Immunology 1990 **69** 104–109

Anti-CD3 antibody-induced expression of both p55 and p75 chains of the high affinity interleukin-2 receptor on human T lymphocytes is inhibited by cyclosporin A

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Accepted for publication 12 September 1989

SUMMARY

The inhibitory effect of cyclosporin (CsA) was investigated on human lymphocytes stimulated by anti-T-cell antibodies (anti-CD3 and -CD2) or mitogenic lectins. Whereas inhibition of cell proliferation (50%) occurred at 10 ng/ml CsA after cell activation via CD3 or CD2, higher CsA concentrations (300 ng/ml) were necessary to inhibit lectin-mediated cell activation (PHA, Con A). Exogenous recombinant interleukin-2 (rIL-2) partially reversed the inhibitory effect on antibody-stimulated cells only; however, at higher CsA concentrations (300 ng/ml) proliferation was again inhibited. Thus, CsA affected IL-2R expression and/or function at higher concentrations (300 ng/ml). CsA had no effect on receptor function as measured on IL-2-dependent cell growth of CTLL cells or preactivated lymphocytes. However, CsA inhibited both high and low affinity receptor expression as shown by [^{125}I]IL-2 equilibrium binding studies on anti-CD3-stimulated cells. Cross-linking studies revealed that both p55 (TAC) and p75 chains of the IL-2R were not induced at low CsA concentrations (10 ng/ml). However, addition of rIL-2 reversed CsA inhibition of IL-2R expression. It is concluded that CsA, at least in anti-CD3-stimulated cells, inhibits IL-2R expression and cell proliferation with similar potency. Exogenous rIL-2 reverses CsA inhibition of IL-2R expression. This might be due to binding of rIL-2 to receptors which escape CsA inhibition, thereby up-regulating receptor expression which is drug resistant.

INTRODUCTION

Cyclosporin A (CsA; SANDIMMUNE®), a cyclic undecapeptide of fungal origin, is a potent immunosuppressive drug now extensively used in organ transplantation (Beveridge, 1986). The focal point of CsA immunosuppressive activity is the inhibition of antigen-induced proliferation of T lymphocytes. The precise mechanism of CsA action is unknown but the drug interferes with early events of T-cell activation including the expression of genes for interleukin-2 (IL-2) and other lymphokines (Reem, Cook & Vilcek, 1983; Krönke *et al.*, 1984; Elliott *et al.*, 1984).

The effect of CsA on other early events of T-cell activation, especially the acquisition of receptors for IL-2 (IL-2R), is controversial. Early reports in mouse and guinea-pig lymphocytes (Bunjes *et al.*, 1981, DosReis & Shevach, 1982) failed to demonstrate any CsA-inhibition of IL-2R expression although a later study in murine thymocytes (Gauchat, Khandjian & Weil, 1986), where both IL-2R mRNA synthesis and membrane expression of receptor were analysed, showed definite CsA sensitivity of receptor expression. In human lymphocytes the reported data has been similarly conflicting. Detection of IL-2R expression by antibodies (Miyawaki *et al.*, 1983; Gelfand, Cheung & Mills, 1987; Bloemena *et al.*, 1988), mRNA probes (Granelli-Piperno, Andrus & Steinman, 1986) and [^{125}I]IL-2 binding/Scatchard analysis (Bloemena *et al.*, 1988) found no CsA inhibition of either high or low affinity receptor expression in lectin-stimulated lymphocytes, although the studies disagreed on whether exogenous IL-2 was capable (Miyawaki *et al.*, 1983) or not (Gelfand *et al.*, 1987; Bloemena *et al.*, 1988) of reversing CsA inhibition of cell proliferation. In contrast to the above, the study by Reed *et al.* (1986), where a combination of all the above techniques were used, showed CsA inhibition of the expression of both high and low affinity IL-2R, although the receptor was found to be less sensitive to the drug than inhibition of IL-2 secretion.

In this present study the sensitivity of IL-2R expression to CsA has been re-addressed using a combination of ligand binding and IL-2-responsiveness analysis. Moreover, [^{125}I]IL-2 cross-linking studies have been performed which allow the detection of both the p55 (Tac protein) and the novel 75,000

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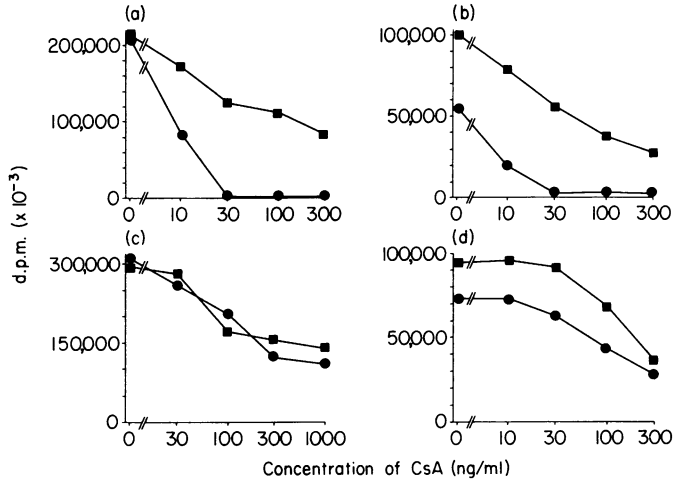

Figure 1. Effect of CsA on PMBC proliferation in the presence and absence of rIL-2. PMBC were stimulated with 2·5 ng/ml OKT3(a), a combination of anti-CD2 antibodies T11.2 and T11.3 (1/800 of ascites) (b), 1 μg/ml PHA (c) or 3 μg/ml Con A (d) in the presence (■) or absence (●) of 20 ng/ml rIL-2 for 3 days before harvesting. Results are representative for at least four independent experiments from different donors.

MW IL-2R (p75) of the high affinity receptor (Sharon *et al.*, 1986; Tsudo *et al.*, 1986; Teshigawara *et al.*, 1987). It is shown that the expression of both chains of IL-2R in antibody (anti-CD3)-activated T lymphocytes was inhibited by CsA. Furthermore, the presence of exogenous IL-2 partially reversed CsA effects on the expression of its receptor.

MATERIALS AND METHODS

Reagents

CsA and rIL-2 were provided by Sandoz Ltd (Basel). [^{125}I]IL-2 was obtained from New England Nuclear (Drieich, FRG). The murine monoclonal antibodies to CD2 (T112 and T113) were a kind gift of Dr E. Reinherz (Boston, MA); OKT3 (anti-CD3) was obtained from Ortho Diagnostics (Raritan, NJ); phytohaemagglutinin (PHA) and concanavalin A (Con A) were from Amimed (Basel, Switzerland) and Sigma (St Louis, MO) respectively.

Cell cultures

Human peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood of healthy volunteers by Ficoll–Hypaque density-gradient centrifugation. Cells were resuspended at 10^6 per ml in complete medium: RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (Gibco, Paisley, Renfrewshire, U.K.), 2 mM L-glutamine, 25 μM β-mercaptoethanol, penicillin (100 U/ml) and streptomycin (100 μg/ml) and cultured in a humified 5% CO_2/95% air, humified atmosphere at 37°. Cells were stimulated with mitogen or antibody, cultured with or without rIL-2 and/or CsA as described. Proliferation assays were performed in flat-bottomed microtitre plates with 10^5 cells in a final volume of 0·2 ml and cultured as above. Six hours before harvesting, each well was pulsed with 0·5 μCi of [^{3}H]thymidine (Amersham International, Amersham, Bucks, U.K). Data are reported as mean d.p.m. of triplicate cultures. The murine CTLL cells (M. Schreier, Sandoz, Basel, Switzerland) were cultured in RPMI media as above except with 50 μM β-mercaptoethanol and 20 ng/ml IL-2. Cells were assayed in microtitre plates at 5×10^4 cells per well. After 20 hr cells were labelled with [^{3}H]thymidine for 6 hr followed by harvesting (as above).

[^{125}I]IL-2 cross-linking studies

Cross-linking of [^{125}I]-rIL-2 (5 nM) to PBMC with disuccinimidyl suberate (DSS; Pierce, rockford, MO) was performed as described previously (Tsudo *et al.*, 1986). Cells grown in 20 ng/ml rIL-2 were washed and recultured without lymphokine for 3 hr before cross-linking. Cross-linked cells (5×10^6) were analysed by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970) followed by autoradiography of the dried gels at −70°. Scanning of autoradiographs was performed using a 2022 LKB Ultrascan laser-densitometer.

[^{125}I]rIL-2 binding assays

Binding assays were performed essentially as described by Robb, Greene & Rusk (1984). Briefly, PBS-washed PBMC (2×10^6) were suspended in 200 μl PBS containing 1% bovine serum albumin with various concentrations of [^{125}I]IL-2 (in triplicate) in the presence or absence of 10 μM unlabelled rIL-2 (to determine non-specific binding) for 1 hr on ice. The cells were isolated by centrifugation (12,000 r.p.m., 90 seconds, at room temperature) through a 200 μl silicone oil cushion (SF 1250, General Electric, Waterford, NY) and frozen on dry ice. The tube tips containing the cell pellets were cut and counted for ^{125}I. The number of binding sites per cell and the apparent dissociation constant (K_a') were determined from the linear portion of Scatchard plots of the data using linear regression analysis.

RESULTS

The effect of exogenous rIL-2 on CsA inhibition of PBMC proliferation

Proliferation of PBMC (Fig. 1), whether induced through the CD2 or CD3 receptors using the relevant monoclonal antibodies, was found to be sensitive to CsA at approximately 10 ng/ml (ID_{50}). The addition of exogenous rIL-2 to antibody-

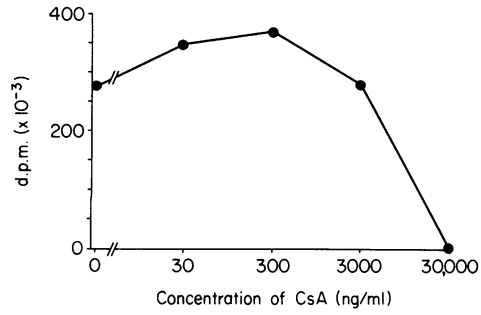

Figure 2. Effect of CsA on the proliferation of IL-2 dependent murine cell line CTLL. CTLL were cultured at 5×10^4 in microtitre plates in total volume of 0·2 ml containing 20 ng/ml rIL-2 with varying concentrations of CsA for 24 hr. Cells were pulsed with 0·5 μCi of [^{3}H]thymidine for 6 hr before harvesting.

stimulated cells resulted in a partial reversal of the CsA effect, but at higher drug concentrations (300 ng/ml) significant inhibition of proliferation was still observed. Activation of PBMC by PHA or Con A (Fig. 1) was also sensitive to CsA, although higher concentrations (100–300 ng/ml, ID_{50}) of the drug were needed. In addition, exogenous rIL-2 failed to reverse the drug's effects on PHA-stimulated cells and had only a minor effect on Con A-stimulated PBMC.

Effect of CsA on IL-2 dependent proliferation of CTLL and PBMC

The IL-2-dependent murine cell line CTLL was treated with CsA to assess the drug effects on IL-2-induced proliferation. As shown in Fig. 2, concentrations up to 3000 ng/ml CsA had no cytostatic effect although above this concentration a total inhibition of cell growth occurred. To investigate CsA effects on IL-2-dependent proliferation of activated PBMC, cells were first stimulated for 2 days with anti-CD3 to allow IL-2R expression, and then washed to remove mitogen and recultured for a third day with 300 ng/ml CsA, a concentration inhibitory to IL-2 responsiveness (Fig. 1a). The results in Table 1 showed that the addition of CsA on the third day (additions Day 3, Table 1) had only a minor inhibitory effect of proliferation (< 10%) regardless of the presence of exogenous rIL-2. When CsA was present during the mitogenic stimulus (additions Days 0–2, Table 1) then a gross inhibition (~90%) of proliferation occurred. The addition of rIL-2 post-activation had only a small effect on reversing the drug's inhibition. The further addition of CsA on the final day of culture did not have any additional effect.

Expression of high and low affinity IL-2R in the presence of CsA

The expression of high and low affinity receptors on PBMC was assessed using [^{125}I]IL-2 binding followed by Scatchard analysis (Fig. 3), or by the cross-linking of [^{125}I]IL-2 to its receptor followed by SDS–PAGE/autoradiography (Fig. 4). Anti-CD3-induced PBMC activation results in the expression of high affinity (3300±940 sites; K_a' 59±23 pM) and low affinity (41,000±9600 sites, K_a' 7±2·5 nM; $n=4$) receptors (Fig. 3); unstimulated control PBMC expressed very few high (103±42) or low (2100±600) affinity binding sites. CsA inhibited dose-dependently the expression of both high and low affinity IL-2

Table 1. Effect of CsA of proliferative responses of PBMC after OKT3 activation

Additions (Days 0–2)	Additions (Day 3)			
	No addition	CsA	IL-2	IL-2±CsA
OKT3	109,209±12,671	98,840±7295	129,858±1087	116,023±2635
OKT3+ CsA	6305±990	9154±1980	13,277±718	12,043±1206

PBMC (1×10^6 cells/ml) were cultured for 2 days (0–2) with OKT3 (2·5 ng/ml) ± CsA (300 ng/ml) and subsequently washed three times with complete medium. The cells were recultured for a third day (Day 3) with/without CsA (300 ng/ml) and/or rIL-2 (20 ng/ml) in microtitre plates (1×10^6 cells/ml) in a total volume of 0·2 ml. The cells were pulsed with [^{3}H]thymidine 6 hr before harvesting.

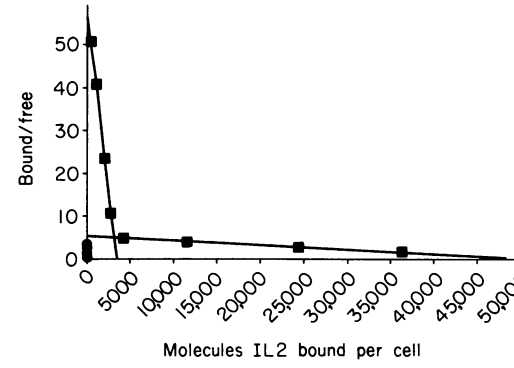

Figure 3. Scatchard analysis of [^{125}I]IL-2 binding to activated PBMC. PBMC (2×10^6/ml) were cultured for 3 days with 2·5 ng/ml OKT3 in the presence (●) or absence (■) of 300 ng/ml CsA. [^{125}I]IL-2 binding studies were performed as described in the Materials and Methods. The data are representative of four independent experiments.

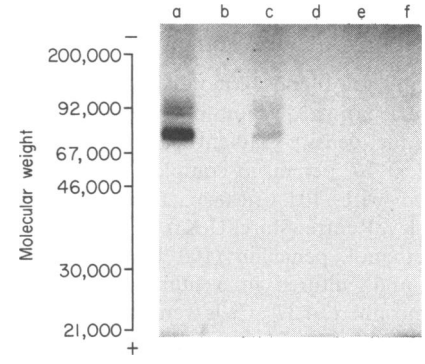

Figure 4. SDS–PAGE/autoradiographic analysis of [^{125}I]IL-2 cross-linked, PBMC stimulated (OKT3) in presence and absence of CsA. PBMC (2×10^6/ml) were cultured with 2·5 ng/ml OKT3 (a, b), 2·5 ng/ml OKT3 plus 300 ng/ml CsA (c, d) or without additions (e, f). Cross-linking studies were performed as described in the Materials and Methods in the absence (a, c, e) or presence (b, d, f) or a 1000-fold excess of unlabelled rIL-2 as a specificity control. Identical results were obtained in three independent experiments.

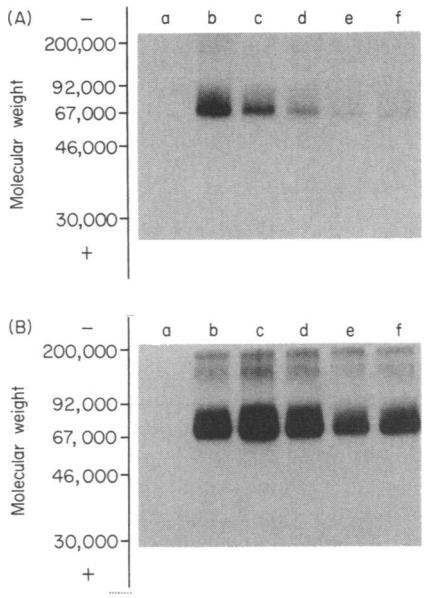

Figure 5. SDS–PAGE/autoradiographic analysis of [^{125}I]IL-2 cross-linked PBMC stimulated in the presence of varying concentrations of CsA in the absence (A) or presence (B) of rIL-2. (A) PBMC (2×10^6/ml) were cultured without (a) or with (b–f) 2·5 ng/ml OKT3 in the presence of 0 (b), 10 ng/ml (c), 30 ng/ml (d), 100 ng/ml (e) and 300 ng/ml (f) CsA for 3 days followed by [^{125}I]IL-2 cross-linking analysis. (B) As above but with cells cultured in media containing 20 ng/ml rIL-2. Experiments were performed with cells from three different donors with similar results.

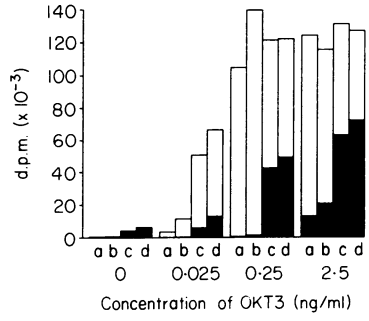

Figure 6. Effect of rIL-2 on CsA inhibition of PBMC stimulation. PBMC (1×10^6/ml) were cultured for 2 days with OKT3 (varying concentration) in the absence (a, b) or presence (c, d) of 20 ng/ml rIL-2. The cells were washed three times with complete medium and recultured without (a, c) or with (b, d) rIL-2 (20 ng/ml) in microtitre plates (1×10^6/ml) in a total volume of 0·2 ml for 24 hr. Cells were pulsed with [^{3}H]thymidine 6 hr before harvesting. (■) Represents the results obtained in the presence of 300 ng/ml CsA during the first 2 days. Data are reported as means of triplicate cultures and are representative for three independent experiments.

binding to the level of unstimulated cells (only values at 300 ng/ml CsA are shown in Fig. 3; 186 ± 85 high affinity sites/cell were calculated). Determination of IL-2R expression at earlier time points (1 and 2 days) showed consistent inhibition of receptor expression by CsA.

Cross-linking of [^{125}I]IL-2 to the IL-2R on anti-CD3-stimulated cells allowed the detection of the p75 chain doublet as well as the p55 chain/TAC (Fig. 4a). No receptor expression was detected on unstimulated cells (Fig. 4e). CsA (300 ng/ml) greatly reduced receptor expression ($< 5\%$ by densitometric scanning) of both p55 and p75 chains (Fig. 4c). Control experiments were performed, with drug added 3 hr before harvesting, and failed to show any effect on IL-2R expression as assessed by cross-linking, indicating that CSA did not simply inhibit the binding and/or cross-linking of IL-2 to its receptor (results not shown).

The effect of exogenous rIL-2 on the dose-dependency of CsA inhibition of IL-2R expression as assessed by [^{125}I]IL-2 cross-linking

Data obtained from Fig. 1 suggested that IL-2R expression, as measured by IL-2 responsiveness, was less sensitive to CsA inhibition than T-cell proliferation. However, receptor-binding studies (Figs 3 and 4) showed nearly total abolition of IL-2R expression at 300 ng/ml CsA. To investigate this further, cross-linking studies were performed on cells exposed to varying doses of CsA in the presence or absence of IL-2. In the absence of IL-2 (Fig. 5A) a 50% inhibition of receptor expression was obtained at approximately 10 ng/ml CsA (estimated by densitometric scanning). In the presence of exogenous lymphokine (Fig. 5B) the expression of IL-2R proved to be much more drug resistant; maximum inhibition of only 40% of control was obtained at the higher drug concentrations.

The effect of rIL-2 on CsA-inhibition of PBMC activation

The results described above showed that IL-2 attenuated CsA inhibition on IL-2R expression. To investigate this further experiments were performed (Fig. 6) to test the effect of exogenous rIL-2, when present with the mitogen, on CsA inhibition of T-cell proliferation and IL-2 responsiveness. Cells were stimulated with varying doses of anti-CD3 in the presence (Fig. 6c, d) and absence (Fig. 6a, b) of rIL-2. Parallel cultures were performed with CsA (300 ng/ml, Fig. 6). After 2 days the cells were washed and recultured without mitogen but plus (Fig. 6b, d) or minus (Fig. 6a, c) rIL-2, and the proliferation assayed. In the absence of mitogen there was no proliferation except for a low level in the presence of rIL-2 which was CsA resistant. At the lowest dose of anti-CD3 (OKT3, 0·025 ng/ml) proliferation in the absence of rIL-2 was only just detectable. (Fig. 6a). The addition of lymphokine post-activation did increase this low level of proliferation (Fig. 6b). Addition of rIL-2 during the mitogenic period (Fig. 6c), however, greatly stimulated proliferation which was slightly enhanced if rIL-2 was additionally present post-mitogen (Fig. 6d). CsA totally inhibited cell activation unless rIL-2 was present with the anti-CD3 (Fig. 6) when a low but significant drug-resistant proliferation occurred.

At a higher anti-CD3 concentration (0·25 ng/ml OKT3) a nearly full proliferation was found in all cases, but drug sensitivity was greatly reduced if rIL-2 was present with the mitogen. Without rIL-2 a complete inhibition was observed (Fig. 6a, b), whereas its presence induced a significant CsA-resistant proliferation (Fig. 6c, d). This effect was more evident at the highest anti-CD3 concentration (2·5 ng/ml) when lymphokine-induced drug resistance increased to $> 50\%$ of control proliferation. This experiment also showed that the 'strength' of the mitogenic stimulus (i.e. the dose of OKT3) had a marked effect of CsA sensitivity.

DISCUSSION

The effect of CsA on IL-2R expression on PBMC upon mitogenic activation has been assessed. The initial experiments were concerned with the effect of exogenous rIL-2 on CsA-inhibited PBMC proliferation. As expected, activation of T cells by anti-CD3 antibodies was very sensitive to the drug (ID_{50} ~10 ng/ml). Similar inhibition was also shown by anti-CD2 stimulation of cells and this is the first demonstration of CsA sensitivity of this activation pathway. In both cases rIL-2 partially reversed CsA inhibition. The similar behaviour of the CD2- and CD3-stimulated cells to CsA inhibition might be expected since there is evidence that the two pathways are interdependent (Alcover *et al.*, 1988). Furthermore, these results suggest that these activation pathways join before the point at which CsA acts. Lectin-induced cell proliferation was at least 10-fold less sensitive to CsA as compared to antibody-activation; exogenous rIL-2 had little effect at reversing the drug's inhibition, in agreement with previously published studies (Gelfand *et al.*, 1987, Bloemena *et al.*, 1988). The differential sensitivity of antibody and lectin-activated cells to CsA inhibition could reflect the triggering of different stimulatory pathways. PHA and Con A bind the T-cell receptor–CD3 complex (Kanellopoulos *et al.*, 1985; Valentine *et al.*, 1985) and presumably activate cells via this pathway. However, lectins do bind to other cell surface determinants, possibly stimulating proliferation which is more CsA-resistant, as has been reported for CD28-activated cells (June *et al.*, 1987).

The studies on antibody-stimulated cells showed that even in the presence of rIL-2, higher concentrations of CsA were able to cause a significant inhibition of T-lymphocyte proliferation. This effect could be due to inhibition of receptor expression, interference with IL-2 binding or IL-2R signal transduction, or a combination of these. The latter two possibilities seem unlikely as CsA did not inhibit the growth of the IL-2-dependent cell line, CTLL, at concentrations up to 3 μg/ml, showing that IL-2 binding and IL-2R signal transduction are not interfered with by the drug. The inhibition at 30 μg/ml can be ascribed to the general cytotoxicity of CsA at this high concentration (Ryffel *et al.*, 1988). Similar drug resistance was shown in preactivated PBMC which proliferate through an autocrine pathway (Meuer *et al.*, 1984), where CsA added after mitogenic stimulation failed to inhibit cell growth. However, when given together with the mitogenic antibody, CsA inhibition was above 90%.

Studies on CsA effects on IL-2R expression were performed on antibody (anti-CD3)-stimulated cells since this form of activation is less complex than using lectins (Weiss *et al.*, 1986). Scatchard analysis of binding data and [^{125}I]IL-2 cross-linking studies showed a near total inhibition of expression of both high and low affinity receptors. The cross-linking data also demonstrated that expression of both the p55 (Tac) and p75 chains of the IL-2R were inhibited. Studies by Dukovich *et al.* (1987) suggested that the p75 chain is constitutively expressed on resting T cells. However, our data failed to detect p75 chain in unstimulated cells and definitely showed inducibility of the p75 chain upon mitogenic stimulation in a manner similar to that of the p55 chain. Furthermore, induction of both chains was CsA sensitive (Figs 4 and 5). The possibility that in the absence of Tac the p75 chain is unable to bind [^{125}I]IL-2 can be discounted as previous studies (Tsudo *et al.*, 1986, 1987; Dukovich *et al.*, 1987) have shown the labelling of p75 chain in cells only expressing this form of the IL-2R.

The dose–response studies (Fig. 5) revealed that IL-2R expression and proliferation have similar sensitivity to CsA (ID_{50} ~10 ng/ml). As with cell proliferation, exogenous rIL-2 partially reversed CsA inhibition of IL-2R expression. IL-2-mediated up-regulation of the expression of its own receptor has been shown previously (Smith & Cantrell, 1984; Depper *et al.*, 1985; Harel-Bellan *et al.*, 1986; Reed *et al.*, 1985). However, the results showed that a primary mitogenic signal (i.e. antibody) was a major requirement for detectable receptor expression. This finding correlates with those below where only minimal proliferation—probably attributable to a small population of pre-activated cells—was observed if resting PBMC were cultured with exogenous rIL-2 alone.

CsA could act by inhibiting the primary mitogenic signal that is required for receptor expression. However, since IL-2 may function as a factor in the expression of its receptor, CsA inhibition of IL-2 could result in reduced IL-2R expression. The final experiment suggested that both factors could be important. In the absence of exogenous rIL-2, CsA was a very potent inhibitor of proliferation and acquisition of IL-2 responsiveness. However, if rIL-2 was provided to the cells during the mitogenic period, so that any indirect effects of CsA due to inhibition of IL-2 expression would be prevented, then a partial protection similar to Fig. 1a and Fig. 5b was seen. The level of this protection was found to be related to the amount of anti-CD3 used for stimulation.

One could envisage a mechanism whereby CsA inhibits IL-2R expression primarily by inhibiting the synthesis of IL-2, thereby removing an essential co-factor for receptor expression. Such a mechanism would explain the higher sensitivity of IL-2R to CsA when receptor expression is assessed in the absence of exogenous IL-2. In the presence of the lymphokine CsA only inhibited the primary activation of the IL-2R gene. If this signal pathway is less sensitive to the drug this would account for the resistance of IL-2R expression and IL-2 responsiveness that was observed in the presence of rIL-2. That the cellular factors regulating IL-2 and IL-2R expression have different sensitivities to CsA has been suggested previously by Reed *et al.* (1986). Such a possibility is not unlikely because requirements for the expression of IL-2 and IL-2 responsiveness are, at least, quantitatively different (Roosnek *et al.*, 1985a; 1985b; Cantrell, Collins & Crumpton, 1988).

It is possible that the above models for CsA effects on IL-2R are too simplistic, as factors other than IL-2, e.g. IL-1 (Kaye *et al.*, 1984) or the Tac-inducing factor recently described (Tagaya *et al.*, 1988), which may be involved in receptor expression, could also be inhibited by the drug. Such possibilities await further studies.

In summary, the sensitivity of IL-2R receptor expression to CsA has been studied using a combination of IL-2 responsiveness, [^{125}I]IL-2 binding and [^{125}I]IL-2 cross-linking. T cells activated by the CD2 pathway were found to be as sensitive as CD3-stimulated cells. It has also been shown that the expression of the IL-2R p75 chain, like the p55 chain, is activation dependent and that the expression of both receptor chains is inhibited by the drug. Exogenous IL-2 is able to partially reverse the effect of CsA, most probably by up-regulating receptor expression. However, it is unclear whether inhibition of IL-2 production by CsA provides a secondary mechanism of in-

hibiting IL-2R expression in addition to a primary effect on the anti-CD3 activation signal to the IL-2R gene. The signal provided by the lymphokine via its receptor to the IL-2R gene appears to be CsA resistant and raises the interesting situation of a gene controlled by CsA sensitive and insensitive pathways.

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