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Modulation of Neonatal Neutrophil Function by Pentoxifylline

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ABSTRACT. Immunomodulating agents are being investigated for treatment of infection in newborn infants where morbidity and mortality remain high despite the continued development of new antibiotics. We studied the effect of the methylxanthine pentoxifylline on polymorphonuclear leukocyte (PMN) chemotaxis, F-actin content, and phagocytic activity as measured by nitroblue tetrazolium reduction and H_2O_2 production in neonates and adults to determine whether pentoxifylline might be useful in augmenting PMN function. The drug was found to have a dose-dependent effect on both neonatal and adult PMN function with enhancement at lower concentrations and suppression at higher concentrations. PMN chemotaxis increased 42% ($p < 0.01$) in neonates and 16% ($p < 0.05$) in adults at 100 μg/mL of pentoxifylline and it decreased 4 and 25%, respectively, at 4000 μg/mL. PMN nitroblue tetrazolium reduction increased by 34% in neonates and 23% ($p < 0.05$) in adults at 100 μg/mL of pentoxifylline and decreased by 52 ($p < 0.01$) and 74% ($p < 0.01$), respectively, at 2000 μg/mL. Similar dose-dependent responses were noted with F-actin content and H_2O_2 production. These and other observations support the hypothesis that pentoxifylline has a broad range of effects on PMN but that a primary effect is alteration of PMN deformability. Pentoxifylline has potential clinical use as an immunomodulator in augmenting impaired PMN function in neonates and other immunocompromised hosts or in suppressing excessive PMN activity in certain disease processes. (*Pediatr Res* 29: 123–127, 1991)

Abbreviations

NBT, nitroblue tetrazolium
PMN, polymorphonuclear leukocyte
NBD, nitrobenzoxadiazole

Newborn infants are at increased risk of morbidity and mortality from infection despite the continued development and use of new broad spectrum antibiotics and improvement in supportive care (1). The mortality rate from sepsis in neonates in the United States is approximately 20% compared to 5 to 10% for older children and adults. A primary reason for this problem is thought to be impairment in neonatal immunity and especially decreased PMN function (1–9). PMN are the major cellular elements that defend against bacterial invasion. Thus, therapeutic strategies that augment host defenses in the neonate such as transfusion of PMN or i.v. Ig have begun to be examined (10–18). The development of immunomodulating drugs that directly stimulate PMN function is an alternative approach that may offer advantages over PMN or i.v. Ig transfusion in regard to specificity of effect, ease of administration, availability, and side effects (10). A number of agents that enhance PMN function have been investigated but none are in routine clinical use and only a few, such as pentoxifylline, have been studied in newborn infants (8, 19–22).

Pentoxifylline is a methylxanthine that was introduced in 1984 for treatment of peripheral vasoocclusive disease. Sheetz, Wang, and Kreutzer were the first to demonstrate that pentoxifylline could enhance adult PMN motility *in vitro* (23). Subsequently, we found that pentoxifylline decreased the morbidity and mortality from experimentally induced *Staphylococcus aureus* infection in neonatal mice, due in part to enhancement of PMN motility (19, 21). To begin to investigate the potential utility of pentoxifylline in human neonates, Hill *et al.* (8) and Krause *et al.* (22) evaluated the *in vitro* effects of this drug on neonatal PMN chemotaxis and noted enhancement of PMN motility. Our study was designed to confirm and extend these findings to include pentoxifylline effect on other PMN responses and to further investigate the mechanism of action of pentoxifylline on PMN function. The results indicate that pentoxifylline can enhance or suppress PMN chemotaxis and phagocytic activity in both neonates and adults depending on the dose and that its effects appear to be mediated in part through alterations in PMN deformability.

MATERIALS AND METHODS

Study population. Blood was obtained from the fetal side of placentas (neonatal cord blood) within 5 min of birth. Placentas were obtained from healthy women whose babies were delivered by repeat cesarean section under regional anesthesia with no complications of pregnancy, labor, or delivery and whose newborns were in good health. Blood was also obtained from healthy adult volunteers on no medication. All blood samples were immediately mixed with preservative-free sodium heparin (50 μL/mL blood) and brought to the laboratory for analysis. Informed consent to sample blood was obtained from all parents or adult subjects in accordance with Hartford Hospital Institutional Review guidelines.

PMN chemotaxis assay. Whole blood was purified using 6% Hetastarch (McGaw Laboratories, Irvine, CA) that yielded a population of cells composed primarily of PMN and monocytes as previously described (24). The PMN were incubated in varying concentrations of pentoxifylline (0, 10, 100, 500, 1000, 2000, and 4000 μg/mL) for 10 min at 37°C before the chemotaxis

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assay. The pentoxifylline used in this assay and all other assays was a generous gift of Hoechst-Roussel Pharmaceuticals, Inc. (Somerville, NJ). It consisted of a powder preparation of the drug without additives. A standard liquid preparation was made by the addition of Medium 199 buffer to the powdered pentoxifylline. PMN chemotaxis was measured using a modified micropore filter assay (24). In brief, 5×10^5 PMN/0.7 mL were placed in the upper compartment of a modified Boyden chamber (Ahlco Corporation, Southington, CT) over cellulose nitrate filters with a diameter of 13 mm and a pore size of 5 μm (Sartorius Filters, Hayward, CA). Stabilized zymosan-activated normal serum (3%) was placed in the lower compartment. The PMN were allowed to penetrate the filter during a 90-min incubation in a humid 5% CO_2 atmosphere at 37°C. The filters were removed, fixed, stained, clarified, and mounted on slides. An automated microsectioning counting technique was used to determine the number of PMN at 10-μm increments from 20 to 120 μm into the filter. Because we used a mixed leukocyte suspension, the first 20 μm of the filter were not examined to avoid inclusion of monocytes and lymphocytes in the cell counts. The mean distance migrated by the PMN in 90 min of incubation time, or LI_{20}, was then determined. A mean LI_{20} value of triplicate determinations was calculated for each blood sample.

PMN actin polymerization assay. Actin is a major protein component of the PMN cytoskeleton that plays a critical role in determining cell deformability, redistribution of cell surface receptors, and cell motility (25). The amount of actin can be measured in its condensed form (F-actin) using NBD phallacidin, a fluorescent probe that specifically binds F-actin. We used NBD phallacidin to determine the effect of pentoxifylline on the F-actin content in neonatal and adult PMN. The effects of pentoxifylline on PMN actin was determined using a standard PMN actin polymerization assay (25). In brief, PMN were separated from whole blood with Hetastarch, washed in Hanks buffer, and standardized to 1.25×10^6 cells/mL. PMN aliquots were then incubated either in Medium 199 buffer or 10^{-8} f-methionylleucylphenylalanine for 10 min at 37°C followed by the addition of varying concentrations of pentoxifylline for 1 min in a gentle shaking water bath. The reaction was stopped by the addition of 37% formalin at 25°C for 15 min. The PMN were then added to a test tube that contained evaporated NBD phallacidin (25 μL/tube) followed by incubation in 10 μL of lysophosphatidylcholine at 37°C for 10 min. The PMN were centrifuged at $150 \times g$ for 5 min, resuspended in Hanks buffer, and immediately analyzed on the flow cytometer at a wavelength of 488 nm.

PMN phagocytosis (NBT) assay. The effect of pentoxifylline on PMN phagocytosis was determined using a NBT assay (26). In brief, PMN were separated from whole blood with Hetastarch, washed in Medium 199, and standardized to 5×10^6 cells/mL. A total of 75 μL each of potassium cyanide and NBT were added to the PMN (300 μL). Aliquots of the PMN solutions were then mixed with either Medium 199 (control) or opsonized zymosan followed by incubation with various concentrations of pentoxifylline for 15 min at 37°C in a shaking water bath. The reaction was stopped with 1 N HCl, decanted, and pyridine was added. This mixture was placed in a boiling water bath for 15 min, centrifuged at $650 \times g$, and the supernatant placed in a glass cuvette for analysis in a spectrophotometer at 550 nm. The average of triplicate samples was obtained and the difference between zymosan and control samples determined.

PMN H_2O_2 production. The effect of pentoxifylline on PMN H_2O_2 production was evaluated using a simple colorimetric assay (27). PMN (4×10^6/mL) separated from whole blood by Ficoll Hypaque followed by dextran sedimentation were incubated in varying concentrations of pentoxifylline with phenol red, horseradish peroxidase, and either opsonized zymosan (0.5 mg/mL) or Hanks buffer in a 37°C water bath for 1 h. The PMN were then centrifuged and the supernatant removed. The supernatant was placed in a glass cuvette after the addition of 10 M NaOH. Spectrophotometric readings were taken at 618 nm. The amount of H_2O_2 produced was determined by comparing the absorbance of the PMN solution with a standard absorbance curve generated by varying concentrations of H_2O_2.

Statistical analysis. Statistical significance of differences between groups was performed using the two-tailed t test with Bonferroni correction (28).

RESULTS

PMN chemotaxis. Pentoxifylline caused a significant increase in neonatal and adult PMN chemotaxis at concentrations of 100 and 500 μg/mL as shown in Figure 1. The percent change in PMN chemotaxis caused by pentoxifylline was significantly greater in neonates than in adults at 100 (42 *versus* 16%, $p = 0.014$) and 500 μg/mL (44 *versus* 30%, $p < 0.01$), indicating that the drug had a greater effect on PMN with impaired PMN chemotaxis. In separate experiments, pentoxifylline was found to inhibit adult (−25%, NS) and neonatal (−4%, NS) PMN chemotaxis at a concentration of 4000 μg/mL. Pentoxifylline was not found to be cytotoxic to PMN as demonstrated by trypan blue exclusion after incubation in 10, 50, 100, 1000, 2000, or 4000 μg/mL of pentoxifylline for 15 min at 37°C.

PMN F-actin polymerization. To help clarify the mechanism of pentoxifylline effect on PMN chemotaxis, neonatal and adult PMN were incubated in buffer or pentoxifylline and tested for F-actin content. In a time-response study, pentoxifylline (500 mg/mL) was found to maximally decrease F-actin after a 60-s exposure. Using this exposure time in PMN pretreated with f-methionylleucylphenylalanine, pentoxifylline was found to significantly decrease the amount of adult and neonatal PMN F-actin in a dose-dependent manner as shown in Figure 2. Pentoxifylline had no significant effect on the F-actin content of resting PMN.

PMN NBT reduction. The effect of pentoxifylline on adult PMN NBT reduction was similar to that on PMN chemotaxis with enhancement at lower concentrations (10–100 μg/mL) and impairment at higher concentrations (1000–2000 μg/mL) (Fig. 3). Pentoxifylline significantly increased adult PMN NBT reduction at 10 and 100 μg/mL ($p < 0.05$ and $p < 0.05$) and significantly decreased it at 1000 and 2000 μg/mL ($p < 0.01$ and $p < 0.01$). There was a similar pattern for neonatal PMN;

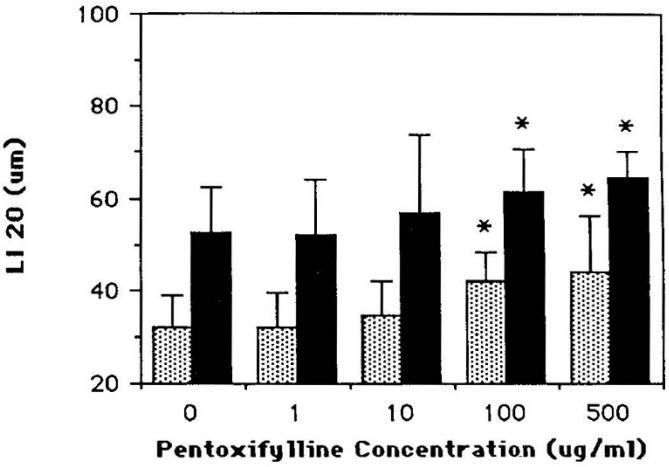

Fig. 1. The effect of pentoxifylline on PMN chemotaxis. PMN were obtained from 10 adults and 10 neonates and incubated in varying concentrations of pentoxifylline, and PMN chemotaxis was determined. Mean PMN chemotactic values (±1 SD) are shown for neonates (*stippled bars*) and adults (*solid bars*). Mean PMN chemotactic values after pentoxifylline exposure were significantly increased compared with control at pentoxifylline concentrations of 100 and 500 μg/mL in neonates ($p < 0.05$ and $p < 0.01$, respectively) and adults ($p < 0.01$ and $p < 0.02$, respectively). *Asterisks* indicate a statistically significant difference from control.

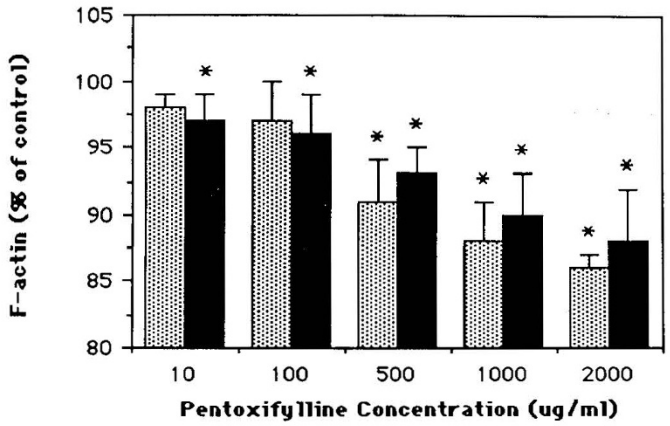

Fig. 2. The effect of pentoxifylline on PMN F-actin. The mean change in PMN F-actin content (mean percent change ± 1 SD) with varying concentrations of pentoxifylline was simultaneously determined in six neonates (*stippled bars*) and six adults (*solid bars*). Pentoxifylline caused a significant decrease in f-methionylleucylphenylalanine-stimulated F-actin content in adult PMN at all concentrations ($p < 0.01$ except at 10 μg/mL where $p < 0.05$) and in neonatal PMN at 500, 1000, and 2000 μg/mL ($p < 0.02$, $p < 0.01$, and $p < 0.01$, respectively). *Asterisks* indicate a statistically significant difference from control.

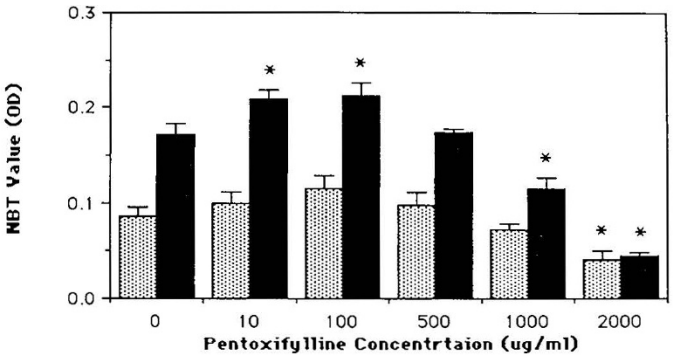

Fig. 3. The effect of pentoxifylline on PMN NBT reduction. The effect of pentoxifylline (mean values ± 1 SEM) in seven neonates (*stippled bars*) and eight adults (*solid bars*) was determined. Pentoxifylline enhanced PMN NBT reduction at lower concentrations (10 and 100 μg/mL) and decreased it at higher concentrations (1000 and 2000 μg/mL). Pentoxifylline significantly increased adult PMN NBT reduction at 10 μg/mL ($p < 0.01$) and 100 μg/mL ($p < 0.05$) and significantly decreased it at 1000 and 2000 μg/mL ($p < 0.01$ for both). There was a similar pattern for neonatal PMN; however, only a decrease at 2000 μg/mL was statistically significant ($p < 0.01$). *Asterisks* indicate a statistically significant difference from control.

however, only the decrease at 2000 μg/mL was statistically significant ($p < 0.01$).

PMN oxidative metabolism. To further clarify the mechanism of action of pentoxifylline on NBT reduction, we examined the effect of pentoxifylline on PMN H_2O_2 production when activated by zymosan. Although there was a similar pattern of pentoxifylline enhancement and impairment of H_2O_2 production as with chemotaxis and NBT production, the only significant effect was impairment of H_2O_2 production in adult PMN at pentoxifylline concentrations of 100 and 1000 μg/mL and in neonatal PMN at a pentoxifylline concentration of 1000 μg/mL (Fig. 4).

DISCUSSION

Pentoxifylline is a xanthine derivative related to caffeine and theophylline. It was discovered in the 1950s, found to decrease blood viscosity in the 1970s, and released for use in peripheral vascular disease in 1984 (29). Initial studies indicated that its

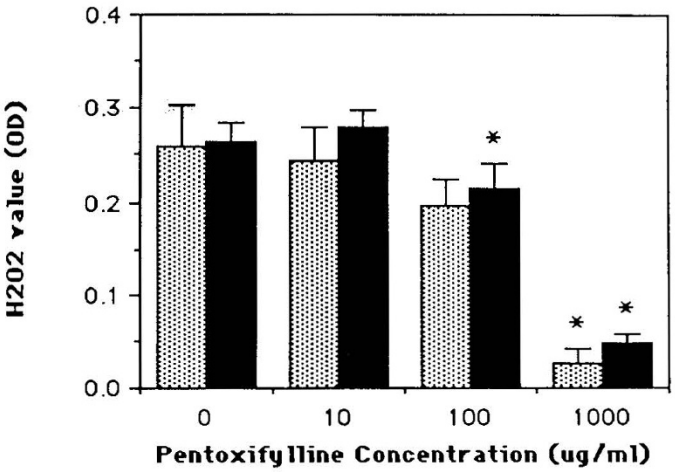

Fig. 4. The effect of pentoxifylline on PMN H_2O_2 production. Neonatal and adult PMN were simultaneously incubated in varying concentrations of pentoxifylline after zymosan stimulation and the amount of H_2O_2 produced determined. The mean H_2O_2 values ± 1 SEM for five consecutive experiments are shown (neonates, *stippled bars*; adults, *solid bars*). Pentoxifylline caused a significant decrease in H_2O_2 production in adult PMN at 100 and 1000 μg/mL ($p < 0.05$ and $p < 0.01$) and in neonatal PMN at 1000 μg/mL ($p < 0.01$). *Asterisks* indicate a statistically significant difference from control.

effect on blood viscosity was due to enhancement of erythrocyte flexibility (30). In 1983, Sheetz, Wang, and Kreutzer presented data suggesting an additional explanation for the hemorheologic properties of the drug: destabilization of the cell membrane of the PMN (23). They also discovered that pentoxifylline could increase PMN motility, suggesting that the drug might be used as an immunomodulator. It was immediately apparent that newborn infants might benefit from pentoxifylline immunomodulation because they had impairment in PMN motility that was thought to contribute to their increased morbidity and mortality from infection (2). We therefore initiated a study of the effects of pentoxifylline on PMN function in newborn mice. The drug was found to enhance both *in vitro* PMN motility and *in vivo* PMN accumulation and to decrease morbidity and mortality from *S. aureus* infection (19, 21). Studies by Hill *et al.* (8) and Krause *et al.* (22) indicated that neonatal PMN chemotaxis was increased by the addition of pentoxifylline. We undertook the present study to determine whether pentoxifylline could enhance PMN phagocytosis and oxidative metabolism as well as chemotaxis in human newborns. The results demonstrate that pentoxifylline can either enhance or suppress PMN chemotaxis and phagocytic activity. In neonates, pentoxifylline enhanced PMN chemotaxis but not phagocytosis. In adults, pentoxifylline enhanced both PMN chemotaxis and phagocytosis. Because H_2O_2 production was not increased by pentoxifylline in adult PMN, enhancement of the NBT reaction of adult PMN may be due to increased engulfment of bacteria (zymosan) rather than increased oxidative metabolism.

The concentration of pentoxifylline required for enhancement of PMN chemotaxis in neonates and adults (≥50 μg/mL) is higher than concentrations of the drug achievable with current dosing guidelines in humans where 2 to 4 mg/kg of pentoxifylline given by the oral route to adults produces average peak drug concentrations of 0.5 to 1 μg/mL (31). The maximum reported dose of pentoxifylline administered to humans to date has been approximately 3 mg/kg given i.v. resulting in a peak blood concentration of 2.5 μg/mL (32). A similar dose in neonatal mice (5 mg/kg s.c.) has been shown to result in a similar peak pentoxifylline blood concentration (1 μg/mL) (21). Maderazo *et al.* (21) found that peak pentoxifylline blood concentrations of ≥50 μg/mL, noted to enhance PMN function in the present study, could be achieved in neonatal mice when given doses of

≥50 mg/kg s.c. Doses of 50 and 100 μg/kg in neonatal mice were well tolerated, augmented PMN function, and decreased morbidity and mortality from infection. Furthermore, a pentoxifylline dose as low as 15 mg/kg s.c. given to neonatal mice was shown to decrease morbidity and mortality from *S. aureus* infection (21). The 15-mg/kg dose in mice could be expected to result in a peak blood concentration of approximately 15–20 μg/mL, a concentration that is lower than the 50 μg/mL of pentoxifylline found necessary in the present study to augment human PMN function. The apparent increased sensitivity of mouse PMN to the effects of pentoxifylline might be due to variability in the pentoxifylline response of PMN in mice and humans; however, other factors may account for the apparent difference. A number of pentoxifylline metabolites that may have similar activity to pentoxifylline were likely present in mice in previous *in vivo* studies but absent in the present *in vitro* study (19, 21, 31). Pentoxifylline has also been shown to affect cells that have significant effects on PMN function, which were likely present in the previous *in vivo* experiments but were absent in the present *in vitro* experiments. For example, Sullivan *et al.* (33) have shown that the effects of macrophage-derived IL-1 and tumor necrosis factor on PMN are inhibited by pentoxifylline, and Hammerschmidt *et al.* (34) have shown that pentoxifylline inhibits the effects of platelet-activating factor on PMN. Finally, pentoxifylline may enhance immune activity through mechanisms other than stimulation of PMN function. In summary, it would appear possible that pentoxifylline could be administered to human neonates in a dose that would enhance PMN function and protect against infection but not result in excessive drug toxicity. Further studies will be necessary to confirm this hypothesis.

The clinical use of pentoxifylline or other PMN immunomodulating agents may be complicated by the fact that although enhancement of PMN function may improve the outcome from infection, it may also increase the possibility of tissue damage due to increased PMN reactivity. For example, several studies have implicated activated PMN in the pathogenesis of bronchopulmonary dysplasia (35, 36). Appropriate timing of immunomodulation therapy might help address this problem. Recent studies in our laboratory have suggested that the PMN chemotactic impairment in newborn infants is only present during the first 2 wk of life (37). Pentoxifylline therapy in newborn infants might therefore only be used in low dosage in the first 2 wk of life to enhance PMN function and thus help combat infection. It might even be considered for use in high dosage after 2 wk of age to suppress PMN activation and thus limit the severity of bronchopulmonary dysplasia. It would appear unlikely, however, that the high pentoxifylline concentrations (≥1000 μg/mL) found necessary in this study to inhibit PMN function could be safely achieved in humans.

Data from our study provide further insight into the mechanism of impaired PMN motility in the neonate. Previous studies have indicated that PMN motility is dependent, in part, on cell deformability and that neonatal PMN have decreased cell deformability compared with adult PMN (20, 38–40). Decreased deformability of the cytoskeleton and decreased fluidity of the cell membrane have been demonstrated in neonatal PMN. Recently, the molecular mechanisms causing the decreased deformability of the neonatal PMN have begun to be investigated. Hilmo and Howard (41), Sacchi *et al.* (42), and Newton *et al.* (20) have shown that the amount of basal F-actin in neonatal PMN is increased compared with that of adults. Actin is a critical structural element of the PMN cytoskeleton and in its polymerized form (F-actin) gives rigidity to the PMN. Rao *et al.* (43) showed that pentoxifylline decreases basal F-actin levels in the adult PMN. Data from Newton *et al.* (20) and from our study indicate that pentoxifylline increases PMN deformability and decreases levels of F-actin in neonatal PMN. There is also evidence that pentoxifylline may act directly to destabilize the cell membrane and thus increase PMN deformability (23, 43, 44).

In summary, the results of this study support the need for further research on the immunomodulating effects of pentoxifylline in both neonates and adults. Further investigation may lead to the clinical use of pentoxifylline for neonatal inflammatory illness and evaluation of its mechanism of action on PMN function may contribute to our understanding of the etiology of neonatal PMN dysfunction.

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