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Dynamics of Dibutyryl Cyclic AMP- and Prostaglandin E_2-Mediated Suppression of Lipopolysaccharide-Induced Tumor Necrosis Factor Alpha Gene Expression

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The regulation of lipopolysaccharide (LPS)-induced tumor necrosis factor alpha (TNF) production by prostaglandin E_2 (PGE_2), forskolin, and dibutyryl cyclic AMP (cAMP) was examined at the cellular and molecular levels. The above three agents could suppress LPS (100 ng/ml)-stimulated TNF production by immunologically activated murine macrophages (Mϕs) in a dose-dependent manner. The concomitant addition of PGE_2, dibutyryl cAMP, or forskolin to LPS-challenged Mϕs resulted in 50% inhibition of TNF production at 10^{-7}, 3×10^{-6}, and 3×10^{-5} M, respectively. Interestingly, delaying the addition of PGE_2 or dibutyryl cAMP by 1.5 h post-LPS stimulation was also effective in suppressing the production of TNF bioactivity, but only dibutyryl cAMP was effective when its addition was delayed by 3 h. Northern (RNA) blot analysis of mRNA isolated from LPS-challenged Mϕs treated with PGE_2 or dibutyryl cAMP corroborated the bioactivity data. The delayed addition of PGE_2 or dibutyryl cAMP by 1.5 h post-LPS stimulation resulted in a suppression of TNF mRNA accumulation by 50 to 70%. These data support the concept that LPS is a potent stimulus for Mϕ-derived TNF production and that this mediator is a very proximal signal in LPS-mediated disease states. Thus, therapeutic approaches that target the suppression of TNF in LPS-dependent disease states may be limited by the rapid expression of this mediator.

Tumor necrosis factor alpha (TNF) is increasingly being recognized as an important mediator involved in the orchestration of a variety of infectious diseases. Compelling evidence from both clinical and experimental animal studies has incriminated TNF as a proximal signal in bacterium-induced septic shock (2, 3, 18). The importance of this mediator is not limited to only bacterial diseases, as recent reports have demonstrated the in vivo production of TNF in patients (17) or animals infected with parasitic protozoans (1). Of particular interest are the high levels of TNF found in experimental mice infected with cerebral malaria (6, 8). Although it is apparent that most infectious agents are capable of inducing elevated levels of TNF in either an in vitro or in vivo setting, bacteria and/or their products (including lipopolysaccharide [LPS] and exotoxins) are important in the induction of TNF-mediated pathology after septic shock. The clinical importance of TNF in bacterial sepsis has been identified in the seminal work by Waage et al. (19), in which elevated levels of TNF were detected in the serum samples of patients with meningococcal septicemia. Interestingly, the TNF concentration in serum was found to directly correlate with patient survival.

Although the above evidence supports the role of TNF in bacterium- or endotoxin-induced shock, the mechanisms involved in the induction and regulation of this cytokine have not been clearly identified. In this study we present data demonstrating that LPS can rapidly induce the expression of macrophage (Mϕ) TNF and that this expression may be suppressed by prostaglandin E_2 (PGE_2) and forskolin (agents that can elevate intracellular levels of cyclic AMP [cAMP]), as well as dibutyryl cAMP. The addition of LPS (in picograms per milliliter) to immunologically activated murine Mϕs can induce the production of TNF bioactivity that peaked by 4 h. In similar studies, TNF mRNA reached a zenith by 3 h post-LPS challenge. The concomitant addition of LPS plus either PGE_2, forskolin, or dibutyryl cAMP suppressed TNF production in a dose-dependent manner. In addition, withholding these suppressing agents for as long as 1.5 h post-LPS challenge could still suppress TNF gene expression by as much as 70%. These data support the theory that TNF is a very proximal mediator and that LPS-induced TNF production may be susceptible to regulation only during a brief temporal window post-Mϕ stimulation.

MATERIALS AND METHODS

Isolation and preparation of murine Mϕs. Immunologically elicited murine Mϕs were recruited by injecting 0.5 ml of complete Freund adjuvant (diluted 1:1 with sterile saline) into the peritoneal cavities of specific pathogen-free female CBA/J mice. The mice were maintained under standard care and were given food and water ad libitum. After 10 to 14 days, the Mϕs were harvested by peritoneal lavage, washed, suspended in RPMI 1640 containing 1 mM glutamine, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 100 U of penicillin per ml, and 100 μg of streptomycin per ml, and allowed to adhere for 2 h before stimulation. After the 2-h adherence, the cultures (>75% Ia antigen positive) were washed and incubated with graded doses of LPS or a single dose of LPS (*Escherichia coli* O111:B4, Sigma Chemical Co., St. Louis, Mo.) in the presence or absence of either PGE_2 (The Upjohn Co., Kalamazoo, Mich.), forskolin (Sigma), or dibutyryl cAMP (Sigma). For TNF mRNA studies, Mϕs were cultured in 100-mm-diameter dishes (20×10^6 cells per dish), while supernatants

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were assessed for TNF production. The adherent cells were greater than 90% mononuclear phagocytes.

Experimental approach. In initial studies, the suppressive effects of PGE_2, at 10^{-6} and 10^{-7} M, were assessed on Mϕ TNF production over a 7-log concentration range of LPS. Either LPS was added alone or PGE_2 and LPS were added simultaneously to the Mϕ cultures; cell-free supernatants were then harvested 4 h later, and the samples were frozen at −20°C until assayed. In subsequent studies, Mϕs were challenged with 100 ng of LPS per ml, and either PGE_2, forskolin, or dibutyryl cAMP was added at time zero. In these studies, full dose-response curves were generated for each agent under study. The ability of PGE_2 or dibutyryl cAMP treatment to suppress TNF production was evaluated by withholding treatment for up to 1.5 h post-LPS (100 ng/ml) addition, and the levels of TNF mRNA and t ity were then determined 3 h after LPS challenge.

TNF determination. The activity of TNF was monitored using a semi-automated LM fibroblast lytic assay. Fibroblasts (5×10^4/0.1 ml) were incubated for 18 h in the presence of 1 μg of dactinomycin and serial 1:2 dilutions of test samples in 96-well flat-bottom microtiter plates. Specificity of lysis was determined by incubating supernatants with rabbit anti-murine TNF. High-titer antibody was raised in our laboratory using recombinant mouse TNF as the immunogen. The antibody fully neutralizes native murine TNF and identifies one band of protein on Western blot (immunoblot) analysis. This treatment blocked the lytic activity of the Mϕ supernatant. The remaining cells were then washed and stained with crystal violet (0.5% in methanol/water [1:4, vol/vol]). The amount of cell lysis was determined using a micro-enzyme-linked immunosorbent assay autoreader at 620 nm. Units of TNF activity were defined using an internal standard of TNF (Cetus Corp., Emeryville, Calif.). In the studies assessing the regulation of TNF production by PGE_2 or dibutyryl cAMP, neither of these agents had an independent effect on the TNF target cells.

TNF mRNA determination. Total RNA from the murine Mϕs was isolated, using a modification of the methods of Chirgwin et al. (5) and Jonas et al. (10). Briefly, Mϕ monolayers were scraped into a solution of 25 mM Tris (pH 8.0) containing 4.2 M guanidine isothiocyanate, 0.5% Sarkosyl, and 0.1 M 2-mercaptoethanol. After homogenization, an equal volume of 100 mM Tris (pH 8.0) containing 10 mM EDTA and 1.0% sodium dodecyl sulfate was added, and the mixture was extracted with chloroform-phenol and chloroform-isoamyl alcohol. The RNA was then alcohol precipitated, and the pellet was dissolved in TE buffer (10 mM Tris, 1 mM EDTA). TNF mRNA was examined by Northern analysis using formaldehyde–1% agarose gels. The separated RNA was transblotted to nitrocellulose, baked, prehybridized, and hybridized with either a nick-translated ^{32}P-labeled human TNF cDNA probe (Cetus) or a ^{32}P-5′-end-labeled 30-mer oligonucleotide for murine TNF, 5′-GTC-CCC-CTT-CTC-CAG-CTG-GAA-GAC-TCC-TCC-3′ (13). Blots were washed and autoradiographed with intensifying screens. The relative sensitivity of the autoradiographs was quantitated by laser densitometry. Equivalent amounts of RNA per gel were assessed by probing for beta-actin (15) and monitoring 28S and 18S rRNA.

Statistics. Complete dose-response curves were initially determined for LPS-induced TNF production and then for the suppression of TNF by either PGE_2, forskolin, or dibutyryl cAMP. Changes in the LPS-induced TNF dose-response curves were assessed using nonlinear least-squares regression to fit a curve. This data is expressed here as the 50% effective concentration (EC_{50}), or the concentration of LPS necessary to generate a half-maximum response. All data are expressed as the mean values ± the standard errors of the mean.

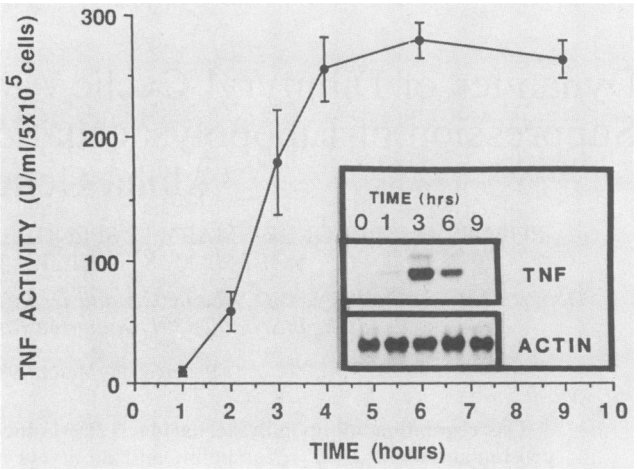

FIG. 1. Kinetic analysis of the induction of TNF mRNA and bioactivity by 30 ng of LPS per ml. Both TNF bioactivity and mRNA were rapidly expressed post-LPS challenge. A peak in TNF production occurring 4 h after LPS treatment and a peak in TNF mRNA expression occurring 3 h after LPS challenge were observed. Note the fairly rapid decay in TNF mRNA.

RESULTS

Kinetics of TNF production and mRNA accumulation by LPS-stimulated Mϕs. The kinetics of TNF production and TNF mRNA levels were assessed at specific time points post-LPS challenge. Mϕs demonstrated an initial delay in the production of TNF for the first hour after LPS (30 ng/ml) stimulation, followed by a rapid increase that plateaued by 4 h (Fig. 1). In comparison studies, TNF mRNA accumulated rapidly after LPS exposure, reaching a peak by 3 h (Fig. 1, insert). After the 3-h time point, the levels of TNF mRNA rapidly declined. The steady-state levels of beta-actin mRNA remained unchanged during LPS stimulation. In all of the studies detailed below, Mϕ samples were examined 3 and 4 h post-LPS challenge to assess TNF mRNA and bioactivity, respectively.

Dose-dependent augmentation of Mϕ TNF by LPS and regulation of TNF production. In subsequent studies, the ability of immunologically activated Mϕs to respond to graded doses of LPS was determined. Murine Mϕs were challenged with various concentrations of LPS, ranging from 1 pg/ml to 1 μg/ml, for 4 h, and the production of TNF bioactivity was determined. Mϕs were shown to produce TNF in response to LPS in a dose-dependent manner (Fig. 2). A 100-pg/ml concentration of this polyclonal activator was found to significantly increase TNF production, while EC_{50} was observed with 1.49 ± 0.33 ng of LPS per ml (n = 14). LPS concentrations above 100 ng/ml were found to induce maximum TNF production. The simultaneous addition of PGE_2 at either 10^{-6} or 10^{-7} M resulted in a suppression of LPS-induced TNF production. The LPS-induced TNF dose-response curve was shifted to the right by using either concentration of PGE_2, with new EC_{50}s of 7.3 ng of LPS per ml ($P < 0.005$) and 27.8 ng of LPS per ml ($P < 0.005$) for 10^{-7}M and 10^{-6} M PGE_2, respectively. Since

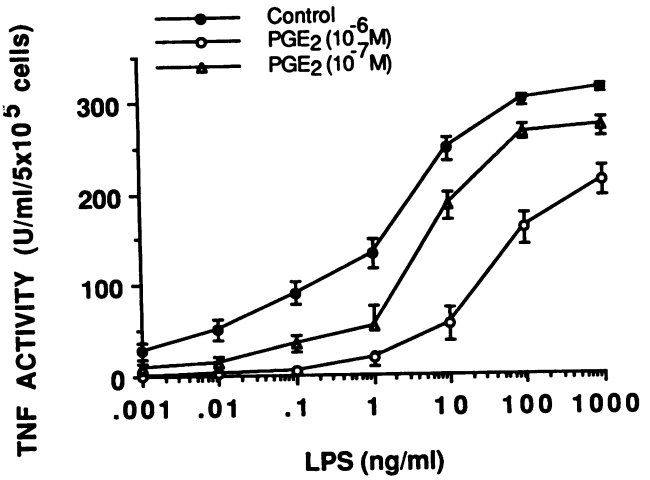

FIG. 2. Effects of PGE_2 on the production of Mϕ TNF in response to graded doses of LPS. Mϕs were treated with LPS with or without 10^{-6} or 10^{-7} M PGE_2. TNF levels were assessed 4 h after the addition of the above reagents. Each point represents the mean of 14 determinations ± the standard error of the mean. The control represents only LPS-treated Mϕs.

PGE_2 has been previously demonstrated to exert its effects by augmenting intracellular levels of cAMP via specific receptor-ligand interactions (7), forskolin (a compound that directly activates adenylate cyclase) and dibutyryl cAMP were examined for their suppressive effects on TNF production (Fig. 3). The concomitant addition of either PGE_2, forskolin, or dibutyryl cAMP with LPS (100 ng/ml) inhibited the production of TNF in a dose-dependent manner. The concentration-effect curves for these three agents demonstrated that PGE_2 was much more potent in suppressing TNF production, followed by dibutyryl cAMP and finally forskolin. Concentrations of PGE_2, dibutyryl cAMP, and forskolin necessary to suppress LPS-induced TNF production by 50% were 10^{-7}, 3×10^{-6}, and 3×10^{-5} M, respectively.

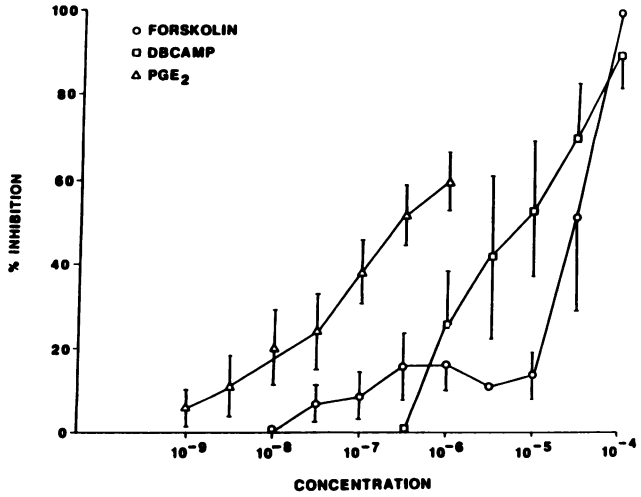

FIG. 3. Effects of graded doses of either PGE_2, forskolin, or dibutyryl cAMP (DBCAMP) on LPS-induced TNF production. All three compounds could suppress LPS-dependent TNF production. PGE_2 was found to be the most potent, followed by dibutyryl cAMP and finally forskolin. Each point represents the mean of 4 to 12 determinations ± the standard error of the mean.

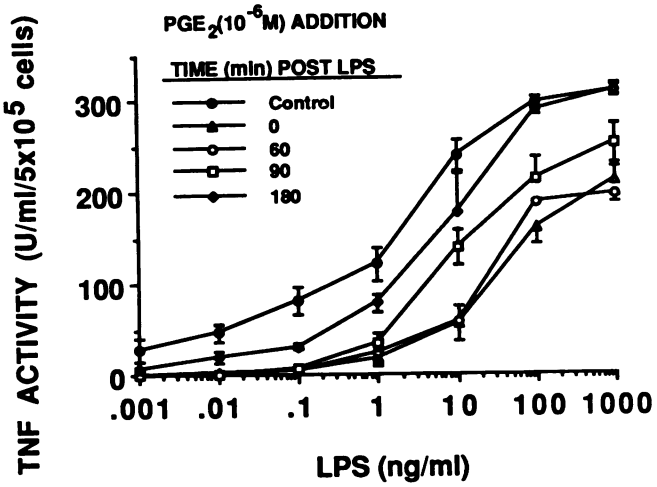

FIG. 4. Effect of delaying the addition of PGE_2 on LPS-induced TNF production. PGE_2 was efficacious in suppressing TNF production in response to various LPS concentrations even when withheld for up to 90 min post-LPS stimulation. Each point represents the mean of 4 to 12 determinations ± the standard error of the mean.

Effects of delayed addition of PGE_2 or dibutyryl cAMP on LPS-induced TNF production. Previous studies in experimental animals have demonstrated that TNF is a very proximal mediator that is rapidly generated in vivo after an LPS challenge (9, 14, 17, 18). Thus, it was of interest to examine the time frame whereby TNF production could be therapeutically altered after an LPS challenge. In these studies, murine Mϕs were treated with LPS (100 ng/ml) at time zero; PGE_2 or dibutyryl cAMP was also added at time zero or was added to the cultures at 60, 90, or 180 min; and the Mϕ supernatants were harvested at a constant time point of 4 h after the original LPS challenge. LPS-stimulated TNF production could be significantly suppressed by delaying the addition of PGE_2 for 90 min, while the addition of PGE_2 180 min after LPS treatment could no longer suppress TNF production (Fig. 4). The addition of PGE_2 at 0, 60, or 90 min shifted the EC_{50}s of the LPS dose-response curve to 27.8 ± 4.7 (n = 9), 14.8 ± 1.9 (n = 5), and 12.6 ± 4.2 (n = 5), respectively ($P < 0.005$ for all points). Delaying the addition of PGE_2 by 180 min did not significantly alter the half-maximal response of the LPS dose-response curve (EC_{50} = 3.76 ± 1.64; n = 5) compared with LPS alone (EC_{50} = 1.14 ± 0.36; n = 10). Similar results were found by delaying dibutyryl cAMP treatment (Fig. 5). In this study, the addition of dibutyryl cAMP at 0, 60, 90, or 180 min post-LPS treatment all significantly suppressed the production of TNF. The LPS dose-response curves for each of the dibutyryl cAMP experiments were shifted to the right with EC_{50}s at 0, 60, 90, and 180 min of 15.5 ± 1.1, 12.3 ± 3.5, 12.4 ± 3.6, and 14.3 ± 4.5 ng of LPS per ml, respectively ($P < 0.005$ for each EC_{50}). Although delaying the addition of dibutyryl cAMP for 180 min did not significantly reduce the amount of TNF produced at the higher, individual LPS concentrations, the entire LPS dose-response curve was significantly shifted to the right. The ability of dibutyryl cAMP to suppress LPS-induced TNF production even when its addition is delayed by 180 min is in contrast to the effects of PGE_2 added 180 min post-LPS challenge. This difference may reflect the potential direct effect of dibutyryl cAMP on the cell that bypasses any receptor-ligand interactions.

Effects of delayed addition of PGE_2 and dibutyryl cAMP on LPS-induced TNF mRNA expression. The next set of studies

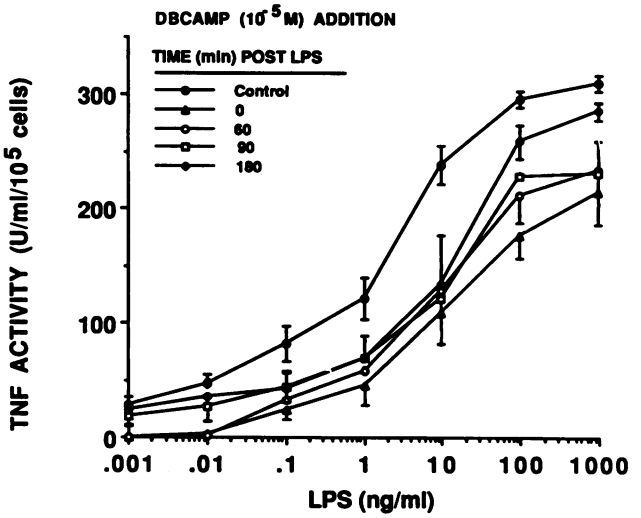

FIG. 5. Effect of delaying the addition of dibutyryl cAMP (DB CAMP) on LPS-induced TNF production. Dibutyryl cAMP (10^{-5} M) was found to be active in inhibiting the production of TNF in response to graded concentrations of LPS when the addition was withheld for up to 180 min. Each point represents the mean of 4 to 9 determinations ± the standard error of the mean.

was designed to determine the effects of delaying the addition of PGE_2 or dibutyryl cAMP on LPS-induced Mϕ TNF mRNA accumulation. As described above, either PGE_2 or dibutyryl cAMP was added to LPS (100 ng/ml)-challenged Mϕs at 0, 30, 60, or 90 min and the mRNA was then extracted at 180 min, the latter time point being the peak in TNF mRNA accumulation (Fig. 1). The addition of PGE_2 90 min after LPS stimulation was still effective in suppressing the accumulation of TNF mRNA (Fig. 6). Northern blot analysis of the TNF mRNA isolated from Mϕs receiving PGE_2 at 0, 30, 60, and 90 min after LPS stimulation demonstrated 70, 55, 65, and 30% reductions in TNF mRNA accumulation, respectively, as assessed by laser densitometry. Dibutyryl cAMP exerted a more dramatic reduction in LPS-induced TNF mRNA accumulation. The addition of dibutyryl cAMP 90 min post-LPS challenge still reduced the accumulation of TNF mRNA by greater than 60%, as

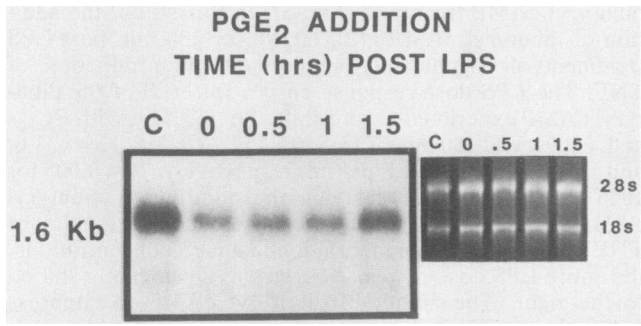

FIG. 6. Northern blot analysis of TNF mRNA isolated from Mϕs treated with LPS at time zero and with 10^{-6} M PGE_2 at the indicated time points (in hours). Equal amounts of total RNA loaded are demonstrated by the inset at the right, which shows 28 and 18S rRNA. The Northern blot is representative of four individual experiments and shows the suppression of TNF mRNA accumulation after the addition of PGE_2. Kb, Kilobases; C, control.

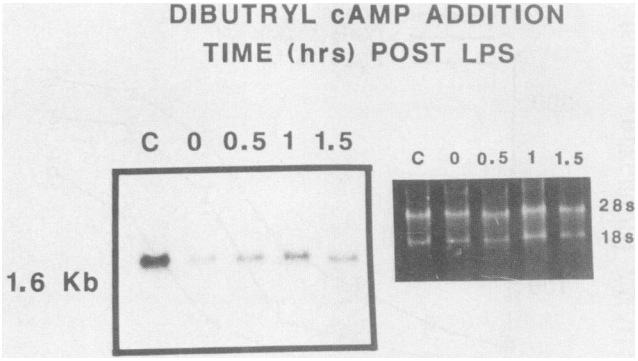

FIG. 7. Northern blot analysis of TNF mRNA isolated from Mϕs treated with LPS at time zero and dibutyryl CAMP at the indicated time points (in hours). rRNA is shown in the insert to the right. The Northern blot demonstrates that delayed addition of dibutyryl cAMP can inhibit the expression of TNF mRNA. The data are representative of four individual studies. Kb, Kilobases; C, control.

determined by laser densitometry (Fig. 7). The Northern blot analysis of the TNF mRNA studies corroborates the earlier studies assessing the production of biologically active TNF.

DISCUSSION

Recent advances in monokine research have increased our understanding of a number of immunologic and physiologic processes. It is now apparent that a variety of Mϕ-derived proteins, including TNF, possess pleomorphic effects that are important in maintaining an effective host defense. Although transformed cells were the original target for TNF (4), subsequent investigations in vitro have demonstrated that TNF is an important activation signal for endothelial cells, fibroblasts, adipocytes, neutrophils, monocytes, and Mϕs (12). The multiple activation events induced by TNF are not limited to an in vitro setting, as this mediator has been incriminated as a key molecular signal involved in chronic inflammation, as well as in the multiple organ pathology of sepsis. The role of TNF in the pathogenesis of LPS-induced shock was initially investigated by Beutler et al. (3), who demonstrated that antibody directed against TNF could protect against the lethal effects of LPS in experimental animals. These studies were extended by Tracey et al. (16), who observed that the lethality of *E. coli*-induced septic shock could be prevented in baboons by pretreating with monoclonal antibody against TNF. Both in vitro and in vivo studies examining the activities of preformed TNF have provided useful information regarding cell activation events, yet mechanisms involved in the regulation of TNF expression are not entirely clear.

In this study we have demonstrated that the in vitro production of TNF bioactivity and mRNA is rapidly expressed post-LPS challenge. Upon Mϕ stimulation with LPS, TNF mRNA and bioactivity peak at 3 and 4 h, respectively. In addition to being rapidly expressed by LPS, the production of TNF by activated Mϕs is very sensitive to low concentrations of LPS, as low levels (picograms per milliliter) can significantly induce the production of TNF. Although the production of TNF is rapidly induced by LPS, this peptide mediator can be modulated by discrete regulatory mechanisms. PGE_2, in a dose-dependent manner, can shift an LPS dose-response curve to the right, demonstrating an inhibition of TNF release by PGE_2 at all LPS concentrations. In an extension of previous studies (11), we now

demonstrate that PGE_2, forskolin, and dibutyryl cAMP can regulate LPS-induced TNF production in a dose-dependent manner. All three of these agents result in an increase in intracellular levels of cAMP. PGE_2, via receptor-ligand interactions, can activate adenylate cyclase, while forskolin can directly activate the cyclase enzyme. Dibutyryl cAMP readily crosses the cell membrane and adds to the intracellular pool of cAMP. Thus, the production of TNF appears to be under the regulation of a cAMP-dependent mechanism. This finding is important since LPS can induce the production of both TNF and endogenous PGE_2 by Mϕs, suggesting an antocrine feedback loop for TNF regulation.

The ability to regulate the production of TNF in response to LPS is defined by a narrow temporal window. The production of LPS-induced in vitro TNF production is susceptible to PGE_2- or dibutyryl cAMP-induced suppression only during a 2- to 3-h time frame post-LPS challenge. Delaying PGE_2 or dibutyryl cAMP treatment for more than 180 min after LPS exposure could no longer cause a significant reduction in TNF levels. The addition of dibutyryl cAMP to Mϕ cultures at 180 min post-LPS challenge could still reduce, to a limited degree, the production of TNF. This finding is of interest, since LPS-induced TNF mRNA reaches a peak at the 180-min time point; thus, this agent may inhibit TNF production at a posttranscriptional point.

In total, these studies demonstrate that TNF is a rapidly induced cytokine that can be regulated only during a brief period post-Mϕ stimulation. Clinically these observations are important, since septic patients are an extremely difficult population to treat and therapy in general has only been of a supportive nature. Our data may provide an insight into the failure of existing therapeutic approaches to control the septic response and suggest that targeting TNF as a regulatory approach may not be in itself warranted.

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