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Bacterial DNA and Lipopolysaccharide Induce Synergistic Production of TNF-α Through a Post-Transcriptional Mechanism1

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LPS is well recognized for its potent capacity to activate mouse macrophages to produce TNF-α, an important inflammatory mediator in bacterial infection-related diseases such as septic shock. We demonstrate here that while inducing only low levels of TNF-α alone, DNA from both Gram-negative and Gram-positive bacteria synergizes with subthreshold concentrations of LPS (0.3 ng/ml) to induce TNF-α in the RAW 264.7 macrophage-like cell line. The bacterial DNA effects are mimicked by synthetic CpG-containing oligodeoxynucleotides, but not non-CpG-containing oligodeoxynucleotides. Pretreatment of macrophages with either DNA for 2–8 h inhibits macrophage TNF-α production in responses to DNA/LPS. However, when pretreatment was extended to 24 h, DNA/LPS synergy on TNF-α is further enhanced. RT-PCR analysis indicates that mRNA levels of the TNF-α gene, however, are not synergistically induced by bacterial DNA and LPS. Analyses of the half-life of TNF-α mRNA indicate that TNF-α message has a longer half-life in bacterial DNA- and LPS-treated macrophages than that in bacterial DNA- or LPS-treated macrophages. These findings indicate that the temporally controlled, synergistic induction of TNF-α by bacterial DNA and LPS is not mediated at the transcriptional level. Instead, this synergy may occur via a post-transcriptional mechanism. The Journal of Immunology, 2001, 166: 6855–6860.

Recent data indicate that purified bacterial DNA can activate macrophages and other inflammatory cells (reviewed in Refs. 1 and 2). Macrophages stimulated with bacterial DNA reportedly produce proinflammatory cytokines such as TNF-α (3, 4), IL-1 (4), IL-6 (5), IL-12 (6–8), IFN-αβ (9, 10), IFN-γ (9), and the reactive nitrogen intermediate, NO (11, 12). Subtle structural differences between bacterial and eukaryotic DNA apparently account for the ability of bacterial DNA to serve as an immune-activating agent. Specifically, bacterial DNA is thought to activate inflammatory cells because of its high content of short sequences with unmethylated CpG dinucleotides (13). In mammalian DNA, CpG-containing sequences occur at a much lower frequency than in bacterial DNA, and the cytosine present in CpG dinucleotides of mammalian DNA is usually methylated (14, 15).

In vivo studies support the concept that bacterial DNA is an important proinflammatory stimulus, as bacterial DNA has been shown to trigger septic shock in d-galactosamine treated mice (16). Also of interest is the finding that bacterial DNA acts synergistically with LPS to induce TNF-α production in vivo, resulting in lethal shock in mice (11, 17). The molecular mechanism(s) by which bacterial DNA acts synergistically with LPS to induce TNF-α production remains to be determined. LPS, a constituent of the Gram-negative bacterial cell wall, is well known as a potent inducer of mouse macrophage activation, resulting in production of many inflammatory mediators, including TNF-α, that play a key role in the development of septic shock (reviewed in Refs. 18 and 19). The induction of TNF-α secretion from mouse macrophages in response to LPS stimulation is controlled at transcriptional, post-transcriptional, and translational levels (17, 20, 21).

In this communication we explore the mechanisms by which LPS and bacterial DNA act synergistically to activate macrophages. We present in vitro data demonstrating that bacterial DNA acts synergistically with substimulatory concentrations of LPS to enhance TNF-α secretion by the murine RAW 264.7 macrophage-like cell line. The observed synergy depends upon the presence of unmethylated CpG residues in DNA and is also dependent upon the temporal order of treatment by LPS and bacterial DNA. Enhanced TNF-α secretion by RAW 264.7 cells simultaneously exposed to bacterial DNA and LPS is not accompanied by enhanced transcription of the TNF-α gene. Analyses of the half-life of TNF-α in differentially treated macrophages suggest that bacterial DNA and LPS act synergistically to enhance TNF-α production through a post-transcriptional event.

Materials and Methods

Materials

Purified LPS from Escherichia coli O111: B4 was obtained from List Biological Laboratories (Campbell, CA). E. coli strain B genomic DNA, salmon sperm genomic DNA, and RNase-free DNase I were purchased from Sigma (St. Louis, MO). Synthetic oligodeoxynucleotides (ODNs; T3, 5'-AACGTT AACGTT AACGTT-3'; C3, 5'-CCATGCGCCATGCG CATGGC-3') were obtained from Sigma-Genosys (The Woodlands, TX). The endotoxin levels in these ODNs are <0.01 ng/μg of DNA based upon the Limulus amebocyte lysate assay.

1 Abbreviations used in this paper: ODN, oligodeoxynucleotide; UTR, untranslated region.
**Culture of macrophages**

The murine macrophage-like cell line RAW 264.7 (American Type Culture Collection, Manassas, VA) was used in all the studies described here. Macrophages were cultured in RPMI 1640 medium (Life Technologies, Grand Island, NY) 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% CO2 environment. Before being stimulated, macrophages were seeded into culture plates and cultured overnight.

**DNA manipulation**

E. coli DNA and salmon sperm DNA (Sigma) were further purified by two-step CsCl ultracentrifugation. DNA from Staphylococcus aureus was extracted exactly as described by Dyer and Iandolo (22). DNA digestion was performed using RNase-free DNase I (2 U/μg of DNA) in buffer (pH 7.6) containing 20 mM Tris-HCl and 20 mM MgCl2 at 37°C for 3 h. The endotoxin levels in these DNA preparations were <0.001 ng/μg of DNA according to the Limulus amebocyte lysate assay.

**TNF-α analysis**

After 20 h of stimulation, TNF-α production in macrophage culture supernatants was analyzed using the ELISA Duoset kit (purchased from R&D Systems, Minneapolis, MN). The protocol from the manufacturer was followed exactly for the assay. All data for TNF-α represent the average of duplicate samples ± SEM. Each experiment was repeated at least twice.

**RNA isolation and RT-PCR analysis**

RAW 264.7 macrophages were stimulated for various periods of time with different combinations of stimuli as described in Results. Total RNA from macrophages was isolated using TRIzol reagent (Life Technologies) according to the manufacturer’s instructions. A total of 1 μg of RNA from each sample was used for RT-PCR using the One-Step RT-PCR kit from Qiagen (Valencia, CA) according to the manufacturer’s protocols. The sequences of the specific primers used in these studies are: mouse TNF-α sense, 5′-GGG AGG TCT ACT TTG GAC TCA TTG C-3′; mouse TNF-α antisense, 5′-ACA TTC GAG CCT CCA GTG ATG TCG G-3′; mouse β-actin sense, 5′-TGT GAT GGT GGG AAT GGG TCA G-3′; and mouse β-actin antisense, 5′-TCT GTG GTT AAC GAC AAC GAT TCC C-3′. PCR products were analyzed using agarose gel electrophoresis, stained with ethidium bromide, and photographed. The photographs were scanned using Adobe Photoshop software (Adobe Systems, San Jose, CA) and analyzed using a GelPro Analyzer (Meyer Instruments, Houston, TX).

**Results**

It has been reported that LPS and bacterial DNA act synergistically to enhance TNF-α production in vivo, which contributes to septic shock in mice (3, 23). Because macrophages are a major source of TNF-α in vivo, we endeavored to reproduce this phenomenon in vitro with cultured mouse macrophages. RAW 264.7 macrophages were exposed to various concentrations of purified E. coli DNA with or without LPS at a subthreshold concentration (0.3 ng/ml; Fig. 1). At this subthreshold concentration LPS by itself either fails to induce TNF-α secretion or induces a minimal response (data not shown). RAW cells stimulated with E. coli DNA alone produced relatively low levels of TNF-α (<2000 pg/ml) even at the highest DNA concentration tested (30 μg/ml) in this study (Fig. 1A, △). However, in the presence of a subthreshold LPS stimulus the TNF-α response to E. coli DNA was dramatically enhanced. For example, TNF-α production induced by 3.0 μg/ml E. coli DNA was increased ~14-fold by adding 0.3 ng/ml LPS (Fig. 1A, △). In the presence of LPS, TNF-α production was dose dependent for E. coli DNA stimuli between 1.0 and 10 μg/ml, then subsequently declined. Importantly, TNF-α secretion in response to E. coli DNA plus a subthreshold LPS stimulus was almost completely abrogated by treatment with DNase I, indicating that the response is specific to DNA (Fig. 1A, △). Salmon sperm DNA, which contains highly methylated CpG dinucleotides, induced a weak TNF-α response by itself and failed to synergize with LPS, suggesting that the macrophage-activating ability of DNA is dependent upon non-methylated CpG dinucleotides (Fig. 1A, ○ and □).

To determine whether the ability of E. coli DNA to act synergistically with LPS was applicable to other bacterial species, we repeated the above experiments with DNA purified from the Gram-positive bacteria, S. aureus. S. aureus DNA alone induced RAW cells to produce only low levels (<2000 pg/ml) of TNF-α (Fig. 1B, △). However, in the presence of a subthreshold LPS stimulus (0.3 ng/ml), S. aureus DNA induced a greatly enhanced

**FIGURE 1.** Induction of TNF-α by E. coli DNA, S. aureus DNA, and synthetic ODNs. RAW 264.7 macrophages were stimulated with E. coli DNA (A), S. aureus DNA (B), or synthetic CpG-containing ODN (C) in either the absence (△) or the presence (○) of 0.3 ng/ml LPS for 20 h before culture supernatants were collected for TNF-α ELISA. Controls include salmon sperm DNA (A and B, ●), salmon sperm DNA plus LPS (A and B, ○), DNase I-treated E. coli DNA plus LPS (A, □), DNase I-treated S. aureus DNA plus LPS (B, □), synthetic non-CpG-containing C3 (C, ●), and C3 plus LPS (C, ○).
The stimulatory activity of *E. coli* and *S. aureus* DNA could be reproduced by a synthetic nonmethylated CpG-containing ODN (termed T3; see Materials and Methods). Although T3 by itself was a more potent stimulus than either *E. coli* or *S. aureus* DNA alone, it still induced only low levels of TNF-α secretion (peaking at ~4000 pg/ml) from RAW macrophages (Fig. 1C, △). However, as with *E. coli* and *S. aureus* DNA, T3-induced TNF-α secretion was markedly enhanced (peaking at ~12,000 pg/ml) in the presence of a subthreshold LPS stimulus (Fig. 1C, △). In marked contrast to the results with T3, treatment of macrophages with a non-CpG-containing ODN (C3) resulted in minimal TNF-α secretion (<1000 pg/ml) in either the absence or the presence of LPS (Fig. 1C, ● and ○).

It has been reported that TNF-α production by mouse macrophages in response to LPS stimulation is controlled at both transcriptional and post-transcriptional levels (17, 20, 21). However, the mechanism by which bacterial DNA plus LPS act synergistically to induce macrophage TNF-α production remains to be determined. To assess whether synergistic induction of macrophage TNF-α by bacterial DNA and LPS is controlled at the level of gene transcription, macrophages were treated for 2 h with *E. coli* DNA or LPS alone or with a combination of *E. coli* DNA plus LPS (along with appropriate controls) as described in Fig. 2. After stimulation, total RNA was extracted from macrophages and subjected to RT-PCR analysis for detection of TNF-α mRNA. TNF-α mRNA levels in macrophages treated with either *E. coli* DNA (1.0 μg/ml) or LPS (0.3 ng/ml) were elevated compared with TNF-α mRNA from control macrophages treated with cell culture medium; recall that LPS at this concentration fails to induce a TNF-α response, and *E. coli* DNA induced low levels of TNF-α secretion (<2000 pg/ml). Simultaneous treatment of macrophages with both *E. coli* DNA and LPS did not further enhance TNF-α mRNA expression compared with treatment with either *E. coli* DNA or LPS alone despite the finding that this combined stimulus markedly enhanced TNF-α secretion. Treatment of *E. coli* DNA with DNase I essentially abrogated the TNF-α mRNA response to *E. coli* DNA alone, but did not alter the TNF mRNA production observed in response to simultaneous *E. coli* DNA plus LPS treatment. Finally, although salmon sperm DNA by itself failed to induce significant TNF-α mRNA production, TNF-α mRNA levels induced by simultaneous treatment with salmon sperm DNA and LPS were comparable to those induced by simultaneous *E. coli* DNA and LPS treatment. Together these data all support the conclusion that *E. coli* DNA and LPS do not act synergistically to enhance TNF-α mRNA production.

In addition to the experiments presented above in which macrophages were stimulated for 2 h before RNA extraction, we also performed experiments in which RNA was extracted after 0.5, 1, and 4 h of stimulation. Varying the period of stimulation did not alter the findings. Simultaneous treatment of macrophages with both *E. coli* DNA and LPS did not further enhance TNF-α mRNA expression compared with treatment with either *E. coli* DNA or LPS alone for any of the stimulation periods tested (data not shown).

The data presented above indicate that although a subthreshold LPS stimulus (0.3 ng/ml) synergizes with bacterial DNA to enhance TNF-α secretion from RAW 264.7 macrophages, LPS and bacterial DNA do not synergize to enhance TNF-α mRNA expression. It has previously been shown that TNF-α gene expression is regulated at both transcriptional and post-transcriptional levels when LPS alone is the stimulus (17, 20, 21). Consequently, it is reasonable to hypothesize that enhanced TNF-α production in response to a combined LPS and bacterial DNA stimulus results from altered post-transcriptional controls. To test this hypothesis, we determined the half-life of TNF-α mRNA in macrophages treated with *E. coli* DNA (Fig. 3A) or LPS alone (Fig. 3B). *E. coli* DNA combined with LPS (Fig. 3C), or salmon sperm DNA combined with LPS (Fig. 3D). To determine the half-life of TNF-α mRNA, transcription was inhibited by treating macrophages with actinomycin D (5.0 μg/ml) after they had been exposed to specific stimuli for a period of 2 h. Total RNA was isolated from macrophages at various time points after actinomycin D treatment and subjected to RT-PCR analysis to assay for TNF-α mRNA. The half-lives of TNF-α mRNA in macrophages stimulated with bacterial DNA alone, LPS alone, bacterial DNA plus LPS, or salmon sperm plus LPS are estimated to be 36, 45, 100, and 45 min, respectively (Fig. 3). Thus, the half-life of TNF-α mRNA in macrophages stimulated with a combination of LPS plus bacterial DNA is 2–3 times that of TNF-α mRNA from macrophages exposed to any of the other stimuli tested. This prolonged half-life of TNF-α mRNA could account at least in part for the synergistic production of TNF-α by macrophages exposed to a combination of bacterial DNA and LPS.

The sequence and timing of exposure to two different stimuli often impact the ability of these stimuli to synergize, presumably because pertinent activating signals must be initiated in a permissive temporal pattern. When multiple stimuli were used in the experiments presented above, macrophages were simultaneously exposed to these stimuli. For the experiments presented in Fig. 4, the sequence and timing of exposure to bacterial DNA and LPS were altered. Pretreatment of macrophages with *E. coli* DNA for 2–8 h before adding *E. coli* DNA plus LPS (0.3 ng/ml) resulted in a time-dependent reduction of TNF-α secretion (Fig. 4, ●). The maximum observed effect in this experiment was when macrophages were pretreated with *E. coli* DNA for 8 h before adding LPS; TNF-α secretion was reduced more than 3-fold compared with macrophages that were not pretreated. Interestingly, when pretreatment with *E. coli* DNA was extended to 24 h, TNF-α secretion was comparable to levels attained in macrophages that were not pretreated (Fig. 4, compare 24 and 0 h points). Macrophages pretreated with LPS for 2–8 h before adding *E. coli* DNA plus LPS also displayed a time-dependent reduction in TNF-α secretion compared with macrophages that were not pretreated (Fig. 4, ○).
LPS pretreatment was attributable to reduced TNF-α4, compare 24 and 0 h points). To levels attained with macrophages that were not pretreated (Fig. 4). Cell viability was not affected by treatment with 5 μg/ml RNA was extracted, and the presence of TNF-α and the fraction of that observed for the controls (Fig. 3). However, the reduction in TNF-α secretion was not as extensive as that observed with E. coli DNA pretreatment (Fig. 4, compare ○ and ●). As with DNA pretreatment, when pretreatment with LPS was extended to 24 h, TNF-α secretion was comparable to levels attained with macrophages that were not pretreated (Fig. 4, compare 24 and 0 h points).

To determine whether the suppressive effect of E. coli DNA or LPS pretreatment was attributable to reduced TNF-α mRNA transcription, macrophages were pretreated with E. coli DNA, LPS, or medium (control) for 8 h before stimulation with E. coli DNA plus LPS for 2 h. After stimulation, total RNA was extracted from macrophages and subjected to RT-PCR analysis for detection of TNF-α specific mRNA. TNF-α mRNA levels in macrophages pretreated with either LPS or E. coli DNA before stimulation with E. coli DNA plus LPS were comparable to TNF-α mRNA levels in macrophages pretreated with medium (Fig. 5), results comparable to those observed earlier regarding the relative quantity of TNF-α secreted by macrophages and the quantity of mRNA produced by macrophages. Pretreatment of macrophages with either LPS or E. coli DNA reduces TNF-α secretion in response to a subsequent LPS plus E. coli DNA stimulus, but appears not to alter transcription of TNF-α mRNA. These data lend additional support to the conclusion that post-transcriptional events account for the synergistic interactions among bacterial DNA, LPS, and macrophages that lead to altered patterns of TNF-α secretion.

Discussion

In the current study we have shown that treatment of RAW macrophages with either bacterial DNA or a subthreshold concentration of LPS (0.3 ng/ml) induced the secretion of relatively low levels of TNF-α protein and enhanced expression of TNF-α mRNA (Figs. 1 and 2). When macrophages were simultaneously treated with bacterial DNA plus LPS, secretion of TNF-α protein was dramatically enhanced, while levels of TNF-α mRNA remained comparable to those attained with either bacterial DNA or LPS stimulation alone (Figs. 1 and 2). However, the half-life of TNF-α mRNA in macrophages treated simultaneously with bacterial DNA plus LPS was substantially longer than that in macrophages treated with bacterial DNA or LPS alone. Additionally, pretreatment of macrophages with either bacterial DNA or LPS alone caused a time-dependent suppression of TNF-α secretion induced by subsequent stimulation with bacterial DNA plus LPS. This suppression of TNF-α secretion was not accompanied by reduced expression of TNF-α mRNA. These findings suggest that...
synergistic interactions among bacterial DNA, LPS, and macrophages leading to enhanced TNF-α secretion are controlled by post-transcriptional events. A synthetic oligonucleotide rich in unmethylated CpG residues also acted synergistically with LPS, whereas both salmon sperm DNA, which contains highly methylated CpG residues, and a synthetic non-CpG-containing oligonucleotide, failed to act synergistically with LPS to enhance TNF-α secretion. These data support the conclusion that unmethylated CpG residues are critical to bacterial DNA’s capacity to act synergistically with LPS to enhance TNF-α secretion by macrophages.

The data we present are consistent with the results of a recent in vivo study by Schwartz et al. (24). Schwartz et al. reported that i.v. pretreatment of mice with CpG-containing oligonucleotides suppressed production of TNF-α and macrophage inflammatory protein-2 in lung lavage fluid in response to inhaled LPS. However, analysis of total lung mRNA indicated that TNF-α and macrophage inflammatory protein-2 mRNA levels were not reduced by pretreatment with CpG oligonucleotides. Thus, this in vivo study shows discrepancies between TNF-α production and TNF-α mRNA transcription following interactions between LPS, CpG DNA, and macrophages. This suggests that modifications in TNF-α production attributable to these interactions in vivo are controlled by post-transcriptional events.

We have previously reported that bacterial DNA and LPS interact synergistically with macrophages to enhance production of NO, another important inflammatory mediator (12). Although the combination of bacterial DNA plus LPS dramatically enhanced the production of both NO and TNF-α, the mechanisms responsible for the synergistic induction of these two inflammatory mediators appear to differ. The synergistic induction of NO is regulated primarily at the transcriptional level, because a combination of bacterial DNA plus LPS enhanced the production of mRNA for the inducible NO synthase gene, which accounts for the synthesis of NO in mouse macrophages (25). In contrast, levels of TNF-α mRNA in macrophages treated with a combination of bacterial DNA plus LPS were similar to those in macrophages treated with either bacterial DNA or LPS alone, suggesting that transcriptional controls do not account for enhanced TNF-α secretion under these conditions. However, the half-life of TNF-α mRNA in macrophages treated with a combination of bacterial DNA plus LPS is 2–3 times that of TNF-α mRNA in macrophages stimulated with either bacterial DNA or LPS alone. This suggests that post-transcriptional controls are responsible for the synergistic induction of TNF-α by bacterial DNA plus LPS.

Expression of the TNF-α gene in mouse macrophages is regulated at multiple levels. Transcriptional (20, 21), post-transcriptional (17, 20), and translational (17, 26–28) controls have all been reported to contribute to regulation of TNF-α gene expression. Stimulation of RAW 264.7 macrophages with LPS resulted in increased transcription and translation of the TNF-α gene, although no apparent change in TNF-α mRNA stability has been observed (20, 28). Transcriptional regulation of the TNF-α gene is mediated primarily by binding of transcription factors (e.g., NF-κB) to the promoter region of the TNF-α gene (29, 30), whereas post-transcriptional and translational regulation is related to the 3' untranslated region (UTR) of TNF-α mRNA. It has been reported that the UA-rich 3' UTR of TNF-α mRNA contributed to instability as well as translation repression of TNF-α mRNA (17, 26–28). Whether the prolonged half-life of TNF-α mRNA in macrophages treated with a combination of bacterial DNA plus LPS is mediated by the 3' UTR of TNF-α mRNA and whether the 3' UTR-mediated translational mechanism also plays a role in the synergistic induction of TNF-α remain to be investigated.

The available experimental evidence suggests that bacterial DNA and LPS may use different signaling transduction pathways, even though both activate common transcription factors such as NF-κB and AP-1 (31–35). Although LPS uses TLR4 as its receptor (36), bacterial DNA uses TLR9 as its receptor that might be dependent on the adaptor protein MyD88 (37–39). Our finding that a temporally controlled synergy exists between bacterial DNA and LPS lends further support to the idea that these two microbial components use different signaling pathways. Whereas LPS alone induces macrophages to produce TNF-α primarily through transcriptional and translational signaling (20, 28), a combination of bacterial DNA plus LPS may activate a signal(s) directed toward post-transcriptional controls, which might contribute to the synergistic production of TNF-α.

Collectively, the data presented in this communication indicate that nonmethylated, CpG-rich, bacterial DNA synergizes with LPS to enhance TNF-α secretion from the RAW 264.7 macrophage cell line cultured in vitro. The synergistic production of TNF-α induced by a combination of bacterial DNA plus LPS appears to be controlled at post-transcriptional levels, as these conditions enhance the half-life of TNF-α mRNA, but do not enhance the transcription of TNF-α mRNA. Our data also showed that pre-exposure of macrophages to either bacterial DNA or LPS resulted in a time-dependent reduction in TNF-α secretion in response to a combined bacterial DNA plus LPS stimulus. This inhibition also appears to be controlled at the post-transcriptional level, as pretreatment with bacterial DNA or LPS reduced TNF-α secretion without impacting TNF-α mRNA levels. These findings provide a mechanistic explanation for previous reports indicating that bacterial DNA and LPS acted synergistically to enhance TNF-α production in vivo, leading to lethal shock in mice (3, 23).

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References

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