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Suppression of delayed-type hypersensitivity reactions and lymphokine production by cyclosporin A in the mouse

A.W. THOMSON, DEBORAH K. MOON & D.S. NELSON *Kolling Institute of Medical Research, The Royal North Shore Hospital of Sydney, St Leonards, New South Wales, Australia*

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SUMMARY

Two consecutive daily i.m. injections of cyclosporin A (Cs A) (>50 mg/kg) inhibited delayed type hypersensitivity (DTH) responses in mice immunized with SRBC. Maximal suppression was observed when Cs A was administered 24 and 48 h after sensitization. Culture of spleen cells from these animals with antigen, insoluble concanavalin A (iCon A) or PHA revealed inhibition of the production of two lymphokines: that inducing macrophage procoagulant activity (MPCA) and macrophage chemotactic factor (LDCF). The inhibitory effect on lymphokine production was not due to depletion of T cells. *In vitro*, 25 ng/ml Cs A suppressed T cell proliferative responses to antigen and mitogen but much higher doses were required to impair the response to LPS. Similar doses of Cs A also suppressed lymphokine production, but the responses of macrophages to these lymphokines was unaffected, even at doses which totally inhibited lymphokine production. Production of interleukin 1 by LPS stimulated macrophages was inhibited by Cs A only at concentrations much greater than those required to suppress lymphokine production.

INTRODUCTION

The novel immune suppressant cyclosporin A (Cs A) prolongs allograft survival in various species (Borel *et al.*, 1976; Calne *et al.*, 1978; Green & Allison, 1978; Tutschka *et al.*, 1979; Homan *et al.*, 1980; Du Toit *et al.*, 1982) and the potential value of Cs A in the clinical control of transplant rejection is under investigation in many centres (Calne *et al.*, 1981; Starzl *et al.*, 1981; Preliminary results of a European multicentre trial, 1982). Despite such observations as indefinite graft survival in rabbits (Green & Allison, 1978; Dunn *et al.*, 1979) and rats (Homan, Fabre & Morris, 1979) following short term Cs A treatment and evidence that Cs A selectively inhibits T cell activation and proliferation (Borel *et al.*, 1977; Burckhardt & Guggenheim, 1979; Gordon & Singer, 1979; Hess & Tutschka, 1980; Andrus & Lafferty, 1982), little is known of its effect on delayed-type hypersensitivity (DTH) reactions.

We have observed that, depending on the treatment regime, Cs A can either suppress or augment DTH reactions to soluble antigen in guinea-pigs (Thomson *et al*, 1983b). In this study, we have examined the influence of Cs A on DTH responses to SRBC in mice and on the production of lymphokines affecting the behaviour of macrophages.

MATERIALS AND METHODS

Animals. Female (C57BL/6J × A/J)F_1 (B6AF1) mice aged 8–12 weeks were provided by the Gore Hill Research Laboratories, Sydney.

Cyclosporin A. Cs A (OL 27-400; Sandoz Ltd., Basle, Switzerland) was dissolved and initially

Correspondence: Dr D.S. Nelson, Kolling Institute of Medical Research, The Royal North Shore Hospital of Sydney, St Leonards, New South Wales 2065, Australia.

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diluted in anhydrous ethanol and used as described elsewhere (Thomson *et al.*, 1983a, 1983b). The final concentration of ethanol in Cs A treated and control cultures was always 0·05%.

Delayed type hypersensitivity (DTH). Mice were injected i.v. with 10^6 washed SRBC in saline and challenged 4 days later under the right hind footpad with $1{\cdot}25 \times 10^8$ SRBC, reactions being assessed by measuring the increase in thickness with a Schnelltaster dial gauge (Nelson & Nelson, 1978).

Spleen cell suspensions. Cells released by teasing were passed through stainless steel gauze, washed twice in Dulbecco's modified Eagle's medium (DME; GIBCO) and suspended in RPMI 1640 containing 60 μg/ml penicillin, 100 μg/ml streptomycin and 2 g/l $NaHCO_3$.

Estimation of T cells. Spleen cells (10^7/ml) were incubated with 1:10 anti-Thy 1.2 alloantiserum (Cedarlane Laboratories, Ontario, Canada) in DME for 60 min at 4°C, washed and resuspended at the same concentration in 1:10 low toxicity rabbit complement (Low-Tox-M; Cedarlane) for 60 min at 37°C. After further washing the remaining viable (non-T) cells were determined by trypan blue exclusion.

PU5 cells. The mouse macrophage cell line PU5 was cultured in DME supplemented with 10% fetal calf serum (FCS; Flow Laboratories) and 0·01 mg/ml asparagine in 75 cm^2 tissue culture flasks (Corning, New York). The cells were detached by agitation and passaged every 2–3 days.

Peritoneal exudate cells (PEC). These were obtained 3 days after intraperitoneal (i.p.) injection of 2 ml sterile thioglycollate broth, pH 6·1 (TG; BBL Microbiology Systems, Cockeysville, Maryland, USA). The peritoneal cavities were lavaged with 6 ml DME containing 10 u/ml preservative free heparin (Commonwealth Serum Laboratories, Melbourne) and the cells washed three times in heparin free DME.

Interleukin 1 (lymphocyte activating factor). IL1 production by adherent resident peritoneal cells in response to *E. coli* lipopolysaccharide (LPS-W; DIFCO) was assayed with mouse thymocytes as previously described (Thomson *et al.*, 1983a).

Solubilized SRBC (sSRBC). Solubilized SRBC (sSRBC) for use in lymphocyte transformation and induction of lymphokine production was prepared according to Waterston (1970) and stored in aliquots at −20°C. It was used in cell cultures at a final concentration of 5%.

Lymphocyte transformation. Triplicate cultures of spleen cells (2×10^6 viable cells/ml) in RPMI 1640 containing 2·5% FCS and various concentrations of PHA (PHA-P; DIFCO), concanavalin A (Con A; Sigma), *E. coli* LPS-W or sSRBC were set up in 96 well round bottomed microculture plates (Nunclon, Denmark). They were maintained for 48 h at 37°C in a humidified atmosphere of 5% CO_2 in air and pulsed 8 h before harvesting with 0·3 μCi ^{3}H-thymidine (6·7 Ci/m mole; New England Nuclear, Boston, Massachusetts, USA). The cultures were harvested with a multiple harvester (Skatron AS, Norway) and counted, in a Packard liquid scintillation counter. Results were expressed as mean counts per minute (ct/min) ± 1 s.d.

Lymphokine production. Lymphokine rich culture supernatants were produced by incubating spleen cells in Linbro multiwell plates (5 or 10×10^6 viable cells/ml RPMI 1640) for 24 h with 50 g/ml Sepharose bound concanavalin A (iCon A; Pharmacia, Uppsala, Sweden) or for 48 h with either 100 g/ml PHA-P or sSRBC. The 48 h cultures were supplemented with FCS (PHA, 10%; sSRBC, 5%). Control supernatants were prepared by adding the appropriate stimulant immediately before harvesting. After centrifugation, the supernatants were aliquoted and stored at −70°C.

Assay of macrophage procoagulant activity (MPCA). Washed PU5 cells ($6{\cdot}7 \times 10^5$) were incubated in a total volume of 0·5 ml RPMI 1640 containing 10% FCS in Nunc minisorb tubes (Nunc, Roskilde, Denmark) with various concentrations of lymphokine. Following 16–20 h incubation at 37°C the cells were washed twice in Hank's balanced salt solution (HBSS), and resuspended in 0·6 ml RPMI 1640. Their ability to shorten the recalcification time (RT) of normal citrated platelet poor mouse plasma (centrifuged at 12,000*g* for 20 min) was determined as described elsewhere for human and mouse systems (Geczy & Meyer, 1982; Geczy, 1983). To 0·2 ml cell suspension was added 0·1 ml plasma and 0·1 ml 0·03 M Ca Cl_2 in 0·9% saline. The clotting times were measured in duplicate, using an automatic coagulometer (Calbiochem, according to Schnitger and Gross, Amelung GmbH, Lemgo, Germany). The results were expressed as % reduction in RT. A decrease in RT of 10% was considered significant, since control supernatants, to which antigen or mitogen was added at the end of the culture period, always gave values below this figure.

Assay of lymphocyte derived chemotactic factor (LDCF). Chemotaxis by mononuclear TG-PEC was assayed in a 48 well micro-chemotaxis chamber (Neuroprobe, Bethesda, Mareyland) (Falk, Goodwin & Leonard, 1980) as described in detail elsewhere (Farram *et al.*, 1982).

Statistics. Student's *t*-test was used.

RESULTS

Treatment in vivo

Administration of Cs A 24 and 48 h after immunization resulted in delayed and dose related suppression of DTH to SRBC (Fig. 1). The delaying effect of the drug was the same for all doses and was most clearly seen in animals given 50 mg/kg. Cs A was also given at different times in relation to immunization. Two successive daily injections of 100 mg/kg starting from day 0, 1, 2 or 3 severely depressed DTH. Depression was less marked when the first of two such doses was withheld until the time of skin testing (day 4). A single injection of 100 mg/kg on day 0 did not inhibit DTH.

Spleen cells removed at 4 days from mice given Cs A 24 and 48 h after immunization showed depressed lymphokine production in response to sSRBC and mitogen. The dose related inhibitory effects of Cs A treatment on production of the lymphokine inducing MPCA and LDCF are shown in Fig. 2 and Table 1. Treatment with 100 mg/kg Cs A substantially inhibited both the generation of MPCA in response to antigen (Fig. 2a) and PHA-induced LDCF production, and almost totally inhibited MPCA production in 24 h iCon A cultures (Fig. 2b).

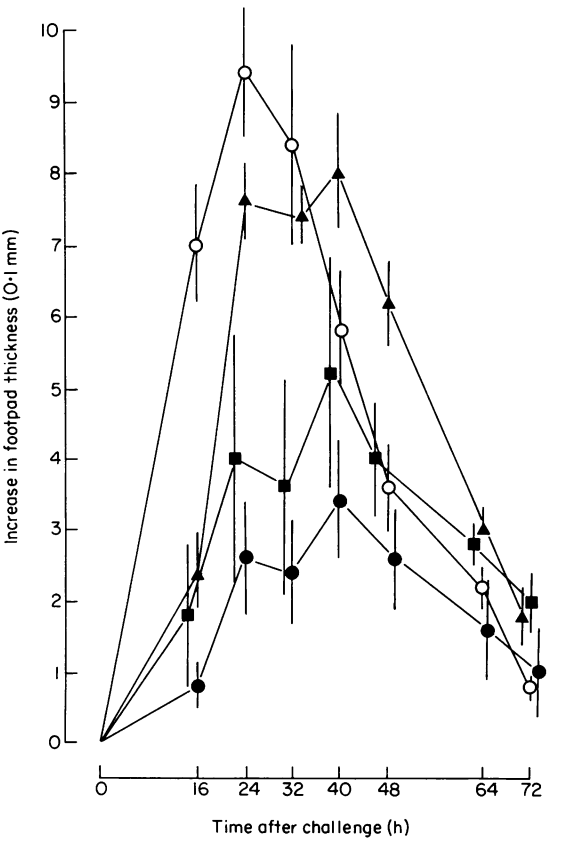

Fig. 1. Effect of Cs A administered 24 and 48 h after immunization on DTH responses to SRBC elicited on day 4 in B6AF1 mice. Results are means ± 1 s.e. obtained from groups of five animals. (○) = vehicle-treated controls; (▲) = 50 mg/kg CsA; (■) = 75 mg/kg CsA; (●) = 100 mg/kg CsA.

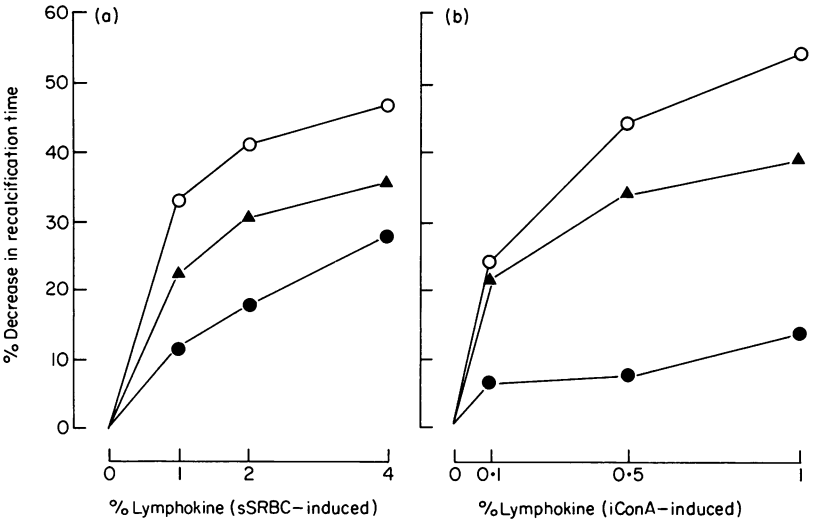

Fig. 2. Procoagulant activity of PU5 cells induced by incubation in lymphokine rich supernatants from (a) sSRBC and (b) iCon A stimulated spleen cells. The spleen cells were derived from groups of three mice immunized with SRBC and treated with either (○) vehicle, (▲) 50 mg/kg Cs A or (●) 100 mg/kg Cs A. The decrease in RT obtained using unstimulated culture supernatants to which sSRBC or iCon A was added at the end of the culture period was always < 10%.

Histologically, spleens from normal and SRBC-immunized mice treated with 100 mg/kg Cs A showed no abnormalities attributable to the drug. Spleen weights, spleen cell numbers and the incidence of Thy 1.2 positive cells were similar in all groups (data not shown).

Treatment in vitro

The inhibitory effect of Cs A on sSRBC and PHA-induced transformation of spleen cells from SRBC immunized mice is shown in Fig. 3. Significant impairment was achieved with 25 ng/ml Cs A. On the other hand, LPS responses were inhibited only at relatively high Cs A concentrations (> 500 ng/ml; $P < 0{\cdot}001$) and then only by about 50%. Inhibition of iCon A-induced lymphokine production, estimated using the MPCA assay, was observed over a similar range of Cs A concentrations to that which depressed lymphocyte transformation (Fig. 4a). In contrast, overnight incubation of the PU5 indicator cells with Cs A, followed by washing, did not affect their basal procoagulant activity or their ability to respond to preformed lymphokine (Fig. 4b). The findings

Table 1. LDCF production by spleen cells from Cs A treated mice

Treatment	Supernatant	Number of macrophages migrating (mean ± 1 s.e.)
Vehicle	Control*	27 ± 1
	LDCF	132 ± 17
CsA (50 mg/kg)	Control	30 ± 3
	LDCF	106 ± 7†
CsA (100 mg/kg)	Control	19 ± 2
	LDCF	54 ± 3‡

* Supernatant to which PHA was added at the end of the culture period. Significance of difference from vehicle treated group: † $P < 0{\cdot}05$; ‡ $P < 0{\cdot}001$.

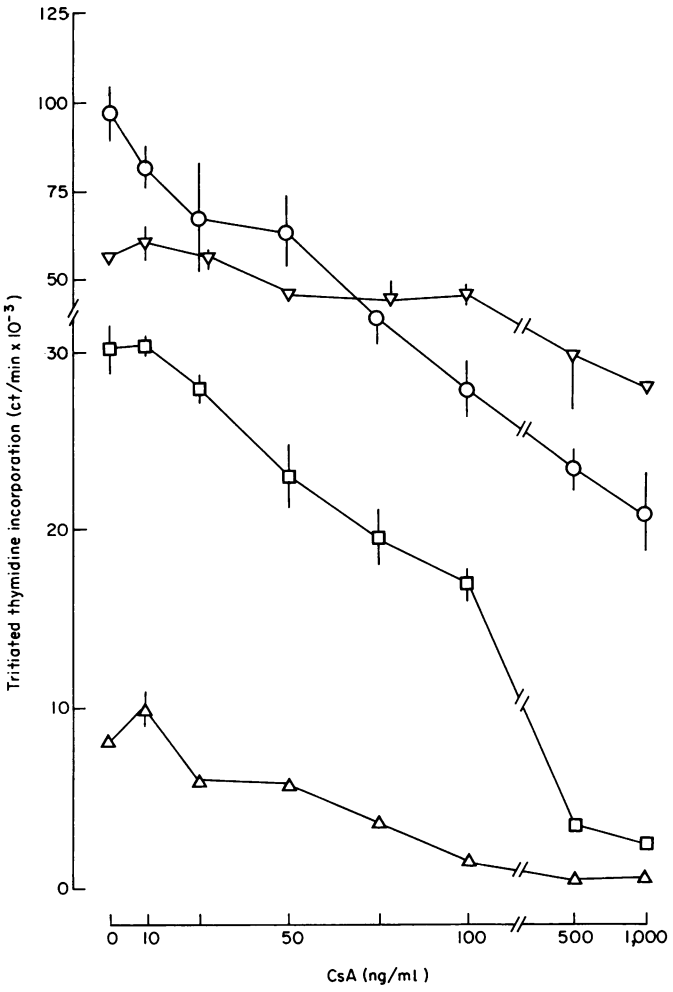

Fig. 3. Mitogenic response of sensitized mouse spleen cells to 5% sSRBC (□), 10 μg/ml PHA (○) and 50 μg/ml LPS (▽) in the presence of various concentrations of Cs A. Thymidine incorporation by unstimulated cells (△) is also shown. Results are means ± 1 s.d.

with LDCF were similar. Although LDCF production by PHA stimulated normal spleen cells was inhibited by Cs A, the drug did not affect the spontaneous migration of peritoneal macrophages or their ability to respond to preformed LDCF. This was apparent whether the drug was initially present in the upper or lower wells of the chemotaxis chamber (data not shown).

Production of LAF by LPS stimulated peritoneal macrophages was inhibited only by concentrations of Cs A 50 times greater than that (100 ng/ml) which severely depressed T cell transformation and lymphokine production (Table 2).

DISCUSSION

We have demonstrated the inhibitory effect of Cs A on the induction and expression of DTH to SRBC, an observation in keeping with previous reports of depressed humoral immune responses to this antigen (Borel *et al.*, 1976, 1977). The treatment regime adopted (two consecutive daily injections of Cs A) is similar to that used by Borel *et al.* (1977) to suppress contact sensitivity to

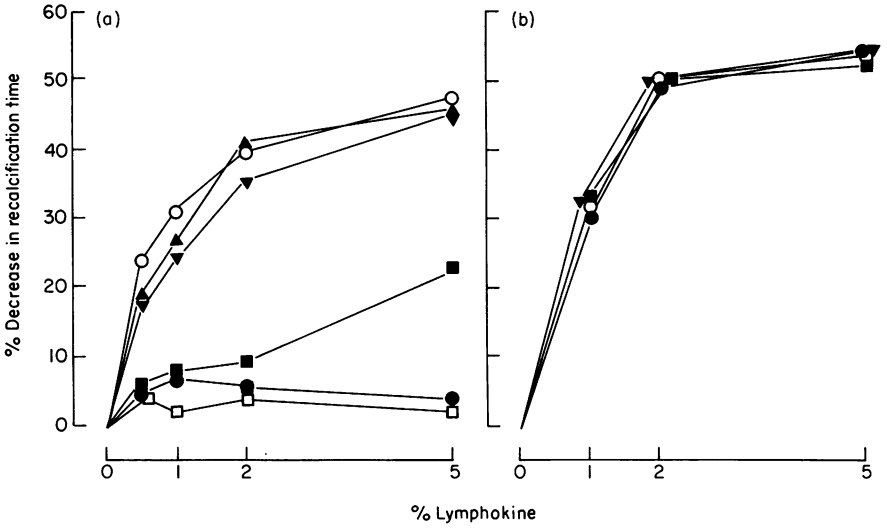

Fig. 4. (a) Procoagulant activity of PU5 cells induced by incubation with iCon A-induced lymphokine, produced in the presence of different concentrations of Cs A. (○) = iCon A supernatant without Cs A, (▲) = 10 ng/ml, (▽) = 50 ng/ml, (■) = 100 ng/ml, (●) = 1000 ng/ml. The effect of control supernatant to which iCon A was added at the end of the culture period is also shown (□). (b) Procoagulant activity of PU5 cells induced by incubation with preformed iCon A-induced lymphokine in the presence of Cs A. (○) = iCon A supernatants without Cs A, (▼) = 50 ng/ml, (■) = 100 ng/ml, (●) = 1000 ng/ml.

Table 2. Effect of Cs A on LPS-induced LAF* production by peritoneal macrophages

Cs A (ng/ml)	^{3}H-TdR incorporation (ct/min × 10^{-3})	Significance
Control	4·1 ± 1·1	—
5,000	1·5 ± 0·5	$P < 0{\cdot}005$
1,000	2·6 ± 1·1	NS
100	3·8 ± 1·4	NS
10	4·1 ± 1·8	NS

Results are means ± 1 s.e. obtained from triplicate cultures in three separate experiments.

* The activity of LAF containing supernatants was determined at a dilution of 15%.

oxazalone in the mouse. In guinea-pigs Cs A caused marked impairment of DTH responses to tuberculin (Borel *et al.*, 1977) and ovalbumin (Thomson *et al.*, 1983b) when administered either throughout the interval between sensitization and skin testing, or solely at the time of skin testing. Paradoxically, DTH in guinea-pigs was augmented when Cs A was given only for the first 4 days after immunization (Thomson *et al.*, 1983b). Cs A also inhibited other experimental cellular immune responses, such as GVH reactivity (Markwick *et al.*, 1979; Tutschka *et al.*, 1979), EAE (Borel *et al.*, 1976; Bolton, Allsopp & Cuzner, 1982) and experimental allergic uveitis (Nussenblatt *et al.*, 1981) in the rat. The drug, when administered to recipients, inhibited the adoptive spleen cell transfer of EAE (Bolton *et al.*, 1982).

Cs A-mediated impairment of DTH and T cell proliferative responses *in vitro* was associated

with marked reductions in lymphokine production. This was not due to a selective reduction in T cells. Production of IL1 by resident peritoneal macrophages was inhibited only by much greater concentrations of Cs A than that required to impair T cell transformation and lymphokine production. Thus reduced availability of IL1 does not account for these effects. Andrus & Lafferty (1982), however, found that Cs A depressed LPS-induced IL1 production by PU5 cells.

Inhibition of lymphokine production seems to be a key factor in the mode of action of Cs A. Others found that Cs A inhibited production of T cell growth factor by alloantigen-activated T cells of mouse (Bunjes *et al.*, 1981; Andrus & Lafferty, 1982) or human (Palacios & Möller, 1981; Hess, Tutschka & Santos, 1982). We have now shown that production of lymphokines affecting macrophage behaviour is also inhibited. The findings with guinea-pigs were similar (Thomson *et al.*, 1983a). There is also evidence that Cs A inhibits release of macrophage activating factor in the mouse (Alberti *et al.*, 1981). The exact mechanism whereby Cs A inhibits T cell activation has yet to be clearly defined, but may centre around the blocking of antigen specific receptors and acquisition of responsiveness to interleukins (Britton & Palacios, 1982; Andrus & Lafferty, 1982).

Suppression of DTH by Cs A thus seems to be effected by reduction in the availability of factors essential not only for the maintenance of T cell proliferation, but also for the recruitment and activation of macrophages, including stimulation of their procoagulant activity. Indeed, activation of the coagulation system plays an integral role in DTH reactions (Nelson, 1965; Colvin *et al.*, 1973) and is correlated both with fibrin deposition on the surfaces of macrophages and with inhibition of macrophage migration (Hopper, Geczy & Davies, 1981). In contrast, in this and other studies Cs A did not directly affect macrophage activities, such as phagocytosis (McIntosh & Thomson, 1980; Paavonen *et al.*, 1981), spontaneous migration (White *et al.*, 1979) or chemotaxis (Thomson *et al.*, 1983a).

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