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Augmentation of delayed-type hypersensitivity to high dose sheep erythrocytes by cyclosporin A in the mouse: influence of drug dosage and route of administration and analysis of spleen cell populations

LAURA M. WEBSTER & A. W. THOMSON *Immunopathology Laboratory, Department of Pathology, University of Aberdeen, Aberdeen, Scotland, UK*

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SUMMARY

When administered by various routes 48 h before a high systemic dose (10^9) of sheep red blood cells (SRBC), Cyclosporin A (CsA) prevented the suppression of delayed-type hypersensitivity (DTH) reactions elicited 4 days later. Augmentation of DTH was observed over a wide range (5–200 mg/kg) and with circulating CsA levels ranging below 45 ng/ml at the time of immunization or antigen challenge. Splenic lymphocytes from vehicle- and CsA-treated mice exhibited good proliferative responses to mitogen *in vitro*, but only those from CsA-treated animals responded to antigen. Expression of DTH was associated with a progessive, 2-fold increase in the absolute numbers of splenic L3T4$^+$ cells, whereas no significant alteration in the number of Lyt-2$^+$ lymphocytes was recorded. B cell and macrophage numbers in the spleen were unaffected by CsA. In contrast to its potentiating effects on cell-mediated immunity, CsA caused profound (up to 100%) suppression of the concomitant production of splenic anti-SRBC IgM-secreting plasma cells. Circulating anti-SRBC antibody levels were also markedly reduced. These data show that CsA can permit induction of T_{DTH}, whilst suppressing T-dependent humoral immunity and without significant change in absolute numbers of Lyt-2$^+$ cells.

Keywords delayed-type hypersensitivity cyclosporin A mouse spleen sheep red blood cells lymphocytes antibody immunopotentiation

INTRODUCTION

Whilst the immunosuppressive properties of cyclosporin A (CsA) in man and other species are now well established (Borel, 1986), it is also recognized that, under well-defined experimental conditions, the drug can augment cell-mediated immune responses. This capacity of CsA, first demonstrated in guinea pigs (Thomson *et al.*, 1983), is dependent on the temporal relationship between drug administration and that of antigen. Thus, high dosage CsA before immunization (Parker, Drössler & Turk, 1984; Aldridge & Thomson, 1986) or short courses of the drug starting at the time of immunization (Thomson *et al.*, 1983), can enhance delayed-type hypersensitivity (DTH) reactions in the guinea pig. It has also been shown that in mice, CsA can prevent high dose antigen-induced suppression of DTH reactions to herpes simplex virus (Altmann & Blyth, 1985), dinitrofluorobenzene (Braida & Knop, 1986), or sheep red blood cells (SRBC) (Webster & Thomson, 1987). These observations have been attributed to inhibition by CsA of the induction of (T) suppressor cells. Moreover, there is recent evidence that *in vitro*, CsA can permit induction of antigen-specific splenic T cells mediating DTH, whilst at the same time, inhibiting antibody production (Shidani, Motta & Truffa-Bachi, 1987)). In the present study, we have examined further the capacity of CsA, when administered before an otherwise tolerogenic dose of SRBC, to augment DTH reactions in the mouse. In particular, we wished to determine the influence of CsA on splenic regulatory T cell populations and the concomitant expression of humoral immunity.

Correspondence: Dr A. W. Thomson, Immunopathology Laboratory, Department of Pathology, University of Aberdeen, Foresthill, Aberdeen AB9 2ZD, Scotland, UK.

MATERIALS AND METHODS

Mice

Closed colony bred MF1 mice (8–12 weeks old) were used throughout. They were bred in the University Animal Department, Foresterhill, Aberdeen, maintained in a temperature-controlled environment and received Oxoid rat and mouse breeding diet with tap water *ad libitum*.

Cyclosporin A

Cyclosporin A (CsA; batch 83601, Sandoz Ltd, Basle, Switzerland) was obtained in powder form and dissolved in absolute ethanol, to which was added 4 parts olive oil (Boots PLC Ltd,

Nottingham, England). After thorough mixing, the CsA solution was administered (0.1 ml) either intraperitoneally (i.p.), subcutaneously (s.c.) or orally (p.o.) by gavage, using a fine gauge intravenous cannula (Portex Ltd., Hythe, UK).

Cyclosporin radioimmunoassay

Blood samples, obtained by cardiac puncture, were collected in capillary containers (Sarstedt) with EDTA (1 mg/ml) as anticoagulant. The samples were frozen immediately at −20°C, thawed and assayed for cyclosporin, using radioimmunoassay kits supplied by Sandoz Ltd, Basle, Switzerland and following the supplier's instructions.

Immunization

Sheep red blood cells (SRBC) obtained from blood in Alsever's solution (Difco Laboratories, West Molesey, Surrey) were washed three times in Dulbecco 'A' phosphate-buffered saline (PBS), pH 7.2. Mice received 10^9 SRBC in 0.2 ml intravenously (i.v.).

DTH assay

Mice were challenged 4 days after immunization with 10^8 SRBC in 0·05 ml PBS under the right hind footpad, the left footpad receiving PBS alone. Reactions were assessed 24 h later, by measuring the increase in dorsoventral thickness of the test over the control footpad, using a spring-gauge caliper (Schnelltaster, H. C. Kroeplin GMbH, Schuluechtern, FRG). All measurements were conducted by the same individual and results expressed as specific increases in footpad thickness (10^{-1} mm, mean ± 1 s.d.).

Preparation of spleen cell suspensions

Spleens were removed 4 days after immunization and placed in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Paisley, Renfrewshire, UK) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. The cell suspension, obtained by disaggregation of chopped tissue over 80-gauge stainless steel mesh, was washed twice by centrifugation (260 **g**, 10 min) in DMEM. A sample was diluted in 0·83% NH_4Cl (PH 7.2) with Trypan blue (0·2% w/v) and the total cell number estimated. Viability always exceeded 95%. For indirect immunofluorescence, the mononuclear cells were separated from the spleen cells suspension by spinning (350 ***g***, 20 min) over Lymphopaque, density 1·086 g/ml (Nyegaard & Co., Oslso, Norway). The mononuclear cells were harvested from the interface, washed ×2 and resuspended at 1×10^7/ml in RPMI-1640 + 10% fetal calf serum (FCS) (Gibco, Paisley, Scotland, UK).

Lymphocyte transformation

Spleen cell suspensions were prepared from individual animals as previously described (McIntosh *et al.*, 1986). The cells were resuspended at 4×10^6/ml in RPMI-1640 + 10% FCS and triplicate 0·2 ml cultures, containing 2×10^5 cells were set up in round-bottomed microculture plates (Nunclon, Denmark), alone or with phytohaemagglutinin (PHA-P; Wellcome, 5 μg/ml) or SRBC (2% v/v). The cultures were maintained for 72 h at 37°C in an atmosphere of 5% CO_2 in air and pulsed 16 h before harvesting with 0·2 μCi [^{3}H]thymidine (Radiochemicals, Amersham). Results are expressed as mean counts per minute (ct/min ± 1 s.d.).

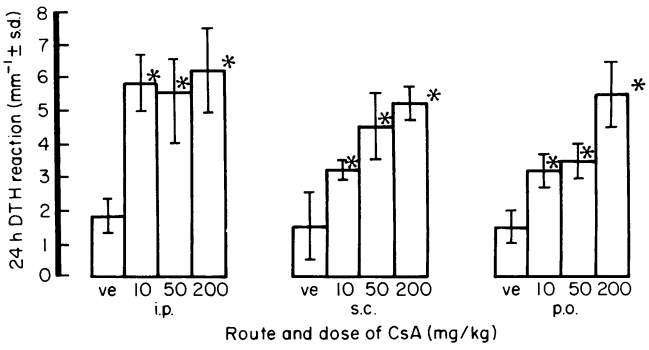

Fig. 1. Effect on DTH reactions of CsA (10, 50 or 200 mg/kg) given intraperitoneally (i.p.), subcutaneously (s.c.) or orally (p.o.) 48 h before immunization with 10^9 SRBC. *$P < 0.01$, significantly greater than vehicle (ve)-treated mice. Results are means ± s.d. obtained from groups of six mice.

Immunofluorescence staining for lymphocytes and macrophages

An indirect immunofluorescence method was employed to demonstrate cell phenotypes, using the appropriate primary rat IgG 2b anti-mouse monoclonal antibodies (anti-L3/T4 ($CD4^+$ helper/inducer T cells), anti-Lyt-2 ($CD8^+$ suppressor/cytotoxic T cells), anti-macrophage (M1/70·15) and rat IgG anti-mouse kappa (OX-20; B cells) (Sera-Lab, Crawley Down, Sussex). On receipt, each antibody was diluted 1:20 in PBS, containing 1% w/v bovine serum albumin (BSA; Sigma Chemical Company Ltd, Poole, Dorset, UK) and 0·1% w/v sodium azide, before storage in aliquots at −70°C. Immediately before use, the antibodies were further diluted, to 1:100, in 0·05 M Tris/HCl (pH 7·6), containing 5% v/v pooled normal rabbit serum (SAPU, Carluke, Lanarkshire, UK). The secondary antibody employed was FITC-conjugated rabbit IgG anti-rat IgG (H and L chains; Miles Scientific, Stoke Poges, Slough, UK), which was preincubated for 45 min at 4°C in Tris/HCl, containing 5% normal mouse serum.

Fifty microlitres of cell suspension (10^7 cells/ml) were mixed with an equal volume of primary antibody (final dilution 1:200) for 1 h at room temperature. After two washes in PBS, the cells were resuspended in 100 μl of secondary antibody (at a final dilution of 1:50) and incubated for 30 min at 4°C. After two further washes, the cells were suspended in 1 ml PBS and maintained on ice until analysis. Controls consisted of omission of the primary layer rat monoclonals (PBS control) and replacement of the primary antibodies by normal rat immunoglobulins.

Flow cytometry

The stained cells were analysed with an 'Epics C' flow cytometer (Coulter Electronics, Luton, England, UK). Lymphoid cells were 'gated/bitmapped' on the basis of their low forward angle (FLS) and 90° light scatter (LS) properties. Analyses were performed with linear mode and logarithmic amplification for FLS, 90° LS and green fluorescence. The fluorescence values of the controls (PBS and normal rat immunoglobulin-incubated cells, with the fluorescent conjugate) were subtracted from all samples by the setting of a 'cursor'. Positive results were those obtained to the right of the cursor. Ten thousand cells were analysed for each sample.

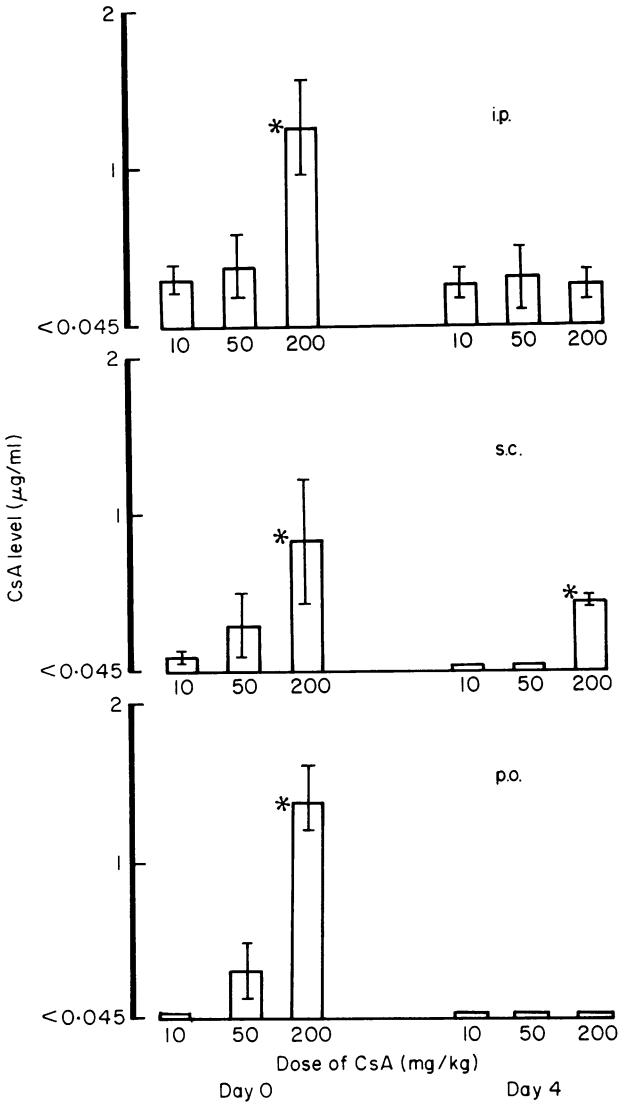

Fig. 2. Whole blood levels of CsA at the time of immunization (day 0) and antigen challenge (day 4). CsA (10, 50 or 200 mg/kg) was given i.p., s.c. or p.o. 48 h before immunization with 10^9 SRBC $*P<0{\cdot}01$, significantly greater than with CsA 50 or 10 mg/kg. Columns without error bars indicate that all values were below the sensitivity limit of the assay (45 ng/ml). Results are means ± s.d. obtained from groups of six mice.

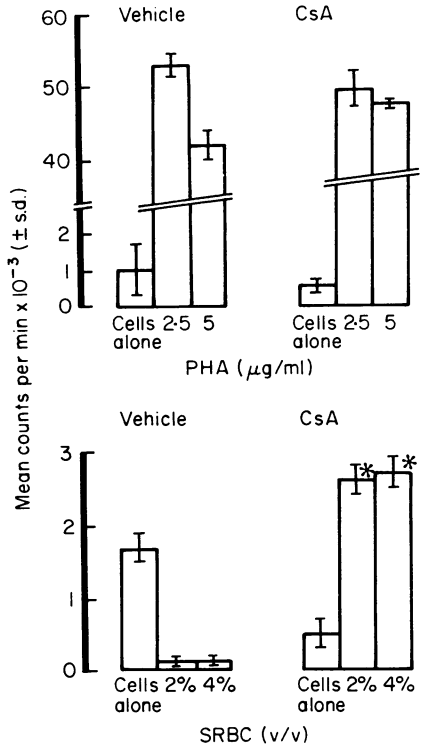

Fig. 3. Transformation of spleen cells from CsA-treated (200 mg/kg, day −2) mice immunized with 10^9 SRBC, in response to PHA or SRBC. Results are means ± s.d. obtained from groups of four mice. $*P<0{\cdot}01$, significantly greater than cells alone.

Assay of antibody-producing cells and circulating antibody

Spleen cell suspensions were prepared as described above and single, antibody (IgM)-producing plasma cells estimated by the plaque assay of Cunningham & Szenberg (1968). Plaques were counted at 40 times magnification, under dark field illumination, using a diamond-etched glass grid. Serum total haemagglutinin titres to SRBC were estimated on heat-inactivated samples, as described by Hudson & Hay (1980).

Statistics

The significance of differences between means was calculated using the Mann–Whitney U-test for DTH results, and Student's *t*-test for all other experiments.

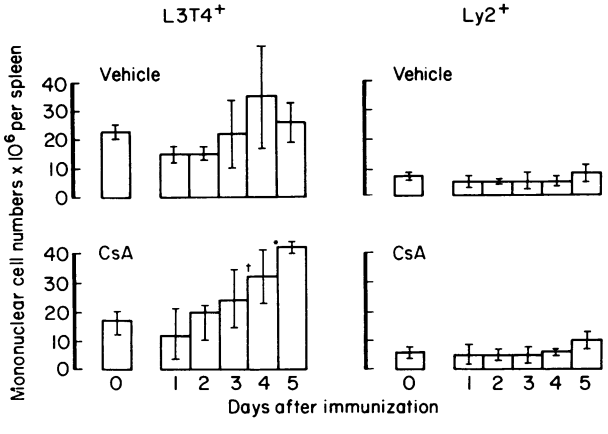

Fig. 4. Absolute L3T4$^+$ and Lyt-2$^+$ cell numbers in spleens of CsA-treated (200 mg/kg, day −2) mice at various times after immunization with 10^9 SRBC. Results are means ± s.d. obtained from groups of four mice. $*P<0{\cdot}01$, significantly higher than L3T4 day 0–3; $\dagger P<0{\cdot}01$, significantly higher than L3T4 day 0–2.

RESULTS

DTH reactions

Drug vehicle-treated animals immunized with 10^9 SRBC and challenged 96 h later, failed to exhibit DTH responses (mean footpad swelling $< 2\ mm^{-1}$). Administration of 200 mg/kg CsA by various routes, 48 h before immunization however, resulted in the expression of good footpad reactions (Fig. 1). These DTH responses were of similar intensity for all routes of drug administration. Lower CsA doses were as effective as 200 mg/kg when given by the intraperitoneal route, but a reduced capacity to prevent suppression of DTH was evident when either 10 or 50 mg/kg was given subcutaneously or orally. Histological examination of DTH reaction sites 24 h after antigen challenge, confirmed the presence of mononuclear cell infiltration related in intensity to that of footpad swelling.

Circulating drug levels

Circulating whole blood CsA levels at the time of immunization (day 0) were related to drug dosage (Fig. 2); concentrations were diminished at 4 days, although there was evidence of persistence of low drug levels in the group given CsA i.p. Notably, CsA levels just above (s.c. route) or below (p.o. route) the sensitivity limit of the assay (45 ng/ml) at the time of immunization, were associated with subsequent expression of moderate DTH reactions.

Lymphocyte transformation

Splenic lymphocytes from either drug vehicle or CsA (200 mg/kg i.p.)-pretreated mice exhibited good proliferative responses to PHA (Fig. 3). In contrast however, only CsA-treated mice showed significant responses to antigen; the corresponding values in vehicle controls were below those of unstimulated cells.

Phenotypic analysis of spleen cell populations

In vehicle control animals, no significant changes in the absolute numbers of $L3T4^+$ or $Lyt\text{-}2^+$ cells estimated by flow cytometry, were observed from days 0–4 following immunization, or on the day after antigen challenge (day 5) (Fig. 4). In CsA-treated mice, however, there was a significant increase (above day 0 values) in the number of $L3T4^+$ cells and a further elevation (to 200% of day 0 value) on day 5. No significant alteration in $Lyt\text{-}2^+$ cells was observed in this group. On day 5, the $L3T4^+ : Lyt\text{-}2^+$ ratio in CsA-treated animals was $6{\cdot}03 \pm 1{\cdot}29$ compared with $3{\cdot}59 \pm 0{\cdot}99$ ($P < 0{\cdot}01$) in vehicle controls. The absolute numbers of splenic B cells and macrophages were not significantly affected by CsA administration (Fig. 5).

Numbers of IgM-producing plasma cells and serum antibody titres

Vehicle pre-treated mice immunized with 10^9 SRBC gave good, 4-day splenic direct PFC (IgM) response (Table 1). In contrast, CsA (5–200 mg/kg) given i.p. 48 h before immunization, caused profound suppression of the humoral response; doses permitting expression of good systemic DTH reactions (Fig. 1) causing >90% suppression of antibody production. Serum anti-SRBC antibody levels on day 8 were also depressed by CsA.

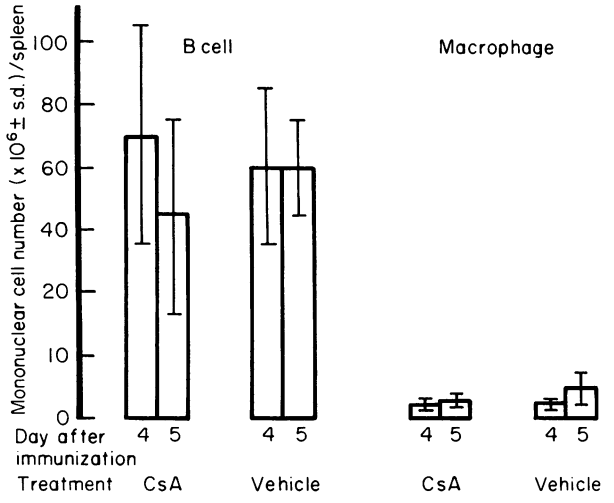

Fig. 5. Absolute B cell ($OX\text{-}20^+$) and macrophage numbers in spleens of CsA-treated mice (200 mg/kg, day −2) immunized with 10^9 SRBC. Results are means ± s.d. obtained from groups of four mice.

Table 1. Effect of CsA* on IgM-producing plasma cells and circulating antibody titres.

Treatment	PFC/spleen†	% reduction	Serum total haemagglutinin titre‡
Vehicle	90,232 ± 4,906	0	1:32
CsA (mg/kg) 5	7,710 ± 407§	91·5	1:8
CsA (mg/kg) 10	1,396 ± 816§	98·5	1:4
CsA (mg/kg) 200	0 ± 0	100	<1:2

* 200 mg/kg i.p., 48 h before immunization.

† Results are means ± 1 s.d. determined 4 days after immunization with 10^9 SRBC i.v.

‡ Antibody titres estimated 8 days after immunization. Results are means from at least three mice, with not more than one well difference between individual mice.

§ $P < 0{\cdot}01$ compared with vehicle controls.

DISCUSSION

In this study, we have confirmed our previous observation that CsA pretreatment of mice can prevent high dose SRBC-induced suppression of DTH (Webster & Thomson, 1987). Our findings are also in accord with recent reports that CsA can potentiate DTH reactions in mice to otherwise tolerogenic doses of herpes simplex virus (Altmann & Blyth, 1985), influenza virus (Schiltnecht & Ada, 1985) or dinitrofluorobenzene (Braida & Knop, 1986). In addition, however, we have shown that this effect of CsA on DTH can be achieved by various routes of drug administration in doses from 10–200 mg/kg. Good DTH reactions were observed with circulating CsA levels ranging below 45 ng/ml (i.e. within and below therapeutic drug levels) at the time of sensitization or antigen challenge. Moreover, the augmentation of DTH which we observed was associated with *in vitro* T_{DTH} responses to antigen, but also with concomitant, profound suppression of humoral immunity to SRBC.

It is well recognized that immunization with a high dose of SRBC causes suppression of antigen-specific DTH reactions in mice (Lagrange, Mackaness & Miller, 1974; Liew; 1977). This impairment of cell-mediated immunity has been attributed to retention within the spleen, of T_H cells involved in antibody production (Lagrange *et al.*, 1974; Kerckhaert, van der Berg & Willers, 1974), or to the induction of T_s cells specific for DTH (Liew, 1977).

Other immunosuppressive agents under well-defined experimental conditions, exhibit the capacity to augment cell-mediated immunity. The augmentory effect of cyclophosphamide (Cy) pretreatment on DTH reactions to high dose SRBC was attributed, initially, to a selective, cytotoxic effect of Cy on B cells, with consequent inhibition of antibody production (Lagrange *et al.*, 1974; Kerckhaert *et al.*, 1974). It was later shown however, that in this model, suppressor (T_s) cell mechanisms were especially sensitive to the drug (Askenase, Hayden & Gershon, 1975; Liew, 1977; Gill & Liew, 1978). With respect to CsA, there are now several reports that fully immunosuppressive levels of CsA can permit priming of T_{DTH} *in vivo* (Thomson *et al.*, 1983; Milon *et al.*, 1984; Altmann & Blyth, 1985; Schiltnecht & Ada, 1985; Aldridge & Thomson, 1986; Braida & Knop, 1986); these observations have been attributed to impairment by the drug of suppressor cell activities and there is evidence that, in mice, the immunoregulatory cells implicated may be T cells (Wick, Müller & Schwartz, 1982; Kaibara *et al.*, 1983; Braida & Knop, 1986; Webster & Thomson, 1987).

Ramshaw *et al.* (1977) and Liew *et al.* (1980) have reported that suppression of DTH in mice immunized with high dose xenogeneic erythrocytes is mediated by suppressor T cells of the Lyt-1^+2^- phenotype. In contrast, the T_s cells for antibody production to SRBC have been found to be Lyt-1^-2^+ (Cantor, Shen & Boyse, 1976; Liew *et al.*, 1980; Flood & Louie, 1984). Lyt-1 is now regarded, however, as a ubiquitous T cell marker and is also expressed on certain murine B lymphocytes (Shimamura *et al.*, 1984). The present investigation conducted using monoclonal antibodies directed specifically against T_H ($L3T4^+$) and T_s (Lyt-2^+) cells indicates that high dose SRBC immunization alone does not significantly affect the absolute numbers of either splenic $L3T4^+$ or Lyt-2^+ cells. CsA before SRBC, however, did increase the absolute number of $L3T4^+$ cells, whilst at the same time suppressing the splenic anti-SRBC humoral response in immunized mice. These data accord in part, with the recent report by Shidani *et al.* (1987) that CsA (1 μg/ml) totally inhibits primary *in vitro* anti-SRBC antibody production by murine spleen cells, whilst permitting the generation of anti-SRBC T_{DTH}. In this latter study, no effect of CsA on generation of T_s (Lyt-2^+) cells was observed.

The present observations add credence to the view that there are CsA-sensitive and CsA-resistant pathways of T lymphocyte activation. Due account must now be taken of the capacity of CsA, in different experimental models and using certain treatment protocols, to allow T_H cell priming and the expression of DTH, possibly by impairment of T_s cells for DTH, the phenotype of which is at present unclear. As shown in this study and that of Shidani *et al.* (1987), doses of CsA which are powerfully suppressive for antibody production can, nevertheless, permit generation of vigorous DTH reactions to the same antigen. Whether the suppression of antibody production by CsA in this study is due to sparing of the relevant T_s (presumably Lyt-2^+ cells), as is well recognized for the maintainance by CsA of donor-specific unresponsiveness to organ grafts, has not been ascertained. It is clearly important that further efforts be made to fully elucidate the action of CsA on regulatory T cell subsets in experimental models, including those of autoimmune disease, in which the more widespread clinical use of various CsA treatment protocols is being contemplated.

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