INFECTION AND IMMUNITY, July 1994, p. 2740–2747
0019-9567/94/$04.00+0

Vol. 62, No. 7

Listeria monocytogenes Infection Enhances Transcription Factor NF-κB in P388D$_1$ Macrophage-Like Cells

NADJA HAUF,[1] WERNER GOEBEL,[1] EDGAR SERFLING,[2] AND MICHAEL KUHN[1]*

Lehrstuhl für Mikrobiologie, Theodor-Boveri-Institut für Biowissenschaften, Universität Würzburg, 97074 Würzburg,[1] and Pathologisches Institut der Universität Würzburg, 97080 Würzburg,[2] Germany

Received 20 December 1993/Returned for modification 3 February 1994/Accepted 12 April 1994

In the present study, we investigated the effect of *Listeria monocytogenes* infection on the cellular level of the transcription factors NF-κB, AP-1, and NF-IL6 in the macrophage-like cell line P388D$_1$ by using electrophoretic mobility shift assays. Infection with *L. monocytogenes* enhanced the formation of two NF-κB-like DNA-protein complexes, C1 and C2, whereas the concentration of AP-1 and NF-IL6 complexes remained unaffected. In supershift assays using NF-κB-specific antibodies, complex C2 was identified to be a p50 homodimer (KBF1) and complex C1 was identified as a p50/p65 heterodimer. Both complexes were formed within 10 min after addition of the bacteria. Since the synthesis of tumor necrosis factor alpha and interleukin-1 occurs at later times, these cytokines cannot be the mediators of enhanced NF-κB formation. Infection experiments with different nonhemolytic mutants of *L. monocytogenes* and the use of the phagocytosis inhibitor cytochalasin B suggest that events prior to invasion and escape of the bacteria from the phagosome into the cytoplasm enhance the nuclear transport of p50/p65 NF-κB components.

Some of the crucial steps in the interaction of pathogenic bacteria with their eucaryotic hosts have been efficiently studied in recent years by using cell culture techniques. These studies have revealed many virulence factors which enable pathogenic bacteria to interact with host cells (20, 21). However, until recently, the eucaryotic cells have been regarded more or less as passive partners in these interactions.

Listeria monocytogenes, a gram-positive bacterium and causative agent of food-borne septicemia and meningitis (54), has been widely used as a model system of facultative intracellular bacteria (12, 40, 48). *L. monocytogenes* can invade, survive, and replicate within nonprofessional phagocytic cells like enterocytes (23) and fibroblasts as well as in professional phagocytes such as macrophages (34, 49). It has been conclusively shown by a number of investigators that the extracellular protein listeriolysin O is absolutely required for intracellular survival and replication (23, 34, 49). The gene encoding listeriolysin O (43) is part of a gene cluster which includes genes encoding a phosphatidylinositol-specific phospholipase C (*plcA*) (35, 41), a metalloprotease (*mpl*) (15, 42), a protein necessary for induction of actin polymerization (*actA*) (16, 31), and a lecithinase (*plcB*) (57). These genes, the internalin gene (22), and several others which have been identified only through their gene products and which are not part of the virulence gene cluster (55) are under the control of the transcriptional activator PrfA (10, 17, 37).

Recent reports have established the in vitro induction of cytokines in fibroblasts, epithelial cells, and macrophages in response to infections with *L. monocytogenes* (4, 25, 26). We have previously used the P388D$_1$ macrophage-like cell line and different mutants of *L. monocytogenes* to study the induction of interleukin-1 alpha (IL-1α), IL-1β, IL-6, and tumor necrosis factor alpha (TNF-α) in response to infection (33). These studies demonstrated that proinflammatory cytokine mRNAs are induced upon infection with *L. monocytogenes*, but nonhemolytic mutants (28, 36), which are unable to escape from the phagosome into the cytoplasm (23), did not induce IL-1α, IL-6, or TNF-α (33). To date, nothing about the mechanisms leading to cytokine mRNA induction has been known.

Analysis of the eucaryotic transcription factors activated during the infection process may provide a first insight into these mechanisms. Different transcription factors may be involved in the regulation of cytokine gene expression. Activator protein 1 (AP-1) (13) is involved in the induction of a variety of target genes in response to stimulation of cell surface receptors that are connected to several different signal transduction pathways (3). Nuclear factor IL6 (NF-IL6) was originally described as a factor necessary for the transcription of the IL-6 gene (1) but also plays a role in the transcriptional regulation of other genes (46). Nuclear factor κB (NF-κB), a pleiotropic mediator of inducible and tissue-specific gene control (38), is involved in the transcription of a variety of genes, many of which are activated during the immune response (e.g., those encoding cytokines [18, 38, 39]). The major form of NF-κB consists of two different subunits, i.e., a 50-kDa DNA binding protein which also forms homodimers, termed KBF1, and a 65-kDa protein (5, 24, 29). The p65 subunit serves as a receptor for inhibitory protein IκB (6, 7, 47), which can prevent NF-κB from binding to its target sequence. In unstimulated cells, NF-κB is located in an inactive form in the cytoplasm because of the complex formation with IκB (7). Different inducers such as lipopolysaccharide, double-stranded RNA phorbol esters, and viruses allow NF-κB to be released from the complex and enter the nucleus. TNF-α, TNF-β, and IL-1 can also activate NF-κB, suggesting mechanisms for regulatory loops (27, 38, 45).

Recently, it was shown that heat-killed *Staphylococcus aureus* and several of its exotoxins as well as the invasive species *Shigella flexneri* induce NF-κB in murine macrophages and HeLa cells, respectively (9, 19). In this report, we present data on the effect of *L. monocytogenes* infection on the generation of nuclear NF-κB complexes in the macrophage-like cell line P388D$_1$. We show that infection with a virulent strain results in the rapid formation of p50/p65 heterodimeric NF-κB com-

* Corresponding author. Mailing address: Lehrstuhl für Mikrobiologie, Theodor-Boveri-Institut für Biowissenschaften der Universität Würzburg, Am Hubland, 97074 Würzburg, Germany. Phone: 49-931-8884421. Fax: 49-931-8884402.

plexes which might be involved in the subsequent induction of TNF-α and IL-1 genes.

MATERIALS AND METHODS

Bacteria. Wild-type *L. monocytogenes* Sv 1/2a strain EGD was provided by S. H. E. Kaufmann (Ulm, Germany). The nonhemolytic *L. monocytogenes* strain M3 derived by transposon Tn*916* insertion into the promoter region of the *hly* gene has been described previously (28). The metalloprotease-negative mutant (*mpl*) of *L. monocytogenes* EGD was constructed through plasmid integration by homologous recombination using the cloned *mpl* gene (14). *L. monocytogenes prfA* (SLCC 53) and *Listeria innocua* Sv 6a (NCTC 11288) were obtained from the Special Listeria Culture Collection of the Institute for Hygiene and Microbiology, Würzburg, Germany. Listeriae used for cell culture infections were grown in brain heart infusion broth (Difco) at 37°C with aeration. Erythromycin (5 μg/ml) and tetracycline (10 μg/ml) were added when mutants *mpl* and M3, respectively, were grown. Mid-log-phase cultures were washed once in phosphate-buffered saline (PBS) and stored at −80°C in aliquots in PBS, with glycerol added to 15% (vol/vol).

Mammalian cell culture and infection. $P388D_1$ macrophages (32) were routinely grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 μg of streptomycin per ml, and 100 U of penicillin per ml (all from Gibco) in a humified 5% CO_2 atmosphere at 37°C. Macrophages were seeded for 48 h prior to infection in tissue culture plates (Greiner) at 5×10^6 cells per plate. Twenty hours prior to infection, the medium was replaced by RPMI 1640 medium supplemented with 0.5% fetal calf serum and 2 mM L-glutamine. The macrophages were infected with listeriae to give a multiplicity of infection of 50 bacteria per eucaryotic cell and incubated for 40 min if not otherwise indicated. They were washed with PBS and incubated further in medium containing gentamicin (50 μg/ml) to kill extracellular bacteria and prevent reinfection. In the experiments using cytochalasin B, the cells were preincubated with 10 μM cytochalasin B (Sigma Chemicals Co., St. Louis, Mo.) for 30 min before the bacteria were added. To inhibit TNF-α and IL-1 activity, the macrophages were infected in the presence of recombinant soluble TNF receptor (sp55) and/or recombinant IL-1 receptor antagonist at concentrations of 1 μg/ml each. Both proteins were kindly provided by P. Scholz (Schering AG, Berlin, Germany).

Preparation of nuclear protein extracts. At the indicated time points following infection, crude nuclear protein extracts were prepared essentially as described by Schreiber et al. (53), with minor modifications. The washed macrophages were scraped from two culture plates and collected by centrifugation (10 min, 4°C, 1,000 × *g*). Pellets were resuspended in 0.6 ml of hypotonic lysis buffer containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-teraacetic acid (EGTA), 1 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride. After the suspension was incubated for 15 min on ice, Nonidet P-40 was added to give a final concentration of 0.5%, and the cells were vortexed for 10 s. The cells were centrifuged (30 s, 4°C, 12,000 × *g*), and nuclear pellets were resuspended in 0.14 ml of buffer containing 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride and vortexed for 30 min at 4°C. After centrifugation (5 min, 4°C, 12,000 × *g*), the supernatant (i.e., nuclear extract) was frozen in aliquots at −80°C.

Preparation of labeled DNA fragments and electrophoretic mobility shift assay (EMSA). The AP-1-specific oligonucleotide was prepared by *Hin*dIII digestion of plasmid $p5xTRE_{coll}$ (2), and the TRE_{coll} fragment was eluted from low-melting-point agarose gels and purified by phenol treatment. The other oligonucleotides (see Table 1) were chemically synthesized and annealed after being heated to 95°C. The complementary strands created 5′ overhanging ends which allowed labeling by the Klenow polymerase (Pharmacia) with [^{32}P]dATP and [^{32}P]dCTP (Amersham).

The binding reactions (53) were performed on ice in a volume of 15 μl and contained 5 μl of 3× binding buffer (60 mM HEPES [pH 7.9], 3 mM dithiothreitol, 3 mM EDTA, 150 mM KCl, 12% Ficoll), 20,000 cpm of ^{32}P-labeled DNA probe, 2 to 4 μg of nuclear proteins, and 0.2 to 2 μg of poly(dI-dC) (Boehringer Mannheim) optimized for each type of reaction. After 30 min on ice, the DNA-protein complexes were separated on native 5% polyacrylamide gels (prerun at 250 V for 1.5 h) in low-ionic-strength buffer (0.4× Tris-borate-EDTA) at 200 V for 2 to 4 h at room temperature. After electrophoresis was performed, the gels were fixed, vacuum dried, and exposed for autoradiography at −70°C for 12 to 36 h. In competition studies, unlabeled oligonucleotides were included in the reaction mixtures in a 1- to 50-fold molar excess.

Supershift experiments. The affinity-purified rabbit anti-p50 and anti-p65 antibodies [NF-κB p50 (A)X and NF-κB p65 (NFL)X, respectively] were supplied at a concentration of 1 mg/ml by Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.). A rabbit anti-p50 antiserum (serum 2) (30) was kindly provided by A. Israel (Institut Pasteur, Paris, France). The monoclonal anti-TNF-α antibody (2 mg/ml) was kindly provided by L. Lemaire (BASF, Ludwigshafen, Germany). Antibodies (2 μg per reaction) were included in the standard reaction mixture and incubated on ice for 20 min before the labeled oligonucleotides were added. Densitometric analysis of DNA-protein complexes from EMSA or supershift assays was performed with an ELSCRIPT 400 densitometer (Hirschmann Instruments).

mRNA detection with RT-PCR. The detection of mRNA with reverse transcription-preceded PCR (RT-PCR) has been described recently in detail (33). Briefly, total RNA was isolated by the guanidine-thiocyanate method (11), and cDNA was synthesized with a first-strand synthesis kit (Stratagene) as described in the supplier's instructions. The PCR was performed as described previously (51). Amplification was performed in a Thermocycler 60/2 (Bio-Med): initial denaturation, 3 min at 91°C; primary denaturation, 1 min at 91°C; primer annealing, 1 min at 60°C; primer extension, 1 min at 72°C; and a prolonged primer extension following the last cycle, 2 min at 72°C. Thirty cycles were always run. For the PCR, fourfold more cDNA was used to detect TNF-α, IL-1α or IL-1β mRNA than to detect β-actin mRNA. The primers used in the PCR are listed in Table 1.

Measurement of IL-1α and TNF-α. The TNF assay using L929 fibroblasts as target cells has been described previously (50). IL-1α was measured by using a commercially available enzyme-linked immunosorbent assay kit (Genzyme).

RESULTS

Transcription factor NF-κB but not AP-1 or NF-IL6 is induced in murine macrophage-like cells upon infection by *L. monocytogenes*. The transcription factors NF-κB, AP-1, and NF-IL6 control the activity of numerous genes in hematopoetic cells. To determine if the generation of these transcription factors is affected by *L. monocytogenes* infection, EMSAs, using

TABLE 1. Sequences of the oligonucleotides used in EMSA and RT-PCR

Assay and oligonucleotide	Sequence(s)[a]	Product (bp)	Source or reference
EMSA			
TRE_{coll}	5′-AGCTTGATGAGTCAGCCG-3′ 3′-ACTACTCAGTCGGCCTAG-5′		Angel et al. (2)
κB consensus	5′-AGCTTCAGAGGGGACTTTCCGAGAGG-3′ 3′-AGTCTCCCCTGAAAGGCTCTCCAGCT-5′		Schreck et al. (52)
κB mutant	5′-AGCTTCAGAGCTCACTTTAAGAGAGG-3′ 3′-AGTCTCGAGTGAAATTCTCTCCAGCT-5′		
NF-IL6 consensus	5′-AGCTTTAAGATTGCACAATGTGACGTCA-3′ 3′-AATTCTAACGTGTTACACTGCAGTTCGA-5′		Natsuka et al. (46)
RT-PCR			
IL-1α			
Sense	5′-CTCTAGAGCACCATGCTACAGAC-3′	308	Murray and Martens (44)
Antisense	5′-TGGAATCCAGGGGAAACACTG-3′		
IL-1β			
Sense	5′-CAGGATGAGGACATGAGCACC-3′	447	Stratagene[b]
Antisense	5′-CTCTGCAGACTCAAACTCCAC-3′		
TNF-α			
Sense	5′-GGCAGGTCTACTTTGGAGTCATTGC-3′	307	Murray and Martens (44)
Antisense	5′-ACATTCGAGGCTCCAGTGAATTCGG-3′		
β actin			
Sense	5′-TGGAATCCTGTGGCATCCATGAAAC-3′	348	Murray and Martens (44)
Antisense	5′-TAAAACGCAGCTCAGTAACAGTCCG-3′		

[a] Putative binding sites are underlined except for the κB mutant, for which mutated bases are underlined.
[b] Primer pairs from Stratagene.

nuclear extracts of $P388D_1$ cells prepared either after 4 h postinfection with *L. monocytogenes* or from uninfected control cells, were performed. A consensus sequence for the binding of AP-1, derived from the phorbol 12-myristate 13-acetate-responsive element of the collagenase gene (TRE_{coll}), exhibited no difference in the complexes formed with nuclear proteins from infected or noninfected $P388D_1$ macrophages (Fig. 1). To examine the specificity of the DNA binding capability induced upon infection, cold double-stranded oligonucleotide was added for competition. A decrease in the amount of bound complex was observed as the concentrations of unlabeled AP-1 consensus sequence increased, while a 10-fold molar excess of a κB consensus oligonucleotide was without effect on the complex formation (Fig. 1).

NF-IL6 has been described to be involved in the regulation of transcription of the IL-6 gene and other stress- and immunologically related genes (1, 46, 56). Constitutive DNA binding activity of nuclear extracts from $P388D_1$ macrophages was also observed by using a NF-IL6-specific oligonucleotide (Fig. 1). Infection of the macrophages with *L. monocytogenes* and subsequent 4-h intracellular growth of the bacteria did not result in any measurable change in the NF-IL6-specific binding activity (Fig. 1).

When a κB consensus site was used in EMSAs with the nuclear extracts of *L. monocytogenes*-infected $P388D_1$ cells, the generation of two complexes (C1 and C2) was found to be elevated. The slower-migrating complex C1 demonstrated a much more elevated binding capability than complex C2 (Fig. 2). Unlabeled DNA demonstrated a dose-dependent inhibition (Fig. 2). A mutated κB double-stranded oligonucleotide changing the GGG motive to CTC at bases 11 to 13 and CC to AA at bases 19 to 20 failed to inhibit the shift at all concentrations tested (Fig. 2). These results confirmed the specificity of the DNA-binding activity.

The most prominent form of transcription factor NF-κB has been described as a heterodimer consisting of two proteins, p50 and p65 (5). A homodimer of p50 named KBF1 (58) which binds to κB motifs has also been identified (8). Antibodies raised against p50 and p65 were used to prove whether complex C2 might represent a p50/p50 homodimer (KBF1) and whether C1 might represent a p50/p65 heterodimer (NF-κB) bound to the κB double-stranded oligonucleotide. Exper-

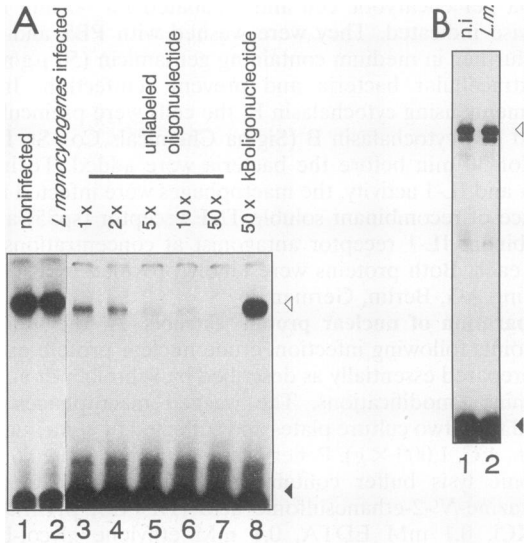

FIG. 1. AP-1- and NF-IL6-like DNA-binding proteins in nuclear extracts from $P388D_1$ macrophages infected with *L. monocytogenes* at 4 h postinfection. (A) AP-1-like complexes in extracts from noninfected (lane 1) and infected (lane 2) cells measured by EMSA. Competition experiments were done with extracts from infected cells and a 1- to 50-fold molar excess of cold TRE_{coll} oligonucleotide (lanes 3 to 7) and a 50-fold molar excess of a nonspecific oligonucleotide (κB consensus site) (lane 8). (B) EMSA using an NF-IL6-specific oligonucleotide and extracts from noninfected (n.i.) (lane 1) and *L. monocytogenes*-infected (*L.m.*) (lane 2) macrophages. Open arrowheads indicate DNA protein complexes; filled arrowheads indicate the free DNA probe.

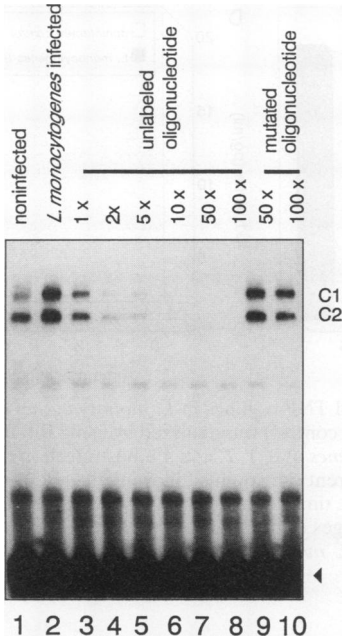

FIG. 2. *L. monocytogenes* infection enhances the generation of NF-κB- or KBF1-like factors in nuclear protein extracts from P388D$_1$ macrophages at 4 h postinfection. EMSAs were performed with a κB oligonucleotide and extracts from noninfected (lane 1) and infected (lane 2) cells. Competition experiments were done with extracts from infected cells, a 1- to 100-fold molar excess of cold oligonucleotide (lanes 3 to 8), and a 50- to 100-fold molar excess of a defective κB oligonucleotide mutated in five bases of the NF-κB consensus site (lanes 9 and 10). C1 and C2 indicate the NF-κB-like DNA-protein complexes. The arrowhead indicates the free DNA probe.

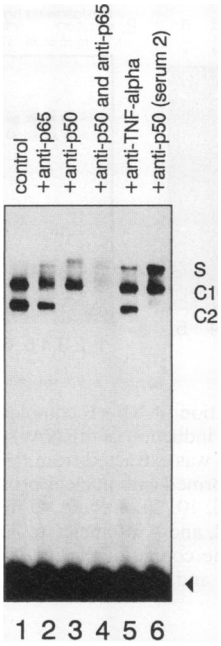

FIG. 3. *L. monocytogenes*-enhanced complexes C1 and C2 contain NF-κB-like features. Supershift experiments were performed to identify the proteins in complexes C1 and C2 induced in P388D$_1$ macrophages infected with *L. monocytogenes*. Nuclear extracts from *L. monocytogenes*-infected macrophages were preincubated with antibodies raised against p65 (lane 2), p50 (lane 3), p50 and p65 (lane 4), and another antiserum directed against human p50 (i.e., serum 2) (lane 6) before the addition of the labeled κB oligonucleotide. The use of antibodies obtained from Santa Cruz Biotechnology resulted in the disappearance of the respective bands (lanes 2, 3, and 4), whereas the use of serum 2 resulted in a supershift (S) of the p50/p50 homodimer (lane 6). Control experiments were done without antibodies (lane 1) or with anti-TNF-α antiserum (lane 5). The arrowhead indicates the free DNA probe.

iments were performed in which the nuclear extracts were preincubated either with anti-p50 antisera, anti-p65 antisera, both antisera simultaneously, or a nonspecific antiserum (anti-TNF-α). The results shown in Fig. 3 and Table 2 clearly support our assumption. Preincubation with anti-p65 antibody led to a significant reduction (85%) of complex C1 (p50/p65) and to a weak and nonspecific reduction of complex C2 (p50/p50), which was also observed when the anti-TNF-α antibody was used. Preincubation with anti-p50 antisera led either to the disappearance of C2 (p50/p50) or to the disappearance of C2 and the simultaneous appearance of a supershifted complex S when antiserum 2 was used. The anti-p50 antibodies NF-κB p50 (NL5)X and serum 2 are unable to bind to p50/p65 complexes in the mouse system (Santa Cruz Biotechnology) (27a). A combination of the antibodies directed against p50 and p65 led to the disappearance of both complexes; however, the anti-TNF-α antiserum had only weak nonspecific activity.

***L. monocytogenes*-induced NF-κB activation appears not to be mediated by IL-1α, IL-1β, or TNF-α.** We have reported recently on the induction of IL-1α, IL-1β, and TNF-α mRNA in P388D$_1$ macrophages after infection with listeriae (33). Since these cytokines are known to activate NF-κB in macrophages (27, 45), *Listeria*-induced NF-κB DNA binding activity might occur by an autocrine mechanism after TNF-α, IL-1α, or IL-1β secretion. To test this possibility, we stimulated P388D$_1$ macrophages with *L. monocytogenes* and measured the induction of mRNAs for IL-1α, Il-1β, and TNF-α. In parallel, TNF-α and IL-1α were measured, and the DNA binding of NF-κB DNA was investigated in EMSAs. The results of these analyses, summarized in Fig. 4, show that enhanced NF-κB binding activity occurs within 10 to 20 min after infection of macrophages. However, cytokine mRNA induction occurred significantly later (initially at 1 h postinfection), and IL-1α and

TABLE 2. Densitometric analysis of the DNA-protein complexes shown in Fig. 3

Antisera[a]	Complex	Area[b]	% Area (relative to control)
Control (no antisera)	C1	554	100
	C2	557	100
Anti-TNF-α	C1	387	70
	C2	245	44
Anti-p65	C1	81	15
	C2	288	52
Anti-p50	C1	232	42
	C2	0	0
Anti-p50 + anti-p65	C1	0	0
	C2	0	0
Anti-p50 (serum 2)	C1	387	70
	C2	0	0
	S	370	66[c]

[a] See text for details.
[b] Area of peak in artificial units after baseline correction.
[c] Percent relative to area of C2.

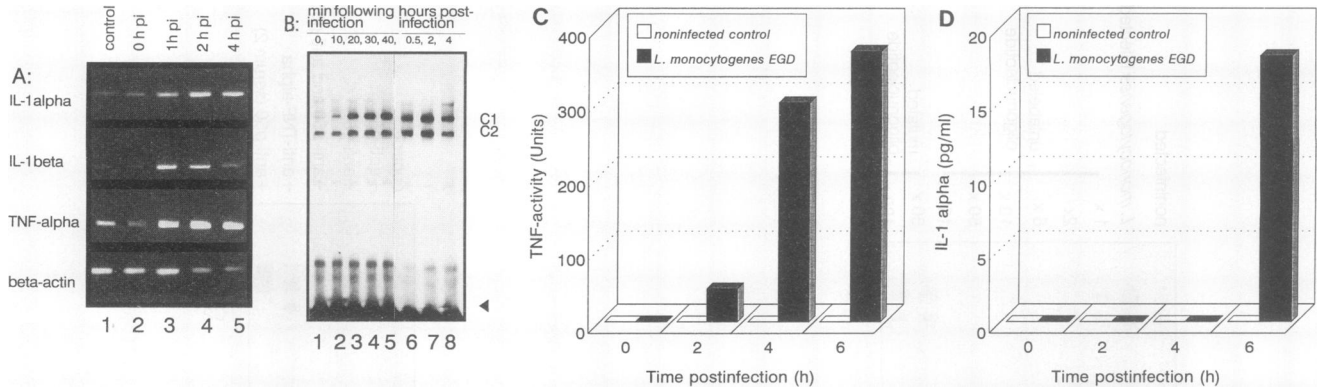

FIG. 4. The generation of NF-κB complexes precedes the induction of IL-1α, IL-1β, and TNF-α genes in *L. monocytogenes*-infected P388D$_1$ macrophages. (A) The induction of mRNAs specific for IL-1α, IL-1β, TNF-α, and β-actin (as control) was analyzed by using RT-PCR as described in the text. Total RNA was extracted from P388D$_1$ macrophages infected with *L. monocytogenes* at 0, 1, 2, and 4 h postinfection (pi), respectively. (B) EMSAs were performed with nuclear protein extracts from P388D$_1$ macrophages at different time points following the addition of the bacteria to the macrophages (0, 10, 20, 30, and 40 min [lanes 1 to 5, respectively]) and at different time points postinfection (after the normal 40-min infection period: 0.5, 2, and 4 h [lanes 6 to 8, respectively]) and with noninfected macrophages (lane 1). The arrowheads indicate the free DNA probe. (C and D) Time course of the induction of TNF-α activity (C) and IL-1α (D) in *L. monocytogenes*-infected macrophages measured as described in Materials and Methods.

TNF-α activity in the supernatant were not detected before 2 h postinfection. Additionally, we infected P388D$_1$ macrophages in the presence of soluble TNF receptor and/or IL-1 receptor antagonist to inhibit potential autocrine TNF-α- and/or IL-1-dependent induction of NF-κB DNA binding activity. As shown in Fig. 5, no inhibition of the formation of complex C1, representing NF-κB, was observed. This suggests that neither IL-1 nor TNF-α acts as an autocrine inducer of early NF-κB activation in P388D$_1$ cells.

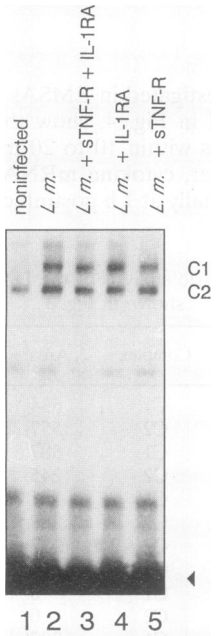

FIG. 5. EMSA with nuclear protein extracts from P388D$_1$ macrophages infected for 40 min and then incubated in the presence of soluble TNF receptor (sTNF-R; lane 5), IL-1 receptor antagonist (IL-1RA; lane 4), soluble TNF receptor and IL-1 receptor antagonist in combination (lane 3) and noninfected (lane 1) and infected (lane 2) control macrophages. The arrowheads indicates the free DNA probe.

Induction of generation of NF-κB DNA by *L. innocua* and mutants of *L. monocytogenes*. What are the signals leading to the induction of NF-κB after infection with *L. monocytogenes*? Although wild-type *L. monocytogenes* appears capable of inducing cytokine mRNAs, we have recently reported that isogenic nonhemolytic mutants of *L. monocytogenes*, which are unable to grow inside macrophages, do not induce IL-1α, IL-6, and TNF-α mRNAs in P388D$_1$ macrophages (33). Escape from the phagosome and subsequent intracellular growth might be the signals for the induction of these proinflammatory cytokines. To determine whether a similar pattern prevails for the generation of NF-κB, we infected P388D$_1$ cells in parallel with the nonhemolytic *L. monocytogenes* strains M3 *hly* and SLCC 53 and the avirulent and nonhemolytic species *L. innocua*. An *mpl* mutant strain of *L. monocytogenes* (14), able to grow inside macrophage-like cells, was also included. As indicated in Fig. 6 and Table 3, the M3 nonhemolytic mutant (with the Tn*916* insertion) and the hemolytic metalloprotease-negative mutant (*mpl*) induced the enhancement of NF-κB generation at levels comparable to those of the wild-type strain. On the other hand, the nonhemolytic strain SLCC 53 (with a deletion in the *prfA* gene) showed only a weak induction of complex C1 (NF-κB) DNA binding activity and no alteration of complex C2. The uptake of the avirulent species *L. innocua* failed to show increased binding.

Pretreatment with cytochalasin B does not inhibit NF-κB induction. The data presented above indicate that early events during the infection of P388D$_1$ macrophages with *L. monocytogenes* might trigger increased NF-κB DNA binding activity. By pretreatment with the actin-depolymerizing drug cytochalasin B, the phagocytic uptake of *L. monocytogenes* by the P388D$_1$ macrophages is inhibited, whereas the adherence of listeriae to the surface of the macrophages is not affected. As shown in Fig. 7, nuclear extracts of cytochalasin B-treated P388D$_1$ macrophages (blocked for phagocytic uptake into cells) exhibited DNA binding activity of NF-κB which was indistinguishable from that of untreated *Listeria*-infected P388D$_1$ macrophages. This increased NF-κB DNA binding activity suggests that adherence and not uptake of the listeriae triggers the elevated levels of NF-κB DNA binding.

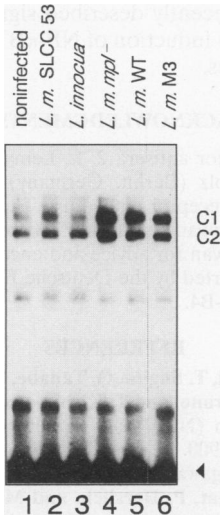

FIG. 6. Induction of NF-κB or KBF1 DNA binding activity in macrophages infected with hemolytic and nonhemolytic *Listeria* strains. An EMSA was performed with nuclear extracts from macrophages infected with *L. monocytogenes* SLCC 53 *prfA* (lane 2), *L. innocua* (lane 3), *L. monocytogenes mpl* (lane 4), *L. monocytogenes* wild type (lane 5), and *L. monocytogenes* M3 *hly* (lane 6) and from noninfected cells (lane 1). Extracts were prepared at 4 h postinfection. The arrowhead indicates the free DNA probe.

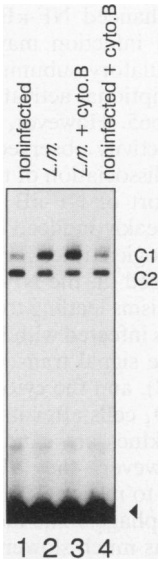

FIG. 7. Cytochalasin B is without effect on the enhancement of NF-κB or KBF1 DNA-protein complexes. P388D$_1$ macrophages were pretreated with 10 μM cytochalasin B (cyto. B) for 30 min before being infected with *L. monocytogenes* (lane 3). The results of infection without pretreatment (lane 2), cells treated with cytochalasin B alone (lane 4), and untreated or noninfected cells (lane 1) are also shown. Nuclear extracts were prepared after 40 min of infection. *L.m.*, *L. monocytogenes*. The arrowhead indicates the free DNA probe.

DISCUSSION

In this study, we demonstrate that the infection of the murine macrophage-like cell line P388D$_1$ with *L. monocytogenes* enhances the formation of nuclear NF-κB complexes, in particular those of p50/p65 heterodimers. By contrast, the generation (DNA binding) of two other transcription factors, AP-1 and NF-IL6, which are also involved in the regulation of cytokine gene expression, was unaffected after infection of P388D$_1$ macrophages with *L. monocytogenes*. Transcription factor AP-1 has been described to be involved in the expression of many constitutively expressed and inducible genes (3). In P388D$_1$ cells, we observed one retarded complex with the TRE$_{coll}$ fragment even without infection. A comparison with a complex induced in HeLa cells after treatment with phorbol 12-myristate 13-acetate and phytohemagglutinin (data not shown) and the results of the competition studies indicated that the protein component of the complex is AP-1. These data were not surprising since P388D$_1$ macrophages appear to be permanently activated as manifested by the relatively high levels of cytokine mRNAs found in noninfected P388D$_1$ macrophages (33). Another transcription factor, NF-IL6, which was reported to be involved in the transcription of the IL-6 gene (1), also appears to possess constitutive DNA binding activity in uninfected cells, and *L. monocytogenes* infection did not result in any measurable change in its DNA binding activity. With regard to our previously reported results on the induction of IL-6 mRNA in *L. monocytogenes*-infected P388D$_1$ macrophages, these data were surprising. Nevertheless, we speculate that NF-IL6 might be necessary for transcription of the IL-6 gene but that the regulation of this cytokine in P388D$_1$ macrophages may be due to other factors, possibly NF-κB.

Transcription factor NF-κB, a pleiotropic mediator of inducible gene control (38), has also been described to be involved in transcriptional regulation of cytokine genes. Gel retardation assays using nuclear extracts from *L. monocytogenes*-infected P388D$_1$ macrophages and a κB consensus motif resulted in increased DNA-protein complex (C1 and C2) formation compared with nuclear extracts from uninfected cells. Of the two complexes, greater DNA binding induction was observed for the slower-migrating complex C1. Competitive inhibition studies established that the binding activity was specific. In competition experiments, an unlabeled κB oligonucleotide competed efficiently, whereas an excess of a mutated κB was without effect on the shift pattern. Several different protein complexes which bind to κB motifs have been described, such as KBF1, a p50 homodimer, and genuine NF-κB, a heterodimer of proteins p50 and p65 (8). The migration patterns of the retarded complexes in our assays suggested that C1 and C2 might represent NF-κB and KBF1, respectively. The blocking experiments using antibodies against p50 and p65 lead to supershifts or the disappearance of C1 and C2, confirming this assumption.

TABLE 3. Densitometric analysis of the DNA-protein complexes shown in Fig. 6

Strain[a]	Complex	Area[b]	% Area (relative to control)
Noninfected control	C1	85	100
	C2	260	100
L. monocytogenes EGD	C1	450	529
	C2	487	187
L. monocytogenes M3 *hly*	C1	388	456
	C2	458	176
L. monocytogenes mpl	C1	565	664
	C2	773	297
L. monocytogenes SLCC 53 *prfA*	C1	132	155
	C2	291	112
L. innocua	C1	79	92
	C2	286	110

[a] *L. monocytogenes* strains used to infect the P388D$_1$ macrophages.
[b] Area of peak in artificial units after baseline correction.

The induction of enhanced NF-κB DNA binding activity after *L. monocytogenes* infection may be due either to the dissociation of the regulatory subunit IκB from the p50/p65 complex or to a transcriptional activation of the genes encoding proteins p50 and p65. However, the rapid induction of NF-κB DNA binding activity observed is more in line with a rapid signal leading to dissociation of the NF-κB–IκB complex and subsequent transport of NF-κB into the nucleus. Since complex C2 was only weakly induced compared with complex C1, and definite physiological roles for KBF1 (C2) have still to be established, we focused on the NF-κB (C1) activity.

What are the mechanisms leading to the induction of NF-κB in macrophage-like cells infected with *L. monocytogenes*? Since NF-κB takes part in the signal transduction pathway of cytokine gene induction (18), and the cytokines IL-1α and TNF-α are produced by P388D$_1$ cells after infection with *L. monocytogenes* (33), both cytokines are candidates for the observed NF-κB induction. However, the NF-κB activation occurs within minutes, leading to maximal NF-κB stimulation 20 min after infection of macrophages. The induction of IL-1α, IL-1β, and TNF-α mRNAs was much slower, and significant TNF-α activity and IL-1α protein were first detected only after 2 and 4 h postinfection, respectively. The addition of soluble TNF receptor and IL-1 receptor antagonist, which blocks TNF- and IL-1 activity very efficiently, did not also affect the induction of NF-κB DNA binding activity. Therefore, we conclude that it is very unlikely that either cytokine mediated NF-κB activation via an autocrine mechanism. A TNF-α-dependent autocrine mechanism for NF-κB activation was also excluded by Busam et al. (9) in their study of the induction of NF-κB in *S. aureus*-exposed murine bone marrow-derived macrophages.

Which mechanisms could account for the rapid induction of NF-κB in *L. monocytogenes*-infected P388D$_1$ macrophages? We have recently shown that nonhemolytic strains of *L. monocytogenes*, which remain entrapped in the phagosome, do not induce a cytokine response in P388D$_1$ macrophages (33). However, the nonhemolytic strain *L. monocytogenes* M3 *hly* induced NF-κB binding activity in infected P388D$_1$ cells. This finding together with the very fast induction of NF-κB DNA binding after infection with the wild-type strain, at time points before the escape of the bacteria from the phagosome into the cytoplasm, implies that events prior to the initiation of bacterial replication inside the cytoplasm trigger NF-κB induction. The mutant SLCC 53 only weakly induced NF-κB DNA binding and was in this respect comparable to the nonhemolytic and nonvirulent species *L. innocua*, which did not enhance NF-κB generation at all. *L. monocytogenes* SLCC 53 is nonhemolytic because of a deletion in the gene encoding the positive regulator PrfA. Invasion in, but not adhesion to, Caco-2 cells is PrfA dependent (55). This suggests that these processes are probably mediated through different bacterial factors, one of which could be the M-protein-like invasion factor internalin (17, 22). However, it is not known whether the listerial factors mediating the interaction of *L. monocytogenes* with enterocytes like Caco-2 cells are the same as those necessary for specific interaction with professional phagocytic cells such as macrophages. Nevertheless, we speculate that NF-κB activation might be already triggered by adherent listeriae through either the surface protein internalin or another as-yet-unknown PrfA-dependent surface protein which should be *L. monocytogenes* specific since NF-κB is not induced by *L. innocua*. The inhibition of the internalization of *L. monocytogenes* by cytochalasin B did not affect NF-κB DNA binding induction, supporting the idea that adhesion of the wild-type bacteria to the surface of the P388D$_1$ macrophages triggers signals leading to NF-κB induction. Experiments are now planned to determine which of the recently described signaling pathways (52) could account for the induction of NF-κB in *L. monocytogenes*-infected macrophages.

ACKNOWLEDGMENTS

We thank A. Israel for antisera 2, L. Lemaire for the anti-TNF-α antibody, and P. Scholz (Berlin, Germany) for the soluble TNF receptor and the IL-1 receptor antagonist. The critical reading of the manuscript by W. R. Schwan is gratefully acknowledged. N.H. is deeply indebted to W. R. Schwan for advice and encouragement.

This work was supported by the Deutsche Forschungsgemeinschaft through grant SFB 165-B4.

REFERENCES

1. **Akira, S., H. Isshiki, T. Sugita, O. Tanabe, S. Kinoshita, Y. Nishio, T. Nakajima, T. Hirano, and T. Kishimoto.** 1990. A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. EMBO J. **9**:1897–1900.
2. **Angel, P., M. Imagawa, R. Chiu, B. Stein, R. J. Imbra, H. J. Rahmsdorf, C. Jonat, P. Herrlich, and M. Karin.** 1987. Phorbol ester-inducible genes contain a common *cis* element recognized by a TPA-modulated *trans*-acting factor. Cell **49**:729–739.
3. **Angel, P., and M. Karin.** 1991. The role of Jun, Fos, and the AP-1 complex in cell proliferation and transformation. Biochim. Biophys. Acta **1072**:129–157.
4. **Arnold, R., J. Scheffer, B. König, and W. König.** 1993. Effects of *Listeria monocytogenes* and *Yersinia enterocolitica* on cytokine gene expression and release from human polymorphonuclear granulocytes and epithelial (HEp-2) cells. Infect. Immun. **61**:2545–2552.
5. **Baeuerle, P. A., and D. Baltimore.** 1988. Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-κB transcription factor. Cell **53**:221–217.
6. **Baeuerle, P. A., and D. Baltimore.** 1988. IκB: a specific inhibitor of NF-κB transcription factor. Science **242**:540–554.
7. **Baeuerle, P. A., and D. Baltimore.** 1989. A 65-kD subunit of active NF-κB is required for inhibition of NF-κB by IκB. Genes Dev. **3**:1689–1698.
8. **Blank, V., P. Kourilsky, and A. Israel.** 1992. NF-κB and related proteins: Rel/dorsal homologies meet ankryn-like repeats. Trends Biochem. Sci. **17**:135–140.
9. **Busam, K., C. Gieringer, M. Freudenberg, and H.-P. Hohmann.** 1992. *Staphylococcus aureus* and derived exotoxins induce nuclear factor κB-like activity in murine bone marrow macrophages. Infect. Immun. **60**:2008–2015.
10. **Chakraborty, T., M. Leimeister-Wächter, E. Domann, M. Hartl, W. Goebel, T. Nichterlein, and S. Notermans.** 1992. Coordinate regulation of virulence genes in *L. monocytogenes* requires the product of the *prfA* gene. J. Bacteriol. **174**:568–574.
11. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**:156–159.
12. **Cossart, P., and J. Mengaud.** 1989. *Listeria monocytogenes*, a model system for the study of intracellular parasitism. Mol. Biol. Med. **6**:463–474.
13. **Curran, T., and B. R. Franza, Jr.** 1988. Fos and jun: the AP-1 connection. Cell **55**:395–397.
14. **Domann, E.** 1992. Molekulargenetische Untersuchungen der Virulenzfaktoren Phospholipase (*plcA*), Listeriolysin (*lisA*), Protease (*mpl*) und Aktin-Bindung (*actA*) und deren koordinierter Regulation durch den positiven Regulator PrfA von *Listeria monocytogenes*. Ph. D. thesis, University of Würzburg, Würzburg, Germany.
15. **Domann, E., M. Leimeister-Wächter, W. Goebel, and T. Chakraborty.** 1991. Molecular cloning, sequencing, and identification of a metalloprotease gene from *Listeria monocytogenes* that is species specific and physically linked to the listeriolysin gene. Infect. Immun. **59**:65–72.
16. **Domann, E., J. Wehland, M. Rohde, S. Pistor, M. Hartl, W. Goebel, M. Leimeister-Wächter, M. Wuenscher, and T. Chakraborty.** 1992. A novel bacteria virulence gene in *Listeria monocytogenes* required for host cell microfilament interaction with homology to the prolin-rich region of vinculin. EMBO J. **11**:1981–1990.

17. **Dramsi, S., C. Kocks, C. Forestier, and P. Cossart.** 1993. Internalin-mediated invasion of epithelial cells by *Listeria monocytogenes* is regulated by the bacterial growth state, temperature and the pleiotropic activator *prfA*. Mol. Microbiol. **9:**931–941.
18. **Drouet, C., A. N. Shakhov, and C. V. Jongeneel.** 1991. Enhancers and transcription factors controlling the inducibility of the tumor necrosis factor-α promoter in primary macrophages. J. Immunol. **147:**1694–1700.
19. **Dyer, R. B., C. R. Collaco, D. W. Niesel, and N. K. Herzog.** 1993. *Shigella flexneri* invasion of HeLa cells induce NF-κB DNA-binding activity. Infect. Immun. **61:**4427–4433.
20. **Falkow, S., R. R. Isberg, and D. A. Portnoy.** 1992. The interaction of bacteria with mammalian cells. Annu. Rev. Cell Biol. **8:**333–363.
21. **Finlay, B. B., and S. Falkow.** 1989. Common themes in microbial pathogenicity. Microbiol. Rev. **53:**210–230.
22. **Gaillard, J. L., P. Berche, C. Frehel, E. Gouin, and P. Cossart.** 1991. Entry of *Listeria monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-positive cocci. Cell **65:**1127–1141.
23. **Gaillard, J. L., P. Berche, J. Mounier, S. Richard, and P. J. Sansonetti.** 1987. In vitro model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line Caco-2. Infect. Immun. **55:**2822–2829.
24. **Ghosh, S., A. M. Gifford, L. R. Riviere, P. Tempst, G. P. Nolem, and D. Baltimore.** 1990. Cloning of the p50 DNA binding subunit of NF-κB: homology to rel and dorsal. Cell **62:**1019–1029.
25. **Havell, E. A.** 1986. Synthesis and secretion of interferon by murine fibroblasts in response to intracellular *Listeria monocytogenes*. Infect. Immun. **54:**787–792.
26. **Havell, E. A., and P. B. Sehgal.** 1991. Tumor necrosis factor-independent IL-6 production during murine listeriosis. J. Immunol. **146:**756–761.
27. **Hohmann, H.-P., R. Remy, B. Pöschl, and A. P. G. M. van Loon.** 1990. Tumor necrosis factor-α and -β bind to the same two types of tumor necrosis factor receptors and maximally activate the transcription factor NF-κB at low receptor occupancy and within minutes after receptor binding. J. Biol. Chem. **265:**15183–15188.
27a. **Israel, A.** Personal communication.
28. **Kathariou, S., P. Metz, H. Hof, and W. Goebel.** 1987. Tn*916*-induced mutations in the hemolysin determinant affecting virulence of *Listeria monocytogenes*. J. Bacteriol. **169:**1291–1297.
29. **Kawakami, K., C. Scheidereit, and R. G. Roeder.** 1988. Identification and purification of a human immunoglobulin-enhancer-binding protein (NF-κB) that activates transcription from a human immunodeficiency virus type 1 promoter in vitro. Proc. Natl. Acad. Sci. USA **85:**4700–4704.
30. **Kieran, M., V. Blank, F. Logeat, J. Vandekerckhove, F. Lottspeich, O. Le Bail, M. B. Urban, P. Kourilsky, P. A. Baeuerle, and A. Israel.** 1990. The DNA binding subunit of NF-κB is identical to factor KBF1 and homologous to the *rel* oncogene product. Cell **60:**1007–1018.
31. **Kocks, C., E. Gouin, M. Tabouret, P. Berch, H. Ohayon, and P. Cossart.** 1992. *L. monocytogenes*-induced actin assembly requires the *actA* gene product, a surface protein. Cell **68:**521–531.
32. **Koren, H. S., B. S. Handwerger, and J. R. Wunderlich.** 1975. Identification of macrophage-like characteristics in a cultured murine cell line. J. Immunol. **114:**894–897.
33. **Kuhn, M., and W. Goebel.** 1994. Induction of cytokines in phagocytic mammalian cells infected with virulent and avirulent *Listeria* strains. Infect. Immun. **62:**348–356.
34. **Kuhn, M., S. Kathariou, and W. Goebel.** 1988. Hemolysin supports survival but not entry of the intracellular bacterium *Listeria monocytogenes*. Infect. Immun. **56:**79–82.
35. **Leimeister-Wächter, M., E. Domann, and T. Chakraborty.** 1991. Detection of a gene encoding a phosphatidylinositol-specific phospholipase C that is co-ordinately expressed with listeriolysin in *Listeria monocytogenes*. Mol. Microbiol. **5:**361–366.
36. **Leimeister-Wächter, M., W. Goebel, and T. Chakraborty.** 1989. Mutations affecting hemolysin production in *Listeria monocytogenes* located outside the listeriolysin gene. FEMS Microbiol. Lett. **65:**23–30.
37. **Leimeister-Wächter, M., C. Haffner, E. Domann, W. Goebel, and T. Chakraborty.** 1990. Identification of a gene that positively regulates expression of listeriolysin, the major virulence factor of *Listeria monocytogenes*. Proc. Natl. Acad. Sci. USA **87:**8336–8340.
38. **Lenardo, M. J., and D. Baltimore.** 1989. NF-κB: a pleiotropic mediator of inducible and tissue-specific gene control. Cell **58:**227–229.
39. **Libermann, T. A., and D. Baltimore.** 1990. Activation of interleukin-6 gene expression through the NF-κB transcription factor. Mol. Cell. Biol. **10:**2327–2334.
40. **Mackaness, G. B.** 1962. Cellular resistance to infection. J. Exp. Med. **116:**381–406.
41. **Mengaud, J., C. Braun-Breton, and P. Cossart.** 1991. Identification of a phosphatidylinositol-specific phospholipase C activity in *Listeria monocytogenes*: a novel type of virulence factor? Mol. Microbiol. **5:**367–372.
42. **Mengaud, J., C. Geoffroy, and P. Cossart.** 1991. Identification of a new operon involved in *Listeria monocytogenes* virulence: its first gene encodes a protein homologous to bacterial metalloproteases. Infect. Immun. **59:**1043–1049.
43. **Mengaud, J., M.-F. Vicente, J. Chenevert, J. M. Pereira, C. Geoffroy, B. Giquel-Sanzey, F. Baquero, J.-C. Perez-Diaz, and P. Cossart.** 1988. Expression in *Escherichia coli* and sequence analysis of the listeriolysin O determinant of *Listeria monocytogenes*. Infect. Immun. **56:**766–772.
44. **Murray, L. J., R. Lee, and C. Martens.** 1990. *In vitro* expression in T cell subsets of the autoimmune MRL/*Mp-lpr/lpr* mouse. Eur. J. Immunol. **20:**163–170.
45. **Nabel, G., and D. Baltimore.** 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature (London) **326:**711–713.
46. **Natsuka, S., S. Akira, Y. Nishio, S. Hashimoto, T. Sugita, H. Isshiki, and T. Kishimoto.** 1992. Macrophage differentiation-specific expression of NF-IL6, a transcription factor for interleukin-6. Blood **79:**460–466.
47. **Nolan, G. P., S. Gosh, H.-L. Liou, T. Tempst, and D. Baltimore.** 1991. DNA binding and IκB inhibition of the cloned p65 subunit of NF-κB, a rel-related polypeptide. Cell **64:**961–969.
48. **Portnoy, D. A., T. Chakraborty, W. Goebel, and P. Cossart.** 1992. Molecular determinants of *Listeria monocytogenes* pathogenesis. Infect. Immun. **60:**1263–1267.
49. **Portnoy, D. A., P. S. Jacks, and D. J. Hinrichs.** 1988. Role of hemolysin for the intracellular growth of *Listeria monocytogenes*. J. Exp. Med. **167:**1459–1471.
50. **Ruff, M. R., and G. E. Gifford.** 1980. Purification and physicochemical characterization of rabbit tumor necrosis factor. J. Immunol. **125:**1671–1677.
51. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Ehrlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
52. **Schreck, R., P. Rieber, and P. A. Baeuerle.** 1991. Reactive oxygen intermediates as apparently widely used messengers in the activation of NF-κB transcription factor and HIV-1. EMBO J. **10:**2247–2258.
53. **Schreiber, E., P. Matthias, M. M. Müller, and W. Schaffner.** 1989. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic Acids Res. **17:**6419.
54. **Seeliger, H. R. P.** 1961. Listeriosis. Hafner, New York.
55. **Sokolovic, Z., J. Riedel, M. Wuenscher, and W. Goebel.** 1993. Surface-associated, PrfA-regulated proteins of *Listeria monocytogenes* synthesized under stress conditions. Mol. Microbiol. **8:**219–227.
56. **Tanabe, O., S. Akira, T. Kamiya, G. G. Wong, T. Hirano, and T. Kishimoto.** 1988. Genomic structure of the murine Il-6 gene. High degree conservation of potential regulatory sequences between mouse and human. J. Immunol. **141:**3875–3881.
57. **Vasquez-Boland, J., C. Kocks, S. Dramsi, H. Ohayon, C. Geoffroy, J. Mengaud, and P. Cossart.** 1992. Nucleotide sequence of the lecithinase operon of *Listeria monocytogenes* and possible role of lecithinase in cell-to-cell spread. Infect. Immun. **60:**219–230.
58. **Yano, O., J. Kanellopoulos, M. Kieran, O. Le Bail, A. Israel, and P. Kourilsky.** 1987. Purification of KBF1, a common factor binding to both H-2 and β2-microglobulin enhancers. EMBO J. **6:**3317–3324.