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Pentoxifylline Modulates Activation of Human Neutrophils by Amphotericin B In Vitro

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The antifungal agent amphotericin B (AmB) alters neutrophil (polymorphonuclear leukocyte [PMN]) function, and this may be the mechanism for some of the adverse effects caused by AmB. AmB is a potent inhibitor of PMN migration, increases PMN adherence and aggregation, and primes PMN for increased oxidative activity in response to a second stimulus. AmB also stimulates mononuclear leukocytes (MNLs) to release inflammatory mediators which augment the effects of AmB on PMN function. In the present study, we observed that the methylxanthine derivative pentoxifylline decreased the effects of AmB on PMN function. AmB (2 μg/ml) priming doubled PMN chemiluminescence stimulated by fMet-Leu-Phe. In the presence of MNLs, AmB priming increased *f*Met-Leu-Phe-stimulated PMN chemiluminescence to 622% of unprimed PMN activity. Pentoxifylline (100 μM) blunted the rise in AmB-augmented PMN chemiluminescence in the presence of MNLs to 282% of unprimed PMN activity, and pentoxifylline metabolites were active at 10 μM. Pentoxifylline (100 μM) also blocked AmB-augmented PMN oxidative activity in whole blood, as measured by nitroblue tetrazolium reduction. In the presence of MNL, AmB (2 μg/ml) doubled the expression of the important PMN adherence factor Mac-1. Pentoxifylline (1 mM) decreased AmB-stimulated PMN Mac-1 expression back to unstimulated amounts. In the presence of MNLs, AmB (2 μg/ml) decreased PMN nondirected and directed migration to fMet-Leu-Phe to 40 and 38% of control PMN migration, respectively. Pentoxifylline (300 μM) counteracted AmB inhibition of nondirected and directed migration to fMet-Leu-Phe, resulting in migration that was 71 and 87% of control PMN migration, respectively. In contrast, the methylxanthine caffeine (100 μM) increased AmB-enhanced chemiluminescence but did not affect AmB-inhibited PMN migration. Pentoxifylline should be evaluated as adjunctive therapy to lessen the inflammatory damage caused by AmB.

The antifungal agent amphotericin B (AmB) has a stimulatory effect on several polymorphonuclear leukocyte (PMN) functions. AmB increases PMN adherence to nylon wool, PMN aggregation (5, 9, 10, 35, 40, 69), and PMN oxidative activity (59). AmB also activates macrophages (15, 34, 48, 66, 67) and induces the production of tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) (17, 25). We have observed that the presence of mononuclear leukocytes (MNLs) exacerbates the inflammatory effects of AmB on PMN function, including AmB priming of the PMN oxidative burst and PMN expression of the adherence integrin Mac-1 (55).

In vivo, AmB treatment has a number of adverse effects including fever, chills, thrombophlebitis, anemia, and nephrotoxicity (12, 39). Nephrotoxicity is evidenced by decreased renal blood flow and glomerular filtration rate (4, 14, 60). Although the role of PMNs in AmB-induced nephrotoxicity has not been fully defined, there is evidence that activated PMNs may contribute to renal necrosis by aggregating within blood vessels and releasing toxic products, especially in the infected host (37, 63). PMNs activated by monokines from AmB-activated macrophages may thus contribute to the adverse effects caused by AmB.

Pentoxifylline, a methylxanthine derivative used for intermittent claudication, has been reported to decrease AmB nephrotoxicity in noninfected and *Candida albicans*-infected rats (36, 62) and in allograft bone marrow recipients (8). We have previously reported that pentoxifylline and its metabolites decrease the inflammatory activity of PMNs elicited by endotoxin-stimulated, MNL-conditioned medium, recombinant human TNF-α, and purified human IL-1β (56–58). In the present study, we examined the effect of pentoxifylline on AmB-altered PMN function. We observed that pentoxifylline (but not the related methylxanthine caffeine) decreased AmB-primed PMN oxidative activity stimulated by fMet-Leu-Phe (fMLP), prevented AmB-stimulated expression of Mac-1, and counteracted AmB inhibition of PMN migration. By these actions, pentoxifylline has the potential to decrease AmB toxicity.

MATERIALS AND METHODS

Materials. A desoxycholate-suspended preparation of AmB (Fungizone) was purchased from Bristol-Myers Squibb Pharmaceuticals (Princeton, N.J.). AmB contains less than 5 endotoxin units of endotoxin per ml by the *Limulus* amebocyte lysate assay, as determined by Bristol-Myers Squibb Pharmaceuticals. fMLP, luminol, cytochrome *c*, catalase, human leukocyte myeloperoxidase (MPO), β-carotene, caffeine, trypan blue, aminophylline, mouse immunoglobulin G2a (IgG2a), kappa myeloma protein, desoxycholate, and nitroblue tetrazolium (NBT) were from Sigma Chemical. Ficoll-Hypaque was purchased from Flow Laboratories (McLean, Va.) and Los Alamos Diagnostics (Los Alamos, N.M.). Hanks balanced salt solution (HBSS), RPMI 1640 medium, fetal bovine serum (FBS), and the *Limulus* amebocyte lysate assay kit were from Whittaker Bioproducts (Walkersville, Md.). Human serum albumin (HSA) was from Cutter Biological (Elkhart, Ind.). Paraformaldehyde was from Eastman Organic Chemicals (Rochester, N.Y.). Anti-Leu-15 and phycoerythrin (PE)-conjugated anti-Leu-M3

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(CD14) were from Becton Dickinson, (Mountain View, Calif.), and fluorescein isothiocyanate-labeled goat anti-mouse IgG $F(ab')_2$ fragments, heavy and light chain specific, were from Cappel, (West Chester, Pa.).

Pentoxifylline [1-(5'-oxohexyl)-3,7-dimethylxanthine] and 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 supplied by Hoechst-Roussel Pharmaceuticals (Somerville, N.J.).

Leukocyte preparation. Purified PMNs (~98% PMNs and >95% viable by trypan blue exclusion) containing <1 platelet per 5 PMNs and <50 pg of endotoxin per ml (*Limulus* amebocyte lysate assay) were obtained from normal heparinized (10 U/ml) venous blood by a one-step Ficoll-Hypaque separation procedure (22). Residual erythrocytes were lysed by hypotonic lysis with 3 ml of iced 0.22% sodium chloride solution for 45 s and then with 0.88 ml of 3% sodium chloride solution for the PMN chemiluminescence assays.

The MNL fraction (>95% viable by trypan blue exclusion) was retained from the Ficoll-Hypaque separation described above. This fraction was composed of monocytes (~15 to 20%) and lymphocytes (~80 to 85%). Mixed leukocyte preparations were made by combining PMNs with MNLs.

MNL-conditioned medium. MNLs (2×10^6/ml of HBSS, 0.1% HSA) were incubated for 90 min 37°C with or without AmB (2 mg/ml) and pentoxifylline. The samples were then iced, and the MNLs were removed by centrifugation, yielding a cell-free MNL-conditioned medium.

Leukocyte chemiluminescence. Luminol-enhanced chemiluminescence is a measure of PMN oxidative activity (16). Chemiluminescence was measured by a procedure modified from one described previously (1). Purified PMNs (1×10^6/ml) or PMNs (1×10^6/ml) added to MNLs (2×10^6/ml) were incubated (37°C for 0 to 90 min) in 1 ml of diluent (HBSS, 0.1% HSA); MNL-conditioned medium; or AmB with or without pentoxifylline, pentoxifylline metabolites, caffeine, and aminophylline. Luminol (500 μM) was then added, and the cells were transferred to a vial (37°C) with a stirring bar. Chemiluminescence was read with a Chronolog photometer (Havertown, Pa.) for 1 min in the absence of fMLP, and then fMLP (1 μM) was added and the chemiluminescence assay continued for an additional 7 min. The relative chemiluminescence (area under the curves) was determined by weighing the cut chart paper. The results are reported as relative fMLP-stimulated chemiluminescence in 7 min. Unprimed pure PMN chemiluminescence in response to fMLP in the absence of MNLs, xanthine derivatives, and AmB was given a value of 100%.

Cell-free luminol-enhanced chemiluminescence was generated by incubating MPO (2.5×10^{-4} mg/ml) with hydrogen peroxide (4.5×10^{-2} mg/ml) and luminol (500 μM) in HBSS.

Leukocyte superoxide release. PMNs (1×10^6/ml), MNLs (2×10^6/ml), or PMNs (1×10^6/ml) plus MNLs (2×10^6/ml) were incubated for 90 min at 37°C in a shaker bath with or without AmB (2 μg/ml) and with or without pentoxifylline, metabolite I, or metabolite V. Then, catalase (0.062 mg/ml to prevent H_2O_2 reoxidation of cytochrome *c* [42]), cytochrome *c* (120 μM), and fMLP (10^{-6} M) were added to the cells; and the samples were incubated for 10 min more at 37°C in a shaker bath. Matched samples containing superoxide dismutase (200 U per sample) were also prepared. The samples were iced and centrifuged ($2,000 \times g$ for 10 min). The optical density of the supernatants was read at 550 nm, and the nanomoles of superoxide dismutase-inhibitable superoxide released in 10 min was calculated with the extinction coefficient of 2.11×10^4 cm^2/mol (61).

Whole-blood PMN NBT test. The NBT test is another assay of the oxidative activity of PMN (2). It was measured as follows, as modified from a previously described procedure (46). Whole blood was drawn with no anticoagulant. AmB (with or without pentoxifylline or pentoxifylline metabolites) was immediately added to some whole-blood samples, and diluent was added to control samples. Two drops of each sample were placed onto a clean coverslip and incubated for 25 min (37°C and 5% CO_2) in a moist chamber. The clot was then washed off with normal saline at 37°C, and the coverslip was inverted onto 3 drops of a 1% NBT solution in HBSS–35% heat-inactivated pooled serum and was incubated for 20 min at 37°C. The coverslip was then washed with normal saline, fixed in methanol, and counterstained with safranin stain. The samples were coded, and the oxidatively active NBT-positive PMNs (blue) were counted under a light microscope.

Mac-1 expression on PMNs. PMN Mac-1 expression was analyzed by using a modification of the method of Pichyangkul et al. (49). Briefly, pure PMN (10^6/ml) or PMNs (10^6/ml) plus MNLs (10^6/ml) were suspended in 1 ml of RPMI 1640 medium–10% heat-inactivated FBS (with or without AmB and pentoxifylline). The cells were incubated for 90 min at 37°C. The samples were then iced and washed with phosphate-buffered saline (PBS)–5% heat-inactivated FBS. Anti-Leu-15 (or control mouse IgG2a, kappa myeloma protein) was added to the cell button, incubated at 4°C for 30 min, fixed with 0.5% paraformaldehyde, and then washed with PBS–5% heat-inactivated FBS. The button was taken up in secondary antibody [fluorescein isothiocyanate-labeled goat anti-mouse IgG $F(ab')_2$ fragments, heavy and light chain specific] washed with PBS–5% heat-inactivated FBS, and washed with PBS containing 1% HSA. Each cell button was suspended in PBS–0.5% paraformaldehyde and stored for up to 3 days in the refrigerator. The fluorescence was read with a fluorescence-activated cell sorter (Epics 753; Epics Coulter, Hialeah, Fla.). The cells were analyzed by light scatter to characterize the cells within the mixed leukocyte preparation into PMN and MNL subpopulations. The PMN population gated by light scatter was analyzed by fluorescence for its Mac-1 content. Fluorescence (as the mean channel intensity) was converted from the 3-decade log scale to a linear scale (1 to 1,000), and then the relative linear fluorescence was compared with unstimulated activity by dividing by the fluorescence of the nonincubated unprimed PMNs and multiplying by 100. There was little detectable fluorescence in those samples that were reacted with the control mouse myeloma protein rather than anti-Leu-15 (less than 1% of PMN fluorescence either in the presence or absence of AmB). For some samples, we selectively analyzed PMN ($CD14^-$ cells) within the light scatter map by staining with PE–anti-Leu-M3, which stains for $CD14^+$ cells (monocytes). We gated for the cells with low PE fluorescence (PMNs) and observed that this did not alter the results either in the absence or the presence of AmB.

PMN migration. Chemotaxis under agarose was quantitated by the method of Nelson et al. (43). Purified PMNs (5×10^6) or PMNs (5×10^6) plus MNLs (3×10^6) were incubated in either diluent (HBSS, 0.1% HSA) or AmB with or without pentoxifylline, pentoxifylline metabolites, or caffeine. Nondirected and directed migrations to fMLP (100 nM) were measured after 2 h of incubation at 37°C, and the results are reported as the distance (in millimeters) that the leading front of PMNs migrated. Microscopic examination of

the stained chemotaxis plates revealed that >99% of the cells within the PMN plus MNL assays which migrated out of the wells by 2 h were PMNs.

Human erythrocyte hemolysis. Human erythrocyte hemolysis was determined by a modification of a method described previously (11). Human erythrocytes from the Ficoll-Hypaque separation were retained (see above). The erythrocytes were washed two times with PBS and diluted to 0.5% in PBS. A 0.5% suspension of erythrocytes in distilled water was made as a 100% hemolysis control. Samples were made with 1 ml of erythrocyte suspension with or without AmB (2 μg/ml), desoxycholate (1.6 μg/ml), pentoxifylline, and caffeine (10 μM). Duplicate samples were incubated (0 to 180 min) at room temperature. Then, the samples were centrifuged at 200 × *g* for 5 min, and the optical density of a 0.5-ml aliquot of the cell-free supernatant was read with a spectrophotometer at 550 nm. The results are reported as percent hemolysis compared with the 100% hemolysis control.

PMN viability. PMN viability was determined by a flow cytometry fluorescence assay (Live/Dead; Molecular Probes Inc, Eugene, Oreg.) that measures plasma membrane integrity with the ethidium homodimer probe (38, 41). PMNs (10^6/ml) were incubated in HBSS–0.1% human serum albumin with or without AmB (2 μg/ml) and with or without pentoxifylline (1 mM) for 90 min at 37°C. The cells were washed and resuspended in calcium and magnesium-free PBS and were stained for 30 min at 37°C with ethidium homodimer (2 μM). The viability was determined with a fluorescence-activated cell sorter (Epics 753; Epics Coulter) as described previously (55).

Statistical analysis. Significance ($P < 0.050$) was determined by two-tailed paired Student's *t* test.

RESULTS

Xanthine modulation of AmB-increased PMN chemiluminescence. We have reported that concentrations of AmB corresponding to peak levels in serum have little effect on PMN viability in vitro (55). We have also noted that AmB has little effect on PMN or MNL chemiluminescence by itself, but that it primes PMN for increased chemiluminescence in response to fMLP. The PMN chemiluminescent response is magnified if MNLs are present during the priming period (55). AmB (2 μg/ml) in the presence of MNLs increased fMLP-stimulated PMN chemiluminescence to 622 ± 94% over fMLP-stimulated pure unprimed PMN chemiluminescence ($P = 0.001$) and 273% over fMLP-stimulated PMN chemiluminescence in the presence of MNLs ($P < 0.001$) (Fig. 1). AmB increased fMLP-stimulated peak and sustained chemiluminescence (Fig. 2A and B).

The MPO inhibitor sodium azide (42, 45) markedly decreased the AmB-enhanced PMN chemiluminescent response (by 86%; $P = 0.005$; Fig. 2C) without affecting PMN viability (96% viable by ethidium homodimer exclusion). The singlet oxygen quencher β-carotene (24) also decreased chemiluminescence (by 42%; $P = 0.011$) without affecting PMN viability (96% viable). This indicates that AmB-enhanced chemiluminescence is both MPO and singlet oxygen dependent.

Pentoxifylline and metabolite I decrease the fMLP-stimulated oxidative activity of unprimed and monokine-primed PMNs (6, 56, 57). In the present study, pentoxifylline (10 μM), pentoxifylline metabolites (10 μM), and caffeine (10 μM) had little effect on the fMLP-stimulated chemiluminescence of pure PMNs, PMNs plus AmB, and PMNs plus

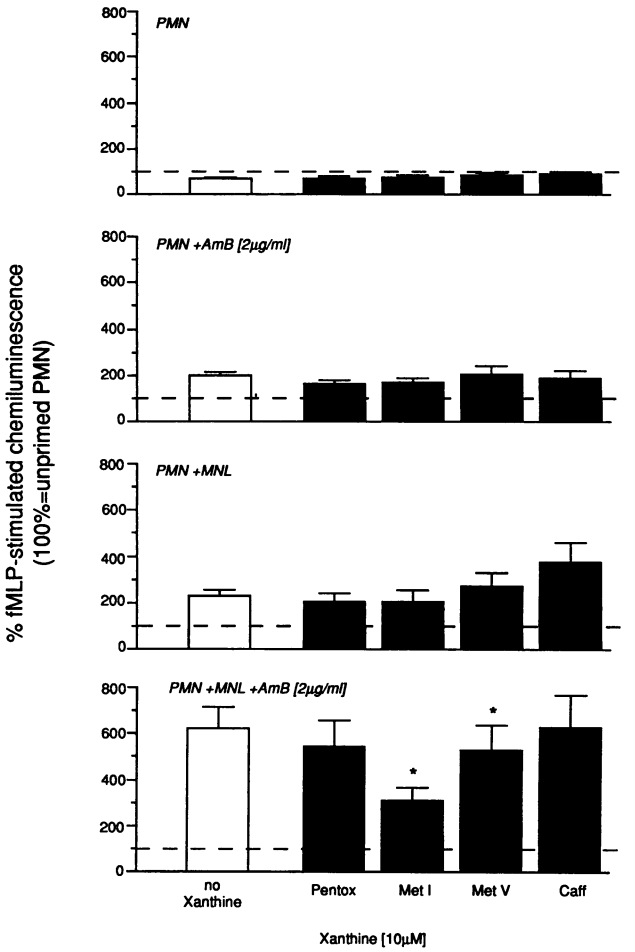

FIG. 1. Effect of AmB on leukocyte luminol-enhanced chemiluminescence: modulation by xanthines. Pure PMNs (1 × 10^6/ml) or PMNs (1 × 10^6/ml) plus MNLs (2 × 10^6/ml) were preincubated in HBSS with or without AmB (2 μg/ml) and with or without xanthine derivatives (10 μM; pentoxifylline [Pentox], pentoxifylline metabolite I [Met I], metabolite V [Met V], caffeine [Caff]) for 90 min at 37°C. The chemiluminescence of fMLP-stimulated unprimed PMNs is set at 100% (– – –). The results are expressed as means ± standard errors of the means ($n = 5$ separate experiments). *, $P < 0.050$; metabolites I and V decreased chemiluminescence compared with the chemiluminescence without the xanthine derivative.

MNLs (no AmB) (Fig. 1). Although pentoxifylline (10 μM) did not significantly reduce PMN chemiluminescence in the presence of AmB and MNLs ($P = 0.115$), metabolite I (10 μM) of pentoxifylline decreased activity by ~50% ($P = 0.008$), and metabolite V (10 μM) decreased fMLP-stimulated PMN chemiluminescence by ~15% in the presence of AmB and MNLs ($P = 0.009$) (Fig. 1). The methylxanthine caffeine (10 μM) did not lower PMN chemiluminescence in MNL plus AmB preparations (Fig. 1). Pentoxifylline metabolite IV (10 μM) did not affect PMN oxidative activity with or without MNLs and AmB (data not shown). Pentoxifylline (100 μM) decreased both the initial AmB-enhanced peak chemiluminescence and the sustained response (Fig. 2D). We observed that pentoxifylline (1 mM) did not affect PMN viability either in the absence or the presence of AmB (2 μg/ml) (99 and 94% viable, respectively).

Pentoxifylline (100 μM) decreased the fMLP-stimulated chemiluminescence of PMNs primed with AmB (2 μg/ml) in

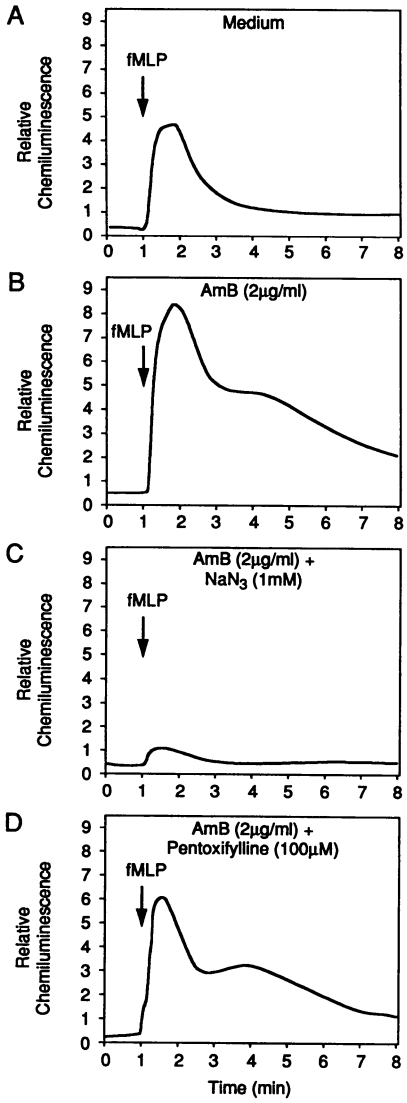

FIG. 2. Effect of AmB on leukocyte luminol-enhanced chemiluminescence: modulation by sodium azide and pentoxifylline. PMNs (1×10^6/ml) plus MNLs (2×10^6/ml) were preincubated in HBSS with or without AmB (2 μg/ml) for 90 min at 37°C and were then stimulated with fMLP (arrow). The traces are from a representative experiment of five separate experiments. (A) Medium; (B) AmB (2 μg/ml); (C) AmB (2 μg/ml) and sodium azide (1 mM); (D) AmB (2 μg/ml) and pentoxifylline (100 μM).

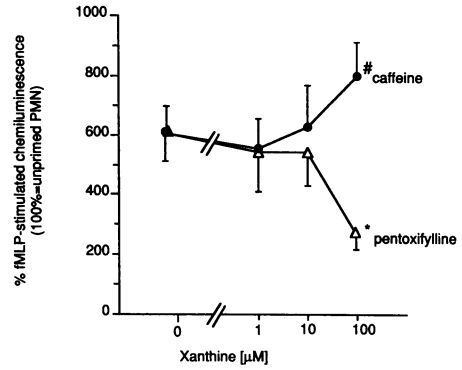

FIG. 3. Effect of AmB on leukocyte luminol-enhanced chemiluminescence in PMN plus MNL preparations: modulation by xanthines. PMNs (1×10^6/ml) plus MNLs (2×10^6/ml) were preincubated in HBSS with or without AmB (2 μg/ml) and xanthine derivatives for 90 min at 37°C. The chemiluminescence of fMLP-stimulated unprimed PMN is set at 100% (– – –). The results are expressed as means ± standard errors of the means ($n = 5$ separate experiments). *, $P < 0.050$; pentoxifylline decreased AmB-augmented chemiluminescence compared with the chemiluminescence without the xanthine derivative. #, $P = 0.006$; caffeine further increased AmB-augmented chemiluminescence compared with the chemiluminescence without caffeine.

the presence of MNLs ($P < 0.050$). In contrast, caffeine (100 μM) increased fMLP-stimulated chemiluminescence in the presence of MNLs and AmB ($P = 0.006$) (Fig. 3). Similar to caffeine, aminophylline tended to increase AmB-primed chemiluminescence. We observed that aminophylline (10 μM) increased AmB-primed (2 μg/ml) PMN plus MNL chemiluminescence in response to fMLP by 45% compared with that obtained with AmB priming alone ($P = 0.001$).

Priming of PMNs with AmB (2 μg/ml for 90 min) tripled fMLP-stimulated PMN chemiluminescence, and priming of PMNs with cell-free MNL-conditioned medium (no AmB) doubled PMN chemiluminescence. The most potent priming was observed with cell-free AmB-stimulated MNL-conditioned medium, which increased fMLP-stimulated PMN chemiluminescence almost 11-fold. The fMLP-stimulated chemiluminescence, as a percentage of unprimed PMN activity, for unprimed PMNs, PMNs plus AmB (2 μg/ml), PMNs plus MNL-conditioned medium, and PMNs plus AmB-stimulated MNL-conditioned medium, were 100, 333 ± 80, 244 ± 55, and 1,078 ± 111 (mean ± standard error of the mean), respectively.

Pentoxifylline (100 μM) halved the chemiluminescence from AmB-stimulated MNL conditioned medium-primed fMLP-stimulated PMN if the pentoxifylline was added either with the AmB during formation of the MNL-conditioned medium or later, at the time that the MNL-conditioned medium was added to the PMNs (544 ± 55% and 467 ± 67% of unprimed PMN chemiluminescence, respectively; $P \leq 0.003$). Thus, pentoxifylline can act even if it is added after the formation of the conditioned medium.

Unlike sodium azide or β-carotene, pentoxifylline does not appear to act by inhibiting MPO and/or by quenching singlet oxygen. We observed that neither pentoxifylline (1 mM) nor its metabolites I and V at 10 μM affected MPO-dependent, luminol-enhanced chemiluminescence in a cell-free system in which we induced chemiluminescence by combining MPO, hydrogen peroxide, and luminol in HBSS (see Materials and Methods). We previously observed that pentoxifylline is not a superoxide scavenger (56).

Xanthine modulation of AmB-stimulated PMN superoxide release. In the absence of priming, superoxide release was below the threshold of detection. Priming with AmB (2 μg/ml for 90 min) increased fMLP-stimulated PMN superoxide release ($P = 0.033$) but did not significantly affect fMLP-stimulated MNL superoxide release. The greatest superoxide release was observed in AmB-primed, fMLP-stimulated PMN plus MNL samples. Pentoxifylline (100 μM) decreased AmB-primed, fMLP-stimulated PMN plus MNL superoxide release from 14.7 to 6.7 nmol/10 min ($P = 0.006$) (Table 1). Pentoxifylline, metabolite I, and metabolite V (10 μM) did not significantly affect AmB-primed, fMLP-stimulated PMN plus MNL superoxide release (11.0 ± 2.3, 11.6 ± 2.0, and 15.3 ± 2.7, nmol of superoxide/10 min, respectively).

TABLE 1. AmB (2 μg/ml) increases PMN superoxide release stimulated by fMLP (10^{-6} M): modulation by pentoxifylline (100 μM)

Amphotericin B	Superoxide release (nmol/10 min)[a]			
	PMN	MNL	PMN + MNL	PMN + MNL + pentoxifylline
Medium	−1.0 ± 0.5	5.5 ± 1.4	11.3 ± 3.3	Not determined
AmB	4.0 ± 1.8[b]	4.1 ± 0.8	14.7 ± 2.9	6.7 ± 1.6[c]

[a] Values are means ± standard errors of the mean (n = 5).
[b] P = 0.033; AmB increased PMN superoxide release compared with that by PMNs in the absence of AmB.
[c] P = 0.006; pentoxifylline (100 μM) decreased AmB-primed PMN plus MNL superoxide release.

Xanthine modulation of AmB-stimulated PMN oxidative activity in whole blood. As another indicator of oxidative activity we measured NBT reduction by PMNs in whole blood. AmB (2 μg/ml) increased the number of NBT-positive PMNs in whole blood from 39 to 53% (P = 0.029). Pentoxifylline (10 μM) did not affect the number of NBT-positive PMNs with or without AmB, but pentoxifylline (100 μM) decreased the number of positive PMNs in the presence of AmB (2 μg/ml) from 53 to 24% positive (P = 0.041) (Fig. 4). Metabolites I, IV, and V (10 μM) did not reduce the number of NBT-positive PMNs with or without AmB (data not shown).

Xanthine modulation of AmB-stimulated PMN Mac-1 expression in the presence of MNLs. The CD11b/CD18 adhesion integrin (Mac-1) is a major adherence factor of phagocytes. We have observed that AmB does not increase the expression of Mac-1 on pure PMNs but that it does increase PMN Mac-1 expression if MNLs are included in the samples (55). In the present study, AmB (2 μg/ml for 90 min) increased PMN Mac-1 expression in preparations containing MNLs to 210% that of PMNs in the absence of AmB (P = 0.003). Pentoxifylline (0.3 to 10 mM) decreased the amount

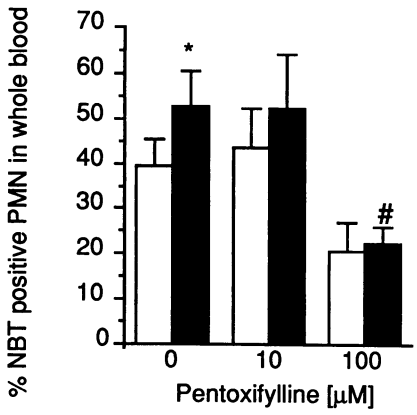

FIG. 4. Effect of AmB on PMN NBT reduction in whole blood: modulation by pentoxifylline. Whole blood was allowed to clot (30 min at 37°C) on glass coverslips with (■) or without (□) AmB (2 μg/ml) and with or without pentoxifylline, and the adherent cells were assayed for NBT reduction. The results are expressed as percent NBT-positive PMNs and are means ± standard errors of the means (n = 7 to 14 separate experiments). *, P = 0.029; AmB increased the number of NBT-positive PMNs. #, P < 0.050; pentoxifylline decreased the number of NBT-positive PMNs compared with the number without pentoxifylline.

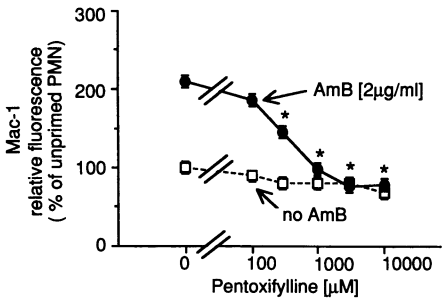

FIG. 5. Effect of AmB on PMN Mac-1 expression: modulation by pentoxifylline. PMNs (10^6/ml) plus MNLs (10^6/ml) in RPMI 1640 medium were preincubated with or without AmB (2 μg/ml) and with or without pentoxifylline for 90 min at 37°C, and then the PMNs were assayed for Mac-1 expression. The results are expressed as relative fluorescence compared with unprimed PMN activity (100%) and are means ± standard errors of the means (n = 3 to 4 separate experiments). *, P < 0.050; pentoxifylline decreased Mac-1 expression compared with that without pentoxifylline.

of AmB-stimulated PMN Mac-1 expression in the presence of MNLs (P < 0.050) (Fig. 5).

Xanthine modulation of AmB-decreased PMN motility. Diminished PMN motility is seen with inflammatory activators such as TNF-α (21, 47, 53) and IL-1β (57). It has been observed previously that AmB diminished PMN motility (9, 35, 69). We have observed that AmB decreases PMN migration and that AmB inhibition is greater in the presence of MNLs (55). In the present study, AmB decreased PMN-directed migration to fMLP from 2.2 ± 0.2 to 0.7 ± 0.3 mm (P < 0.001). When MNLs were included in the samples, AmB (2 μg/ml) decreased PMN-directed migration to fMLP from 1.6 ± 0.8 to 0.4 ± 0.1 mm (P < 0.001). Caffeine did not affect AmB-inhibited PMN migration, and pentoxifylline (micromolar to millimolar concentrations) counteracted AmB-inhibited PMN (with or without MNLs)-directed migration to fMLP (Fig. 6).

AmB (2 μg/ml) decreased PMN-nondirected migration (from 0.7 ± 0.1 to 0.2 ± 0.0 mm; P = 0.001). When MNLs were included in the samples, AmB (2 μg/ml) decreased PMN-nondirected migration from 0.5 ± 0.1 to 0.2 ± 0.0 mm (P = 0.010). Pentoxifylline (1 mM) (but not caffeine) counteracted the effects of AmB on PMN-nondirected migration in both the presence and the absence of MNLs (0.4 ± 0.0 and 0.3 ± 0.0 mm, respectively; P < 0.050).

Xanthines have little effect on AmB-stimulated human erythrocyte hemolysis. AmB lyses erythrocytes by binding to the cholesterol in the plasma membrane and causing the formation of small pores which allow passage of monovalent cations (13, 31). Interaction of AmB with mammalian cell membranes (including PMN plasma membranes) can be blocked by the addition of exogenous cholesterol (18), and AmB-induced hemolysis can be lessened by the addition of the H_2O_2 scavenger catalase (11). Desoxycholate is the detergent in which AmB is suspended. Desoxycholate (1.6 μg/ml, the concentration in AmB [2 μg/ml]) caused a small amount of hemolysis in 3 h (14 ± 1% hemolysis compared with 5 ± 2% hemolysis in the control preparations; P = 0.010). In contrast, AmB (2 μg/ml) caused 65 ± 11% hemolysis in 3 h (P < 0.001). We observed that pentoxifylline and caffeine had little effect on AmB (2 μg/ml)-stimulated hemolysis. Both pentoxifylline (100 to 1,000 μM), and caffeine (1,000 μM) minimally decreased AmB-stimulated human erythrocyte hemolysis (Fig. 7). Thus, it does not appear that

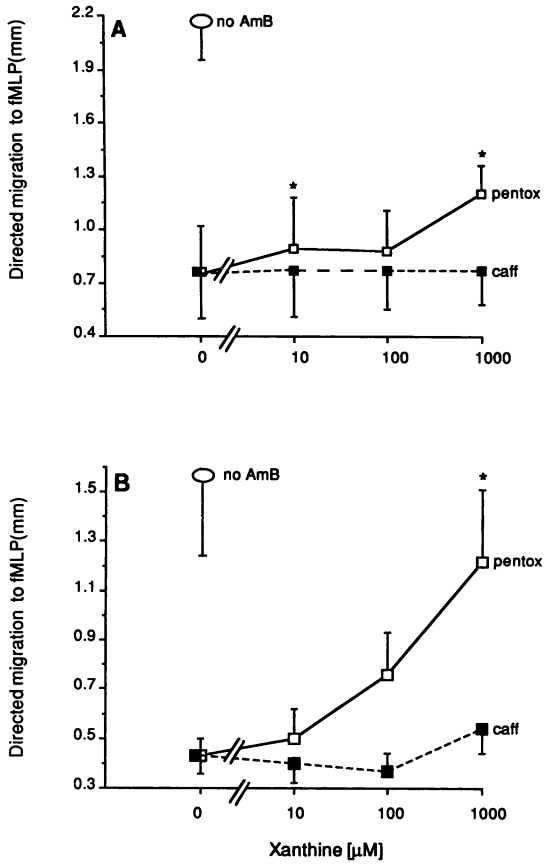

FIG. 6. Pentoxifylline (but not caffeine) counteracted AmB-inhibited PMN directed migration to fMLP (100 nM). Pure PMNs (5×10^6/ml) (A) or PMNs (5×10^6/ml) plus MNLs (3×10^6/ml) in HBSS–0.1% HSA (B) were preincubated with or without AmB, pentoxifylline (pentox), and caffeine (caff) for 90 min at 37°C; and then PMN migration was assayed under agarose. The results are expressed as leading-front PMN migration (in millimeters) and are means ± standard errors of the means ($n = 6$ separate experiments). *, $P < 0.050$; pentoxifylline counteracted AmB-inhibited PMN-directed migration.

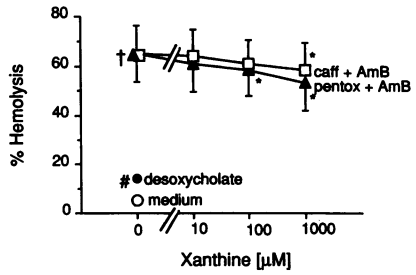

FIG. 7. AmB-induced human erythrocyte hemolysis is only slightly affected by pentoxifylline or caffeine. Erythrocytes in PBS were incubated (~25°C) for 180 min with or without AmB (2 μg/ml), desoxycholate (1.6 μg/ml), pentoxifylline (pentox), and caffeine (caff). The results are reported as percent hemolysis compared with a 100% hemolysis control in distilled water. The results are expressed as means ± standard errors of the means of duplicate samples from four separate experiments. #, $P = 0.010$; incubation with desoxycholate increased erythrocyte hemolysis. ##, $P < 0.001$; incubation with AmB increased erythrocyte hemolysis. *, $P < 0.050$; pentoxifylline and caffeine decreased AmB-induced erythrocyte hemolysis.

pentoxifylline acts to modify cell function by competitively binding AmB or by scavenging H_2O_2.

DISCUSSION

AmB increases pulmonary leukostasis stimulated by zymosan-activated plasma or phorbol myristate acetate (5). AmB administered 1 h prior to infusion of human PMNs preincubated with nylon wool fiber stimulates severe pulmonary reactions in rabbits (10). Lethal pulmonary reactions have been observed with the simultaneous administration of leukocyte transfusions and AmB in patients who were receiving leukocyte transfusions because of gram-negative septicemia (68). These studies indicate that AmB can exacerbate the inflammation mediated by activated leukocytes.

Our observations indicated that AmB can directly activate PMNs (e.g., AmB primes PMNs for increased chemiluminescence in response to fMLP) and can indirectly activate PMNs by stimulating the production of inflammatory mediators from MNLs (55). Pentoxifylline could be acting by blocking the production of mediators and/or by blocking the action of the mediators on PMNs. Pentoxifylline has been reported to block TNF-α production from endotoxin-stimulated macrophages and monocytes (20, 28, 54). Others have observed a release of TNF-α from AmB-stimulated macrophages and monocytes (17, 25).

Results of our experiments indicate that pentoxifylline acts predominantly at the level of the PMNs in the chemiluminescence experiments rather than altering mediator production by the MNLs, since the time of addition did not affect the ability of pentoxifylline to block priming by AmB-stimulated MNL-conditioned medium (i.e., pentoxifylline blocked PMN chemiluminescence just as well if it was added with the AmB to the MNLs during conditioned medium formation or later, when it was added with cell-free AmB-stimulated MNL-conditioned medium). We and others (6, 27, 29, 56–58) have observed that pentoxifylline diminishes the action of a number of inflammatory agents on PMN function, including endotoxin-stimulated MNL-conditioned medium, recombinant human TNF-α, purified human IL-1β, platelet activating factor, and fMLP.

The chemiluminescence response is dependent on cell activities that occur subsequent to superoxide release. These events result in the release of other toxic oxygen metabolites, including hydrogen peroxide (produced spontaneously and catalyzed by superoxide dismutase), hypochlorous acid (produced spontaneously and catalyzed by MPO), and singlet oxygen, which stimulates luminol-enhanced chemiluminescence. The sustained chemiluminescence response observed when PMNs were primed with AmB and then stimulated with fMLP may be a reflection of these other processes that occur within the PMNs after the initial production of superoxide. The differences in the responsiveness of the different assays for oxidative activity (i.e., cytochrome *c* assay, NBT reduction, and chemiluminescence) to modulation by pentoxifylline metabolites I and V may be an indication that these metabolites act at later points in PMN activation.

The expression of the PMN adherence integrin Mac-1 is stimulated by a number of inflammatory agents, including TNF-α (49). In the present experiments, AmB elicited the production of an MNL mediator(s) which stimulated PMN Mac-1 expression. The expression of Mac-1 on PMN makes the PMNs more prone to both aggregation and adherence.

AmB has been reported to increase both PMN adherence and aggregation (5, 9, 10, 35, 40, 69). By blocking AmB-induced Mac-1 expression on PMNs, pentoxifylline has the potential to block AmB-induced inflammatory tissue damage.

In vivo, pentoxifylline has been shown to decrease the action of inflammatory agents on host tissues in several different systems. Pentoxifylline decreases inflammatory tissue damage in animal models of endotoxic shock (33, 44, 52, 65), hemorrhagic shock (3, 64), IL-2 induced lung injury (30), and bacterial meningitis (50). Pentoxifylline decreased nephrotoxicity associated with AmB and cyclosporin treatment in patients receiving allograft bone marrow transplants (8).

The vasoconstricting action of AmB can be counteracted with the methylxanthine aminophylline (32, 51). Pentoxifylline has vasodilating activity on constricted blood vessels that is equal to that of aminophylline (26). Thus, vasodilation could contribute to pentoxifylline's in vivo activity. Since aminophylline and pentoxifylline appear to have opposite effects on AmB-induced PMN oxidative activity, it may be possible to sort out the contributions from vasoconstriction and PMN oxidative activity by comparing the effects of aminophylline and pentoxifylline in AmB-treated animals. This may be especially interesting in an infected host, in which the PMNs have the potential to be additionally primed by microbial factors.

Pentoxifylline is active in vivo at levels lower than the concentrations that are active in vitro. This apparent discrepancy might be explained by the added presence of pentoxifylline metabolites and the longer exposure times of mature and developing PMNs to pentoxifylline and its metabolites in vivo. Intravenous infusion into human allograft bone marrow recipients at 0.6 mg/kg of body weight per h for 7 days results in levels of pentoxifylline, metabolite I, and metabolite V in plasma of ~400, ~700, and ~1,200 ng/ml, respectively. The total concentration of pentoxifylline plus metabolites is ~8 μM (7).

Ex vivo PMNs from volunteers who received a single oral dose of pentoxifylline (400 mg) had a diminished chemiluminescence response to both fMLP- and zymosan-activated serum. There was a strong correlation between the levels of the principle metabolites (but not pentoxifylline) in serum and chemiluminescence. The most pronounced effect was observed 1.5 h after receiving the pentoxifylline (23). This same laboratory has since reported (19) that metabolites I, IV, and V at concentrations of as low as 0.3 μM are able to reduce PMN in vitro chemiluminescence in response to fMLP. At that concentration, metabolites IV and V reduced PMN lactoferrin release, and metabolite V reduced PMN Mac-1 expression. Thus, there is increasing evidence that the achievable levels of pentoxifylline and metabolites in serum can modulate both in vitro and in vivo activated PMN function.

By decreasing PMN oxidative activity and Mac-1 expression and by counteracting the adverse effects of AmB on PMN migration, pentoxifylline could be useful in ameliorating the toxicity of AmB.

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