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Effects of Cyclosporin A on growth and polyamine metabolism of MOLT-4 T-lymphoblastic leukaemia cells

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Summary We have examined the effects of Cyclosporin A (CsA) on growth and polyamine metabolism of MOLT-4, human T lymphoblastic leukaemia cells to ascertain the role of the polyamine biosynthetic pathway in the antitumour action of CsA. We observed that CsA had a dose-dependent inhibitory effect on growth of the cells *in vitro*, decreasing protein content, cell number and the rate of incorporation of ^{3}H-thymidine into the cells. However, CsA treatment had no significant effect on intracellular polyamine levels in the cells. Contrary to previous reports, simultaneous addition of the diamine, putrescine, with CsA did not block or lessen the growth inhibitory effects of CsA. On the other hand, ornithine decarboxylase activity, the rate limiting enzyme of polyamine biosynthesis which converts ornithine to putrescine, was decreased by CsA treatment. This decrease appeared to be reversible and contrasts with the inhibition by α-difluoromethylornithine, which is irreversible and can be overcome by addition of putrescine. This suppression of ornithine decarboxylase by CsA is more likely to occur by indirect effects on translation and/or transcription rather than a direct effect on the enzyme. It may be a contributory factor in the overall antiproliferative effects of CsA but is more likely to be a response to these growth inhibitory effects rather than a direct effect of the drug.

Cyclosporin A (CsA) is the immunosuppressive agent most commonly used in the clinical management of allograft rejection. Its immunosuppressive action has been well documented (Borel *et al.*, 1977; Granelli-Piperno *et al.*, 1986; Hess *et al.*, 1982; Thomson *et al.*, 1983) and is attributed to the inhibition of CD4$^+$ T helper lymphocyte activation and the production of growth promoting lymphokines. Because of its specificity for T-cells, it is currently undergoing evaluation as a treatment for various autoimmune disorders and as an experimental anti-cancer agent against malignant T-cells. Various studies have shown selective cytostatic and cytolytic effects of CsA on malignant T-cells in culture, including freshly isolated human T-leukaemia/lymphoma cells (Totterman *et al.*, 1982; Foa *et al.*, 1986). Morever, we have previously reported that CsA inhibits the development of the leukaemic phase in rats injected with the Roser T-cell leukaemia (Thomson *et al.*, 1988). The mechanism(s) underlying these and other growth inhibitory effects of CsA on cancer cells (reviewed by McLachlan *et al.*, 1990, and Twentyman, 1988) are not well understood.

Fidelus and Laughter (1986) showed that in the murine T-cell lymphoma EL4, low doses of CsA inhibited the activity of ornithine decarboxylase (ODC), the rate limiting enzyme of polyamine biosynthesis. Furthermore, Saydjari *et al.* (1987) reported inhibition of growth of two animal tumours *in vitro* by CsA and by α-difluoromethylornithine (DFMO), an irreversible inhibitor of ODC. Moreover, they found that they could overcome the inhibitory effect of both CsA and DFMO by addition of the diamine putrescine, suggesting that both drugs were blocking the conversion of ornithine to putrescine by ODC, resulting in depletion of intracellular polyamines. On the other hand a study of the effects of DFMO and CsA on cytolytic T lymphocyte (CTL) induction (Bowlin *et al.*, 1989) indicated that the drugs may inhibit different processes required for CTL induction. However since the naturally occurring polyamines, putrescine, spermidine and spermine, are known to be essential for optimal growth and differentiation of cells (Heby, 1981), and elevated levels are found in many tumours (Kingsnorth *et al.*, 1984*a*, *b*) the possibility that the anti-tumour activity of CsA may be mediated via depletion of polyamines is worthy of investigation.

In this study, we show that CsA produces a dose-dependent anti-proliferative effect against MOLT-4 human T-lymphoblastic leukaemia cells. We also report that CsA decreases ODC activity but that putrescine, the product of ODC action does not reverse the growth inhibitory effects of CsA.

Materials and methods

Chemicals

[Methyl-^{3}H]-thymidine, (25 Ci mmol^{-1}) and DL-[^{14}C]-ornithine hydrochloride, (58 mCi mmol^{-1}), were obtained from Amersham International, UK. Pyridoxal-5-phosphate and DL-ornithine hydrochloride were obtained from Sigma Chemical Co., Poole, UK. Dithiothreitol was obtained from Aldrich Chemical Co. Ltd., Dorset, UK.

Cell culture

MOLT-4, human T-lymphoblastic leukaemia cells, were supplied by the European Collection of Animal Cell Cultures, Porton Down, Salisbury, UK. The cells were maintained as a suspension culture at 37°C in an atmosphere of CO_2/air (1:19) in RPMI 1640 medium supplemented by 10% foetal calf serum (Gibco-BRL, Paisley, Scotland).

Effects of drugs on cell growth

CsA (batch 161412, Sandoz, Basle, Switzerland) was provided in powder form. It was serially dissolved initially in absolute ethanol and subsequently, in RPMI-1640. D-L-α-difluoromethylornithine (DFMO)-HClH$_2$O (a kind gift from Merrell Dow Research Institute, Strasbourg, France), provided as powder, was dissolved in 0.9% w/v saline solution for addition to cell cultures. Cells were seeded in triplicate cultures at 3×10^5 cell ml^{-1} in 24 well plates. Drug solutions were added at time of plating and cells were exposed continuously during the experiments. The final ethanol concentration was 0.1%. Control cells were treated with drug vehicle. Protein was measured in mg/well by the method of Lowry *et al.* (1951). Cell number and viability was determined using an Improved Neubauer Haemocytometer and Trypan blue exclusion.

The rate of [methyl ^{3}H]-thymidine incorporation was also determined. Cells were given a 1 h pulse of [methyl ^{3}H]-TdR (0.2 μCi ml^{-1}), harvested mechanically, and the amount of

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radioactivity in the cells was measured by liquid scintillation counting (Wallace & Keir, 1981).

Generation times (G_T) of the cells were calculated according to the formula:

$$G_T = \log 2 (\Delta t) / \log (N_t/N_o)$$

where Δt = time in culture between counts, N_t = final count and N_o = first count.

Measurement of polyamines

Cells were harvested by centrifugation (13,000 r.p.m., 4 min) and the pellet washed twice in ice-cold phosphate buffered saline (PBS) before extraction of polyamines with 0.2 M $HClO_4$ (Wallace *et al.*, 1984). Polyamines were measured by the h.p.l.c. methcd of Wallace *et al.* (1988) and protein content was determined by the method of Lowry *et al.* (1951).

Extraction and assay of ornithine decarboxylase (ODC)

Extraction Cells were harvested and washed twice in ice-cold saline buffer (0.9% w/v NaCl, 100 mM Hepes, 1 mM Dithiothreitol [DTT]), swollen on ice for 5 min in hypotonic buffer (100 mM Hepes, 1 mM DTT), then disrupted by homogenisation. The homogenate was then centrifuged at 4°C for 20 min at 40,000 g_{av} in an MSE Prepspin 50 Ultracentrifuge, using a 10 × 10 titanium rotor, to remove insoluble cell debris. The supernatant containing the soluble proteins, including ODC, was assayed immediately.

Assay The activity of ODC was measured by the release of [^{14}C]-CO_2 from ^{14}C-ornithine hydrochloride (58 mCi mmol^{-1}) (Russell & Synder, 1968). The reaction mixture contained, in a final volume of 1 ml, 100 mM Hepes, pH 7.2 at 37°C, 1 mM DTT, 50 μM pyridoxal-5-phosphate, 0.2 mM ornithine hydrochloride, 0.15 μCi ^{14}C-ornithine hydrochloride and 0.3 ml test enzyme preparation or 0.3 ml of partially purified *E. coli* ODC solution.

Statistics

The significance of differences between the means was calculated using ANOVA/Dunnett's test.

Results

Exposure of MOLT-4 cells to a range of concentrations of CsA from 0.1 μg ml^{-1} to 10 μg ml^{-1} showed that the growth of the cells was inhibited in a dose-dependent manner. The observed effects of CsA treatment were significant decreases in cell number (Figure 1) and viability (Table I), and in protein content and ^{3}H-TdR incorporation (Results not shown). A dose of 10 μg ml^{-1} CsA had marked toxic effects on the cells with cell viability reduced to less than 40% after 96 h in culture. However when the cells were washed after 96 h treatment, fresh medium added, and recovery assessed by the rate of ^{3}H-TdR incorporation into the cells, it was observed that the increases in the rate of DNA synthesis with time in the remaining viable cells in all the treatment groups were comparable (Table II). The generation times of the treatment groups were all within 40 ± 6 h (Results not shown) after the drug was removed and fresh medium added.

Individual polyamine concentrations in cells treated with 1 μg ml^{-1} and 5 μg ml^{-1} CsA were virtually unchanged compared to controls after 48 h and 96 h in culture and no significant alterations in total polyamine content were observed (Table III).

Simultaneous addition of putrescine at concentrations of 0.1 mM, 1 mM and 10 mM with CsA at 1 μg ml^{-1} and 5 μg ml^{-1} did not affect the ability of CsA to inhibit growth of MOLT-4 cells (Table IV), although in contrast, the growth inhibitory effects of DFMO were completely reversed by putrescine at all the concentrations studied.

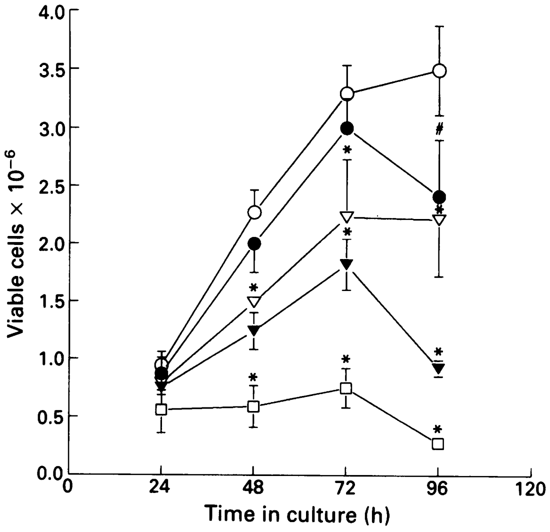

Figure 1 Effect of range of concentrations of CsA on the growth of MOLT-4 cells grown in culture over 96 h. Growth measured as the number of viable cells × 10^{-6}. Means ± 1 s.d. of triplicate cultures. ○, Control; ●, 1 μg ml^{-1}; ▽, 2.5 μg ml^{-1}; ▼, 5 μg ml^{-1}; □, 10 μg ml^{-1}; *$P<0.01$; #, $P<0.05$.

Table I Viability of MOLT-4 cells in culture during and after CsA treatment

	Treatment			*Recovery*	
	48 h	*96 h*	*(% viability)*	*24 h*	*72 h*
Control	90	71	Cells	97	78
1 μg ml^{-1} CsA	90	69	washed and	87	83
2.5 μg ml^{-1} CsA	82	65	fresh medium	90	85
5 μg ml^{-1} CsA	84	54	added	78	86
10 μg ml^{-1} CsA	60	38		73	84

% viability of cells measured by trypan blue exclusion.

Table II Recovery of MOLT-4 cells in culture following 96 h treatment with a range of CsA concentrations

	Time from end of treatment (h)			
96 h treatment	*0 h*	*24 h*	*48 h*	*72 h*
Control	204 ± 22	385 ± 30	1982 ± 504	1146 ± 70
1 μg ml^{-1} CsA	148 ± 47	759 ± 95*	2601 ± 80	901 ± 223
2.5 μg ml^{-1} CsA	227 ± 77	591 ± 42*	2635 ± 177*	1007 ± 81
5 μg ml^{-1} CsA	173 ± 67	649 ± 92**	2973 ± 170**	1089 ± 218
10 μg ml^{-1} CsA	204 ± 75	274 ± 41	3522 ± 172**	1094 ± 151

Cells were treated with CsA for 96 h, washed and fresh medium added. Recovery was assessed by ^{3}H-TdR incorporation into acid-insoluble fraction of cells (DPM 10^{-5} viable cells). *$P<0.05$, **$P<0.01$ compared to controls.

Table III Polyamine concentrations in MOLT-4 cells treated with CsA *in vitro*

	48 h	*96 h*
Control		
Putrescine	5.99 ± 0.73	0.65 ± 0.16
Spermidine	8.90 ± 0.93	8.57 ± 1.02
Spermine	8.08 ± 0.53	9.68 ± 0.73
Totals	22.98 ± 2.18	18.88 ± 1.91
1 μg ml^{-1} CsA		
Putrescine	4.24 ± 0.50*	0.75 ± 0.09
Spermidine	8.49 ± 0.55	7.78 ± 1.60
Spermine	7.28 ± 0.58	8.35 ± 1.99
Totals	20.01 ± 1.43	16.89 ± 3.67
5 μg ml^{-1} CsA		
Putrescine	4.98 ± 0.51	1.70 ± 0.03
Spermidine	10.12 ± 0.68	7.68 ± 0.91
Spermine	9.07 ± 0.82	8.93 ± 0.39
Totals	24.17 ± 1.96	18.31 ± 1.20

Amount of polyamines expressed in nmol mg^{-1} protein. Results are means ± 1 s.d. of triplicate assays. *$P<0.05$ compared to controls.

Table IV Effects of addition of putrescine with CsA and DFMO treatments over 96 h in culture

	CsA		*DFMO*
	1 $\mu g\ ml^{-1}$	5 $\mu g\ ml^{-1}$	2.5 mM
Control	0.46 ± 0.06	0.49 ± 0.07	0.54 ± 0.04
Drug alone	0.35 ± 0.04*	0.31 ± 0.01*	0.43 ± 0.01*
0.1 mM Put	0.37 ± 0.02*	0.31 ± 0.01*	0.53 ± 0.02
1 mM Put	0.35 ± 0.01*	0.34 ± 0.03*	0.58 ± 0.04
10 mM Put	0.36 ± 0.02*	0.29 ± 0.04*	0.60 ± 0.02

Results expressed as mg protein/well. Means ± 1 s.d. of triplicate assays. *$P < 0.01$ compared to controls. Put = putrescine.

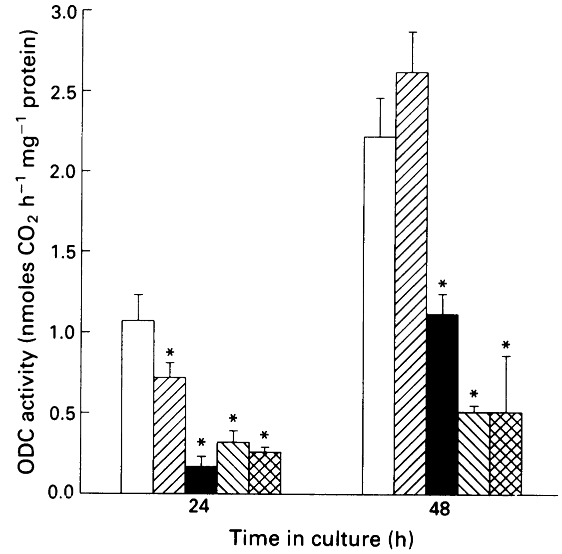

Figure 2 Effects of CsA and DFMO treatment on ODC activity in MOLT-4 cells in culture, measured by the release of ^{14}C-CO_2 from ^{14}C-ornithine. Results are means ± 1 s.d. of triplicate assays. □, Control; ▨, 2.5 $\mu g\ ml^{-1}$ CsA; ■, 5 $\mu g\ ml^{-1}$ CsA; ▧, 2.5 mM DFMO; ▩, 5 mM DFMO; *$P < 0.01$.

Figure 2 shows that ornithine decarboxylase (ODC) activity in the MOLT-4 cells was inhibited by treatment with DFMO (2.5 mM and 5 mM) as expected after 24 h and 48 h in culture. CsA (2.5 $\mu g\ ml^{-1}$ and 5 $\mu g\ ml^{-1}$) also lowered ODC activity after 24 h treatment with the higher concentration being the more effective. After 48 h in culture however, the ODC activity in the cells treated with 2.5 $\mu g\ ml^{-1}$ CsA appeared to have been fully restored to the same levels as the controls and the suppression of activity in the cells treated with 5 $\mu g\ ml^{-1}$ was markedly less than at 24 h.

Discussion

We have investigated the effects of CsA on growth and polyamine metabolism of MOLT-4 cells in culture and compared the results to those obtained with the well established inhibitor of ODC, α-DFMO. Our data show that CsA inhibits cell growth in a dose-dependent manner demonstrated by the observed reductions in cell number, cell viability, protein content and ^3H-TdR incorporation in cultures treated with a range of CsA concentrations.

Polyamines are known to be essential for cell growth and it has been suggested that depletion of polyamines was a potential mode of action for the growth inhibitory effects of CsA. However despite suggestions that polyamine biosynthesis may be involved in the antitumour action of CsA (reviewed, McLachlan *et al.*, 1990), we observed no changes in intracellular polyamine content following addition of the drug to cultures. In contrast to previous reports (Saydjari *et al.*, 1987) where the growth inhibitory effects of CsA could be overcome by the addition of putrescine, simultaneous addition of putrescine with CsA in our model did not reverse the effects of CsA on cell growth. We were however, able to reverse the actions of DFMO on intracellular polyamine content and cell growth by addition of putrescine. The lack of effect of CsA on intracellular polyamine levels is consistent with the results from our *in vivo* study (Smart *et al.*, 1989), where we found that CsA did not deplete polyamine levels, nor did it enhance the polyamine depletion, seen with DFMO treatment, in blood mononuclear cells or in various tissue samples from the tumour hosts.

Despite the lack of a long term effect of CsA on polyamine content, CsA treatment did decrease ODC activity in MOLT-4 cells transiently (Table IV), suggesting that either CsA has a reversible effect on ODC or that there is a decreasing availability of CsA. Since CsA is known to bind to various molecules within the cell, such as cyclophilin (Merker & Handschumacher, 1984; Quesniaux *et al.*, 1988; Ryffel, 1990) and the 170 Kd membrane P-glycoprotein which functions as a drug efflux pump in multidrug resistant cells (Foxwell *et al.*, 1990; Nooter *et al.*, 1990), decreased availability may be a major reason for the transient effect. DFMO is a 'suicide' inhibitor of ODC and binds irreversibly to the active site of the enzyme molecule (Metcalf *et al.*, 1978) thus producing a direct effect on enzyme activity. On the other hand the reduction of ODC activity observed in CsA treated cells is more likely to be a consequence of the antiproliferative mechanism(s) of CsA on these cells.

It has been proposed by Sigal *et al.* (1990), that CsA inhibits Ca^{2+}-associated signal transduction pathways which may play a major role in the cascade leading to lymphokine production rather than directly inhibiting lymphokine mRNA transcription. These signals may also be linked to the rise in ODC activity which has been shown to be an integral event regluating lymphocyte DNA synthesis (Kay & Lindsay, 1973; Klimpel *et al.*, 1979).

The intracellular binding protein cyclophilin is generally thought to be involved in the mode of action of CsA and has recently been shown to possess peptidyl-prolyl *cis-trans* isomerase (PPIase) activity which is inhibited by CsA binding (Takahashi *et al.*, 1989; Fischer *et al.*, 1989). Therefore it is possible that elements involved in the activation cascade may be 'conformationally' regulated by peptidyl-prolyl bond isomerisation. Alternatively, it may also be that intracellular metabolism of CsA may convert CsA to a derivative which has no effect on ODC.

Since CsA treatment does not affect intracellular polyamine content and its effects cannot be reversed by the addition of putrescine it seems more likely that the link between CsA effects and ODC activity is casual rather than specific and may exist for other enzymes whose functions, like that of ODC, are so closely linked to cell proliferation.

A recent report by Bowlin *et al.* (1989), concerning the effects of CsA and DFMO on the induction of cytolytic T lymphocytes *in vitro* and *in vivo*, demonstrated enhanced inhibition by combination of the drugs. The explanation proposed for this result was that the inhibition of interleukin-2 (IL-2) production by CsA was augmented by a decreased ability of polyamine-depleted cells to respond to IL-2. We hope that further studies on the effects of CsA, either on its own or in combination with DFMO, examining uptake and intracellular distribution of bound and free drug in different cellular compartments will help to clarify these observations and assist in design of possible therapeutic modalities.

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