The production of nitric oxide by macrophages has been implicated as a host defense mechanism against microbial pathogens and tumor cells. Recent reports have implicated interferon-α/β (IFN-α/β) as an autocrine/paracrine signal critical for the induction of murine iNOS. In this report we have systematically investigated the role of IFN-β in the induction of iNOS in the murine macrophage cell line, RAW 264.7. First, we demonstrate that IFN-β expression is highly up-regulated, and is secreted in response to lipopolysaccharide (LPS). Treatment of RAW macrophages with LPS results in a time-dependent phosphorylation of STAT-1 on both tyrosine residue 701 (Tyr-701) and serine residue 727 (Ser-727) that is consistent with the timing of endogenous IFN-β expression. LPS also induces interferon regulatory factor-1 expression with similar kinetics. We further demonstrate that exogenous IFN-β accelerates the induction of iNOS by LPS. The acceleration of iNOS induction is observed at the levels of transcription, protein expression, and NO formation. Accordingly, we propose that the cytokine environment of macrophages may determine the rate and magnitude of nitric oxide production, thereby regulating the cytotoxic response to pathogen challenge.

Activated macrophages produce nitric oxide (NO), a free radical species that mediates cytotoxic and cytostatic effects against pathogenic microbes and tumor cells (1–3). The enzyme responsible for the production of NO by macrophages is the inducible isoform of nitric-oxide synthase (iNOS), which catalyzes the oxidation of one of the equivalent guanidino nitrogens of arginine to form NO and citrulline (4). Compared with the neuronal and endothelial isoforms of NOS (nNOS and eNOS, respectively), iNOS generates high concentrations of NO, which accounts for the cytotoxic and cytostatic effects of NO on target cells (5). The regulation of NO production by iNOS has been extensively studied in murine macrophages, and occurs primarily at the level of transcription. The gene encoding iNOS is transcriptionally silent, but is dramatically induced by bacterial lipopolysaccharide (LPS). LPS activates Toll-like receptor-dependent signaling pathways in macrophages, including the transcription factor, NF-κB (6). However, maximal induction is not achieved by LPS alone. The induction of iNOS by LPS can be synergized by the addition of specific cytokines, including tumor necrosis factor-α, interleukin-1β, and interferon-γ (IFN-γ) (7). This synergy is reflected in the promoter region of the murine iNOS gene, which contains oligonucleotide motifs for the binding of both LPS- and several cytokine-induced transcription factors (8).

Both type I (IFN-α/β) and type II (IFN-γ) interferons mediate the induction of target genes via the activation of the JAK-STAT signaling pathway (9). Signal transducer and activator of transcription (STAT) proteins are a family of transcriptional activators that interact with IFN receptors and associated Janus kinase (JAK) proteins. The activation of STAT proteins is regulated by phosphorylation, which occurs in response to ligand-induced dimerization of IFN receptors, and the consequential activation of associated protein kinases. It has been reported that phosphorylation of the transcription factor, STAT-1 mediates the synergy between the IFN- and LPS-induced pathways in the induction of iNOS in murine macrophages (10). Accordingly, it has been proposed that murine macrophages secrete IFN-α/β in response to stimulation with LPS, and that these IFNs have an autocrine/paracrine function preceding the induction iNOS (11–13). More recently, it was shown that murine macrophages lacking the IFNAR1 component of the IFN-β receptor do not express iNOS protein in response to LPS alone, thereby implicating IFN-β as a critical element in the processes leading to LPS-stimulated NO production (14).

IRF proteins also mediate the transcriptional responses to IFN stimulation. IRF-1 and IRF-2 have opposing roles in cellular responses to IFNs. IRF-1 acts as a transcriptional activator, whereas IRF-2 represses transcription in a competitive fashion by occupying the same DNA-binding site as IRF-1 (15, 16). Typically, IRF-1 is not expressed or is undetectable. However, IRF-1 can be induced by a variety of stimuli, including IFN-β (17). Both the expression of IRF-1, and the presence of a crucial IRF-1-binding site within the promoter of the iNOS gene are necessary for the induction of iNOS in murine macrophages (18, 19). IRF-2 is constitutive in many cell types, but the regulation of its expression and its role in regulating iNOS in murine macrophages is less well defined.

In this report we systematically examine the events leading to the induction of iNOS in LPS-treated RAW macrophages. The LPS-induced expression of IFN-β is correlated with the activation of STAT and IRF proteins, transcription of the iNOS gene, iNOS protein levels, and finally, to the generation of NO. In doing this we have evaluated the role of IFN-β as an autocrine/paracrine mediator and provide both support and new insight into the existing model.
Role of Interferon-β in Murine iNOS Induction

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—RAW 264.7 macrophages (obtained from ATCC) were cultured in 10-cm dishes containing Dulbecco's modified Eagle's medium (Mediatech) supplemented with 50 mM HEPES, 4 mM L-glutamine, 1.2 mM l-arginine, 10 IU/ml penicillin, 10 μg/ml streptomycin, 250 ng/ml amphotericin B, and 10% fetal bovine serum. Cells were grown to confluence, then split using 0.05% trypsin, 0.53 mM EDTA and plated at a density of 3 x 10^5 cells/dish. Viability was assessed using trypan blue dye exclusion, and was typically greater than 95%. Cultures were maintained until passage 20, and then discarded. LPS (Escherichia coli serotype 0128:B12, Sigma) was suspended in sterile water and added to obtain the desired concentration. Recombinant murine IFN-β was obtained from Research Diagnostics, Inc., and rabbit anti-mouse IFN-β polyclonal antibody was obtained from PBL Biomedical Laboratories.

Ribonuclease Protection Assay—Following treatment with experimental agents, cells were harvested using a cell scraper and collected by centrifugation. Total RNA was extracted using the RNeasy Mini kit (Qiagen) and quantified by absorbance at 260 nm. 15 μg of RNA was dried in a Speed-Vac (Savant) and stored at −80 °C. Radiolabeled antisense RNA was synthesized using the Riboprobe transcription kit (Promega) and [α-32P]CTP. Templates for antisense RNA for murine iNOS and IFN-γ were purchased from Ambion. The template for murine GAPDH was purchased from Pharmingen. The template for murine IFN-β was generated by RT-PCR cloning. Briefly, RNA from RAW macrophages stimulated with LPS for 3 h was reverse transcribed using the Superscript premilation system (Life Technologies, Inc.), then subjected to 30 rounds of PCR using Ready-To-Go PCR beads (Amersham Bioscience, Inc.). The primers used for PCR were designed to amplify a 312-nucleotide sequence within the IFN-β coding region (forward primer: 5'-AACACATTATTCTCAGCACTG-3' and reverse primer: 5'-ATTCTAGGGCTCAACTGAC-3'). The PCR product was cloned into the pGEM-T vector (Promega), amplified, and linearized with NcoI. Ribonuclease protection assay (RPA) was performed using the RPA-III kit (Ambion), but substituting RNase ONE (Promega) for the supplied RNases. Protected fragments with the following lengths: iNOS, 249; IFN-γ, 210; IFN-β, 312; GAPDH, 97, were resolved using a 5% polyacrylamide:bisacrylamide (19:1) TBE (25 mM Tris borate, 2.5 mM EDTA, 8 M urea) gel. Gels were dried and subjected to autoradiography and PhosphorImager analysis (Molecular Dynamics).

Protein Immunoblots—Following treatment with experimental agents, cells were washed with phosphate-buffered saline, scraped, and collected by centrifugation. For total cell extracts, cells were lysed in RIPA buffer (50 mM Tris, pH 7.4, 1% Igepal CA-630, 0.25% deoxycholic acid, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 mM activated Na3VO4, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A). Nuclear extracts were prepared based on a modification of published protocols (20, 21). Cells pellets were resuspended in 0.5 ml of cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) and 50 μl of Igepal CA-630 extracted. Nuclei were pelleted by centrifugation at 1,000 x g for 5 min at 4 °C. Nuclear proteins were extracted in buffer B (10 mM HEPES, pH 7.9, 2.5 mM NaCl, 1.5 mM MgCl2, 0.1 mM EDTA, 0.1 mM EGTA, 25% glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) and 50 μl of Igepal CA-630 were added. Nuclei were resuspended and centrifuged at 14,000 x g for 10 min at 4 °C to remove debris. Protein concentrations were determined by the Bio-Rad assay. 5 μg of protein per sample was resolved by SDS-PAGE, and electroblotted onto a nitrocellulose membrane. Membranes were blocked with blocking buffer (20 mM Tris, pH 7.6, 140 mM NaCl, 0.1% Tween 20, 5% nonfat dry milk) prior to incubation with primary antibody diluted in blocking buffer. After washing 3 times in TBS containing 0.1% Tween 20, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody. Chemiluminescent detection with luminal was performed according to manufacturer's protocol (Santa Cruz Biotechnology). Antibodies were obtained from the following sources: anti-p-STAT-1α, anti-p-STAT-1 (Y701), anti-IRF-1, anti-IRF-2, and anti-iNOS, and all secondary antibodies were purchased from Santa Cruz Biotechnology; anti-phospho-STAT-1 (Ser-727) was purchased from Upstate.

Virul Inhibition Assay—For quantifying units of interferon-β in cell culture media, samples were submitted to PBL Biomedical Laboratories for analysis. To assay for interferon content, samples were added to cultures of murine L929 fibroblasts and challenged with encephalomyocarditis virus (22, 23). One interferon unit per ml is defined as that quantity which inhibits the cytopathic effect of encephalomyocarditis virus by 50%.

RESULTS

LPS Induces a Time-dependent Induction in IFN-β mRNA Expression and Protein Secretion in RAW Macrophages—IFN-β is a critical and necessary component of the signaling processes leading to induction of the iNOS gene in murine macrophages (13, 14). We sought to determine the capacity of RAW macrophages to express and secrete IFN-β in response to LPS. Because IFN-γ can also initiate signaling events activated by IFN-β, we investigated the expression of IFN-γ as well. Cells were cultured in 100-mm plates to a subconfluent density of 5 x 10^6/cm^2, and treated with LPS (100 ng/ml). A RPA was used to monitor either IFN-β or IFN-γ transcripts in a series of time points following induction. Total RNA was extracted at 0.5, 1, 2, 4, 6, and 8 h after treatment, and simultaneously probed with [α-32P]radiolabeled antisense constructs for IFN-β or IFN-γ, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (loading control) transcripts. Data reveal that IFN-β mRNA was not expressed in untreated cells. Addition of LPS induced IFN-β mRNA within 1 h, peak levels were attained by 4 h, and levels subsequently declined over the remaining time points (Fig. 1). This observation suggests the potential existence of a regulatory mechanism for a timed and limited expression of IFN-β. In contrast, IFN-γ mRNA is constitutively expressed in RAW macrophages, but at a low level compared with the LPS- inducible IFN-β expression (data not shown). Also, addition of LPS had no effect on the basal expression of IFN-γ mRNA. This lack of induction suggests that IFN-γ does not
share the autocrine/paracrine functions of IFN-β in LPS-treated macrophages.

The amount of IFN-β secreted into the culture medium was measured by viral inhibition assay on murine L929 cells challenged with encephalomyocarditis virus. RAW macrophages at a density of 5 × 10⁶/cm² were treated with LPS, and the medium was sampled at 1, 4, and 8 h and assayed for IFN-β. At 1 h after LPS addition, IFN-β was below the detection limit of the assay (<6 units/ml). At 4 h, the mean level of IFN-β was 530 units/ml, and at 8 h the mean level was 2561 units/ml (Fig. 2). The highly inducible expression and the timing of IFN-β in response to LPS agree with and support the current model of an autocrine/paracrine role for IFN-β in macrophage induction.

**LPS Induces IFN-β-dependent Signaling Pathways in RAW Macrophage—**Binding of IFN-β to functional Type I-IFN receptor results in activation of JAK-STAT signaling pathways. Phosphorylated STAT proteins regulate gene transcription by binding to conserved sequences in the promoters of IFN-induced genes, including iNOS (24). The promoter of the murine iNOS gene contains GAS elements, which mediate the binding of STAT homo- and heterodimers (25). Phosphorylated STAT-1 is a protein that is common to many functional STAT complexes. Therefore, we sought to determine, by Western blot analysis, the kinetics of STAT-1 phosphorylation in response to LPS, as an example of the autocrine/paracrine activation of an IFN-dependent signaling process. RAW macrophages at a density of 5 × 10⁶/cm² were treated with either LPS (100 ng/ml), or as a positive control for IFN-dependent pathway activation, IFN-β (100 units/ml) for various time periods. Nuclear extracts were prepared and analyzed by Western blot using a STAT-1 antibody. We observed a time-dependent phosphorylation in response to LPS, observed as a shift in the mobility of the STAT-1α band (Fig. 3). The phosphorylated band appeared 2 h following treatment with LPS, and remained elevated. The p-STAT-1α band was also observed, although sooner and with greater intensity, in samples treated with IFN-β.

Two phosphorylation sites, Tyr-701 and Ser-727, differentially regulate STAT-1 (26, 27). Because phosphorylation of both sites is required for maximal transcriptional activity, we wanted to determine if one or both sites are phosphorylated in response to LPS. To this end, Western blots were performed using antibodies specific for tyrosine- and serine-phosphorylated forms of STAT-1. We observed that STAT-1 is phosphorylated on both residues Tyr-701 and Ser-727 in LPS-treated cells (Fig. 3), and that the approximate 2-h delay in phosphorylation corresponds with the timing of endogenous IFN-β synthesis. As anticipated, treatment of macrophages with IFN-β alone also causes phosphorylation of STAT-1 on both Tyr-701 and Ser-727 residues, but with comparatively rapid kinetics, as phosphorylation was observed within 30 min of IFN-β addition. Based on the similar kinetics and intensity of tyrosine and serine phosphorylation, we hypothesize that the mobility shift in the upper blot of Fig. 3 likely represents STAT-1α that is phosphorylated on both residues.

IFN-stimulated STAT activation mediates the expression of a second family of transcription factors, the IRF proteins (28). Since a critical role for IRF-1 has been described for the expression of iNOS in murine macrophages, we sought to determine the effect of LPS on the levels of IRF-1, and on its antagonistic counterpart, IRF-2. RAW macrophages at a density of 5 × 10⁵/cm² were treated with either LPS (100 ng/ml), or as a positive control, IFN-β (100 units/ml). Nuclear extracts were prepared and analyzed by Western blot using antibodies against IRF-1 and IRF-2. In unstimulated macrophages IRF-1 is not expressed at a detectable level (Fig. 4). Following treatment with LPS, however, a dramatic time-dependent increase in the levels of IRF-1 is observed that correlates with the timing of STAT-1 phosphorylation. As anticipated, IRF-1 is expressed sooner in samples treated with IFN-β. The relative delay of IRF-1 expression in LPS-treated samples is likely related to the time needed for the synthesis and secretion of IFN-β as an autocrine/paracrine signal. In contrast to IRF-1, the level of IRF-2 was relatively high in nuclear extracts from unstimulated macrophages. The basal expression of IRF-2 was unaffected by either LPS or IFN-β, suggesting that the induction of IRF-1 rather than inhibition of IRF-2 expression mediates the IFN-dependent expression of murine iNOS.

Neutralizing Antibody against IFN-β Attenuates the Expression of iNOS mRNA in LPS-treated RAW Macrophages—Having shown that IFN-β is induced and secreted in response to LPS, we next studied the effect of blocking IFN-β with neutralizing antibodies on the time course of iNOS induction. RAW macrophages at a density of 5 × 10⁶/cm² were treated with either LPS (100 ng/ml), or a combination of LPS (100 ng/ml) and a polyclonal anti-IFN-β neutralizing antibody (1000 neutralization units/ml). The addition of neutralizing antibody did not totally block, but did significantly attenuate the induction of iNOS mRNA as determined by RPA (Fig. 5). Accordingly, the magnitude of iNOS expression was lower in the presence of neutralizing antibody than with LPS alone at all time points measured. Curiously, whereas iNOS transcription in antibody-treated samples was not totally blocked, expression did plateau at 6 h after induction. In contrast, iNOS expression in samples treated with LPS only continued to rise over the course of the experiment. This observation implies that IFN-β-dependent signals function in initiating transcription, as well as in the continued generation of iNOS message at later time points.

**IFN-β Accelerates the Timing of iNOS mRNA Expression, Protein Expression, and NO Generation in LPS-treated RAW Macrophages—**Detectable levels of iNOS transcript in RAW macrophages are typically observed between 2 and 4 h following treatment with LPS (100 ng/ml). The recent reports implicating IFN-β as an autocrine signal in the transcriptional activation of iNOS by LPS motivated us to investigate the effect of exogenous IFN-β on the timing of iNOS induction. RAW macrophages were cultured in 100-mm plates to a density of 5 × 10⁵/cm², and treated with LPS (100 ng/ml) or a combination of LPS (100 ng/ml) and IFN-β (100 units/ml). These were
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Expression of IRF-1 and IRF-2 in RAW macrophages following treatment with either LPS or IFN-β. C (control) indicates expression in uninduced cells. Treatment with LPS results in a time-dependent induction of IRF-1, whereas no difference is observed in the expression of IRF-2. For these experiments, RAW macrophages were treated with either LPS (100 ng/ml) or IFN-β (100 units/ml) and incubated for the indicated times. Cells were lysed and nuclear proteins extracted according to protocol. 5 μg of nuclear extract was used per sample for immunoblot analysis.

Fig. 3. Phosphorylation of STAT-1 in RAW macrophages following treatment with either LPS or IFN-β. C (control) indicates expression in uninduced cells. Treatment with LPS results in a time-dependent phosphorylation of STAT-1 on residues Tyr-701 and Ser-727. Phosphorylated STAT-1α is observed as a shifted band in the upper blot. The levels of tyrosine (Y701) and serine (S727) phosphorylated STAT-1 are represented in the middle and lower blots, respectively. These phospho-specific gels correlate with the timing and intensity of the p-STAT-1α band, suggesting the shifted STAT-1α band represents both serine and tyrosine phosphorylation. For these experiments, RAW macrophages were treated with either LPS (100 ng/ml) or IFN-β (100 units/ml) and incubated for the indicated times. Cells were lysed and nuclear proteins extracted according to protocol. 5 μg of nuclear extract was used per sample for immunoblot analysis.

Fig. 4. Expression of IRF-1 and IRF-2 in RAW macrophages following treatment with either LPS or IFN-β. C (control) indicates expression in uninduced cells. Treatment with LPS results in a time-dependent induction of IRF-1, whereas no difference is observed in the expression of IRF-2. For these experiments, RAW macrophages were treated with either LPS (100 ng/ml) or IFN-β (100 units/ml) and incubated for the indicated times. Cells were lysed and nuclear proteins extracted according to protocol. 5 μg of nuclear extract was used per sample for Western blot analysis.

subsequently assayed by RPA for the presence of iNOS mRNA in a series of time points following induction (Fig. 6A). Total RNA was extracted at 2, 4, 6, 8, and 10 h after treatment, and simultaneously probed with 32P-radiolabeled antisense constructs complementary to sequences within the iNOS and GAPDH (loading control) transcripts. The data reveal that IFN-β accelerates the induction of iNOS mRNA by LPS, with significant levels of iNOS mRNA being detected within 2 h following treatment. PhosphorImager analysis of the RPA gel illustrates the effect of IFN-β plus LPS in hastening the production of iNOS transcript, in contrast to treatment with LPS alone. Also, IFN-β synergizes with LPS to enhance the level of iNOS mRNA expression as much as 5.7-fold over LPS alone at the 4-h time point, and maintaining a higher level of expression throughout the experiment.

We also investigated the effect of IFN-β on iNOS protein expression in response to LPS. RAW macrophages were again cultured in 100-mm plates at a density of 5 × 10^5/cm^2, and treated with LPS (100 ng/ml) or both LPS (100 ng/ml) and IFN-β (100 units/ml). Cells were lysed with RIPA buffer and samples analyzed by Western blot using a commercially available polyclonal antibody against murine iNOS. LPS-treated RAW macrophages express detectable levels of iNOS protein within 4–6 h following stimulation. Treatment with LPS and IFN-β together accelerated the rate of protein expression, with significant levels of iNOS being detected by Western blot analysis within the first 2 h (Fig. 6B). This temporal shift in protein expression to a time point ~3 h earlier than treatment with LPS alone concurs with the data obtained for iNOS transcript expression. Likewise, the synergy between IFN-β and LPS is observed in the magnitude of iNOS protein expression, as indicated by the density of the iNOS bands. This indicates that the effects of IFN-β in accelerating and synergizing iNOS expression in response to LPS is affected at the level of transcription, but maintained at the level of protein expression.

To determine if the acceleration of iNOS induction was maintained to the level of enzyme catalytic activity, we monitored formation of the iNOS product, NO, using a modified Clark-type NO-electrode. RAW macrophages at a density of 1 × 10^6/cm^2 were induced with either LPS (1 μg/ml) or LPS (1 μg/ml) + IFN-β (100 units/ml). Addition of LPS alone resulted in the detection of NO at ~6 h following induction (Fig. 7). A steady-state level of NO generation was achieved by about 9 h, with a maximum detected concentration of 305 nM. Addition of 1-γN^2-monomethylarginine (1 mM) caused a sharp decline in the level of NO, with levels returning to baseline within 15 min of addition, indicating that the source of NO in this experiment was due to enzymatic generation by NOS. In contrast, addition of LPS plus IFN-β resulted in detectable NO levels within 3 h of induction. This observation is consistent with the temporal shifts seen in both mRNA and protein expression of iNOS. A steady-state level of NO formation was achieved within 6 h, and the maximum NO concentration observed was 316 nM. Again, the source of this NO was enzymatic, as indicated by the
total ablation of detectable NO following the addition of L-N\textsuperscript{G}-monomethylarginine.

**DISCUSSION**

The production of NO by macrophages has been implicated as a host defense mechanism against pathogens and neoplastic cells. Expression of iNOS leads to the high-output production of NO, for which several mechanisms of cellular toxicity have been proposed. For example, NO has been shown to inhibit the activity of the iron-sulfur containing respiratory enzyme, aconitase, and heme-containing proteins such as cytochrome c oxidase (1, 29). It can also cause the disruption of zinc-containing proteins, such as metallothionein and zinc-finger containing transcription factors (30). Also, NO may elicit a cytostatic effect by inhibiting ribonucleotide reductase and therefore, DNA synthesis (31). Cytostasis of target cells is also achieved by NO-mediated nitrosation of ornithine decarboxylase, which is the rate-determining enzyme in polyamine biosynthesis (32–34). Furthermore, the cellular damage related to macrophage-derived NO has been reported to induce apoptosis in target tumor cells (35).

Given the significance of NO in host defense, we sought to systematically investigate the mechanisms and timing of events leading from challenge with a pathogenic stimulus (LPS) to the generation of NO by macrophages. In this report, we present a comprehensive study on the induction of iNOS by LPS in RAW macrophages, measuring and correlating events leading from signal transduction to iNOS transcription, protein expression, and product formation. We believe one of the values of this study is in unifying several individually addressed phenomena into a single investigation, in which a cellular response has been followed from the initiation of signaling cascades to the production of a cytotoxic effector molecule. Previous reports on the mechanisms of iNOS induction in macrophages clearly implicate IFN-\(\beta\) as a critical intermediate (12–14, 36, 37). Based on the evidence that we have obtained, and on these earlier reports, a current model for the signaling events mediating the expression of iNOS in response to LPS is presented in Fig. 8. According to this model, the expression of iNOS is regulated by a combination of LPS- and IFN-induced transcription factors. One aspect of this model that we have previously examined is the necessary role of NF-\(\kappa B\) for the induction of iNOS in LPS-treated RAW macrophages (38). In the present study, we sought to confirm the role of endogenous IFN-\(\beta\) as an autocrine/paracrine activator of iNOS expression, and to determine the timing of downstream events, including activation of the JAK/STAT and IRF signaling pathways, iNOS transcription and translation, and ultimately NO generation.

Activation of NF-\(\kappa B\) by LPS mediates transcription of IFN-\(\beta\) (39–42). In our observations, IFN-dependent signaling pathways are activated in response to LPS in a time-dependent manner that correlates with the expression and secretion of IFN-\(\beta\). Accordingly, we have seen that STAT-1 is phosphorylated on Tyr-701 and Ser-727 in LPS-treated RAW cells within the same time frame as IFN-\(\beta\) expression. Similar findings have been reported elsewhere. In particular, in the Bac1.2F5 macrophage LPS treatment also causes phosphorylation of STAT-1 on Ser-727. This requires activation of the p38 mitogen-activated protein kinase pathway, as demonstrated by use of the p38 mitogen-activated protein kinase inhibitor, SB203580 (43). Moreover, it has been reported that p38 kinase activity is stimulated by IFN-\(\alpha/\beta\) (44). It is possible that the serine phosphorylation of STAT-1 we observe in RAW cells is partly mediated by a p38-dependent pathway.

We also demonstrate that LPS induces a time-dependent induction in IRF-1 synthesis. It has been previously reported in that expression of IRF-1 is essential for the induction of iNOS in murine macrophages. First, macrophages from \(\text{IRF-1}^{-/-}\) mice fail to express iNOS in response to infection with Mycobacterium bovis (18) Furthermore, experiments with iNOS re-
porter constructs in RAW macrophages indicate that the IRF-element at nucleotides −913 to −923 is necessary for the synergistic effects of IFN-γ (19). Some reports indicate that exceptions to this model may exist, and deserve study in RAW macrophages. For example, in J774 macrophages, the protein synthesis inhibitor cyclohexamide did not block, but rather potentiated transcription of the IRF-1 gene in response to LPS, implying that IRF-1 could be induced by an undefined IFN-independent mechanism (45).

However, the role of IFN-β as an autocrine/paracrine mediator of iNOS activation in RAW macrophages was confirmed by the use of antibody against IFN-β. Simultaneous incubation with IFN-β-neutralizing antibody and LPS attenuated the induction of iNOS mRNA compared with LPS alone. This result...
is consistent with earlier investigations in both RAW macrophages and other cell types (12, 13, 37). Also consistent with these reports was our observation that iNOS expression was inhibited, but not completely blocked, by IFN-β-antibody treatment. The inability to completely abolish iNOS expression with neutralizing antibody against IFN-β implies to us that in RAW cells, additional LPS-dependent pathways to iNOS gene expression may be operating. Perhaps, as demonstrated in the Balb.2F5 macrophage line, LPS may also be capable of stimulating STAT phosphorylation though an interferon-independent mechanism (43).

Seeing that the endogenous secretion of IFN-β stimulates transcription of the iNOS gene in RAW cells, we hypothesized that bypassing this step by the addition of exogenous IFN-β should accelerate the induction of iNOS by LPS. Indeed, macrophages treated with LPS and IFN-β expressed iNOS mRNA 2–3 h sooner than macrophages treated with LPS alone. In our observations, this corresponds to the time required for the synthesis and secretion of IFN-β in LPS-treated macrophages, which supports an autocrine/paracrine role for endogenous IFN-β. The acceleration of iNOS induction by IFN-β is also observed at the level of iNOS protein expression, and at the level NO generation by means of an NO electrode. Also, we believe this to be the first report in which an NO electrode has been used to continuously monitor NO levels produced by cultured macrophages following activation.

The acceleration of iNOS induction by exogenous IFN-β suggests that the cytokine environment of macrophages may determine the rate of iNOS induction in response to LPS. Acting as a co-stimulatory signal, IFN-β both accelerates and potentiates the induction of iNOS in response to LPS. In contrast, it has been reported by others that preincubating murine macrophages with IFN-β attenuates iNOS induction in response to LPS (46, 47). The explanations for this phenomenon differ in the two reports. In one case, IFN-β was indicated to attenuate subsequent NF-κB activation by LPS. Alternatively, the other report provides evidence for an enhanced turnover of phosphorylated STAT-1 subsequently to a prolonged treatment with IFN-β. These observations, in conjunction with what we and others report, suggests a novel dual role for IFN-β in mediating iNOS transcription that depends upon the concomitant levels of bacterial endotoxin. Accordingly, we propose that when LPS is present as a co-stimulatory signal, IFN-β accelerates and potentiates the induction of iNOS. As infection levels decline and LPS is low or absent, IFN-β attenuates macrophage activation. We believe that this is an interesting model for macrophage autoregulation that deserves further study in vitro and in animal models.

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