INFECTION AND IMMUNITY, Jan. 1999, p. 206–212 Vol. 67, No. 1
0019-9567/99/$04.00+0

Gamma Interferon Augments Macrophage Activation by Lipopolysaccharide by Two Distinct Mechanisms, at the Signal Transduction Level and via an Autocrine Mechanism Involving Tumor Necrosis Factor Alpha and Interleukin-1

THOMAS K. HELD,[1,2] XIAO WEIHUA,[3] LIANG YUAN,[2] DHANANJAYA V. KALVAKOLANU,[3,4,5] AND ALAN S. CROSS[2,3]*

Abteilung für Innere Medizin m.S. Hämatologie und Onkologie, Virchow-Klinikum der Humboldt-Universität, 13357 Berlin, Germany,[1] and Division of Infectious Diseases, Department of Medicine,[2] Department of Microbiology & Immunology,[4] Molecular and Cellular Biology Program,[5] and Greenebaum Cancer Center, Program in Oncology,[3] University of Maryland School of Medicine, Baltimore, Maryland 21201

Received 20 January 1998/Returned for modification 10 April 1998/Accepted 17 September 1998

When given in the presence of gamma interferon (IFN-γ), otherwise nontoxic doses of lipopolysaccharide (LPS or endotoxin) become highly lethal for mice. The mechanisms of this synergistic toxicity are not known. We considered the possibility that an interaction between the LPS-induced NF-κB and IFN-γ-induced JAK-STAT pathways at the pretranscriptional level may enhance the LPS-induced signals. To test this hypothesis, we incubated murine macrophage RAW 264.7 cells with IFN-γ for 2 h before addition of different doses of LPS. Consistent with the synergistic induction of inducible nitric oxide synthase mRNA and nitric oxide production by a combination of LPS and IFN-γ, IFN-γ strongly augmented LPS-induced NF-κB activation and accelerated the binding of NF-κB to DNA to as early as 5 min. In agreement with this, IFN-γ pretreatment promoted rapid degradation of IκB-α but not that of IκB-β. Inhibition of protein synthesis during IFN-γ treatment suppressed LPS-initiated NF-κB binding. A rapidly induced protein appeared to be involved in IFN-γ priming. Preincubation of cells with antibodies to tumor necrosis factor alpha or the interleukin-1 receptor partially reduced the priming effect of IFN-γ. In a complementary manner, LPS enhanced the activation of signal-transducing activator of transcription 1 by IFN-γ. These data suggest novel mechanisms for the synergy between IFN-γ and LPS by which they cross-regulate the signal-transducing molecules. Through this mechanism, IFN-γ may transform a given dose of LPS into a lethal stimulus capable of causing sepsis. It may also serve a beneficial purpose by enabling the host to respond quickly to relatively low doses of LPS and thereby activating antibacterial defenses.

Lipopolysaccharide (LPS) of gram-negative bacteria induces a diverse array of biologic responses in mammalian cells and initiates inflammatory, complement, and coagulation cascades. These responses may be an important defense against invading gram-negative bacteria, but when excessive, such responses to LPS may devolve into sepsis (7). When a given amount of LPS is administered in the presence of other agonists, such as interleukin-1 (IL-1) and gamma interferon (IFN-γ), there may be enhanced lethality (14). IFN-γ also plays an important role in the lethal response to LPS, particularly in mediating the lethal Shwartzman reaction (17). In contrast, IFN-γ is a key cytokine in host defenses against obligate and facultative intracellular organisms (23) and enhances the ability of peritoneal macrophages and Kupffer cells to phagocytose and kill virulent *Escherichia coli* (6). Thus, IFN-γ may participate in both the beneficial and detrimental effects of LPS. A better understanding of the mechanisms by which such sensitizing agents enhance LPS activity may therefore provide potential targets for the limitation of LPS-initiated responses. Potential mechanisms of LPS synergy with cytokines such as IFN-γ (16) and IL-1 (4, 37, 38) include upregulation of putative LPS receptors on cells by cytokines (39), induction of autocrine and paracrine responses by cytokines (22), or enhanced expression of downstream response factor genes (26).

LPS activates the NF-κB family of transcription factors (28). In resting cells, NF-κB is complexed in the cytoplasm by an inhibitory protein, IκB. Signal-induced phosphorylation and subsequent proteolytic degradation of IκB frees NF-κB from such complexes. Following this, NF-κB rapidly translocates to the nucleus, binds to the κB element of target genes, and activates the expression of previously quiescent genes (reviewed in reference 2). In contrast, IFN-γ employs the JAK-STAT pathway for its signal transduction (10). Binding of IFN-γ to its receptor results in recruitment of two Janus tyrosine kinases, JAK1 and JAK2, which induce the tyrosine phosphorylation of a dormant cytoplasmic protein, signal-transducing activator of transcription 1 (STAT1). STAT1 then migrates to the nucleus and binds to the IFN-γ-activated site of cellular genes whose products mediate IFN-γ effects (10). Although they activate different sets of genes, IFN-γ and LPS both are known to induce IFN-γ regulatory factor 1, which is essential for induction of inducible nitric oxide synthase (iNOS) (19). It is not known, however, whether the synergistic effect of LPS and IFN-γ may occur at a more proximal site

* Corresponding author. Mailing address: Greenebaum Cancer Center, University of Maryland School of Medicine, 22 S. Greene St., Baltimore, MD 21201. Phone: (410) 328-2565. Fax: (410) 328-6896. E-mail: across@umcc01.umcc.ab.umd.edu.

than the induction of common sets of genes. In the present study, we determined whether IFN-γ, which is known to enhance LPS lethality, alters the intracellular signalling responses of the LPS-initiated NF-κB pathway. Here, we show that pretreatment of macrophages with IFN-γ augments DNA binding of NF-κB in response to LPS and increases the expression of the gene for iNOS, as well as the production of nitric oxide. In turn, LPS affects the IFN-γ signal transduction pathway by increasing the activation of STAT1 binding to DNA. Finally, we present evidence that the interaction between IFN-γ and LPS not only occurs at the signal transduction level but may involve the induction of factors which act in an autocrine fashion.

MATERIALS AND METHODS

Cell culture and reagents. Murine macrophage RAW 264.7 (RAW) cells were grown in Dulbecco modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum, 2.4 mM L-glutamine, 60-U/ml penicillin, 60-U/ml streptomycin, 0.55 mM 2-mercaptoethanol, and 40 mM HEPES buffer. Cells were maintained for no longer than 6 weeks to avoid unresponsiveness to either IFN-γ or LPS. Cells were incubated with recombinant murine IFN-γ (Genzyme) and/or LPS from *E. coli* O55:B5 (Sigma) as described in Results. Monoclonal anti-TNF-α and anti-IL-1 receptor (IL-1R) antibodies (Genzyme) were used at a concentration of 2.5 μg/ml.

Gene expression analyses. (i) Transfection and luciferase assays. Cells (0.5×10^6) were mixed with 5 μg of plasmid DNA carrying the palindromic IFN response element (pIRE)-regulated luciferase gene (21), 2 μg of a β-actin–β-galactosidase reporter (internal control), and 13 μg of salmon sperm DNA as the carrier. DNA was electroporated, and cells were rested for 14 h prior to stimulation with the indicated agents. Cells were treated with IFN-γ (50 U/ml) and/or LPS (100 ng/ml) and incubated for an additional 10 h. Equal amounts of cell extracts (50 μg) from individual samples were assayed for luciferase activity (1). Luciferase activity was normalized to β-galactosidase activity to correct for variations in transfection efficiency.

(ii) EMSAs. Preparation of nuclear and cytosolic extracts (11) and electrophoretic mobility shift assays (EMSAs) were performed as described previously. κB and pIRE oligonucleotides (21–23) were labeled with ^{32}P as described earlier (40). Double-stranded wild-type κB (5′ gaagcttGGGGACTCTCCCtttg 3′) and mutant κB (5′ gaagcttGGCGACTCTCCCtttg 3′) oligonucleotides were employed in these studies. Only the sense strand sequences are shown. In each case, the complementary antisense strand was annealed to the sense strand prior to the experiments. The core NF-κB binding site is in italicized uppercase letters. The mutated base is underlined. The pIRE oligonucleotide was based on the sequence present in the ICSBP promoter (21). Each experiment was repeated at least three times.

(iii) Western blotting. For Western blotting experiments, equal amounts of cytosolic protein (20 μg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto a polyvinylidene difluoride membrane (0.45 μm; Millipore), and IκB was detected by using rabbit anti-IκB immunoglobulin G (IgG; Santa Cruz) after blocking of nonspecific binding sites with 0.2% Tween 20 and 1% bovine serum albumin in Dulbecco phosphate-buffered saline. After washing, bound rabbit anti-IκB IgG was detected with horseradish peroxidase-labeled goat anti-rabbit IgG (Sigma) and subsequent enhanced chemiluminescence (Pierce).

RESULTS

Pretreatment with IFN-γ augments DNA binding of NF-κB in response to LPS. Because LPS and IFN-γ exert their effects through the induction of cellular genes, we measured the induction of iNOS mRNA by reverse transcription-PCR. Consistent with previous data (15, 25, 31), iNOS mRNA was robustly induced when cells were cotreated with IFN-γ and LPS (data not shown). In addition, elevation of the iNOS mRNA level correlated with an increase in its enzyme activity (data not shown). Since LPS is known to activate DNA binding of NF-κB and the iNOS promoter contains a functional κB site, we investigated whether pretreatment of macrophages with IFN-γ augmented the NF-κB binding to DNA. RAW cells were pretreated with either IFN-γ or nothing for 2 h and subsequently stimulated with various amounts of LPS. Nuclear extracts were then prepared after 30 min of LPS stimulation and assessed for the amount of NF-κB by electrophoretic mobility shift assay (EMSA) using a ^{32}P-labeled κB oligonucleotide based on the sequences between −85 and −76 of the murine iNOS promoter (13, 43). In untreated cells, there was no activation of DNA binding of NF-κB (Fig. 1A, lane 1). A typical binding pattern was shown in Fig. 1. Formation of two complexes was detected with this probe. The upper band was recognized by antibodies raised against the p65 and p50 subunits of NF-κB (data not presented), which supershifted these complexes. The lower band was supershifted by antibodies specific to the p50 subunit. LPS alone caused a slight activation of NF-κB binding at higher concentrations (Fig. 1A, lanes 3 and 5), consistent with the residual increase of iNOS mRNA and the slight increase in NOS activity observed with LPS alone (data not

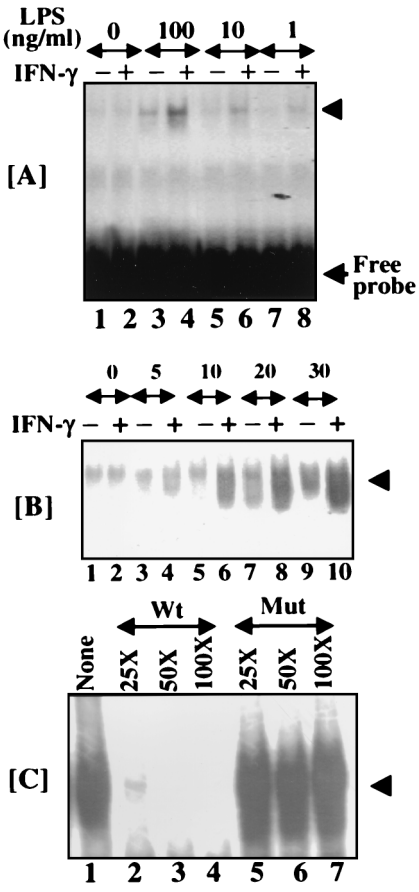

FIG. 1. (A) Pretreatment with IFN-γ enhances the DNA binding activity of NF-κB in response to LPS. RAW 264.7 cells were preincubated with IFN-γ (50 U/ml) for 2 h and then stimulated with the indicated amounts of LPS for 30 min. Nuclear extracts (20 μg) were then analyzed by EMSA using a ^{32}P-labeled oligonucleotide specific for an NF-κB site of the murine iNOS promoter. (B) Kinetics of activation of NF-κB by LPS. Cells were incubated with IFN-γ (50 U/ml) for 2 h before addition of LPS (100 ng/ml; indicated by the horizontal arrows; the values above the arrows indicate the lengths of LPS treatment in minutes). After additional incubation with LPS for the indicated times, nuclear extracts were prepared and analyzed by EMSA using a ^{32}P-labeled oligonucleotide based on a functional κB site of the murine iNOS promoter. Only the region corresponding to NF-κB binding is shown. The arrowheads indicate specific NF-κB complexes. (C) Specific DNA binding of NF-κB. All of the extracts are from cells stimulated with IFN-γ (50 U/ml) for 2 h and with LPS (100 ng/ml) for 20 min (similar to panel B, lane 8). Extracts in lane 1 were not pretreated with an unlabeled oligonucleotide before analysis with a ^{32}P-labeled oligonucleotide. Nuclear extracts were preincubated with 25-, 50-, and 100-fold molar excesses of unlabeled oligonucleotides. The wild-type (Wt; lanes 2 to 4) and mutant (Mut; lanes 5 to 7) oligonucleotides used are described in Materials and Methods.

shown). IFN-γ alone did not induce the DNA binding of NF-κB (lane 2). Preincubation of cells with IFN-γ, however, strongly augmented the LPS-initiated NF-κB binding in a dose-dependent manner (Fig. 1A, lanes 4, 6, and 8). This increase correlated with the enhanced expression of iNOS mRNA and nitrite production observed under these conditions (data not shown).

Since synergy between IFN-γ and LPS may also manifest itself as more rapid induction of NF-κB by LPS, we next determined the kinetics of IFN-γ and LPS synergy. There was a basal level of DNA binding of NF-κB which did not alter significantly until 20 min post LPS treatment (Fig. 1B, lanes 1, 3, 5, and 7, respectively). The relatively high basal levels of DNA binding in this experiment and others (see below) may be due to variable levels of nonspecific stimuli present in the different batches of media and serum used and the passage number of the cells. However, priming with IFN-γ strongly accelerated NF-κB binding to DNA as early as 5 min (Fig. 1B, lane 4), significantly higher than the basal level (lane 1). IFN-γ alone did not increase the DNA binding (Fig. 1B, lane 2). This increase in the DNA binding activity of NF-κB persisted for up to 30 min (Fig. 1B, lanes 6, 8, and 10). LPS alone caused a level that was slightly higher at 20 and 30 min than that at earlier time points (lanes 7 and 9) but clearly lower than that of the IFN-pretreated controls. There was a progressive decrease in NF-κB activation at later time points (120 min; data not shown), which was consistent with the known loss of functional activity of NF-κB over time. Specific NF-κB complex formation was demonstrated by the ability of the wild type, but not the mutant, κB oligonucleotide to compete out the binding (Fig. 1C, compare lanes 2 to 4 to lanes 5 to 7).

IFN-γ promotes rapid IκB-α degradation. In the light of the above observations, we examined whether enhancement of NF-κB activation was due to accelerated degradation of IκB in IFN-γ-primed macrophages. Unstimulated or IFN-pretreated (2 h) cells were treated with LPS for 30 min, and cytosolic extracts were prepared in the presence of a protease inhibitor (phenylmethylsulfonyl fluoride) to avoid nonspecific degradation of IκB. The same amount of cytosolic protein from each sample was then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then subjected to Western blotting. As shown in Fig. 2A (top), some IκB-α degradation occurred in the absence of IFN-γ priming when cells were stimulated with a high dose of LPS (compare lanes 4 and 1). IFN-γ per se did not cause detectable degradation of IκB-α (lane 2) compared to unstimulated cells (lane 1). However, priming of the cells with IFN-γ strongly enhanced LPS-regulated IκB-α degradation (Fig. 2A, lanes 3 and 5). Interestingly, priming with IFN-γ or treatment with LPS did not affect the degradation of IκB-β under these conditions (Fig. 2A, bottom). These observations prompted us to examine whether IκB degradation was accelerated in the presence of IFN-γ. Indeed, in IFN-γ-primed cells, LPS induced rapid degradation of IκB-α in a time-dependent manner until 20 min (Fig. 2B, top, lanes 5, 7, 9, and 11). Although LPS alone caused degradation of IκB-α (lanes 4, 6, 8, and 10), it was consistently lower than what was observed in IFN-γ-primed cells. After 30 min, IκB-α levels rose again, consistent with the previously described autoregulation of NF-κB/IκB-α (30). Indeed, when more NF-κB was activated (LPS and IFN-γ), more IκB-α was resynthesized (compare lanes 12 and 13). In agreement with the data shown in Fig. 2A, levels of IκB-β were unaffected under these conditions (Fig. 2B, bottom).

Effect of protein synthesis inhibition on IFN-γ priming. Previous reports demonstrated a synergistic effect of IFN-γ and LPS on nitric oxide production in murine macrophages

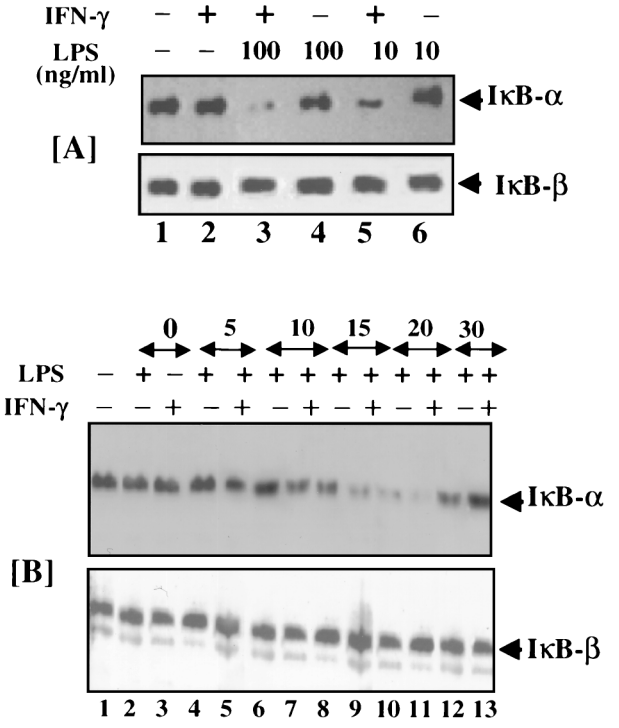

FIG. 2. (A) Pretreatment with IFN-γ enhances the dose-dependent degradation of IκB-α in response to LPS. RAW cells were treated as described in the legend to Fig. 1, and cytosolic extracts were prepared in the presence of phenylmethylsulfonyl fluoride. They were analyzed for the presence of IκB-α and IκB-β by Western blotting as described in Materials and Methods. (B) Kinetics of IκB-α degradation in RAW cells induced by LPS with or without priming by IFN-γ. Cells were treated as described in the legend to Fig. 1. Cytosolic extracts were prepared at the indicated times after addition of LPS and analyzed as described above. The values above the arrows in panel B indicate the times (minutes) of LPS exposure. A minus or plus sign indicates the absence or presence, respectively, of the given agent during incubation.

(15, 25). This effect was thought to be due to the enhanced expression of iNOS mRNA requiring de novo protein synthesis. However, the requirement of de novo protein synthesis during IFN-γ priming was not demonstrated (25). Since IFN-γ alone did not promote IκB-α degradation (Fig. 2A, lane 2, and 2B, lane 3) and IFN-γ priming augmented the LPS-induced IκB degradation, we investigated whether IFN-γ induced a protein factor that was responsible for rapid activation of NF-κB. Therefore, we examined the effect of the protein synthesis inhibitor cycloheximide (CHX) on the priming of IFN-γ. As shown in Fig. 3A, 30 min of IFN-γ priming strongly enhanced LPS activated NF-κB binding (compare lanes 5 and 3). CHX alone did not induce NF-κB binding (lane 2), and it did not inhibit LPS-inducible NF-κB binding (lane 4). However, CHX blocked the hyperactivated NF-κB binding elicited by treatment with IFN-γ and LPS (Fig. 3A, lane 6). These data suggest that an IFN-γ-stimulated, rapidly synthesized protein may contribute to the priming observed with IFN-γ.

Involvement of TNF-α and/or IL-1 in the priming effect of IFN-γ. The above data indicate that IFN-inducible proteins may contribute to the synergy between IFN-γ and LPS. Under the conditions of IFN-γ priming, the induction of other cytokines may account for some of these effects. We therefore determined whether IFN-γ modulation of LPS-regulated NF-κB activation involved known cytokines. For this purpose, we tested the effects of antibodies against the IL-1 receptor (IL-1R) and/or tumor necrosis factor alpha (TNF-α) on the

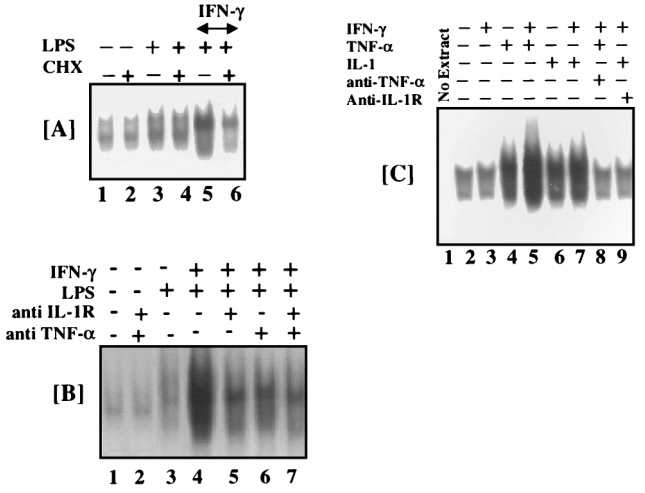

FIG. 3. (A) Effect of CHX on induction of the DNA binding of NF-κB by LPS with or without pretreatment with IFN-γ. RAW 264.7 cells were primed with IFN-γ (50 U/ml) for 30 min in the presence or absence of CHX (25 μg/ml) (lanes 4 and 5). LPS (100 ng/ml) was then added, and the mixture was incubated for 20 min. Nuclear extracts were prepared and analyzed by EMSA as described in the legend to Fig. 1. (B) Effect of antibodies against TNF-α or IL-1R on the induction of DNA binding of NF-κB by LPS with or without priming by IFN-γ. An anti-TNF-α antibody (2.5 μg/ml) and/or an anti-IL-1R antibody (2.5 μg/ml) were added to the culture 30 min before priming with IFN-γ (50 U/ml) for 60 min. LPS (100 ng/ml) was then added for an additional 20 min before the preparation of nuclear extracts. Extracts were then analyzed by EMSA as described in the legend to Fig. 1. (C) Synergistic activation of NF-κB by TNF-α or IL-1 in association with IFN-γ (50 U/ml). Cells were treated with the indicated agents as described above. Cells were stimulated with recombinant IL-1 (25 ng/ml) and TNF-α (10 ng/ml) for 20 min in this experiment. In all cases, only the relevant region of the autoradiogram is shown.

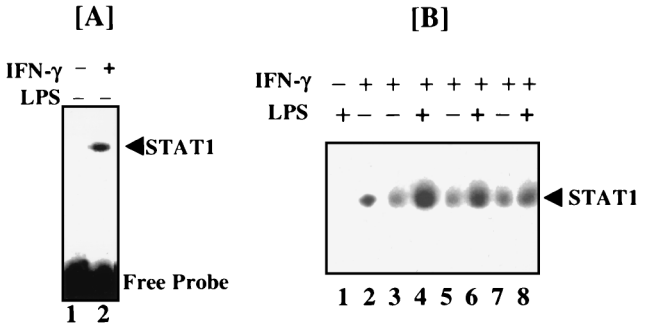

FIG. 4. Effect of pretreatment with IFN-γ on the induction of DNA binding of STAT1 by LPS. (A) Pattern of activated STAT1 binding to the pIRE by IFN-γ. (B) Where indicated, cells were treated with IFN-γ (50 U/ml) for 2 h prior to LPS treatment for 30 min. The LPS concentrations were 100 (lanes 1 and 4), 10 (lane 6), and 1 (lane 8) ng/ml. Nuclear extracts were analyzed by EMSA using a ^{32}P-labeled pIRE probe for STAT1 binding. Only the relevant region of the blot is shown.

priming activity of IFN-γ because these two cytokines are known to activate NF-κB (2, 28). Antibody against IL-1R was chosen to block the α and β isoforms of IL-1. As shown in Fig. 3B, the combination of IFN-γ and LPS treatments strongly activated NF-κB DNA binding activity compared to that in untreated cells, which again showed a basal level of NF-κB activation (compare lanes 1 and 4). Although LPS alone caused a slight activation of NF-κB (lane 3), it strongly induced NF-κB in IFN-γ-primed cells (lane 4). Antibodies against both IL-1R and TNF-α each blocked the hyperactivation of NF-κB binding in IFN-γ-primed, LPS-treated cells (compare lanes 5 and 6 to lane 4). These antibodies themselves had no effect on the basal DNA binding activity of NF-κB (lane 2). Preincubation of cells with both antibodies together did not reduce the DNA binding of NF-κB beyond that obtained with either antibody alone (lane 7). In the presence of either or both of the antibodies, notable activation of NF-κB still occurred (compare lanes 5 to 7 to lanes 1 and 2). These results suggest the existence of an autocrine loop involving key cytokines (IL-1 or TNF-α and perhaps others), which may contribute to the priming effect of IFN-γ on LPS-regulated NF-κB activation.

Because anti-IL-1R and -TNF-α antibodies greatly inhibited LPS–IFN-γ-induced gene expression, we examined whether these cytokines, in association with IFN-γ, also augmented NF-κB activation. Treatment with IFN-γ did not cause intense NF-κB binding compared to that of untreated cells (Fig. 3C, compare lanes 2 and 3). Similarly, addition of exogenous TNF-α or IL-1 clearly induced NF-κB (lanes 4 and 6). In IFN-γ-pretreated cells, both IL-1 and TNF-α robustly activated NF-κB (lanes 5 and 7), compared to either IL-1 or TNF-α alone (lanes 4 and 6). Addition of an anti-IL-1R or -TNF-α antibody blocked hyperactivation of NF-κB (lanes 8 and 9). Thus, the antibodies were functional in blocking their cognate cytokines.

Effect of LPS on IFN-γ-stimulated gene expression. Since IFN-γ augmented LPS-stimulated pathways, we examined whether LPS also modulated signals induced by IFN-γ in a corresponding manner. In these experiments, nuclear extracts after various treatments were incubated with a ^{32}P-labeled pIRE oligonucleotide, and an EMSA was performed to detect the binding of STAT1 to this element. Previously, this element has been shown to bind to STAT1 in many cell types (21, 35). A typical pattern of STAT1 activation is shown in Fig. 4A. In the absence of any stimulus, there was no activation of STAT1 binding to pIRE (Fig. 4A, lane 1). As anticipated, IFN-γ activated STAT1 binding to pIRE (Fig. 4A, lane 2). To examine the effect of LPS on IFN-γ-stimulated STAT1 activation, cells were costimulated with various doses of LPS in the presence of a fixed dose of IFN-γ (50 U/ml). In contrast to IFN-γ (lane 2), LPS alone did not induce STAT1 binding to pIRE (lane 1). However, in the presence of increasing doses of LPS, IFN-γ strongly activated STAT1 (Fig. 4B, lanes 4, 6, and 8). The identity of this band as STAT1 was ascertained by supershifting of this band with STAT1-specific antibodies (data not shown).

If LPS and IFN-γ act synergistically to increase STAT1 binding to DNA, this should be reflected by increased transcription of genes regulated by IFN-γ. We therefore transfected the cells with a luciferase reporter gene regulated by pIRE. In untreated cells, no significant induction of luciferase activity was noted (Fig. 5, bar 1). Incubation of the cells with LPS caused a slight but not significant increase in luciferase activity (bar 2). As anticipated, incubation of the cells with IFN-γ caused a high level of induction (bar 3). However, with IFN-γ and LPS, luciferase expression was synergistically induced (bar 4). Thus, LPS enhanced the activation of STAT1 by IFN-γ and the consequent gene transcription.

Since the LPS–IFN-γ combination induced STAT1-dependent gene expression better than IFN-γ alone and the above-described studies indicated involvement of IL-1 and TNF-α in the synergistic actions of LPS and IFN-γ, we next examined whether these two cytokines also augmented pIRE-driven gene expression in association with IFN-γ. RAW cells were transfected with a pIRE-luciferase construct and treated with various combinations of cytokines. As expected, IFN-γ induced luciferase expression (Fig. 6, bar 2). Although neither TNF-α nor IL-1 induced gene expression (bars 3 and 4), they caused a significant increase in luciferase expression in association

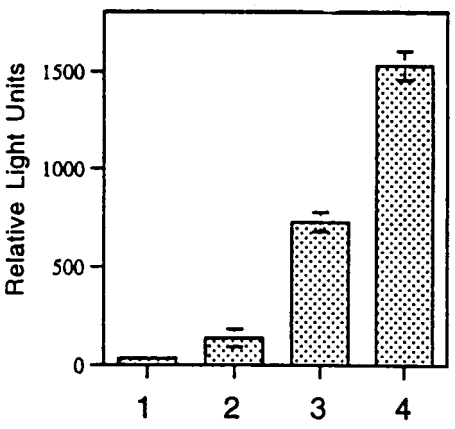

FIG. 5. Synergistic induction of IFN-γ-responsive gene promoter. RAW cells were transfected with a luciferase reporter gene driven by the pIRE. Cell extracts were prepared and assayed for luciferase activity as described earlier (19). The data are means ± the standard errors of the means of triplicate measurements. Bars: 1, no treatment; 2, LPS (100 ng/ml); 3, IFN-γ (50 U/ml); 4, IFN-γ plus LPS.

with IFN-γ (bars 5 and 6) compared to IFN-γ alone (bar 2). Incubation of cells with antibodies specific to IL-1R or TNF-α inhibited the synergistic induction. IFN-γ-inducible levels occurred under these conditions (bars 7 and 8). Anti-TNF-α antibodies did not inhibit IL-1–IFN-γ-induced expression (bar 9). Similarly, anti-IL-1R antibodies failed to inhibit TNF-α–IFN-γ-inducible gene expression (bar 10). These observations indicated the functional specificity of the antibodies.

DISCUSSION

LPS may be required for activation of host defenses against bacteria commonly found in sepsis (4). However, an excessive host response to LPS may result in collateral tissue injury and sepsis (7). Consequently, it would be advantageous to have mechanisms that limit the biological effects of endotoxin. In the event of transient endotoxin exposure, inflammatory responses are limited. In contrast, it may be advantageous for

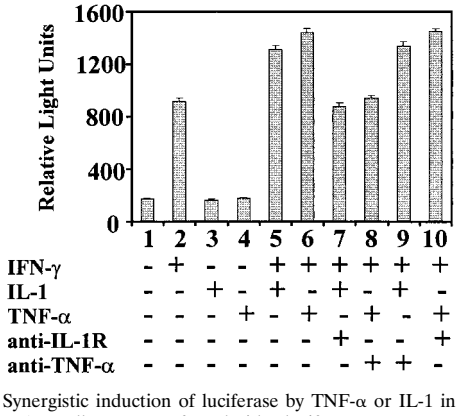

FIG. 6. Synergistic induction of luciferase by TNF-α or IL-1 in association with IFN-γ. RAW cells were transfected with a luciferase reporter gene driven by the pIRE. Cell extracts were prepared and assayed for luciferase activity as described earlier (19). The data are means ± the standard errors of the means of triplicate measurements. The various treatments applied (for 16 h) are indicated at the bottom. A minus or plus sign indicates the absence or presence, respectively, of the given agent during incubation. An anti-TNF-α antibody (2.5 μg/ml) and/or an anti-IL-1R antibody (2.5 μg/ml) were added to the culture 30 min prior to treatment with the indicated cytokines. IFN-γ (50 U/ml), IL-1 (25 ng/ml), and TNF-α (10 ng/ml) were used in the experiment.

LPS to initiate a vigorous inflammatory response once gram-negative bacteria have gained a foothold in the host. During active infection, there is an expression of inflammatory cytokines (IL-1, TNF-α, and IFN-γ) such that the superimposition of LPS in this milieu may enable the host to mount a more vigorous response to the pathogen. Thus, the synergy between LPS and inflammatory cytokines such as IFN-γ may represent an important regulatory mechanism by which the host is sure of a significant, ongoing infection before it activates potent effector responses (8). We have shown that the translocation of NF-κB to the nucleus by LPS in IFN-γ-primed cells is a dose-dependent event. This synergistic interaction may enable the host to rapidly mobilize its antibacterial defenses at much lower levels of LPS and minimize collateral tissue injury and sepsis. Consequently, an understanding of the mechanisms by which the synergistic interactions between endotoxin and proinflammatory cytokines occur may provide important insight not only into the development of antibacterial defense mechanisms but also into how those mechanisms decompensate into an excessive septic response.

In this study, we investigated the mechanisms by which IFN-γ enhances the effects of LPS on macrophages. Both LPS and IFN-γ are important in the lethal outcome of bacterial infection. The role of IFN-γ in aggravating the host response to LPS has been demonstrated in vitro (18, 27, 29) and in vivo (20, 33, 42). Consistent with previous observations (12, 15, 25, 31, 41), we have observed that priming of murine macrophages with IFN-γ significantly enhances the expression of iNOS mRNA with a subsequent increase in the production of nitrite in response to LPS (data not shown). Nitrite is an important mediator of not only antimicrobial but also immunosuppressive and host-damaging activities (reviewed in reference 26). However, the exact manner in which these molecules interplay is not completely understood.

To understand these mechanisms, we first investigated whether priming of macrophages with IFN-γ had any effect on the DNA binding activity of NF-κB induced by subsequent stimulation with LPS. Indeed, priming by IFN-γ rendered the macrophages more responsive. Lower doses of LPS were sufficient for induction of NF-κB DNA binding activity (Fig. 1A), and these responses were rapid in IFN-γ-primed cells (Fig. 1B). These data are consistent with the enhanced iNOS mRNA expression and nitrite production. In a complementary manner, LPS augmented IFN-γ-activated STAT1 DNA binding. STAT1 binding to pIRE in response to IFN-γ was enhanced further when macrophages were incubated with LPS (Fig. 4B, lanes 4, 6, and 8). Previously, LPS was shown to activate STAT3 (44). Indeed, STAT3 associates with IFN receptors (32). Consistent with these data, we have observed enhancement of STAT1 and STAT3 binding to the *sis*-inducible element of the c-*jun* promoter (data not shown). These observations suggest yet another level of synergy between LPS- and IFN-γ-regulated pathways. More importantly, our results identify a novel interaction between IFN-γ and LPS at the pretranscriptional level, in contrast to the previously described posttranscriptional effects (25). The fact that TNF-α and IL-1 also enhance IFN-γ responses (Fig. 6) may indicate that these LPS-inducible cytokines contribute to the enhanced responses. The molecular mechanism of such actions needs to be defined further.

IκB, the natural inhibitor of NF-κB, exists in mammalian cells in three isoforms, α, β, and ε (34). Although the role of IκB-ε is uncertain, IκB-α is specific for NF-κB, and IκB-β inhibits the DNA binding of the related c-Rel protein (34). Thus, the enhancement of LPS-initiated DNA binding of NF-κB by IFN-γ should reflect the corresponding changes in

IκB. Indeed, we demonstrated that IFN-γ strongly enhanced the LPS-regulated degradation of IκB-α in a time- and dose-dependent manner (Fig. 2A and B). Interestingly, only IκB-α, not IκB-β, was affected. These data suggest a specific effect of IFN-γ priming on one of the isoforms. Since NF-κB was rapidly activated by LPS in IFN-γ-primed cells and at least 30 min of priming was required, we therefore determined whether an IFN-γ-induced protein factor was responsible for the observed synergy. Indeed, blockade of protein synthesis during IFN-γ priming suppressed the synergistic activation of NF-κB (Fig. 3A). It has recently been shown that IFN-induced PKR (protein kinase R) is essential for NF-κB activation in response to certain stimuli, such as poly(I · C) (24). The fact that IFN-γ augments the ability of LPS to superstimulate NF-κB raises the possibility that IFN-stimulated PKR participates in this process. Blockade with CHX probably prevents accumulation of the optimal levels of PKR required for synergy with LPS.

Alternatively, IFN-γ may induce the production and/or release of other cytokines, like IL-1 or TNF-α, which enhance LPS-mediated effects (2, 3, 5, 28). Preincubation of cells with monoclonal antibodies raised against IL-1R or TNF-α at saturating concentrations only partially blocked the LPS-regulated NF-κB activation (Fig. 3B). Furthermore, cotreatment with both of the antibodies neither completely blocked NF-κB activation nor reduced it below the levels achieved with the single antibodies (Fig. 3B, lane 6). Thus, IFN-γ-stimulated factors other than IL-1 and TNF-α may synergize with LPS. The fact that IFN-γ enhances IL-1- and TNF-α-activated NF-κB binding to DNA is consistent with this notion (Fig. 3C). Furthermore, these experiments also indicate the functional specificity of the antibodies used in this study. These observations also suggest a linear pathway in which IFN-γ stimulates the expression of one of these cytokines (IL-1 or TNF-α) which, in turn, activates the other. Therefore, blockade of one will block the action of the other. In an analogous manner, priming with IFN-γ increases the amount of IL-1 release upon subsequent stimulation with LPS in endothelial cells (27). Also, IFN-γ is known to augment the TNF receptor expression (36). IFN-γ also increases the binding of LPS to macrophages via changes in the membrane phospholipid fatty acid composition (9) and thus may enhance the effects of LPS, even at a level before the signal transduction cascade. However, this increase in binding of LPS required 18 h of treatment with IFN-γ (9), unlike the rapid priming effect of IFN-γ seen in our studies. Although our studies indicate the interactions between the disparate signaling pathways and consequent hyperstimulation of cytokine responses in a macrophage cell line, the occurrence of these phenomena in primary macrophages needs to be addressed further. Our future investigations are directed on this line.

In summary, we present evidence that (i) IFN-γ-induced priming of macrophages enhanced their response to LPS and occurred at the level of signal transduction (i.e., pretranscription) and (ii) this involved at least two distinct mechanisms, i.e., a direct interaction between the signal transduction pathways employed by IFN-γ and LPS and an autocrine loop that uses TNF-α and IL-1. By sensitizing itself to very low doses of LPS, the host may respond more efficiently to an incipient infection by gram-negative organisms.

ACKNOWLEDGMENTS

T.K.H. was supported by a grant from the Deutsche Forschungsgemeinschaft (He 2759/1-1). D.V.K. is supported by grants by from the National Cancer Institute.

REFERENCES

1. **Ausubel, F. M., R. Brent, D. D. Kingston, J. G. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1994. Current protocols in molecular biology. Wiley-Interscience, New York, N.Y.
2. **Baeuerle, P. A., and T. Henkel.** 1994. Function and activation of NF-kappa B in the immune system. Annu. Rev. Immunol. **12:**141–179.
3. **Bonizzi, G., E. Dejardin, B. Piret, J. Piette, M. P. Merville, and V. Bours.** 1996. Interleukin-1 beta induces nuclear factor kappa B in epithelial cells independently of the production of reactive oxygen intermediates. Eur. J. Biochem. **242:**544–549.
4. **Cannon, J. G., R. G. Tompkins, J. A. Gelfand, H. R. Michie, G. G. Stanford, J. W. van der Meer, S. Endres, G. Lonnemann, J. Corsetti, B. Chernow, et al.** 1990. Circulating interleukin-1 and tumor necrosis factor in septic shock and experimental endotoxin fever. J. Infect. Dis. **161:**79–84.
5. **Crofford, L. J., B. Tan, C. J. McCarthy, and T. Hla.** 1997. Involvement of nuclear factor kappa B in the regulation of cyclooxygenase-2 expression by interleukin-1 in rheumatoid synoviocytes. Arthritis Rheum. **40:**226–236.
6. **Cross, A., L. Asher, M. Seguin, L. Yuan, N. Kelly, C. Hammack, J. Sadoff, and P. Gemski, Jr.** 1995. The importance of a lipopolysaccharide-initiated, cytokine-mediated host defense mechanism in mice against extraintestinally invasive Escherichia coli. J. Clin. Investig. **96:**676–686.
7. **Cross, A. S., and S. M. Opal.** 1995. Endotoxin's role in gram-negative bacterial infection. Curr. Opin. Infect. Dis. **8:**156–163.
8. **Cross, A. S., J. C. Sadoff, N. Kelly, E. Bernton, and P. Gemski.** 1989. Pretreatment with recombinant murine tumor necrosis factor/cachectin and murine interleukin-1α protects mice from lethal bacterial infection. J. Exp. Med. **169:**2021–2027.
9. **Darmani, H., J. Parton, J. L. Harwood, and S. K. Jackson.** 1994. Interferon-g and polyunsaturated fatty acids increase the binding of lipopolysaccharide to macrophages. Int. J. Exp. Pathol. **75:**363–368.
10. **Darnell, J. E. J., I. M. Kerr, and G. R. Stark.** 1994. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science **264:**1415–1421.
11. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
12. **Dileepan, K. N., J. C. Page, Y. Li, and D. J. Stechschulte.** 1995. Direct activation of murine peritoneal macrophages for nitric oxide production and tumor cell killing by interferon-gamma. J. Interferon Cytokine Res. **15:**387–394.
13. **Ding, A., S. Hwang, H. M. Lander, and Q. W. Xie.** 1995. Macrophages derived from C3H/HeJ (Lpsd) mice respond to bacterial lipopolysaccharide by activating NF-kappa B. J. Leukocyte Biol. **57:**174–179.
14. **Doherty, G. M., J. R. Lange, H. N. Langstein, H. R. Alexander, C. M. Buresh, and J. A. Norton.** 1992. Evidence for IFN-gamma as a mediator of the lethality of endotoxin and tumor necrosis factor-alpha. J. Immunol. **149:**1666–1670.
15. **Gao, J., D. C. Morrison, T. J. Parmely, S. W. Russell, and W. J. Murphy.** 1997. An interferon-gamma-activated site (GAS) is necessary for full expression of the mouse iNOS gene in response to interferon-gamma and lipopolysaccharide. J. Biol. Chem. **272:**1226–1230.
16. **Hamilton, T. A., N. Bredon, Y. Ohmori, and C. S. Tannenbaum.** 1989. IFN-gamma and IFN-beta independently stimulate the expression of lipopolysaccharide-inducible genes in murine peritoneal macrophages. J. Immunol. **142:**2325–2331.
17. **Heremans, H., J. Van Damme, C. Dillen, R. Dijkmans, and A. Billiau.** 1990. Interferon gamma, a mediator of lethal lipopolysaccharide-induced Shwartzman-like shock reactions in mice. J. Exp. Med. **171:**1853–1869.
18. **Johnston, P. A., D. O. Adams, and T. A. Hamilton.** 1985. Regulation of the Fc-receptor-mediated respiratory burst: treatment of primed murine peritoneal macrophages with lipopolysaccharide selectively inhibits H_2O_2 secretion stimulated by immune complexes. J. Immunol. **135:**513–518.
19. **Kamijo, R., H. Harada, T. Matsuyama, M. Bosland, J. Gerecitano, D. Shapiro, J. Le, S. I. Koh, T. Kimura, S. J. Green, T. W. Mak, T. Taniguchi, and J. Vilcek.** 1994. Requirement for transcription factor IRF-1 in NO synthase induction in macrophages. Science **263:**1612–1615.
20. **Kamijo, R., J. Le, D. Shapiro, E. A. Havell, S. Huang, M. Aguet, M. Bosland, and J. Vilcek.** 1993. Mice that lack the interferon-gamma receptor have profoundly altered responses to infection with bacillus Calmette-Guerin and subsequent challenge with lipopolysaccharide. J. Exp. Med. **178:**1435–1440.
21. **Kanno, Y., C. A. Kozak, C. Schindler, P. H. Driggers, D. L. Ennist, S. L. Gleason, J. E. Darnell, Jr., and K. Ozato.** 1993. The genomic structure of the murine ICSBP gene reveals the presence of the gamma interferon-responsive element, to which an ISGF3α subunit (or similar) molecule binds. Mol. Cell. Biol. **13:**3951–3963.
22. **Kasama, T., R. M. Strieter, N. W. Lukacs, P. M. Lincoln, M. D. Burdick, and S. L. Kunkel.** 1995. Interferon gamma modulates the expression of neutrophil-derived chemokines. J. Investig. Med. **43:**58–67.
23. **Kaufmann, S. H.** 1993. Immunity to intracellular bacteria. Annu. Rev. Immunol. **11:**129–163.
24. **Kumar, A., J. Haque, J. Lacoste, J. Hiscott, and B. R. Williams.** 1994. Double-stranded RNA-dependent protein kinase activates transcription fac-

tor NF-kappa B by phosphorylating I kappa B. Proc. Natl. Acad. Sci. USA **91:**6288–6292.
25. **Lorsbach, R. B., W. J. Murphy, C. J. Lowenstein, S. H. Snyder, and S. W. Russell.** 1993. Expression of the nitric oxide synthase gene in mouse macrophages activated for tumor cell killing. Molecular basis for the synergy between interferon-gamma and lipopolysaccharide. J. Biol. Chem. **268:**1908–1913.
26. **MacMicking, J., Q. W. Xie, and C. Nathan.** 1997. Nitric oxide and macrophage function. Annu. Rev. Immunol. **15:**323–350.
27. **Miossec, P., and M. Ziff.** 1986. Immune interferon enhances the production of interleukin 1 by human endothelial cells stimulated with lipopolysaccharide. J. Immunol. **137:**2848–2852.
28. **Muller, J. M., H. W. Ziegler-Heitbrock, and P. A. Baeuerle.** 1993. Nuclear factor kappa B, a mediator of lipopolysaccharide effects. Immunobiology **187:**233–256.
29. **Nagao, S. I., K. Sato, and Y. Osada.** 1986. Synergistic induction of cytotoxicity in macrophages by murine interferon-gamma and biological response modifiers derived from microorganisms. Cancer Immunol. Immunother. **23:**41–45.
30. **Noble, P. W., C. M. McKee, M. Cowman, and H. S. Shin.** 1996. Hyaluronan fragments activate an NF-kappa B/I-kappa B alpha autoregulatory loop in murine macrophages. J. Exp. Med. **183:**2373–2378.
31. **Ohshima, H., M. Tsuda, H. Adachi, T. Ogura, T. Sugimura, and H. Esumi.** 1991. L-Arginine-dependent formation of N-nitrosamines by the cytosol of macrophages activated with lipopolysaccharide and interferon-gamma. Carcinogenesis **12:**1217–1220.
32. **Pfeffer, L. M., J. E. Mullersman, S. R. Pfeffer, A. Murti, W. Shi, and C. H. Yang.** 1997. STAT3 as an adapter to couple phosphatidylinositol 3-kinase to the IFNAR1 chain of the type I interferon receptor. Science **276:**1418–1420.
33. **Redmond, H. P., K. D. Chavin, J. S. Bromberg, and J. M. Daly.** 1991. Inhibition of macrophage-activating cytokines is beneficial in the acute septic response. Ann. Surg. **214:**502–508. (Discussion, 508–509.)
34. **Siebenlist, U.** 1997. NFκB/IκB proteins: their role in cell growth, differentiation and development. Biochim. Biophys. Acta **1332:**R7–R13.
35. **Stark, G. R., I. M. Kerr, B. R. G. Williams, R. H. Silverman, and R. D. Schreiber.** 1998. How cells respond to interferons. Annu. Rev. Biochem. **67:**227–264.
36. **Tannenbaum, C. S., J. A. Major, and T. A. Hamilton.** 1993. IFN-gamma and lipopolysaccharide differentially modulate expression of tumor necrosis factor receptor mRNA in murine peritoneal macrophages. J. Immunol. **151:**6833–6839.
37. **Ulich, T. R., K. Guo, S. Yin, J. del Castillo, E. S. Yi, R. C. Thompson, and S. P. Eisenberg.** 1992. Endotoxin-induced cytokine gene expression in vivo. IV. Expression of interleukin-1 alpha/beta and interleukin-1 receptor antagonist mRNA during endotoxemia and during endotoxin-initiated local acute inflammation. Am. J. Pathol. **141:**61–68.
38. **Urbaschek, R., and B. Urbaschek.** 1983. Tumor necrosis factor and interleukin 1 as mediators of endotoxin-induced beneficial effects. Rev. Infect. Dis. **5:**199–207.
39. **Wan, Y., P. D. Freeswick, L. S. Khemlani, P. H. Kispert, S. C. Wang, G. L. Su, and T. R. Billiar.** 1995. Role of lipopolysaccharide (LPS), interleukin-1, interleukin-6, tumor necrosis factor, and dexamethasone in regulation of LPS-binding protein expression in normal hepatocytes and hepatocytes from LPS-treated rats. Infect. Immun. **63:**2435–2442.
40. **Weihua, X., V. Kolla, and D. V. Kalvakolanu.** 1997. Interferon gamma-induced transcription of the murine ISGF3gamma (p48) gene is mediated by novel factors. Proc. Natl. Acad. Sci. USA **94:**103–108.
41. **Weisz, A., S. Oguchi, L. Cicatiello, and H. Esumi.** 1994. Dual mechanism for the control of inducible-type NO synthase gene expression in macrophages during activation by interferon-gamma and bacterial lipopolysaccharide. Transcriptional and posttranscriptional regulation. J. Biol. Chem. **269:**8324–8333.
42. **Williams, J. G., G. J. Jurkovich, G. B. Hahnel, and R. V. Maier.** 1992. Macrophage priming by interferon gamma: a selective process with potentially harmful effects. J. Leukocyte Biol. **52:**579–584.
43. **Xie, Q. W., Y. Kashiwabara, and C. Nathan.** 1994. Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. J. Biol. Chem. **269:**4705–4708.

Editor: J. R. McGhee