In vitro effects of oxpentifylline on inflammatory cytokine release in patients with inflammatory bowel disease

J M Reimund, S Dumont, C D Muller, J S Kenney, M Keding, R Baumann, P Poindron, B Duclos

Abstract

Background—Inflammatory cytokines, including tumour necrosis factor- α (TNF- α) and interleukin (IL)-1 β , have been implicated as primary mediators of intestinal inflammation in inflammatory bowel disease.

Aim—To investigate the in vitro effects of oxpentifylline (pentoxifylline; PTX; a phosphodiesterase inhibitor) on inflammatory cytokine production (1) by peripheral mononuclear cells (PBMCs) and (2) by inflamed intestinal mucosa cultures from patients with Crohn's disease and patients with ulcerative colitis.

Methods—PBMCs and mucosal biopsy specimens were cultured for 24 hours in the absence or presence of PTX (up to 100 μ g/ml), and the secretion of TNF- α , IL-1 β , IL-6, and IL-8 determined by enzyme linked immunosorbent assays (ELISAs).

Results—PTX inhibited the release of TNF- α by PBMCs from patients with inflammatory bowel disease and the secretion of TNF- α and IL-1 β by organ cultures of inflamed mucosa from the same patients. Secretion of TNF- α by PBMCs was inhibited by about 50% at a PTX concentration of 25 μ g/ml (IC₅₀). PTX was equally potent in cultures from controls, patients with Crohn's disease, and those with ulcerative colitis. The concentrations of IL-6 and IL-8 were not significantly modified in PBMCs, but IL-6 increased slightly in organ culture supernatants.

Conclusions—PTX or more potent related compounds may represent a new family of cytokine inhibitors, potentially interesting for treatment of inflammatory bowel disease.

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Keywords: inflammatory bowel disease, inflammatory cytokines, oxpentifylline, cyclic nucleotide phosphodiesterase inhibitors.

Inflammatory cytokines – tumour necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β) and interleukin-6 (IL-6) – and neutrophil activating peptide-I/interleukin-8 (IL-8) have been shown to be important mediators during the development and perpetuation of intestinal inflammation in inflammatory bowel diseases. Raised concentrations of TNF- α in serum, as

well as raised faecal concentrations² and increased TNF- α production by peripheral blood mononuclear cells (PBMCs),³ lamina propria mononuclear cells (LPMCs),^{4,5} and organ cultures of biopsy specimens from inflamed⁵ or morphologically normal inflammatory bowel disease intestinal mucosa⁶ have been reported. Similarly high expressions of IL-1 β ,³⁻⁷ IL-6,^{8,9} and IL-8,¹⁰ have been found in both Crohn's disease and ulcerative colitis. Neutralisation of TNF- α by anti-TNF- α antibodies has recently been suggested to be of therapeutic benefit in Crohn's disease by both open^{11,12} and controlled therapeutic trials.¹³ Therefore cytokine inhibition seems a rational target in treatment of inflammatory bowel disease.

Numerous points of therapeutic intervention to inhibit the synthesis or the action of cytokines are possible.¹⁴ Among them, modulation of cytokine transcription by oxpentifylline (pentoxifylline; PTX), a phosphodiesterase (PDE) inhibitor, has been explored.^{15,16} Inhibitors of PDE generate high intracellular cAMP concentrations, which are known to inhibit expression of TNF- α .^{15,16} PTX, the 1-5-oxohexyl analogue of the methylxanthine theobromine, was initially characterised as a haemorrhheological agent and has been used in vascular disorders for the past 20 years. PTX has well known pharmacokinetics with minimal side effects.

The present work was designed to evaluate whether PTX reduces the spontaneous and stimulated production of TNF- α , IL-1 β , IL-6, and IL-8 by PBMCs and organ cultures of colon biopsy specimens in patients with inflammatory bowel disease.

Methods

PATIENTS

A total of 27 patients (Crohn's disease: n=18; ulcerative colitis: n=9) were included in this study. They were prospectively included when entering our gastroenterology unit. Diagnosis was established according to the criteria of Lennard-Jones.¹⁷ The Table shows the clinical data and treatments at the time of inclusion. In Crohn's disease, activity was assessed according to Van Hees *et al*,¹⁸ and in ulcerative colitis by the criteria of Truelove and Witts.¹⁹ No patient had associated disease or was complaining of extraintestinal manifestations of inflammatory bowel disease or extraintestinal

Service
d'Hépatogastro-
entérologie et
d'Assistance Nutritive,
CHRU Haute-pierre,
67098 Strasbourg
Cedex, France
J M Reimund
R Baumann
B Duclos

Département
d'Immunologie,
Immuno-
pharmacologie et
Pathologie, Centre de
Recherches
Pharmaceutiques,
67401 Illkirch
Graffenstaden, France
J M Reimund
S Dumont
P Poindron

Unité de Recherche
INSERM U 381,
Avenue Molière, 67000
Strasbourg Cedex,
France
J M Reimund
M Keding
B Duclos

Laboratoire de
Biophysique, U.R.A.
n°491 du CNRS, Centre
de Recherches
Pharmaceutiques,
67401 Illkirch
Graffenstaden, France
C D Muller
J S Kenney

Correspondence to:
Dr Jean Marie Reimund,
Département
d'Immunologie,
Immunopharmacologie et
Pathologie, Centre de
Recherches
Pharmaceutiques, 74 Route
du Rhin, 67401 Illkirch
Graffenstaden, France.

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Clinical characteristics and treatment at the time of inclusion of the patients with Crohn's disease (CD) or ulcerative colitis (UC)

	Patients with CD (n=18)	Patients with UC (n=9)
Age (mean (range))	31 (21–57)	38 (23–66)
Sex (men/women)	8/10	5/4
Disease activity (n):		
Severe	8	3
Moderate	8	6
Mild	2	0
Bowel involvement (n):		
Ileum alone	3	—
Colon alone	7	—
Ileocolonic disease	8	—
Whole colon	—	6
Left colon	—	3
Current treatment:		
Steroids >10 mg/day	8	3
5-Aminosalicylic acid	5	2
Both	—	4
No medication	5	—

Disease activity was assessed by the index of Van Hees *et al*¹⁸ for patients with CD and by the criteria of Truelove and Witts¹⁹ for patients with UC.

infection. Serological studies for *Yersinia enterocolitica* and cytomegalovirus, and stool cultures including search for *Clostridium difficile* were negative in all cases. Peripheral blood was taken and biopsies were performed in fasting patients free of medication for at least 12 hours.

PERIPHERAL BLOOD MONONUCLEAR CELLS

In 14 patients (Crohn's disease: n=10; ulcerative colitis: n=4) and in six healthy subjects (control group) PBMCs were separated as previously described by Bøyum.²⁰ Briefly, peripheral blood diluted with Ca²⁺ and Mg²⁺ free (CMF) Hank's balanced salt solution (HBSS; Gibco BRL, Cergy, France) containing 100 IU heparin/ml was layered over Ficoll Hypaque (Pharmacia AB, Uppsala, Sweden) and centrifuged for 40 minutes at 400 g. Cells harvested from the interface were washed three times in HBSS-CMF and resuspended at a final concentration of 2×10⁶/ml in a culture medium consisting of RPMI 1640 (Gibco) supplemented by 10% heat inactivated foetal calf serum (FCS, Gibco), 2mM L-glutamine (Gibco), penicillin (100 IU/ml), and streptomycin (100 µg/ml) (Diamant, Paris, France). Human PBMCs were incubated with doses of PTX (Sigma, Paris, France) ranging from 1 µg/ml to 100 µg/ml, with or without simultaneous activation by 1 µg/ml lipopolysaccharide from *Salmonella abortus equi* (LPS, Sigma) and 1 µg/ml of phytohaemagglutinin (PHA, Difco, Detroit, MI, USA) in 24 well culture plates (Falcon, PolyLabo, Strasbourg, France) for 24 hours at 37°C in a humidified 5% CO₂/95% air atmosphere. Supernatants were then removed, filtered, and stored at -80°C until cytokine analysis. Cell viability was assessed by the trypan blue exclusion test.

INTESTINAL BIOPSY SPECIMENS FROM PATIENTS AND TISSUE CULTURE

In 17 patients with inflammatory bowel disease colon biopsy specimens were obtained during colonoscopy (Crohn's disease, n=11; ulcer-

ative colitis, n=6) from macroscopically involved areas of intestinal mucosa (grades 3/4) according to Wardle *et al*.²¹ All patients required a colonoscopy for clinical reasons. In each patient eight biopsy specimens were taken. Two were fixed in formalin for histological assessment and six placed in HBSS-CMF supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin (Diamant) at 4°C for organ culture. After collection, the biopsy specimens were transferred to the laboratory. Within three hours after collection, the tissue was gently washed three times in CMF-Hank's medium with added penicillin and streptomycin, blotted carefully, weighed (range 3–12 mg), and placed in 24 well tissue culture plates in 1 ml culture medium with increasing concentrations of PTX (1 to 100 µg/ml). After 24 hours of culture at 37°C in a humidified 95% air/5% CO₂ atmosphere, supernatants were removed, filtered, and stored at -80°C until cytokine analysis.

Structural integrity of biopsy specimens was assessed by standard histology. Biopsy specimens from each patient were fixed in 10% buffered formalin and embedded in paraffin wax after dehydration, clearing, and impregnation. Serial 4 µm sections were stained with haematoxylin and eosin. Lactate dehydrogenase release in supernatants of cultured tissues was also determined.

IMMUNOASSAYS FOR CYTOKINES

Culture supernatants were assayed with two-site enzyme linked immunosorbent assays (ELISAs) specific for human interleukins IL-1β, IL-6, IL-8, and TNF-α. Antibodies used were a gift from J Kenney, Antibody Solutions, Half Moon Bay, CA, USA. Quantitative evaluation of monocyte secreted interleukins was achieved by ELISAs using conditions described by Kenney *et al*,²² with slight modifications as described here. Tween (500 µl/l PBS) was substituted for thimerosal for the preparation of incubation, blocking, and washing buffers. Polyvinyl chloride plates (Costar, No 2596) were coated with 50 µl per well of antibodies (15 µg/ml) and incubated overnight at 4°C. After the usual wash and non-specific saturation steps, 25 µl standard or sample were added to 25 µl biotinylated monoclonal antibody (2 µg/ml) and incubated for two hours at room temperature. After washing steps, 50 µl of a peroxidase-streptavidin dilution (1:3000 in PBS Triton) were added and the mixture incubated for one hour at room temperature. A colorimetric reaction (optical density at 450 nm) using *o*-phenylenediamine dihydrochloride as peroxidase substrate was performed after four washing steps. Concentrations (pg/ml) of unknown samples were computed by interpolation with a standard curve run on each plate using four parameters logistics analysis.

STATISTICS

Data are expressed as means (SEM) of percentage of basal cytokine secretion. The

symbol *n* refers to the number of experiments. ELISAs were performed on duplicate samples. Results were compared using Student's *t* test for normally distributed data. For non-normally distributed data, the Mann-Whitney U test, or the Kruskal Wallis test if more than two groups were compared, was used. The level of significance was taken as $p < 0.05$.

Results

CELL AND TISSUE VIABILITY

Cell viability as assessed by the trypan blue exclusion test and structural integrity appreciated by standard histology was not affected by any drug concentration or LPS/PHA activation. Lactate dehydrogenase concentrations were significantly lower in organ culture supernatants than in the supernatants of uncultured biopsy specimens, indicating satisfactory tissue viability.

Figure 1: Effect of PTX on IL-8, IL-6, IL-1β, and TNF-α release by PBMCs isolated from patients with inflammatory bowel disease. The effect of PTX (1, 10, 25, 50, and 100 μg/ml) on cytokine production by PBMCs was evaluated by ELISA as described in Methods in the absence (A) or presence (B) of 1 μg/ml LPS and 1 μg/ml PHA. Results (mean (SEM), *n*=14) express cytokine production as the percentage of control (100% without activation by LPS/PHA: IL-8, 20.1 (3.8) ng/ml, IL-6, 3.8 (0.7) ng/ml, IL-1β, 546.1 (181.5) pg/ml, TNF-α, 654.4 (78.5) pg/ml; 100% in presence of LPS/PHA: IL-8, 18.9 (3.1) ng/ml, IL-6, 8.1 (2.4) ng/ml, IL-1β, 1343.8 (398.4) pg/ml, TNF-α, 1427.2 (375.1) pg/ml; * $p < 0.001$).

IN VITRO EFFECTS OF PTX ON SPONTANEOUS AND STIMULATED CYTOKINE PRODUCTION BY PBMCs

Spontaneous TNF-α and IL-1β production by PBMCs did not differ between cells obtained from controls, patients with Crohn's disease, or patients with ulcerative colitis ($p < 0.45$ and $p < 0.56$ respectively) in control plates without PTX. Despite a trend toward higher IL-1β and TNF-α production after activation by LPS/PHA the difference did not reach significance (for example, for TNF-α: 891.6 (118.7) *v* 1142.8 (232.5) *v* 1995.9 (164.7) pg/ml respectively in controls, patients with Crohn's disease, and patients with ulcerative colitis). Stimulated IL-6 production and both spontaneous and stimulated IL-8 production by PBMCs were higher in patients with Crohn's disease or ulcerative colitis than in controls. This difference, however, was not significant. Neither disease activity, nor steroid or mesalazine treatment influenced the spontaneous or stimulated cytokine production by PBMCs.

When PTX was used, TNF-α concentrations in the supernatants of PBMCs from patients with inflammatory bowel disease were found to be significantly reduced after 24 hours of culture compared with basal production ($IC_{50} \sim 25$ mg/ml; $p < 0.0001$) (Fig 1A). The effect of PTX on TNF-α production by PBMCs from patients with inflammatory bowel disease was the same in the absence or presence of activation by LPS/PHA (Fig 1B) except for TNF-α at 100 μg/ml (43% of the basal production without LPS/PHA *v* 26% with LPS/PHA; $p < 0.05$). The pattern of the inhibition of PBMC TNF-α production by PTX in the presence or absence of simultaneous incubation with LPS/PHA did not differ between controls, patients with Crohn's disease, or patients with ulcerative colitis (Fig 2A and 2B).

PTX did not change the production of IL-1β by PBMCs from patients with inflammatory bowel disease either in the absence or presence of LPS/PHA ($p = 0.86$ without activation by LPS/PHA and $p = 0.17$ with LPS/PHA; Fig 1A and 1B). No difference was found between PBMCs from controls, patients with Crohn's disease or patients with ulcerative colitis (for example, $p = 0.63$ for PTX=100 μg/ml with LPS/PHA).

Despite a trend toward higher IL-6 secretion in the presence of PTX, this difference was not significant ($p = 0.29$; Fig 1A). These results were not modified after LPS/PHA activation ($p = 0.10$; Fig 1B). Concentrations of IL-6 in the supernatants of PBMC cultures did not differ between controls, patients with Crohn's disease, and patients with ulcerative colitis.

Concentrations of IL-8 in the cell culture supernatant, in the presence or absence of activation by LPS/PHA (Fig 1A and 1B), were not modified by PTX in controls, patients with Crohn's disease, or patients with ulcerative colitis.

The effect of PTX upon cytokine production (particularly TNF-α production) did not differ according to disease activity, or steroid or mesalazine treatment (data not shown).

Figure 2: Comparison of the effect of PTX on TNF- α release by PBMCs isolated from healthy controls (non-inflammatory bowel disease (non-IBD), $n=6$) and patients with Crohn's disease (CD, $n=10$), or ulcerative colitis (UC, $n=4$). The effect of PTX (1, 10, and 100 $\mu\text{g/ml}$) on cytokine production by PBMCs was evaluated by ELISA as described in Methods in the absence (A) or presence (B) of 1 $\mu\text{g/ml}$ LPS and 1 $\mu\text{g/ml}$ PHA. Results (mean (SEM)) express cytokine production as the percentage of control (100% without LPS/PHA: non-inflammatory bowel disease, 698.2 (114.7) pg/ml, Crohn's disease, 736.5 (98.8) pg/ml, ulcerative colitis, 490.3 (93.9) pg/ml; 100% with LPS/PHA: non-inflammatory bowel disease, 891.6 (118.7) pg/ml, Crohn's disease, 1142.8 (232.5) pg/ml, ulcerative colitis, 1995.9 (164.7) pg/ml).

CYTOKINE CONCENTRATIONS IN THE ORGAN CULTURE SUPERNATANTS

Spontaneous cytokine production by intestinal biopsy specimens was not different in regard to disease activity (for example, $p<0.84$ for TNF- α), steroid treatment (for example,

Figure 3: Effect of PTX on IL-1 β and TNF- α concentrations in the supernatants of organ cultures of inflamed intestinal mucosa from patients with inflammatory bowel disease ($n=17$). The effect of PTX (1, 10, 25, 50, and 100 $\mu\text{g/ml}$) on cytokine production was evaluated by ELISA as described in Methods. Results (mean (SEM)) express cytokine production as the percentage of control (100%: IL-1 β , 81.9 (23.4) pg/ml; TNF- α , 75.2 (19.9) pg/ml; * $p<0.001$, ** $p<0.01$).

$p<0.28$ for TNF- α), or mesalazine treatment (for example, $p<0.16$ for TNF- α).

As found in PBMC cultures, PTX inhibited TNF- α release significantly in organ cultures of intestinal mucosa from patients with inflammatory bowel disease ($\text{IC}_{50} \sim 25$ $\mu\text{g/ml}$; $p<0.0001$) (Fig 3). Concentrations of PTX exceeding 25 $\mu\text{g/ml}$ were not able to enhance this inhibitory effect. No difference was seen between patients with Crohn's disease and those with ulcerative colitis ($p=0.33$). The concentration of TNF- α inhibition by PTX was not influenced by disease activity ($p=0.10$), steroid ($p=0.57$), or mesalazine ($p=0.59$) treatment.

Concentrations of IL-1 β were significantly decreased in the presence of PTX (about 40% of IL-1 β released spontaneously; $p<0.0001$; Fig 3) by contrast with the effect in PBMC cultures. The effect of PTX was similar in patients with Crohn's disease and those with ulcerative colitis ($p=0.93$).

Concentrations of IL-6 in the organ culture supernatants of inflamed mucosa from patients with inflammatory bowel disease increased significantly in the presence of PTX (144% compared with spontaneous release; $p<0.0025$).

Concentrations of IL-8 were not modified in the presence of PTX ($p=0.18$).

Discussion

The present study shows the ability of PTX to inhibit (1) the release of TNF- α by PBMCs isolated from patients with inflammatory bowel disease and (2) the secretion of both TNF- α and IL-1 β in the organ culture supernatants of inflamed intestinal mucosa from the same patients. The inhibition of TNF- α secretion by PBMCs from patients with inflammatory bowel disease was around 50% at a PTX concentration of 25 $\mu\text{g/ml}$ (IC_{50}), with similar potency in PBMCs from healthy controls. When PBMCs from patients with inflammatory bowel disease were activated by LPS/PHA, the potency of PTX to inhibit TNF- α was further increased (up to 75% of control values) in accord with other reports.²³ The inhibitory effect of PTX was similar in Crohn's disease or ulcerative colitis when checked in PBMCs or in organ cultures from patients with these diseases. This cytokine inhibiting effect did not differ between patients with active, moderate, or mild disease activity, and was not influenced by previous treatment with mesalazine or steroids. This finding may be partially explained because steroids and PTX probably inhibit TNF- α synthesis at distinct points in the signalling pathway; this has been suggested by Han *et al.*,²⁴ who showed in RAW 264.7 macrophages that PTX blocked TNF- α mRNA accumulation whereas dexamethasone strongly inhibited translational derepression, a mechanism not reported for PTX. These authors also showed that combined application of dexamethasone and PTX enhanced the inhibition of TNF- α synthesis compared with each drug alone.

Since inflammatory cytokines have been established as key mediators in the cascade of

inflammatory events associated with inflammatory bowel disease, immunity modifying drugs have been developed, with some of them currently showing promising effects in animal models of experimental colitis or in humans with inflammatory bowel disease. This was, for example, shown by using IL-1ra in a rabbit model of formalin immune complex colitis,²⁵ and by using chimeric antitumour necrosis factor monoclonal antibodies in active steroid refractory patients with Crohn's disease.¹¹⁻¹³ Such treatments, although effective, are costly and not always well tolerated in humans. Furthermore, their efficacy and their safety in long term use remains to be established.¹³ The results, however, strongly suggest that inflammatory cytokines, in particular TNF- α and IL-1 β , are of major importance in the pathogenesis of inflammatory bowel disease, leading to the evaluation of other cytokine modulating molecules. Whether these drugs act exclusively through cytokine inhibition or involve multiple immune and inflammatory mechanisms remains to be determined. However, as cytokines appear as regulators at the initial steps of intestinal immune response, their inhibition may be a cornerstone in inflammatory bowel disease treatment.

Our results suggest that PDE inhibitors such as PTX may potentially be interesting in the field of cytokine inhibition in patients with Crohn's disease or ulcerative colitis. The difference between response in PBMCs, which was dose related, and the absence of a dose-response effect in organ cultures remains to be explained. It may be related to the fact that organ cultures represent a complex system in which multiple cells interact. This difference may disappear using lamina propria mononuclear cells instead of organ cultures. Studies are currently in progress in our department to clarify this. Inhibition of IL-1 β in organ cultures but not in PBMCs may also be the result of multiple cell-cell interactions in biopsy specimens. In fact, inhibition of IL-1 β production by PBMCs – as well as down regulation of IL-6 and IL-8 release – by PTX has been reported in vivo under specific culture conditions,²⁶ and described for IL-1 β by Bienvenu *et al*²⁷ using the whole blood model. Such data suggest that inhibiting TNF- α may result in the inhibition of IL-1 β , or cytokines with late expression such as IL-8.

As well as the role of TNF- α and IL-1 β in the pathogenesis of inflammatory bowel disease, other cytokines seem to be potential targets in the treatment of Crohn's disease and ulcerative colitis. Previous studies have shown increased numbers of IL-2 and IFN- γ secreting cells, especially in Crohn's disease.²⁸ These studies have hypothesised that a Th1-like profile of lymphokine production may exist in the mucosal lesions of Crohn's disease. Animal studies have indicated that the inhibitory properties of PTX are particular to Th1-like CD4⁺ T cells.²⁹⁻³⁰ Furthermore, Novak and Rothenberg²³ and Betz and Fox³¹ recently suggested that Th1 and Th2 cells could be distinguished by their ability to maintain a low cAMP concentration (Th1)

and high cAMP concentration (Th2), respectively. Such a balanced effect may explain the increase in IL-6 in some of our experiments and also that reported for normal PBMCs.³²⁻³³ The differential effect of PTX on TNF- α on the one hand and other cytokines on the other is currently not explained as the exact mechanisms of action of PTX are not known. One hypothesis was a selectivity in the induction of cytokine gene expression. Signals such as LPS, or effects of transcription factors on gene expression, vary largely from one gene to another. For TNF- α , it is suspected that PTX modifies NF- κ B activation or blocks the ras/raf/MEK/MAPK pathway,³⁴⁻³⁵ by enhancing intracellular cAMP concentrations.¹⁵⁻³⁶⁻³⁷ By contrast, an increase in intracellular cAMP has been suggested to activate the IL-6 gene.³⁸

Various other immunological properties of PTX may be of interest regarding the current knowledge of immunopathogenesis of inflammatory bowel disease. This is the case for inhibition of tissue factor mRNA expression,³⁹⁻⁴⁰ decrease in cytokine induced intercellular adhesion molecule-1,⁴¹ or reduction in fibroproliferation.⁴² Our data need to be extended further by in vitro and in vivo studies in humans and in animal models of inflammatory bowel disease before considering the question of therapeutic relevance of PTX or other PDE inhibitors in patients with inflammatory bowel disease. Since we began our work,⁴³ open studies including a few patients with ulcerative colitis and Crohn's disease have been reported, with conflicting results.⁴⁴⁻⁴⁵ Treatment schedules usually used doses in the range of 1200–2400 mg/day which allowed the achievement of apparently adequate plasma concentrations of PTX,⁴⁶ but showed no or only mild clinical results. Data on tissue concentrations are missing, but may explain contrasting results between in vivo studies and the present results.

In conclusion, our data show that PTX down regulates in vitro TNF- α and IL-1 β production by PBMCs and intestinal organ cultures from patients with inflammatory bowel disease and indicate a potential interest in this drug and related compounds for treatment of Crohn's disease and ulcerative colitis.

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