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PENTOXIFYLLINE INHIBITS Vγ9/Vδ2 T LYMPHOCYTE ACTIVATION OF PATIENTS WITH ACTIVE BEHÇET'S DISEASE *IN VITRO*

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The aim of this study is to evaluate the *in vitro* effect of pentoxifylline (PTX) on T Vγ9/Vδ2 lymphocyte function in Behçet's disease (BD). We investigated the effect of PTX on Vγ9/Vδ2 T cell expansion and expression of TNFRII receptor and perforin content before and after PTX addition by means of FACS analysis lymphocyte cultures from patients with active and inactive BD and healthy subjects. The addition of PTX at a concentration of 1 mg/ml determined a significant inhibition of cell expansion, a down regulation of TNF receptor expression and inhibited the PMA-induced degranulation of perforin. Taken together these data indicate that PTX is capable of interfering with Vγ9/Vδ2 T cell function in BD, and although cell culture models cannot reliably predict all of the potential effects of the drug *in vivo*, our results encourage the possibility that this drug may find use in a range of immunological disorder characterized by dysregulated cell-mediated immunity.

Behçet disease (BD) is a multisystemic inflammatory disorder characterized by recurrent oral and genital aphthous ulcers, involvement of the eyes and skin lesions.

Various immune abnormalities have been observed in this disease. T cell-mediated immune responses are thought to play a major part in the immunopathogenesis of BD (1-3). Increased levels of circulating activated γδ T cells have been reported in BD (4-5) and, in particular, Vγ9/Vδ2 T lymphocytes, that represent the major part of circulating γδ T cells, are activated in patients with active BD and express increased levels of receptors for TNF and IL-12 (6).

Pentoxifylline (PTX), a methylxantine derivate, is a non-selective phosphodiesterase inhibitor that has been recently used in the treatment of some immune-mediated disorders such as rheumatoid arthritis and multiple sclerosis (7-8). The rationale of this kind of therapy is that PTX has been shown to inhibit TNF-α production and to promote a deviation toward type-2 cytokine production (9). There is also evidence that PTX inhibits the release of IL-1, IL-6 and IL-8 by human peripheral blood mononuclear cells (10). PTX seems to be active in the treatment of patients with BD (11-12) and plays a role in the suppression of neutrophil activation in active BD both *in vivo* and *in vitro* (12).

The aim of the present study is to investigate the effect of PTX on Vγ9/Vδ2 T cells. In particular, we studied the *in vitro* effect of PTX on Vγ9/Vδ2 T cell proliferation, after phosphoantigen stimulation, and TNF-RII expression. To analyse whether PTX could also interfere with the cytotoxic activity of these cells, we evaluated the content of the cytoplasmic

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granule-associated molecule perforin (Pf) before and after PTX exposure.

MATERIALS AND METHODS

Patients

Heparinized venous blood was obtained from five patients with active BD (2 males and 3 females; mean age 33 ± 7 years), five patients with inactive BD (3 males and 2 females; mean age 36 ± 8 years) and five healthy subjects (2 males and 3 females; mean age 28 ± 3 years). Patients were classified according to the International Study Group for Behçet's disease (14). The activity of BD was assessed by the presence of at least 2 major criteria. At the time of sampling the active patients were using colchicine and low dose prednisone. Human studies committee approval and individual informed consent from each subject were obtained.

Monoclonal antibodies and flow cytometry

Monoclonal antibody (mAb) specific for human surface antigen anti-CD3 phycoerythrin (PE), anti-TCR Vδ2 fluorescein isothiocyanate (FITC, PharMigen, San Diego, Ca, USA) and PE-conjugated anti-TNF-RII (R&D system, Minneapolis, MN, USA) were used as previously reported (6). Anti-human perforin PE (Ancell Corporation, Bayport, MN, USA) was used for perforin determination.

Cell separation and expansion in vitro of Vγ9/Vδ2 T lymphocytes

PBMC were obtained from each individual by separating heparinized venous blood on Ficoll (Euroclone, Wetherby, Yorkshire, UK). The cells were washed in RPMI-1640 medium (Euroclone), and cultured in 24-well plates (Costar, Cambridge, MA, USA) at a concentration of 5 x 10^5, cells/ml in RPMI-1640 supplemented with 10% foetal calf serum (Euroclone), hepes 20 mmol/l (Euroclone), 2 mmol/l L-glutamine (Euroclone) and penicillin/streptomycin 100 U/ml (Sigma, St Louis, USA) at 37°C and at 0.5% CO_2. For the expansion of Vγ9/Vδ2 T lymphocytes, PBMC were cultured for 10 days in medium alone or in the presence of dimethylallyl pyrophosphate (DMAPP; Sigma; 0.5 mmol/l, final concentration). After 72 hours the cultures were supplemented with a 0.5 ml medium containing 20 U/ml interleukin-2 (IL-2; Genzyme, Cambridge, MA, USA). Every 72 hours, 0.5 ml medium was replaced with a 0.5 ml fresh medium. After 10 days, the cells were washed three times in medium, and expansion of Vγ9/Vδ2 T cells was assessed using FACScan, as described previously (6). The absolute number of Vγ9/Vδ2 T cells in each culture was calculated according to the following formula: % Vγ9/Vδ2 positive cells before culture x total cell count/100. The Vγ9/Vδ2 expansion factor (EF) was then calculated by dividing the absolute number of Vγ9/Vδ2 T cells in specifically stimulated cultures by the absolute number of Vγ9/Vδ2 T cells cultured in the absence of any antigen.

Expression of tumor necrosis factor receptor II by Vγ9/Vδ2 T lymphocytes

The expression of TNF receptor II on Vγ9/Vδ2 T cells from the peripheral blood of patients with BD and normal subjects, was studied using anti TNF receptor II PE (R&D systems, Minneapolis, MN, USA) and anti V δ2 TCR FITC simultaneously (6). The number of TNFRII molecules (MESF; molecular equivalents of soluble fluorochrome) was calculated by FACS analysis of cells stained with saturating amounts of PE labelled anti-TNFRII mAb of known PE/protein ratio and comparing the staining with a standard curve of microbeads labelled with defined numbers of PE molecules (Quantum Fluorescence Kit, Sigma). The analysis was done using Quickal Program for MESF Units for Windows.

Intracytoplasmic content of Perforin

For the evaluation of the presence of Perforin in the cytoplasmic granules of Vγ9/Vδ2 T cells, 3 x 10^5 cells were stained with anti V δ2 TCR FITC and after washing fixed with 4% paraphormaldeyde for 30 minutes. After 2 washes with permeabilization buffer (saponine containing) the cells were incubated at 4°C for 45 minutes with PE labelled monoclonal anti-Perforin antibody. The specificity of the reaction was confirmed by using appropriate isotype-matched antibody as negative control. The number of perforin molecules (MESF) was calculated as above.

Vγ9/Vδ2 T lymphocyte studies in the presence/absence of Pentoxifylline (PTX)

In order to examine the effects of PTX on Vγ9/Vδ2 expansion, TNFRII expression and perforin content, PTX was freshly dissolved in medium, filtered with 0.2 μm Acrodisc (Gelmann, Ann. Arbor, MI) and added at various concentrations (0.1, 1 and 2 mg/ml). For Vγ9/Vδ2 expansion studies, PTX was added at the beginning of cell cultures. For TNFRII and perforin studies, PTX was added at the 9th day of culture and in some experiment at the 7th day. The PBMC viability was assessed in all the experiments by Trypan blue exclusion and was found to be >90% in both unstimulated and stimulated culture independently of the duration of exposure and of incubation time. Vγ9/Vδ2 T cell expansion was also measured after co-addition of exogenous human TNFα (10 ng/ml = 100 U/ml; Genzyme). Perforin content was determined before and after degranulation induced by phorbol myristic acetate (PMA; Sigma; 1 ng/ml) and anti human CD3

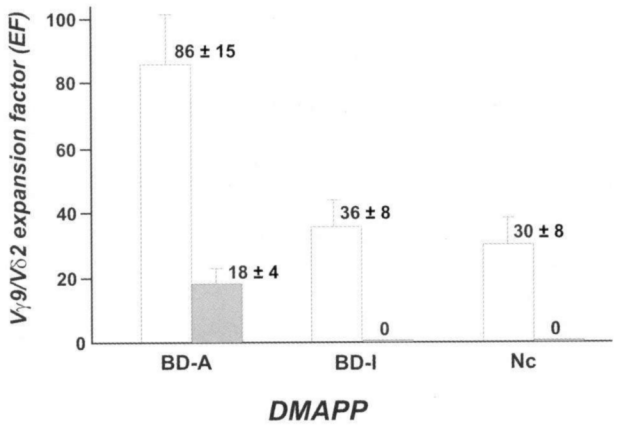


Fig. 1. *Expansion of Vγ9/Vδ2 T lymphocytes from patients with active (BD-A) or inactive (BD-I) Behçet's disease and healthy controls (Nc) in response to dimethylallylp yrophosphate (DMAPP). The Vγ9/Vδ2 expansion factor (EF) was calculated by dividing the absolute number of Vγ9/Vδ2 T cells in specifically DMAPP stimulated cultures by the absolute number of Vγ9/Vδ2 T cells cultured in the absence of any antigen. Grey bars indicate the EF obtained adding pentoxifylline (PTX, 1 mg/ml) in the DMAPP cultures of active patients. In both patients and controls after PTX there was a complete suppression of expansion. Results were expressed as mean ± SD. The EF difference between DMAPP and DMAPP plus PTX was statistically significant (p< 0.001; Student t test for paired data).*

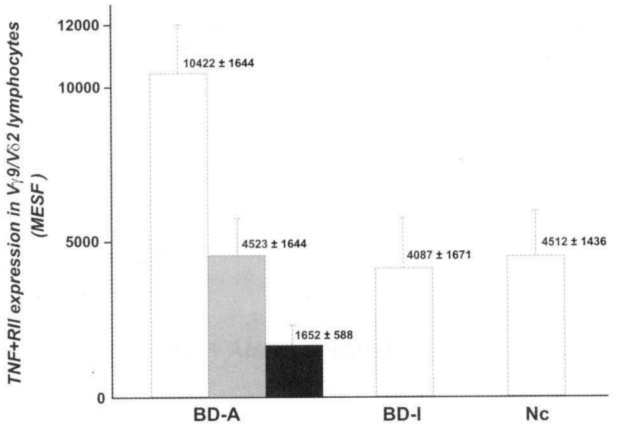


Fig. 2. *Effect of PTX on TNFRII expression of Vγ9/Vδ2 T lymphocytes from patients with active (BD-A) or inactive (BD-I) and healthy controls (NC). Levels of TNFRII were expressed as MESF (molecular equivalents of soluble fluorochrome). Grey and black bars indicate results obtained adding PTX at the 9th and at the 7th day of culture, respectively.*

(Sigma; 1 μg/ml) in the presence or not of PTX. Actinomycin D (Sigma; 1 μ/ml) as inhibitor was also added in some experiments. Briefly, cells were washed in medium and re-supended at 3 x 10^5 cells/ml in medium containing anti-CD3 and PMA. Cells were then incubated for 5 hours, washed again and labelled for the perforin expression measurement.

RESULTS

Statistics

Kruskal-Wallis test followed by a Dunnett's post hoc analysis was used to compare continuous variables in the different groups while Wilcoxon rank test was used to evaluate effect of PTX addition on Vγ9/Vδ2 T cell expansion, TNFRII expression and perforin content. A p value < 0.05 was considered to be significant.

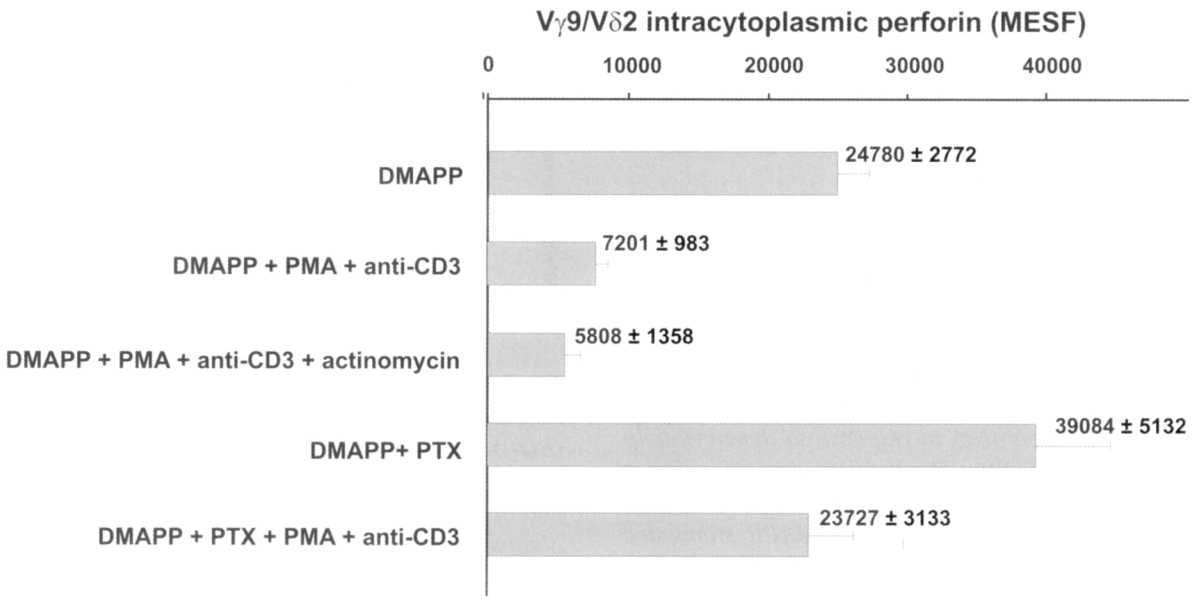
Effect of PTX on Vγ9/Vδ2 T cell expansion

The EF of Vγ9/Vδ2 T lymphocytes (Fig. 1) from active BD patients (86 ± 15) was significantly higher than that from inactive patients (36 ± 8, p<0.001) and healthy controls (30 ± 8, p<0.001). PTX incubation determined a dose-dependent inhibition of cell expansion. At a concentration of 1 mg/ml there was a significant inhibition of cell expansion (18 ± 4, p<0.001). No effect was observed with a concentration of 0.1 mg/ml whereas after 2 mg/ml of PTX there was a complete block of cell expansion (not shown). No effects were observed with the addition of TNFα.

Effect of PTX on TNFRII expression on Vγ9/Vδ2 T lymphocytes

The expression of TNFRII (Fig. 2) was up-regulated in active patients (MESF; 10,422 ± 1,694, p<0.001) in comparison with both inactive patients (4,087 ± 1,671) and controls (4,512 ± 1,436). The addition of 1 mg/ml PTX to cell cultures from active patients at the 7th and 9th day determined a signifi-

Fig. 3. *Effect of PTX on perforin content of Vγ9/Vδ2 T lymphocytes from patients with active BD under various conditions. Perforin levels were expressed as MESF (molecular equivalents of soluble fluorochrome).*



cant decrease of TNF RII expression (4,523 ± 1,232 and 1,652 ± 588 respectively, $p<0.001$). Similar results were observed in inactive patients and controls (not shown).

Effect of PTX on perforin content

High levels of intra-cytoplasmic perforin (Fig. 3) (24,780 ± 2,772, $p< 0.01$) were found in lymphocyte cultures (in the presence of DMAPP) from active patients in comparison with those of inactive patients (4,711 ± 1,413) and controls (5,067 ± 1,063). Experimental studies with PTX were performed in active patients only. After degranulation of Vγ9/Vδ2 T cells with PMA plus anti-CD3, perforin content was 7,201 ± 983; the co-addition of actinomicin to inhibit protein synthesis determined a further reduction of perforin content (5,808 ± 1,358). Perforin content in cells pre-treated with PTX was 39,084 ± 5,132 ($p< 0.01$ vs untreated cultures). After co-incubation with PMA levels were 23,727 ± 3,133.

DISCUSSION

In previous studies we demonstrated an increased proliferation of Vγ9/Vδ2 T lymphocytes from patients with active BD together with an up regulation of TNF RII (6). The role of Pentoxifylline (PTX) has been postulated in the treatment of BD according to some clinical and experimental evidence (11-13).

PTX, a methylxantine derivative, is a non-selective phosphodiesterase inhibitor that has been recently used in the treatment of some immune-mediated disorders such as rheumatoid arthritis and multiple sclerosis (7-8). PTX is poorly active as an immunosuppressant but prevents the synthesis of pro-inflammatory cytokines (namely IL-1, TNF-α, IL-12, IL-2) (9-10, 15) and has inhibitory effects on the T lymphocyte expression of activation antigens, CD25 (IL-2R alpha-chain), CD69 (activation–inducer molecule) and CD98, induced by PHA (16).

The results obtained in the present study indicate that PTX may interfere with Vγ9/Vδ2 T cell function in BD. In particular, the addition of PTX was able to suppress Vγ9/Vδ2 T cell expansion and TNFRII expression induced by DMAPP. Both expansion and TNFRII expression in our study were not modified by the addition of exogenous human TNFα, indicating that the inhibition of Vγ9/Vδ2 T cell expansion could be due mainly to the down-regulation of TNFRII. Indeed, PTX might interfere with early Vγ9/Vδ2 T cell activation events being able to inhibit the rise in intracellular Ca and the activation of the Na/H antiporter induced by PHA and phorbol esters (16).

Our previous observations point for an increased cytotoxic potential of Vγ9/Vδ2 T lymphocytes in BD

(17). In the present study we also analysed whether PTX could interfere with the cytotoxic activity of these cells, evaluating the expression of the cytoplasmic granule-associated molecules perforin. Increased perforin content was found to be significantly higher in Vγ9/Vδ2 T cells of patients with active BD, but not of inactive patients or controls. Upon activation with PMA a significant decrease of perforin content was observed in Vγ9/Vδ2 T cells of both patients and controls, suggesting an active process of exocytosis. PTX addition, in fact, was followed by an increase in cell perforin content and by a block in the degranulation effect of PMA. In this regard PTX seems to inhibit the mechanism of granule exocytosis, a process mediated by a rise in intracellular Ca and PKC activation and inhibited by the increase of cAMP levels (18-20).

In conclusion, despite a relatively large body of information on PTX effects in αβ T cells, very little is known on its effects on γδ T cells. Data collected in this study point to a critical role of PTX in the regulation of cellular activation mechanism of Vγ9/Vδ2 T cells. Although cell culture models cannot reliably predict all of the potential effects of the drug *in vivo*, our results encourage the possibility that this drug may find widespread use in a range of immunological disorder characterized by dysregulated cell-mediated immunity. PTX might be considered, in particular, as a supporting drug in the treatment of BD patients, especially when administered in concert with other already established immunosuppressant drugs, and suggest that inhibition of γδ activation and therefore of proinflammatory cytokine production, may provide an interesting therapeutic strategy for BD.

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