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Pentoxifylline Downregulates Nitric Oxide and Tumor Necrosis Factor-α Induced by Mycobacterial Lipoarabinomannan in a Macrophage Cell Line[1]

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Pentoxifylline (PTX) is a phosphodiesterase inhibitor that is FDA approved for the treatment of peripheral vascular disease ([38]). There has been enthusiasm for the use of PTX as a potential anti-inflammatory agent because of its ability to downregulate the powerful proinflammatory molecule tumor necrosis factor-alpha (TNF-α) ([19, 24, 33]). Reports in mice ([29, 30, 31]) and humans ([30]) indicate possible efficacy in the treatment of allergic contact dermatitis, rheumatoid arthritis and *Mycobacterium avium* exacerbation in late-stage HIV infection ([5, 21]). In addition, two promising reports have appeared in the treatment of leprosy reactions ([26, 27, 35]) where TNF-α has been identified as a major proinflammatory molecule ([19, 25]). Lipoarabinomannan (LAM), the major carbohydrate antigen in mycobacterial cell walls, has been shown to induce TNF-α and nitric oxide (NO) in mouse macrophages, including the mouse macrophage cell line RAW 264.7 ([28]). These findings provide us with an *in vitro* model to further explore the effects of PTX on the macrophage at the cellular and molecular level.

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MATERIALS AND METHODS

Reagents. Lipopolysaccharide (LPS) W *(Escherichia coli* 0111:B4) was purchased from Difco Laboratories, Detroit, Michigan, U.S.A. Arabinose lipoarabinomannan (ara-LAM), a rapidly growing *Mycobacterium* sp. or *M. tuberculosis* H37Ra, was a gift from Dr. John Belisle, University of Colorado, Fort Collins, Colorado, U.S.A. Murine iNOS cDNA probe and murine TNF-α probe were graciously provided by Dr. Carl Nathan, Cornell University, New York, New York, U.S.A. and Chiron Corporation, Emeryville, California, U.S.A., respectively. RPMI 1640, murine recombinant interferon-gamma (rIFN-γ), fetal calf serum (FCS), penicillin and streptomycin and RNA size markers were obtained from Gibco-BRL, Grand Island, New York, U.S.A. Nylon membranes (Genescreen Plus) for Northern blot analyses were purchased from Dupont, Boston, Massachusetts, U.S.A., and Tri-Reagent was purchased from Molecular Research Center, Cincinnati, Ohio, U.S.A. Agarose was obtained from FMC Bioproducts, Rockland, Maine, U.S.A. Herring sperm DNA was purchased from Roche Molecular Biochemicals, Indianapolis, Indiana, U.S.A. and Denhardt reagent and ^{32}P-dCTP was from Amersham Pharmacia, Piscataway, New Jersey, U.S.A. Acrylamide/bis solution (30%), molecular standards of protein and glycine were purchased from Bio-Rad Laboratories, Hercules, California, U.S.A. Nitrocellulose membrane for Western blot analysis was obtained from Schlecher-Schuell, Keene, New Hampshire, U.S.A. Rabbit anti-mouse iNOS was from BD Transduction Laboratories, San Diego, California, U.S.A., and alkaline phosphatase

labeled sheep anti-rabbit antibody was from Roche Molecular Biochemicals. PTX, formaldehyde, formamide and morpholinopropanesulfonic acid were obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A. Murine recombinant TNF-alpha (rTNF-α) and monoclonal hamster anti-murine TNF-α were purchased from R&D Systems, Minneapolis, Minnesota, U.S.A., and polyclonal rabbit anti-murine TNF-α was purchased from Endogen, Cambridge, Massachusetts, U.S.A.

Cell culture and stimulation. RAW 264.7 cells, an Abelson leukemia virus-transformed murine macrophage cell line (American Type Culture Collection, Rockville, Maryland, U.S.A.) were cultured in RPMI 1640 containing 10% heat-inactivated fetal calf serum (FCS) and antibiotics (complete medium). Cells were grown in complete RPMI 1640 for maintenance and in complete DMEM without phenol red for nitrite and TNF-α measurements. Cells (2×10^5/well) were added to a 96-well plate and adhered overnight for functional assays. Various amounts of PTX (0.05 mg/ml, 0.1 mg/ml, 0.5 mg/ml and 1.0 mg/ml) and activators (LPS 1 μg/ml or LAM 1 μg/ml containing IFN-γ 50 U/ml) were added and incubated for 24 hr. One-hundred μl were used for nitrite determination and 50 μl for TNF-α measurement. For Western and Northern analyses, 1×10^7 cells were cultured in Petri dishes. Cell viability, monitored by both trypan blue exclusion and MTT viability assays, was unaffected by the concentrations of PTX used in these studies.

Nitrite determination. Nitrite was measured by the method described previously ([2, 17]). Briefly, 100 μl of the supernatant from a 96-well plate culture was mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride and 2.5% phosphoric acid) and incubated at room temperature for 10 min. The optical density (OD) of each sample was read at 550 nm by a microplate reader (Cambridge Technology, Inc., Cambridge, Massachusetts, U.S.A.). The calibration curve was prepared using sodium nitrite as a standard.

TNF-α measurement by ELISA. TNF-α secretion was measured by ELISA as described previously ([17, 34]). Briefly, 50 μl of hamster anti-mouse TNF-α monoclonal antibody (R&D Systems), diluted 1:500 in carbonate buffer (pH 9.6), was incubated in 96-well ELISA plate (Dynatech Laboratories, Chantilly, Virginia, U.S.A.) overnight at 4°C. After washing the plate, 2% BSA in PBS-Tween 20 (T-PBS, 0.1 M PBS with 0.05% Tween) was added, and the plate was incubated at 37°C for 2 hr. The plate was washed with T-PBS and 50 μl of either various concentrations of standard murine rTNF-α or diluted samples were added to the plates. After incubation at 4°C overnight and washing with T-PBS, 50 μl of rabbit anti-mouse TNF-α polyclonal antibody, diluted 1:500 in T-PBS, was added and the plate was incubated at 37°C for 1.5 hr. After washing, the plate was incubated with 50 μl of goat anti-rabbit immunoglobulin G horseradish peroxidase conjugate (Zymed Laboratories, San Francisco, California, U.S.A.) at a 1:5000 dilution for 1 hr. The color was developed with 0-phenylenediamine dihydrochloride and H_2O_2 in citrate buffer (pH 5.0). The plate was read at 490 nm by an MR 600 microplate reader (Dynatech Laboratories). The concentration of TNF-α released into the medium was calculated by using murine rTNF-α as a standard.

Western blot analyses. Western blot analysis was performed by the method described previously ([18]). Briefly, activated RAW 264.7 cells were washed with Hanks balanced salt solution (HBSS). The cells were lysed with HBSS containing 1.0% Nonidet P-40, aprotinin (10 μg/ml), antipain (5 μg/ml), and PMSF (0.2 mM). After centrifugation, cell lysates (50 μg protein) were diluted in SDS-containing sample preparation buffer, electrophoresed on 7.5% SDS polyacrylamide gels, and transferred overnight to nitrocellulose filters. The nitrocellulose filters were blocked with a solution containing 2% BSA and 2% normal sheep serum for 1 hr at room temperature and washed with 0.2% Tween 20 in PBS once for 5 min. The blot was incubated with rabbit inducible nitric oxide synthase (iNOS) antiserum (1/5000 dilution) as a primary antibody for 2 hr at 37°C, and washed three additional times. After incubation with alkaline phosphatase-labeled sheep anti-rabbit antibody for 1.5 hr at 37°C the blot was again washed three times. The reaction product was visualized using nitro-

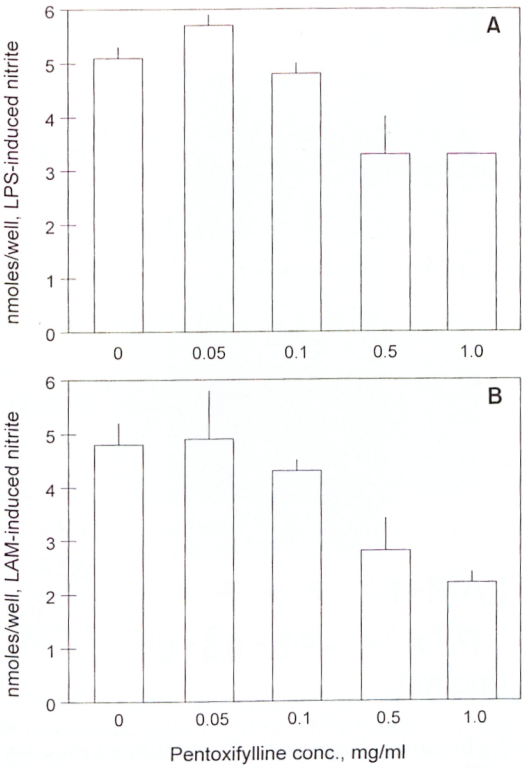

FIG. 1. Inhibition of NO by pentoxifylline (PTX). **A** = RAW 264.7 cells were activated with LPS and IFN-γ. Simutaneously, various concentrations of PTX were added to the cells. After 24 hr, supernatants were collected and nitrite measured using Griess reagent and sodium nitrite as a standard. **B** = RAW 264.7 cells were activated with LAM and IFN-γ. Each point represents the mean of triplicate samples ± S.D. One mg/ml as well as 0.5 mg/ml of PTX were significantly decreased compared to the control ($p < 0.05$). Similar results were obtained in three additional independent experiments.

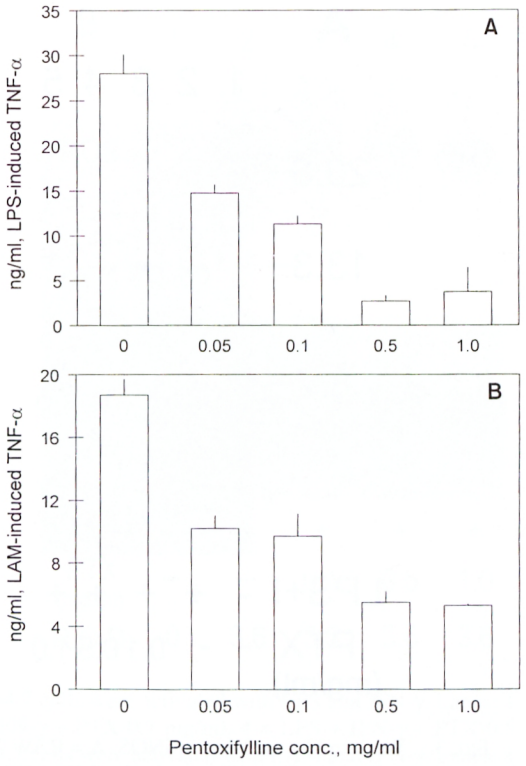

FIG. 2. Inhibition of TNF-α secretion by PTX. **A** = RAW 264.7 cells were activated with LPS and IFN-γ. Simultaneously, various concentrations of PTX were added. After 24 hr, supernatants were collected and the secretion of TNF-α measured by ELISA using recombinant murine TNF-α as a standard. **B** = RAW 264.7 cells were activated with LAM and IFN-γ. Each point represents the mean of triplicate samples ± S.D. All PTX groups were significantly decreased compared to the control ($p < 0.05$). Similar results were obtained in three additional independent experiments.

blue tetrazolium and 5-bromo-4-chloro-3-indolyphosphate.

Northern blot analyses. Total RNA was extracted from RAW 264.7 cells with Tri-Reagent ([1]) before being size-fractioned by electrophoresis in 1.5% formaldehyde agarose gel and transferred overnight to a nylon membrane ([18]). Blots were prehybridized in hybridization buffer (50% formamide, 5× SSPE, 2× Denhardt reagent, 0.5% SDS and 100 μg/ml denatured herring sperm DNA) followed by hybridization with [^{32}P]dCTP random prime-labeled cDNA of iNOS at 42°C for 12–20 hr. The RNA blots were washed once with 2× SSPE containing 0.1% SDS for 10 min at room temperature followed by two washes with 1× SSPE containing 0.1% SDS for 15 min at 65°C. RNA hybridized with iNOS cDNA was visualized after autoradiography using Kodak XAR-5 film developed at –70°C.

Statistical analysis. Significant differences between control and experimental groups were analyzed by the Student's paired *t* test (Statistica; StatSoft, Inc., Tulsa, Oklahoma, U.S.A.). A significant difference was considered as $p < 0.05$.

RESULTS

In order to determine the inhibitory effect of PTX on NO production and TNF-α se-

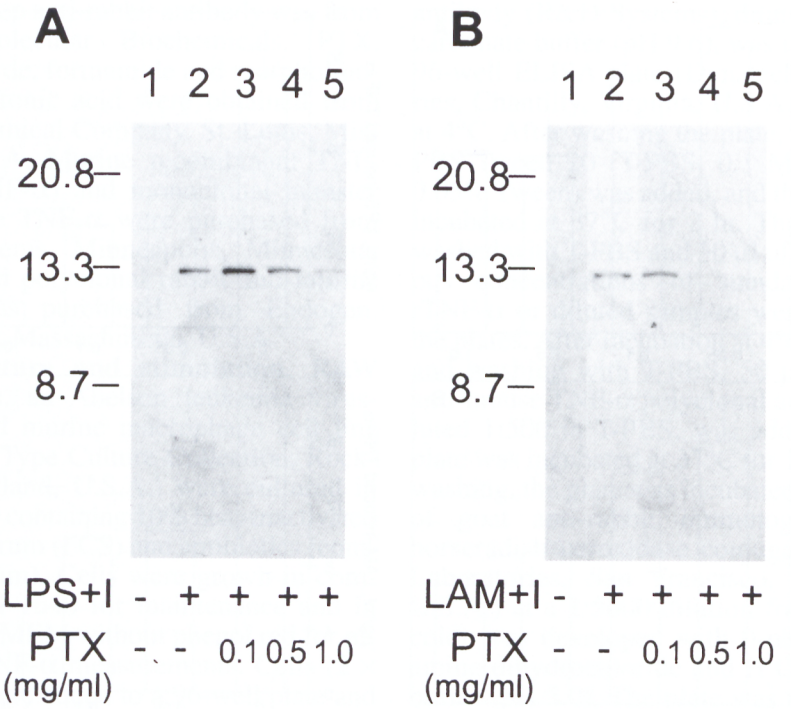

FIG. 3. Western blot analyses of iNOS. **A** = RAW 264.7 cells were activated with LPS and IFN-γ. Lanes: 1 = unactivated cells, 2 = LPS + IFN-γ only, 3 = LPS + IFN-γ + PTX 0.1 mg/ml, 4 = LPS + IFN-γ + PTX 0.5 mg/ml, 5 = LPS + IFN-γ + PTX 1.0 mg/ml. **B** = RAW 264.7 cells were activated with LAM and IFN-γ. Lanes: 1 = unactivated cells, 2 = LAM + IFN-γ only, 3 = LAM + IFN-γ + PTX 0.1 mg/ml, 4 = LAM + IFN-γ + PTX 0.5 mg/ml, 5 = LAM + IFN-γ + PTX 1.0 mg/ml. Similar results were obtained in two additional independent experiments.

cretion in LAM activated RAW 264.7 cells, various amounts of PTX (0.05 mg/ml, 0.1 mg/ml, 0.5 mg/ml and 1.0 mg/ml) were used simultaneously when the cells were activated with IFN-γ (50 U/ml) and either LPS (1 μg/ml) or LAM (1 μg/ml). PTX inhibited NO induced by LPS (Fig. 1A) ([10]). PTX inhibited the production of NO induced by LAM in a dose-dependent manner (Fig. 1B). Even though 0.05 mg/ml and 0.1 mg/ml of PTX was not effective, 0.5 mg/ml and 1.0 mg/ml significantly inhibited NO production ($p < 0.01$). The inhibition of TNF-α was greater than that of NO by PTX when the cells were activated with either LPS and IFN-γ or LAM and IFN-γ (Figs. 2A and 2B). Even as little as 0.05 mg/ml of PTX inhibited the secretion of TNF-α significantly ($p < 0.001$). In contrast, NO was not significantly inhibited at either 0.05 mg/ml or 0.1 mg/ml PTX.

Since PTX inhibited the production of NO induced by LAM or LPS in RAW 264.7 cells, iNOS protein was examined by Western blot analyses. RAW 264.7 cells were treated with PTX (0.1, 0.5 and 1.0 mg/ml) in the presence of either LPS or LAM with IFN-γ and separated by SDS-PAGE after cell lysis. iNOS proteins induced by LPS or LAM with IFN-γ were inhibited by PTX (Figs. 3A and 3B). At 0.1 mg/ml of PTX, NOS protein induced by LPS and IFN-γ was actually increased. iNOS protein induced by LAM and IFN-γ was decreased in a dose-dependent manner. Untreated cells did not show an iNOS protein band.

Since iNOS and TNF-α proteins were inhibited by PTX, Northern blot analyses were performed to determine whether PTX could inhibit transcription of mRNA of iNOS and TNF-α. After extraction of total RNA and separation of total RNA by agarose gel, specific ^{32}P-labeled cDNA probes were used to detect the mRNA of iNOS and TNF-α, using α-actin as a control. All cell preparations for Northern blot analyses were activated with LPS or LAM, including IFN-γ, for 16 hr, which gives

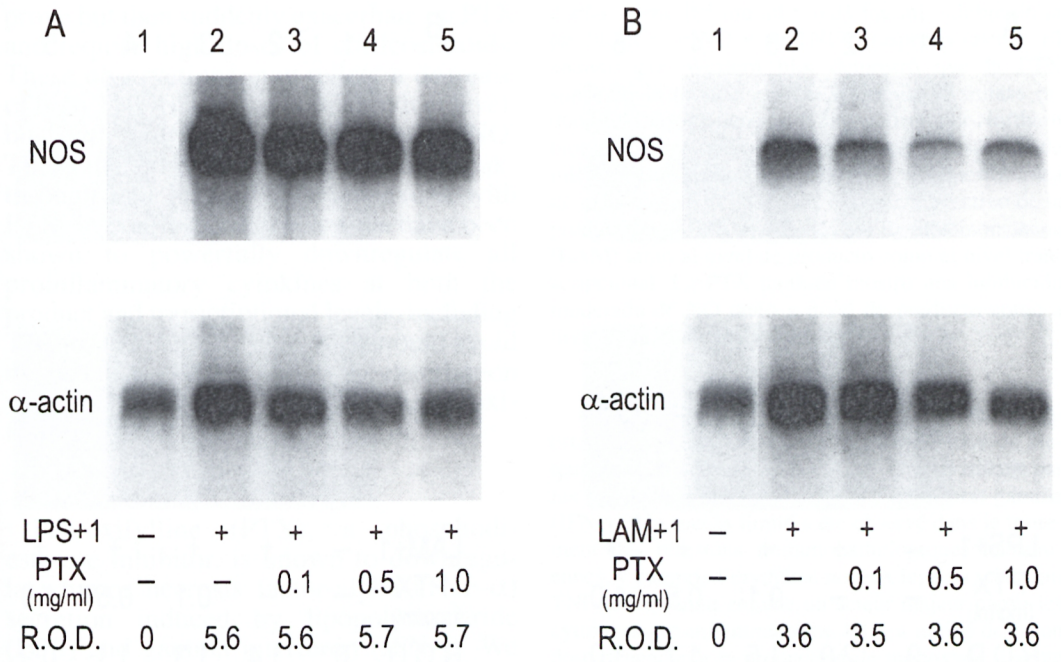

FIG. 4. Northern blot analyses of iNOS. **A** = RAW 264.7 cells were activated with LPS and IFN-γ. Lanes: 1 = unactivated cells, 2 = LPS + IFN-γ only, 3 = LPS + IFN-γ + PTX 0.1 mg/ml, 4 = LPS + IFN-γ + PTX 0.5 mg/ml, 5 = LPS + IFN-γ + PTX 1.0 mg/ml. **B** = RAW 264.7 cells were activated with LAM and IFN-γ. Lanes: 1 = unactivated cells, 2 = LAM + IFN-γ only, 3 = LAM + IFN-γ + PTX 0.1 mg/ml, 4 = LAM + IFN-γ + PTX 0.5 mg/ml, 5 = LAM + IFN-γ + PTX 1.0 mg/ml. Similar results were obtained in two additional independent experiments. Relative optical density (ROD) was performed using α-actin as a control.

maximum expression of iNOS mRNA ([18]). When the data were normalized using relative optical density (ROD), PTX failed to inhibit the transcription of iNOS induced by LPS at 0.5 mg/ml or 1.0 mg/ml (Fig. 4A). Similarly, when the data were normalized using ROD, iNOS mRNA induced by LAM was not inhibited at any dose tested (Fig. 4B). PTX inhibited the transcription of TNF-α induced by LPS as well as by LAM (Figs. 5A and 5B).

DISCUSSION

Our findings with LPS and PTX are in keeping with prior studies that show a downregulation of TNF-α at both the product and transcriptional levels and a downregulation of NO at the product level ([4, 10, 12, 36]). In addition, we extended these findings to show similar effects when macrophages are activated with LAM, the major carbohydrate antigen of mycobacterial cell walls ([28, 32]). Our findings also show some downregulation of iNOS with PTX at the translational level when activated with LPS or LAM. In fact, at low concentrations of PTX there was a slight increase of iNOS at the translational level (Fig. 3A) when the cells were activated with LPS.

Our findings indicate a possible problem with the use of PTX as an immunosuppressive or anti-inflammatory agent in dermatology. NO as an important proinflammatory component of the immune system ([14, 20]) is not suppressed by PTX at the transcriptional level. Furthermore, even at the product level, high concentrations (0.5–1.0 mg/ml) were required to suppress NO production. However, TNF-α was inhibited by PTX at 50 μg/ml. The possible benefit of PTX as an anti-inflammatory/immunosuppressive agent has not been limited to dermatology, but has been used in diverse inflammatory conditions such as cerebral malaria ([6]) and bacterial meningitis ([37]). As discussed by Van Furth, *et al.*, some controversy exists on the efficacy of PTX on suppressing IL-6 and IL-8 ([37]). Neuner, *et al.* showed a decrease in IL-8 and IL-6 as well as TNF-α when leukocytes were pretreated

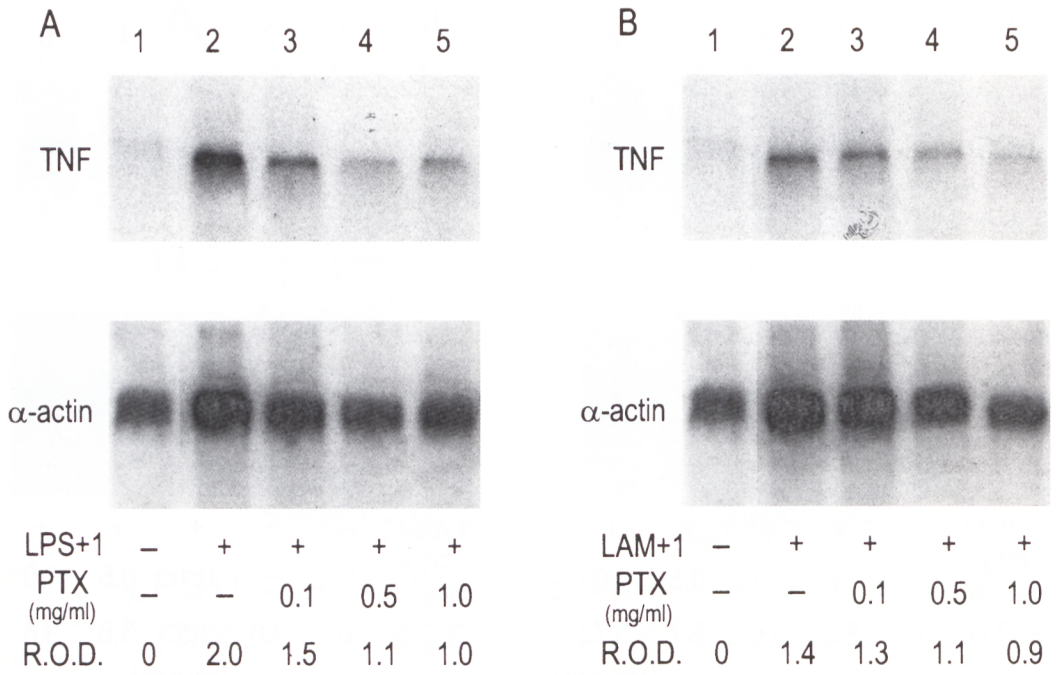

FIG. 5. Northern blot analyses of TNF-α. **A** = RAW 264.7 cells were activated with LPS and IFN-γ. Lanes: 1 = unactivated cells, 2 = LPS + IFN-γ only, 3 = LPS + IFN-γ + PTX 0.1 mg/ml, 4 = LPS + IFN-γ + PTX 0.5 mg/ml, 5 = LPS + IFN-γ + PTX 1.0 mg/ml. **B** = RAW 264.7 cells were activated with LAM and IFN-γ. Lanes: 1 = unactivated cells, 2 = LAM + IFN-γ only, 3 = LAM + IFN-γ + PTX 0.1 mg/ml, 4 = LAM + IFN-γ + PTX 0.5 mg/ml, 5 = LAM + IFN-γ + PTX 1.0 mg/ml. Similar results were obtained in two additional independent experiments. Relative optical density (ROD) was performed using α-actin as a control.

for 24 hr ([16]). However, in keeping with prior studies without 24 hr pretreatment, IL-6 and IL-8 were not downregulated in contrast to TNF-α. In fact, the mechanism of PTX as an anti-inflammatory/immunosuppressive agent is not as simple as analyzing only product and transcriptional levels. Han, *et al.* have shown that dexamethasone strongly impedes translational derepression of NO and TNF-α in sharp contrast to PTX ([7]).

Finally, since LAM plays a role in macrophage activation, we need to interpret our findings in the mycobacterial model at the clinical, immunologic and molecular levels. LAM preparations from nonvirulent species of mycobacteria have been shown to induce elevated levels of TNF-α at the product level; whereas virulent (e.g., *M. tuberculosis* Erdman and *M. leprae*) induce the TNF-α message in the absence of product ([22]). Future studies need to address the structural (e.g., mannose capping of virulent LAMs) and functional relationships to mycobactericidal activity in IFN-γ-primed macrophages. Leprosy has a spectrum of immunologic disease ([22]) from a Th1 dominant tuberculoid form (IFN-γ positive) to a Th2 dominant lepromatous form (IFN-γ negative) ([13, 32]), including two types of reactions [type 1 or reversal reactions and type 2 or erythema nodosum leprosum (ENL) reactions], both of which are currently best treated with corticosteroids or in type 2 reactions, thalidomide and/or corticosteroids ([11, 25, 26]). PTX has been suggested to theoretically benefit ENL reactions ([19, 24]) and, indeed, there have been two reports of possible benefit, although both reports included patients who required additional corticosteroids ([7, 8]).

This has also been our experience in the New York regional program, and similar experience at the National Hansen's Disease Center (personal communication, Dr. Leo Yoder, National Hansen's Disease Center, Carville, Louisiana, U.S.A.) that some patients while receiving PTX appear to im-

prove but then suddenly exacerbate on PTX and require high doses of corticosteroids. These observations may reflect a decrease of both TNF-α and NO at the product level, but with only partial inhibition of NO. Thus, the possibility of a sudden breakthrough and leprosy reaction exists with PTX. In contrast, corticosteroids have been shown to powerfully downregulate all proinflammatory cytokines at both the product and transcriptional levels, including TNF-α and iNOS ([3, 8, 9]), which would avoid the problem of a late (60 days) excerbation with 20% of patients treated with pentoxifylline, as described by Nery, *et al.* ([15]).

SUMMARY

Pentoxifylline (PTX), a phosphodiesterase inhibitor, is known to downregulate tumor necrosis factor-alpha (TNF-α) secretion induced by lipopolysacchride (LPS) and gamma interferon (IFN-γ). We have had limited success in treating leprosy reactions, including erythema nodosum leprosum (ENL), in which TNF-α has been identified as a major proinflammatory cytokine. PTX inhibited production of NO (IC50 ≅ 1.0 mg/ml) and TNF-α (IC50 ≅ 0.05 mg/ml) in a dose-dependent fashion. As little as 0.5 mg/ml of PTX decreased NO production and 0.01 mg/ml of PTX inhibited TNF-α production. Western blot analyses demonstrated that iNOS was suppressed by PTX. Northern blot analyses showed significant reduction of TNF-α mRNA. We conclude that PTX is an effective inhibitor of lipoarabinomannan (LAM)-induced TNF-α production at both the product and transcriptional levels in our macrophage cell line. PTX also showed moderate inhibition of NO at the product level as well as translation of iNOS.

RESUMEN

Se sabe que la pentoxifilina (PTX), un inhibidor de fosfodiesterasas, disminuye la secreción de factor de necrosis tumoral alfa (TNFα) inducida por lipopolisacárido (LPS) e interferón gamma (IFNγ). Nosotros hemos tenido un éxito limitado en el tratamiento de reacciones leprosas, incluyendo eritema nodoso leproso, en las cuales el TNFα se ha identificado como la principal citocina proinflamatoria. La PTX inhibió la producción de óxido nítrico (NO) (IC50 = 1.0 mg/ml) y de TNFα IC50 = 0.05 mg/ml) en forma dosis-dependiente. Cantidades de PTX tan pequeñas como 0.5 mg/ml y 0.01 mg/ml, inhibieron la producción de NO y de TNFα, respectivamente. El análisis por Western blot demostró que la PTX suprimió la producción de oxido-nítrico sintasa inducible (iNOS) mientras que el análisis por Northern blot indicó una reducción significante en la síntesis de mRNA para TNFα. Concluimos que en nuestra línea de macrófagos, la PTX es un inhibidor efectivo de la producción de TNFα inducida por lipoarabinomanana (LAM) tanto al nivel de producto como al nivel transcripcional. La PTX también mostró una inhibición moderada de NO tanto a nivel de producto como a nivel de la traducción de iNOS.

RÉSUMÉ

Pentoxifylline (PTX), un inhibiteur des phosphodiestérases, est capable de diminuer la sécrétion du facteur alpha de nécrose des tumeurs (TNF-α) induite par les lipopolysaccharides (LPS) et l'interféron gamma (IFN-γ). Un succès limité a été observé dans le traitement des réactions immunopathologiques hansénniennes, y compris l'érythème noueux lépreux, chez qui TNF-α est connu comme un acteur majeur parmi les cytokines pro-inflammatoires. PTX a inhibé de façon dose-dépendante la production de monoxyde d'azote (NO) (concentration inhibitrice 50% ou IC50 ~ = 1,0 mg/ml) et de TNF-α (IC50 ~ = 0,05 mg/ml). Une quantité aussi faible que 0,5 mg/kg et que 0,01 mg/kg de PTX a diminué la production de NO et inhibé celle de TNF-α, respectivement. Une analyse par Western-Blot a montré que la présence de la synthétase inductible du monoxyde d'azote (iNOS) était réprimée par PTX. Une analyse par Northern blot a montré une réduction significative de l'ARNm du TNF-α. Nous concluons que PTX est un inhibiteur efficace de la production de TNF-α induite par le lipoarabinomannane (LAM) à la fois au niveau du produit que celui de la transcription sur notre lignée cellulare macrophagique. PTX exerce également une activité inhibitrice modérée sur la production de NO, ainsi que sur la traduction de iNOS.

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