCl_2$), 5 μl of 0.1 M dithiothreitol, 1 μl (20 U/μl) of RNase Block II, 3 μl of deoxynucleoside triphosphates (dNTPs; 25 mM each), and 1 μl of Moloney murine leukemia virus reverse transcriptase (20 U/μl). The mixture was then incubated at 37°C for 1 h and stored at −20°C.

PCR analysis (39). PCR primer pairs specific for murine β-actin, TNF-α, and IL-1β were purchased from Stratagene. All other primers were synthesized at the lab facilities. cDNA prepared as described above was amplified in 1.5-ml tubes in the presence of 1 μM (final concentration) 5′ and 3′ primers, dNTPs (200 μM each), and 2.5 U of *Taq* DNA polymerase (Promega) in a final volume of 100 μl of 1× *Taq* DNA polymerase buffer containing 10 mM Tris-Cl (pH 8.5), 1.5 mM $MgCl_2$, and 50 mM KCl. PCR was performed with an initial denaturation step of 3 min at 91°C, and then 30 cycles were run as follows: 1 min of denaturation at 91°C, 1 min of annealing at 60°C, and a 1-min extension at 72°C. The reaction products were visualized by electrophoresis of 15 μl of the reaction mixture in a 2% agarose gel containing 0.5 μg of ethidium bromide per ml.

The specificities of the amplified bands were confirmed by

TABLE 2. Expected sizes of the PCR products

Cytokine detected	Primer pair name	PCR product (bp)	
		mRNA specific	pMCQ specific
IL-1α	I1A	308	138
IL-1β	I1BS[a]	447	NA[b]
IL-6	IL6	154	288
TNF-α	TNF	307	438
	TNFS[a]	276	NA
β-Actin	ACT	348	264
	ACTS[a]	245	NA

[a] Primers from Stratagene.
[b] NA, not applicable.

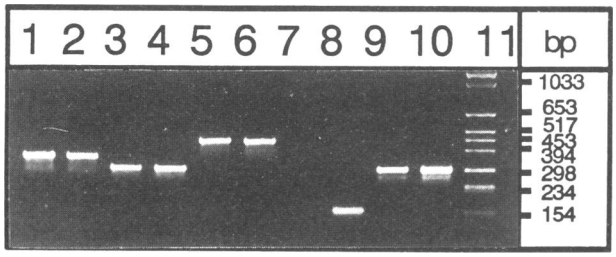


FIG. 1. Detection of mRNAs specific for β-actin (lanes 1 and 2), IL-1α (lanes 3 and 4), IL-1β (lanes 5 and 6), IL-6 (lanes 7 and 8), and TNF-α (lanes 9 and 10) in noninfected P388D$_1$ macrophages (lanes 1, 3, 5, 7, and 9) and P388D$_1$ macrophages infected with *L. monocytogenes* EGD (lanes 2, 4, 6, 8, and 10). PCR was performed as described in the text with 2 μl of cDNA, and the following primer pairs were used: ACT (lanes 1 and 2), I1A (lanes 3 and 4), I1BS (lanes 5 and 6), IL6 (lanes 7 and 8), and TNF (lanes 9 and 10) (see Table 2 for details on primer pairs). Lane 11 shows the molecular size standards. The numbers on the right represent the sizes of the molecular size standards (in base pairs).

their predicted sizes (see Table 2) based on a molecular weight standard (MWM VI; Boehringer). Densitometric analysis of the PCR products was performed with an ELSCRIPT 400 gel scanner (Hirschmann). Samples without cDNA were always included in the amplification reactions to check for contamination. All primer pairs used correspond to sequences in different exons of the representative genes; therefore, DNA-contaminated RNA preparations would produce additional products of higher molecular weight which were actually never observed.

Quantification of mRNA by competitive PCR (34). For quantification of cytokine mRNA expression, equal amounts of cDNA were amplified in the presence of 1 μl of fivefold serially diluted control plasmid pMCQ (kindly provided by I. Autenrieth, Institut for Medical Microbiology and Hygiene, Würzburg, Germany, with the permission of T. Blankenstein, Universitätsklinikum Steglitz, Berlin, Germany). This plasmid contains sequences specific for β-actin, IL-1α to IL-6, TNF-α, lymphotoxin, and INF-γ (34). Through the array ot the sequences and a linker between 5′ and 3′ sequences, the plasmid gives, upon amplification with the appropriate primers, products of known size which are different from the products obtained through the simultaneous amplification of the cDNAs (Table 2). All primer sequences for use in competitive PCR with plasmid pMCQ have been described by Murray et al. (32). Table 2 summarizes the sizes of the different expected PCR products.

TNF, IL-1α, and IL-6 assays. The TNF bioassay using L929 fibroblasts as target cells has been described in detail (37).

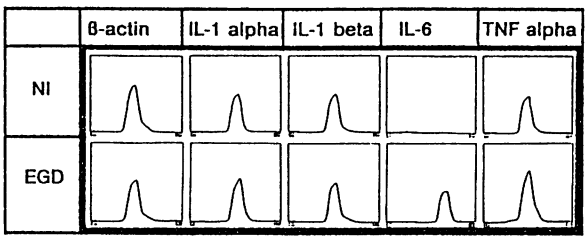
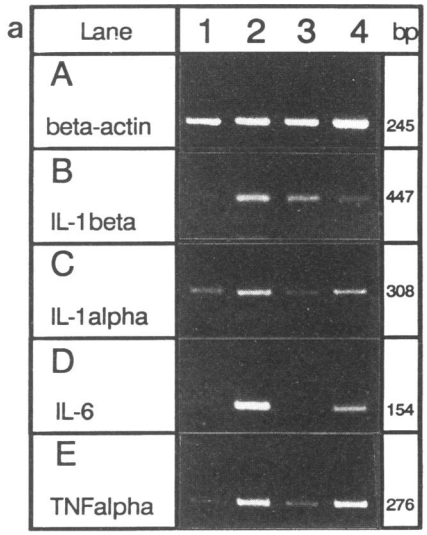


FIG. 2. Densitometric analysis of the PCR products (bands) shown in Fig. 1. NI, noninfected; EGD, infected with *L. monocytogenes* EGD.



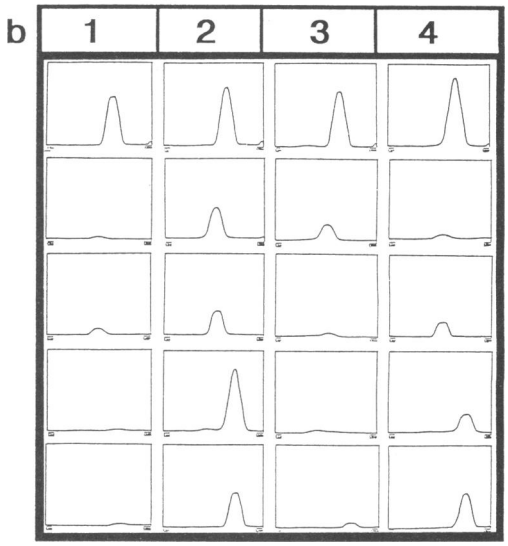


FIG. 3. (a) PCR detection of β-actin and cytokine mRNAs in *Listeria*-infected P388D$_1$ macrophages. The following primer pairs (see Table 2) and cDNA concentrations were used: A, ACTS (2 μl of 1:40-diluted cDNA); B, I1BS (8 μl of 1:40-diluted cDNA); C, I1A (8 μl of 1:40-diluted cDNA); D, IL6 (1 μl of cDNA); E, TNFS (4 μl of 1:40-diluted cDNA). Macrophages were infected with *L. monocytogenes* EGD (lane 2), heat-killed *L. monocytogenes* EGD (lane 3), *L. ivanovii* (lane 4), or PBS (lane 1). The lengths of the individual PCR products (in base pairs) are shown on the right. (b) Densitometric analysis of the PCR products (bands) shown in panel a.

Briefly, 5×10^4 L929 fibroblasts, cultured in fetal calf serum- and glutamine-supplemented RPMI medium in a 5% CO_2 atmosphere, were seeded (0.1 ml) in each well of a 96-well flat-bottom microtiter plate (Greiner). Supernatants from macrophages (see above) were twofold serially diluted in RPMI medium containing 2 μg of actinomycin (Sigma) per ml. The diluted supernatant (0.1 ml) was then added to the L929 cells, and they were incubated at 37°C for 24 h. The killing of the L929 cells could be observed directly with a microscope or after staining the remaining viable cells with crystal violet. One unit of TNF is defined as the reciprocal of the dilution of a preparation that results in 50% survival of the cells. The assay was calibrated with the use of mouse recombinant TNF-α, and

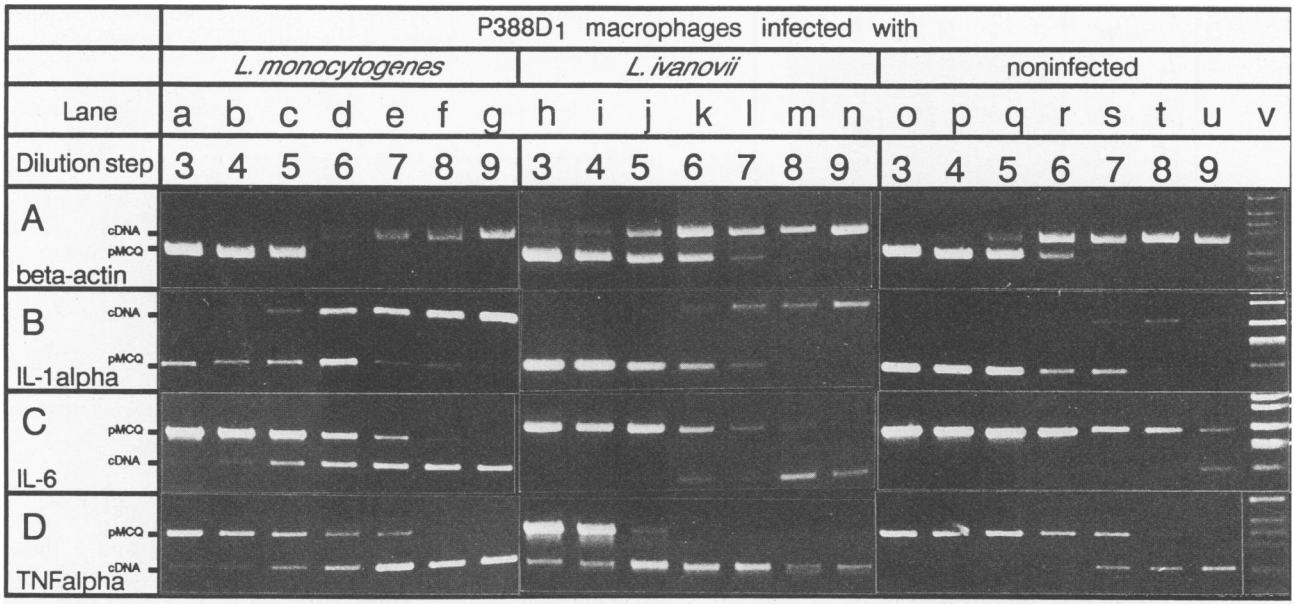


FIG. 4. Results of competitive PCR to quantify the induction of cytokine mRNAs in *L. monocytogenes*- and *L. ivanovii*-infected $P388D_1$ macrophages. Plasmid pMCQ (1 μg/μl) was fivefold serially diluted. The following cytokines were tested: β-actin (A), IL-1α (B), IL-6 (C), and TNF-α (D). The dilution steps used in the PCR are indicated by numbers. The primer pairs and cDNA volumes used were identical to those described in the legend to Fig. 3 with the following exceptions: primers ACT and TNF were used instead of ACTS and TNFS, respectively, and TNF-α-specific amplification used 8 μl of 1:40-diluted cDNA.

its specificity was controlled with a rabbit anti-mouse TNF antibody, both kindly provided by L. Lemaire (BASF, Ludwigshafen, Germany).

IL-1α (Genzyme) and IL-6 (Endogen) in the supernatants of infected macrophages were measured by using enzyme-linked immunosorbent assay kits as described in the manufacturer's instructions.

RESULTS

Induction of IL-1α, IL-1β, IL-6, and TNF-α mRNA in $P388D_1$ macrophages infected by *L. monocytogenes* and *L. ivanovii*. To investigate the role of macrophages in the induction of inflammation in listerial infections, we infected the mouse macrophage-like cell line $P338D_1$ and analyzed mRNA levels at 4 h postinfection by using standardized cDNA amplification by PCR. Low concentrations of TNF-α, IL-1α, and IL-1β mRNA and even lower levels of IL-6 could already be detected in noninfected macrophages (Fig. 1 and 2). Since basal levels of these cytokines were detected, care was taken to optimize the amount of cDNA used in the PCR to see the differences present in the mRNA levels.

Amplification of 2 μl of cDNA (diluted 1:40) with β-actin-specific primers (i.e., ACTS) resulted in bands of indistinguishable intensities with all RNA preparations, indicating that there were no significant differences in the amounts of RNA in the different preparations (Fig. 3). IL-1β mRNA was induced upon infection with viable and heat-killed *L. monocytogenes* (Fig. 3). *L. ivanovii*-infected cells showed only low levels of IL-1β mRNA induction (Fig. 3).

Induction of IL-1α mRNA seemed to be differentially regulated, and the background level of IL-1α was relatively high in noninfected macrophages. *L. monocytogenes* and *L. ivanovii*, but not heat-killed *L. monocytogenes*, induced IL-1α mRNA (Fig. 3).

mRNA for IL-6 was barely detected in noninfected macrophages, but a very significant induction took place upon infection with *L. monocytogenes* and *L. ivanovii*. This induction did not occur with heat-killed *L. monocytogenes* (Fig. 3). The pattern of induction of TNF-α mRNA was very similar to that found for IL-6 and IL-1α. *L. monocytogenes* and *L. ivanovii* strongly induced TNF-α mRNA (Fig. 3).

To obtain more quantitative data on the levels of induction of cytokine mRNAs after infection of $P388D_1$ macrophages, we performed a competitive PCR method. This technique allows titration and quantitative comparisons of mRNA levels by the use of a plasmid containing cytokine-specific sequences. mRNA-derived cDNAs and the plasmid pMCQ as an internal standard were coamplified. Through the use of a serially diluted plasmid, one can read directly from the gel at which point of dilution equal intensities of cDNA- and plasmid-derived PCR products are present. Such a competitive PCR was applied to the mRNAs of *L. monocytogenes*- and *L. ivanovii*-infected macrophages.

Figure 4 shows that the levels of β-actin mRNA were identical in all three preparations (equal band intensities at dilution step 6 for *L. monocytogenes*-infected, *L. ivanovii*-infected, and uninfected preparations [lanes d, k, and r, respectively]).

Twenty-five times more IL-1α mRNA was found in *L. monocytogenes*-infected macrophages than in noninfected cells (equal band intensities at dilution steps 6 [lane d] and 8 [lane t], respectively). *L. ivanovii* infection induced a fivefold induction of IL-1α mRNA (equal band intensities at step 7 [lane 1]). The induction of IL-6 mRNA upon infection is dramatic. Equal band intensities at dilution step 6 (lane d) after *L. monocytogenes* infection compared with those at step 9 (lane u) in noninfected macrophages represent a 125-fold induction, and *L. ivanovii* infection resulted in a 25-fold induction (step 7, lane 1).

The TNF-α mRNA levels were about 25 times higher in the *L. monocytogenes*-infected macrophages than in the control

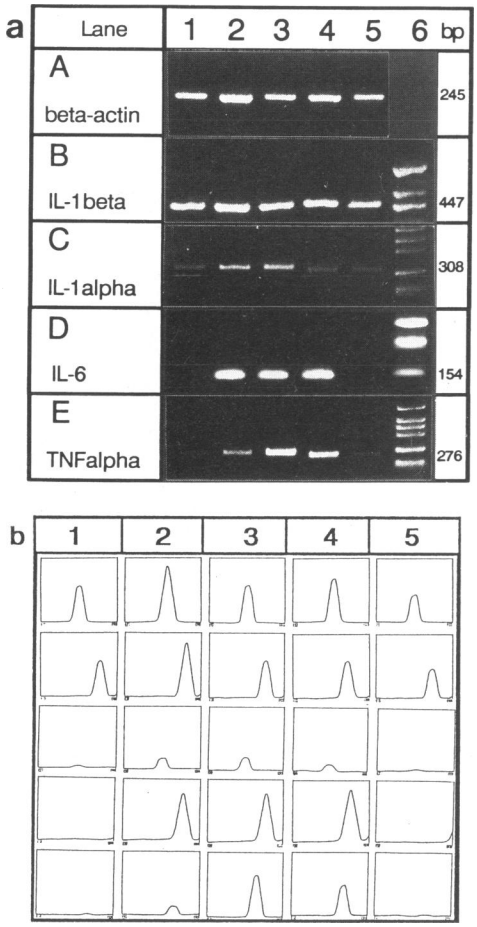


FIG. 5. (a) Detection of β-actin and cytokine mRNAs in P388D$_1$ macrophages infected with *L. monocytogenes* mutants. Macrophages were infected with *L. monocytogenes prfA* (lane 1), *L. monocytogenes plcA* (lane 2), *L. monocytogenes mpl* (lane 3), *L. monocytogenes actA* (lane 4), and *L. monocytogenes* M3 *hly* (lane 5). Primer pairs and cDNA volumes used were the identical to those described in the legend to Fig. 3. Lane 6 contains the molecular weight standards. (b) Densitometric analysis of the PCR products (bands) shown in panel a. Lanes 1 to 5 are the same as those described for panel a.

TABLE 3. Intracellular growth and calculated generation times of different *Listeria* strains inside P388D$_1$ macrophages

Listeria strain	No. of listeriae per cell[a]		Generation time (h)
	1 h[b]	6 h	
L. monocytogenes			
EGD	1.8	5.7	3.1
M3 hly	3.2	2.7	No growth
prfA	2.1	2.1	No growth
plcA	1.8	7.4	2.5
mpl	2.7	7.3	3.4
actA	2.2	9.9	2.3
L. ivanovii	1.4	4.6	2.9

[a] Macrophages were infected as described in the text and fixed and stained with giemsa at the indicated time points. The listeriae per cell were counted microscopically.
[b] Postinfection time.

(equal band intensities at steps 6 [lane d] and 8 [lane t], respectively). Moreover, *L. ivanovii* infection caused a 125-fold induction of TNF-α mRNA (equal intensities at step 5 [lane j]).

Induction of cytokine mRNA by *L. monocytogenes* requires entry into the cytoplasm. With the help of *L. monocytogenes* virulence gene mutants, it was possible to search for steps in the infectious cycle which could be essential for the induction of cytokine mRNAs. The mRNAs detected in P388D$_1$ macrophages infected with five different mutants are shown in Fig. 5. The levels of β-actin mRNA in the different RNA preparations are quite similar (Fig. 5). Mutants in the *plcA*, *mpl*, and *actA* genes (Fig. 5) all induced IL-1α, IL-6, and TNF-α mRNAs to levels more or less comparable to those induced by wild-type *L. monocytogenes* (Fig. 3). The nonhemolytic strain *L. monocytogenes* M3 *hly* with a transposon insertion in the *hlyA* gene (17) failed to induce IL-1α, IL-6, and TNF-α mRNA above the background level (Fig. 5). Infection with the *L. monocytogenes prfA* mutant in which all genes of the virulence gene cluster are transcriptionally silent (3) gave the same results as infection with the M3 *hly* mutant (Fig. 5). Although both nonhemolytic mutants of *L. monocytogenes* (M3 *hly* and *prfA*) are taken up by the macrophages with the same efficiency

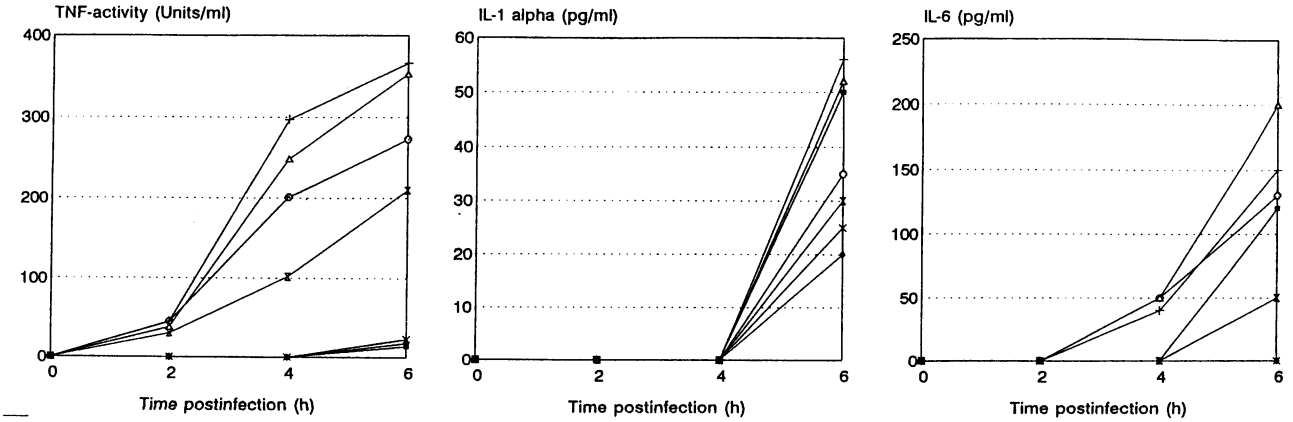


FIG. 6. Induction and time course of activity of TNF IL-1α, and IL-6 in the supernatant of P388D$_1$ macrophages infected with different mutants of *L. monocytogenes* compared with those infected with the wild-type strain and *L. ivanovii*. Infection of macrophages and cytokine measurement were performed as described in Materials and Methods. Symbols: +, *L. monocytogenes* EGD; ■, *L. ivanovii*; ×, *L. monocytogenes hly*; ◆, *L. monocytogenes prfA*; △, *L. monocytogenes plcA*; ⧖, *L. monocytogenes actA*; ○, *L. monocytogenes mpl*.

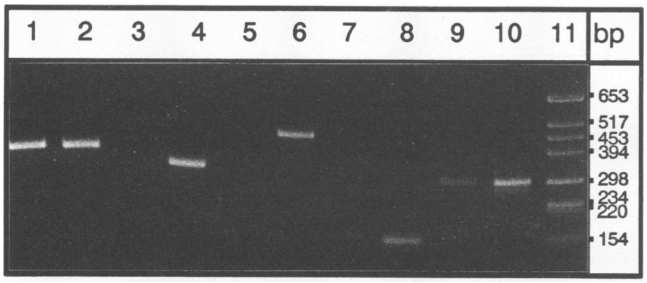


FIG. 7. Induction of cytokine mRNAs in bone-marrow-derived macrophages. mRNAs specific for β-actin (lanes 1 and 2), IL-1α (lanes 3 and 4), IL-1β (lanes 5 and 6), IL-6 (lanes 7 and 8), and TNF-α (lanes 9 and 10) in noninfected macrophages (lanes 1, 3, 5, 7, and 9) and macrophages infected with *L. monocytogenes* EGD (lanes 2, 4, 6, 8, and 10). PCR was performed as described in the text with 2 μl of cDNA, and the following primer pairs were used: ACT (lanes 1 and 2), I1A (lanes 3 and 4), I1BS (lanes 5 and 6), IL6 (lanes 7 and 8), and TNF (lanes 9 and 10). Lane 11 contains the molecular size standards. The numbers to the right represent the molecular size standards (in base pairs).

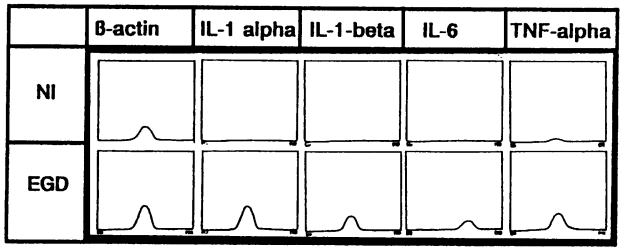


FIG. 8. Densitometric analysis of the PCR products (bands) shown in Fig. 7 NI, noninfected; EGD, infected with *L. monocytogenes* EGD.

as wild-type *L. monocytogenes* (Table 3), they are unable to lyse the phagosomal membrane. Therefore, they cannot escape into the cytoplasm of the host cell and do not replicate (Table 3).

The transcription of the IL-1β gene seemed again to be differentially regulated since all of the mutant strains tested were indistinguishable (hemolytic and nonhemolytic ones) in their levels of IL-1β mRNA induction (Fig. 5).

Levels of cytokines in the supernatants of *Listeria*-infected P388D$_1$ macrophages correspond in most cases to mRNA levels. To identify whether the levels of cytokine mRNAs detected by PCR directly correspond to activity, we measured TNF, IL-1α, and IL-6 in the supernatants of *Listeria*-infected macrophages at different time points postinfection. None of the cytokines was detectable in the supernatant of noninfected P388D$_1$ macrophages or macrophages infected with heat-killed *L. monocytogenes*. This was surprising because TNF-α- and IL-1α-specific mRNAs were always detectable. Infection with *L. monocytogenes* resulted in a strong induction of TNF and IL-6 which was time dependent through 6 h. TNF activity was first detectable at 2 h and significantly increased between 2 and 4 h postinfection. IL-6 in the supernatant first appeared at 4 h and dramatically increased between 4 and 6 h postinfection. The levels of IL-1α in the supernatant were low, and IL-1α was not detectable before 6 h postinfection (Fig. 6).

More or less the same amounts and similar time courses of cytokine production were found in macrophages infected with the *plcA*, *mpl*, and *actA* mutants, with the *actA* mutant being always somewhat less effective in cytokine induction. The nonhemolytic strains (*L. monocytogenes prfA* and *L. monocytogenes* M3 *hly*) induced only a weak increase in TNF activity, which was first detectable at 4 h postinfection and reached less than 15% of the induction observed with macrophages infected with wild-type *L. monocytogenes*. These two strains also did not induce any measurable IL-6 (Fig. 6). In two cases, however, there was no clear correlation between the mRNA level in the macrophages and the amount of cytokine detected in the supernatant after infection. *L. ivanovii* induced significant amounts of TNF-α mRNA (Fig. 3), but TNF activity was found only in low amounts at 6 h postinfection (Fig. 6). On the other hand, IL-1α mRNA levels were low in P388D$_1$ macrophages infected with the nonhemolytic strains M3 *hly* and *prfA*, but IL-1α levels in the supernatant reached about 50% of those found with the wild-type strain.

Proinflammatory cytokines are also induced in *L. monocytogenes*-infected bone-marrow-derived macrophages. To confirm the induction of cytokine mRNAs in normal macrophages, we prepared bone-marrow-derived macrophages from the *Listeria*-susceptible mouse strain BALB/c. The differentiated macrophages were infected, and cytokine-specific mRNAs were detected as described in Materials and Methods. As shown in Fig. 7 and 8, all four cytokine mRNAs tested were induced upon infection. Background levels of the cytokine mRNAs were hardly detectable when IL-1α-, IL-1β-, and IL-6-specific primer pairs were used in the PCR. Only some TNF-α-specific mRNA was detectable even in noninfected bone-marrow-derived macrophages. Cytokine activity in the supernatants of *L. monocytogenes*-infected bone-marrow-derived macrophages was also detectable as outlined in Fig. 9. TNF activity appeared first at 2 h postinfection and finally reached levels comparable to those found in P388D$_1$ macrophages. In contrast, only low amounts of IL-1α were detectable at 6 h postinfection with *L. monocytogenes*. On the other hand, large amounts of IL-6 (1,250 pg/ml) were found in the supernatant of bone-marrow-derived macrophages infected with *L. monocytogenes* at 6 h postinfection.

DISCUSSION

We have shown that the infection of the mouse macrophage-like cell line P388D$_1$ and bone-marrow-derived macrophages with different *Listeria* strains could induce a number of cytokine-specific mRNAs. By the use of a competitive PCR, we were able to quantify the amounts of mRNA and present relative values for the steady-state mRNA levels in the P388D$_1$ macrophages before and after infection with *Listeria* strains.

The P388D$_1$ cell line possesses most of the characteristics of normal macrophages (20). It has been shown, however, that the cells produce low amounts of IL-1 activity even without stimulation (1). Accordingly, we were able to detect IL-1α- and IL-1β mRNAs in the noninfected macrophages although at relatively low levels. Nevertheless, we were able to demonstrate induction of cytokine mRNAs after *Listeria* infection.

Interestingly, we found different responses with different *Listeria* species. Only *L. monocytogenes*, and not *L. ivanovii*, induced IL-1β mRNA. *L. ivanovii*, which is able to grow inside macrophages, caused at best a weak induction of IL-1β mRNA; however, this species did elicit elevated levels of the other cytokines, including TNF-α mRNA, comparable to those found after infection with *L. monocytogenes*. It was, therefore, quite astonishing that in the TNF activity assays little to no TNF activity was detected in the supernatant of P388D$_1$ macrophages after infection with *L. ivanovii*. This contrasted with the high levels of TNF activity found in the supernatant of

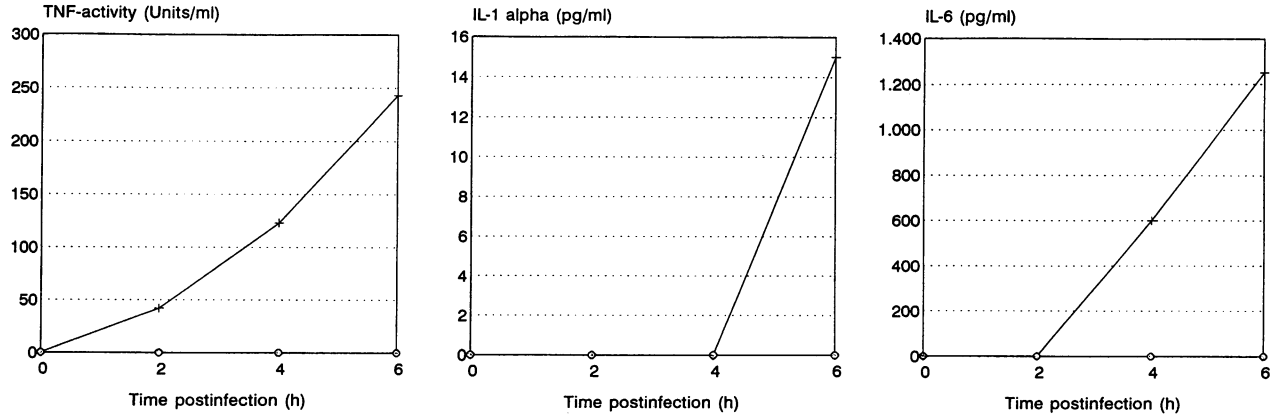


FIG. 9. Induction and time course of TNF activity, IL-1α, and IL-6 in the supernatant of bone-marrow-derived macrophages infected with *L. monocytogenes*. Infection of macrophages and cytokine measurement were performed as described in Materials and Methods. Symbols: ○, control; +, *L. monocytogenes* EGD.

L. monocytogenes-infected P388D_1 macrophages. We hypothesized that the addition of recombinant IL-1β probably could induce the synthesis of active TNF in *L. ivanovii*-infected macrophages, but neither IL-1β alone nor IL-1β in combination with *L. ivanovii* infection could induce any TNF activity (data not shown). We do not know what this species-specific difference in the host cell response means, but it may be one of the reasons for the differences in virulence observed with these organisms.

Low levels of TNF-α mRNA were also detected in noninfected P388D_1 macrophages, showing that these macrophage-like cells are permanently activated to some extent. On the other hand, we never detected any TNF activity in the supernatants of noninfected P388D_1 cells. These data are in agreement with the low translational activity of TNF-α mRNA (2, 22). It is also known that stimulation with lipopolysaccharide increases the translation rate per unit of TNF-α mRNA up to 200-fold in macrophages (11), which shows that TNF activity is indeed regulated at both the transcriptional and translational levels. Such translational activation could also account for the low levels of TNF activity observed in *L. monocytogenes prfA*-infected P388D_1 macrophages without any increase in TNF-α mRNA levels. Unfortunately, it is not known which mechanism(s) in *L. ivanovii*-infected macrophages could repress efficient translation of the TNF-α mRNA or the transport of the protein into the supernatant. Proteolytic degradation of the TNF protein could also account for the missing TNF activity in the supernatant of the *L. ivanovii*-infected P388D_1 macrophages. IL-6 mRNA synthesis and IL-6 levels in the supernatant were strongly induced after infection with hemolytic and intracellularly growing *Listeria* spp., with inductions of 125- and 25-fold for *L. monocytogenes* and *L. ivanovii*, respectively. IL-6 mRNA was found in barely detectable levels in noninfected P388D_1 cells. This finding implies that the expression of this particular cytokine is strictly controlled in P388D_1 macrophages, supporting the observations of Iizawa et al. (15) that, in contrast to IL-1α and TNF-α mRNA, no IL-6 mRNA was found in the spleens and livers of noninfected mice.

The use of bacterial mutants allowed us to analyze whether specific bacterial factors are necessary for a specific host response. Therefore, we used mutants which are deficient in different steps of the infection cycle. Indeed, a striking correlation between hemolytic activity of the *Listeria* spp. and the property to induce cytokine mRNAs (IL-1α, IL-6, and TNF-α) was found in P388D_1 macrophages. The two nonhemolytic mutants used, *L. monocytogenes prfA*, in which all genes of the virulence regulon are not expressed (3, 26), and *L. monocytogenes* M3 *hly*, in which the gene coding for listeriolysin O is inactivated by a transposon insertion in the promoter region (17), are both unable to induce the cytokine mRNAs mentioned above as well as IL-6 and TNF-activity in the supernatant. These data imply that the lysis of the phagosome membrane and the subsequent escape of the *Listeria* spp. into the host cytoplasm, for which listeriolysin O is absolutely required, are the critical steps necessary for the induction of IL-1α, IL-6, and TNF-α mRNAs. Additionally, it became obvious that mutants in the genes coding for the metalloprotease, phospholipase C, and *actA* are not significantly impaired in the induction of cytokine mRNAs. Mouse macrophages and epithelial cells can be stimulated to produce IL-6 by staphylococcal exotoxin and *Escherichia coli* P fimbria, respectively (9, 14, 44). It was therefore surprising that, in our system, phagocytosis of *Listeria* spp. without intracellular growth did not induce IL-1α, IL-6, or TNF-α mRNA. Phagocytosis and the intracellular presence of the bacteria are sufficient to induce only IL-1β mRNA synthesis. IL-1β was also the only cytokine which was induced by heat-killed *L. monocytogenes*, further supporting the view that the transcriptional activation of the IL-1β gene does not require intracellular growth of the *Listeria* spp. inside the P388D_1 macrophages. The IL-1α levels in the supernatant of infected P388D_1 macrophages were always very low, indicating a low translational efficiency of the IL-1α mRNA. The application of a competitive PCR allowed us to calculate mRNA levels relative to β-actin mRNA as an internal standard. It is important to note that the basal levels of IL-1α and TNF-α mRNA in P388D_1 macrophages were in the same range but IL-6 mRNA levels in noninfected cells were extremely low. Interestingly, the strongest induction (125-fold) was observed in the case of this strictly regulated cytokine.

The rapid increase of cytokine mRNAs for IL-1α, IL-1β, IL-6, and TNF-α in the cultured macrophage-like cell line P388D_1 after infection with intracellularly growing *Listeria* spp., especially *L. monocytogenes*, might resemble the first step in vivo in controlling listerial infection. The simultaneous release of these synergistically active inflammatory cytokines might contribute to initiate the onset of T-cell-mediated restriction of listerial growth after infection.

It is known that the macrophages in the liver and spleen are very rapidly colonized after infection of laboratory animals with *L. monocytogenes*. To determine whether such natural macrophages respond to listerial infection in vitro, we prepared bone-marrow-derived macrophages and infected them with *L. monocytogenes*. The levels of all four cytokine mRNAs tested (IL-1α, IL-1β, IL-6, and TNF-α) were significantly elevated after infection with *L. monocytogenes* at 4 h postinfection in bone-marrow-derived macrophages. Additionally, we showed that TNF activity and IL-6 in the supernatant of infected bone-marrow-derived macrophages also increase. The high levels of IL-6 present in the supernatant underline the importance of this particular cytokine. In contrast, enhanced transcription of the IL-1α gene is not followed by efficient translation and/or transport since we found only minimal amounts of IL-1α in the supernatant, which implies a limited significance of this cytokine in establishing an antilisterial host response. These data obtained by infection of natural macrophages correspond well to the results we got by infection of the $P388D_1$ macrophage-like cell line. Permanent macrophage-like cell lines can therefore be regarded as useful model systems for the behavior of normal macrophages in vitro.

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