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Modification of delayed-type hypersensitivity reactions to ovalbumin in cyclosporin A-treated guinea-pigs

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Summary. Cyclosporin A (Cs A) administered daily (25 mg/kg *per os*) to outbred guinea-pigs for 2 weeks following immunization with ovalbumin (OVA; CsA 0–13) caused profound suppression of 14-day delayed-type hypersensitivity (DTH) skin reactions. Very marked impairment of DTH was also found when Cs A was given for the first time 24 hr before skin testing and at 6 and 24 hr thereafter. In contrast, Cs A given on days 0–4 following OVA immunization (Cs A 0–4) caused dose-related potentiation of 14-day skin responses.

These changes in the magnitude and character of DTH *in vivo* were accompanied by striking alterations in lymphocyte transformation responses and in the extent of macrophage migration inhibition and lymphokine production. Whereas Cs A (0–13) caused almost total suppression of the mitogenic responses of lymph node cells to PHA and antigen, OVA-induced migration inhibition and production of the lymphokine inducing macrophage procoagulant activity (MPCA), Cs A (0–4) augmented these responses to OVA, but did not affect lymphocyte transformation or lymphokine production in response to mitogen.

Strain 13 guinea-pigs treated with Cs A (0–4) showed depressed Arthus, but augmented DTH responses to OVA. This significant increase in cell-mediated immunity could be passively transferred using spleen and peritoneal exudate cells, suggesting that under these circumstances Cs A (0–4) may interfere with the generation of a population of suppressor cells which regulate DTH reactions in the guinea-pig.

Abbreviations: Cs A, cyclosporin A; Cs A (0–4), cyclosporin A administered on days 0–4 after immunization; Cs A (0–13) cyclosporin A administered on days 0–13 following immunization; DTH, delayed-type hypersensitivity; iCon A, insoluble concanavalin A; LNC, lymph node cells; MPCA, macrophage procoagulant activity; OVA, ovalbumin; PEC, peritoneal exudate cells; p.o., *per os*; PHA, phytohaemagglutinin; TG, thioglycollate.

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INTRODUCTION

Much of the current interest in cyclosporin A (Cs A) as a prospectively important immunotherapeutic agent is founded on its ability to prolong allograft survival in laboratory animals (Borel, Feurer, Gubler & Stähelin, 1976; Green & Allison, 1978; Homan, Fabre, Williams, Millard & Morris, 1980; Jamieson, Burton, Bieber, Reitz, Oyer, Stinson & Shumway, 1979). Since delayed-type hypersensitivity (DTH) reactions play an important role in the mediation of graft rejection, it would seem important that the influence of Cs A on mechanisms underlying the generation of DTH be studied extensively both *in vivo* and *in vitro*.

Although Borel, Feurer, Magnée & Stähelin (1977) described impaired DTH and contact sensitivity reactions in Cs A-treated animals, there has been no subsequent attempt to analyse further these drug-induced immunological changes. In view of the inhibitory effect of Cs A on T-cell proliferation (Borel & Wiesinger, 1977; Burckhardt & Guggenheim, 1979; Gordon & Singer, 1979; Palacios, 1981), its effects on lymphokine production (Thomson, Moon, Geczy & Nelson, 1982) and the importance of different T-cell subpopulations in the generation and control of cell-mediated immune responses (Silver & Benacerraf, 1974; Gershon, 1974; Janeway, Sharrow & Simpson, 1975; Huber, Devinsky, Gershon & Cantor, 1976; Asherson & Zembala, 1976; Turk, 1980) we have embarked on a study of DTH to ovalbumin (OVA) in Cs A-treated guinea-pigs. During the course of this investigation, it has become evident that, depending on the treatment regime, Cs A may depress or augment DTH responses.

MATERIALS AND METHODS

Animals

Outbred and strain 13 guinea-pigs originally obtained from the National Institutes of Health, Bethesda, Md. were used. They were bred in the Royal North Shore Hospital Animal House, received pelleted diet, supplemented with green vegetables and hay and weighed 400–600 g.

Cyclosporin A

Cyclosporin A (Cs A; OL 27-400) provided by Dr J. F. Borel (Sandoz Ltd, Basel, Switzerland) was dissolved initially in anhydrous ethanol. A 10% solution of the Cs A-containing ethanol in olive oil was then prepared and 0·3 ml/400 g administered to the conscious guinea-pig by gastric intubation using an intravenous cannula. Vehicle controls received 10% ethanol in olive oil.

Immunization

Animals were immunized with five times crystallized ovalbumin (OVA, Sigma Chemical Company) dissolved in sterile physiological saline (2 mg/ml) and emulsified with an equal volume of complete Freund's adjuvant (Commonwealth Serum Laboratories, Parkville, Victoria). Each footpad was injected with 0·2 ml.

Skin tests

Skin tests were performed on the shaved flank by intradermal injection of 100, 50, 10 or 1 μg OVA dissolved in 0·1 ml of saline. Reactions were assessed at intervals by measuring the increase in skin-fold thickness with a 'Schnelltaster' dial gauge (System Kröplin, Germany). The average thickness of the skin-fold on both sides of the reaction was subtracted from the skin-fold thickness at the test site. The extent of erythema (mean of two diameters) was also recorded.

Histology

Skin reaction sites were excised and fixed for 24 hr in Bouin's fluid. Paraffin sections were cut at 5 μm and stained with haematoxylin and eosin.

Lymphocyte transformation and macrophage migration inhibition tests

The mitogenic responses of lymph node cells to PHA-P (Difco) and OVA and the extent of antigen-induced macrophage migration inhibition observed using oil-induced peritoneal exudate cells (PEC) were determined using the methods described in the accompanying paper (Thomson *et al.*, 1982).

Production of lymphokine

Lymphokine-rich supernatants were prepared by stimulating sensitized lymph node cells (LNC; 10^7 viable cells/ml) with 50 μg/ml Sepharose-bound concanavalin A (iCon A, Pharmacia, Uppsala, Sweden) for 24 hr in serum-free RPMI 1640 (Gibco, Grand Island, New York) containing 60 μg/ml penicillin, 100 μg/ml streptomycin, 2 g/l $NaHCO_3$ and 5×10^{-5}M 2-mercaptoethanol. Cultures were also set up with 50 μg/ml OVA for 48 hr in the presence of $2{\cdot}5 \times 10^5$/ml viable autologous PEC and 5% foetal calf serum (Flow Laboratories). Control supernatants were prepared by adding the iCon A or OVA immediately before harvesting. All cultures were maintained at 37° in 5% CO_2 in air. Supernatants were stored at −70° until assayed.

Assay of macrophage procoagulant activity (MPCA)

The ability of iCon A and OVA-induced lymphokine supernatants to promote the procoagulant activity of thioglycollate (TG)-induced PEC from normal guinea-pigs was determined using the method of Geczy & Hopper (1981), as described in the preceding paper (Thomson *et al.*, 1982).

Cell transfer

Passive transfer of DTH in strain 13 guinea-pigs was achieved by intracardiac injection of 10^8 viable spleen cells and 10^8 viable PEC in 2 ml of Hanks's balanced salt solution. The cells were pooled from donors immunized with OVA 12 days before. Skin tests were performed immediately after cell transfer.

Statistics

Student's *t* test was used to determine the significance of differences between means. Values of $P < 0{\cdot}05$ were considered significant.

RESULTS

As may be seen in Fig. 1a, daily administration of 25 mg/kg Cs A *per os* (p.o., Cs A 0–13) to OVA-immunized outbred guinea-pigs resulted in profound suppression of both antibody-dependent Arthus reactivity and delayed hypersensitivity responses evoked by skin testing at 14 days. Marked, but less pronounced suppression of skin reactions was also observed in animals which received Cs A for the first time only 1 day before challenge and at 6 and 24 hr after skin testing.

In contrast, animals which received 10 or 25 mg/kg p.o. on days 0–4 (Cs A 0–4) following immunization showed dose-related augmentation of DTH responses to OVA (Fig. 1b). The potentiating effect of the drug was apparent at 24 hr and more clearly evident in the higher dose group at 48 hr, at which time the skin reactions were about 150% more intense than those in vehicle-treated controls. An additional feature was that skin responses in the animals given 25 mg/kg Cs A (0–4) were often haemorrhagic. This degree of reaction intensity was not observed in any animals which had not received Cs A.

The potentiating effect of both doses of Cs A (0–4) on the degree of induration at the test site 24 hr after OVA injection was evident over a range of antigen concentrations (Fig. 2); this was accompanied by increased erythema in the high dose group, but the lower dose of drug (10 mg/kg) did not consistently increase the accompanying erythematous reactions. Histological examination of skin test sites at 48 hr confirmed the presence of an increased perivascular mononuclear cell infiltrate in the Cs A (0–4) treated animals. This was particularly pronounced at 25 mg/kg.

Table 1 shows the mitogenic responses of lymph node lymphocytes from vehicle and Cs A-treated animals (25 mg/kg) to both PHA and OVA. As anticipated from the results of the skin tests, lymphocyte transformation in the Cs A (0–13) group was almost totally suppressed in response to PHA and was

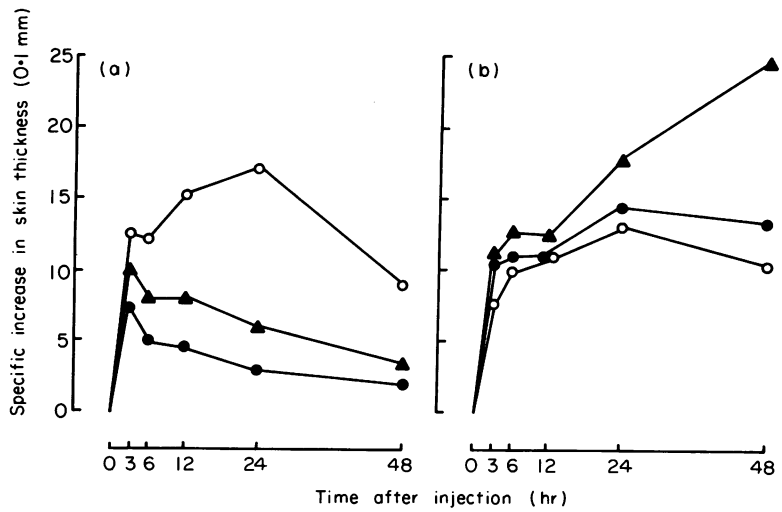

Figure 1. Effects of Cs A on 14-day DTH skin responses to 10 μg OVA in outbred guinea-pigs. (a) Suppression of induration: (○) vehicle, days 0–13; (●) 25 mg/kg Cs A, days 0–13; (▲) 25 mg/kg Cs A −24, +6 and +24 hr relative to skin test. (b) Potentiation of induration; (○) vehicle, days 0–4; (●) 10 mg/kg Cs A, days 0–4; (▲) 25 mg/kg Cs A, days 0–4. Results are means obtained from groups of eight guinea-pigs.

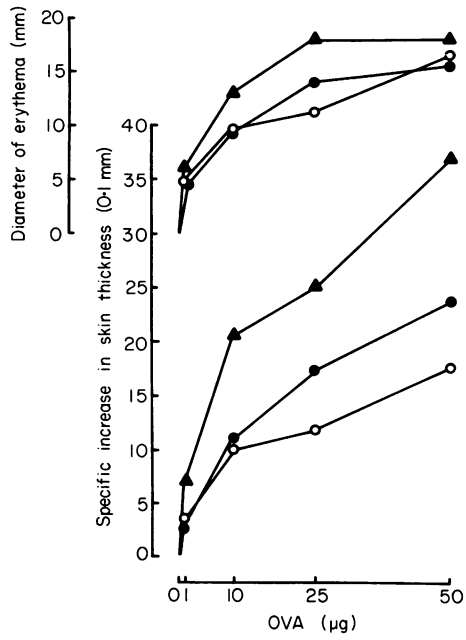

Figure 2. Dose-related potentiation by Cs A of skin reactions to various concentrations of OVA. (○) Vehicle, days 0–4; (●) 10 mg/kg Cs A days 0–4; (▲) 25 mg/kg days 0–4. Results are means obtained from groups of eight guinea-pigs.

completely inhibited in response to OVA. In the Cs A (0–4) group, no significant effect on PHA-induced transformation in comparison to vehicle-treated controls was found. However, in keeping with the increased skin reactions observed in this group, there was a marked increase in the mitogenic response to OVA, particularly at the higher dose of 10 μg, where a highly significant three-fold increase in stimulation index was observed.

In order to determine whether the changes in lymphocyte responses described above were accompanied by alterations in any other *in vitro* measure of cell-mediated immunity, the macrophage migration inhibition test was performed on PEC from animals in each group. A wide range of antigen concentrations was used in order to discriminate carefully between the groups and it is clear from the results (Table 2) that migration inhibition was more marked in the Cs A (0–4) group at all doses of OVA. It was particularly striking that cells from these animals were sensitive to lower antigen concentrations than PEC from vehicle-treated controls. In contrast, no significant macrophage migration inhibition in response to OVA was observed in the Cs A (0–13) group.

Determination of lymphokine activity in supernatants from iCon A and OVA-stimulated LNC from each group gave results which reflected the foregoing increases in lymphocyte transformation and macrophage migration inhibition in response to antigen. Although no significant increase in production by LNC of the lymphokine promoting MPCA in response to iCon A was observed in the Cs A (0–4) group, there was a significant increase (at 10% lymphokine) in the response to OVA (Table 3). In the Cs A (0–13) group, no lymphokine activity in response to either mitogen or antigen could be detected using this sensitive assay.

To ascertain whether increased DTH responses could be passively transferred with cells from the Cs A (0–4) group, strain 13 guinea-pigs were immunized

Table 1. Effect of Cs A treatment on lymphocyte transformation 14 days after immunization with OVA

	Vehicle control		Cs A (days 0–4)*		Cs A (days 0–13)	
	c.p.m. $\times 10^{-3}$	SI	c.p.m. $\times 10^{-3}$	SI	c.p.m. $\times 10^{-3}$	SI
No stimulant	2·17±0·53†	—	2·32±0·43	—	1·57±0·50	—
PHA (1 μg)	78·97±21·58	34·7±2·9	57·85±17·94	23·2±3·3	3·87±0·19	4·4±1·5§
PHA (0·25 μg)	10·55±4·23	4·0±0·9	8·51±6·19	3·3±0·5	1·32±0·39	0·9±0·1§
OVA (10 μg)	9·16±2·49	4·2±0·6	30·45±7·71	13·0±1·9§	0·86±0·17	0·7±0·1§
OVA (0·1 μg)	4·43±1·35	2·0±0·4	8·00±0·90	4·7±1·3‡	1·37±0·25	1·1±0·2‡

* Cs A given p.o. (25 mg/kg).

† Results are means ±1 SE obtained from groups of four guinea-pigs.

Statistically significant enhancement/suppression of the control response: (‡) $P<0·01$; (§) $P<0·001$.

Table 2. Effect of Cs A treatment on OVA-induced macrophage migration inhibition 14 days after immunization

Treatment	OVA (μg/ml)	Migration*: animal no. 1	2	3	Mean migration inhibition (%)
Vehicle control	0	17·2±4·0	17·6±0·9	24·6±2·1	—
	10	5·7±0·8	9·1±0·8	15·6±2·2	51
	1	9·9±1·1	14·1±1·1	19·5±1·5	28
	0·3	16·2±2·5	16·7±0·9	19·5±0·7	11
	0·1	15·5±1·3	16·5±1·7	21·7±1·8	9
	0·03	17·3±0·2	16·9±1·5	22·5±1·2	2
Cs A† (days 0–4)	0	37·9±1·2	16·6±1·5	26·2±2·1	—
	10	12·8±1·2	4·2±0·4	8·8±1·7	69
	1	29·1±3·8	9·0±0·9	14·0±2·7	39
	0·3	33·5±1·8	11·4±1·2	13·0±1·5	31
	0·1	35·8±2·3	14·4±1·9	19·7±2·8	15
	0·03	39·6±1·6	13·4±2·2	19·5±2·1	13
Cs A (days 0–13)	0	18·9±0·7	16·9±1·0	17·4±2·2	—
	10	13·7±1·5	16·6±2·3	18·6±2·4	8
	1	20·0±1·4	18·2±2·0	19·2±2·9	−10

* Results are expressed as the mean weight (mg ±1 SE) of the projected areas of the migrating cells.
† Cs A given p.o. (25 mg/kg).

Table 3. Effect of Cs A treatment on iCon A and OVA-induced production of the lymphokine inducing MPCA

Treatment	iCon A (% lymphokine) Control*	4	10	OVA (% lymphokine) Control*	4	10
Vehicle control	4·8±2·2†	15·7±4·0	22·5±6·6	9·3±1·7	13·5±5·1	16·6±5·1
Cs A (days 0–4)‡	5·5±4·3	13·2±5·4	20·5±9·9	11·9±3·3	16·2±5·4	26·0±4·1§
Cs A (days 0–13)	4·3±4·1	6·6±4·0§	6·0±2·7§	11·3±1·0	7·0±4·6	8·4±5·4§

* Dilution (10%) of culture supernatant from unstimulated cells.
† Results are mean (±1 SD) percentage reductions in the recalcification time of guinea-pig plasma in the presence of TG-induced PEC incubated for 18 hr with iCon A or OVA-stimulated LNC culture supernatants. Four guinea-pigs per group.
‡ Cs A given p.o. (25 mg/kg).
§ Statistically significant enhancement/suppression of the vehicle control response: $P<0·01$.

with OVA and treated with Cs A or vehicle as described for outbreds. In the Cs A group, skin testing resulted in a significant depression of the Arthus-like reaction (Table 4) and although there was no difference between the groups at 24 hr, there was strong potentiation of the DTH reaction at 48 hr. Transfer of 2×10^8 cells from donors treated with Cs A (0–4) gave rise to significantly enhanced skin responses in comparison with the results obtained in recipients of cells from the vehicle-treated group (Table 5).

Table 4. Effect of Cs A on DTH skin responses to OVA in strain 13 guinea-pigs

Time after injection (hr)	Induration*		Erythema†	
	Vehicle	Cs A‡	Vehicle	Cs A
6	29·1±1·8	18·1±1·8§	25·4±1·6	17·1±0·5§
24	31·0±2·5	29·0±1·9	26·0±2·3	22·3±0·9
48	9·8±3·5	22·6±4·1§	2·0±0·7	14·1±1·6§

* Induration: increase in skin thickness (0·1 mm)

† Erythema: diameter of the reaction (mm). Results are means ± 1 SE.

‡ CsA (25 mg/kg) or vehicle (10% EtOH in olive oil) administered p.o. on days 0–4.

§ $P < 0.001$ compared with vehicle-treated controls.

DISCUSSION

Our results show that daily oral administration of Cs A (25 mg/kg) markedly depresses DTH responses to a soluble protein antigen in the guinea-pig when the Cs A is given throughout the interval between immunization and skin challenge at 14 days. We have also shown that profound suppression of DTH is obtained when the same dose of Cs A is given only around the time of skin testing (−24, +6, 24 hr). This latter observation is in keeping with the work of Borel *et al.* (1977), who found that Cs A (80–100 mg/kg) given i.p. only at −5 and +6 hr relative to skin testing at 5–6 weeks suppressed DTH responses to tuberculin in BCG-immunized guinea-pigs. Although these authors did not examine the effect of other treatment regimes on the tuberculin response, they did report that Cs A given on days 0–4 markedly suppressed contact sensitivity to oxazolone tested on day 9 and to DNCB tested on day 10 in mice and guinea-pigs, respectively. These observations taken together with our finding that Cs A inhibits lymphokine production (Thomson *et al.*, 1982) are consistent with suppression by Cs A of T cell-mediated events in the induction and/or expression of cell-mediated immunity. They are also in agreement with previous reports of depressed cellular immune responses in Cs A-treated animals, including graft-versus-host reactivity (Markwick, Chambers, Hobbs & Pegrum, 1979) and experimental allergic encephalomyelitis (Bolton, Allsopp & Cuzner, 1982). We had expected that the adoption of a similar treatment regime to that which had been reported by Borel *et al.* (1977) to depress contact sensitivity responses might impair DTH reactions in OVA-immunized guinea-pigs. As the present study shows, however, such a regime (Cs A 0–4) caused augmentation of subsequently evoked DTH responses to the same antigen in this species. This observation we believe is of importance, not only because of the magnitude of the effect seen *in vivo* and *in vitro*, but also since it was specific to antigen responses (and not to mitogen). It may also be of some clinical significance.

The immunosuppressive effect of Cs A appears to be dependent on its continuous administration. Others, for example, have shown that skin allografts are retained by Cs A-treated animals only as long as the

Table 5. Effect of Cs A on the transfer of DTH to OVA in strain 13 guinea-pigs

Source of transferred cells*	Time (hr) after injection	DTH reaction in recipients†	
		Induration (0·1 mm)	Erythema (mm)
Vehicle-treated donors	17	4·5±0·6	5·8±1·5
	24	7·0±0·8	10·5±1·9
	48	2·3±0·3	3·2±0·9
Cs A-treated donors‡	17	13·7±1·0§	16·3±1·1§
	24	17·0±0·3§	19·0±0·8§
	48	5·7±0·6§	9·3±1·7§

* Spleen cells $(100 \times 10^6) + 100 \times 10^6$ PEC were transferred 12 days after immunization, by intracardiac injection in 2 ml of HBSS.

† Results are means ±1 SE obtained from groups of four animals injected intradermally with 50 μg OVA immediately after cell transfer.

‡ Cs A (25 mg/kg) was administered p.o. on days 0–4 following immunization.

§ All results in Cs A treated group significantly higher ($P < 0.001$) than vehicle controls.

drug is administered (Deeg, Storb, Gerhard-Miller, Shulman, Weiden & Thomas, 1980; Lems, Capel & Koene, 1980; Borel & Meszaros, 1980). Conceivably, withdrawal of Cs A 4 days after immunization of guinea-pigs with OVA in CFA may, in the presence of such chronic antigen stimulation, have permitted a normal state of delayed hypersensitivity to have developed and even exceeded, that in vehicle-treated controls. However, there is no evidence elsewhere in the literature that withdrawal of Cs A from immunized animals is associated with such a 'compensatory overshoot' in immune reactivity. Moreover, our *in vitro* tests have shown that the extent of background lymphocyte proliferation and lymphokine production was not affected by Cs A (0–4). On the other hand, the results of the cell transfer experiment suggest that cells which would otherwise restrain the DTH response to OVA in Cs A-treated animals have failed to become activated. This finding may therefore constitute the first evidence for an inhibitory effect of Cs A on the generation of suppressor cells *in vivo*. Recent reports concerning the influence of Cs A on cell proliferation *in vitro* may support this conclusion. Thus Palacios (1981) has found that Cs A inhibits the generation of human suppressor T cells, activated in the autologous mixed lymphocyte reaction, which can inhibit the proliferative response to PHA or alloantigens. The same author has reported that Cs A inhibits the generation of human suppressor cell function stimulated by Con A (Palacios, 1982). Further circumstantial evidence is consistent with an inhibitory effect of Cs A on a cell population(s) normally suppressing immune reactivity. Klaus (1981) has reported paradoxical two–three-fold enhancement of antibody responses to certain thymus-independent antigens by immunosuppressive doses of Cs A in mice; and White, Plumb & Calne (1981) have found significantly elevated IgG levels in Cs A-treated renal allograft recipients. In addition, Bueding, Hawkins & Cha (1981) found that a 5-day course of Cs A increased the resistance of infected mice to *Schistosoma mansoni* proliferation and speculated that augmentation of host defences against the parasite by Cs A might be responsible. In contrast to these findings, it has been claimed that selective sparing of suppressor cells may underlie Cs A-induced abrogation of allograft rejection (Hutchinson, Shadur, Duarte, Strom & Tilney, 1981) and mixed lymphocyte reactivity (Hess & Tutschka, 1980).

There is evidence that under the appropriate circumstances, certain other immunosuppressive drugs can augment cell-mediated immune responses. All forms of DTH, including the tuberculin reaction in animals immunized with FCA alone (Dwyer, Parker & Turk, 1981), the Jones-Mote reaction and contact sensitivity in the guinea-pig (Katz, Parker & Turk, 1974) and DTH responses to sheep red blood cells in mice (Lagrange, Mackaness & Miller, 1974; Askenase, Hayden & Gershon, 1975) are potentiated by pretreatment of the animals with the alkylating agent cyclophosphamide. The injection of methotrexate (which inhibits folic reductase) also enhances PHA responses and GVH reactivity in mice five days later (Orbach-Arbouys & Castes, 1979). In both these instances it seems likely that effector cells freed from the modifying influence of suppressor cells produce stronger cell-mediated reactions. In contrast to cyclophosphamide and methotrexate, however, CsA is not cytotoxic at immunotherapeutic dosage. The exact mechanism whereby Cs A augments DTH under the circumstances described in this study and the nature of the putative suppressor cell deleted by Cs A is an intriguing and important topic worthy of further investigation.

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