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Inhibition of the Inflammatory Action of Interleukin-1 and Tumor Necrosis Factor (Alpha) on Neutrophil Function by Pentoxifylline

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Inflammatory cytokines, including interleukin-1 and tumor necrosis factor, are produced by monocytes and macrophages in response to microorganisms and microbial products such as endotoxins. The cytokines stimulate neutrophil adherence, degranulation, and superoxide production but inhibit neutrophil migration. We studied the modulation of cytokine-induced neutrophil activation by pentoxifylline and its principle metabolites. Lipopolysaccharide-stimulated mononuclear-leukocyte-conditioned medium containing inflammatory cytokines, purified human interleukin-1, or recombinant human tumor necrosis factor increased neutrophil adherence to nylon fiber, primed neutrophils for increased superoxide production in response to *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP), increased neutrophil lysozyme release stimulated by FMLP, and decreased directed migration of neutrophils to FMLP. Pentoxifylline and its principle metabolites at or near therapeutically achievable levels were able to counteract these effects. Pentoxifylline inhibited the increase in free intracellular calcium in polymorphonuclear leukocytes stimulated by FMLP and increased binding of FMLP to neutrophils at 37°C but not at 4°C. By blocking the inflammatory action of interleukin-1 and tumor necrosis factor on neutrophils, pentoxifylline may diminish the tissue damage caused by neutrophils in such conditions as septic shock, adult respiratory distress syndrome, cardiopulmonary bypass lung damage, and myocardial reperfusion injury.

Inflammatory cytokines, including interleukin-1 (IL-1) and tumor necrosis factor (alpha) (TNF), are produced by mononuclear leukocytes in response to numerous agents, including microorganisms, microbial products (such as endotoxins), lipopolysaccharide (LPS), C5a, and lymphokines (for a review, see references 6 and 10). They induce systemic responses including fever (3) and release of acute-phase proteins from the liver (32). They stimulate and enhance neutrophil degranulation and superoxide production and can decrease chemotaxis (2, 13, 20, 21, 34, 35, 38; G. W. Sullivan, H. T. Carper, J. A. Sullivan, and G. L. Mandell, Clin. Res. 35:657A, 1987). Intense, generalized, or chronic production of inflammatory cytokines can lead to tissue damage (28).

IL-1 activity has been detected in the tissues of patients with Crohn's disease (40) and in the synovial fluid of patients with rheumatoid and nonrheumatoid inflammatory joint disease (47). Elevated levels (50 to 1,000 pg/ml [~200 to 4,400 U/ml]) of TNF in the serum of patients with renal allograft rejection (25), parasitic diseases (33), or meningococcal disease (44) have been reported. Endotoxin stimulates the production of both TNF and IL-1. These cytokines, acting in part on neutrophils, may cause much of the tissue damage observed with infection (28).

Inhibition of the effects of these cytokines on polymorphonuclear leukocytes (PMNs) may be beneficial for a variety of inflammatory diseases, including septic shock and adult respiratory distress syndrome.

We previously reported that pentoxifylline [1-(5-oxohexyl)-3,7-dimethylxanthine] enhanced neutrophil chemotaxis (41). Further study indicated that this effect was due to inhibition of the action of the inflammatory cytokines on neutrophil function. We propose that tissue damage in vivo may be diminished by the ability of pentoxifylline and its metabolites to decrease the activity of these cytokines on neutrophils.

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MATERIALS AND METHODS

Materials. Pentoxifylline, metabolite I [1-(5-hydroxyhexyl)-3,7-dimethylxanthine], metabolite IV [1-(4-carboxybutyl)-3,7-dimethylxanthine], and metabolite V [1-(3-carboxypropyl)-3,7-dimethylxanthine] were provided by Hoechst-Roussel Pharmaceuticals, Inc., Somerville, N.J.

(i) IL-1. Purified human monocyte IL-1 (ph-IL-1) and diluent were purchased from Cistron Biotechnology, Pine Brook, N.J. More then 90% had isoelectric points between 6.8 and 7.0 (data supplied by Cistron). The diluent was phosphate-buffered saline with 0.01% bovine serum albumin. Diluent and ph-IL-1 contained <50 pg of LPS per μg as determined by *Limulus* amebocyte lysate assay (performed by Cistron). One lymphocyte-activating factor (LAF) unit of IL-1 activity is defined as the amount of IL-1 which causes one-half of the maximal incorporation of [^{3}H]thymidine by murine (C3H) thymocytes with concanavalin A (0.5 μg/ml) (data from Cistron).

(ii) rh-TNF. Recombinant human TNF (alpha) (rh-TNF) was purchased from Genzyme Corp., Boston, Mass. It was produced in *Escherichia coli* and was purified by phenyl Sepharose chromatography and fast-protein liquid chromatography to a final purity of greater than 99% as determined by analysis on sodium dodecyl sulfate-polyacrylamide gels stained with both Coomassie brilliant blue R250 and a silver stain. It had a molecular mass of 36,000 daltons as determined by gel filtration on Superose 12 and consisted of two dimers of 17,000 daltons each. It was supplied in sterile phosphate-buffered saline containing 0.1% bovine serum albumin as a carrier protein (data supplied by Genzyme). Just before use, the rh-TNF was diluted in Hanks balanced salt solution (HBSS) containing 0.1% human serum albumin.

(iii) Other chemicals. Dimethyl sulfoxide, *N*-formyl-L-

methionyl-L-leucyl-L-phenylalanine (FMLP; 10 mM stock solution in dimethyl sulfoxide; stored in 20-μl portions at −70°C), heparin, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), Percoll, allopurinol, cytochrome *c* type VI from horse heart, and superoxide dismutase from bovine liver (SOD; stock solutions [5 mg/ml in HBSS] stored in 100-μl portions at −70°C) were purchased from Sigma Chemical Co., St. Louis, Mo. Neutrophil isolation medium (NIM) was purchased from Los Alamos Diagnostics, Inc., Los Alamos, N. Mex. Phorbol myristate acetate (PMA) was purchased from Consolidated Midland, Brewster, N.Y. HBSS, minimum essential medium, and medium 199 were purchased from Whittaker, M. A. Bioproducts, Walkersville, Md. Dulbecco phosphate-buffered saline was purchased from GIBCO Laboratories, Grand Island, N.Y. The *Limulus* amebocyte lysate test was purchased from Associates of Cape Cod, Inc., Woods Hole, Mass. Scrubbed nylon fiber (3 denier, type 200) was purchased from Fenwal Laboratories, Deerfield, Ill. Litex agarose type HSA was purchased from Accurate Chemical and Scientific Corp., Hicksville, N.Y., and LPS (from *E. coli* K235) was purchased from List Biological Laboratories, Inc., Campbell, Calif. The calcium probe Fura 2/AM {1-[2-5-carboxyoxazol-2-yl-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-pentaacetoxymethyl ester} was purchased from Calbiochem-Behring, La Jolla, Calif. Tritiated FMLP and Bray solution were purchased from Dupont, NEN Research Products, Boston, Mass.).

PMN Preparation. Purified PMNs (~98% PMNs; >95% viable as determined by trypan blue exclusion) containing less than one platelet per five PMNs and <50 pg of LPS per ml (as determined by *Limulus* amebocyte lysate assay) were obtained from normal, heparinized (10 U/ml) venous blood by a one-step Ficoll-Hypaque separation procedure in NIM unless otherwise stated (12). The PMNs were washed three times with HBSS or minimal essential medium. Residual erythrocytes were lysed (by hypotonic lysis with 3 ml of an iced, 0.22% sodium chloride solution for 45 s followed by 0.88 ml of a 3% sodium chloride solution for the PMN oxidative burst assays.

LPS-MCM. LPS-stimulated mononuclear leukocyte-conditioned medium (LPS-MCM) was prepared by incubating washed, mixed mononuclear leukocytes (3×10^6/ml; ~15 to 20% monocytes and ~80 to 85% lymphocytes) obtained from neutrophil isolation medium separation in medium 199 containing 10% fresh autologous serum for 18 h at 37°C in 10% CO_2 with or without LPS (5 ng/ml) in Lab-Tek Flaskettes (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.). The suspension was centrifuged at 150 × *g* for 10 min, and then the supernatant was filtered (0.45-μm pore size) and frozen at −70°C. Undiluted control medium containing 10% autologous serum and LPS (5 ng/ml) in medium 199 contained ~1 U of IL-1 per ml and ~20 U of TNF per ml. Undiluted LPS-MCM contained ~1,400 U of IL-1 per ml according to the D10 cell assay (performed by John Castracane of Endogen Inc., Boston, Mass.) and ~350 U of TNF per ml according to an in vitro cytotoxicity assay (John Castracane, Endogen) (14).

PMN adherence. PMN adherence was determined by a modification of the method of MacGregor et al. (24). Purified PMNs (5×10^6 in a total volume of 0.1 ml) were incubated for 30 min at 37°C with or without pentoxifylline, LPS, LPS-MCM, rh-TNF, ph-Il-1, or diluent. After incubation, 0.9 ml of HBSS containing 10 μl of autologous serum was added. The cell suspensions were applied to prewarmed (37°C), 60-mg nylon columns packed to the 0.3-ml mark on a plastic 1-ml syringe. The columns were allowed to elute for 30 min at 37°C, and the number of PMNs in both the pre- and postelution samples was determined by doing microscopic counts. The results were expressed as percent PMN adherence to the nylon.

Chemotaxis. Chemotaxis under agarose was quantitated by the method of Nelson et al. (30). Purified PMNs (5×10^6) were incubated for 15 min in minimal essential medium or HBSS (60 to 90 μl) with or without pentoxifylline, its metabolites, or other methyl xanthines. The total volume was then brought up to 0.1 ml for 30 min at 37°C in medium containing LPS, rh-TNF, LPS-MCM, ph-IL-1, or diluent. The migration towards FMLP (100 nM) was measured after incubation for 2 h at 37°C.

PMN oxidative burst. Cytochrome *c* reduction was determined by the following method. Purified PMNs (4×10^6) were suspended and incubated as described above for chemotaxis except with or without SOD (200 U per sample). HBSS (0.4 ml) and cytochrome *c* (50 μl; final concentration, 120 μM) were added to all samples, and FMLP or PMA was added as specified. The samples were incubated for 10 min more at 37°C and then iced and centrifuged at 2,000 × *g* for 10 min). The optical density of the supernatants was read at 550 nm, and the nanomoles of SOD-inhibitable superoxide per 10^6 PMN was calculated with the extinction coefficient of 2.11×10^4 cm^2/mmol (42).

Superoxide scavenger activity test. Superoxide scavenging was quantitated by a method modified from that of Goldstein et al. (15). HBSS (1 ml) containing cytochrome *c* (5 μM), xanthine (0.1 mM), and xanthine oxidase (3.0 mU) was incubated with or without pentoxifylline (300 μg/ml), allopurinol (300 μg/ml), or SOD (200 U/ml) for 3 min at 37°C. The optical density was read at 550 nm both before and after incubation, and the nanomoles of superoxide released per 10 min per 10^6 PMN was calculated.

Degranulation. Purified PMNs (4×10^6) were suspended and incubated as described above for chemotaxis. After incubation with or without pentoxifylline and cytokines, 0.9 ml of HBSS with or without FMLP (100 nM) or PMA (10 ng/ml) was added, and the samples were incubated for an additional 10 min. The samples were then iced and centrifuged at 2,000 × *g* for 15 min. Lysozyme release into the supernatant was quantitated by the measurement of changes in the optical density of a suspension of *Micrococcus lysodeikticus* (39) after addition of the supernatants. The activity is reported in micrograms of lysozyme per milliliter of medium.

Cytosolic calcium. Cytosolic calcium fluxes were monitored by a method modified from that of Murata et al. (29). PMNs (3×10^6 per ml) were prepared from heparinized whole human blood by gravity sedimentation followed by Percoll purification (11). The PMNs were tumbled at 37°C for 1 h with 10 μM Fura 2/AM in loading buffer of the following composition: 138 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 0.1 mM EGTA, 5 mM Na_2PO_4, 5 mM $NaCO_3$, 5.5 mM glucose, and 20 mM HEPES.

The PMNs were then centrifuged for 10 min at 150 × *g* and suspended in HBSS containing 1% heat-inactivated autologous serum held at room temperature for 2 h. The Fura 2/AM-loaded PMNs were centrifuged for 5 min at 150 × *g*, taken up in HBSS containing 1% heat-inactivated autologous serum with or without pentoxifylline, and then agitated for 30 min at 37°C in a water bath.

The fluorescence of the PMNs was read with an Aminco-Bowman spectrophotofluorometer (model J4-8202; Aminco-Bowman, Silver Spring, Md.) at an excitation wavelength of 340 or 380 nm and an emission wavelength of 510 nm. FMLP (1 nM) was added, and the fluorescence was read again after 45 s. The intracellular free calcium concentration was calculated by the method of Grynkiewicz et al. (16) as reported by Murata et al. (29).

FMLP binding. FMLP binding was assayed by the following method modified from that of Williams et al. (46). PMNs were suspended (1×10^6 to 5×10^6/ml) in incubation buffer (1.7 mM KH_2PO_4, 8 mM Na_2HPO_4, 117 mM NaCl, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$ [pH 7.2]). Cell suspension (150 μl) was incubated with 10 nM [^{3}H]FMLP for 10 min at 4 or 37°C. The reaction was stopped by the addition of 2 ml of buffer at 4°C, and the cells were harvested by vacuum filtration of the mixture through glass fiber filters (no. 30 glass, 25-mm diameter; Scheicher and Schuell, Inc., Keene, N.H.). The filters were washed with 10 ml of cold buffer, vacuum dried, and placed into 15 ml of Bray counting solution; radioactivity was assessed by liquid scintillation counting. Other samples were prepared the same way, except that 10 μM nontritiated FMLP was added before the [^{3}H]FMLP to measure nonspecific binding. The specific binding was calculated by subtracting the nonspecific binding from the total binding, and the data were expressed as the number of [^{3}H]FMLP molecules bound per PMN.

Statistics. The results were reported as the mean ± the standard error of the mean. *P* values were determined by a two-tailed Student *t* test. Curve fitting was done by using Cricket Graph (Cricket Software, Malvern, Pa.), and regression analysis was done by using Statworks (Cricket).

RESULTS

PMN adherence. Adherence of PMNs to nylon fibers was enhanced by inflammatory cytokines and diminished by pentoxifylline at concentrations as low as 10 μg/ml.

There was 64.1 ± 2.7% (n = 16) PMN adherence to nylon when PMNs were incubated for 30 min at 37°C in diluent. Incubation of the PMNs for 30 min with pentoxifylline (300 μg/ml) decreased PMN adherence to 54.0 ± 3.9% (n = 6; P = 0.031.

(i) LPS-MCM. Incubation with LPS (5 ng/ml) did not significantly affect PMN adherence (65.8 ± 3.9% [n = 9; P = 0.431]). In contrast, LPS-MCM increased PMN adherence to nylon from 59.0 ± 9.0% without LPS-MCM to 87.2 ± 2.0% with 40% LPS-MCM (5 ng of LPS per ml) (Fig. 1). Pentoxifylline (10 μg/ml) decreased PMN adherence enhanced by undiluted LPS-MCM from 83.7 ± 1.8 (n = 3) to 75.3 ± 4.9% (n = 3; P = 0.024).

(ii) rh-TNF (alpha). Incubation of PMNs with rh-TNF (100 to 10,000 U/ml) increased PMN adherence to nylon from 64.1 ± 2.7% (in diluent) to 89.5 ± 1.9% (in TNF [10,000 U/ml]; $P < 0.050$) (Fig. 2). Pentoxifylline (300 μg/ml) decreased PMN adherence augmented by rh-TNF (100 U/ml) from 80.1 ± 4.1 to 73.4 ± 2.9% (n = 6; P = 0.036).

(iii) ph-IL-1. IL-1 (100 U/ml) increased PMN adherence to nylon from 64.1 ± 2.7% (n = 16) to 79.6 ± 3.2% (n = 11; P = 0.001). Pentoxifylline (300 μg/ml) decreased adherence enhanced by IL-1 to 60.4 ± 4.1% (n = 6; P = 0.002).

PMN migration. Inflammatory cytokines diminished directed migration of PMNs to FMLP. Pentoxifylline in concentrations as low as 1 μg/ml restored the chemotactic activity of PMN.

(i) LPS-MCM. LPS (100 ng/ml) decreased directed migration of PMNs from 2.33 ± 0.05 to 1.85 ± 0.01 mm (n = 6; P = 0.001). PMNs in medium containing 2 ng of LPS per ml had a directed migration under agarose to FMLP of 2.93 ± 0.06 mm (n = 10). In contrast, incubation of PMNs in 40% LPS-MCM (containing 2 ng of LPS per ml) decreased directed migration to FMLP to 2.02 ± 0.10 mm. Pentoxifylline (100 μg/ml) restored LPS-MCM-inhibited directed migration of PMNs to FMLP to 2.26 ± 0.06 mm (P = 0.005). A primary metabolite of pentoxifylline, metabolite I (at 0.1, 1.0, and 100 μg/ml), significantly restored directed migration ($P < 0.050$) (Fig. 3). Metabolite IV restored directed migration to 2.25 ± 0.11 mm (P = 0.035). Metabolite V did not affect LPS-MCM-inhibited directed migration to FMLP.

LPS-MCM did not significantly affect random migration ($P > 0.050$).

(ii) rh-TNF (alpha). TNF (100 to 1,000 U/ml) reduced directed migration of PMNs to FMLP ($P < 0.050$) (Fig. 4A) but not random migration ($P > 0.050$). Pentoxifylline (1 to 100 μg/ml) and metabolite I (10 to 100 μg/ml) restored the directed migration inhibited by TNF (1,000 U/ml) ($P < 0.050$) (Fig. 4B and C).

(iii) ph-IL-1. IL-1 (150 U/ml) decreased directed migration of PMNs to FMLP from 2.30 ± 0.13 to 1.57 ± 0.12 mm (P =

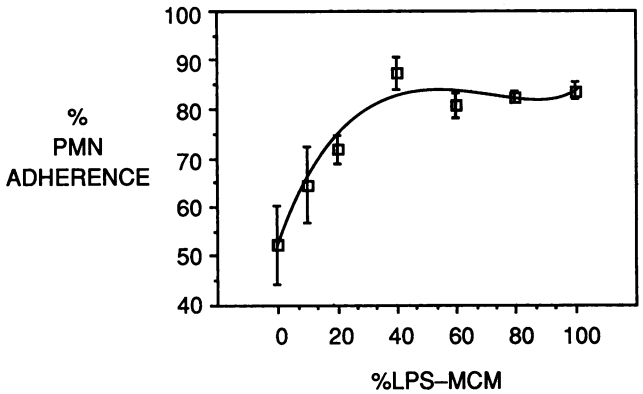

FIG. 1. Effect of incubation with LPS-MCM for 30 min at 37°C on PMN adherence to nylon. The results are expressed as the percent PMN adherence to the nylon at LPS-MCM concentrations between 0 and 100%. Data are means ± standard errors of the means of at least three separate experiments.

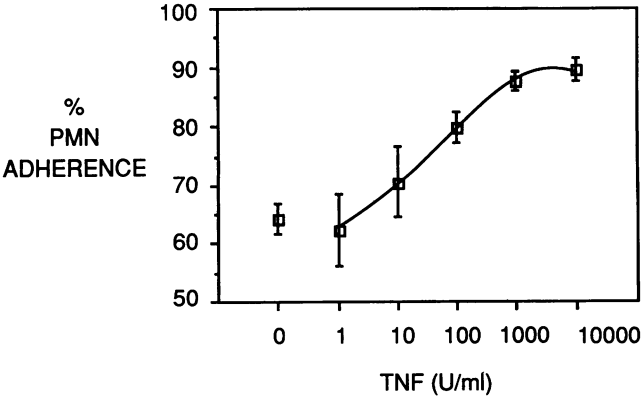

FIG. 2. Effect of incubation with rh-TNF (alpha) for 30 min at 37°C on PMN adherence to nylon. The results are expressed as the percent PMN adherence to the nylon at rh-TNF (alpha) concentrations between 0 and 10,000 U/ml. Data are means ± standard errors of the means of at least six separate experiments.

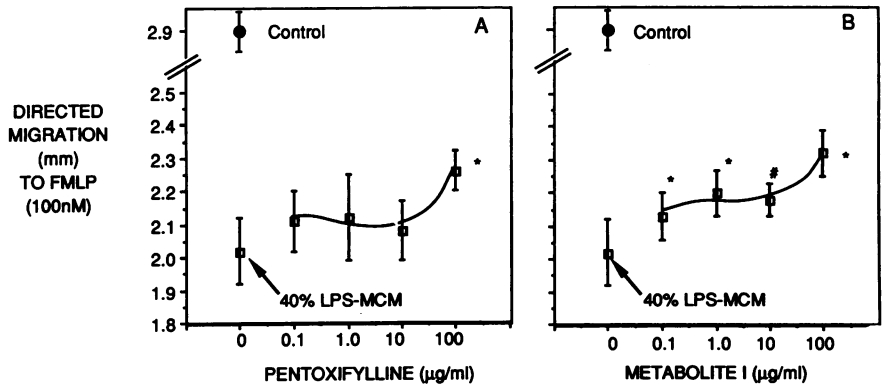

FIG. 3. Effect of incubation with 40% LPS-MCM for 30 min at 37°C on directed migration of PMNs to FMLP (100 nM). Modulation by 0 to 100 μg of pentoxifylline (A) or metabolite I (B) per ml. The results are presented as the distance in millimeters migrated by the leading front towards FMLP. Data are means ± standard errors of the means of at least five separate experiments. Symbols: *, $P < 0.050$; #, $P = 0.056$ compared with migration in the absence of pentoxifylline or metabolite I. (Directed migration in the presence of LPS [2 ng/ml] was 2.93 ± 0.06 mm [$n = 5$].)

0.001). IL-1 did not affect random migration ($P > 0.050$). Pentoxifylline and metabolites I, IV, and V (each at 100 μg/ml) restored the directed migration toward FMLP which had been inhibited by IL-1 (150 U/ml) to 2.39 ± 0.11, 2.25 ± 0.06, 2.26 ± 0.10, and 2.17 ± 0.10 mm, respectively ($n = 3$; $P < 0.005$).

Superoxide production. Inflammatory cytokines primed PMNs for increased superoxide production after stimulation with FMLP. Pentoxifylline and metabolites in concentrations as low as 100 μg/ml diminished this priming activity.

Pentoxifylline (100 μg/ml) did not affect unstimulated PMN superoxide production (0.26 ± 0.07 nmol/10 min per 10^6 PMNs without pentoxifylline and 0.28 ± 0.11 nmol/10 min per 10^6 PMNs [$n = 5$] with pentoxifylline [$P = 0.878$]) or superoxide production stimulated by PMA (10 ng/ml) (2.19 ± 0.71 nmol/10 min per 10^6 PMNs without pentoxifylline and 2.07 ± 0.70 nmol/10 min per 10^6 PMNs [$n = 5$] with pentoxifylline [100 μg/ml; $P = 0.577$]). Pentoxifylline (100 μg/ml) did reduce superoxide production stimulated by FMLP (100 nM) from 1.68 ± 0.37 to 0.67 ± 0.11 nmol/10 min per 10^6 PMNs. [$n = 5$; $P = 0.038$).

(i) PMNs primed with LPS-MCM. Incubation of PMNs with as much as 800 ng of LPS per ml for 30 min at 37°C resulted in the release of only 1.47 ± 0.34 nmol/10 min per 10^6 PMNs ($n = 5$). Priming the PMNs with a small amount of LPS (0.25 ng/ml) increased FMLP (100 ng/ml)-stimulated release slightly from 1.68 ± 0.37 to 2.17 ± 0.70 nmol/10 min per 10^6 PMNs ($n = 6$; $P = 0.032$). Incubation of PMNs with 40% LPS-MCM resulted in the release of only 0.85 ± 0.32 nmol/10 min per 10^6 PMNs ($n = 5$). In contrast, LPS-MCM appreciably primed PMNs for increased superoxide production in response to FMLP (100 nM) (Fig. 5A). LPS-MCM (5%; 0.25 ng of LPS per ml) tripled superoxide production stimulated by FMLP (100 nM) to 6.52 ± 1.75 nmol/10 min per 10^6 PMNs ($P = 0.011$ compared with superoxide production in nonconditioned medium containing LPS). Pentoxifylline and metabolite I (100 and 300 μg/ml, respectively) reduced LPS-MCM-primed superoxide production ($P < 0.050$) (Fig. 5B and C).

(ii) rh-TNF (alpha). TNF (10,000 U/ml) increased superoxide production from 0.62 ± 0.13 to 1.91 ± 0.28 nmol/10 min per 10^6 PMNs ($P = 0.007$) (Fig. 6A). Pentoxifylline (100 μg/ml) decreased superoxide production stimulated by TNF (10,000 U/ml) to 1.05 ± 0.38 nmol/10 min per 10^6 PMNs ($P = 0.008$).

TNF (10 to 10,000 U/ml) primed PMNs for increased superoxide production in response to 100 nM FMLP ($P < 0.050$) (Fig. 6A). Metabolite I (100 μg/ml) inhibited TNF (10 U/ml)-primed superoxide production ($P = 0.030$) (Fig. 6C).

(iii) ph-IL-1. IL-1 (50 U/ml) stimulated production of 0.39 ± 0.13 nmol of superoxide per 10 min per 10^6 PMNs ($n = 5$). Pentoxifylline (100 μg/ml) did not affect IL-1-stimulated superoxide production (0.46 ± 0.10 nmol/10 min per 10^6 PMNs [$n = 5$; $P = 0.588$]).

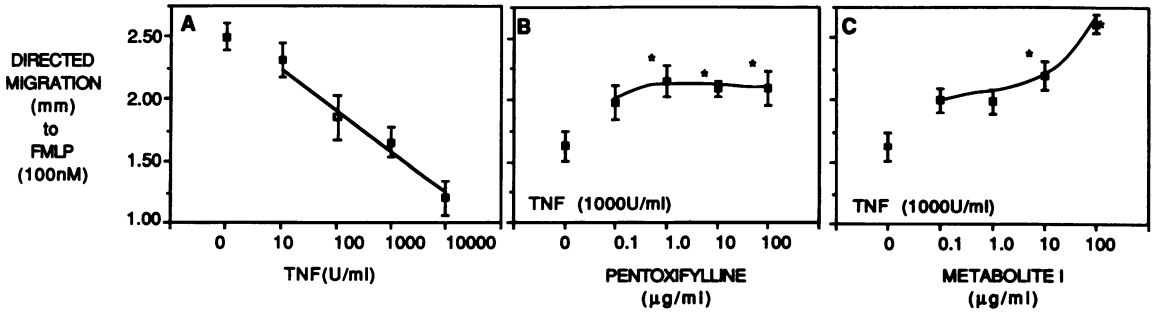

FIG. 4. Effect of incubation with rh-TNF (0 to 10,000 U/ml) for 30 min at 37°C on directed migration of PMNs to 100 nM FMLP (A). Modulation of rh-TNF (1,000 U/ml) inhibited directed migration of PMNs by 0 to 100 μg of pentoxifylline (B) or metabolite I (C) per ml. The results are presented as the distance in millimeters migrated by the leading front towards FMLP. Data are means ± standard errors of the means of at least five separate experiments. Symbol: *, $P < 0.05$ compared with migration without pentoxifylline or metabolite I.

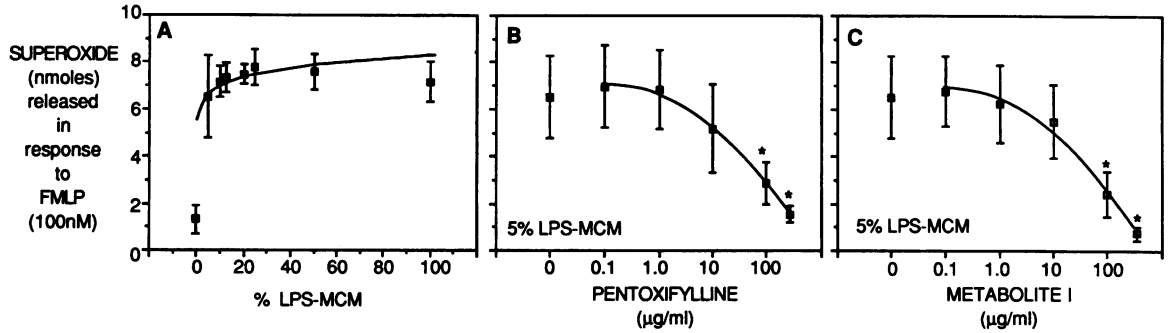

FIG. 5. Effect of priming PMNs (4×10^6) with LPS-MCM for 30 min at 37°C on superoxide production stimulated with 100 nM FMLP for 10 min at 37°C (A). Modulation of PMN superoxide production primed with 5% LPS-MCM for 30 min at 37°C and stimulated with FMLP for 10 min at 37°C by 0 to 300 μg of pentoxifylline (B) or metabolite I (C) per ml. Data are means ± standard errors of the means of at least five separate experiments. Symbol: *, $P < 0.050$ compared with superoxide production without pentoxifylline or metabolite I.

IL-1 (50 U/ml) primed PMNs for increased superoxide production in response to FMLP (100 nM) from 1.81 ± 0.43 to 4.39 ± 0.6 nmol/10 min per 10^6 PMNs ($P = 0.001$). Pentoxifylline and metabolite I (100 and 300 μg/ml, respectively) decreased ph-IL-1-primed, FMLP-stimulated superoxide production ($P < 0.050$) (Fig. 7). Metabolites IV and V (300 μg/ml) decreased IL-1-primed FMLP-stimulated superoxide production to 1.90 ± 0.75 ($n = 5$) and 1.78 ± 0.69 ($n = 4$) nmol/10 min per 10^6 PMNs ($P = 0.039$ and $P = 0.040$, respectively, compared with values obtained in the absence of metabolite IV or metabolite V).

Test for superoxide scavenger activity. Allopurinol, another xanthine derivative, is a xanthine oxidase inhibitor, and SOD is a superoxide scavenger. Superoxide production by the xanthine-xanthine oxidase system in the presence of HBSS was 8.33 ± 0.33 nmol/3 min per ml ($n = 3$); in the presence of pentoxifylline (300 μg/ml), it was 10.19 ± 0.90 nmol/3 min per ml ($n = 2$); in the presence of allopurinol (300 μg/ml), it was −0.030 ± 0.44 nmol/3 min per ml ($n = 3$); and in the presence of SOD (200 U/ml), it was −0.95 ± 0.33 nmol/3 min per ml ($n = 3$). Thus, pentoxifylline is neither a xanthine oxidase inhibitor nor a superoxide scavenger.

Degranulation. Inflammatory cytokines increase the release of granule enzymes from PMNs stimulated with FMLP. Pentoxifylline in concentrations as low as 1 μg/ml inhibited granule enzyme release in these cells.

Unstimulated PMNs released 1.14 ± 0.10 μg of lysozyme per ml ($n = 8$). When stimulated with FMLP (100 nM), PMNs released 1.57 ± 0.16 μg of lysozyme per ml ($n = 8$). When stimulated with PMA (1 ng/ml), PMNs released 1.62 ± 0.27 μg of lysozyme per ml ($n = 4$). Pentoxifylline (100 μg/ml) slightly decreased FMLP (100 nM)-stimulated release to 1.39 ± 0.12 μg of lysozyme per ml ($n = 8$) and did not significantly affect PMA (1 ng/ml)-stimulated release (1.38 ± 0.24 μg of lysozyme per ml [$n = 4$; $P = 0.034$ and 0.228, respectively, compared with lysozyme release without pentoxifylline]).

(i) PMNs treated with LPS-MCM. LPS (1 ng/ml) stimulated release of 0.62 μg of lysozyme per ml. Treatment of PMNs with medium containing LPS (1 ng/ml) followed by stimulation with FMLP (100 nM) resulted in the release of 1.60 ± 0.19 μg of lysozyme per ml ($n = 10$). LPS-MCM (20%; 1 ng of LPS per ml) stimulated the release of 0.79 ± 0.12 μg of lysozyme per ml. Treatment with 20% LPS-MCM further increased lysozyme release in response to 100 nM FMLP to 2.31 ± 0.11 μg/ml ($n = 10$; $P < 0.001$ compared with lysozyme release following treatment with nonconditioned medium containing LPS [1 ng/ml] followed by FMLP [100 nM]). Pentoxifylline (1 and 100 μg/ml) decreased lysozyme release from LPS-MCM-treated PMNs stimulated with 100 nM FMLP ($P < 0.050$) (Fig. 8).

(ii) rh-TNF. Treatment with TNF (100 to 10,000 U/ml) increased lysozyme release stimulated by FMLP ($P < 0.050$) (Fig. 9). Pentoxifylline (300 μg/ml) decreased lysozyme release from PMNs treated with TNF (100 and 1,000 U/ml) and stimulated with FMLP (100 nM) from 3.18 ± 0.24 ($n = 13$) and 2.20 ± 0.14 ($n = 8$) to 2.85 ± 0.26 ($n = 8$) and 2.02

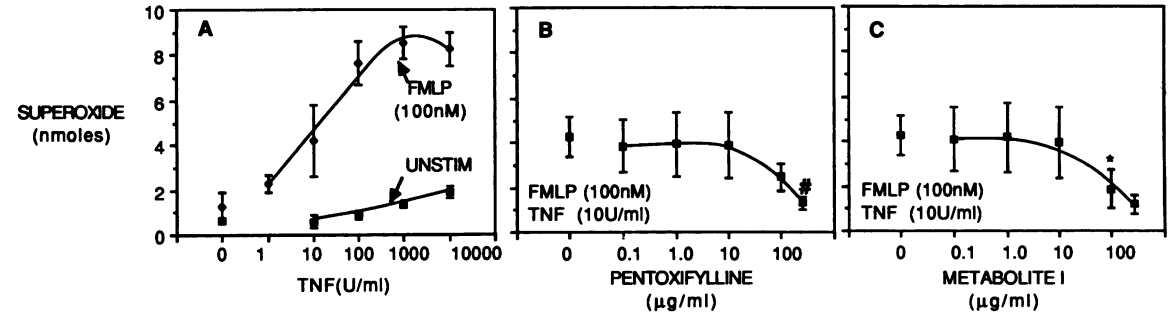

FIG. 6. Effect of priming PMNs (4×10^6) with rh-TNF (0 to 10,000 U/ml) for 30 min at 37°C on unstimulated superoxide production and on superoxide production stimulated with 100 nM FMLP for 10 min at 37°C (A). Modulation of PMN superoxide production primed with TNF (10 U/ml) for 30 min at 37°C and stimulated with FMLP for 10 min at 37°C by 0 to 300 μg of pentoxifylline (B) or metabolite I (C) per ml. Data are means ± standard errors of the means of at least six separate experiments. Symbols: *, $P = 0.03$ compared with superoxide production without metabolite I; #, $P = 0.078$ compared with superoxide production without pentoxifylline.

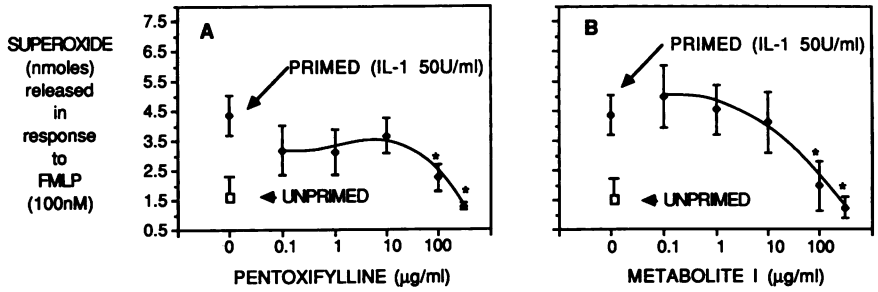

FIG. 7. Effect of priming PMNs (4×10^6) with ph-IL-1 (50 U/ml) for 30 min at 37°C on superoxide production stimulated with 100 nM FMLP for 10 min at 37°C (A). Modulation of superoxide production primed with IL-1 (50 U/ml) for 30 min at 37°C and stimulated with FMLP for 10 min at 37°C by 0 to 300 μg of pentoxifylline (A) or metabolite I (B) per ml. Data are means ± standard errors of the means of at least four separate experiments. Symbol: *, $P < 0.05$ compared with superoxide production without pentoxifylline or metabolite I.

± 0.2 ($n = 8$) μ/ml, respectively ($P = 0.025$ and 0.025, respectively).

(iii) ph-IL-1. ph-IL-1 (100 U/ml) did not affect lysozyme release by unstimulated PMNs (0.82 ± 0.89 μg/ml with IL-1 diluent and 1.01 ± 0.10 μg/ml with IL-1 [$n = 5$]). ph-IL-1 did not cause PMNs stimulated with FMLP (100 nM) to release more lysozyme (1.21 ± 0.19 μg/ml without IL-1 treatment and 1.45 ± 0.07 μg/ml with IL-1 treatment [$n = 5$; $P > 0.050$).

Cytosolic calcium. Preliminary studies indicated that pentoxifylline had its greatest effect on calcium mobilization in PMNs at levels of FMLP (1 nM) at the threshold for stimulating the oxidative burst. Resting PMNs had a low free-cytosolic-calcium concentration (60.63 ± 19.49 nM [$n = 5$]). Stimulation with FMLP (1 nM) increased free cytosolic calcium to 115.07 ± 18.96 nM ($n = 5$; $P = 0.001$). Pentoxifylline (300 μg/ml) decreased the calcium concentration of PMNs stimulated with FMLP to 33.11 ± 11.90 nM ($n = 5$; $P = 0.025$) (Fig. 10).

FMLP binding. At 4°C, $1.23 \times 10^3 \pm 0.20 \times 10^3$ molecules of FMLP were bound per PMN ($n = 7$). Pentoxifylline (1 mM; 278 μg/ml) did not affect FMLP binding ($1.25 \times 10^3 \pm 0.26 \times 10^3$ molecules of FMLP per PMN [$n = 7$; $P = 0.890$]). At 37°C, FMLP binding increased to $5.73 \times 10^3 \pm 0.95 \times 10^3$ molecules of FMLP per PMN ($n = 15$; $P = 0.002$). Pentoxifylline (1 mM) further increased binding at 37°C to $7.74 \times 10^3 \pm 0.68 \times 10^3$ molecules of FMLP per PMN ($n = 7$; $P = 0.002$ compared with FMLP binding without pentoxifylline).

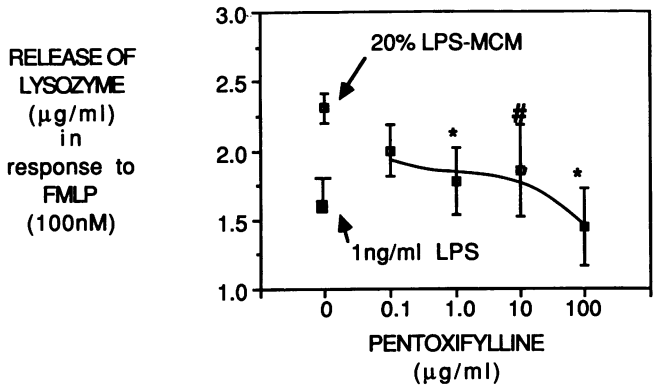

FIG. 8. Effect of incubation in 20% LPS-MCM for 30 min at 37°C on lysozyme released by PMNs (4×10^6) stimulated with 100 nM FMLP for 10 min at 37°C and modulation by pentoxifylline (0 to 100 μg/ml). Data are means ± standard errors of the means of at least four separate experiments. Symbols: *, $P < 0.050$; #, $P = 0.102$ compared with lysozyme release without pentoxifylline.

DISCUSSION

At the site of tissue injury or infection, the production of inflammatory cytokines may benefit the host by promoting the accumulation of PMNs and activating their microbicidal functions. However, prolonged, disseminated, or excessive activation of PMNs can initiate microvascular injury resulting in increased vasopermeability, hemorrhage, and thrombosis (28).

The LPS-induced cytokine preparations could contain a number of additional endogenous substances, including complement factors, cyclooxygenase and lipoxygenase products, and other monokines and lymphokines. The altered PMN function (both stimulated and inhibited) in the presence of LPS-induced cytokines is probably the net result of the interaction of several endogenous factors.

PMNs from patients on hemodialysis (23) and patients with bacteremic shock (43) are more adherent to nylon fibers than are normal PMNs. In our experiments, pentoxifylline decreased adherence stimulated by LPS-MCM, TNF, and IL-1. This suggests that pentoxifylline could be effective in preventing PMN-induced vascular damage induced by cytokines in vivo. In a recent clinical study, pentoxifylline treatment reduced the incidence of shunt thromboses by 50% in hemodialysis patients (31).

The decrease in PMN adherence observed with pentoxifylline cannot be explained by a decrease in PMN viability. PMNs incubated with a pentoxifylline concentration 10 times that which caused a decrease in adherence are viable and active, as indicated by their migration.

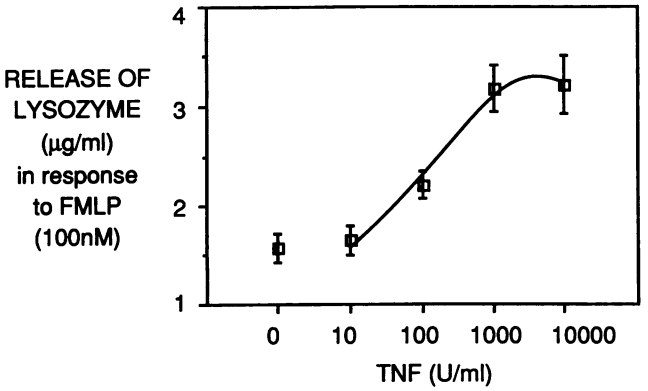

FIG. 9. Effect of exposure to rh-TNF (0 to 10,000 U/ml) for 30 min at 37°C on lysozyme released by PMNs (4×10^6) stimulated with 100 nM FMLP for 10 min at 37°C. Data are means ± standard errors of the means of at least five separate experiments.

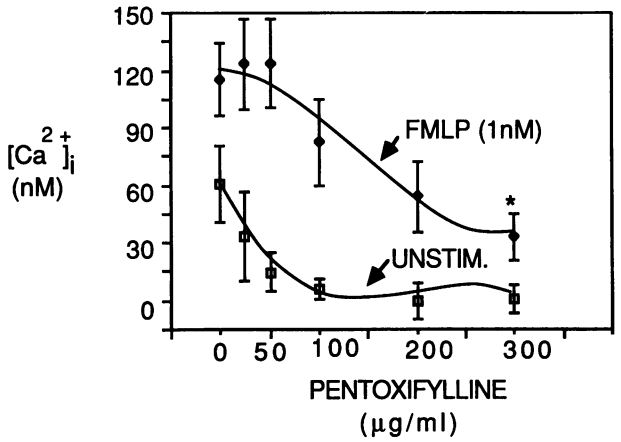

FIG. 10. Effect of pentoxifylline (0 to 300 μg/ml) on the cytosolic ionic calcium concentration ($[Ca^{2+}]_i$) of unstimulated PMNs and PMNs stimulated with 1 nM FMLP for 45 s at 37°C. Data are means ± standard errors of the means of at least five separate experiments. Symbol: *, $P = 0.025$ compared with cytosolic ionic calcium concentration without pentoxifylline.

Pentoxifylline has been shown to increase PMN migration to zymosan-activated serum in vitro and PMN migration in vivo in mice (41). In mice, more PMNs were found at the site of *Staphylococcus aureus* skin infections when the mice had been pretreated with pentoxifylline. Mouse survival was also significantly increased by pentoxifylline treatment (41). In a rat peritonitis model, pentoxifylline increased survival (8).

Pentoxifylline is a phosphodiesterase inhibitor and raises cyclic AMP (cAMP) levels in PMNs (5, 17). Substances which substantially increase PMN cAMP levels (including isoproterenol [18], dibutyrl cAMP [18], prostaglandin E_1 [18], cholera toxin [18], and *E. coli* toxin [1]) decrease PMN chemotaxis. In contrast, Issekutz et al. (19) have reported that some of these same substances can counteract the inhibitory action of LPS on directed migration of PMNs in a mixed leukocyte preparation containing monocytes. In addition, the phosphodiesterase inhibitor isobutylmethylxanthine has been reported to restore chemotaxis in vitro of PMNs from patients with juvenile periodontitis (a disease often associated with the gram-negative bacterium *Actinobacillus actinomycetemcomitans*) (9).

Hill et al. have reported that neonate PMNs, which have a lower chemotactic response to FMLP, have a depressed cAMP elevation in response to FMLP and that pentoxifylline was effective in both raising neonate PMN cAMP levels and increasing neonate PMN chemotaxis (17).

Pentoxifylline decreased superoxide production by PMNs stimulated with FMLP but not by those stimulated with PMA. This suggests that pentoxifylline may act through a pathway bypassed by PMA, such as the one responsible for the turnover of inositol phospholipids. Inositol triphosphate and diacylglycerol are produced from phosphatidylinositol 4,5-biphosphate (PIP_2) by the action of phospholipase C (for a review, see reference 4). Inositol triphosphate can stimulate mobilization of calcium (26), and diacylglycerol activates protein kinase C (27) and can be degraded further to arachidonic acid (for a review, see reference 4). PMA bypasses this pathway and activates protein kinase C directly (7). Sheetz et al. (37) reported that pentoxifylline caused an increase in PIP_2 and its precursor, phosphatidylinositol 4-phosphate, in the PMN cell membrane. We found that pentoxifylline blunted the rise in free intracellular calcium which normally follows FMLP stimulation. Increased free-calcium levels appear to modulate PMN superoxide production (29). It has been proposed that cAMP-dependent kinases can inactivate phospholipase C (45; M. Verghese, C. D. Smith, and R. Snyderman, Clin. Res. **33**:566A, 1985). Thus, pentoxifylline may act by slightly raising cAMP levels, which activates kinases and inhibits phospholipase C function. Decreased turnover of PIP_2 to inositol triphosphate and diacylglycerol results in less calcium mobilization, arachidonic acid formation, and protein kinase C activation.

Pentoxifylline did not affect binding of FMLP to PMNs at 4°C but did increase FMLP binding at 37°C. Hill et al. have reported that, although pentoxifylline did not significantly affect binding of FMLP to neonate PMNs at 0°C, it did enhance concanavalin A receptor mobility (in the presence of 1 μM colchicine) in neonate PMNs at 37°C (17). Sheetz et al. (36) demonstrated that PIP_2 increases the lateral mobility of glycoproteins in erythrocyte membranes. Since pentoxifylline increases the amount of PIP_2 in the PMN membrane (37) and makes the membrane more deformable (22), we propose that the change in the phospholipid layer of the cell membrane makes the PMN membrane more fluid and promotes receptor lateral mobility and internalization at 37°C.

Pentoxifylline and its metabolites at or near therapeutically achievable levels (S. K. Puri, H. B. Lassman, I. Ho, and R. Sabo, Clin. Pharmacol. Ther. **41**:204, 1987) modulate PMN adherence, superoxide production, degranulation, and migration altered by mononuclear leukocyte-produced inflammatory cytokines. There is relatively little effect on the function of PMNs not preactivated. Since activated PMNs are mediators of a wide variety of inflammatory diseases, pentoxifylline should be evaluated to determine if it can prevent inflammatory damage in clinical situations such as septic shock and adult respiratory distress syndrome.

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