Necessity and Sufficiency of Beta Interferon for Nitric Oxide Production in Mouse Peritoneal Macrophages

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Bacterial lipopolysaccharide and some cytokines can activate macrophages to secrete nitric oxide. Macrophage-derived nitric oxide is a key cytotoxic factor for microbialidal and tumoricidal processes. We report here that a monoclonal antibody specific for beta interferon inhibited lipopolysaccharide-induced nitric oxide production in thioglycollate-elicited C3H/HeJ peritoneal macrophages and macrophage-like cell line RAW 264.7. In addition, exogenous added beta interferon enabled lipopolysaccharide-hyporesponsive thioglycollate-elicited C3H/HeJ peritoneal macrophages to produce nitric oxide in response to lipopolysaccharide. These data support the concept that beta interferon provides an essential signal(s) for lipopolysaccharide-triggered nitric oxide production by mouse macrophages. Heat-killed Staphylococcus aureus, a gram-positive bacterium which was unable to initiate nitric oxide production in thioglycollate-elicited C3H/HeJ peritoneal macrophages in vitro, promoted nitric oxide formation in the presence of beta interferon, suggesting that beta interferon may be a general cofactor necessary for bacteria-derived stimulus-induced nitric oxide production in these macrophages. However, neither beta interferon nor tumor necrosis factor alpha, alone or in combination, triggered nitric oxide production in thioglycollate-elicited mouse peritoneal macrophages, demonstrating that these macrophage-derived cytokines, while necessary, were not sufficient by themselves for the induction of nitric oxide production in these cells. On the other hand, gamma interferon and tumor necrosis factor alpha acted together to induce nitric oxide production in vitro in the absence of lipopolysaccharide in thioglycollate-elicited mouse peritoneal macrophages, indicating that these two types of interferons provided different signals during the activation of these macrophages.

Nitric oxide (NO), synthesized from l-arginine, is a potent effector molecule produced by many mammalian cell types (36). For example, NO accounts for the biological activity of the endothelium-derived relaxing factor in endothelial cells (41). In addition, macrophage-derived NO functions as a cytotoxic molecule for invading microorganisms (1, 2, 22, 25, 39) as well as tumor cells (16, 34, 48). NO released from Kupffer cells and hepatocytes has inhibitory effects on the total protein synthesis of these cells (9, 10). Recent reports have suggested that NO synthesis in these cells may serve to reduce hepatic damage during acute murine endotoxemia (26). Large amounts of NO formation may cause significant nitrosation of amines, which have been implicated in carcinogenesis (31, 35).

Regulatory mechanisms for NO production were recently studied intensively, and two types of NO synthases were characterized (45). One is constitutive and Ca2+ and calmodulin dependent. The other is Ca2+ and calmodulin independent and inducible after immunological activation of macrophages, endothelial cells, and some other cell types. Bacterial lipopolysaccharide (LPS) can increase both blood NO concentration in vivo and production of NO by macrophages in vitro (46). All three species of interferon (IFN), i.e., alpha interferon (IFN-α), IFN-β, and IFN-γ, are able to augment LPS-induced NO production (4, 13, 47). In addition, IFN-γ in combination with interleukin-1α (IL-1α) and/or tumor necrosis factor alpha/beta (TNF-α/β) can interact synergistically to initiate NO production (4, 13). However, among the cytokines tested to date, including IL-1β, IL-2, IL-3, IL-4, IL-6, IFNs, macrophage colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, TNF-α/β, and transforming growth factor beta (TGF-β), IFN-γ is the only cytokine which has been reported to be capable of inducing NO production by itself under some experimental conditions in certain murine macrophages (13, 17, 47). On the other hand, cytokines such as TGF-β-1, TGF-β-2, TGF-β-3, IL-10, and macrophage deactivating factor have been shown to inhibit the induction of NO production (12, 22). Recent reports demonstrated that IL-4 blocked inducible NO production (3). The anti-inflammatory reagents dexamethasone and hydrocortisone have also been shown to prevent NO formation (15, 30). These studies reflect the fact that inducible NO production is tightly regulated and that a delicate functional balance among various microbial stimuli, host-derived cytokines, and hormones in the microenvironment is very important for this regulation. The significance of this regulation has been particularly implicated in a recent report showing that high-dose IL-2 therapy, which is known to induce IFN-γ, TNF-α, and IL-1 in vivo, causes a striking increase in circulating levels of NO as well as urinary excretion of stable NO metabolites in patients with advanced cancer (28).

Several lines of evidence indicate that different regulatory pathways may exist for inducible NO production. First, LPS-induced NO formation has been observed with both nude mice and CBA/N mice, indicating that neither functional T cells nor mature B cells are required for this LPS-initiated response in vivo (4). In addition, different macrophage cell lines respond differentially to IFN-γ activation in terms of NO production (47); only two cell lines tested in that study (PLS-1.8 and RAW 264.7) can synthesize NO in response to IFN-γ, while the other two (WEHI-2 and J774A.1) cannot, although all of these four cell lines can
produce NO upon LPS activation. Furthermore, macrophage deactivating factor and TGF-β have been shown to partially block NO release in macrophages activated with IFN-γ plus TNF-α; however, these inhibitors are significantly less efficient in the downregulation of IFN-γ plus LPS-induced NO formation (12). Finally, the induction of NO formation by IFN-γ plus muramyl dipeptide can be suppressed almost completely by an anti-TNF-α neutralizing antibody, while the same antibody can only modestly inhibit NO formation induced by the combination of IFN-γ plus LPS (17).

Macrophages activated with bacterial stimuli, such as LPS, can secrete a variety of cytokines, including TNF-α (40) and IFN-β (7, 27). Both of these cytokines function as autocrine or paracrine regulators for macrophage activation (14, 34). In this manuscript, we have further defined the important regulatory role of the macrophage-derived cytokine IFN-β in bacterial stimulus-induced NO production. We have been able to demonstrate here that, in LPS-initiated NO production by thioglycolate (TG)-elicited mouse peritoneal macrophages, IFN-β provides a required signal which appears to be functionally different from that provided by IFN-γ. We have also shown that IFN-β complements heat-killed Staphylococcus aureus for NO production, implicating the generality of IFN-β-mediated regulation for bacterial stimulus-initiated NO release in mouse TG-elicited peritoneal macrophages. Moreover, a monoclonal antibody (MAb) against IFN-β is able to inhibit LPS-induced NO production in the macrophage-like cell line RAW 264.7, therefore suggesting that IFN-β has autocrine regulatory functions not only in TG-elicited mouse peritoneal macrophages but also in other mouse macrophages. (This research was conducted in partial fulfillment of the Ph.D. dissertation requirements for X. Zhang in the Department of Microbiology, Molecular Genetics and Immunology at the University of Kansas Medical Center.)

MATERIALS AND METHODS

Animals. C3HeB/FeJ and C3H/HeJ female mice were obtained from the Jackson Laboratory (Bar Harbor, Maine) and used at the ages of 4 to 16 weeks.

Reagents. Fetal bovine serum (FBS) was purchased from Intergen Co. (Purchase, N.Y.) and heated at 56°C for 1 h. RPMI 1640 with glutamine and N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) was obtained from Whitaker M. A. Bioproducts (Walkersville, Md.). Penicillin, streptomycin, glutamine, and Hanks’ balanced salt solution without Ca2+ and Mg2+ (HBSS) were products of JRH Biosciences Inc. (Lenexa, Kans.). Cell culture medium used for culturing macrophages in vitro was RPMI 1640 containing 10% FBS, an additional 4 mM glutamine, 200 U of penicillin per ml, and 200 U of streptomycin per ml. Escherichia coli O111:B4 wild-type smooth LPS (S-LPS) was prepared as lipid A-rich fraction II of phenol-extracted E. coli O111:B4 (38). Ra-LPS, purified from the Salmonella minnesota R600 mutant (42), was a product of List Biological Laboratories Inc. (Campbell, Calif.). The particular lot of Ra-LPS used in this experiment is reported by the distributor to contain less than 1.6% (by weight) protein. LPS stock solutions at a concentration of 1 or 5 mg/ml in pyrogen-free sterile H2O were diluted into working concentrations with cell culture medium immediately before use. The endotoxin level in each component of the cell culture medium was tested with a Limulus amebocyte lysate kit at a sensitivity of 0.5 endotoxin unit per ml (Pyrotell, Woods Hole, Mass.), and negative results (LPS, <0.5 endotoxin unit per ml) were obtained with all of the reagents at working concentrations. S. aureus was generously provided by Chia Y. Lee (Kansas University Medical Center, Kansas City) and heated for 2 h at 100°C. Rat MAbS (immunoglobulin G1 [IgG1]) specific for mouse IFN-β and IFN-α were both purchased from Seikagaku America Inc. (Rockville, Md.) and had neutralizing titers of 1:400,000 and 1:500, respectively. The neutralizing titers of these antibodies were confirmed with L929 fibroblasts challenged with vesicular stomatitis virus and established to be close to the titers reported by the manufacturer (33b). Purified mouse IFN-β (specific activity, 5 × 107 U/ml) was purchased from Lee Biomolecular Research Laboratories Inc. (San Diego, Calif.). According to the distributor, this IFN-β product was prepared and evaluated as containing less than 0.5% IFN-α and no detectable IFN-γ (32). Recombinant mouse IFN-γ (specific activity, 2.3 × 108 U/ml) was a generous gift from Genentech Inc. (San Francisco, Calif.). Recombinant mouse TNF-α (specific activity, 8 × 104 U/ml) was purchased from Genzyme (Cambridge, Mass.).

Macrophages. Mice were injected intraperitoneally with 1.5 ml of 4% (wt/vol) Brewer TG (Difco Laboratories, Detroit, Mich.). Peritoneal cells were harvested 5 days later by lavage with cell culture medium and seeded into 24- or 96-well cluster plates (Costar Corp., Cambridge, Mass.). The cells were incubated in 0.5 ml of culture medium at 37°C in a humidified 5% CO2 incubator. After overnight incubation, nonadherent cells were removed by washing the plates twice with HBSS. These TG-elicited peritoneal macrophages were then activated with LPS or S. aureus in the presence or absence of various concentrations of IFNs or anti-IFN MAbS. At various times following activation, the cell culture supernatants were collected, and TNF-α production and NO production were assessed.

The purity of the peritoneal macrophage population was evaluated by immunochromatography with MAb specific for a macrophage surface antigen, F4/80 (generously provided by Judy L. Pace, Kansas University Medical Center, Kansas City) (6). In brief, adherent peritoneal cells were washed twice with phosphate-buffered saline (PBS) (pH 7.4) and air dried for 1 h at room temperature. The cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature and then washed twice with PBS. The fixed cells were immunochromatographically stained with 11.6 μg of MAb against F4/80 per ml by use of a Histostain-SP kit (Zymed Laboratories Inc., South San Francisco, Calif.). Rat IgG served as an antibody control. Ninety-nine percent of the adherent peritoneal cells were positively stained with MAb against F4/80 and therefore were recognized as macrophages.

Macrophage-like cell line RAW 264.7 (American Type Culture Collection) was cultured in Spinner flasks containing 25 mM HEPES-buffered RPMI 1640 medium (GIBCO/BRL, Grand Island, N.Y.) with 10% (vol/vol) FBS (HyClone, Logan, Utah), 2 mM glutamine (JRH Biosciences), 100 μg of streptomyacin (Sigma, St. Louis, Mo.) per ml, and 100 U of penicillin (Bristol-Myers Squibb, Princeton, N.J.) per ml.

TNF-α assay. TNF-α production was quantified by a cytotoxicity assay with TNF-α-sensitive cell line L929 as described previously (51). In brief, L929 cells were cultured in 96-well plates (Costar) for 24 h at 37°C in a humidified 5% CO2 incubator. The cells were then treated with 5 μg of actinomycin D (Merck Sharp & Dohme, West Point, Pa.) per ml for 3 h. Serial twofold dilution of macrophage culture supernatants was then carried out directly on the L929 cell
cultures, and the plates were incubated overnight at 37°C in a humidified 5% CO₂ incubator. The extent of killing of L929 cells mediated by macrophage culture supernatants was assessed by the 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyltetrazolium bromide (MTT) assay (11). In brief, an MTT stock solution (5 mg/ml in PBS) was filtered and diluted into a 1-mg/ml working solution with RPMI 1640 without phenol red (IRH Biosciences) immediately before use. L929 cells treated with macrophage culture supernatants were washed twice with HBSS and then incubated with 100 µl of the MTT working solution for 3 h at 37°C in a humidified 5% CO₂ incubator. The 96-well plates were centrifuged at 1,520 x g for 5 min. The blue product of the reaction was solubilized with 100 µl of isopropanol (Fisher Scientific, Fair Lawn, N.J.) per well, and the A₅₇₀ was measured on an MR700 Microplate reader (Dynatech Laboratories Inc., Chantilly, Va.). One unit of TNF-α activity was defined as the amount of TNF-α required to lyse 50% of the L929 cells in the assay (44).

The cytotoxicity assay in L929 cells with TG-elicited peritoneal macrophage culture supernatants was carried out in the absence or presence of rabbit neutralizing polyclonal antibody against murine TNF-α (1.48 × 10⁵ neutralizing units per ml; generously provided by R. E. McCallum, Department of Medical Microbiology and Immunology, Texas A & M University). The anti-TNF-α antibody completely abolished macrophage culture supernatant-dependent cytotoxic activity (9a). These results therefore confirm that the cytotoxic activity in macrophage culture supernatants was solely due to TNF-α.

Analysis of NO production. NO production was assessed by measuring nitrite, a stable metabolic product of NO, in macrophage culture supernatants (47). In brief, Griess reagent (1:1 [vol/vol] mixture of 0.1% N-[1-naphthyl]ethylenediamine dihydrochloride [Sigma] in H₂O-1% sulfanilamide [Sigma] in 5% H₂PO₄) was added to 96-well round-bottomed microtiter immunoassay plates (Dynatech Laboratories) at 100 µl per well. An equal volume of macrophage culture supernatants was then mixed with the Griess reagent, and the A₅₇₀ was determined on an MR700 Microplate reader. The nitrite amount was calculated from an NaNO₂ standard curve.

RESULTS

Role of endogenous IFNs in LPS-induced NO production in mouse macrophages. Rat IgG1 neutralizing MAbs for mouse IFN-β and IFN-α were used to assess the regulatory functions of the macrophage-derived type I IFNs (IFN-β and IFN-α) in inducible NO formation by mouse macrophages in response to LPS activation in vitro. These two MAbs are highly specific; they have no reported cross-reactivities with each other, and neither of them can recognize IFN-γ (29). In addition, we have confirmed that, at any of the concentrations used in the studies to be reported, neither of these two MAbs preparations was capable of initiation of NO production in macrophages (data not shown). In parallel experiments, therefore, macrophages were activated with 100 ng of E. coli O111:B4 S-LPS per ml for 24 h in the presence of various concentrations of anti-IFN-β or anti-IFN-α MAb. NO production induced by LPS in the absence of these MAbs served as a control.

As shown by the data in Fig. 1A, the presence of the anti-IFN-β MAb effectively inhibited LPS-induced NO production by C3HeB/FeJ TG-elicited peritoneal macrophages in a dose-dependent fashion. Approximately 30% reduction

FIG. 1. Effects of anti-IFN MAbs on LPS-induced NO production in mouse macrophages. (A) TG-elicited C3HeB/FeJ mouse peritoneal cells were seeded into 24-well plates at 5 × 10⁵ cells per well. After overnight adhesion, these cells were washed twice and then activated with 100 ng of E. coli O111:B4 S-LPS per ml in the presence or absence of various dilutions of the anti-IFN-β or anti-IFN-α MAb in 0.5 ml of cell culture medium. (B) RAW 264.7 cells were seeded into 96-well plates at 1 × 10⁶ cells per well. After 2 h of adhesion, the cells were washed and then activated with 100 ng of LPS per ml in the presence or absence of various dilutions of the MAbs in 150 µl of cell culture medium. After 24 h of stimulation, the macrophage culture supernatants were collected, and 100 µl of macrophage culture supernatant per well was used for the assessment of NO production. Data are the averages of triplicate determinations ± standard errors of the means. The statistical analysis was done by use of the Student t test (21): * P < 0.025; ** P < 0.01; *** P < 0.005.
in NO formation was observed with as little as a 1:300,000 dilution of the antibody (35 ng of protein per ml); 50% inhibition was obtained with an approximately 1:3000 to 1:10,000 dilution of the antibody (350 to 1,050 ng of protein per ml); and a 1:300 dilution of the antibody (35 μg of protein per ml) blocked up to 90% of LPS-induced NO production. Similarly, NO formation by LPS-stimulated RAW 264.7 cells was inhibited by the presence of the anti-IFN-β MAb. As shown in Fig. 1B, a 30% reduction in NO production was observed with a 1:300,000 dilution of the antibody. At the highest concentration tested (105 μg/ml), the anti-IFN-β MAb reduced LPS-induced NO formation by 60%. In contrast, the isotype-matched anti-IFN-α MAb had no detectable effects on LPS-initiated NO formation at equivalent protein concentrations in comparison with the anti-IFN-β MAb. According to the distributor, the titer of the anti-IFN-α MAb is about 800-fold lower than that of the anti-IFN-β MAb (see Materials and Methods). However, at the highest concentration tested (1:100 dilution; 0.1 mg of protein per ml), the anti-IFN-α MAb still did not exhibit any inhibitory effects, while the anti-IFN-β MAb at an equivalently equivalent titer (1:300,000 dilution; 350 ng of protein per ml) reduced NO production to about 50% of the control level for TG-elicited peritoneal cells and 40% of the control level for RAW 264.7 cells. These results suggest that endogenous IFN-β, but not IFN-α, may be required for LPS-induced NO formation in mouse TG-elicited peritoneal macrophages as well as the macrophage-like cell line RAW 264.7.

Effects of exogenous IFNs on LPS-induced responses in C3H/HeJ TG-elicited peritoneal macrophages. As an alternative approach to addressing the requirement for IFN-β in LPS-induced NO production, we used C3H/HeJ TG-elicited peritoneal macrophages as target cells. The C3H/HeJ mouse strain is a mutant inbred strain which has been demonstrated to have an inherited defect in its ability to respond to LPS. This is a global defect which is manifested in B and T lymphocytes, fibroblasts, and macrophages (37). Although the mutant gene (Lps) has been mapped to a locus on chromosome 4 (43), the precise mechanism(s) for hypersensitivity to LPS has not been defined to date. It has been reported that some LPS-dependent responses can be elicited in vitro by R-chemotype LPS in C3H/HeJ mouse cells (19, 20, 52). We have confirmed these observations, and the results are shown in Table 1. Relatively high concentrations of Ra-LPS, purified from S. minnesota R60, can induce TNF-α production in cultures of C3H/HeJ TG-elicited peritoneal macrophages. However, neither S-LPS nor Ra-LPS, even at a concentration as high as 10 μg/ml, can ever initiate NO production in these macrophages. Purified lipid A lacks this ability as well (data not shown). Therefore, it was of interest to investigate whether IFNs might complement the ability of LPS to induce NO production in C3H/HeJ TG-elicited peritoneal macrophages.

We therefore carried out experiments in which exogenous IFN-β was added together with 10 μg of E. coli O111:B4 S-LPS or S. minnesota R60 Ra-LPS per ml to C3H/HeJ TG-elicited peritoneal macrophage cultures and the cells were activated for 18 h. The results obtained from these experiments are summarized in Table 1. The addition of exogenous IFN-β (e.g., 100 U/ml) enhanced Ra-LPS-induced TNF-α formation (about 15-fold) and enabled S-LPS to initiate the production of TNF-α. The most important point to be made from the data shown in Table 1, however, is the fact that the addition of exogenous IFN-β was able to correct the LPS hyporesponsiveness of C3H/HeJ macrophages for LPS-dependent NO production with both S-LPS and Ra-LPS, therefore demonstrating the critical role of IFN-β in inducible NO formation in response to LPS in mouse TG-elicited peritoneal macrophages.

The effect of exogenous IFN-γ on these LPS-induced responses in C3H/HeJ TG-elicited peritoneal macrophages was also examined with similar experimental procedures, and the results of these studies are summarized in Table 2. Like IFN-β, IFN-γ can restore the capacity of LPS to induce NO production, in addition to enhancing the effects of LPS on the induction of TNF-α production. Therefore, the necessary signal(s) required for LPS-initiated NO formation in murine TG-elicited peritoneal macrophages can be supplied by IFN-γ as well. It should be noted, however, that under the experimental conditions described, neither preparation of IFN was capable of eliciting NO production in the absence of LPS (data not shown).

Effects of IFN-β on S. aureus-induced NO production in C3HeB/FeJ TG-elicited peritoneal macrophages. We previously reported that in C3HeB/FeJ macrophages, heat-killed S. aureus, a gram-positive bacterium, does not induce NO formation under experimental conditions in which the production of other inflammatory mediators, such as TNF-α, can readily be initiated (51). We therefore questioned whether IFN-β might also be able to act as a cofactor for S. aureus-induced NO production in macrophages. In these

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TABLE 1. Effects of IFN-β on LPS-induced responses in C3H/HeJ macrophagesa

<table>
<thead>
<tr>
<th>IFN-β dose (U/ml)</th>
<th>TNF-α (U/ml)b</th>
<th>Nitrite (μM)b</th>
<th>TNF-α (U/ml)b</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>&lt;10 ≤ 1.2</td>
<td>0.9 ± 0.1</td>
<td>0.7 ± 0.1</td>
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</tr>
<tr>
<td>100</td>
<td>76 ± 5</td>
<td>1.5 ± 0.1</td>
<td>2.5 ± 0.1</td>
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</tr>
<tr>
<td>1,000</td>
<td>223 ± 31</td>
<td>18.5 ± 0.2</td>
<td>35.7 ± 0.7</td>
<td></td>
</tr>
</tbody>
</table>

a TG-elicited C3H/HeJ mouse peritoneal cells were seeded into 24-well plates at 5 × 105 cells per well. After overnight adhesion, these cells were washed twice and then activated with 10 μg of E. coli O111:B4 S-LPS or S. minnesota R60 Ra-LPS per ml in the presence of various concentrations of IFN-β in 0.5 ml of cell culture medium for 18 h.

b Averages of duplicate determinations ± standard errors.

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TABLE 2. Effects of IFN-γ on LPS-induced responses in C3H/HeJ macrophagesa

<table>
<thead>
<tr>
<th>IFN-γ dose (U/ml)</th>
<th>TNF-α (U/ml)b</th>
<th>Nitrite (μM)b</th>
<th>TNF-α (U/ml)b</th>
<th>Nitrite (μM)b</th>
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<tr>
<td>0</td>
<td>&lt;10 ≤ 1.2</td>
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<td>0.7 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>392 ± 28</td>
<td>7.2 ± 0.1</td>
<td>28.7 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>452 ± 50</td>
<td>31.1 ± 0.6</td>
<td>48.2 ± 0.8</td>
<td></td>
</tr>
</tbody>
</table>

a TG-elicited C3H/HeJ mouse peritoneal cells were seeded into 24-well plates at 5 × 105 cells per well. After overnight adhesion, these cells were washed twice and then activated with 10 μg of E. coli O111:B4 S-LPS or S. minnesota R60 Ra-LPS per ml in the presence of various concentrations of IFN-γ in 0.5 ml of cell culture medium for 18 h.

b See Table 1, footnote b.

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See Table 1, footnote c.
experiments, C3HeB/FeJ TG-elicited peritoneal macrophages were stimulated with heat-killed *S. aureus* in the presence of various concentrations of IFN-β for 24 h. Cultures receiving no IFN-β served as controls. As shown by the data in Fig. 2, *S. aureus* in the absence of IFN-β was able to stimulate the production of TNF-α. However, the addition of IFN-β provided a necessary signal(s) for *S. aureus*-induced NO production in a dose-dependent manner. Therefore, these results suggest that IFN-β may serve as a general cofactor which is required for inducible NO formation in mouse TG-elicited peritoneal macrophages. In contrast to NO production, *S. aureus*-induced TNF-α production, as measured in 6-h culture supernatants from the same macrophage cultures, was not significantly affected by the addition of exogenous IFN-β. This phenomenon may suggest either that IFN-β is not absolutely required for *S. aureus*-induced TNF-α formation or, alternatively, that the dose of *S. aureus* used in these experiments may have been sufficiently high to already have maximized TNF-α levels and therefore that added exogenous IFN-β would not have been capable of enhancing TNF-α production to any further extent under such experimental conditions.

**Effects of cytokines on NO production in the absence of LPS in C3HeB/FeJ TG-elicited macrophages.** It has been reported that the presence of an anti-TNF-α antibody in cultures of LPS-stimulated macrophages can partially inhibit macrophage-dependent NO production induced by IFN-γ and LPS (17). We therefore questioned whether LPS might trigger NO formation indirectly in macrophages via the induction of TNF-α and IFN-β, which would then act in a coordinate fashion to induce NO formation. To address this question,

![Graph](https://via.placeholder.com/150)

**FIG. 2.** Effects of IFN-β on *S. aureus*-induced responses in C3HeB/FeJ macrophages. TG-elicited C3HeB/FeJ mouse peritoneal cells were seeded into 24-well plates at 5 × 10⁵ cells per well. After overnight adhesion, these cells were washed twice and then activated with heat-killed *S. aureus* at a concentration of 50 bacteria per macrophage in the presence or absence of various concentrations of IFN-β in 0.5 ml of cell culture medium. After 6 h of stimulation, a volume of 220 μl from 500 μl of the culture supernatant in each well was collected for the TNF-α assay. Then, 100 μl of fresh culture medium was added to each well, and the macrophage cultures were incubated for an additional 18 h. Then, 100 μl of cell culture supernatant per well was collected, and NO production was assessed. Data for TNF-α production (Δ) are the averages of duplicate determinations ± standard errors. Data for the amounts of nitrite (○) are the averages of triplicate determinations ± standard errors of the means.

<table>
<thead>
<tr>
<th>TNF-α (U/ml)</th>
<th>NO produced (μM) ±d in the presence of the following concn, in U/ml of IFN-β:</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0</td>
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<td>50</td>
<td>0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0</td>
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<tr>
<td>100</td>
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<td>0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0</td>
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</table>

* Data for the amounts of nitrite are averages of triplicate determinations ± standard errors of the means.

C3HeB/FeJ TG-elicited peritoneal macrophages were treated with various concentrations of recombinant TNF-α and/or IFN-β, alone or in combination, for 18 h. Macrophages activated with *E. coli* O111:B4 S-LPS in parallel wells served as a positive control for these experiments. As shown by the data in Table 3, under experimental conditions in which significant amounts of NO production could be readily initiated by 100 ng of *E. coli* O111:B4 S-LPS per ml, neither TNF-α nor IFN-β, alone or in combination, was capable of inducing any significantly detectable levels of NO formation.

It was shown previously that, in C3H/He LPS-responsive TG-elicited peritoneal macrophages, LPS-induced NO production becomes detectable after 12 h of activation, while substantial amounts of NO metabolites produced by these macrophages are present in culture supernatants after 24 h of LPS stimulation. NO formation increases continuously and reaches plateau levels at about 48 h postactivation (47). It is possible, in the experiments shown in Table 3, that the time allowed for the activation of macrophages with these cytokines was insufficient or that the concentrations of these cytokines used were inappropriate for the detection of any stimulatory effects. However, when we carried out similar experiments in which cells were incubated with higher concentrations of IFN-β (from 500 to 50,000 U/ml) and/or TNF-α (from 1,250 to 10,000 U/ml), alone or in combination, or for longer times of culturing (24 h), similar results were obtained. Neither of the two cytokines was capable of initiating detectable NO formation (data not shown). These results demonstrate that TNF-α plus IFN-β is not sufficient to account for LPS-triggered NO formation in mouse TG-elicited peritoneal macrophages, although both of these cytokines appear to be required for optimal LPS-induced macrophage responses.

In contrast to the results obtained with IFN-β, we confirmed the result reported by others that IFN-γ, in combination with TNF-α, can trigger a significant amount of NO formation in mouse TG-elicited peritoneal macrophages in the absence of LPS (Table 4). Other investigators have reported that IFN-γ by itself is able to initiate NO formation in TG-elicited peritoneal macrophages after 48 h of stimulation (47). We were not, however, able to detect any NO production induced by IFN-γ alone. This result may have been due in part to the shorter activating time (24 h) relative
to that in the earlier published studies. Nevertheless, it is clear from these results that IFN-γ synergistically interacts with TNF-α to induce NO formation, while under similar experimental conditions, IFN-β does not. These data would therefore support the concept that the signals provided by IFN-β and IFN-γ for inducible NO production in mouse TG-elicited peritoneal macrophages are not identical.

**DISCUSSION**

In this study, we showed that an anti-IFN-β neutralizing MAb significantly reduced LPS-initiated NO production in C3HeB/FeJ TG-elicited peritoneal macrophages and that the addition of exogenous recombinant IFN-β was capable of restoring the ability of LPS-hyporesponsive C3H/HeJ TG-elicited peritoneal macrophages to respond to LPS by producing NO. These results therefore demonstrate that IFN-β is a critical autocrine regulator for LPS-induced NO formation in mouse TG-elicited peritoneal macrophages. Furthermore, exogenous IFN-β enabled heat-killed *S. aureus* to stimulate NO production in mouse TG-elicited peritoneal macrophages, suggesting that IFN-β may be required not only for the LPS-induced response but also for other bacterial stimulus-induced NO production as well. The fact that the neutralizing MAb against IFN-β was also able to inhibit LPS-induced NO production in macrophage-like cell line RAW 264.7 demonstrated that IFN-β has autocrine regulatory functions in other macrophages. The requirement for IFN-β in NO production is consistent with the results of other laboratories showing that LPS-induced activation of bone marrow culture-derived mouse macrophages for tumor cell killing can be abolished by a polyclonal antisera specific for IFN-α/β (34).

Our studies have suggested that a neutralizing MAb to IFN-β, but not IFN-α, inhibits LPS-induced NO formation. One of the possible reasons for the relative inefficiency of the anti-IFN-α MAb in inhibiting NO production is that LPS-stimulated TG-elicited peritoneal macrophages appear to produce predominantly IFN-β, and not IFN-α (7, 27). Alternatively, the neutralizing titer of the anti-IFN-α antibody used in our experiments may not be sufficiently high to allow the detection of any inhibitory effects. A recent report suggests that both subspecies of type I IFNs (IFN-α and IFN-β) share a common component (8). It has been shown that IFN-α is able to augment LPS-initiated NO production (13). Whether IFN-α can provide the same necessary signal(s) required for LPS-dependent NO formation remains to be unequivocally established. Nevertheless, the anti-IFN-α antibody can be considered an antibody isotype-matched negative control, suggesting that the inhibition of NO production by the anti-IFN-β antibody is unlikely to be due to nonspecific effects of immunoglobulin per se. Rather, these results more likely reflect a specific autocrine regulatory role of IFN-β in this LPS-induced macrophage response. However, the possibility that an IFN-β-IgG immune complex partially contributes to the observed inhibitory effects cannot be totally excluded on the basis of these results.

The inability of C3H/HeJ TG-elicited peritoneal macrophages to produce NO in response to any preparations of LPS is consistent with the fact that the same macrophages are incapable of killing the TNF-α-resistant P815 tumor cell line, even when stimulated with R-chemotype LPS (19); these results are also consistent with the observation that C3H/HeJ macrophages are susceptible to infection with vesicular stomatitis virus (49). Both observations suggest that LPS cannot activate these macrophages to generate IFN-β. Of interest, C3HeB/FeJ TG-elicited peritoneal macrophages stimulated with heat-killed *S. aureus* appear to behave phenotypically like C3H/HeJ TG-elicited peritoneal macrophages incubated with LPS in terms of NO production. Therefore, it would not be unreasonable to speculate that heat-killed *S. aureus* is unable to provide the appropriate triggering signal necessary to initiate IFN-α/β production in these cells. Some studies, however, have indicated that certain components of *S. aureus*, including staphylococcal exotoxin, toxic shock syndrome toxin 1, and lipoteichoic acid, can, under some conditions, induce NO production in macrophages (5, 18, 50). These findings would suggest that whole killed bacterial particles and purified individual components from these bacteria may have different immunostimulatory activities. Recent studies from our laboratories with gram-negative bacteria and the bacterial constituents lead to the same conclusion (33a).

We have demonstrated here that neither IFN-β nor TNF-α, alone or in combination, can induce detectable NO secretion under experimental conditions in which LPS can initiate a substantial amount of NO production. These results indicate that a costimulatory signal(s) provided exclusively by bacterial stimuli, such as LPS, in addition to those provided by IFN-β and TNF-α, is required for macrophage NO production. The fact that IFN-γ plus TNF-α, in contrast, can initiate NO production in the absence of LPS clearly indicates that the signals provided by these two types of IFN for NO production are not identical. IFN-γ has been shown to induce IFN-β production in peritoneal macrophages from normal LPS-responsive mice (*lps*), as assessed by the accumulation of IFN-β mRNA in hybridization assays and the secretion of IFN-β protein in neutralization assays with MAbs (23, 24). Moreover, IFN-γ alone stimulated IFN-β production by posttranscriptional mechanisms in peritoneal macrophages from C3H/HeJ LPS-hyporesponsive mice (*lps*), whereas LPS did not induce IFN-β production in these *lps* peritoneal macrophages (24, 49). Therefore, the defect of C3H/HeJ macrophages in response to LPS is likely to be, as least in part, due to the inability of these cells to produce IFN-β upon LPS stimulation. The fact that IFN-γ is able to promote the production of IFN-β may account, at least in part, for the IFN-γ complementation of LPS in the induction of NO production by C3H/HeJ peritoneal macrophages. IFN-γ may also provide positive signals.
for LPS-induced TNF-α production, another way in which this cytokine could render LPS more potent in generating NO production in C3H/HeJ peritoneal macrophages.

In animal models of endotoxemia, acute hypotension has been attributed to vigorous, unregulated NO synthesis in macrophages and endothelial cells following transcriptional enhancement of the inducible NO synthase (45). Thus, studies on regulatory mechanisms of macrophage-derived cytokines for LPS-induced NO production would be of benefit in the design of potential immunological therapies to treat this disease. On the other hand, cancer patients, especially those being treated with chemical therapies, would be more likely to be susceptible to infection than normal individuals. Therefore, selective augmentation of the production of NO by host defense cells, especially macrophages, by exogenous IFNs and other cofactors may be of importance to cancer patients, in terms of both protection against microbial infection and induction of host responses to tumor cells. Therefore, in both cases, it is clear that further elucidation of the role of the cytokine regulatory network in the control of inducible NO production will be of considerable value in understanding the functional roles of macrophages in both infectious diseases and cancer.

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