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Effects of Cyclosporin A on expression of IL-2 and IL-2 receptors in normal and multiple sclerosis patients

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SUMMARY

The effects of the immunosuppressant Cyclosporin A (CsA) on T cell activation *in vivo* and *in vitro* were examined using the monoclonal antibody, anti-Tac, for interleukin 2 (IL-2) receptors and 3.9C2, for a peptide fragment of human IL-2. Peripheral blood lymphocytes (PBL) were stimulated with PHA in the presence of CsA. The expression of IL-2 receptors and production of IL-2 were reduced. PBL from CsA-treated MS patients had significantly lower proportions of Tac^+ cells compared with untreated patients. This inhibition was not reflected in the CSF lymphocyte populations from CsA-treated patients and indicates the urgent need for an immunosuppressant drug which can enter the CNS in sufficient concentrations to inhibit local T cell activation.

Keywords multiple sclerosis IL-2 IL-2 receptors Cyclosporin A

INTRODUCTION

Cyclosporin A (CsA) is a fungal cyclic undecapeptide with potent immunosuppressive properties widely used in preventing graft rejection and recently in treatment of certain autoimmune diseases such as juvenile diabetes mellitus (Feutren *et al.*, 1986). CsA has an inhibitory effect on T lymphocyte proliferation and interferes with lymphocyte responses to allogeneic histocompatibility antigens (Borel *et al.*, 1977; Wiesinger & Borel, 1979). Certain T cell functions such as help and cytotoxicity are blocked by CsA. However, the exact mechanism by which CsA exerts its immunosuppressive influence on T cells remains unclear, although it is thought to act during the early stage of T cell activation (Orosz *et al.*, 1983; Shevach, 1985; Gauchat, Khandjian & Weil 1986). Detailed studies have been performed on the effects of CsA on IL-2 receptor expression following the recent availability of monoclonal antibodies such as anti-Tac, (Uchiyama *et al.*, 1981a, b). Miyawaki *et al.* (1983) and Solbach *et al.* (1985) measured the levels of the Tac expression by mitogen-stimulated human lymphocytes. The former group showed that although proliferation was inhibited by CsA at 5 μg/ml, the expression of the Tac antigen was unaffected. In contrast, data from the latter study indicated that CsA reduced the intensity of Tac antigen expression on cells of all sizes. In addition, Lillehoj, Malek & Shevach (1984) using the antibody 7D4, directed against the murine IL-2 receptor, found inhibition of IL-2 receptor expression, IL-2 production and proliferative responses after 72 h culture of T cells with Con A and CsA.

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Larsson (1980) did not find any inhibition of IL-2 production by Con A-stimulated murine spleen cells. Others reported an abrogation of IL-2 production by T lymphocytes when incubated with CsA (Bunjes *et al.*, 1981; Orosz *et al.*, 1983). Inhibition of the production of other lymphokines such as interleukin 3 and γ-interferon by CsA has been found for T helper lymphocytes stimulated with Con A (Herold *et al.*, 1986). Although Kronke *et al.* (1984), using cDNA gene probes, have shown the molecular effects of CsA to include an inhibition of IL-2 mRNA synthesis, a reduction in either cytoplasmic or membrane-bound IL-2 has not yet been demonstrated.

Using immunofluorescence techniques and monoclonal antibodies specific for IL-2 (3.9C2) and its receptor (anti-Tac), we have studied the effects of CsA *in vivo* and *in vitro*. CsA inhibited both the production of IL-2 and expression of the IL-2 receptor on PHA-activated human peripheral blood lymphocytes (PBL) *in vitro*. In addition, the effects of CsA on IL-2 receptor expression *in vivo* were studied using lymphocytes from peripheral blood and cerebrospinal fluid (CSF) of MS patients undergoing treatment with CsA. We found a significant decrease in Tac binding in the blood but not the CSF lymphocytes of CsA-treated patients.

MATERIALS AND METHODS

Patients with MS. Patients (16 male, 27 female) with MS were from a double-blind study of CsA treatment for MS. All patients had definite MS (Poser *et al.*, 1983) with active disease, having at least one relapse per year in the preceding 2 years or a steady progression of their disorder.

CsA was given as a drinking solution containing 100 mg per ml of Cyclosporin dissolved in olive oil and librafil (Sandoz, Switzerland). The dosage was 10 mg per kg per day in divided dosage for the first two months of the trial, reducing to 8 mg per kg per day for the next 22 months over 2 years. Control subjects received the carrier oil alone. The dosage of CsA received varied according to adverse effects, especially renal function, as monitored by creatinine clearance but, on average, the patients received 10 mg per kg per day for the first 2 months and approximately 7 mg per kg per day for the next 22 months. The whole blood trough levels of CsA taken 12 h after the last dose were widely variable, ranging from 300–600 ng per ml for months 3–24 of the trial. No patient had a level less than 70 ng per ml at any time. Blood was obtained at three monthly intervals throughout the trial, and CSF at the beginning of the trial and after 24 months.

Cells. PBL and CSF lymphocytes from healthy laboratory controls and MS patients were obtained from whole heparinized blood and CSF as previously described by Bellamy *et al.* (1985). None of the donors were receiving any form of drug therapy.

Antibodies. Anti-Tac (anti-CD25), UCHT1 (anti-CD3) and 3.9C2 (anti IL-2) were donated by Dr T. Waldmann, NIH, Dr P. C. L. Beverley, London, UK, and Dr C. Lewis, Glaxo Group Research, Middlesex respectively. All the antibodies were used at optimal concentrations as determined by previous titration experiments. The anti-Tac antibody was supplied as ascites fluid and used at a final dilution of 1:10000. UCHT1, as culture supernatant, was added to cells undiluted, and 3.9C2 was diluted 1:10 before use.

Immunofluorescent staining of lymphocytes. For *in vitro* studies, PBL at 10^6/ml were incubated in RPMI 1640 medium (GIBCO Europe, Paisley, UK) containing 10% A^+ human serum (for optimal growth), 2 mM L-glutamine, 25 mM HEPES buffer, 50 μg/ml gentamicin, 25 IU/ml sodium heparin and 1 mM sodium pyruvate (Sigma Chemical Co., England) in the presence of 1 μg/ml PHA-P (Difco Labs, UK) and a range of doses of CsA (100 ng/ml–5 μg/ml) for varying periods of time (0–72 h).

CsA (kindly donated by Dr J. Borel, Sandoz, Switzerland) was dissolved at 1 mg CsA in 1·2 ml premixed solvent of 1 ml pure ethanol with 0·2 ml TWEEN 80 (Sigma, Poole, UK) and was diluted at least 200 times in A^+ serum-containing culture medium, before adding to lymphocytes. Control cultures were incubated with premixed solvent alone to ensure lymphocyte viability.

For immunofluorescence staining, the PBL were harvested at various times following addition of CsA and air-dried cytocentrifuge spreads prepared. When staining with UCHT1 and anti-Tac antibodies, cytocentrifuge spreads were fixed in acetone for 10 min and washed in PBS before adding antibody. Slides for 3.9C2 were fixed in 90% ethanol for 2 min, and washed in PBS before

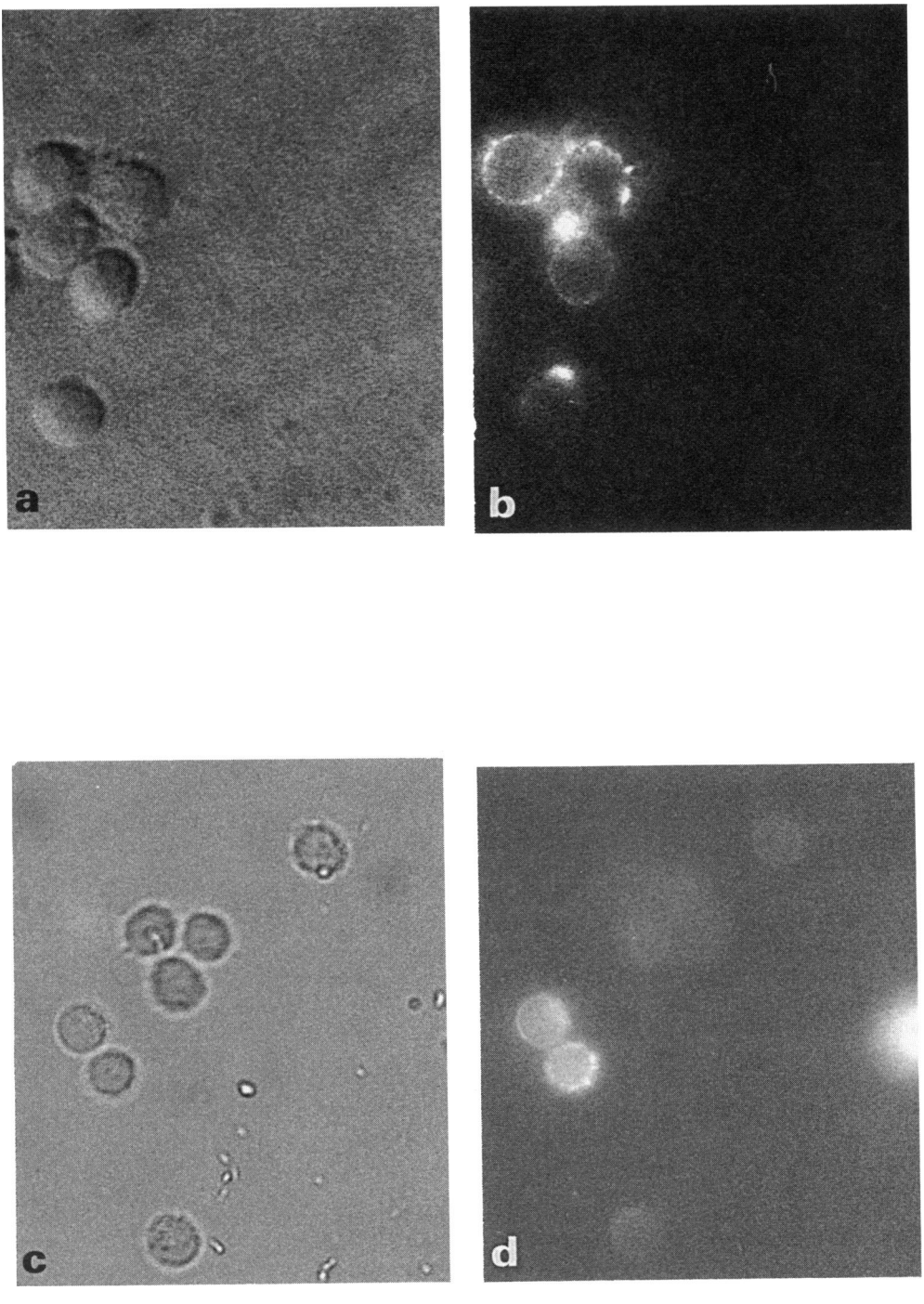

Fig. 1. PHA-stimulated lymphocytes were examined by phase contrast for (a) Tac and (c) 3.9C2 and by immunofluorescence technique for (b) Tac and (d) 3.9C2 expression (× 630).

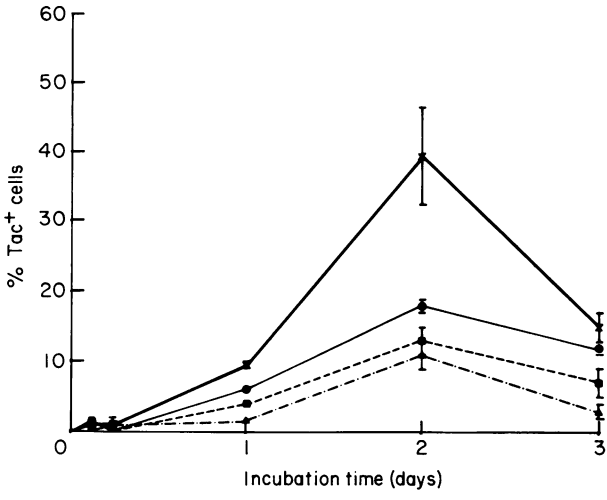

Fig. 2. Levels of IL-2 receptor-bearing cells at days 0–3 following PHA-stimulation of normal PBL, in the presence of CsA at (▲) 5000 ng/ml, (■) 1000 ng/ml, (●) 100 ng/ml and (×) no CsA.

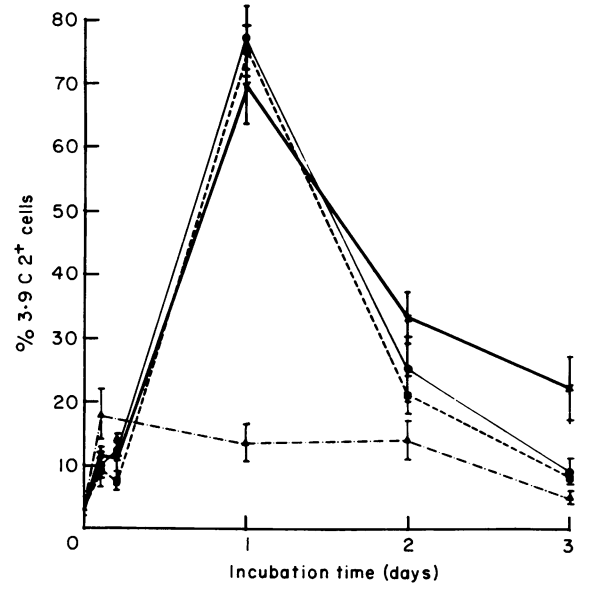

Fig. 3. Levels of IL-2-bearing cells at days 0–3 following PHA-stimulation of normal PBL, in the presence of CsA at (▲) 5000 ng/ml, (■) 1000 ng/ml, (●) 100 ng/ml and (×) no CsA.

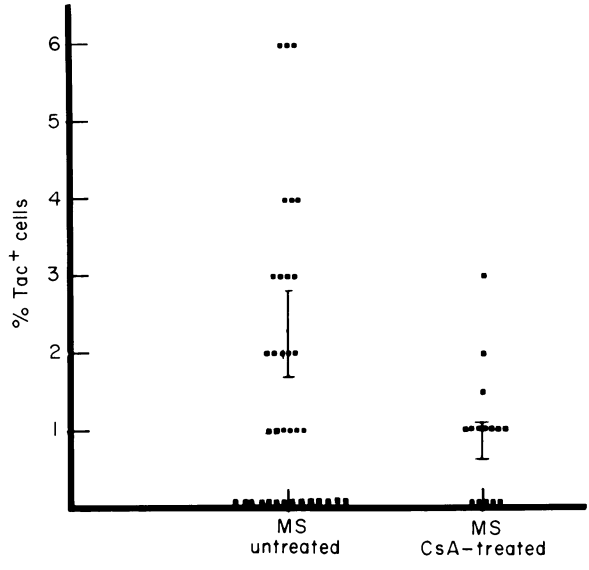

Fig. 4. Levels of Tac$^+$ cells in peripheral blood of CsA-treated and control MS patients.

staining. Monoclonal antibodies were applied for 30 min in a 100 μl volume, slides washed twice in RPM1 1640 medium containing 5% newborn bovine serum (Flow Laboratories, Irvine, UK) and 100 μl of fluorescein-conjugated rabbit anti-mouse Ig (FITC-RaMIg) (Nordic Immunological Laboratories, Maidenhead, UK) (diluted 1:100) added for 30 min. After washing three times, the preparations were mounted and viewed by eye (by two observers) using a fluorescent microscope (Leitz Dialux 20EB). Four counts of more than 300 cells were performed on each slide. Some MS PBL were stained in suspension and analysed on a Fluorescence Activated Cell Sorter (FACS IV, Becton Dickinson Ltd, UK). Aliquots of CSF cells were stained as previously described (Bellamy *et al.*, 1985).

RESULTS

Effect of CsA on expression of IL-2 and its receptor in vitro

IL-2 receptor. The effect of CsA on PHA-induced expression of IL-2 receptor expression was studied. The expression of IL-2 receptors, determined microscopically on normal PBL cultured with PHA (Fig 1a, b) was inhibited as shown in Fig. 2. Due to the slight variability in activation kinetics between different blood samples, the data shown are from a single sample. However, similar results have been found with PBL from four normal controls and three MS patients in remission.

No detectable Tac$^+$ cells (i.e. $<1\%$) were present in resting PBL cultures (0 h) as in normal controls (Bellamy *et al.*, 1985). Significant numbers of IL-2 receptor positive cells (9·5%) were first found after 24 h in culture with PHA (Fig. 2). The levels of Tac$^+$ cells reached maximal levels at 48 h (39%) declining to 15% at 72 h. However, expression of Tac antigen was inhibited by all doses of CsA tested (even with the lowest dose of 100 ng/ml). This dose-dependent inhibition reduced the number of IL-2 receptor-bearing cells in the cultures to less than 1·5% at 24 h ($P<0{\cdot}0005$), 3% at 72 h ($P<0{\cdot}01$), and 11% at 48 h ($P<0{\cdot}01$).

The proportion of cells stained with UCHT1, a pan-T cell marker, remained unchanged.

IL-2. The monoclonal antibody 3.9C2 was used as a marker for IL-2. Both cytoplasmic and membrane-bound IL-2 stains brightly in human PHA-stimulated cultures (Fig. 1c, d), but does not with resting PBL, EBV lines or B cell hybridomas (V.L. Calder, unpublished observation). IL-2 can

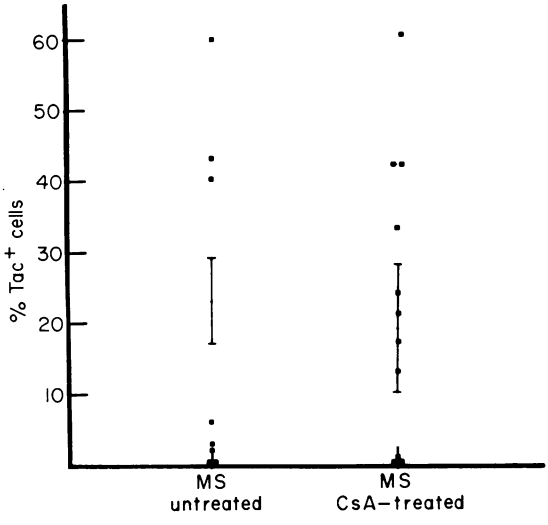

Fig. 5. Levels of Tac^+ cells in cerebrospinal fluid samples of CsA-treated and control patients.

be observed within the cytoplasm of PBL cultured in the presence of PHA for 24 h, but most staining is located on or near the cell membranes (Fig. 1c, d).

Levels of $3.9C2^+$ cells (Fig. 3) in resting PBL were very low (3%), but this increased to approximately 10% after 3–6 h incubation with mitogen. By 24 h, 70% of all cells were $IL\text{-}2^+$, (over 90% of all T cells) and considerable amounts of cytoplasmic staining were seen, compatible with the finding that only 10% of the cells were expressing IL-2 receptors at that time. The expression of IL-2 decreased to 33% of cells at 48 h and 22% at 72 h. Thus the maximum expression of IL-2 occurred earlier than IL-2 receptor expression. Pretreating the cells with CsA had no effect on its inhibitory capacity (data not shown).

Effect of CsA on IL-2 receptor expression in vivo

We have previously demonstrated a range in values of Tac^+ cells of 0–9% in the peripheral blood of MS patients with a small but significant increase in mean value, compared with normal controls (Bellamy *et al.*, 1985). In the present study, levels of Tac^+ cells in PBL of MS patients were significantly reduced ($P > 0{\cdot}01$) by treatment with CsA in comparison to untreated controls (Fig. 4). CsA treatment *in vivo* resulted in a smaller range in the proportion of Tac^+ cells (0–6%) with values of $2{\cdot}3 \pm 0{\cdot}5\%$ in 35 untreated MS patients compared with $0{\cdot}9 \pm 0{\cdot}2\%$ in 15 CsA-treated MS patients. The proportion of Tac^+ cells in the few CSF ($n = 8$) from CsA-treated MS patients studied (Fig. 5) gave mean values of $19 \pm 9\%$ (range 0–60%) which were not significantly different in comparison with 11 untreated MS patients (mean values of $23 \pm 6\%$; range 0–60%). None of the patients in this study showed any gross blood–brain barrier damage since the concentrations of albumin and IgG in the CSF were in the normal range.

DISCUSSION

This paper reports on the effects of CsA on expression of IL-2 and its receptor by PHA-stimulated human PBL *in vitro* and its effects on IL-2 receptor expression by lymphocytes from CsA-treated MS patients *in vivo*. A novel anti-IL-2 monoclonal, 3.9C2, has been used to examine the kinetics of IL-2 expression by PHA blasts. $IL\text{-}2^+$ cells reached maximal levels at 24 h, correlating with peak transcriptional activity of the gene coding for IL-2 (Efrat & Kaempfer, 1984; Kronke *et al.*, 1985). The subsequent decline in transcriptional activity of the IL-2 gene and the corresponding drop in

expression of the IL-2 receptor indicates the transient expression of the genes involved in early T cell activation.

We observed inhibition of IL-2 expression in mitogen-stimulated PBL cultures, by CsA. At 72 h, cultures showed a decrease in $3.9C2^+$ cells even at the lowest dose of CsA tested (100 ng/ml). This provides definitive evidence for an inhibitory effect of CsA on IL-2 production as CsA blocks IL-2 gene expression at the level of mRNA transcription (see Kronke *et al.*, 1984; 1985).

IL-2 receptor-bearing cells induced by PHA peaked at 48 h (39%) and were slightly lower (around 50% maximum) than in previous studies (Cantrell & Smith, 1983; Cotner *et al.*, 1983). Incubation with CsA caused dose-dependent inhibition of the expression of the receptor for IL-2 at 24, 48 and 72 h (Fig. 2). Exposure of PHA blasts to CsA (5 μg/ml) for 72 h reduced levels of both IL-2 and its receptor to resting values. The inhibitory effect of CsA on IL-2 receptor expression may be direct or indirect via the lack of IL-2. However a previous observation that IL-2 can induce expression of IL-2 receptors would support the hypothesis that CsA directly inhibits IL-2 receptor expression (Smith & Cantrell, 1985; Depper *et al.*, 1985). Miyawaki *et al.* (1983) and Solbach *et al.* (1985) did not observe a decrease in the number of Tac^+ lymphocytes after CsA exposure but different experimental techniques were used. CsA undoubtedly interferes at several stages early in the process of T cell activation (Shevach, 1985). This may vary between species, the dose of CsA used and the method for inducing lymphocyte activation.

CsA has been used successfully to prevent and treat experimental allergic encephalomyelitis (Borel *et al.*, 1977; Ryffel, Gotz & Heuberger, 1982; Bolton *et al.*, 1982). In our patients, it has been found that CsA had a marginally beneficial effect upon the course of disease, the rate of decline being significantly slower in the treated group (P. Rudge, pers. comm.). CsA significantly reduced the levels of Tac^+ cells seen in PBL of MS patients, indicating an effect *in vivo* but the proportion of Tac^+ cells in the CSF was not reduced. Entry of CsA may be impeded by an intact blood–brain barrier as negligible levels of CsA have been detected in the CSF of treated patients (less than 5% of serum concentrations) (Palestine *et al.*, 1985; M. L. Cuzner, pers. comm. in our patients), and in CsA-treated mice (Fazakerley & Webb, 1985). Since IL-2 receptor expression on lymphocytes within the CSF appears to be unchanged, it implies that there is insufficient CsA to suppress endogenous lymphocyte activation in the brain.

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REFERENCES

Bellamy, A.S., Calder, V.L., Feldmann, M. & Davison, A.N. (1985) The distribution of interleukin-2 receptor bearing lymphocytes in multiple sclerosis: evidence for a key role of activated lymphocytes. *Clin. exp. Immunol.* **61,** 248.

Bolton, C., Borel, J.F., Cuzner, M.L., Davison, A.N. & Turner, A.M. (1982) Immunosuppression by Cyclosporin A of experimental allergic encephalomyelitis. *J. neurol. Sci.* **56,** 147.

Borel, J.F., Feurer, C., Magnee, C. & Stahelin, H. (1977) Effects of the new anti-lymphocytic peptide Cyclosporin A in animals. *Immunol.* **32,** 1017.

Bunjes, D., Hardt, C., Rollinghoff, M. & Wagner, H. (1981) Cyclosporin A mediates immunosuppression of primary cytotoxic T cell responses by impairing the release of interleukin 1 and interleukin 2. *Eur. J. Immunol.* **11,** 657.

Cantrell, D.A. & Smith, K.A. (1983) Transient expression of interleukin 2 receptors: consequences for T cell growth. *J. exp. Med.* **158,** 1895.

Cotner, T., Williams, J.M., Christenson, L., Shapiro, H.M., Strom, T.B. & Strominger, J. (1983) Simultaneous flow cytometric analysis of human T cell activation, antigen expression and DNA content. *J. exp. Med.* **157,** 461.

Depper, J.M., Leonard, W.J., Drogula, C., Kronke, M., Waldmann, T.A. & Greene, W.C. (1985) Interleukin 2 (IL-2) augments transcription of the interleukin-2 receptor gene. *Proc. natn Acad. Sci. USA.* **82,** 4230.

Efrat, S. & Kaempfer, R. (1984) Control of biologically active interleukin 2 messenger RNA formation in induced human lymphocytes. *Proc. natn Acad. Sci. USA.* **81,** 2601.

Fazakerley, J.K. & Webb, H.E. (1985) Cyclosporin, blood–brain barrier, and multiple sclerosis. *Lancet* **ii,** 889.

Feutren, G., Assan, R., Karsenty, G., Du Rostu, H., Sirmai, J., Papoz, L., Vialettes, B., Vexiau, P., Rodier, M., Lallemand, A. & Bach, J.-F. (1986)

Cyclosporin increases the rate and length of remissions in insulin-dependent diabetes of recent onset. *Lancet* **i,** 119.

GAUCHAT, J.-F., KHANDJIAN, E.W. & WEIL, R. (1986) Cyclosporin A prevents induction of the interleukin 2 receptor gene in cultured murine thymocytes. *Proc. natn Acad. Sci. USA.* **83,** 6430.

HEROLD, K.C., LANCKI, D.W., MOLDWIN, R.L. & FITCH, F.W. (1986) Immunosuppressive effects of Cyclosporin A on cloned T cells. *J. Immunol.* **136,** 1315.

KRONKE, M., LEONARD, W.J., DEPPER, J.M., ARYA, S.K., WONGSTAAL, F., GALLO, R.C., WALDMANN, T.A. & GREENE, W.C. (1984) Cyclosporin A inhibits T cell growth factor gene expression at the level of mRNA transcription. *Proc. natn Acad. Sci. USA.* **81,** 5214.

KRONKE, M., LEONARD, W.J., DEPPER, J.M. & GREENE, W.C. (1985) Sequential expression of genes involved in human T lymphocyte growth and differentiation. *J. exp. Med.* **161,** 1593.

LARSSON, E.-L. (1980) Cyclosporin A and dexamethasone suppress T cell responses by selectively acting at distinct sites of the triggering process. *J. Immunol.* **124,** 2828.

LILLEHOJ, H.S., MALEK, T.R. & SHEVACH, E.M. (1984) Differential effect of Cyclosporin A on the expression of T and B lymphocyte activation antigens. *J. Immunol.* **133,** 244.

MIYAWAKI, T., YACHIE, A., OHZEKI, S., NAGAOKI, T. & TANIGUCHI, N. (1983) Cyclosporin A does not prevent expression of Tac antigen, a probable TCGF receptor molecule, on mitogen-stimulated human T cells. *J. Immunol.* **130,** 2737.

OROSZ, C.G., ROOPENIAN, D.C., WIDMER, M.B. & BACH, F.H. (1983) Analysis of cloned T cell function. II Differential blockade of various cloned T cell functions by Cyclosporin. *Transpl.* **36,** 706.

PALESTINE, A.G., ROBERGE, F., CHAROUS, B.L., LANE, H.C., FAUCI, A.S. & NUSSENBLATT, R.B. (1985) The effect of Cyclosporine on immunization with tetanus and keyhole limpet hemocyanin (KLH) in humans. *J. clin. Immunol.* **5,** 115.

POSER, C.M., PATY, D.W., SCHEINBERG, L., MCDONALD, W.I., DAVIS, F.A., EBERS, G.C., JOHNSON, K.P., SIBLEY, W.A., SILBERBERG, D.H. & TOURTELLOTTE, W.W. (1983) New diagnostic criteria for multiple sclerosis: guidelines for research protocols. *Ann. Neurol.* **13,** 227.

RYFFEL, B., GOTZ, U. & HEUBERGER, B. (1982) Cyclosporin receptors on human lymphocytes. *J. Immunol.* **129,** 1978.

SHEVACH, E.M. (1985) The effects of Cyclosporin A on the immune system. *Ann. Rev. Immunol.* **3,** 397.

SMITH, K.A. & CANTRELL, D. (1985) Interleukin-2 regulates its own receptors. *Proc. natn Acad. Sci. USA.* **82,** 864.

SOLBACH, W., HEEG, K., VON STELDERN, D.U., ROLLINGHOFF, H. & WAGNER, H. (1985) On the partial suppression of IL 2 receptor expression and the prevention of lectin-induced lymphoblast formation by Cyclosporin A. *Clin. exp. Immunol.* **60,** 501.

UCHIYAMA, T., BRODER, S. & WALDMANN, T.A. (1981a) A monoclonal antibody (anti-Tac) reactive with activated and functionally mature human T cells. 1. Production of anti-Tac monoclonal antibody and distribution of Tac (+) cells. *J. Immunol.* **126,** 1393.

UCHIYAMA, T., NELSON, D.L., FLEISHER, T.A. & WALDMANN, T.A. (1981b) A monoclonal antibody (anti-Tac) reactive with activated and functionally mature T cells. II. Expression of Tac antigen on activated cytotoxic killer T cells, suppressor cells, and on one of two types of helper T cells. *J. Immunol.* **126,** 1398.

WIESINGER, D. & BOREL, J.F. (1979) Studies on the mechanism of action of Cyclosporin A. *Immunobiol.* **156,** 454.