Effect of pentoxifylline on the course of systemic *Candida albicans* infection in mice


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Pentoxifylline can decrease the production of tumour necrosis factor alpha (TNFα) by endotoxin-stimulated macrophages and may improve survival in animals with overwhelming bacterial sepsis. In this study various doses of pentoxifylline were administered to mice with systemic *Candida albicans* infection to determine its effect on serum TNFα levels, organ fungal burden, and host survival. Intraperitoneal injections of pentoxifylline at 20 mg/kg every 8 h did not affect these endpoints. However, fungal counts were significantly higher in kidneys of animals that received 30 and 60 mg/kg of pentoxifylline every 8 h when compared to controls. Injection of 60 mg/kg of pentoxifylline at 8 h intervals also significantly shortened mean survival from 5.8 to 3.8 days (*P* = 0.01). Pentoxifylline did not affect peripheral WBC counts, serum TNFα and interleukin-6 levels, or the density of neutrophils in tissues. *In vitro*, pentoxifylline decreased the production of TNFα by *C. albicans*-stimulated macrophages in a dose-dependent manner, but only at concentrations greater than 100 mg/L. In contrast, pentoxifylline suppressed TNFα production by endotoxin-stimulated macrophages at concentrations as low as 10 mg/L. Thus, higher doses of pentoxifylline are detrimental in systemic *C. albicans* infection. However, the detrimental effect is not mediated by alterations in serum TNFα or interleukin-6 levels or the aggregation of neutrophils in tissues.

Introduction

Tumour necrosis factor alpha (TNFα) is a 17-kDa polypeptide cytokine (Beutler *et al.*, 1985) that is produced by mononuclear phagocytes in response to Gram-positive and Gram-negative bacteria, certain viruses and fungi (Beutler *et al.*, 1986; Havell, 1987; Strieter *et al.*, 1988; Allendoerfer *et al.*, 1993). In overwhelming bacterial infections, TNFα initiates a series of metabolic and haemodynamic responses that lead to sepsis syndrome and septic shock (Tracey *et al.*, 1987). Administration of anti-TNFα antibody
preceding bacterial infection decreases the severity of end-organ injury and improves survival (Tracey et al., 1987; Hinshaw et al., 1990; Opal et al., 1990).

Pentoxifylline is a methylxanthine compound that has many notable properties. It can decrease the rheologic properties of blood and, thus, is prescribed for the treatment of ischaemic peripheral vascular disease (Ward & Clissold, 1987). In addition, pentoxifylline can decrease the production of the cytokines TNFα and interleukin-1 by mononuclear phagocytes in response to bacterial endotoxin (Strieter et al., 1988; Saez-Llorens et al., 1990). It can also attenuate the production of superoxide radicals by endotoxin-stimulated macrophages and neutrophils (Bessler et al., 1986; Sullivan et al., 1988). In vivo, pentoxifylline decreases serum concentrations of TNFα in animals infected with Gram-negative bacteria and improves host survival (Noel et al., 1990).

We have shown that mice produce TNFα in response to systemic infection with the fungus Candida albicans (Louie et al., 1994A). However, in contrast to the deleterious effect of this cytokine in bacterial sepsis, the TNFα that is produced by the host in response to systemic C. albicans infection has a beneficial role. It augments the capacity of the host to inhibit the growth of C. albicans in organs and prolongs host survival (Louie et al., 1994B). These beneficial effects of endogenous TNFα persist in the face of amphotericin B or fluconazole therapy (Louie et al., 1995). Although we have demonstrated that pentoxifylline can decrease the production of TNFα by C. albicans-stimulated RAW 264.7 macrophages in vitro (Louie et al., 1994A), it is unclear whether pentoxifylline can decrease the TNFα that is produced in animals in response to systemic C. albicans infection. If an in-vivo effect is seen, it may potentially have a deleterious effect on outcome.

In this study we investigated the effect of pentoxifylline on the fungal burden in the kidney and on survival of mice that were systemically infected with C. albicans. We also evaluated the impact of pentoxifylline on the host inflammatory response to C. albicans infection in tissues and peripheral WBC counts. Finally, we correlated these findings with serum TNFα and interleukin-6 (IL-6) levels to determine whether the effect of pentoxifylline on the course of systemic candidiasis is mediated by an attenuation of cytokine production.

Material and methods

C. albicans

Strain 88-689-6 was isolated from the blood of a neutropenic patient. The microorganism was maintained on Sabouraud-dextrose agar (BBL Microbiology Systems, Cockeysville, MD, USA) at 22°C until use. For each study, two to three colonies of C. albicans were subcultured on to potato-dextrose agar (BBL) and incubated at 35°C for 48 h. A fungal suspension was prepared with sterile, pyrogen-free, phosphate-buffered saline (Gibco-BRL Inc. Grand Island, NY, USA) and quantified with a haemocytometer. For the in-vitro studies, the suspension was diluted to the required concentrations with complete DMEM. Complete DMEM consisted of Dulbecco’s modified Eagle’s medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 5% fetal calf serum (Sigma), 100,000 IU/L penicillin, 100 mg/L streptomycin, L-glutamate, pyruvate, and non-essential amino acids. For the in-vivo studies, the fungal suspension was diluted with sterile, pyrogen-free phosphate-buffered saline (PBS) to the desired concentration.
Morphologic examination demonstrated that >99% of the organisms were blastoconidia. The viability of the yeast was >95% by trypan blue exclusion and quantitative cultures. The endotoxin concentration in the fungal suspension was <0.05 EU (endotoxin units) per mL by competitive endotoxin ELISA (PyroChek competitive lipopolysaccharide ELISA, ALerCHEK, Inc., Portland, ME, USA).

**Mice**

Female, 18–20 gram NYLAR mice were raised at the Animal Research Facility of the Wadsworth Center for Laboratories and Research (Griffin Laboratories, Guilderland, NY, USA). These outbred Swiss mice were housed in hanging metal cages and received food and water ad libitum. Animal experimentation was conducted in accordance to the guidelines established by the Institutional Animal Care and Use Committees of the investigators’ respective institutions.

**Pentoxifylline**

Pentoxifylline powder was provided by Dr. William Novick Jr (Hoeschst-Roussel, Somerville, NJ, USA). The potency of the drug was confirmed by the manufacturer just prior to the initiation of this study. Pentoxifylline was dissolved in sterile, pyrogen-free water to produce a stock solution of 20,000 mg/L. The stock solution was passed through a 0.45 μm filter (Lida Manufacturing, Kenosha, WI, USA). For the in-vitro studies, the drug was prepared in and diluted to the desired concentrations with complete DMEM. For the in-vivo studies the drug was diluted to the desired concentrations with PBS. The drug was used immediately after preparation. The solutions contained less than 0.25 EU of endotoxin per mL by limulus amoebocyte lysate (LAL) assay (Whittaker MA Bioproducts, Walkersville, MD, USA).

**Systemic candidiasis model**

For each in-vivo experiment mice were divided into four groups of 28 animals. Group I received pentoxifylline 20 mg/kg; group II pentoxifylline 30 mg/kg; group III pentoxifylline 60 mg/kg; and group IV received PBS. Pentoxifylline or PBS was administered every 8 h in 0.2 mL volumes via the intraperitoneal route. Forty-eight hours after the first injection of pentoxifylline or PBS, all mice were intravenously infected with 2 x 10⁶ C. albicans blastoconidia via the lateral tail vein. Two to three mice from each group were killed by CO₂ asphyxiation at 0, 2, and 4 days of infection. Animals were killed immediately before the next scheduled pentoxifylline injection. A sample of blood was obtained from each mouse by cardiac puncture for WBC determination. Clotted blood was cultured non-quantitatively on Mueller-Hinton agar (BBL) for fungi and bacteria. After 48 h of incubation at 35°C, individual colonies were characterized as fungi or bacteria by Gram's staining and quantified. Mice were observed twice daily for survival. Earlier studies demonstrated that mice died within 4 h after becoming moribund. Thus, moribund mice were killed. This study was conducted twice.

Preliminary studies demonstrated that the intraperitoneal administration of pentoxifylline at doses of 20, 30, and 60 mg/kg every 8 h for 14 days was not toxic to
healthy mice. The animals remained well for at least 28 days so there was no need to include non-infected pentoxifylline controls in the above studies.

**Effect of pentoxifylline on serum TNFα and IL-6 levels**

For animals that received 0, 20, 30, or 60 mg/kg of pentoxifylline every 8 h, blood was collected by cardiac puncture from mice that were killed at 0 and 48 h of infection. Animals were killed immediately prior to a scheduled pentoxifylline injection. The blood from each group was pooled and allowed to clot on ice. The serum was separated from the clot by centrifugation and stored at −70°C. The serum was evaluated for TNFα and IL-6 by ELISA (see below). This portion of the study was conducted twice.

To further define the effect of pentoxifylline on serum TNFα and IL-6 levels, a third trial was performed. Mice were injected intraperitoneally with 60 mg/kg of pentoxifylline or PBS every 8 h beginning 48 h before they were intravenously infected with $2 \times 10^6$ cfu of *C. albicans*. On days 0, 1, and 2 after infection, 2 to 3 mice were killed by CO$_2$ asphyxiation at 0, 1, 2, 3, and 4 h after pentoxifylline or PBS injection and the serum was collected and assayed for cytokine content as described.

**Semi-quantitative organ cultures**

On days 2 and 4 of each in-vivo experiment, the right kidney was aseptically collected from killed mice. Semi-quantitative organ cultures were conducted, in duplicate, by plating serial dilutions of homogenized tissue on to Sabouraud-dextrose agar (BBL) supplemented with 100,000 IU of penicillin and 100 mg of streptomycin per litre of agar (Sigma). After 48 h of incubation, at 35°C, the colonies were counted. To monitor for bacterial suprainfection, 0.1 mL (0.1 g) of homogenized tissue was plated on Mueller-Hinton agar (BBL) supplemented with 2 mg/L of amphotericin B (Sigma). These plates were incubated for 18–24 h, then read. The semi-quantitative cultures reproducibly detected $\geq 100$ organisms per gram of tissue.

In preliminary studies we found that pentoxifylline at concentrations up to 1000 mg/L of agar did not inhibit the growth of *C. albicans*.

**Histology**

On days 0, 2, and 4 of infection, a portion of the right kidney was collected from killed animals and preserved in 10% buffered formalin (Fisher Scientific Inc., Rochester, NY, USA) for histology. Sections were processed with haematoxylin-eosin, periodic acid-Schiff, Gomori’s methenamine-silver, and trichrome stains. The size and number of foci of *C. albicans* in tissues, the ratio of blastoconidia to pseudohyphae in these foci, and the intensity of the inflammatory response in tissues were assessed semi-quantitatively by a single investigator (J. K. S.). This investigator was blinded to the treatments administered to the experimental groups.

**NYLAR peritoneal macrophages**

Peritoneal elicited macrophages (PEMs) were collected from NYLAR mice five days after the animals were injected intraperitoneally with 0.5 mL of sterile, pyrogen-free thioglycollate broth (BBL). The animals were killed by CO$_2$ asphyxiation. PEMs were
harvested by rinsing the exposed peritoneal cavity with complete DMEM. The PEMs were washed with complete DMEM, quantified by haemocytometer, and diluted to $1 \times 10^6$ cells per mL with medium. Then 1 mL aliquots of the cell suspension were added to wells of 24-well tissue culture plates (Corning Glass Works, Corning, NY, USA) and incubated at 37°C, 5% CO$_2$ for 2 h. The wells were vigorously washed with complete DMEM to remove non-adherent cells. The macrophages were used immediately in the in-vitro study described below.

**In-vitro study**

Peritoneal-elicited macrophages were incubated with 0, 1, 10, 50, 100, 500, or 1000 mg/L of pentoxifylline for 1 h. Then the medium was replaced with complete DMEM which contained the desired concentrations of pentoxifylline and $2 \times 10^4$ cfu of *C. albicans* or 2.5 μg/L of *Escherichia coli* 055:B5 lipopolysaccharide (Sigma). After the cultures were incubated at 37°C, 5% CO$_2$ for 0, 3, 6, and 24 h, the supernatants were collected, centrifuged, collected, and immediately stored at $-70^\circ$C. Supernatants were tested for TNFα and IL-6 content by ELISA. Wells containing complete DMEM, *C. albicans*, *E. coli* endotoxin, or incremental concentrations of pentoxifylline, incubated with and without macrophages, served as controls. This study was conducted twice.

**TNFα and IL-6 quantification**

For the in-vitro and in-vivo studies, samples from each group and time were analyzed for TNFα and IL-6 with ELISAs. Each sample was assayed in duplicate and the results were averaged. The TNFα and IL-6 ELISAs were purchased from Genzyme Inc. (Cambridge, MA, USA) and Endogen Inc. (Boston, MA, USA), respectively. The TNFα and IL-6 ELISAs detected only biologically active TNFα and IL-6, respectively (manufacturers' instruction manuals). Pentoxifylline, *C. albicans* and *E. coli* endotoxin, alone and in combination, did not alter the capacity of the TNFα and IL-6 ELISAs to detect known amounts of TNFα and IL-6, respectively, in complete DMEM and normal mouse serum. The lower limit of detection of the TNFα ELISA was 50 ng/L. The sensitivity of the IL-6 ELISA was $\geq 15$ ng/L. The coefficient of variance of the TNFα and IL-6 ELISAs were 8% and 9%, respectively.

**Endotoxin analysis**

All reagents, *C. albicans* suspensions, cell-culture supernatants, and serum samples were tested for endotoxin with a competitive lipopolysaccharide ELISA (ALerCHEK) or by LAL assay (Whittaker MA Bioproducts). The lower limit of the ELISA and LAL endotoxin assays were 0.05 and 0.25 EU/mL, respectively.

**Statistical analysis**

The significance of differences between TNFα and IL-6 levels, mean survival of infected animals, peripheral WBC counts, and organ colony counts in experimental and control groups was determined by using the Student-Neuman-Keuls test for multiple comparisons. A difference was considered statistically significant at $P < 0.05$. Error bars in graphs represent 1 S.E.M.
Results

Effect of pentoxifylline on survival of infected mice

The survival results of two separate trials were similar. Therefore, the results were combined. The mean survival of mice that received 20 mg/kg of pentoxifylline was similar to that of controls (5.7 and 5.8 days, respectively, \( P > 0.05 \)). As demonstrated in Figure 1, mean survival of animals that were given 8 h injections of 30 and 60 mg/kg of pentoxifylline progressively decreased (5.0 and 3.8 days, respectively, versus 5.8 days for infected controls). However, the difference was statistically significant only for animals that received the highest dosage (\( P = 0.01 \) for recipients of pentoxifylline 60 mg/kg every 8 h when compared with controls). Non-infected mice that received 20, 30, and 60 mg/kg of pentoxifylline every 8 h for 14 days remained well for a 28 day observation period.

Quantitative organ cultures

On days 2 and 4 of infection colony counts in kidneys were significantly higher in recipients of 30 and 60 mg/kg of pentoxifylline than controls (Table). The fungal burden in kidneys of recipients of 20 mg/kg of pentoxifylline were similar to controls.

Blood cultures

On days 2 and 4 of infection, non-quantitative cultures of blood of controls and pentoxifylline recipients all yielded *Candida* (data not shown).
Table. Fungal counts \((\times 10^4 \text{ cfu/g} \pm \text{s.e.m.})\) in kidneys of mice two and four days after intravenous infection with \(2 \times 10^6\) cfu of *Candida albicans*. Mice received intraperitoneal injections of the specified doses of pentoxifylline (Pentox) or phosphate-buffered saline (PBS) at 8 h intervals beginning 48 h prior to fungal injection.

<table>
<thead>
<tr>
<th></th>
<th>Day 2</th>
<th>Day 4</th>
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<tbody>
<tr>
<td>PBS control</td>
<td>38.25 ± 3.42</td>
<td>142.67 ± 10.13</td>
</tr>
<tr>
<td>Pentox 20 mg/kg</td>
<td>42.37 ± 4.48</td>
<td>136.24 ± 9.31</td>
</tr>
<tr>
<td>Pentox 30 mg/kg</td>
<td>80.50 ± 9.37*</td>
<td>293.46 ± 7.38*</td>
</tr>
<tr>
<td>Pentox 60 mg/kg</td>
<td>66.00 ± 9.70*</td>
<td>312.51 ± 9.78*</td>
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*P < 0.05 when compared with control.

*P < 0.01 versus control.

**Serum cytokine levels**

Serum concentrations of TNFα and IL-6 for animals that received 20, 30, or 60 mg/kg of pentoxifylline at 8 h intervals were similar to those of controls on days 1, 2, and 3 of infection. The results of day 2 of infection for two trials are shown in Figure 2. TNFα and IL-6 were not detected in the serum of non-infected animals that received either pentoxifylline or PBS. Endotoxin was not detectable in any of the serum samples. All organ and blood cultures were sterile for bacteria.

**Histologic studies**

The number and size of *C. albicans* foci in kidneys were similar in all experimental groups on days two and four of infection. The proportion of fungi that were blastoconidia and pseudohyphae or hyphae was not affected by pentoxifylline. Also, the exuberant neutrophil inflammatory response in tissues was similar (data not shown).

**Peripheral WBC count**

The peripheral WBC counts in infected mice that received 20, 30, or 60 mg/kg of pentoxifylline at 8 h intervals were similar to those of controls throughout infection (data not shown).

**In-vitro studies**

*E. coli* endotoxin, at 2 μg/L, stimulated peritoneal-elicited macrophages to produce greater amounts of TNFα than \(2 \times 10^6\) cfu of *C. albicans* (Figure 3). Pentoxifylline suppressed the production of TNFα by endotoxin-stimulated macrophages in a dose-dependent manner. The effect was seen at concentrations of pentoxifylline as low as 10 mg/L (Figure 3(a)). Pentoxifylline also suppressed the production of TNFα by *C. albicans*-stimulated macrophages in a dose-dependent manner. However, the effect of pentoxifylline was seen only for drug concentrations of \(\geq 100\) mg/L (Figure 3(b)).
Figure 2. Serum tumour necrosis factor alpha (TNFα; (a) and interleukin-6 (IL-6; (b)) levels in mice that were killed 2 days after intravenous infection with *C. albicans*. Each animal received intraperitoneal injections of phosphate-buffered saline (PBS) or 20, 30, or 60 mg/kg of pentoxifylline (Pentox) at 8 h intervals beginning 2 days prior to infection. Mice were killed 8 h after the most recent injection of drug. The results represent the average of two trials. The differences between groups were not statistically significant. Of note, TNFα and IL-6 levels between groups also were similar on days 0 and 1 of infection (data not shown). TNFα and IL-6 were not detected in the serum of well animals that received PBS or pentoxifylline (data not shown).

Discussion

In this study we demonstrated that the administration of pentoxifylline 30 and 60 mg/kg at 8 h intervals resulted in an increase in fungal colony counts in the kidneys of mice with systemic *C. albicans* infection. Pentoxifylline therapy was associated with a dose-dependent decrement in mean host survival, although the difference was statistically significant only for the highest dosage evaluated. The detrimental effects of pentoxifylline were not associated with a decrease in serum levels of TNFα or IL-6 and were not mediated by an attenuation of the inflammatory response in tissues.

Others have shown that pentoxifylline can reduce the transcription of the TNFα mRNA that is produced by endotoxin-stimulated macrophages (Han, Thompson & Beutler, 1990; Doherty et al., 1991). This, in turn, results in a decrease in TNFα concentrations in cell culture supernatants. In addition, in a rat model of *E. coli* endotoxaemia, Noel et al. (1990) demonstrated that treatment with a single intravenous injection of 50 or 100 mg/kg of pentoxifylline decreased TNFα levels in
Effect of pentoxifylline in systemic candidiasis

serum. The lower TNFα concentrations were associated with improved survival. However, in the current study we found that pentoxifylline, when given at doses as high as 60 mg/kg every 8 h, did not affect TNFα levels in the serum of mice with systemic candidiasis. We assayed for TNFα in serum of animals at 0, 1, 2, 3, 4, and 8 h after pentoxifylline administration on days 0, 1, and 2 of infection and could not detect a difference in TNFα levels between pentoxifylline recipients and controls at any time. In a murine model of E. coli sepsis, Doherty et al. (1991) reported that serum TNFα levels were decreased in mice at 2 h after a single 50 mg/kg injection of pentoxifylline and this finding suggests our inability to detect a reduction in TNFα levels in mice that received 60 mg/kg of pentoxifylline at 8 h intervals was not due to a sampling error. If the doses of pentoxifylline we examined were capable of attenuating the TNFα that is produced in response to systemic C. albicans infection, this effect should have been observed in our study.
We found that pentoxifylline attenuated the production of TNFα by endotoxin-stimulated macrophages \textit{in vitro} at concentrations as low as 10 mg/L. Similarly, Strieter \textit{et al.} (1988) reported that pentoxifylline at concentrations as low as 2.8 mg/L inhibited mRNA expression and production of TNFα by endotoxin-stimulated mononuclear phagocytes. However, in the current study we found that pentoxifylline concentrations of at least 100 mg/L were required to decrease the production of TNFα by \textit{C. albicans}-stimulated macrophages. Lechner \textit{et al.} (1993) noted that, \textit{in vivo}, pentoxifylline significantly decreased TNFα levels in serum of rats that were infected with \textit{E. coli} but not in animals infected with \textit{C. albicans}. These in-vitro and in-vivo findings suggest that \textit{C. albicans} and Gram-negative bacteria stimulate macrophages to produce TNFα by different pathways.

In single-dose pharmacokinetics studies Honess, Dennis & Bleehen (1993) reported that an intraperitoneal injection of 100 mg/kg of pentoxifylline is required to achieve a peak serum concentration of 100 mg/L in mice. Honess also reported that in mice the serum half-life of pentoxifylline is 7.5 min. If our in-vitro data are predictive of in-vivo activity, Honess's data suggest that the intraperitoneal injection of pentoxifylline, at doses as high as 60 mg/kg every 8 h, are not sufficient to achieve the serum concentrations of pentoxifylline that are required to attenuate the production of TNFα by \textit{C. albicans}-infected mice. Doses of pentoxifylline higher than 60 mg/kg every 8 h (180 mg/kg per day) were not examined because this regimen resulted in an increase in fungal counts in kidneys and shortened mean survival.

The increased fungal burden in kidneys was not mediated by an attenuation of serum TNFα or IL-6 levels nor by a quantitative reduction in the neutrophil response at foci of infection in tissues. However, it is possible that other immunomodulating effects of pentoxifylline were responsible for these findings. Pentoxifylline can decrease the production of interleukin-1, interleukin-2, and interferon-gamma by macrophages (Saez-Llorens \textit{et al.}, 1990; Thanhauser \textit{et al.}, 1993). We did not examine the effect of pentoxifylline on these cytokines. However, these cytokines may augment host defences against \textit{C. albicans} and other pathogens. \textit{In vitro}, pentoxifylline also can attenuate the stimulatory effect of TNFα, interleukin-1, and bacterial endotoxin on neutrophils at concentrations between 1 and 100 mg/L (Bessler \textit{et al.}, 1986; Hammerschmidt \textit{et al.}, 1988; Sullivan \textit{et al.}, 1988). This is manifested by a reduction in neutrophil oxidative burst, degranulation, and the adherence of polymorphonuclear phagocytes to nylon fibers in response to stimulation with N-formyl-L-methionyl-L-leucyl-L-phenylalanine (Bessler \textit{et al.}, 1986; Hammerschmidt \textit{et al.}, 1988; Sullivan \textit{et al.}, 1988). Thus, although we did not observe a decrease in the aggregation of neutrophils in tissues or a reduction of serum TNFα or IL-6 levels in pentoxifylline recipients, it is possible that the adverse effects of the higher doses of pentoxifylline examined were due to the effect of this drug on neutrophil function or the production of other cytokines.

In summary, the administration of pentoxifylline at doses of \( \geq 30 \text{ mg/kg} \) every 8 h to mice that were systemically infected with \textit{C. albicans} resulted in an increase in fungal colony counts in kidneys. In addition, 8 h injections of 60 mg/kg of pentoxifylline shortened the survival of the infected host. The effects of pentoxifylline were not mediated by an alteration in TNFα or IL-6 production nor by a quantitative reduction in the neutrophil response in tissues. This study suggests that pentoxifylline should be used with caution in patients that are at risk for systemic candidiasis.
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References


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