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PENTOXIFYLLINE INHIBITS GRANZYME B AND PERFORIN EXPRESSION FOLLOWING T-LYMPHOCYTE ACTIVATION BY ANTI-CD3 ANTIBODY

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Abstract—Pentoxifylline (PTX), a methylxanthine derivative, is known to inhibit the production of the Th1 cytokines interleukin-2, tumour necrosis factor-α and interferon-γ. Because these cytokines play an important role in promoting the development of cell-mediated immunity, we hypothesized that PTX would also interfere with the generation of cytotoxic effector cells in response to an immunological stimulus. In this study we used a mouse model system to investigate the effect of PTX on the induction of non-specific killer lymphocytes by anti-CD3 monoclonal antibody. Anti-CD3-induced T-cell proliferation and the generation of anti-CD3-activated killer (AK) cells was inhibited in a dose-dependent fashion by PTX (25–100 μg/ml). The inhibitory effect of PTX could not be attributed to a defect in the recognition/adhesion phase of cytolysis because AK cells generated in the presence of PTX conjugated normally with P815 tumour target cells. However, AK cell expression of the cytoplasmic granule-associated cytolytic effector molecules granzyme B and perforin was markedly reduced when AK cells were induced in the presence of PTX. In contrast, PTX had no effect on AK cell expression of Fas ligand, a cell-surface cytolytic effector molecule which is involved in granule-independent cytotoxicity. PTX thus has a profound inhibitory effect *in vitro* on the induction of granule-dependent cytolytic effector mechanisms in a mouse model system. © 1997 International Society for Immunopharmacology.

Keywords: pentoxifylline, killer lymphocyte, granzyme B, perforin, Fas ligand

The xanthine-derived phosphodiesterase inhibitor pentoxifylline (PTX) has been widely used in the past as a haemorrheological drug to treat peripheral vascular disorders and cerebrovascular disease (Ward & Clissold, 1987). More recently, PTX has shown promise as an anti-inflammatory agent for the treatment of adult respiratory distress and septic shock syndromes (Zabel *et al.*, 1989; McDonald, 1991). In addition, PTX is able to ameliorate life-threatening systemic toxicities associated with bone marrow transplantation (Bianco *et al.*, 1991) and lymphokine-activated killer cell therapy of metastatic renal cell carcinoma (Thompson *et al.*, 1994). Because it is likely that the clinical usage of PTX will increase in the future, it is important to understand better how PTX interacts with the immune system.

The anti-inflammatory activity of PTX has been attributed to the ability of PTX to downregulate the production of certain proinflammatory cytokines. PTX inhibits the *in vitro* synthesis of tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin (IL)-2 by phytohaemagglutinin-stimulated human peripheral blood mononuclear cells but fails to affect IL-6 production (Funk *et al.*, 1995). Similarly, in rat $CD4^+$ T-cell cultures PTX has been shown to suppress mitogen-induced TNF-α and IL-2 secretion while allowing normal IL-4 and IL-6 production to occur (Rott *et al.*, 1993). In mice, PTX reduces the release of TNF-α and IL-2 following the administration of anti-CD3 monoclonal antibody (mAb) (Alegre *et al.*, 1991). It is now well established that cytokine-producing $CD4^+$ T-helper lymphocytes can be divided into two subsets: Th1 cells which secrete primarily TNF-α, IFN-γ and IL-2; and Th2 cells which elaborate mainly IL-4, IL-5, IL-6, and IL-10 (Mosmann & Coffman, 1989). PTX, therefore, appears to downregulate the biosynthesis of Th1 cytokines while leaving Th2 cytokine production intact. It is believed that PTX modulates Th1 cytokine synthesis by increasing intracellular cyclic adenosine monophosphate (cAMP) levels in Th1 lymphocytes (Rott *et al.*, 1993; Semmler *et al.*, 1993). Th1 cytokine pro-

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duction being much more sensitive to inhibition by cAMP than is Th2 cytokine synthesis (Novak & Rothenberg, 1990).

Th1 cytokines are crucial for the development of cellular immune responses. For example, IFN-γ and IL-2 are required for the proliferation and differentiation of cytotoxic T-lymphocyte (CTL) precursors into functional effector cells following antigenic stimulation (Maraskovsky *et al.*, 1989), whereas TNF-α acts synergistically with IL-2 to promote an optimal induction of CTL generation (Robinet *et al.*, 1990). TNF-α and IL-2 also upregulate the expression of adhesion molecules involved in lymphocyte trafficking and effector-target cell conjugate formation (Pober *et al.*, 1987; Kovach *et al.*, 1994). We therefore reasoned that the selective inhibition of Th1 cytokine synthesis by PTX might have profound inhibitory effects on the generation of CTL responses.

The aim of this study was to investigate the effect of PTX on non-specific killer lymphocyte induction in mouse spleen cell cultures stimulated with anti-CD3 mAb. We tested the influence of PTX on anti-CD3-induced T-cell proliferation and anti-CD3-activated killer (AK) cell induction. In addition, we determined the effect of PTX on granzyme B, perforin, and Fas ligand (Fas-L) expression by AK cells. Granzyme B and perforin are cytoplasmic granule-associated cytolytic effector molecules involved in calcium-dependent target cell destruction by cytolytic effector cells whereas the cell-surface expression of Fas-L is required for calcium-independent cytolysis (Podack, 1995).

EXPERIMENTAL PROCEDURES

Mice

Male (6–8 weeks old) C57BL/6 (H-2^b haplotype) mice were purchased from Charles River Laboratory (Lasalle, PQ) and were maintained in our animal care facilities on standard mouse chow and water supplied *ad libitum*.

Cell lines, medium, and reagents

RPMI–1640 medium (ICN Pharmaceuticals Canada Ltd., Montreal, PQ) was supplemented with 10 mM *L*-glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin (all ICN), and 5% heat-inactivated (at 56°C for 30 min) fetal calf serum (Gibco BRL, Burlington, ON, Canada). Hereafter, this will be referred to as complete RPMI–1640 medium. P815 mastocytoma cells (H-2^d haplotype) were obtained from the American Type Culture Collection, Rockville, MD, U.S.A. The hybridoma (clone 145–2C11) producing hamster anti-mouse CD3 mAb (Leo *et al.*, 1987) was a generous gift from Dr J. Bluestone (University of Chicago, Chicago, IL, U.S.A.). All cell lines were maintained by *in vitro* passage in complete RPMI–1640 medium. A 1 mg/ml stock solution of PTX (Sigma Chemical Co., St Louis, MO, U.S.A.) was prepared in complete RPMI–1640 medium and stored at −20°C. Boc–Ala–Ala–Asp thiobenzyl ester was obtained from Enzyme Systems Products (Dublin, CA, U.S.A.). Dithiobis(2-nitrobenzoic acid) was purchased from Boehringer Mannheim (Laval, PQ, Canada).

AK cell cultures

To generate AK cells, spleen cells from C57BL/6 mice were depleted of erythrocytes by osmotic shock, washed extensively with phosphate buffered saline (PBS), and cultured in complete RPMI–1640 medium (4×10^6 cells/ml) containing anti-CD3 hybridoma culture supernatant (1/20 final dilution). PTX (25–100 μg/ml) was added to test cultures at the initiation of culture. Following 48 h of culture at 37°C in a humidified atmosphere containing 5% CO_2, AK cells were collected, washed three times by centrifugation to remove residual anti-CD3 mAb and cytolytic activity was measured. In this culture system peak cytolytic activity is reached at 48 h of culture (Kaiser *et al.*, 1993). Prior to use in experiments, AK cell viability was confirmed by the trypan blue dye exclusion test.

Cellular proliferation assay

Cultures consisted of 2.5×10^5 C57BL/6 spleen cells (minus erythrocytes) in a 0.2 ml volume of complete RPMI–1640 medium alone, or containing anti-CD3 mAb (1/20 final dilution of hybridoma culture supernatant) alone or with PTX at the desired concentration. All cultures were performed in quadruplicate in 96-well round-bottomed microtitre plates (Sarstedt Inc., St Laurent, PQ, Canada) and maintained at 37°C in a 5% CO_2 humidified atmosphere for 48 h to generate peak lymphoproliferative responses (Fitzpatrick *et al.*, 1994). During the last 6 h of incubation the cultures were pulsed with 0.5 μCi of tritiated thymidine ([^{3}H]TdR; specific activity 65 Ci/mmol; ICN) to measure DNA synthesis. Cultures were harvested onto glass fibre mats using a Titertek multiple sample harvester (ICN) and [^{3}H]TdR incorporation was determined by liquid scintillation counting. Data from replicate cultures are expressed as mean counts/minute (cpm) plus or minus the standard deviation (S.D.).

Cytolytic ^{51}Cr-release assay

P815 target cells ($2–5 \times 10^6$) were incubated for 1 h at 37°C with 100 μCi (0.1 ml) of [^{51}Cr]sodium chromate (specific activity 250–500 mCi/mg Cr; ICN), washed three times with PBS, and resuspended in complete RPMI–1640 medium at a concentration that would yield 5×10^3 target cells/well. Equal numbers of viable control and PTX-treated AK cells in complete RPMI–1640 medium were added to wells of a 96-well "V"-bottomed microtitre plate (Sarstedt Inc.), serially diluted to produce the desired effector:target cell ratio, and radiolabelled P815 target cells were added to achieve a final volume of 0.2 ml/well. Plates were then incubated for 4 h at 37°C in a 5% CO_2 humidified atmosphere, centrifuged at 400**g** for 5 min, and 0.1 ml volumes of supernatant were collected from the wells. ^{51}Cr released from lysed target cells was measured by gamma counting with a Beckman Gamma 8000 counter. Percentage lysis was calculated according to the formula

$$\%\text{lysis} = (E - S)/(M - S) \times 100$$

where E is the release from experimental samples, S is the spontaneous release, and M is the maximum release upon lysis with 10% sodium dodecyl sulfate. Data are presented as the mean percentage lysis of triplicate samples (±S.D.).

Conjugate formation assay

P815 target cells were stained by incubation at 37°C for 10 min in the presence of neutral red dye (1 mg/ml in complete RPMI–1640 medium). The dye stains the cytoplasmic granules of P815 cells, allowing them to be readily distinguished from AK cells, without affecting cell viability as assessed by trypan blue dye exclusion. Stained P815 cells and viable AK cells (control or PTX-treated) were washed and resuspended at 5×10^6 cells/ml in complete RPMI–1640 medium. Complete RPMI–1640 medium, AK cells, and P815 cells (all 0.1 ml) were added to 12 × 75 mm round-bottomed polystyrene tubes (Becton Dickinson, Lincoln Park, NJ, U.S.A.) which were then centrifuged at 100**g** for 5 min and incubated for 30 min at 37°C in 5% CO_2. The tubes were then placed on ice and the conjugates were resuspended five times using a pipetman set at 200 μl. Unbound AK cells and AK cells conjugated to P815 cells were enumerated by microscopic examination in a haemocytometer. At least 100 AK cells were counted. Each sample was counted in triplicate. The percentage of conjugated AK cells was calculated. Data are presented as the mean percentage of conjugated AK-T cells (±S.D.).

Colorimetric granzyme B assay

Granzyme B activity in the cytosolic fraction of AK cells was measured using the granzyme B-specific synthetic substrate Boc–Ala–Ala–Asp thiobenzyl ester (Poe *et al.*, 1991). AK cell lysates were prepared by combining 10^6 AK cells with 0.25 ml of lysis buffer (PBS containing 0.5% NP40 and 0.4 mM Ca^{2+}) and incubating on ice for 30 min. A 180 μl volume of the reaction mixture (PBS with 0.2 mM Boc–Ala–Ala–Asp thiobenzyl ester and 0.1 mM 5,5′-dithiobis(2-nitrobenzoic acid)) was added to 20 μl of cell lysates in wells of a flat-bottomed microtitre plate (Sarstedt Inc.). After 90 min at room temperature the absorbance at 405 nM was read using a Titertek plate reader (ICN). An absorbance of 0.01 was arbitrarily defined as 1 unit of esterolytic activity. Data are presented as mean units of esterolytic activity/10^6 cells in quadruplicate samples (±S.D.).

Semiquantitative reverse transcription polymerase chain reaction (RT–PCR)

AK cells were harvested after 48 h of culture in the absence or presence of PTX, and total cellular RNA was isolated using Trizol reagent (Gibco BRL) according to the supplier's instructions. Single strand cDNA was synthesized from 0.5 μg RNA with 200 units of Moloney murine leukemia virus-derived reverse transcriptase (Gibco BRL) in the presence of 5 mCi ^{32}P–dCTP (Du Pont Canada Inc., Mississauga, ON, Canada). Amplification of equal amounts of cDNA was ensured by monitoring ^{32}P–dCTP incorporation during reverse transcription. Polymerase chain reaction (PCR) was carried out in an automatic DNA thermal cycler (MJ Research Inc., Watertown, MA, U.S.A.). Each reaction used 5×10^4 cpm of cDNA, 2.5 units Taq polymerase (Gibco BRL), 200 μM deoxynucleotide triphosphates (Gibco BRL), and 50 nM (β-actin) or 500 nM (Fas-L, granzyme B or perforin) gene-specific 5′ and 3′ primers (Table 1) in a final volume of 50 μl. Gene-specific primers for Fas-L (Arase *et al.*, 1995), granzyme B and perforin (Nakajima *et al.*, 1994) were obtained from Gibco BRL whereas the β-actin primers were a generous gift from Dr A. Stadnyk (Dept. Pediatrics, Dalhousie University). The specificity of the individual primers was verified by Southern blot analysis using ^{32}P-labelled cDNA probes for Fas-L (kindly provided by S. Nagata, Dept. Molecular Biology, Osaka Bioscience Institute), perforin (generously provided by K. Okumura, Dept. Immunology, Juntendo University School of Medicine), granzyme B (kindly provided by R. C. Bleackley, Dept. Biochemistry, University of Alberta), and β-actin. PCR conditions for granzyme

Table 1. Gene-specific primer sequences used for PCR

mRNA	5′	3′	RNA fragment length [base pairs (bp)]
β-actin, mouse	CTG GAG AAG AGC TAT GAG C	TTC TGC ATC CTG TCA GCA ATG	241
Perforin, mouse	GTC ACG TCG AAG TAC CTG GTG	ATG GCT GAT AGC CTG TCT GC	205
Fas-L, mouse	ATG GTT CTG GTG GCT CTG GT	GTT TAG GGG CTG GTT GTT GC	362
granzyme B, mouse	GCC CAC AAC ATC AAA GAA CAG	AAC CAG CCA CAT AGC ACA CAT	216

B, perforin and β-actin amplifications were 92°C for 30 s, 57°C for 30 s, and 72°C for 1 min for a total of 22, 29 and 25 cycles, respectively. For Fas-L amplifications, PCR conditions were 94°C for 1 min, 64°C for 1 min, and 72°C for 1.5 min for a total of 34 cycles. The number of PCR cycles chosen for Fas-L, granzyme B, perforin, and β-actin amplifications were previously determined to generate PCR product during the exponential phase of amplification. Reverse transcription (RT)–PCR performed under these conditions has been shown to yield reasonably accurate data about relative changes in mRNA levels, providing reliable detection of two-fold or greater differences, even without especially prepared internal standards (Singer-Sam *et al.*, 1990). PCR products were resolved on a 1.7% agarose gel containing 0.01% (v/v) ethidium bromide and were visualized by UV light illumination. The detected cDNA was compared to a 100 bp ladder (Promega Corp., Madison, WI) and quantified by densitometric scanning with a Macintosh Color OneScanner. PCR product levels, as determined by densitometric scans, were normalized relative to steady state expression of β-actin.

Statistical analysis

Data analysis was performed using the Instat statistics program (GraphPad Software Inc., San Diego, CA, U.S.A.). The statistical significance of data from representative experiments was tested using the *t* statistic evaluation and Student's *t* distribution. *P* values less than 0.05 were considered to be statistically significant. Each experiment was repeated at least three times.

RESULTS

PTX inhibits anti-CD3-induced T-cell proliferation

The immunomodulatory capacity of PTX was tested on the proliferative response of T-cells in C57BL/6 whole spleen cell cultures to mitogenic anti-CD3 mAb. According to previous kinetic studies, peak cellular proliferation, as measured by [^{3}H]TdR incorporation, occurs at 48 h of culture (Fitzpatrick *et al.*, 1994). As shown in Fig. 1, PTX exerted a significant inhibitory effect on 48 h anti-CD3-induced T-cell proliferation at all concentrations tested when PTX was present from the initiation of culture. The inhibition of DNA synthesis was dose-dependent, ranging from 19% to 53% suppression as the concentration of PTX was increased from 25 to 100 μg/ml. The inhibitory effect of PTX was not due to non-selective toxicity because spleen cell viability, as assessed by trypan blue staining, was unaffected by PTX.

PTX inhibits AK cell induction

The stimulation of mouse spleen lymphocytes with anti-CD3 mAb results in the activation of killer T-cells with non-specific, major histocompatibility complex-unrestricted cytolytic activity against a range of tumour target cells, including P815 mastocytoma cells (Stankova *et al.*, 1989). The peak cytolytic activity of AK cells against P815 cells is observed at 48 h of culture (Kaiser *et al.*, 1993). When PTX (25–100 μg/ml) was present from the initiation of culture, C57BL/6-derived AK cell induction at 48 h was significantly inhibited in a dose-dependent fashion (Fig. 2). At an effector:target cell ratio of 50:1, 100 μg/ml PTX reduced P815 lysis by 73% ($P=0.001$ vs. no PTX treatment). All AK cell populations used in the cytotoxicity assay were adjusted to contain the same number of viable effector cells.

PTX fails to affect effector-target cell conjugation

We next tested whether AK cells induced in the presence of PTX were able to conjugate normally to

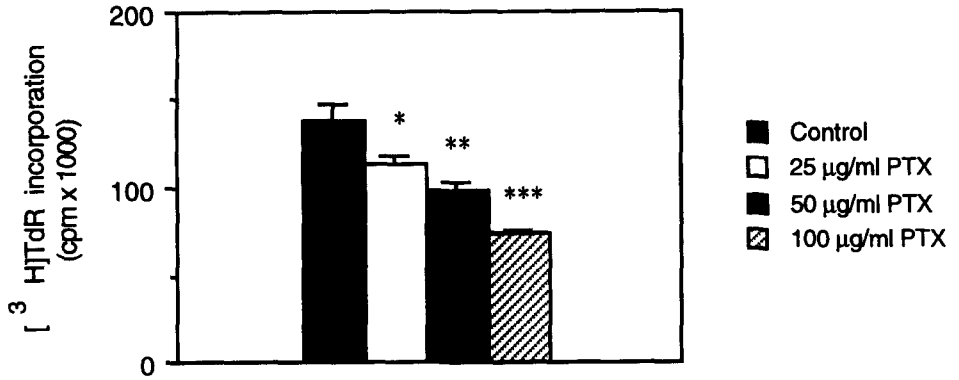

Fig. 1. Effect of PTX on anti-CD3-induced T-cell proliferation. Spleen cells were stimulated with anti-CD3 mAb in the absence or presence of 25, 50, or 100 µg/ml PTX. Following 48 h of culture, DNA synthesis was measured by [^{3}H]TdR incorporation. Data are expressed as mean cpm ±S.D. The background response of spleen cells cultured in medium alone was 450±34 cpm. Statistical significance was determined by Student's *t*-test; $*P=0.0024$; $**P=0.0002$; $***P<0.0001$ in comparison to the control response. Results from one representative experiment out of three are shown.

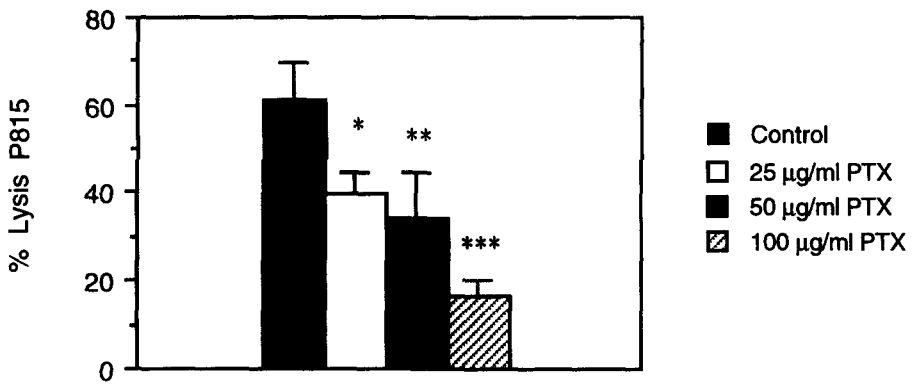

Fig. 2. Effect of PTX on AK cell induction. To generate AK cells, spleen cells were stimulated with anti-CD3 mAb in the absence or presence of 25, 50, or 100 µg/ml PTX. Following 48 h of culture, cytolytic activity against P815 mastocytoma cells at a 50:1 effector:target cell ratio was measured in a 4-h ^{51}Cr-release assay. Data are expressed as mean percentage lysis ±S.D. Statistical significance was determined by Student's *t*-test; $*P=0.0178$; $**P=0.0237$; $***P=0.001$ in comparison to the control response. Results from one representative experiment out of four are shown.

P815 tumour cells. No detectable difference in effector-target cell conjugate formation was observed when control AK cells and AK cells generated in the presence of 100 µg/ml PTX were compared. For example, in one experiment typical of three separate experiments, 24.0±2.9% of control AK cells bound to P815 tumour cells whereas 24.4±1.9% of PTX-treated AK cells conjugated with P815 target cells.

PTX inhibits granule-associated cytolytic effector molecule expression by AK cells

AK cells employ both granule-dependent (e.g. granzyme B and perforin) and granule-independent (e.g. Fas-L) cytolytic effector mechanisms to kill tumour target cells, although cytolysis of P815 mastocytoma cells is mediated mainly through granule-dependent means (Fitzpatrick *et al.*, 1996). We used semiquantitative RT–PCR to determine the effect of PTX on granzyme B, perforin, and Fas-L mRNA expression by AK cells. This procedure has been shown to detect changes in mRNA levels of two-fold or greater reliably, without the use of internal standards (Singer-Sam *et al.*, 1990). Spleen cells at a concentration of 4×10^6 cells/ml were stimulated with anti-CD3 mAb for 48 h in the presence or absence of 100 µg/ml PTX and the synthesis of granzyme B, perforin, and Fas-L was determined. Previous studies established that peak expression of mRNAs coding for these cytolytic effector molecules occurs at 48 h of culture (Prendergast *et al.*, 1992; and our own unpublished data). A β-actin PCR was performed to control for equal loading of PCR products onto the agarose gel (Fig.

3d). PTX strongly inhibited anti-CD3-induced granzyme B (Fig. 3a and e) and perforin (Fig. 3b and e) mRNA expression relative to the untreated control AK cells. In contrast, PTX treatment upregulated Fas-L expression by AK cells (Fig. 3c and e).

A colorimetric granzyme B assay revealed that granzyme B protein levels in AK cells induced in the presence of 100 μg/ml PTX were dramatically reduced. In one experiment (representative of three independent experiments) 10^6 untreated control AK cells harboured 21.7 ± 1.9 units of granzyme B activity, whereas an equal number of PTX-treated AK cells contained only 11.9 ± 0.7 units of granzyme B activity (55% reduction, $P < 0.0001$).

DISCUSSION

Although PTX is of considerable clinical interest because of its anti-inflammatory activity (Zabel *et al.*,

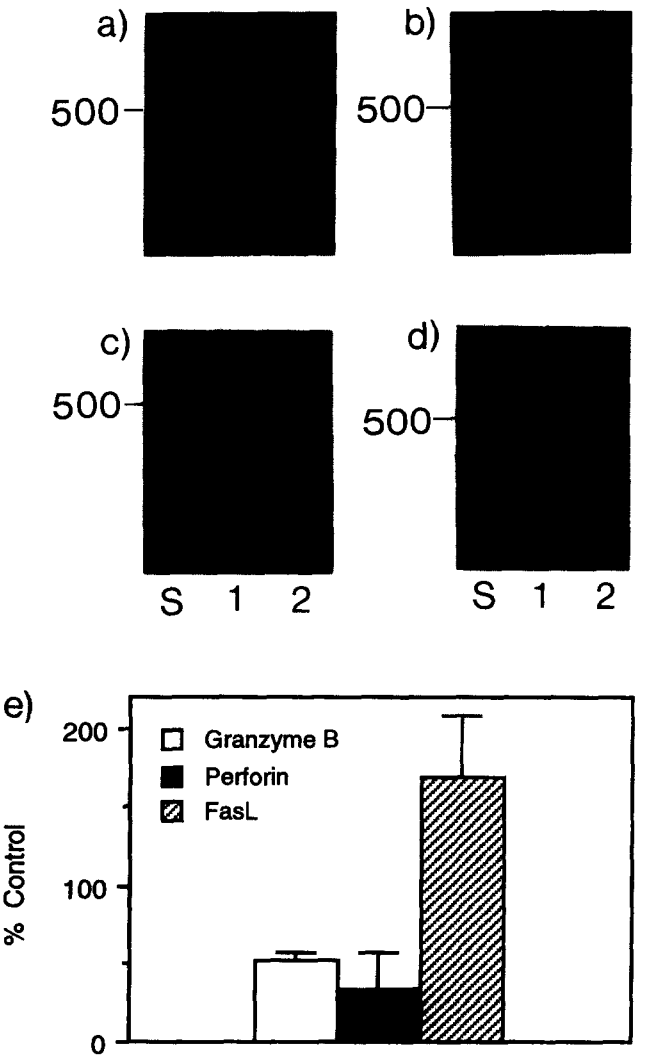

Fig. 3. Effect of PTX on mRNA expression by AK cells. Spleen cells were stimulated with anti-CD3 mAb in the absence (1) or presence (2) of 100 μg/ml PTX. Following 48 h of culture, mRNA was isolated and reverse transcribed. A semiquantitative PCR with primers specific for granzyme B (a), perforin (b), Fas-L (c), or β-actin (d) was performed and ethidium bromide-stained cDNA was visualized under UV light. The detected cDNA was compared to a molecular weight standard (S) consisting of a 100 bp ladder to confirm PCR product size. One representative experiment out of three. (e) PCR products from three independent experiments were quantified by densitometric scanning and granzyme B, perforin, and Fas-L expression was normalized relative to the steady-state expression of β-actin. Data are expressed as percentage of control (AK cells induced in the absence of PTX) ± S.D., $n = 3$.

1989; McDonald, 1991) as well as its haemorrheological properties (Ward & Clissold, 1987), little is known about the effect of PTX on T-cell-mediated cytotoxic immune reactions. In this study we examined the influence of PTX on mouse T-cell proliferation and non-specific killer T-cell induction in response to mitogenic anti-CD3 mAb. We demonstrated that both proliferative and cytotoxic immune reactions are inhibited in a dose-dependent fashion by PTX. In contrast to many recent *in vitro* studies which have used very high concentrations of PTX (up to 1 mg/ml) to study PTX-induced immunomodulation (Rieneck *et al.*, 1993; Tilg *et al.*, 1993; Weiss *et al.*, 1995), we elected to use lower concentrations of PTX (25–100 μg/ml) which more closely approximate typical plasma levels of PTX, including active metabolites, observed *in vivo* (Funk *et al.*, 1995). In agreement with previous investigations (Rott *et al.*, 1993; Tilg *et al.*, 1993), we failed to detect any effect of PTX on lymphocyte viability. Furthermore, PTX-treated AK cells conjugated normally to tumour target cells. Taken together, these findings indicate that the inhibitory activity of PTX is not simply due to non-selective toxic effects on T-lymphocytes.

The reduced cytolytic activity of AK cells generated in the presence of PTX could not be attributed to a defect in the binding stage of the lytic process because PTX-treated AK cells and control AK cells bound equally well to P815 tumour target cells. AK cell adhesion to P815 mastocytoma cells is largely dependent on the interaction of LFA-1 on the effector cell population with corresponding ligands on the target cells (unpublished observations). Our finding that PTX has no effect on the ability of AK cells to adhere to P815 target cells is therefore consistent with a recent report that PTX, at concentrations identical to those employed in our study, has almost no effect on the percentage of LFA-1-bearing lymphocytes in phytohaemagglutinin-stimulated human peripheral blood mononuclear cell cultures, and only slightly reduces LFA-1 density on positive lymphocytes (Funk *et al.*, 1995). Constitutive expression of LFA-1 by mouse TK-1 T-lymphoma cells is similarly unaffected by a 1 mM (~250 μg/ml) concentration of PTX (Weiss *et al.*, 1995). However, it must be noted that the inability of PTX to affect LFA-1-dependent AK cell binding to P815 mastocytoma cells should not be extrapolated to other combinations of effector cells and target cells, where adhesion molecules other than LFA-1 may play an important role in effector-target cell conjugate formation.

AK cells kill P815 mastocytoma cells primarily through the secretion of granule-associated granzyme B and perforin molecules, which require extracelluar calcium in order to induce cytolysis (Fitzpatrick *et al.*, 1996). Our data indicate that PTX downregulates granzyme B and perforin mRNA expression by AK cells. The inhibitory effect of PTX on granzyme B gene induction was confirmed at the protein level by a colorimetric assay of granzyme B enzymatic activity in AK cell lysates. Both PCR analysis and the colorimetric enzyme assay indicated that granzyme B expression was reduced by approximately 50%. We conclude that most, if not all, inhibition of AK cell induction by PTX can be attributed to the reduced expression of cytolytic effector molecules involved in granule-dependent cytolysis. However, a granule- and calcium-independent cytolytic pathway also exists which involves Fas-L on the effector cell triggering the transmission of an apoptotic signal to the target cell nucleus through Fas on the target cell surface (Podack, 1995). Because anti-CD3 mAb induces Fas-L expression by mouse T-lymphocytes (Suda *et al.*, 1995), AK cells also have the potential to kill Fas-bearing target cells through granule-independent means. We observed that Fas-L mRNA expression by AK cells induced in the presence of PTX is upregulated, suggesting that the ability of AK cells to kill Fas-bearing target cells is retained, or may even be enhanced, following exposure to PTX.

The underlying mechanism(s) by which PTX inhibits anti-CD3-induced T-cell proliferation and AK cell induction is not completely understood. T-lymphocyte-elaborated cytokines are clearly crucial for the regulation of cellular immune responses. T-cell proliferation following activation by anti-CD3 mAb requires either IL-2 or IL-4, although 500 units/ml of IL-4 is needed to elicit the same proliferative response as 10 units/ml of IL-2 (Fitzpatrick *et al.*, 1994). IL-2 and IFN-γ are essential for CTL development (Maraskovsky *et al.*, 1989), at least in part because of the ability of IL-2 and IFNγ to induce perforin expression (Lui *et al.*, 1990). In combination with IL-2, TNF-α enhances CTL activity (Robinet *et al.*, 1990), and by itself is able to effect a moderate increase in perforin activity (Lui *et al.*, 1990). IL-2 has also been shown to upregulate granzyme B expression in a mouse CTL clone (Lui *et al.*, 1990), whereas in a previous study we demonstrated the importance of IFN-γ during the induction of granzyme B expression by mouse AK T-cells (Fitzpatrick *et al.*, 1996). Therefore, reduced Th1 cytokine production (TNF-α, IFN-γ and IL-2) in anti-CD3-stimulated spleen cell cultures as a result of a PTX-mediated inhibition of phosphodiesterase activity and the subsequent increase in intracellular levels of cAMP (Rott *et al.*, 1993; Funk *et al.*, 1995) would be expected to suppress not only anti-CD3-induced T-cell proliferation but also AK cell induction

by indirectly blocking the synthesis of granule-associated cytolytic effector molecules. Our data suggest that this is indeed the case. Reduced expression of the high-affinity IL-2 receptor in the presence of PTX (Funk *et al.*, 1995) might also contribute to the immunomodulatory effect of PTX. In addition, PTX has been reported to upregulate prostaglandin E_2 synthesis by human peripheral blood mononuclear cells (Reed & DeGowin, 1992). Because prostaglandin E_2 is known to inhibit Th1 cytokine production (Betz & Fox, 1991) and the induction of CTL responses (Wrenshall *et al.*, 1994), a possible role for the PTX-induced production of prostaglandin E_2 in the suppression of anti-CD3-induced T-cell proliferation and AK cell induction is also indicated. Because Fas-L expression is directly inducible by T-cell receptor engagement and is not dependent on NF-κB activation (Anel *et al.*, 1995), it is not surprising that PTX, a known blocker of NF-κB action (Biswas *et al.*, 1993), fails to inhibit Fas-L gene transcription. The reason for upregulated Fas-L expression in the presence of PTX is not yet clear. One possibility is that PTX treatment may induce transcription factors that control Fas-L gene expression (Takahashi *et al.*, 1994).

PTX is widely acknowledged to be an established and safe drug with a broad therapeutic dose range (Ward & Clissold, 1987). The present finding that PTX is able to inhibit the expression of certain gene products involved in CTL function may have important implications in clinical situations such as organ transplantation, where it is desirable to suppress cell-mediated immune responses directed against foreign tissue. It is believed that both granzyme B and perforin play a major role in the immunopathogenesis of acute cellular rejection because the intragraft expression of CTL-derived granzyme B and perforin transcripts is highly restricted to acutely rejecting cardiac and renal allografts (Legros-Maida *et al.*, 1994; Lipman *et al.*, 1994). It is therefore reasonable to speculate that PTX might be able to prolong allograft survival by down-regulating granzyme B and perforin expression in alloreactive graft-infiltrating CTL. Future studies will investigate the immunosuppressive potential of PTX *in vivo* in rodent models of allogeneic heart and kidney transplantation.

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