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A Specific Sequence of Stimulation Is Required To Induce Synthesis of the Antimicrobial Molecule Nitric Oxide by Mouse Macrophages

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Nitric oxide production by macrophages required either simultaneous or sequential exposure to gamma interferon and lipopolysaccharide; exposure to lipopolysaccharide followed by exposure to gamma interferon gave little response. The apparently evanescent nature of the lipopolysaccharide signal, necessitating persistent stimulation, could be essential to down-regulating nitric oxide production after bacteria are cleared in vivo.

Macrophages play an essential role in immunity to many microbial pathogens in vivo. Their capacity to effect microbiostatic or cytolytic activity against several microorganisms is dependent on the generation of nitric oxide (NO) (1, 3, 4, 19). NO is derived in macrophages from the terminal guanido nitrogen of L-arginine (5, 11) through a reaction catalyzed by an inducible enzyme, nitric oxide synthase. Nitric oxide synthase can be induced in macrophages by exposing them to gamma interferon (IFN-γ) and bacterial lipopolysaccharide (LPS) (2, 14, 15). We show in this report that the sequence of stimulation with IFN-γ and LPS can profoundly influence the extent to which NO synthesis is induced. Further, we provide evidence that the sequence requirement reflects a difference in the longevity of intracellular signals induced by the two stimuli. These findings may have important implications for negative regulation of NO synthesis and immunity to microorganisms in vivo.

In all experiments, macrophages were cultured in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered minimal essential medium (Auto-Pow MEM; Flow Laboratories, McLean, Va.) containing 10% fetal bovine serum (JRH Biosciences, Lenexa, Kans.). Recombinant murine IFN-γ was provided by Paul Trotta (Schering-Plough, Bloomfield, N.J.). Natural, highly purified murine IFN-β was purchased from Lee Biomolecular (San Diego, Calif.). LPS, as the lipid-A-rich fraction II of phenol-extracted *Escherichia coli* O11:B4 (12), was kindly provided by David C. Morrison of our institution. All tissue culture media and reagents, except LPS, were negative for contaminating endotoxin at a sensitivity level of 15 pg/ml, as determined by an assay with *Limulus* amebocyte lysate (10).

Bone marrow culture-derived macrophages were prepared as described previously (9). For use in assays, macrophages were seeded into the wells of 96-well plates at a population density of 6×10^4 per well and incubated for 2 h at 37°C in 5% CO_2 in air to allow them to adhere. Macrophage cultures were stimulated as indicated in the figure legends, after which the culture medium was aspirated and 200 μl of fresh medium was added per well. The monolayers were then incubated for 16 to 20 h at 37°C. Synthesis of NO was assayed colorimetrically as the accumulation of NO_2^- in macrophage culture supernatants by reaction with the Griess reagent, as described previously (16); NO reacts with molecular oxygen to form NO_2, which subsequently reacts with water to yield the stable products NO_2^- and NO_3^- in equimolar amounts. The concentration of NO_2^- was determined from a standard curve generated with $NaNO_2$.

Macrophages were exposed to various concentrations of IFN-γ and to 1 ng of LPS per ml, added either simultaneously or sequentially. In the latter instance, monolayers were exposed to the first compound for 2 h, after which the monolayers were washed once with medium. The second stimulus was then added for another 2 h. Except where indicated otherwise, the duration of exposure to any one stimulus was identical, i.e., 2 h. As shown in Fig. 1, simultaneous stimulation of macrophages with IFN-γ and LPS for 2 h induced significant levels of NO release. Likewise, exposure to IFN-γ followed by stimulation with LPS permitted efficient induction of NO synthesis, yielding levels of NO comparable to those seen after 2 h of simultaneous stimulation with these compounds. In contrast, exposure of macrophages to LPS prior to stimulation with IFN-γ resulted in poor induction of NO release, an effect not overcome by even the highest concentration of IFN-γ used, i.e., 100 U/ml.

Normal levels of NO synthesis by macrophages pretreated with LPS could not be restored by even prolonged subsequent stimulation (up to 16 h) with IFN-γ (data not shown). Inclusion of 10^{-6} M indomethacin in cultures of LPS-pretreated macrophages (17) failed to enhance NO release induced by subsequent stimulation with IFN-γ, indicating that the low level of NO synthesis was not attributable to prostaglandin-mediated inhibition (data not shown).

Preexposure of macrophages to low levels of LPS can prevent subsequent LPS-mediated induction of other macrophage secretory products, e.g., tumor necrosis factor alpha, by selectively rendering the macrophage refractory to LPS stimulation (18). To confirm that the macrophages were not in some way irreversibly desensitized by preexposure to LPS, macrophages were treated with LPS for 2 h and then stimulated with IFN-γ and LPS. As shown in Fig. 2, this resulted in good induction of NO synthesis, although the magnitude of NO release at every dose of IFN-γ tested was approximately 20% less than that produced by macrophages not subjected to LPS pretreatment.

One explanation that could account for our results is that induction of NO synthesis by IFN-γ and LPS requires continued stimulation of the macrophage with LPS. To test this hypothesis directly, we stimulated macrophages first with both IFN-γ and LPS. The monolayers were then washed, and either IFN-γ or LPS was added for an addi-

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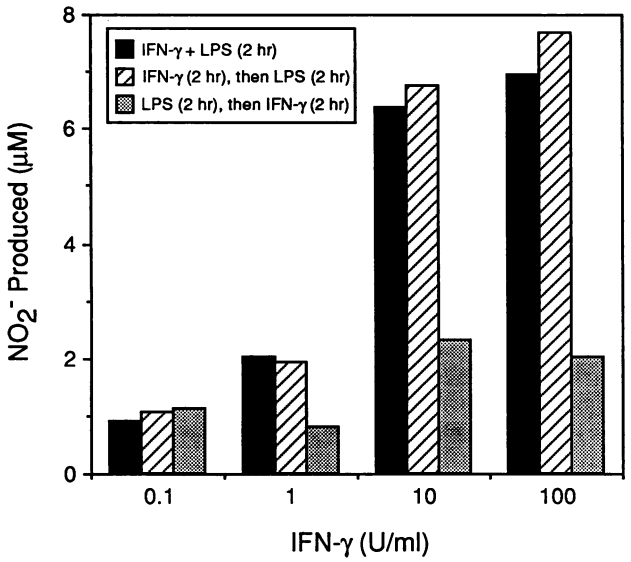

FIG. 1. The effect on NO synthesis of the order in which macrophages were stimulated with IFN and LPS. Macrophages were stimulated for 2 h either sequentially or simultaneously with 1 ng of LPS per ml and various concentrations of IFN-γ, as indicated in the figure. The duration of exposure to either IFN-γ or LPS was identical irrespective of the stimulation sequence. When sequentially treated, monolayers were washed prior to the addition of the second stimulus. Following the respective stimulation protocols, monolayers were washed, fresh medium was added, and the cultures were incubated an additional 16 to 20 h at 37°C, after which NO_2^- accumulation in culture supernatants was assayed as a measure of NO synthesis. The results shown are representative of three independent experiments.

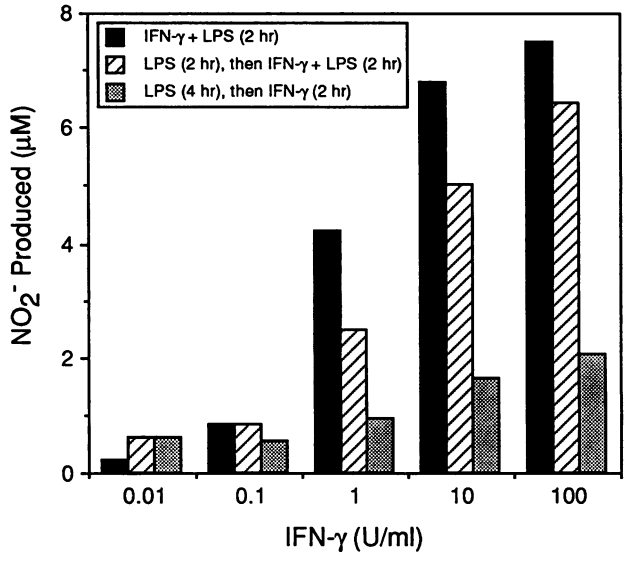

FIG. 2. Preexposure of macrophages to LPS alone did not induce desensitization to subsequent stimulation of NO synthesis by IFN-γ and LPS. Macrophages were treated with IFN-γ and LPS, with the mode and duration of treatment indicated in the figure. After the final stimulation, all monolayers were washed, fresh medium was added, and the cultures were incubated an additional 16 to 20 h at 37°C, after which NO_2^- accumulation in culture supernatants was assayed as a measure of NO synthesis. The results shown are representative of two independent experiments.

tional 2 or 4 h. Figure 3 shows that macrophages costimulated with IFN-γ and LPS for 2 h produced approximately 5 μM NO_2^-. Sustained treatment with IFN-γ of cells initially costimulated with IFN-γ and LPS failed to further enhance NO synthesis. By contrast, sustained exposure to LPS increased the quantity of NO synthesized by greater than twofold relative to that induced by an initial 2-h costimulation with IFN-γ and LPS, yielding NO_2^- levels that were comparable to those produced by macrophages costimulated with IFN-γ and LPS for the entire activation phase of the experiment, i.e., 4 or 6 h.

These observations suggest that the order in which macrophages encounter IFN-γ and LPS can profoundly affect the extent to which NO synthesis is induced in these cells. It is likely that the need for such sequential stimulation reflects a fundamental difference in the nature of the signal transduced in the macrophage by IFN-γ versus that of LPS; i.e., the signal transduced by LPS appears to be very evanescent, necessitating the continued presence of LPS for the maintenance of the signal. Therefore, when cells are exposed to LPS before they are stimulated with IFN-γ, the LPS-induced signal has decayed to the point that it is insufficient to trigger NO synthesis irrespective of the dose of IFN-γ to which the macrophage is subsequently exposed. Poor synthesis of NO is the result. In contrast, the signal induced by IFN-γ is apparently more sustained, because macrophages initially costimulated with IFN-γ and LPS and subsequently treated with LPS alone produced 75 to 80% as much NO as those cells costimulated for the duration of the activation phase of the experiment (Fig. 3). Similar requirements for a specific stimulation sequence with respect to activation of macrophages by IFN-γ and LPS for tumor cell killing have previously been reported by other investigators (7, 13). The basis for their observations may also reside in induction of NO synthesis, because susceptible tumor cells can be killed by exposure to NO (6, 8).

In the context of a gram-negative bacterial lesion in vivo,

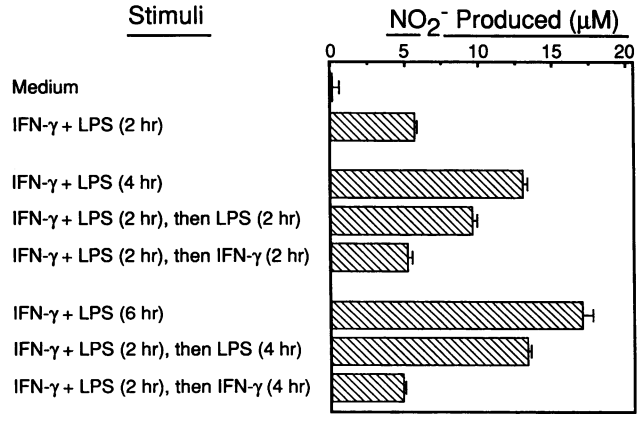

FIG. 3. Continued stimulation with LPS, but not IFN-γ, was required for sustained synthesis of NO by activated macrophages. Macrophages were costimulated with 1 U of IFN-γ per ml and 1 ng of LPS per ml for either 2, 4, or 6 h. Those monolayers initially costimulated for 2 h were then washed, and either IFN-γ or LPS alone was added for an additional 2 or 4 h, as indicated in the figure. All monolayers were then washed and fresh medium was added, and the cultures were then incubated to allow NO_2^- to accumulate, as described in the legend to Fig. 1. The results shown are representative of two independent experiments.

a sequential requirement for the induction of NO synthesis may prevent inappropriate release of NO by stimulated macrophages: significant synthesis would only occur as long as endotoxin persisted in the inflammatory milieu. In the absence of endotoxin, subsequent stimulation of macrophages with even high concentrations of IFN-γ would likely be ineffective at further augmenting production of NO. Such a regulatory mechanism could play an important role in minimizing indiscriminate tissue damage incurred during the course of an inflammatory response.

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