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CELLULAR AND MOLECULAR REGULATION OF TUMOR NECROSIS FACTOR-ALPHA PRODUCTION BY PENTOXIFYLLINE

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Tumor necrosis factor-alpha (TNF), a mononuclear phagocyte (MO)-derived peptide, is increasingly being recognized for its pleomorphic immunologic effects. A number of investigations have demonstrated that lipopolysaccharide (LPS) can induce TNF synthesis, yet mechanisms that regulate TNF expression at the cellular and molecular levels have not been fully elucidated. In this study, we present data demonstrating pentoxifylline, a methylxanthine, is efficacious in suppressing LPS-induced MO-derived TNF at the level of both TNF mRNA accumulation and TNF supernatant bioactivity. Pentoxifylline, at a dose of 1 x 10^{-5}M, suppressed the production of both biologically active TNF and TNF mRNA expression by more than 50%. Furthermore, additional methylxanthines and dibutyryl cAMP have similar effects on TNF expression. These data support the mechanism for this suppressive effect is via the generation of intracellular cAMP.

Tumor necrosis factor-alpha (TNF) is a mononuclear phagocytic cell (MO)-derived protein that is being recognized as an important mediator in a variety of physiological and immunological processes (1,2). Systemic effects of TNF were first described for its oncolytic activity on solid murine tumors (3), but subsequent investigations have demonstrated an expanded role for TNF in various diseases. For example, it is now apparent that TNF is one of the more proximal mediators responsible for the pathogenesis of endotoxin-induced shock (4,5,6). At the cellular level, TNF has significant pleomorphic effects on cells which have been shown to orchestrate inflammatory processes; including endothelial cells, fibroblasts, polymorphonuclear leukocytes, blood monocytes and macrophages, adipocytes, lymphocytes, and osteoclasts (2,7,8). The effects of TNF on different cellular populations place this monokine in a pivotal role in modulating acute and chronic inflammatory states.

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Mechanisms that regulate TNF expression at the cellular and molecular levels have not been fully clarified. Investigations have demonstrated LPS challenged murine M0 populations can concomitantly produce both monokine polypeptides (interleukin-1 and TNF), as well as prostaglandin E_2 (PGE_2), a metabolite of the cyclooxygenase pathway of arachidonate acid (9). The concomitant synthesis of PGE_2 is an important component in this system and may serve as an autocoid for transcriptional and post-transcriptional regulation of TNF (10) via the second messenger cAMP. Data regarding TNF expression by other pharmacologic agents which may mediate their effects through cAMP is lacking. In the present study we demonstrate that pentoxifylline is efficacious in suppressing TNF at the level of both TNF mRNA accumulation and TNF supernatant bioactivity. Furthermore, we demonstrate that certain methylxanthines, as well as dibutyryl cAMP, have similar suppressive effect on TNF expression. Evidence provided via Northern blot analysis and bioassay, shows that pentoxifylline and other methylxanthines can induce significant regulation of LPS-induced TNF production.

Materials and Methods

Macrophage Populations: Immunologically-activated murine (CBA/J) macrophages (M0) were recruited by injecting 0.5 ml of complete Freund's adjuvant (CFA) (diluted 1:1 with sterile saline) into the peritoneal cavity. After 14 days M0 populations were harvested and plated on either 35 mm or 100 mm culture plates (Costar, Cambridge, MA) at a concentration of 1 x 10^6 cells/ml. The cells were allowed to adhere for 2 hrs at 37°C in 5% CO_2/95% humidified air, and washed twice with complete media prior to further stimulation. Various concentrations of test compound were added concomitantly with the LPS challenge. At specific time points, supernatants were harvested and total RNA extracted as described below.

Reagents: Pentoxifylline was the generous gift of Hoechst-Roussel Pharmaceuticals, Inc. Stock pentoxifylline was prepared at 5 x 10^{-2}M in complete media. Serial dilutions of pentoxifylline were prepared in complete media. PGE_2 was the generous gift of Upjohn Co. PGE_2 stock was prepared at 10^{-2}M in ethanol. Serial dilutions of PGE_2 were prepared in complete media. Stock LPS (E. coli 0111:B4, Sigma Chemical Co., St. Louis, MO) was prepared at a concentration of 200 ug/ml in complete media. Theophylline, theobromine, isobutyl methyl xanthine and dibutyryl cAMP (Sigma Chemical Co) were prepared at 10^{-2}M stock solution.

Tumor Necrosis Assay:

The TNF bioactivity was quantitated in cell-free supernatants and standardized to the number of adherent cells. Bioactivity of TNF was monitored using a semi-automated LM fibroblast lytic assay (11). LM cells (5 x 10^4/100 µl) were cultured in 96 well flat bottom microtiter plates for 18 hrs at 37°C, 5% CO_2/95% humidified air in the presence of 5 ug/ml actinomycin D with serial 1:2 dilutions of test samples. After incubation, plates were washed in phosphate buffered saline and the remaining cells were stained with crystal violet (0.5% in methanol/water [1:4 v/v]). The quantity of cell lysis was determined using a microELISA autoreader. Units of TNF activity were defined using an internal standard of human recombinant TNF with a specific activity of 10^7 units/mg protein (Cetus Corporation, Emeryville,CA). To establish specificity of cell lysis for TNF, several test samples were incubated concomitantly with specific, rabbit anti-murine TNF neutralizing antibody prior to the addition of samples to the targets.

Northern Blot Analysis:

Total RNA from murine M0 were isolated at various time points using a modification of the method of Chirgwin *et al* (12) and Jonas *et al* (13). Briefly, M0 monolayers were scraped into a solution of 25 mM Tris, pH 8.0 containing 4.2M 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% SDS was added and the RNA was extracted with chloroform-phenol and chloroform-isoamyl alcohol. The RNA was alcohol precipitated, separated by formaldehyde/1% agarose gels, and transblotted to nitrocellulose. The baked Northern blots were prehybridized and the hybridized with ^{32}P-5'end labeled oligonucleotide probes for TNF (14) and beta-actin (15), respectively (gifts of Dr. Glen Andrews and Henry Showell, Pfizer Pharmaceutical Co, Groton, CT). The nucleotide sequence for TNF was the 25-mer 5'-GGT-CAC-CCT-TCT-CCA-GCT-GGA-AGA-C-3' and the βeta-actin probe was 42-mer 5'-GGC-TGG-GGT-GTT-GAA-GGT-CTC-AAA-CAT-GAT-CTG-GGT-CAT-CTT-3'. Stringency washed blots were placed at -70°C with intensifying screens and the developed autoradiographs quantitated using laser densitometry (Ultrascan XL, LXB Instruments, Inc., Houston, TX). Equivalent amounts of total RNA/well were assessed by monitoring 28S and 18S rRNA and accumulation of actin mRNA.

Results

Pentoxifylline Suppression of LPS-induced M0-Derived TNF Bioactivity: Cell-free supernatants from LPS (10 ug/ml) treated MO in the presence or absence of pentoxifylline were collected and analyzed for TNF bioactivity. As shown in Figure 1, pentoxifylline suppressed LPS-induced TNF production in a dose-dependent manner. Specificity for TNF was demonstrated by neutralization of TNF-induced cytotoxicity of LM cells with anti-murine TNF antibody. Pentoxifylline in concentrations equivalent to those found in serial dilutions did not interfere with the cytotoxicity of preformed TNF in the bioassay. Other methylxanthines, such as theobromine, isobutyl methylxanthine, and theophylline, were also shown to suppress the LPS-induced TNF production. As shown in Table 1, the ability of the

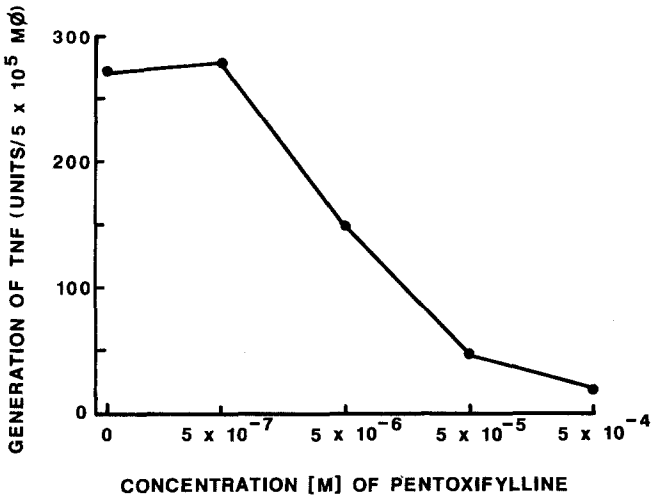

Figure 1 Pentoxifylline suppression of LPS-induced MO-derived TNF bioactivity in a dose-dependent manner. Pentoxifylline significantly reduced TNF production by greater than 50% at 1 x 10-^{5}M.

Table 1. Effects of various compounds associated with an increase in intracellular cAMP on LPS-induced TNF-alpha production. M0s were incubated with the above test compounds concomitant with LPS. Cell-free supernatants were then collected and assayed for TNF activity. The data presented represents the concentration of compound that would induce an approximate 50% reduction in the LPS response.

Sample	% Suppression
LPS (1ug/ml)	-
LPS + pentoxifylline (2.5×10^{-5}M)	57%
LPS + isobutyl methylxanthines (5×10^{-5}M)	54%
LPS + theophylline (1×10^{-4} M)	52%
LPS + theobromine (1×10^{-4} M)	47%
LPS + prostaglandin E_2 (1×10^{-7} M)	60%
LPS + dibutyryl cAMP (5×10^{-5})	61%

above methyl xanthines to block LPS-dependent TNF production is compared to pentoxifylline, PGE_2, and dibutyryl cAMP.

Pentoxifylline Suppression of LPS-Induced M0-derived TNF mRNA Accumulation: To establish the effects of pentoxifylline on TNF mRNA expression, Northern blot analyses were performed. Adherent M0 were incubated in presence of LPS (10 ug/ml) alone or LPS in the presence of graded doses of pentoxifylline. Total RNA was extracted after 3 hr incubation. As shown in Figure 2, Northern blot analysis demonstrated a reduction in TNF mRNA in response to various concentrations of pentoxifylline. The level of TNF mRNA, as assessed by laser densitometry, was reduced by 60% and 45% in response to 5×10^{-5}M and 5×10^{-6}M, pentoxifylline, respectively. Since methylxanthines and PGE_2 can promote the accumulation of intracellular cAMP, it was of interest to establish the role of dibutyryl cAMP on LPS-induced TNF mRNA expression. Thus, Northern blot analyses of TNF mRNA from M0-treated with LPS alone or in the presence of dibutyryl cAMP were performed. As shown in Figure 3, dibutyryl-cAMP significantly suppressed the ability of LPS to induce TNF mRNA expression. The accumulation of TNF mRNA in response to either 1 ng/ml or 100 ng/ml LPS were susceptible to dibutyryl-cAMP modulation.

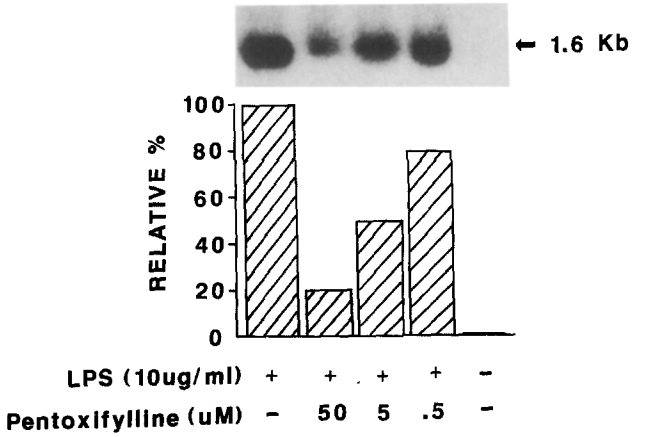

Figure 2 Northern blot analysis of pentoxifylline suppression of LPS-induced TNF mRNA, as assessed by laser densitometry. Expression of TNF mRNA was reduced by 60% and 45% in response to 5 x 10^{-5}M and 5 x 10^{-6}M pentoxifylline, respectively. MOs were treated with LPS 3 hrs prior to mRNA isolation.

Discussion

TNF has become increasingly recognized as an important mediator of diverse physiological and immunological processes. TNF *in vivo* exhibits characteristics which support this cytokine as a proximal mediator for the pathogenesis of septic shock (4,5,6). At the cellular level, TNF is an important cytokine orchestrating a variety of local immune responses, resulting in pleomorphic effects on cellular constituents of inflammation. In this study, we demonstrate that pentoxifylline, a methylxanthine, dose-dependently suppressed of LPS-induced TNF production at the level of mRNA expression. Other methylxanthines,

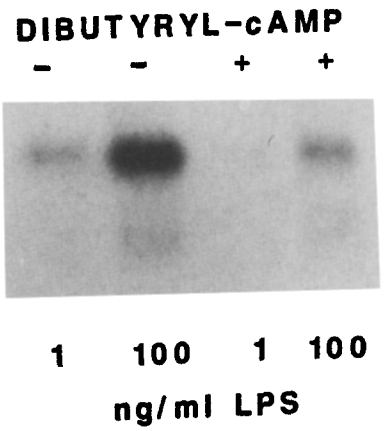

Figure 3 Northern blot analysis of dibutyryl cAMP suppression of LPS-induced TNF mRNA. Dibutyryl cAMP, 10^{-5}M, resulted in significant suppression of both 1 and 100 ng/ml LPS. MOs were treated with LPS 3 hrs prior to mRNA isolation.

such as theobromine, isobutyl methylxanthine, and theophylline, as well as PGE_2 and dibutyryl cAMP, were found to be efficacious in the suppression of TNF. Pentoxifylline was initially demonstrated to clinically abrogate of claudication in patients with peripheral vascular occlusive disease (16). This pharmacologic phenomenon is attributed to enhanced vascular wall production of prostacyclin (PGI_2) (17) and intracellular cAMP in platelets, polymorphonuclear leukocytes and monocytes (17,18). Since cAMP can function in intracellular signal translocation and regulate monokine production (19), this mechanism may be involved in pentoxifylline-induced inhibition of TNF production.

The pharmacologic effects of pentoxifylline may have important implications in clinical disease states where the local and systemic modulation of TNF may be essential for the control of specific inflammatory processes. Investigations regarding the initiation of septic shock have provided data which establishes TNF as the proximal mediator in the pathogenesis of this disorder (4,5,6). It is interesting to speculate on the potential pharmacologic intervention with pentoxifylline, as a means to treat septic shock.

In conclusion, pentoxifylline can dose dependently suppress TNF production at both the mRNA and bioactivity levels. Other methylxanthines, including theobromine, isobutyl methylxanthine, and theophylline, as well as PGE_2 and dibutyryl cAMP were demonstrated to have similar effects in the suppression of TNF. These findings are important with regard to understanding mechanisms that regulate the production of MO-derived TNF.

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