Pentoxifylline decreases *in vivo* and *in vitro* tumour necrosis factor-alpha (TNF-α) production in lepromatous leprosy patients with erythema nodosum leprosum (ENL)

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SUMMARY

Increasing evidence has implicated TNF-α as a pivotal molecule involved in the systemic inflammatory manifestations of ENL, an acute inflammatory complication that may occur in the chronic course of leprosy. In the present study, the mechanism of action of pentoxifylline (PTX) as an alternative therapy for management of leprosy reactions has been evaluated. The effect of PTX on TNF-α production was examined in leprosy patients at the protein level and at the transcriptional level as well. Treatment of ENL patients with PTX (1200 mg daily) ameliorated the systemic symptoms and favoured the evolution of reactional leprosy lesions. Serum TNF-α was assayed before and during treatment with PTX in 15 patients. The increased TNF-α levels seen in the circulation during the reaction were dramatically reduced within 3–7 days of therapy. No significant effect on serum IL-6 was noted. *In vitro* TNF-α production was assayed upon culture stimulation with *Mycobacterium leprae*. A reduction of inducible TNF-α in peripheral blood mononuclear cells (PBMC) was seen after 1–2 weeks of *in vivo* administration of PTX. Furthermore, no effect of the drug on IL-10 secretion was detected in these cultures. A kinetic analysis of the expression of TNF-α and IL-6 mRNA at the site of leprosy lesion was performed in six reactional patients by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR). The amount of TNF-α mRNA was increased in the tissue during ENL compared with before the reaction, and decreased thereafter following treatment for reaction (either PTX or thalidomide). These data suggest that PTX inhibits TNF-α production in ENL patients both *in vivo* and *in vitro*, and it may be useful in the treatment of leprosy patients undergoing ENL.

Keywords pentoxifylline tumour necrosis factor-alpha erythema nodosum leprosum thalidomide RT-PCR

INTRODUCTION

All information accumulated in the past few years has implicated cytokines in the pathogenic mechanisms of several human diseases. Since then, TNF-α has been considered a key molecule in the intricate net of interaction between blood leucocytes and tissue cells. TNF-α is thought to be a proximal factor of the inflammatory response, and it has been considered an important mediator in a wide variety of acute and chronic inflammatory disease states [1–3].

Our first demonstration that leprosy patients undergoing reactional inflammatory episodes (ENL) present increased TNF-α and IL-1β levels in their sera [4] has been confirmed by other groups [5,6]. ENL develops more frequently towards the lepromatous pole of leprosy and it is characterized by symptoms resembling septic syndrome accompanied by both local and systemic inflammation.

Increased evidence has so far implicated TNF-α as a pivotal molecule involved in the inflammatory manifestations of ENL and in mediating tissue damage in leprosy. It has been demonstrated that cells from these patients release enhanced amounts of TNF-α when stimulated *in vitro*, compared with unreactional lepromatous patients or normal individuals [7,8]. In addition, increased expression of TNF-α mRNA and TNF-α protein has been identified in reactional leprosy lesions [9].

IL-6, also a proinflammatory cytokine, has also been found in the serum of leprosy patients with reaction [10], probably implicating this cytokine in the complex set of responses seen in these reactional leprosy patients, such as enhanced acute-phase response. Accordingly, it has been reported that levels of C-reactive protein (CRP) and serum amyloid A (SAA) are markedly elevated in ENL patients [11].

On the other hand, increased production of IL-10 has been recently implicated in the functional impairment of the immune response. IL-10 has a profound inhibitory role by decreasing

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macrophage function. It inhibits antigen presentation and cytokine synthesis by monocytes [12–14]. Furthermore, it has been suggested that IL-10, released mainly from *Mycobacterium leprae*-infected macrophages [15], could be involved in mediating the anergic state seen in the lepromatous form of leprosy [15,16].

Because of the inflammatory symptoms and of neurological complications that are very often associated with these reactional episodes in leprosy, they have to be considered as a clinical emergency, and treatment with thalidomide and/or steroids needs to be promptly initiated. It was first shown by our group that thalidomide, the drug of choice for treatment of ENL, inhibits TNF-α production either *in vivo* or *in vitro* [10,17]. The effect of thalidomide was specific, and it suppressed lipopolysaccharide (LPS)- and mycobacteria-induced monocyte-derived TNF-α production *in vitro*, at the level of both TNF mRNA expression and protein secretion. The drug seemed to enhance TNF-α mRNA degradation [18] and, *in vivo*, it was also demonstrated to down-modulate the expression of activation molecules (intercellular adhesion molecule-1 (ICAM-1), HLA-DR) in the reactional tissue when the reactional episode subsided [17]. Nevertheless, the predominant side-effects of corticosteroids and the teratogenic activity of thalidomide have limited their use in reactional patients, mainly females of child-bearing age. The use of other anti-inflammatory drugs for treatment of reactional leprosy patients is therefore warranted.

The methylxanthine PTX is a drug of known haemorrheological activity, used clinically for treatment of intermittent claudication and other conditions involving defective regional microcirculation [19]. Following the demonstration that PTX suppress induction of TNF-α in endotoxin-treated murine macrophages [20], and in healthy human volunteers given endotoxin [21], the drug has been used as a therapeutic tool in many other immunological based disorders in which TNF seems to play a major role, with a clinically beneficial effect in some cases [22,23].

We have previously reported our preliminary clinical observations on the use of PTX as an alternative therapy for management of leprosy reactions [24]. Although PTX is not as rapidly acting as thalidomide, treatment of ENL patients with the former drug alleviated the clinical manifestations of reaction in most of the patients tested. In the present study, the effect of PTX on TNF-α production has been examined in these patients, both *in vivo* and *in vitro*, at the protein level and also at the transcriptional level. Here, cytokine gene expression has been evaluated in leprosy biopsies before and after treatment with PTX, compared with thalidomide, by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR).

PATIENTS AND METHODS

Patient population
Leprosy patients from the Leprosy Out-Patient Unit, Oswaldo Cruz Foundation (Rio de Janeiro, Brazil) were diagnosed according to the Ridley–Jopling classification [25]. A total of 18 multibacillary patients (14 males and four females), 15–64 years old (mean ± s.d. 34·9 ± 15 years), who were undergoing acute inflammatory reactional episodes, was included in the study. Patients were classified as lepromatous leprosy (LL, $n = 13$) and borderline lepromatous (BL, $n = 5$). All were lepromin-negative, having a bacterial index (BI) ranging from 2·0 to 5·5 + (mean BI ± s.d. 4·2 ± 1·1 +), and had been treated with multidrug therapy (MDT) as recommended by WHO [10]. The clinical features of each patient are summarized

in Table 1. Seven ENL patients were newly diagnosed leprosy patients who had not received any kind of treatment prior to reaction (patients 3, 6, 10, 14–16, 18). In these individuals, MDT was initiated while patients were still on treatment for reaction (either PTX or thalidomide). Four patients (patients 5, 11–13, 17) developed ENL when they had already completed the 2 years of chemotherapy and were under surveillance (Surv). For the other patients (patients 1, 2, 4, 7–9), MDT was continued throughout the study. For treatment of the reactional episode, patients ($n = 16$) were prescribed with PTX 400 mg (Hoechst do Brasil, S.A., Sao Paulo, Brazil), three times a day, for a 2-month period. A simultaneous analysis was performed in two lepromatous patients who developed ENL (patients 17 and 18, Table 1) and were treated with thalidomide (300 mg/day) until total remission of the inflammatory symptoms.

Skin biopsies
After informed consent, biopsies (6 mm punch) were obtained from the patients at the time of leprosy diagnosis and at the onset of the reactional episode. Biopsies were processed for histological and PCR analysis. For RT-PCR, fresh biopsy samples were collected at diagnosis (unreactional leprosy lesion, two patients), at the onset of the reactional episode (ENL lesion, six patients), and after 3 and/or 7 days of PTX (reactional lesion, four patients) or thalidomide (two patients) therapy.

Serum samples
Serum was collected for determination of circulating cytokine levels during ENL (day 0), and at different periods (3–7, 10–14, 30 and 60 days) after PTX administration. Serum was collected aseptically, processed under sterile conditions, stored in 0·5-ml aliquots and kept frozen until use (−20°C).

Culture stimulation
Armadillo-derived *M. leprae* antigen, provided by Dr R. J. W. Rees (IMMLEP Bank, The National Institute of Medical Research, Mill Hill, UK), was used at 10 μg/ml. In some cultures, LPS (from *Salmonella minnesota* R595; Sigma Chemical Co., St Louis, MO) 1 μg/ml was used as a positive control for TNF-α induction. For *in vitro* use, PTX (Sigma) was dissolved in PBS pH 7·0 and used in the cell cultures at final concentrations of 25 and 50 μg/ml. Concentrations of contaminating endotoxin in stock solutions, culture medium, and antigens were estimated by the Limulus amoebocyte lysate assay (LAL; Whittaker M.A. Bioproducts, Walkersville, MD). Reagents contained < 10 pg/ml endotoxin.

Cell preparation and culture
Heparinized venous blood was collected for *in vitro* tests and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll–Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density centrifugation. Cells were suspended in RPMI 1640 medium (GIBCO Labs, Gaithersburg, MD), supplemented with 10% human AB serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (GIBCO, complete medium), and cultured (1×10^6 cells) onto a 24-well plate (Costar Corp., Cambridge, MA) for *in vitro* cytokine stimulation (PBMC cultures). For monocyte cultures, PBMC were allowed to adhere for 2 h at 37°C. After this period, non-adherent cells were removed by rinsing four times with warmed RPMI medium and cultures replaced with 1 ml complete medium. The purity of monocytes was > 95% as determined by morphological examination and non-specific esterase staining.

Both the PBMC and the attached monocyte-enriched population were then further incubated in the presence or absence of TNF-α-inducing agonists (either *M. leprae* or LPS) for 18–20 h, when culture supernatants were harvested, centrifuged, and kept frozen until use (−20°C).

Cytokine determination
Concentration of TNF-α, IL-6 and IL-10 in supernatants and serum samples was determined using commercial specific ELISA kits (IL-6, R&D Systems, Inc., Minneapolis, MN; IL-10, Endogen Inc., Boston, MA; TNF-α, Innogenetics Inc., Antwerp, Belgium) processed according to the manufacturers' specifications. Serial samples from a given patient were assayed at the same time. Cytokine levels are expressed as pg of protein/ml. The detection limit of the assay was 5 pg/ml for TNF-α, 3 pg/ml for IL-10, and 0·4 pg/ml for IL-6.

RNA isolation and cDNA synthesis
Frozen biopsies were recovered, when dermis and epidermis were separated, and the tissues homogenized in a Politron PT-3000 in 3 ml of Trizol (GIBCO). Total RNA was extracted according to the manufacturer's instructions. Spectrophotometric analysis at 260 nm was used to measure the purity and concentration of RNA. One microgram of total RNA obtained from the dermis was reverse-transcribed into cDNA. The RT reaction mixture was performed by mixing 1 μg RNA with 1 μg of an oligo-dT primer (United States Biochemical Corp., Cleveland, OH), in a volume of 8 μl, and incubated for 10 min at 65°C [26]. After cooling on ice, a solution containing 5× RT buffer, 40 U RNAsin (Promega Biotec, Madison, WI), 10 mM DTT (Boehringer Mannheim Biochemicals, Indianapolis, IN), 100 μg/ml acetylated bovine serum albumin (BSA; Promega Biotec), 250 μM of each dNTP (Perkin Elmer Cetus, Emeryville, CA), and SuperScript II RNAse H⁻ reverse transcriptase (GIBCO) at 200 U, in a final volume of 20 μl, was added to the tubes, which were incubated for 1 h at 42°C. Finally, tubes were heated to 95°C for 10 min, and 80 μl of dH₂O were added to the mixture. Samples were stored at −20°C until further use. This reaction was always performed simultaneously for parallel samples from one experiment.

PCR conditions
Cytokine-specific oligonucleotide primer pairs TNF-α, IL-6 and β-actin were purchased (Stratagene Cloning System, La Jolla, CA) or synthesized (Macromolecular Resources, Fort Collins, CO). The primers were RNA-specific in that both the 5′ and 3′ primers span the junctions of two exons, thus precluding amplification of genomic DNA. PCR primer sequences (5′ and 3′) for the cytokines and the control β-actin were as follows: TNF-α, CGGGACGTG-GAGCTGGCCGAGGAG, CACCAGCTGGTTATCTCTCAGC-TC; IL-6, ATGAACTCCTTCTCCACAAGCGC, GAAGAGCC-CTCAGGCTGGACTG; β-actin, TGACGGGGGGTCACCCACA-CTGTGCCCATCTA, CTAGAAGCATTTGCGGTGGGACGG-ATGGAGGG. cDNA (5 μl) was added to 20 μl of a solution consisting of 1 U *Taq* DNA polymerase (Perkin Elmer), 200 μM dNTPs, 1 mM primers, and PCR buffer (1·5 mM MgCl₂, 50 mM KCl, 10 mM Tris–HCl pH 8·3, 0·01% gelatin, and 0·1% Triton X-100). The reaction mixture was overlaid with a drop of mineral oil, and PCR was performed in a DNA thermocycler 480 (Perkin Elmer) for 35 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 1·5 min. PCR product (5 μl) was subjected to electrophoresis on 2% agarose gels and visualized by staining with ethidium bromide. Specificity of the amplified bands was validated by their predicted size. PCR-assisted mRNA amplification was performed simultaneously for all samples of each patient included in the study.

Hybridization of PCR product
To confirm the specificity of PCR amplification, and for semi-quantitative analysis, PCR products were transferred to Hybond-N nylon membranes (Amersham Corp., Arlington Heights, IL) and hybridized with a radioactive oligonucleotide probe complementary to sequences internal to those recognized by the specific primers. Sequences of the probes were: β-actin, CTGCTGACC-GAGGCCCCCCTGAACCCC; TNF-α, TGAGGGTTTGCTA CATGGGCTA; IL-6, TCTTGTTACATGTCTCCTTTCTCAG. The probe was labelled at the 5′ end with T4 polynucleotide kinase (Boehringer) and ³²P-γATP (6000 Ci/mM; Amersham). Blots were hybridized with probe overnight, washed twice for 15 min with 2× SSC and 0·5% SDS, followed by 0·5× SSC and 0·1% SDS, at 42°C, and then exposed to x-ray film (ECL Hyper-film; Amersham). Densitometer analysis was performed by scanning the images from autoradiograms (Quick Scan; Helena Labs, Beverly, MA). The relative amount of PCR product present in an individual sample was expressed as percentage relative to the most intense band, assigned the value of 100. To make sure that the samples contained identical quantities of cDNA, β-actin was used in order to normalize the exact levels of input cDNA present among the different samples tested.

Validity of PCR amplification for semiquantitative comparison of cytokine mRNA expression
A number of controls were employed to permit semiquantitative comparison of cytokine mRNA expression in the biopsy samples studied. Sensitivity of PCR was equivalent for each cytokine based on titration of plasmid cDNA (serial 10-fold dilutions). There was a log-linear correlation between the number of starting copies of plasmid and the yield of PCR products across the range of plasmid concentrations investigated. Linearity of PCR amplification was also demonstrated by varying the number of PCR cycles. Repeat PCR analysis of the same samples yielded reproducible results. These studies indicate that our PCR conditions were not within the plateau phase of amplification. In addition, precautions were taken to avoid cross-contamination of samples, including use of ali-quotted reagents, pipettes dedicated for assembling PCR reactions, and use of aerosol-resistant pipette tips.

Statistical analysis
The variation observed in cytokine levels in patients' samples during PTX treatment was assayed through the Friedman ANOVA test (Statsoft Inc., 1995; STATISTICA for Windows (Computer Program Manual) Tulsa, OK) [27]. For the Friedman ANOVA by ranks test, the interpretation of results is similar to that of repeated measures ANOVA, to which it is a non-parametric alternative. All data are given as mean ± s.e.m. For statistical analysis of paired samples, the Wilcoxon signed-rank test was used. The significance level adopted was $P < 0.05$.

RESULTS

As previously reported [24], reactional leprosy patients treated with PTX (1200 mg daily) experienced, within the first week of treatment, some degree of relief to total regression of their

Table 1. Clinical features of ENL patients enrolled into the study

Patient	Diagnosis (ENL status)*	Sex	Age (years)	BI	MDT† (months)	Immunological assays‡	Serum TNG-α levels (pg//ml)§
1	LL (II)	M	52	4·6 +	12	SR/SN/PCR	100
2	LL (II)	F	40	5·5 +	1	SR/SN/PCR	100
3	LL (II)	M	30	5·5 +	0¶	SR/PCR	60
4	LL (II)	M	26	4·0 +	9	SR/SN/PCR	62
5	BL (I)	F	50	4·8 +	17 Surv**	SN	0††
6	LL (I)	F	31	4·2 +	0	SR/SN	38
7	LL (II)	M	33	5·2 +	2	SR	150
8	LL (II)	M	20	5·5 +	9	SR	160
9	LL (II)	F	15	5·0 +	8	SR	60
10	LL (III)	M	28	4·3 +	0	SR	53
11	BL (III)	M	64	2·0 +	10 Surv	SR	60
12	BL (II)	M	23	4·0 +	2 Surv	SR	80
13	LL (II)	M	61	2·0 +	36 Surv	SR	180
14	LL (II)	M	36	3·8 +	0	SR/SN	70
15	LL (II)	M	24	4·6 +	0	SR/SN	200
16	BL (II)	M	20	2·8 +	0	SR/SN	82
17‡‡	LL (II)	M	50	4·0 +	1	PCR	80
18‡‡	BL (III)	M	26	3·3 +	0	PCR	ND

BI, Bacterial index at the time of leprosy diagnosis.

* Clinical status of the ENL episode: I, presence of ENL nodules only; II, presence of ENL nodules + systemic symptoms; III, presence of erythema multiforme-like lesions accompanied or not by systemic symptoms.

† Duration of multidrug therapy (MDT) before development of reaction.

‡ Assays performed during the study: SR, measurement of TNF-α and IL-6 on serum samples by ELISA; SN, detection of TNF-α and IL-10 on cell culture supernatants by ELISA; PCR, semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis on tissue samples.

§ Levels of TNF-α detected in the serum at the onset of the ENL episode; ND, not done.

¶ Patients who developed ENL before initiation of MDT.

** Patients who developed reaction after completion of MDT (surveillance (Surv)). Number indicates the period in months of development of reaction after discontinuation of the 2 years of MDT.

†† Kinetics of serum TNF-α values during the follow up was not obtained for this patient and therefore not included in the study.

‡‡ Patients 17 and 18, ENL patients who were treated with thalidomide; ENL patients numbered 1–16 were treated with pentoxifylline (PTX).

systemic symptoms, which included fever, malaise, headache, and insomnia. Patients reported an increased sense of well-being, with no adverse side-effects. In addition, a decrease in the inflammatory aspects of ENL skin lesions was noted after 7–14 days. Our data showed that oral PTX is safe and well tolerated in patients with leprosy.

Effect of oral PTX on circulating cytokines
Serum TNF-α levels were assayed before and during treatment with PTX in 15 patients. Elevated amounts of TNF-α were found in the serum of reactional patients who presented systemic symptoms (Table 1) [7]. As shown in Fig. 1, LL patients had increased TNF-α levels (mean ± s.e.m. 101 ± 14·8 pg/ml) in the circulation during the reaction (ENL, day 0), and treatment with PTX dramatically reduced TNF-α values already within 3–7 days of treatment (Fig. 1a,b). The reduction in mean TNF-α noted after 7 days (24·4 ± 5·4 pg/ml (mean ± s.e.m.)) was statistically significant (Fig. 1d, $P = 0.006$, Friedman ANOVA test). In four patients (Fig. 1b), although systemic clinical manifestations were impaired with PTX, baseline serum levels rose again in the course (after 2–3 weeks) of PTX therapy. In these patients, the further appearance of ENL nodules, noted during PTX, in the absence of systemic symptoms [24], did not comprise a major clinical complaint. No significant difference was observed in the increase in mean TNF-α values noted after 14 ($P = 0.102$) and 30 days of treatment

($P = 0.414$) compared with 7 days of PTX (Fig. 1d). Nevertheless, when compared with the values seen at the onset of reaction, the reduction in TNF-α was still significant ($P < 0.02$ and 0·05, respectively). Moreover, mean TNF-α at the end of the study (total of 8 weeks under PTX) was 13·3 ± 7·6 pg/ml, still a significant decrease (86·8% of baseline levels) in TNF-α compared with values obtained at the onset of the study (Fig. 1d, $P = 0.004$). In addition, on an individual basis, the decrease in systemic TNF-α paralleled the improvement in patients' clinical symptomatology (not shown). In two patients, who seemed to be refractory to PTX, serum TNF-α was found to be even higher after 1 week of PTX (Fig. 1c), when treatment with thalidomide and/or steroid was initiated and improvement of their clinical condition was noted 4 days later. Therefore, TNF-α values from these two patients were not included in the previous analysis.

We were then interested to determine whether PTX affected the levels of IL-6 detected in the serum of leprosy patients with reaction. Similarly to what is observed for TNF-α, levels of IL-6 (382 ± 187 pg/ml) were significantly ($P < 0.002$, Wilcoxon rank test) enhanced in the serum of leprosy patients with reaction ($n = 13$) compared with levels obtained from the same patients before the reaction (61 ± 32 pg/ml). However, although in all patients but one, levels of IL-6 were diminished following PTX (not shown), there were substantial variations in IL-6 values between subjects (range 64–1890 pg/ml at day 0). Hence, no
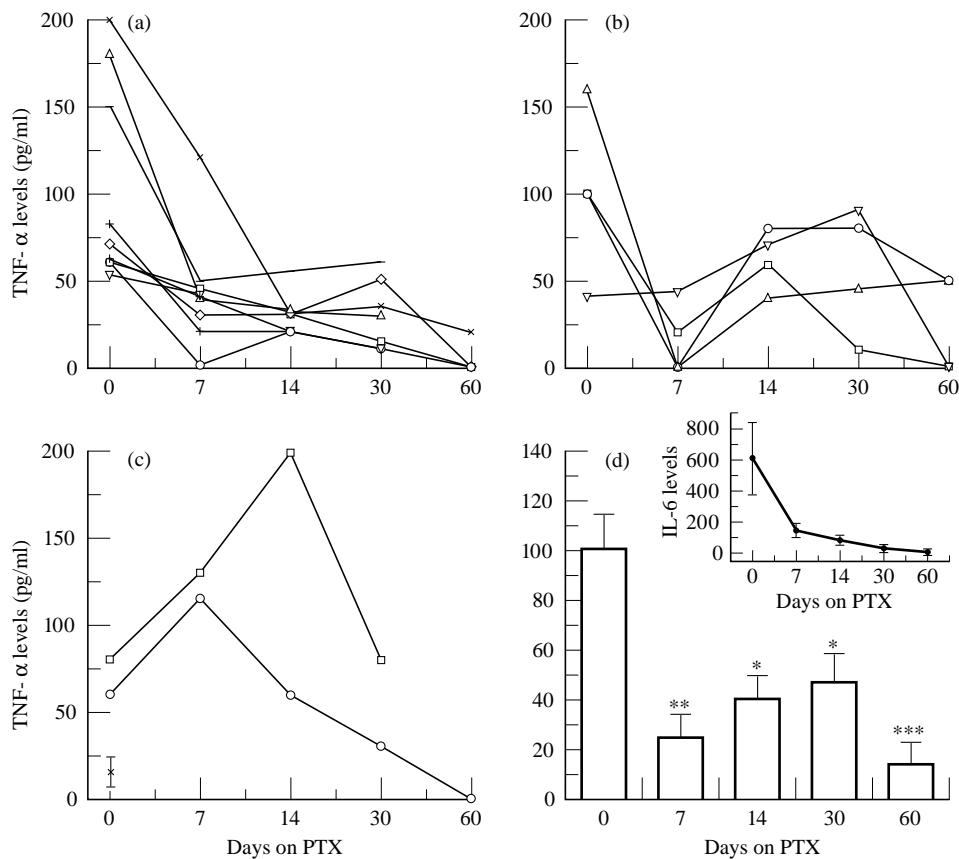
Fig. 1. Levels of TNF-α were assayed in the serum of 15 reactional patients who had been treated with 1200 mg pentoxifylline (PTX) daily for a total of 2 months. Serum was collected at the onset of the reaction episode and during treatment with PTX. (a) TNF-α values were promptly reduced within the first week of treatment ($n = 9$). (b) In four patients, although systemic symptoms were impaired by PTX, baseline serum TNF levels rose again in the course of PTX therapy. (c) There are two patients who were refractory to PTX. Closed symbols represent the period when treatment with thalidomide and/or steroids was initiated in order to control the inflammatory manifestations of reaction. Cut-off value for positive response = 45 pg/ml, which is 3 s.d. above mean values found in (*) healthy donors ($n = 10$, mean ± s.d.). (d) Serum TNF-α levels of reactional leprosy patients. Data are mean ± s.e.m. of 13 patients. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$, significant differences compared with levels obtained at the onset of the reactional episode (day 0). Variations in TNF levels between 7 and 14 or 7 and 30 days of PTX therapy were not significant ($P > 0.05$, Friedman ANOVA test). Inset: levels of IL-6 in the serum of these same patients assayed before and during treatment with PTX. Data are mean ± s.e.m. No significant differences in mean IL-6 were noted ($P > 0.05$, Friedman ANOVA test).
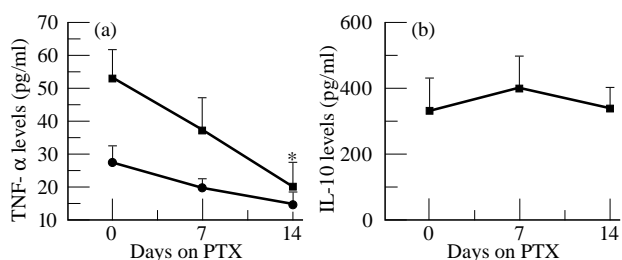
Table 2. Effect of *in vitro* pentoxifylline (PTX) on *Mycobacterium leprae-* or lipopolysaccharide (LPS)-induced cytokine production by cultured mononuclear cells

Culture stimulation	PTX*	TNF-α†		IL-6		IL-10 PBMC
		PBMC	Monocytes	PBMC	Monocytes	
M. leprae	−	15200 ± 2279	6648 ± 214	3430 ± 123	2929 ± 86	1856 ± 87
(10 μg/ml)	+	1761 ± 853 (88·4%)‡	1507 ± 239 (77·3%)	3386 ± 38	2590 ± 327	1794 ± 267
LPS	−	17109 ± 479	ND	ND	4401 ± 22	2072 ± 218
(1 μg/ml)	+	1131 ± 303 (93·4%)	ND	ND	4374 ± 55	2104 ± 128

*Human cells (peripheral blood mononuclear cells (PBMC) or adherent monocytes) were cultured and stimulated *in vitro* with *M. leprae* or LPS in the presence or absence of 50 μg/ml (178 μM) PTX. After 20 h of culture, supernatants were harvested and levels of TNF-α, IL-6 and IL-10 were determined through specific ELISAs.

†Levels of cytokines are expressed in pg/ml and are mean ± s.d. of four individual experiments. ND, Not done.

‡Numbers in parentheses represent percentage inhibition of cytokine secretion. Mean TNF-α background valves were 38 ± 36 pg/ml for PBMC and 46·5 ± 27 pg/ml for monocytes; 78 ± 56 pg/ml and 71 ± 17·8 pg/ml for IL-6; 33·5 ± 92 pg/ml and 34·9 ± 11 pg/ml for IL-10, respectively.

Fig. 2. Effect of pentoxifylline (PTX) therapy on *in vitro* (a) TNF-α and (b) IL-10 release from (■) peripheral blood mononuclear cells (PBMC) and (●) monocytes stimulated with *Mycobacterium leprae*. Data are mean ± s.e.m. of eight individual experiments. *Significant difference (*P* < 0·05) compared with levels obtained at the onset of the reactional episode (day 0).

significant differences (*P* > 0·05, Friedman ANOVA test) in mean IL-6 were detected at any time point tested during treatment with PTX (Fig. 1d, inset).

Effect of PTX on induced cytokine production
It has been previously reported that PTX inhibits LPS-induced TNF-α production by cultured monocytes *in vitro* [28]. So, at first, we intended to determine whether the drug was able to interfere with *M. leprae*-induced TNF-α production *in vitro* compared with its effects when LPS was used as the stimulus. PTX used at a concentration of 50 μg/ml (178 μM) induced a range of 77–88% inhibition of *in vitro* TNF-α release following *M. leprae* stimulation of PBMC/monocyte cultures (Table 2). The same effect was noted when the cells were stimulated with LPS in the presence of the drug. However, no inhibitory action of *in vitro* PTX was

observed on either IL-6 or IL-10 secretion in those cultures (Table 2). The next step was then to determine whether PTX administered orally to leprosy patients induced a similar phenomenon in *in vitro* TNF-α secretion. Likewise, the production of TNF-α by PBMC and isolated monocytes cultured overnight following stimulation with *M. leprae* was assayed for this group. Since the most pronounced effect of PTX on serum TNF-α was observed during the first week of treatment, most immunological *in vitro* assays were then performed at this period. *In vitro* TNF-α production by PBMC and purified monocytes was assayed in eight patients who had been receiving oral PTX. Interestingly, a reduction in inducible TNF-α was seen in PBMC cultures stimulated with *M. leprae* from six patients, after 1–2 weeks of PTX therapy. The percentage reduction of baseline mean TNF-α values was 61·9% for PBMC and 41·2% for monocytes after 14 days of treatment. The decay in mean TNF-α released *in vitro* by PBMC observed after 14 days of PTX was statistically significant (*P* < 0·014, Friedman ANOVA test, Fig. 2a). Nevertheless, it was observed that in monocyte cultures, although on an individual basis there was a tendency for decreased TNF-α secretion, the differences in mean TNF-α values were not statistically significant after 7 or 14 days of PTX compared with day 0 (*P* = 1·0 and 0·414, respectively).

Since IL-10 is a well known cytokine involved in down-modulating macrophage functions, we then investigated the possibility that PTX could be additionally leading to the inhibition of TNF-α production secondary to an increased induction of IL-10 release in these cultures. In the same way, there was a trend toward increased *in vitro* IL-10 production in *M. leprae*-stimulated PBMC cultures obtained from patients after 7 days of PTX therapy (Fig. 2b, *P* = 0·058, Friedman ANOVA test). Additionally, the differences
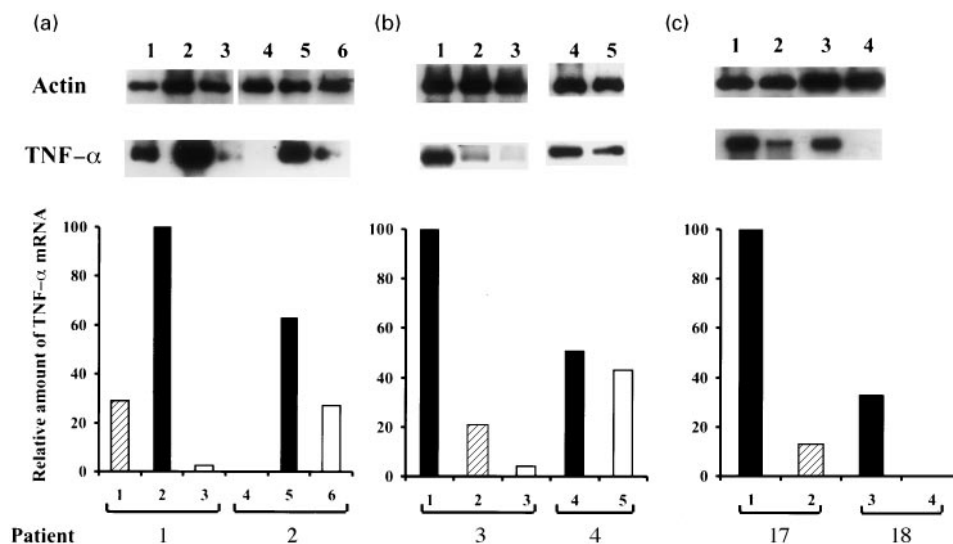
Fig. 3. TNF-α mRNA expression in leprosy lesions evaluated by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR). (a) Skin biopsies were taken from two patients before (lanes 1 and 4), at the onset of the reactional episode (ENL, lanes 2 and 5), and after 7 days of pentoxifilline (PTX) (lanes 3 and 6). mRNA from different samples was reverse transcribed and the cDNA derived from lesions was normalized to yield equivalent β-actin products. The relative amounts of TNF-α mRNA were compared among different samples from each individual patient and assessed as percentage of the most intense band for each experiment. (b) Biopsies were taken from two other patients at the onset of the ENL episode (lanes 1 and 4), and after 3 (lane 2) or 7 (lanes 3 and 5) days of PTX treatment. Biopsy samples were processed as described above for RT-PCR and semiquantitative analysis. (c) TNF-α mRNA expression during thalidomide treatment evaluated by semiquantitative RT-PCR as described. Skin biopsies were taken from two patients at the onset of the reactional episode (ENL, lanes 1 and 3), and following 3 (lane 2) or 7 (lane 4) days of treatment with thalidomide.
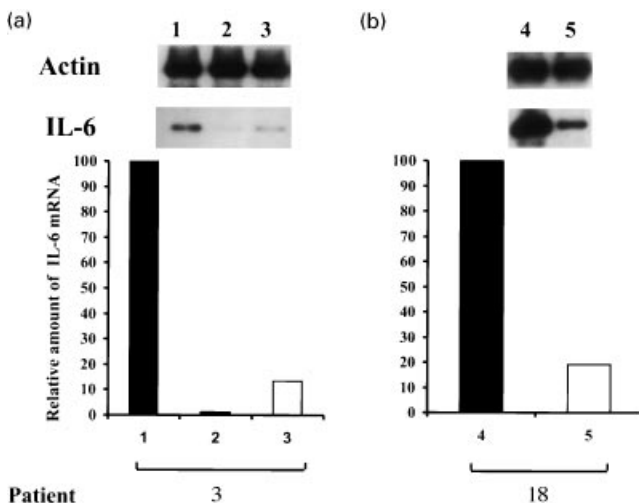
Fig. 4. IL-6 mRNA expression in leprosy lesions evaluated by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR). One representative experiment for each group of patients is shown. Skin biopsies were taken from leprosy patients at the onset of the ENL episode (lanes 1 and 4), and after 3 (lane 2) or 7 days (lanes 3 and 5) of treatment with (a) pentoxifilline (PTX) or (b) thalidomide. mRNA from tissue samples was reverse transcribed and the cDNA normalized to yield equivalent β-actin products. The relative amount of IL-6 mRNA was compared among different samples from each individual patient and assessed as percentage of the most intense band.

in mean IL-10 values between 0 (ENL) and 14 days of PTX were no longer significant ($P = 1 \cdot 0$).

Cytokine mRNA expression at the lesion site
Modulation of cytokine gene expression in the tissue by PTX and thalidomide was also analysed. Since PTX and thalidomide have been shown to inhibit TNF-α production *in vivo* and *in vitro*, a kinetic analysis of the expression of TNF-α mRNA at the site of leprosy lesion was performed in six ENL patients by semiquantitative RT-PCR (four patients treated with PTX and two patients treated with thalidomide). Histologically, ENL lesions contain dense cellular infiltrates extending from the lower dermis into the subcutaneous fat, composed predominantly of mononuclear cells and neutrophils. Although the epidermis of the reactional tissue also shows profound histological modifications, at this stage cytokine gene expression at the dermis was assessed.

In two patients (patients 1 and 2), biopsies were collected before, at the onset of the ENL episode, and after 1 week of treatment with PTX. The amount of TNF-α mRNA was increased in the tissue during ENL compared with before the reaction, and decreased thereafter following PTX treatment (Fig. 3a). For the other two patients (patients 3 and 4), biopsies were collected during the reactional episode and at 3 and/or 7 days of PTX therapy (Fig. 3b). The high amount of TNF-α mRNA present in the lesion during ENL decreased at different rates following 1 week of PTX. The decay in the relative amount of TNF-α message varied from three- to a 50-fold decrease in three patients tested (patients 1–3). In the other patient (patient 4), a slight variation in the amount of TNF-α mRNA was noted at the lesion site following PTX therapy. Biopsies taken 3 days after PTX also showed a reduction in TNF-α message expression (Fig. 3b, patient 3).

As shown in Fig. 3c, biopsies taken from two patients (patients

17 and 18) treated with thalidomide were assayed for TNF-α mRNA expression, at the onset of the reactional episode (ENL) and during treatment with thalidomide. Similarly to what has been observed for PTX, a decreased expression of TNF-α message at the lesion site was noted after both 3 and 7 days of thalidomide therapy compared with before treatment.

Furthermore, we were interested to determine whether the expression of IL-6 mRNA at the lesion site was affected following treatment for reaction (Fig. 4). The *in vivo* expression of IL-6 gene was down-regulated *in vivo* during therapy with PTX (Fig. 4a) or thalidomide (Fig. 4b). The relative amount of IL-6 message was reduced by five- to 50-fold after 3 or 7 days of treatment.

DISCUSSION

In the present study, we were able to demonstrate that oral PTX inhibits *in vivo* and *in vitro* TNF-α production in leprosy patients with ENL. The high levels of TNF-α seen in the serum of reactional patients decreased following PTX treatment in 13 out of the 15 patients assayed. These findings are in agreement with our own previous studies [4,7], as well as those from others [5,6] which have confirmed that TNF-α is overproduced during the reactional states and is a key mediator of systemic symptoms and of tissue damage in leprosy in both reversal reaction (RR) and ENL [9,10,29]. All available data have shown that reactional leprosy patients present high circulating TNF-α levels [4–7], and that cells from these patients secrete elevated amounts of TNF-α following *in vitro* stimulation as well [7,8,30].

More interesting is the fact that here modulation of TNF-α mRNA expression at the lesion site (reactional tissue) was also achieved following patients' treatment with PTX. In addition, treatment of ENL patients with thalidomide also inhibited TNF-α gene expression at the reactional lesion. To provide meaningful comparisons between different samples, we normalized the cDNAs to the β-actin PCR product, a marker for all cells. Although we carefully controlled for levels of input cDNA, the RT-PCR analysis used is purely semiquantitative and only allows comparisons of the relative amounts of cytokine mRNA present in the lesions of the same patient.

This is the first time that inhibition of TNF-α gene expression in the tissue by PTX and thalidomide has been demonstrated. Previous studies showed that PTX therapy inhibits TNF-α mRNA expression in the PBMC of AIDS patients [31], and in mononuclear cells isolated from patients with cancer [32]. In a longitudinal analysis, we have shown that the amount of TNF-α mRNA was increased in the tissue during ENL, compared with before the reaction, and decreased following treatment for reaction. This effect correlated with the increased TNF-α values seen in these patients' sera during the reactional episode, which were reduced after PTX. These data further suggest a direct relationship between increased *in vivo* production and release of TNF-α and the clinical symptoms presented by these patients during the reaction. In a similar way, in a very recent publication, Khanolkar-Young *et al.* [9] have demonstrated an increased expression of TNF-α mRNA (by *in situ* hybridization) and TNF-α protein in macrophages infiltrating the skin and peripheral nerves in reversal reaction. In our hands, immunohistochemical analysis of skin biopsies with anti-TNF MoAbs revealed higher numbers of TNF-α-positive cells in lepromatous reactional lesions, either RR or ENL (Sarno *et al.* unpublished data).

The ability of *M. leprae* and its fractions to induce the release

of TNF-α by mononuclear cells *in vitro* was recently demonstrated by our group and others [7,8,30]. Our present data show that PTX added *in vitro* to the cultures significantly inhibited TNF-α release by *M. leprae*-stimulated PBMC and monocytes, similarly to its effect on LPS-stimulated cultures. In addition, *M. leprae*-induced TNF-α production *in vitro* by PBMC was also down-modulated following oral administration of PTX. Unexpectedly, whereas there was a trend toward TNF-α inhibition, no significant effect of PTX therapy on *in vitro* TNF-α release by cultured monocytes was detected. The reason for the differential effect of the drug on PBMC and monocytes *in vivo* is not clear. Note that purified monocytes produced less TNF-α than PBMC (Table 2 and Fig. 2a) containing an equivalent number of monocytes. This suggests a possible positive cooperation (cell–cell interaction) between monocytes and lymphocytes in the production of TNF-α, which could lead to a differential effect of the drug on these cells. PTX has been shown to modulate TNF-α production via inhibition of TNF-α mRNA transcription [20,28] and to decrease the activity of the transcription factor NF-κB [33]. The presumed mechanism of action of PTX is secondary to the inhibition of phosphodiesterase resulting in an intracellular increase of cAMP [28,34]. Hence, it is possible that different signals are then triggered through these different cell types by one and the same drug.

The fact that PTX has been largely used for treatment of vascular disorders suggests that other non-immune cells have a major role through the release of inflammatory factors such as TNF and IL-6. This fact raises the possibility that PTX might exert its effect also on cell types other than monocytes/macrophages, such as epithelial cells, keratinocytes, endothelial cells, and fibroblasts [35], all of them recently described as TNF-producing cells.

Increased serum levels of IL-1β and IL-6 in the sera of leprosy patients with reaction have also been previously reported [4,10]. Here, we have further confirmed and extended these observations. Although there was a trend toward lower IL-6 values following PTX therapy, the increased levels of IL-6 detected in the sera of reactional leprosy patients were not significantly affected by oral treatment with PTX. Likewise, and consistent with findings in previous studies [18,36], PTX when added to cell cultures did not affect the release of IL-6 following *M. leprae* or LPS *in vitro* stimulation. On the other hand, IL-6 mRNA expression in the reactional lesion was reduced following *in vivo* treatment for reaction (either PTX or thalidomide). Since we could not see a direct effect of both drugs on the *in vitro* release of IL-6 [17,18], these data, together with the observed tendency of PTX to inhibit serum IL-6, indicate that, *in vivo*, inhibition of TNF-α by these drugs does have an impact on the synthesis of other inflammatory cytokines. As TNF-α is known to modulate synthesis of other inflammatory molecules [1], inhibition of TNF *in vivo* could ultimately lead to a reduction in other inflammatory mediators, including IL-6.

An additional mechanism by which PTX would lead to the inhibition of TNF-α release could be through the induction of an increased production of IL-10. IL-10 has been shown to inhibit cytokine synthesis by monocytes, namely TNF-α, IL-1, IL-6, IL-8, IL-12 [13,37,38]. Interestingly, following patients' oral administration, PTX resulted in a trend toward significantly increased IL-10 secretion by stimulated PBMC after 7 days of therapy. However, no effect of the drug when added *in vitro* to cell cultures has been noted. Again, we cannot exclude that this *in vivo* effect is the result of the natural resolution of the inflammatory response.

Curiously, it was recently reported that PTX inhibited IL-10 production by human T cells infected with HIV [39].

The diverse biological effects of TNF-α make it a prime suspect in either the initiation and/or amplification of tissue injury in leprosy reaction. We have postulated that TNF-α is involved in mediating nerve damage in leprosy [29]. Limiting the development of reactions and deformities in leprosy patients constitutes a major effort in controlling the disease. Thalidomide and/or steroids have been used in these patients in order to control the clinical manifestations of reaction. Because of their side-effects, the use of alternative drugs for treatment of ENL which down-regulate TNF production seems a logical approach to management of reaction.

In conclusion, we demonstrate here that PTX decreased systemic TNF-α levels in these reactional patients, suppressed induction of TNF-α protein by mononuclear cells *in vitro*, and down-regulated TNF-α and IL-6 mRNA expression at the lesion site. This study constituted an open controlled pilot trial on the use of PTX for treatment of leprosy patients with ENL reaction [24]. Although the effect of PTX in ENL might be of no longer lasting benefit compared with thalidomide or steroids, this pilot study suggests that PTX may be useful as an alternative therapy for ENL patients, mainly females of child-bearing age, and in patients whose treatment with steroids is not well tolerated. Controlled clinical trials are needed to confirm these expectations.

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