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# Differential effects of pentoxifylline on the production of tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) by monocytes and T cells

L. SCHANDENÉ,* P. VANDENBUSSCHE,† A. CRUSIAUX,* M.-L. ALÈGRE,‡ D. ABRAMOWICZ,‡§ E. DUPONT,* J. CONTENT† & M. GOLDMAN*§ *Departments of *Immunology and §Nephrology, Hôpital Erasme and ‡Laboratoire Pluridisciplinaire de Recherche Expérimentale Biomédicale, Université Libre de Bruxelles and †Pasteur Institute of Brabant, Brussels, Belgium*

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## SUMMARY

Pentoxifylline (PTX) is a methylxanthine compound known to inhibit the production of tumour necrosis factor-alpha (TNF-α) by monocytic cells. In this study, we found that PTX differentially regulates the production of TNF-α and interleukin-6 (IL-6). Indeed, PTX at high concentrations triggers the production of IL-6 but not of TNF-α by peripheral blood mononuclear cells (PBMC). Further experiments indicated that monocytes are responsible for this PTX-induced IL-6 production. When PBMC were stimulated with LPS, PTX was found to inhibit the secretion of TNF-α as well as the accumulation of TNF-α messenger RNA (mRNA). In contrast, no inhibitory effect was observed on the induction of IL-6. Similar results were obtained when PBMC were stimulated with OKT3 monoclonal antibody (mAb). In addition, the *in vivo* administration of PTX in transplant patients receiving the first dose of OKT3 allowed to decrease the systemic release of TNF-α but not of IL-6. Since monocytes represent a major source of TNF-α and IL-6 in these settings, additional experiments were performed *in vitro* on purified T cells stimulated with the CLB-T3/3, an anti-CD3 mAb which does not require the presence of accessory cells to activate T cells. In this system, PTX was found to inhibit the secretion of both TNF-α and IL-6 by T cells. We suggest that cAMP could be involved in these differential effects of PTX on production of TNF-α and of IL-6.

## INTRODUCTION

Tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) are recognized as important mediators in a variety of inflammatory processes.[1,2] It is therefore of interest to identify pharmacological compounds able to suppress TNF-α and/or IL-6 production. Pentoxifylline (PTX), a methylxanthine known for many years for its haemorrheological properties, has recently been found to prevent or attenuate the release of TNF-α induced by bacterial lipopolysaccharides (LPS) *in vitro*[3] or *in vivo*.[4] Studies on murine macrophage cell lines revealed that this effect of PTX on TNF-α production is exerted at the transcriptional level.[5,6] On the other hand, there is evidence that PTX does not affect the production of IL-6 triggered by LPS.[4,7]

The present study was designed to delineate the effects of PTX on the production of TNF-α and IL-6 by peripheral blood mononuclear cells (PBMC). First, we observed that high doses of PTX trigger IL-6 secretion by PBMC. This led us to determine the influence of PTX on the spontaneous secretion of IL-6 by isolated monocytes and T cells. We then compared the modulation by PTX of the TNF-α and IL-6 release induced *in vitro* by LPS or anti-CD3 monoclonal antibodies. The *in vivo* effects of PTX on the production of those cytokines were also analysed in transplant patients receiving OKT3 as primary immunosuppression.[8]

Abbreviations: mAb, monoclonal antibody; m-PDS, methylprednisolone; PBMC, peripheral blood mononuclear cells; PTX, pentoxifylline.

Correspondence: Dr M. Goldman, Dept. of Immunology, Hôpital Erasme, 808, route de Lennik, B-1070 Brussels, Belgium.

## MATERIALS AND METHODS

*Cells*

PBMC were prepared by centrifugation of heparinized venous blood from healthy adult donors on Lymphoprep (Nycomed, Oslo, Norway).

Monocytes were isolated as described elsewhere.[9] Briefly, mononuclear cell suspensions were allowed to clump by low speed centrifugation at 4°. The clumps separated by sedimentation on ice-cold foetal calf serum (FCS; Myoclone, Gibco, Life Technologies, Paisley, Renfrewshire, U.K.) were resuspended in RPMI-1640 containing 5% FCS. These cell preparations routinely contained more than 90% monocytes.

To purify T cells, lymphocyte fractions were further depleted of B cells and monocytes by incubation with optimal numbers of immunomagnetic beads coated with anti-CD19 and anti-DR mAb (Dynabeads, Dynal, Oslo, Norway) at 4° for 30 min. The PBMC/Dynabeads mixture was placed in a magnetic separator. T cells contained in the supernatants were harvested, washed and resuspended for immunophenotyping and cytokine assays. The purity of T cells was >95% as determined by immunofluor-

escence flow cytometry after staining with anti-CD2 mAb. The percentage of monocytic cells expressing CD14 antigen in this preparation was below 0·5%.

*Reagents*

PTX (Trental$^R$) was provided by Hoechst Pharmaceuticals (Frankfurt, Germany) and methylprednisolone (m-PDS) (Solumedrol$^R$) by Upjohn (Kalamazoo, MI). LPS from *Escherichia coli* 0111:B4 was purchased from Sigma Chemical Co (St Louis, MO) and the OKT3 mAb from Ortho Biotech (Raritan, NJ). The CLB-T3/3 anti-CD3 mAb was kindly provided by Dr R. van Lier (Central Laboratory of the Netherlands Red Cross, Amsterdam, The Netherlands). All these reagents as well as the culture medium (RPMI-1640 containing 5% FCS) containing less than 10 pg/ml endotoxin as determined with the Limulus assay (LAL-QCL-1000, Whittacker MA, Bioproducts, Walkersville, MD).

*Culture conditions*

Monocytes, PBMC or T cells were cultured in 96-well flat-bottom microtitre plates (Nunc, Roskilde, Denmark) in a 5% $CO_2$ atmosphere. Stimulation of PBMC ($10^6$/ml) or monocytes ($10^6$/ml) was induced by LPS (10 ng/ml) or by OKT3 (10 ng/ml) in fluid phase. Purified T cells ($2 \times 10^6$/ml) were stimulated by solid-phase CLB-T3/3 mAb. For coating of the mAb, wells were incubated for 2 hr with 6 $\mu$g of CLB-T3/3 in ascitis form followed by two washings with RPMI-1640. After 24 hr of culture, supernatants were collected, centrifuged at 3000 ***g*** for 15 min and stored at $-20°$ until assayed for TNF-$\alpha$ and IL-6.

*TNF-$\alpha$ and IL-6 assays*

The levels of TNF-$\alpha$ and IL-6 in the culture supernatants and in serum samples were determined using an immunoradiometric (IRMA) and an immunoenzymatic (EASIA) assay, respectively (IRE, Medgenix, Fleurus, Belgium). These sandwich assays are based on the use of several mAb directed against distinct epitopes of TNF-$\alpha$ or IL-6. The limits of sensitivity are 15 pg/ml for TNF-$\alpha$ and 20 pg/ml for IL-6.

*Northern blot analysis*

PBMC ($2 \times 10^6$/ml) were stimulated with LPS in the presence or absence of either PTX or m-PDS in serum-free RPMI-1640 for 4 hr in 6-well plates (Nunc). Total RNA was extracted by the guanidine isothiocyanate (GIT) method. Briefly, cells were first lysed in GIT buffer. The lysate was layered onto a caesium chloride cushion and spun for 16 hr at 174,000 ***g*** in a SW 41 Beckman rotor (Beckman Instruments, Fullerton, CA) at 20°. RNA pellets were redissolved in 0·3 mol/l Na acetate, and aliquots were electrophoresed under denaturating conditions on a 1% formaldehyde/agarose gel. After separation, 6 $\mu$g RNA was transferred to a nylon membrane (Hybond- N, Amersham, International Ltd, Little Chalfont, Bucks, U.K.) and hybridized with a $^{32}$P-labelled DNA probe prepared by random priming labelling of a 1138 base pair (bp) DNA fragment from the human TNF-$\alpha$ copy DNA (cDNA) provided by Dr W. Fiers (Rijksuniversiteit, Ghent, Belgium).

For detection of IL-6 mRNA, the $^{32}$P-labelled DNA probe was a 480 bp fragment (*Eco*R1-*Hin*dIII) of the human IL-6 cDNA.[10] Hybridization was done at 65° in Rapid Hybridization Buffer (Amersham). After stringent washing, the membranes were exposed to X-ray film (Kodak; X Omatic, Eastman Kodak, Rochester, NY) for 12 hr at $-70°$. As control, the membranes were hybridized with glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) $^{32}$P-labelled cDNA probe.

*Patients*

Fourteen kidney transplant recipients were studied from the time of surgery until 24 hr after transplantation. The initial immunosuppressive protocol consisted in all patients of an intravenous bolus of 8 mg/kg of m-PDS followed 3 hr later by 10 mg of OKT3 given intravenously. In addition to m-PDS and OKT3, seven patients received PTX as an intravenous bolus of 20 mg/kg immediately before the OKT3 injection followed by a continuous infusion of 20 mg/kg/day. Serum samples were collected 90 min, 9 hr and 24 hr after the administration of OKT3 and assayed for TNF-$\alpha$ and IL-6 as described above.

*Statistical analysis*

The Student's *t*-test was used for statistical analyses.

## RESULTS

### PTX triggers IL-6 production by monocytes

In the presence of medium alone, the background levels of IL-6 and TNF-$\alpha$ in the 24 hr culture supernatants of PBMC from normal donors were $30 \pm 10$ pg/ml (mean $\pm$ SD) and $60 \pm 10$ pg/ml, respectively. As shown in Fig 1, PTX stimulates in a dose-dependent manner the secretion of IL-6 by PBMC from normal donors. In contrast, the levels of TNF-$\alpha$ in the same culture supernatants remained in the background range despite the PTX concentration added (data not shown). In order to determine the nature of the cells secreting IL-6 under the influence of PTX, these experiments were repeated on highly purified T cells or monocytes. The data presented in Table 1 demonstrate that PTX at a concentration of 4 mM stimulated monocytes but not T cells to produce IL-6. In each set of experiments, the absence of endotoxin contamination of the

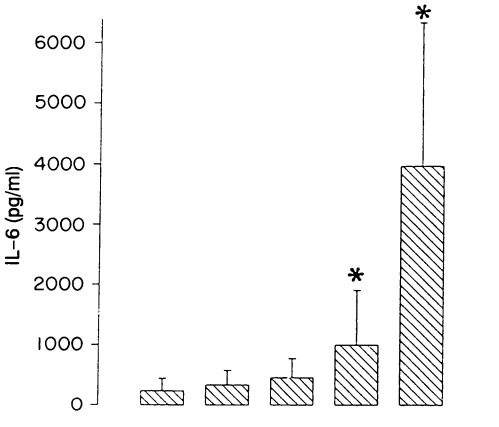


**Figure 1.** PTX stimulates IL-6 production by PBMC. PBMC ($1 \times 10^6$/ml) were incubated with PTX at concentrations ranging from $4 \times 10^{-3}$ to 4 mM. Supernatants were collected at 24 hr for determination of IL-6 levels as described in Materials and Methods. Data represent the mean $\pm$ SD of four independent experiments. $*P < 0{\cdot}01$ as compared with IL-6 levels obtained in the absence of PTX.

**Table 1.** Pentoxifylline stimulates the secretion of IL-6 by isolated monocytes

| | PTX (mM) | Monocytes* | T cells* |
|---|---|---|---|
| IL-6 (pg/ml)† | — | 183 | 56 |
| | 4 | 2873 | 74 |
| TNF-α (pg/ml)† | — | 150 | 50 |
| | 4 | 150 | 50 |

* Purified monocytes ($1 \times 10^6$/ml) were incubated for 24 hr in the absence or in the presence of 4 mM PTX.

† Levels of IL-6 and TNF-α in culture supernatants were determined as described in Materials and Methods. Data from one out of four experiments which yielded similar results are shown.

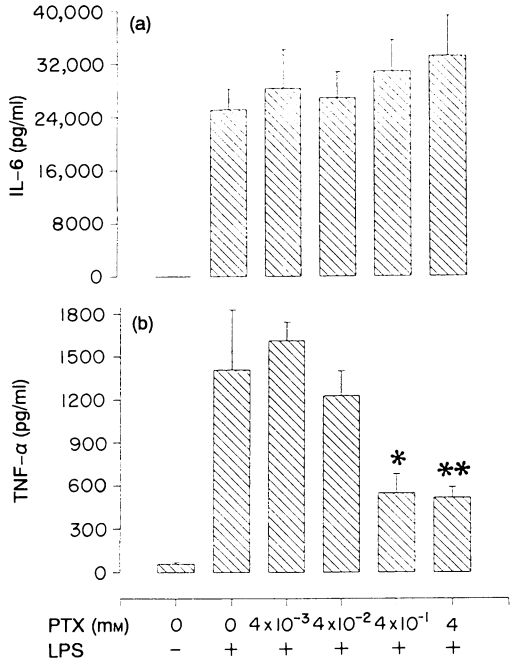


**Figure 2.** Effects of PTX on the *in vitro* production of IL-6 and TNF-α induced by LPS. PBMC ($1 \times 10^6$/ml) were stimulated with 10 ng/ml of LPS in the presence of various concentrations of PTX. Supernatants were collected at 24 hr for determination of IL-6 (a) and TNF-α (b). Data represent the mean ± SD of six independent experiments. *$P < 0.005$, **$P < 0.001$ as compared with TNF-α levels obtained in the presence of LPS and in the absence of PTX.

PTX preparation was ascertained as described in Materials and Methods.

### Differential effects of PTX on *in vitro* induction of TNF-α and IL-6 by LPS

When we analysed the influence of PTX on the *in vitro* production of cytokines triggered by LPS, we first confirmed the data of Waage *et al.*[7] that the drug mitigated the TNF-α release in a dose-dependent manner with a significant inhibition beginning at a concentration of $4 \times 10^{-1}$ mM (Fig. 2b). In contrast, PTX had no inhibitory effect on the production of IL-6 whatever the concentration used (Fig. 2a).

In order to define the site of action of PTX and m-PDS, Northern blot analysis for IL-6 and TNF-α mRNA was performed on PBMC stimulated by LPS. The experiments presented in Fig. 3 established that both PTX and m-PDS blocked the accumulation of TNF-α mRNA. In contrast, only m-PDS reduced accumulation of IL-6 mRNA.

### Differential effects of PTX on anti-CD3-triggered TNF-α and IL-6 release

Since the OKT3 mAb induces the release of TNF-α and IL-6 *in vitro* and *in vivo*, we were interested to determine the influence of PTX in both settings. First, we found that PTX inhibited in a dose-dependent manner the production of TNF-α induced *in vitro* by OKT3. On the other hand, the secretion of IL-6 was unaffected by PTX even at concentrations as high as 4 mM (Fig. 4).

Similar observations were made when we analysed the *in vivo* effects of PTX in kidney transplant recipients receiving their first dose of OKT3. As previously reported, the first administration of OKT3 was followed by a major increase in serum levels of TNF-α (mean ± SD of peak levels at 90 min: 611 ± 68 pg/ml, $n = 7$) and IL-6 (mean ± SD of peak levels at 9 hr: 410 ± 152 pg/ml, $n = 7$). The infusion of PTX starting 1 hr prior the injection of the mAb significantly reduced the peak serum levels of TNF-α (255 ± 48 pg/ml, $n = 7$, $P < 0.005$) whereas the IL-6 levels were not modified (496 ± 228 pg/ml, $n = 7$, NS).

As monocytes participate to the production of TNF-α and IL-6 after stimulation with OKT3,[11,12] these experiments did not allow conclusions to be drawn about the effects of PTX on the production of these cytokines by T cells. In order to investigate this question, highly purified T cells were stimulated with solid-

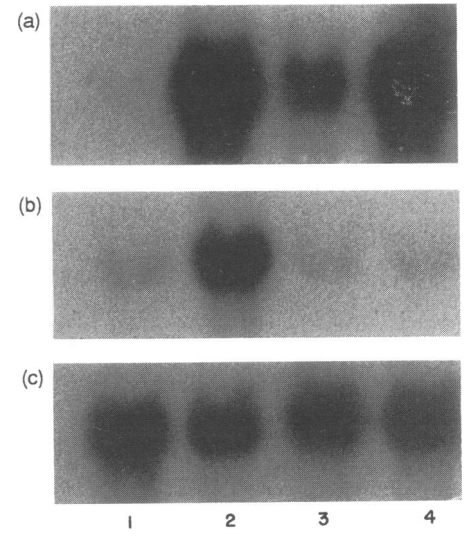


**Figure 3.** Effects of PTX and m-PDS on the accumulation of IL-6 and TNF-α mRNA induced by LPS. PBMC ($2 \times 10^6$/ml) were incubated in serum-free medium (lane 1), with LPS (10 ng/ml) in the absence (lane 2) or in the presence of either 4 mM PTX (lane 4) or $3 \times 10^{-1}$ mM m-PDS (lane 3). After Northern blotting, extracted RNA was probed for IL-6 (a), TNF-α (b), IL-6 and GAPDH (c) as control for RNA loading.

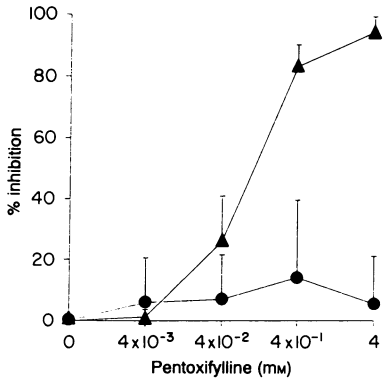


**Figure 4.** Effects of PTX on the *in vitro* production of TNF-α (▲) and IL-6 (●) induced by the OKT3 mAb. PBMC ($1 \times 10^6$/ml) were stimulated with 10 ng/ml OKT3 in the presence of various concentrations of PTX. Supernatants were collected after 24 hr of culture for determination of TNF-α and IL-6 levels. Results are expressed as the mean ± SD of inhibition percentages in seven independent experiments. The levels (mean ± SD) of TNF-α and IL-6 induced by OKT3 in the absence of PTX was 3550 ± 1687 pg/ml and 4139 ± 3753 pg/ml, respectively.

**Table 2.** Pentoxifylline reduces the production of IL-6 and TNF-α by purified T cells stimulated by the CLB-T3/3 mAb

| PTX (mM) | IL-6* (pg/ml) | TNF-α* (pg/ml) |
|---|---|---|
| 0 | 941 | 4300 |
| 4 | 43 | 43 |
| $4 \times 10^{-1}$ | 421 | 1400 |
| $4 \times 10^{-2}$ | 1002 | 3350 |
| $4 \times 10^{-3}$ | 929 | 4300 |

* Purified T cells ($2 \times 10^6$/ml) were incubated in plastic wells coated with the CLB-T3/3 anti-CD3 mAb in the presence of various concentrations of PTX. After 24 hr of culture, supernatants were assayed for IL-6 and TNF-α as described in Materials and Methods. Data from one out of four experiments which yielded similar results are shown.

phase CLB-T3/3, an anti-CD3 mAb which does not require the presence of accessory cells to activate T cells.[13] We first confirmed the data of van Lier *et al.* indicating that this mAb triggers the production of IL-6 and TNF-α by T cells (ref. 13; R. van Lier, personal communication). The absence of significant monocyte contamination among the T cells was confirmed by the fact that their production of IL-6 remained at the background level after incubation with LPS. In this system independent of accessory cells, PTX inhibited the production of both TNF-α and IL-6 by T cells (Table 2).

## DISCUSSION

Our data first demonstrate that high doses of PTX trigger the secretion of IL-6 but not of TNF-α by normal human monocytes, in the absence of any other stimulus. As a methylxanthine, PTX exerts its pharmacological effects by inhibiting intracellular cyclic nucleotide phosphodiesterase, thus increasing the intracellular concentration of cAMP.[14] It is possible that the induction by PTX of monocytic IL-6 involves cAMP since cAMP-responsive DNA elements have been functionally implicated in IL-6 gene expression.[15] Furthermore, cAMP elevating agents have been reported to induce the expression of IL-6 mRNA in human fibroblasts[16,17] and monocytes.[18]

When we analysed the effects of PTX on the production of cytokines by PBMC triggered by LPS *in vitro*, we also found that PTX exerted differential effects on the production of IL-6 and TNF-α. Indeed, PTX blocked the secretion of TNF-α in a dose-dependent manner while IL-6 secretion was not significantly modified. These data are in agreement with those previously reported by Waage *et al.*[7] Our Northern blot analyses indicated that the inhibition of TNF-α production by PTX was associated with a decreased accumulation of specific mRNA, suggesting that the drug might act at the transcriptional level. Blockade of TNF-α gene transcription by PTX has indeed been clearly demonstrated in murine macrophages[5,19] and there is evidence that this effect is mediated by cAMP.[19-22] In contrast with PTX, m-PDS efficiently inhibited the accumulation of both TNF-α and IL-6 mRNA. This is consistent with the data indicating that corticosteroids regulate cytokine genes both at the translational and at the transcriptional levels.[5,19,23,24]

Although the direct pathogenic role of IL-6 is controversial, IL-6 participates in the acute phase response,[1] and both TNF-α and IL-6 have been found to be involved in murine models of septic shock[25] and cerebral malaria.[26] Moreover IL-6 is a potent stimulator of B cells and there is evidence that it plays a pathogenic role in multiple myeloma and B-lymphoproliferative disorders.[1,27] The differential effects of PTX on IL-6 and TNF-α should therefore be taken into account when one considers the therapeutic use of the drug in septic shock or in other clinical settings in which both inflammatory cytokines are involved. The cytokine release syndrome induced by the OKT3 mAb represents one of these situations. Indeed, we and others found that the first injection of OKT3 in organ transplant recipients results in a massive release of TNF-α, IFN-γ and IL-2 in the blood stream.[8,28] More recently, high serum levels of IL-6 were also found in these patients.[27]

As a murine IgG2a, OKT3 is able to cross-link the human FcγRI receptors when it is bound by its variable regions to CD3+ cells.[11] Therefore, the production of cytokines induced by OKT3 is caused not only by polyclonal T-cell activation but also by the cross-linking of the FcγRI receptors of monocytic cells.[11,12] Since cytokines are most probably responsible for the first dose reactions induced by OKT3,[8,28] their inhibition should improve the clinical tolerance of the drug. High doses of corticosteroids already proved to be of some efficacy in this respect.[28,29] In a pilot trial in which kidney transplant recipients received PTX—in addition to m-PDS—immediately before and after the first injection of OKT3, we observed that PTX significantly reduced the release of TNF-α but without modifying the incidence of the side-effects of OKT3.[30] This could be related to the inability of PTX to inhibit OKT3-induced IL-6 release. Similar observations were made in a murine model of OKT3 toxicity[30] and in the present study when we analysed the production of TNF-α and IL-6 by unseparated PBMC stimulated by OKT3 *in vitro*. To get more insight into the effects of PTX on the secretion opf cytokines by activated T cells, we conducted further experiments using an anti-CD3 mAb which does not require the presence of monocytic cells to induce T-cell

activation.[13] In this system, PTX inhibited the secretion of TNF-α as well as IL-6 by purified T cells.

Taken together, our observations indicate that PTX modulates the production of IL-6 and TNF-α in different ways depending on their cellular origin. This suggests that drugs acting on adenylate cyclase might selectively regulate the production of cytokines by activated leucocytes.

## ACKNOWLEDGMENTS

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