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CUTTING EDGE

Cutting Edge: Bacterial DNA and LPS Act in Synergy in Inducing Nitric Oxide Production in RAW 264.7 Macrophages[1]

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LPS is well recognized for its potent capacity to activate mouse macrophages to produce NO, an important inflammatory mediator in innate host defense. We demonstrate here that, although inducing little NO alone, DNA from both Gram-negative and Gram-positive bacteria synergizes with subthreshold concentrations of LPS (0.3 ng/ml) to induce NO in cultures of RAW 264.7 macrophages. The effects of the DNA are mimicked by synthetic CpG-containing oligodeoxynucleotides but not by non-CpG-containing oligodeoxynucleotides. This synergistic activity is not inhibited by neutralizing Abs against IFN. Preincubation of macrophages with DNA for 8–24 h suppresses subsequent synergistic macrophage responses to DNA/LPS, whereas prolonged pretreatment with LPS enhances synergy. RT-PCR analysis indicates that the mRNA levels of the inducible NO synthase gene are also coordinately suppressed or induced. These findings indicate that temporally controlled, synergistic interactions exist between microbial DNA and LPS in the induction of macrophage NO via enhanced inducible NO synthase gene expression. ***The Journal of Immunology*****, 1999, 163: 4095–4099.**

Bacterial LPS is well known as an inducer of mouse macrophage activation, resulting in the production of NO, a radical gas with both beneficial (e.g., microbial and tumor cell killing) and potentially detrimental (e.g., tissue damage and septic shock) effects (1–3). The induction of NO secretion from mouse macrophages is due to increased expression of the inducible NO synthase (*iNOS*)[3] gene in response to LPS stimulation (3). Recent data indicate that purified bacterial DNA can also activate immune inflammatory cells, including macrophages (reviewed in Ref. 4). One of the key structural features that serves to distinguish bacterial vs eukaryotic DNA is that the former often contains short sequences with unmethylated CpG dinucleotides flanked by two 5′ purines and two 3′ pyrimidines (5). In contrast, in eukaryotic DNA, these CpG-containing sequences occur at a much lower frequency, and the cytosine residues in the CpG dinucleotide are usually selectively methylated (6). These subtle differences between bacterial DNA and eukaryotic DNA appear to be sufficient to provide the former with the necessary biochemical difference so as to render them capable of serving as immune activating agents (5, 7, 8). Macrophages have been reported, in this respect, to respond to bacterial DNA by producing proinflammatory cytokines such as TNF-α, IL-1, and IL-6 (9–11) as well as the secretion of NO from IFN-γ-primed mouse macrophages (12).

Here we present data demonstrating that bacterial DNA can synergize with subthreshold concentrations of LPS (0.3 ng/ml) in inducing the murine RAW 264.7 macrophage-like cell line to secrete NO. The observed synergy appears to depend upon the temporal order of treatments by LPS and bacterial DNA, and upon the presence of CpG residues, and most likely results from up-regulated expression of the *iNOS* gene.

Materials and Methods

Materials

Purified LPS from *Escherichia coli* O111:B4 was purchased from List Biological Laboratories (Campbell, CA). Neutralizing rat anti-mouse IFN-γ mAb and control rat IgG were obtained from PharMingen (San Diego, CA) (with endotoxin levels of <0.01 ng/ml). Neutralizing rabbit anti-mouse IFN-$\alpha\beta$ Ab and control rabbit IgG were gifts of Dr. William J. Murphy (University of Kansas Medical Center; described in Ref. 13). RNase-free DNase I was purchased from Sigma (St. Louis, MO). Synthetic oligodeoxynucleotides (ODNs) (T3, 5′-AAC GTT AAC GTT AAC GTT-3′; C3, 5′-CCA TGG CCA TGG CCA TGG-3′) were obtained from Genosys (The Woodlands, TX). The endotoxin levels in these ODNs are <0.01 ng/μg of DNA, based upon *Limulus* amoebocyte lysate assay.

Isolation and culture of macrophages

Female C57BL/6 mice from The Jackson Laboratory (Bar Harbor, ME) were used at 6–8 wk. Mice were injected i.p. with 1.5 ml of 4% Brewer

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[3] Abbreviations used in this paper: iNOS, inducible NO synthase; ODN, oligodeoxynucleotide.

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thioglycollate (Difco, Detroit, MI), and peritoneal macrophages were harvested 5 days later by lavage with RPMI 1640 culture medium (Life Technologies, Grand Island, NY). Both C57BL/6 peritoneal macrophages and the murine macrophage-like cell line RAW 264.7 (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 medium supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% heat-inactivated FBS (endotoxin content of <0.06 ng/ml) (Sigma) at 37°C in a humidified, 5% CO_2 environment.

DNA manipulation

DNA from *E. coli* strain B and salmon testes was purchased from Sigma and further purified by two-step CsCl ultracentrifugation. DNA from *Staphylococcus aureus* was purified exactly as described by Dyer and Iandolo (14). DNA digestion was performed using RNase-free DNase I (2 U/μg of DNA) at 37°C for 2–3 h in buffer (pH 7.6) containing 20 mM Tris-HCl and 20 mM $MgCl_2$. The endotoxin levels in these DNA preparations were <0.001 ng/μg of DNA.

Nitrite assays

The NO in culture supernatants was measured as concentrations of nitrite using Griess reagent (15) and quantitated by comparison with a standard curve generated using sodium nitrite. The data presented represent averages of triplicate cultures ± SEM and are representative of at least three similar experiments.

RNA isolation and RT-PCR analysis

RAW 264.7 macrophages (2.5×10^6 cells/well) were seeded into six-well tissue culture plates and incubated for 2.0 h to allow for adherence. After simulation for 2.5 h with various stimuli as described in *Results*, total RNA was isolated using Trizol reagent (Life Technologies) exactly according to the manufacturer's instructions. A total of 1 μg of total RNA from each sample was used for reverse transcription using the GeneAmp RNA PCR kit (Perkin-Elmer, Branchburg, NJ). The manufacturer's protocols were followed exactly for both reverse transcription and PCR. The sequences of the specific primers used in these studies are: mouse *iNOS* sense, 5′-TCA CTG GGA CAG CAC AGA AT-3′; mouse *iNOS* antisense, 5′-TGT GTC TGC AGA TGT GCT GA-3′; mouse β-actin sense, 5′-TGT GAT GGT GGG AAT GGG TCA G-3′; mouse β-actin antisense, 5′-TTT GAT GTC ACG CAC GAT TTC C-3′. PCR products were subjected to agarose gel electrophoresis, stained with ethidium bromide, and photographed. The photographs were scanned using Adobe PhotoShop software (Adobe Systems Incorporated, San Jose, CA) and analyzed using a GelPro Analyzer (Meyer Instruments, Houston, TX).

Results and Discussion

Both bacterial DNA and LPS have been reported to be activators of macrophages; however, the signal transduction pathways employed by bacterial DNA and LPS have been suggested to be different (16–21). Therefore, we first investigated whether bacterial DNA and LPS might synergize with each other to stimulate an exaggerated mouse macrophage response, as assessed by an increased production of NO. To evaluate this possibility, RAW 264.7 macrophages were stimulated with various concentrations of *E. coli* DNA either in the absence or presence of 0.3 ng/ml of LPS, which, at this concentration, stimulated little or no detectable production of NO by itself (Fig. 1*A*). As shown by the data in Fig. 1*A*, *E. coli* DNA, by itself, was at best only a weak inducer of NO even at concentrations as high as 10 μg/ml (Fig. 1*A*, controls). In the presence of 0.3 ng/ml of LPS, however, NO production was dramatically enhanced (Fig. 1*A*, ○). NO induction by *E. coli* DNA at a concentration as low as 1.0 μg/ml in the presence of 0.3 ng/ml of LPS was at least 20-fold higher compared with that induced by either 1.0 μg/ml of *E. coli* DNA or 0.3 ng/ml of LPS alone. Of importance, this enhanced production of NO was completely abrogated by pretreatment with DNase I (Fig. 1*A*, controls). As a control for these studies, a eukaryotic source of DNA, specifically DNA obtained from salmon sperm, neither induced NO by itself nor was capable of enhancing the mouse macrophage response to LPS (Fig. 1*A*, controls). These observations also extend to other microbial DNA preparations (e.g., Gram-positive DNA and synthetic CpG-containing oligonucleotides). Similar to the results found using *E. coli* DNA, *S. aureus* DNA by itself also did not induce significant production of NO, even at concentrations as high as 10 μg/ml (Fig. 1*A*, controls). However, in the presence of 0.3 ng/ml of LPS, NO production can again be shown to be dramatically enhanced by *S. aureus* DNA (Fig. 1*A*, ▲). As anticipated, the enhanced production of NO by the DNA was completely abolished by treatment of DNase I (Fig. 1*A*, controls). Essentially parallel results to those obtained with *E. coli* and *S. aureus* DNA were obtained using the CpG-containing ODN (T3). By itself, this oligonucleotide induced little production of NO in RAW 264.7 macrophages, but in the presence of 0.3 ng/ml of LPS, a high level of NO production was observed (Fig. 1*A*, △). In contrast, a second ODN (C3), which does not contain CpG dinucleotides, induced little NO yield either with or without the presence of LPS (Fig. 1*A*, controls).

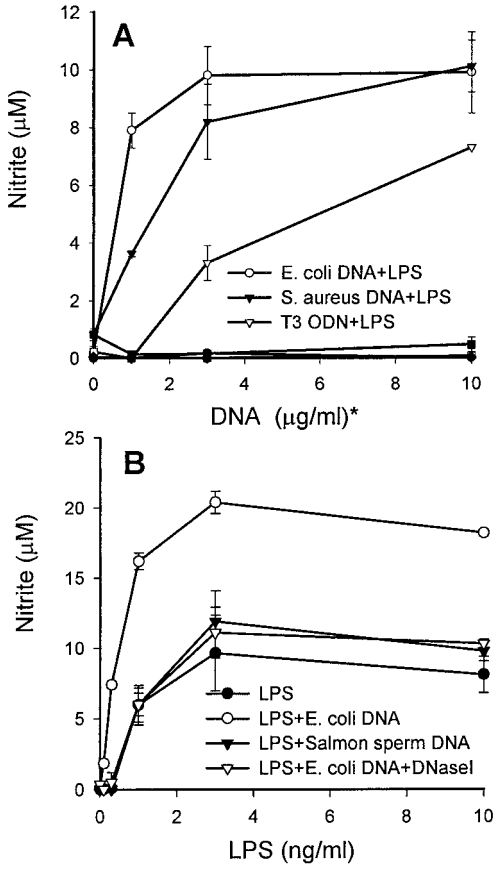

FIGURE 1. Induction of NO by *E. coli* DNA, *S. aureus* DNA, synthetic ODNs, and LPS. RAW 264.7 macrophages were subjected to various stimuli for 20 h before culture supernatants were taken for nitrite assay. *A*, Macrophages were stimulated with various concentrations of *E. coli* DNA (○), *S. aureus* DNA (▲), and CpG-containing T3 ODNs (△) in the presence of 0.3 ng/ml of LPS. Controls (not completely listed in the figure due to space limit) include various concentrations of salmon sperm DNA plus 0.3 ng/ml of LPS, non-CpG C3 ODN plus LPS, DNase I-treated *E. coli* DNA plus LPS, DNase I-treated *S. aureus* DNA plus LPS, *E. coli* DNA, *S. aureus* DNA, salmon sperm DNA, C3 ODNs, and DNase I-digested *E. coli* DNA or *S. aureus* DNA. The nitrite levels induced by controls were <0.5 μM. *, Concentrations of T3 and C3 are expresses as micromolar concentrations. *B*, Macrophages were stimulated with different concentrations of LPS (●), LPS plus 1 μg/ml of *E. coli* DNA (○), LPS plus DNase I-treated *E. coli* DNA (△), or LPS plus salmon sperm DNA (▲).

The data summarized above indicate that subactivating concentrations of LPS (0.3 ng/ml) can potentiate bacterial DNA for NO

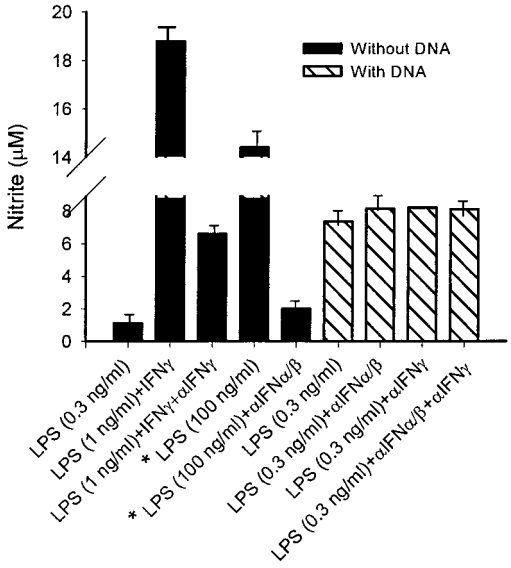

FIGURE 2. Effects of neutralizing Abs against IFN on *E. coli* DNA plus LPS-induced NO production. RAW 264.7 macrophages were treated as indicated in the figure. In samples involving neutralizing Abs, anti-IFN-$\alpha\beta$ (0.3 μg/ml), anti-IFN-γ (1 μg/ml), or a combination of both were incubated with macrophages for 30 min before the addition of stimuli. IFN-γ and *E. coli* DNA were used at 100 U/ml and 3 μg/ml, respectively. LPS was used at 0.3–100 ng/ml. The data indicated with an asterisk were obtained from C57BL/6 peritoneal macrophages exclusively to demonstrate that anti-$\alpha\beta$ is biologically functional. Control Abs showed no effect on NO induction by *E. coli* DNA plus LPS (data not shown).

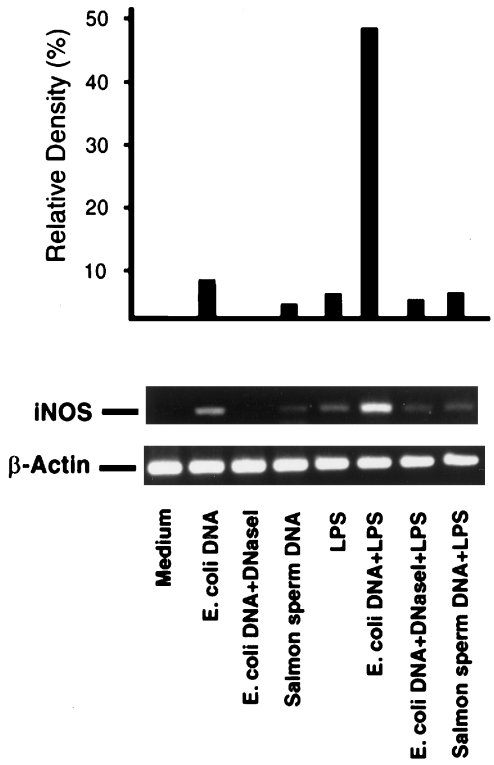

FIGURE 3. Induction of mouse *iNOS* mRNA by *E. coli* DNA and LPS. RAW 264.7 macrophages were incubated with culture medium alone, medium containing 1 μg/ml of *E. coli* DNA, DNase I-treated *E. coli* DNA, or salmon sperm DNA either in the absence or in the presence of 0.3 ng/ml of LPS for 2.5 h before RNA isolation for RT-PCR analysis (30 PCR cycles), as described in *Materials and Methods*. The *top panel* of the figure shows the densitometric scanning data from the amplified *iNOS* mRNA normalized against β-actin controls.

production in RAW 264.7 macrophages. To examine whether the reverse would also occur (that is, whether low concentrations of *E. coli* DNA would potentiate the LPS-mediated induction of NO production), RAW 264.7 macrophages were stimulated with LPS in the absence or presence of substimulatory concentrations of *E. coli* DNA (e.g., 1.0 μg/ml). As shown by the data in Fig. 1*B*, when macrophages were treated with relatively low concentrations of LPS alone (e.g., 0.3–10.0 ng/ml), increasing amounts of NO production were detected in response to increasing concentrations of LPS until a plateau of LPS-dependent NO secretion was attained at ~3.0 ng/ml of LPS. However, in the presence of 1.0 μg/ml of *E. coli* DNA (itself only weakly stimulatory), the LPS-induced NO production, as expected, was significantly potentiated. Once again, the findings that DNase I treatment almost completely ablated the observed enhancement of LPS-induced NO production by *E. coli* DNA and that salmon sperm DNA at equivalent concentrations did not significantly enhance LPS-induced NO production provide support for the conclusion that the observed effects are most likely microbial DNA-specific (Fig. 1*B*). Collectively, these data presented in Fig. 1 would strongly support the conclusion that microbial DNA and LPS can synergize in their immunostimulatory capacities to promote the release of NO from RAW 264.7 macrophages.

Because bacterial DNA has been reported to induce both type I and type II IFN from mouse spleen cells in vitro (22, 23), and because IFN has been known to synergize with LPS to induce NO production in mouse macrophages (24, 25), it was of interest to examine whether the observed synergy between bacterial DNA and LPS in their ability to induce NO synthesis might reflect the ability of DNA to induce production of autocrine IFN. To test this hypothesis, RAW 264.7 macrophages were treated with 3 μg/ml of *E. coli* DNA plus 0.3 ng/ml of LPS in the presence of neutralizing Abs against mouse IFN-$\alpha\beta$, IFN-γ, or both. Neither Abs against IFN-$\alpha\beta$, IFN-γ (used at 0.3 μg/ml and 1 μg/ml, respectively), or the combination of both significantly inhibited NO production in response to *E. coli* DNA plus 0.3 ng/ml of LPS, despite the fact that the two Abs were potent inhibitors of NO production in LPS- and LPS plus IFN-γ-stimulated mouse macrophages, respectively (Fig. 2). These findings would suggest that the observed synergy between LPS and bacterial DNA is not mediated through the production of macrophage-derived IFN.

The NO production in mouse macrophages in response to activation signals is controlled by expression of the mouse *iNOS* gene, which, in turn, is primarily regulated at the level of transcription (26). To investigate whether the synergistic induction of NO by bacterial DNA and LPS is attributable to increased transcription of the *iNOS* gene, macrophages were treated with various stimuli as described in the legend to Fig. 3. RNA isolated from these stimulated samples was then subjected to RT-PCR analysis. As shown by the data in Fig. 3, treatment of macrophages for 2.5 h with *E. coli* DNA (1.0 μg/ml) alone or LPS (0.3 ng/ml) alone induced only a modest increase of *iNOS* mRNA, consistent with the finding reported above for detectable NO in culture supernatants. However, when *E. coli* DNA and LPS were added to macrophages together, a readily detectable level of *iNOS* mRNA was observed, and this potentiated induction of *iNOS* mRNA was ablated by pretreatment of the *E. coli* DNA with DNase I. As controls, culture medium alone or salmon sperm DNA in the absence or presence of LPS induced little or no production of *iNOS* mRNA. In this experiment, a parallel set of macrophages was treated and cultured

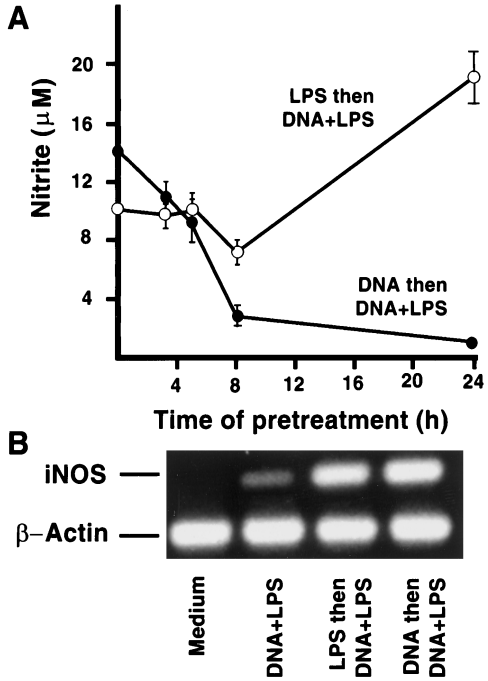

FIGURE 4. Effects of *E. coli* DNA and LPS pretreatment on induction of NO and *iNOS* mRNA in macrophages in response to *E. coli* DNA plus LPS. *A*, RAW 264.7 macrophages were preincubated with 3 μg/ml of *E. coli* DNA or 0.3 ng/ml of LPS for various amounts of time as shown in the figure. They were then washed twice with culture medium and continually incubated with 3.0 μg/ml of *E. coli* DNA plus 0.3 ng/ml of LPS for 20 h before assaying nitrite from the supernatants. *B*, RAW 264.7 macrophages were either unpretreated or pretreated with 3 μg/ml of *E. coli* DNA or 0.3 ng/ml of LPS for 24 h before exposure to *E. coli* DNA (3 μg/ml) plus LPS (0.3 ng/ml) for 2 h. Total RNA was then isolated for RT-PCR analysis of *iNOS* mRNA as described in Fig. 3, except that 24 PCR cycles were used in this experiment.

for 20 h, followed by assay of culture supernatants for nitrite exactly as described earlier. The results obtained are fully consistent with the *iNOS* mRNA levels shown in Fig. 3, in that little NO was induced by either *E. coli* DNA alone or LPS alone, but together *E. coli* DNA plus LPS elicited significant induction of NO (data not shown).

As synergy between two distinct activating agents usually requires an appropriate timing/coordination of initiation triggering signals, we tested whether such a temporal requirement also exists between bacterial DNA and LPS. Pretreatment of macrophages with *E. coli* DNA 2–4 h before addition of LPS (0.3 ng/ml) resulted in a modest reduction in NO production compared with that observed in macrophages treated simultaneously with *E. coli* DNA and LPS. In contrast, macrophages pretreated with *E. coli* DNA for 8 h before addition of LPS showed NO production that was greatly reduced relative to simultaneous addition, and when macrophages were pretreated with *E. coli* DNA for 24 h, the synergy between *E. coli* DNA and LPS on NO production was essentially completely abolished (Fig. 4*A*, filled circles). On the other hand, pretreatment of macrophages with LPS 2–8 h before the addition of *E. coli* DNA caused no statistically significant changes in NO secretion. Interestingly, however, when macrophages were pretreated with LPS for 24 h, NO production was dramatically increased (Fig. 4*A*, ○). To test whether the differential NO production reflects expression of *iNOS* mRNA, RNA isolated from LPS- or *E. coli* DNA-pretreated macrophages was subjected to RT-PCR analysis, as described in the legend to Fig. 4. Interestingly, whereas LPS pretreatment resulted in, as anticipated, increased *iNOS* mRNA levels, *E. coli* DNA pretreatment did not cause the expected reduction in *iNOS* mRNA levels. Instead, increases in the mRNA levels in *E. coli* DNA-pretreated macrophages were found to be comparable with those in LPS-pretreated macrophages. This occurs despite the fact that, in a parallel set of macrophages, the secreted NO levels were enhanced with LPS pretreatment but inhibited with *E. coli* DNA pretreatment (data not shown). Nevertheless, these results support the conclusion that the temporal relationship between additions of the activating signals of DNA and LPS to macrophages has a profound effect upon the ability of these cells to generate and secrete NO. The precise molecular mechanism(s) that regulate these differential responses, however, is clearly complex and will require additional studies.

The available experimental evidence would support the concept that LPS and bacterial DNA most likely use different signal transduction pathways to induce host responses, as assessed by the production of inflammatory mediators, but that these pathways may involve shared transcription factors such as NF-κB and AP-1 (16–21). Our finding that a temporally controlled synergy rather than additivity exists between bacterial DNA and LPS would further support the concept that these two bacterial components do, in fact, use different signaling pathways. Additional experimental evidence supporting this hypothesis derives from the fact that such synergy between bacterial DNA and LPS also exists for the induction of TNF-α both in vivo and in vitro (Refs. 9 and 27 and our manuscript in preparation). Our finding that biologically active neutralizing Abs against IFN did not have detectable effects upon bacterial DNA plus LPS-induced NO would be consistent with the conclusion that the bacterial DNA signal is most likely not mediated by an autocrine macrophage production of IFN. Synergistic induction of NO by bacterial DNA and LPS, nevertheless, does coincide with the increase of *iNOS* mRNA levels, suggesting that NO induction involves an enhanced expression of the *iNOS* gene. Recently, Cowdery et al. (28) reported that a CpG-containing oligonucleotide induced IL-12 p40 gene promoter activity as well as the activation of transcription factor NF-κB, suggesting that CpG-containing oligonucleotides and/or bacterial DNA may act at the transcriptional level to induce gene expression. However, whether or not increased expression of the *iNOS* gene induced by bacterial DNA plus LPS occurs either exclusively or primarily at the transcriptional level remain to be clarified.

Of particular interest is the finding that prolonged pretreatment of macrophages with *E. coli* DNA dramatically suppressed *E. coli* DNA plus LPS-induced production of NO, although *iNOS* mRNA levels in *E. coli* DNA-pretreated macrophages remain comparable with those in LPS-pretreated macrophages. This suggests that *E. coli* DNA pretreatment regulates NO production via a different mechanism (e.g., posttranscriptional and/or posttranslational) from that of LPS pretreatment. Although these different mechanisms remain to be clarified, the suppressive effect of *E. coli* DNA pretreatment on NO production does coincide with a recent in vivo study by Schwartz et al. (29). In that study, Schwartz et al. found that systemic pretreatment of mice with bacterial DNA or CpG-containing ODNs suppressed the airway inflammatory response to inhaled LPS as manifested by reduced production of TNF-α and macrophage inflammatory protein-2. However, whether or not the suppressive effect of bacterial DNA on these two studies occurs via a similar molecular mechanism remains to be investigated. The finding that low concentrations of LPS can potentiate bacterial DNA on NO induction in cultured macrophages also points out the necessity of using highly purified bacterial DNA in both in vitro and in vivo studies, because contamination of a DNA preparation by even a small amount of LPS may enable bacterial DNA to exert

an exaggerated stimulatory effect on target cells compared with the effect seen for bacterial DNA by itself.

In summary, our data indicate that a strong synergy and antagonism exists between bacterial DNA and LPS on NO production in the mouse macrophage cell line RAW 264.7. Such synergy and antagonism is temporally controlled and is not mediated by autocrine production of IFN. The synergistic induction of NO by bacterial DNA and LPS coincides with the *iNOS* mRNA level, whereas the antagonistic production of NO does not, suggesting that different molecular mechanisms are involved. These findings may be of considerable significance in vivo, because NO production has been known to be important in controlling bacterial infection and tumor cell growth, causing tissue damage, and mediating septic shock (1, 2), and because it is likely that both microbial constituents would be anticipated to be present within the microbial milieu of a nidus of infection. Understanding the molecular mechanism of the synergy and antagonism between bacterial DNA and LPS can be anticipated to contribute to the development of therapeutic strategies against both tumor growth and diseases related to bacterial infection.

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