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Tumor necrosis factor alpha (TNF-α) is a critical cytokine that mediates the toxic effects of bacterial superantigens like staphylococcal enterotoxin B (SEB) and toxic shock syndrome toxin 1 (TSST-1). Pentoxifylline, an anti-inflammatory agent that inhibits endotoxemia and lipopolysaccharide (LPS)-induced release of TNF-α, was tested for its ability to inhibit SEB- and TSST-1-induced activation of human peripheral blood mononuclear cells (PBMCs) in vitro and toxin-mediated shock in mice. Stimulation of PBMCs by SEB or TSST-1 was effectively blocked by pentoxifylline (10 mM), as evidenced by the inhibition of TNF-α, interleukin 1β (IL-1β), gamma interferon (IFN-γ), and T-cell proliferation. The levels of TNF-α, IL-1α, and IFN-γ in serum after an SEB or TSST-1 injection were significantly lower in mice given pentoxifylline (5.5 mg/animal) versus control mice. Additionally, pentoxifylline diminished the lethal effects and temperature fluctuations elicited by SEB and TSST-1. Thus, in addition to treating endotoxemias, the cumulative in vitro and in vivo data suggest that pentoxifylline may also be useful in abrogating the ill effects of staphylococcal enterotoxins and TSST-1.

Staphylococcal exotoxins are among the most common etiological agents that cause toxic shock syndrome (2, 34). The disease is characterized by fever, hypotension, desquamation of skin, and dysfunction of multiple organ systems (6, 34). Staphylococcal toxic shock syndrome toxin 1 (TSST-1) and the distantly related enterotoxins are superantigens that potently stimulate T-cell proliferation and cytokine production (16). These toxins bind directly to the major histocompatibility complex (MHC) class II molecules on antigen-presenting cells and subsequently stimulate T cells that express specific Vβ elements on T-cell receptors (7, 16, 34, 35). In vitro and in vivo studies show that these superantigens induce high levels of various proinflammatory mediators, including tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), and interleukin 1 (IL-1) (14, 16, 17, 23, 27, 28, 36). These cytokines upregulate the expression of MHC class II as well as adhesion molecules and possess potent immunoenhancing properties (18). Further evidence for the pivotal role of TNF-α in superantigen-induced shock was revealed by experiments with transgenic knockout mice (3, 37) and neutralizing antibodies against TNF-α (22, 23).

Pentoxifylline is a methylxanthine derivative that inhibits the production of TNF-α by endotoxin-stimulated monocytes/macrophages at the transcriptional level (11, 25) and is effective in reducing serum TNF-α levels in mice with endotoxic shock (32). Recently, pentoxifylline was reported to inhibit adhesion and activation of human T cells (10). The drug has been well characterized and subsequently used in clinical settings for years with few deleterious side effects. This study was undertaken to determine the effect of pentoxifylline on staphylococcal enterotoxin B (SEB)- and TSST-1-induced cytokine production from human peripheral blood mononuclear cells (PBMCs). The therapeutic efficacy of pentoxifylline in superantigen-induced toxic shock was further examined via an in vivo murine model (36).

MATERIALS AND METHODS

Reagents. Purified SEB and TSST-1 were obtained from Toxin Technology (Sarasota, Fla.). The endotoxin content of these preparations was <1 ng of endotoxin/mg of protein, as determined by the Limulus amoebocyte lysate assay (BioWhittaker, Walkerville, Md.). Escherichia coli lipopolysaccharide (LPS; O55:B5) was purchased from Difco Laboratories (Detroit, Mich.). Human (h) recombinant (r) TNF-α, peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) and peroxidase-conjugated anti-goat IgG were obtained from Boehringer-Mannheim (Indianapolis, Ind.). Antibodies against hTNF-α were purchased from R&D Systems (Minneapolis, Minn.). hrIL-1β was kindly provided by J. Oppenheim (National Cancer Institute, Frederick, Md.). hrIFN-γ and antibodies against hIL-1β were obtained from Collaborative Research (Boston, Mass.). Anti-hIFN-γ IgG, with and without biotin, were obtained from Pharmingen (San Diego, Calif.). Mouse (m) rTNFα, anti-mTNF-α IgG, mIL-1α, and anti-mIL-1α IgG were obtained from Biosource (Camarillo, Calif.). mIFN-γ and anti-mIL-1α IgG were obtained from Genzyme (Boston, Mass.). Pentoxifylline and all other reagents were from Sigma (St. Louis, Mo.).

Cell cultures. Human PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation of heparinized blood from healthy human donors. The PBMCs (105 cells/ml) were cultured at 37°C in 24-well plates containing RPMI 1640 medium and 10% heat-inactivated fetal bovine serum. The cells were incubated with SEB or TSST-1 (100 ng/ml) for 16 h, and the supernatants were harvested and analyzed for TNF-α, IL-1β, and IFN-γ by an enzyme-linked immunosorbent assay (ELISA) as described below. Pentoxifylline, when present, was added simultaneously with the exotoxins.

Human T-cell proliferation assays. PBMCs (104 cells/well) were plated in triplicate with SEB or TSST-1 (100 ng/ml), with or without various concentrations of pentoxifylline, for 48 h at 37°C in 96-well microtiter plates. The cells were pulsed with 1 μCi of [3H]thymidine (New England Nuclear, Boston, Mass.) per well during the last 5 h of culture as described previously (17). The cells were harvested onto glass fiber filters, and the incorporated [3H]thymidine was measured by liquid scintillation.

Murine model of superantigen-induced toxic shock. Male BALB/c mice (18 to 22 g; Harlan Sprague-Dawley, Frederick, Md.) were kept in a pathogen-free environment. A sterile temperature-identification transponder (IPTT-100; Bio-Medic Data Systems, Maywood, N.J.) was implanted subcutaneously into each animal and the temperature was monitored hourly (37) after an initial intraperitoneal (i.p.) injection of SEB or TSST-1 (1 μg/mouse), followed 4 h later by an LPS injection (80 μg/mouse) as described previously (36, 44). Pentoxifylline (5.5 mg/animal) was given i.p. at the designated time points. Temperature data were calculated as the mean temperature reading ± standard deviation for each group (n = 5 mice per group). The total number of mice dead versus alive was recorded at 72 h.

Sera were collected and pooled from each group (n = 5 mice per group and
Differences were considered significant if \( P < 0.05 \). Pentoxifylline was injected at 4 h. The levels of cytokines in sera were detected by ELISA as described previously (36).

## Statistical analysis.

The cytokine data were expressed as the mean reading ± standard deviations for duplicate samples from three experiments. Differences were considered significant if \( P < 0.05 \).

### RESULTS

**Pentoxifylline inhibits SEB- and TSST-1-induced cytokines from hPBMCs.**

Previous in vitro studies indicate that pentoxifylline prevents endotoxin-induced TNF-\( \alpha \) production by monocytes/macrophages (11). Since proinflammatory cytokines like TNF-\( \alpha \) play an important role in superantigen-induced shock, the effect of pentoxifylline was examined with hPBMCs incubated with SEB or TSST-1. Figure 1 shows that pentoxifylline effectively blocked in a dose-dependent manner the production of TNF-\( \alpha \), IL-1\( \beta \), and IFN-\( \gamma \) from PBMCs incubated with either toxin, achieving total inhibition of all three mediators at 10 mM compared to the level of inhibition for controls incubated with toxin alone (\( P < 0.05 \)). With 1 mM pentoxifylline, TNF-\( \alpha \) was inhibited by 100% in SEB-stimulated PBMCs and 78% in TSST-1-stimulated cells. The levels of inhibition of IL-1\( \beta \) and IFN-\( \gamma \) were 57 and 65%, respectively, for SEB-stimulated PBMCs and 99 and 55%, respectively, for TSST-1-stimulated cells. Lower concentrations of pentoxifylline were not effective in blocking the induction of these three cytokines in SEB-stimulated cells. However, TNF-\( \alpha \) and IL-1\( \beta \) were reduced by 55% in TSST-1-stimulated PBMCs with 0.1 mM pentoxifylline, whereas no inhibition of IFN-\( \gamma \) was observed with this concentration.

**Human T-cell proliferation due to SEB or TSST-1 is inhibited by pentoxifylline.**

In addition to increasing cytokine levels, superantigens are also potent activators of T-cell proliferation. Therefore, the effect of pentoxifylline on SEB- and TSST-1-induced proliferation of T cells was examined next. The results show that pentoxifylline significantly decreased superantigen-induced proliferation of T cells in a dose-dependent manner, with maximal inhibition (92 to 94%) achieved at the same concentration (10 mM) that was most effective at blocking cytokine production (Fig. 2). The lack of proliferation or cytokine release from PBMCs was not due to lethal effects of superantigen and drug, as determined by trypan blue exclusion.

**Pentoxifylline attenuates serum cytokine levels in vivo.**

On the basis of the strong inhibitory effects of pentoxifylline on superantigen-mediated cytokine production and T-cell proliferation in vitro, the potential therapeutic role of pentoxifylline in vivo was further investigated in mice. Previous studies (38, 44, 45) optimized the doses of SEB, TSST-1, and LPS required for a murine model of lethal toxic shock and accompanied temperature fluctuations recorded within 12 h (37). Elevated serum levels of TNF-\( \alpha \), IL-1\( \alpha \), and IFN-\( \gamma \) are a prominent feature of toxic shock mediated by superantigens (22), and these results are clearly evident in a murine model (36, 38, 44, 45). Therefore, the in vivo effect of pentoxifylline on serum cytokine concentrations was examined in mice injected with SEB or TSST-1. Peak levels of IL-1\( \alpha \), TNF-\( \alpha \), and IFN-\( \gamma \) were reduced by 40, 94, and 99%, respectively, in mice.
among mice injected with 1 μg of SEB or TSST-1 plus 80 μg of LPS (37). However, relative to the temperature increases among controls treated with SEB or TSST-1 plus LPS, there were less dramatic decreases in temperature among pentoxifylline-treated animals (data not shown). The results of protection against temperature fluctuations essentially paralleled the lethality results.

**DISCUSSION**

Pentoxifylline has been used for many years to treat peripheral vascular disease because it affects erythrocyte shape, platelets, and plasma viscosity (8, 42). Further investigations showed that the drug also potently affects endotoxin-induced release of TNF-α, a proinflammatory cytokine that plays an important role in superantigen-mediated shock (22). The present study demonstrated that pentoxifylline effectively inhibited superantigen-mediated production of TNF-α, IL-1β, and IFN-γ by human PBMCs in vitro. These results also mimic the human in vivo responses of the drug against LPS stimulation (25). Inhibition of these cytokines by pentoxifylline evidently occurs at the transcriptional level and can last for up to 5 days after the final pentoxifylline dose (11, 25). This extended protection may be due to an upregulation of cytokine receptors that are shed from the cell surface when the drug is no longer present. Besides decreasing the levels of proinflammatory cytokines in vitro, superantigen-induced proliferation of T cells was also completely blocked by pentoxifylline in our study. This effect is likely a result of inactivation of β1 integrins on T lymphocytes, as suggested by a recent study (10). Pentoxifylline also inhibits the expression of activation markers CD25, CD69, and CD98 on T lymphocytes stimulated with mitogens (10). Neutrophil functions such as adherence, degranulation, and superoxide production induced by the inflammatory cytokines TNF-α and IL-1 are also blocked by pentoxifylline (41). Thus, pentoxifylline has a broad spectrum of effects and interferes with the activation of multiple cell types of the immune system.

The percentage of mice that were alive 72 h after the toxin injection. Group size (n = 10) is based on cumulative data from two experiments. Pentoxifylline (5.5 mg/animal) was given at the designated time points after the toxin injection. *Toxin (1 μg/mouse) was injected i.p. followed 4 h later by the injection of LPS (80 μg/mouse). There were no lethal effects among mice given PBS plus LPS or those given SEB or TSST-1 plus PBS. **The protective effects of pentoxifylline that are statistically significantly different (P < 0.05) from the effects of no treatment for control animals not given pentoxifylline (No Drug).

### Table 1. Protective effects of pentoxifylline in vivo

| Toxinb | No drug   | 1 h | 2 h | 3 h | 4 h |
|--------|-----------|-----|-----|-----|-----|
| TSST-1 | 10        | 70% | 20% | 100%| 100%|
| SEB    | 20        | 70% | 50% | 80% | 50% |

FIG. 3. Peak levels of TNF-α (A), IL-1α (B), and IFN-γ (C) in mice treated with (i) SEB plus PBS, (ii) PBS plus LPS, (iii) PBS plus LPS and pentoxifylline (PENTOX), (iv) SEB plus LPS, (v) SEB plus LPS and pentoxifylline, (vi) TSST-1 plus LPS, or (vii) TSST-1 plus LPS and pentoxifylline. Values represent the means ± standard deviations for duplicate samples.

Treated with TSST-1, LPS, and pentoxifylline compared with the levels in mice treated with TSST-1 plus LPS but not pentoxifylline (Fig. 3). The concentrations of the same cytokines in serum were inhibited 86% (IL-1α) and 99% (TNF-α and IFN-γ) in mice given SEB, LPS, and pentoxifylline compared to the concentrations in the sera of mice treated only with SEB plus LPS (Fig. 3). Pentoxifylline also reduced the serum TNF-α levels by 99% among controls treated with phosphate-buffered saline (PBS) and LPS but had no effect on reducing IL-1α concentrations.

**Pentoxifylline diminishes superantigen-mediated shock in mice.** Table 1 shows that pentoxifylline significantly increased the survival rate among LPS-potentiated mice previously injected with SEB or TSST-1. However, mice were not protected if pentoxifylline was given 4.25 h after the SEB or TSST-1 injection (data not shown). Additional data on temperature fluctuations in LPS-potentiated mice injected with TSST-1 or SEB, with and without pentoxifylline, were collected. Normally, there is a 6 to 9°C decrease in temperature within 12 h

Previous studies with mice showed that pentoxifylline can be given 24 h before or up to 4 h after an LPS injection and still confer significant protection in a dose-dependent manner (32).
The same report reveals that increasing concentrations of pentoxifylline also prevent the production of TNF from murine macrophages incubated in vitro with LPS. We witnessed a similar dose effect of pentoxifylline incubated with PBMCs and SEB or TSST-1. Our in vivo studies also showed that pentoxifylline given up to 4 h after an SEB or TSST-1 injection still afforded considerable protection against mortality and temperature fluctuations. However, when the drug was given 4.25 h after the toxin, which was only 15 min after administration of the LPS dose, there was no protection in our toxic shock model. These results revealed a finite time frame in which the drug is efficacious. The lack of protection with pentoxifylline given after LPS administration suggests that the drug is extremely effective in abrogating the priming effect of SEB or TSST-1 on cytokine induction in this murine toxic shock model. The serum cytokine and mortality data reveal the protective effects of pentoxifylline following an injection of SEB or TSST-1. Besides mice, pentoxifylline effectively prevents LPS-induced mortality and diminishes serum TNF levels in rats (26). Similar results were reported from studies with guinea pigs (9), dogs (43), and chimpanzees (20), thus suggesting that this drug is efficacious in various species.

Our in vivo studies showed that the beneficial effects of pentoxifylline in superantigen-induced shock included increased survival, minimal temperature change, and a dramatic decrease in serum TNF-a and IL-6 levels (31), which correlates well with the results of a previous study with rats injected intravenously with endotoxin (26). Pentoxifylline may also abrogate the potentially lethal sequelae associated with bacterial meningitis via inhibition of TNF-α and IL-1β release from microglial cells (5). In conclusion, the promising findings of this study indicate that pentoxifylline has the potential to mitigate SE- and TSST-1-mediated shock in humans. Pentoxifylline protects mice against staphylococcal infections (41) and may be particularly useful among patients suffering from severe infections due to the ever increasing numbers of antibiotic-resistant bacteria (12) that produce one or more toxic superantigens.

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necrosis factor-α by human peripheral blood mononuclear cells. Immunology 83:262–267.
26. Noel, P., S. Nelson, R. Bokulic, C. Bagby, H. Lippton, G. Lipcomb, and W. Summer. 1990. Pentoxifylline inhibits lipopolysaccharide-induced serum tumor necrosis factor and mortality. Life Sci. 47:1023–1029.
27. Parsonnet, J., and Z. A. Gillis. 1988. Production of tumor necrosis factor by human monocytes in response to toxic shock syndrome toxin-1. J. Infect. Dis. 158:1026–1033.
28. Parsonnet, J., R. K. Hickman, D. D. Eardley, and G. B. Pier. 1985. Induction of human interleukin-1 by toxic shock syndrome toxin-1. J. Infect. Dis. 151:514–522.
29. Pettit, G. W., M. R. Elwell, and P. B. Jahrling. 1977. Possible endotoxia in rabbits after intravenous injection of Staphylococcus aureus enterotoxin B. J. Infect. Dis. 135:646–648.
30. Priest, B. P., P. M. Schlievert, and D. L. Dunn. 1989. Treatment of toxic shock syndrome with endotoxin-neutralizing antibody. J. Surg. Res. 46:527–531.
31. Refsum, S. E., M. I. Halliday, G. Campbell, M. McCaigue, B. J. Rowlands, and V. E. Boston. 1996. Modulation of TNFs and IL-6 in a peritonitis model using pentoxifylline. J. Pediatr. Surg. 31:926–930.
32. Schade, U. F. 1990. Pentoxifylline increases survival in murine endotoxin shock and decreases formation of tumor necrosis factor. Circ. Shock 31:171–181.
33. Schlievert, P. M., and D. W. Watson. 1978. Group A streptococcal pyrogenic exotoxin: pyrogenicity alteration of blood-brain barrier and separation of sites for pyrogenicity and enhancement of lethal endotoxin shock. Infect. Immun. 21:753–763.
34. Schlievert, P. M. 1993. Role of superantigens in human disease. J. Infect. Dis. 167:997–1002.
35. Scholl, P., A. Diez, W. Mourad, J. Parsonnet, R. S. Geha, and T. Chatila. 1989. Toxic shock syndrome toxin-1 binds to major histocompatibility complex class II molecules. Proc. Natl. Acad. Sci. USA 86:4210–4214.
36. Stiles, B. G., S. Bavari, T. Krakauer, and R. G. Ulrich. 1993. Toxicity of staphylococcal enterotoxins potentiated by lipopolysaccharide: major histocompatibility complex class II molecule dependency and cytokine release. Infect. Immun. 61:5333–5338.
37. Stiles, B. G., Y. G. Campbell, R. M. Castle, and S. A. Grove. 1999. Correlation of temperature and toxicity in murine studies of staphylococcal enterotoxins and toxic shock syndrome toxin 1. Infect. Immun. 67:1521–1525.
38. Stiles, B. G., T. Krakauer, and P. F. Bonventre. 1995. Biological activity of toxic shock syndrome toxin 1 and a site-directed mutant, H185A, in a lipopolysaccharide-potentiated mouse lethality model. Infect. Immun. 63:1229–1234.
39. Stone, R. L., and P. M. Schlievert. 1987. Evidence for the involvement of endotoxin in toxic shock syndrome. J. Infect. Dis. 155:682–689.
40. Sugiyama, H., E. M. McKissic, M. S. Bergdoll, and B. Heller. 1964. Enhancement of bacterial endotoxin lethality by staphylococcal enterotoxin. J. Infect. Dis. 114:111–118.
41. Sullivan, G. W., T. N. Patselas, J. A. Redick, and G. L. Mandell. 1984. Enhancement of chemotaxis and protection of mice from infection. Trans. Assoc. Am. Physicians 97:337–345.
42. Ward, A., and S. P. Clissold. 1987. Pentoxifylline: a review of its pharmacodynamic and pharmacokinetic properties, and its therapeutic efficacy. Drugs 34:50–97.
43. Welsh, C. H., D. Lien, G. S. Worthen, and J. V. Weil. 1988. Pentoxifylline decreases endotoxin-induced pulmonary neutrophil sequestration and extravascular protein accumulation in the dog. Am. Rev. Respir. Dis. 138:1106–1114.
44. Woody, M. A., T. Krakauer, and B. G. Stiles. 1997. Staphylococcal enterotoxin B mutants (N23K and F44S): biological effects and vaccine potential in a mouse model. Vaccine 15:133–139.
45. Woody, M. A., T. Krakauer, R. G. Ulrich, and B. G. Stiles. 1998. Differential immune responses to staphylococcal enterotoxin B mutations in a hydrophobic loop dominating the interface with major histocompatibility complex class II receptors. J. Infect. Dis. 177:1013–1022.