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Cyclosporin A: *in vivo* and *in vitro* suppression of rat T-lymphocyte function

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Summary. The immunosuppressive effect of cyclosporin A (CS-A) was investigated in RIC-Sprague-Dawley rats. *In vivo*, CS-A totally abolished the formation of antibodies to the hapten dinitrophenyl (DNP) in rats immunized with DNP-keyhole limpet haemocyanin. *In vitro*, the effect of CS-A was investigated in spleen cell cultures stimulated by concanavalin A, phytohaemagglutinin or lipopolysaccharide. The suppression due to CS-A was more pronounced in cultures set up with cells from rats fed the drug than in spleen cell cultures from control animals supplemented with serum containing CS-A. Purified by filtration through Degalan-rat Ig-anti IgG columns, T lymphocytes from CS-A treated rats were no longer suppressed by CS-A serum in contrast to purified T cells obtained from control rats. Thus, CS-A seems to interfere with the mitogenic triggering of a subpopulation of T lymphocytes resulting in a functional clonal deletion.

INTRODUCTION

The fungal metabolite Cyclosporin A (CS-A) (Dreyfuss, Härri, Hofmann, Kobel, Pache & Tscherter, 1976) is a cyclic endecapeptide (Ruegger, Kuhn, Lichti, Loosli, Hugenin, Quiquerez & Von Wartburg, 1976) which suppresses both humoral and cell-mediated allergic reactions (Borel, Feurer, Gubler & Stähelin, 1976; Borel & Wiesinger, 1977). Furthermore, we have recently shown that CS-A reduces dental caries in gnotobiotic rats (Guggenheim, Hefti & Burckhardt, 1978). This unexpected finding prompted us further to investigate the mode of action of CS-A both *in vivo* and *in vitro*. Experiments were designed to elucidate the effect of CS-A on helper T cells *in vivo* and to show whether *in vitro* the impaired activation of lymphocytes stimulated by T-cell mitogens was due to CS-A present in the serum of treated animals and/or to CS-A bound to the lymphocytes. Results are presented indicating that CS-A blocks T-helper cell function necessary for the antibody response to the hapten DNP (reviewed by Katz & Benacerraf, 1972) and suppresses the activation/stimulation induced by the T-cell mitogens concanavalin A (Con A) and phytohaemagglutinin (PHA). In contrast, the activation due to lipopolysaccharide (LPS), a rodent B-cell mitogen, was hardly affected at all. Further data demonstrate that CS-A influenced a subpopulation of T lymphocytes, pointing to a functional clonal deletion as the most probable mode of action.

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MATERIALS AND METHODS

Cyclosporin A

Cyclosporin A (Batch 76903) was a generous gift from Sandoz Ltd, Biological and Medical Research Division, Basel, Switzerland.

Outline of the experiment

Thirty-two conventional inbred RIC-Sprague Dawley rats originating from three litters were divided into two groups when they were 27 days old. They were kept in stainless steel cages without bedding. The control group was fed the carbohydrate-rich powdered diet 2000a (56% sucrose, 28% skim milk, 8% wheat flour, 5% yeast extract, 2%Gevral Protein® Lederle, 1% sodium chloride). The experimental group ate the same diet supplemented with cyclosporin A (214 mg/kg). On days 28 and 29 all rats were orally superinfected with cultures of *Streptococcus mutans* OMZ 176 and *Actinomyces viscosus* Ny 1 twice daily in order to induce caries lesions. The results of the caries-scoring, however, will be reported elsewhere. The animals were weighed at the start of the experiment and at sacrifice on day 54 or 55 as indicated below. The total amount of food consumed by each group was determined at the end of the experiment. Culture conditions for bacteria as well as general procedures related to animal experimentation have been previously described (Regolati, Guggenheim & Mühlemann, 1972).

Immunization

Four animals of each group were immunized on day 47 by an intraperitoneal injection of 400 μg of DNP-KLH (Calbiochem 324 121, Lot 440008) adsorbed to alumina. One week later, these rats were individually bled to obtain serum samples. Another four animals of each group were exsanguinated to obtain serum pools after both treatments. The two serum pools were sterilized by filtration and used in the cell cultures set up the following day.

Cell cultures

On day 55, the remaining eight animals of each group were sacrificed. Their spleens were aseptically removed and pooled according to treatments. Spleen cell suspensions were obtained in Dulbecco's phosphate buffered saline (D-PBS) (Oxoid Ltd, London, England). The erythrocytes were lysed by a short hypotonic shock. The cell suspensions were then washed twice with D-PBS and resuspended in EHAA-Medium (Click, Benck & Alter, 1972). T lymphocytes were collected after filtration of the spleen cell suspensions through Degalan rat Ig-anti-IgG columns as described (Burckhardt, 1978). Cell cultures were set up in a factorial arrangement. Concanavalin A (Calbiochem 234567, Lot 210073) phytohaemagglutinin-P (Difco 3110-57, Lot 602205), and lipopolysaccharide (*E. coli*, 055:B 5 Difco 3120-25, Lot 640552) were dissolved in saline (4 mg/ml) and diluted with EHAA-Medium resulting in the final concentrations indicated in the Results section. One hundred microlitres of mitogen or medium only were dispensed in flat-bottom microtitre plates (M 29 ART, C.A. Greiner, Nurtingen, GFR). Half a million spleen cells or purified T lymphocytes were then dispensed in 0·1 ml. Finally, 20 μl of a serum dilution were added yielding a final serum concentration of 1 or 3% of either pool.

Spleen cell cultures were incubated for 48 h and T lymphocyte cultures for 96 h as described before (Burckhardt, 1978). The uptake of methyl [^{3}H]-thymidine ([^{3}H]-dThd, NET 027 A, 2mCi/mM, Lot 1014-079, New England Nuclear Corporation, Boston, Massachusetts) was measured and calculated as previously reported in detail (Burckhardt, Guggenheim & Hefti, 1977).

Passive haemagglutination test

Serum antibody titres against DNP were measured in a microtitre system using 25 μl volumes and two-fold dilution steps. DNP-bovine serum albumin (Calbiochem 324101, Lot 440002) was coupled to tanned sheep erythrocytes at 10 mg/ml in a 5% (v/v) cell suspension.

RESULTS

Influence of cyclosporin A on weight increase

During the entire experimental period, animals of both groups were in good health as judged by general inspection. The rats kept on the diet containing 214 mg/kg CS-A, however, showed a significantly reduced increase of body weight at the time of sacrifice (Table 1). This might be largely due to a lower food consumption, the difference being about 10% (data not shown).

Suppression of T-helper cell function by cyclosporin A

The primary response to the hapten DNP was tested by immunizing rats of each group with DNP-KLH and measuring the serum antibody titres to DNP-BSA-coupled erythrocytes 1 week later. The control group showed a mean agglutination titre ($\log_2$) of $5{\cdot}7 \pm 0{\cdot}6$ (SD) whereas in the CS-A group no anti-DNP antibodies could be detected. Within the limits of error of the method used, this indicates that CS-A inhibits the T-helper cell population necessary for an anti-DNP response because CS-A does not directly affect B lymphocytes (Borel, Feurer, Magnée & Stähelin, 1977).

Table 1. Weight increase of RIC-Sprague-Dawley rats from day 27 to day 54/55 fed diet 2000a with or without cyclosporin A

	Weight increase (g) $\bar{x} \pm SD$	
	Control	CS-A
Males (*n*)	135·0 ± 3·0 (8)	115·2 ± 13·3 (9)*
Females (*n*)	92·8 ± 3·8 (8)	87·9 ± 3·9 (7)†

* $P < 0.01$
† $P < 0.05$

Inhibition of the proliferation induced by adding Con A or PHA but not LPS to spleen cell cultures

In a factorial arrangement, spleen cells from both treatments were cultured in the presence of serum from both groups. This set-up revealed the effect of CS-A upon both the control cultures and the mitogen stimulated cells. Table 2 shows the [^{3}H]-dThd uptake by spleen cell cultures in the presence of medium only. It is evident that both cell populations had lower background proliferation in the presence of 3% serum of either source than in 1% serum.

Table 2. [^{3}H]-dThd uptake by spleen cell cultures in the absence of mitogen measured after 48 h of culture

Final concentration	Serum used	Spleen cells cultured*	
		Control	CS-A
% v/v		dpm × 10^{-3}	dpm × 10^{-3}
1	Control	28·9 ± 1·2†	22·4 ± 0·7
	CS-A	24·7 ± 1·3	19·1 ± 1·2
3	Control	9·8 ± 0·4	10·1 ± 0·6
	CS-A	8·4 ± 0·4	7·7 ± 0·4

* 5×10^5 cells were set up in a final volume of 0·22 ml of EHAA-medium in mirotitre plates.
† Mean values and standard errors of nine replications.

The suppressive effect of CS-A was of another order of magnitude if the spleen cell cultures were stimulated by Con A (Fig. 1) or by PHA (Fig. 2). The [^{3}H]-dThd uptake by cells from CS-A treated animals was reduced to 12 and 22% of the value obtained with spleens cells from control rats at 0·5 μg Con A per culture. At the lower dose, the respective values ranged from 46 to 55%. The peak response of spleen cells from rats fed CS-A seemed to be shifted towards lower concentrations as has been previously observed in other experiments (unpublished results). The two mitogen concentrations chosen in this experiment were appropriate to reveal such an effect. With a suboptimum dose of 5 μg PHA per culture a very strong reduction

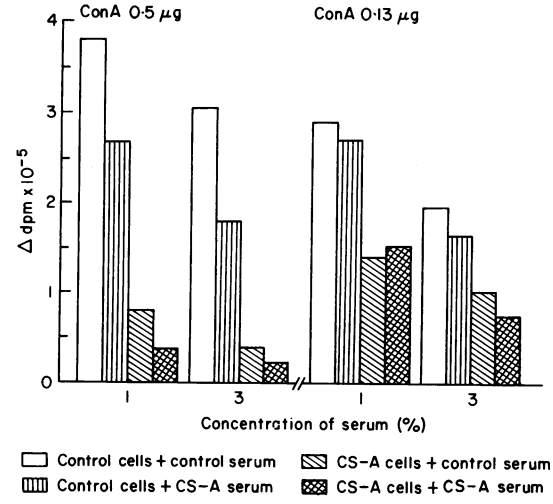

Figure 1. [^{3}H]-dThd uptake by 5×10^5 spleen cells from hours 24–48 of culture. The data are presented as the difference between cultures with (triplicates) and without (nine replications) mitogen: Δ dpm $\times 10^{-5}$. The figures indicate the amount of mitogen per culture. The mean variation coefficient ± SD was 12·4 ± 7·1%.

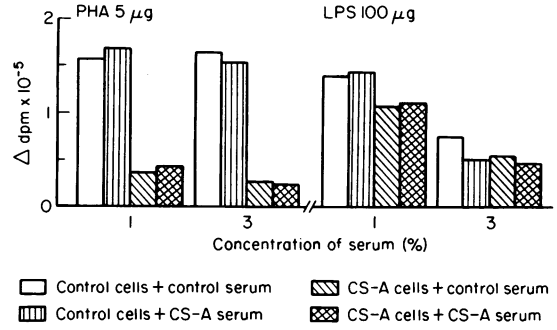

Figure 2. [^{3}H]-dThd uptake by 5×10^5 spleen cells from hours 24–48 of culture. The data are presented as the difference between cultures with (triplicates) and without (nine replications) mitogen: Δ dpm $\times 10^{-5}$. The figures indicate the amount of mitogen per culture. The mean variation coefficient ± SD was 9·6 ± 4·0%.

to 14 and 24% of the level in the control was observed.

The effects of CS-A in serum were less clear-cut. A variable suppression with cells of both treatments stimulated by Con A was observed or even a slight enhancement when PHA was used as a mitogen. In cultures stimulated by LPS, however, only a minor suppression due to CS-A was detected (Fig. 2). This is in accordance with the results reported from mice (Borel & Wiesinger, 1977).

Effect of cyclosporin A on T lymphocyte cultures

In order to know whether the inhibitory effect of CS-A observed in spleen cell cultures (Figs 1 and 2) was due to a direct action of CS-A on T cells and not mediated by another cell population, T lymphocytes were purified by filtration through Degalan rat Ig anti-rat IgG columns. The passing cell population from both animal groups contained less than 0·5% surface IgG-positive cells when tested with a $F(ab')_2$ anti-rat IgG-fluorescein isothiocyanate conjugate. There was, however a different recovery of T lymphocytes; 13·6% T cells were recovered from control rats and 8·8% from rats fed CS-A. The latter value was clearly below the usual recovery (Mean recovery ± SD: 19·0 ± 6·4%, $n=21$) in this laboratory. Table 3 summarizes the uptake of [^{3}H]-dThd measured after 96 h in the presence of 0·5 μg Con A. It is evident that the passing T-cell population from CS-A treated rats was not suppressed at all by CS-A-containing serum. In contrast, the T lymphocytes from control animals produced in the presence of 1% CS-A serum a strongly diminished response which was only 39% of that reached in the presence of control serum.

Table 3. Influence of serum from control or CS-A rats on T-cell cultures stimulated by 0·5 μg Con A

Final conc.	Serum used	T cells cultured*	
% v/v		control	CS-A
1	control	100†	309
	CS-A	39	333
3	Control	126	380
	CS-A	77	350

* 5×10^5 T cells were set up in a final volume of 0·22 ml of EHAA-medium in microtitre plates.

† Measured on day 4 of culture, the Δ dpm value ($63·1 \times 10^3$) of control T cells in 1% control serum was set as 100%

The mean variation coefficient ± SD was 9·1 ± 3·6%.

DISCUSSION

The present data demonstrate that the fungal metabolite CS-A selectively suppresses rat T-lymphocyte functions. The results confirm the *in vivo* T-cell suppressive action of CS-A (Borel *et al.*, 1976; Borel *et al.*, 1977) using another strain of rats and that antibody formation against the DNP-hapten is dependent on helper T cells (Katz & Benacerraf, 1972). A lower food consumption accompanied by a small but significant retardation of the weight increase (Table 1) was observed as a side effect. Because these two parameters severely influence the outcome of experimental caries in rats, they have to be considered in further investigations, e.g. by reducing the dose of CS-A to the amount previously used without a side effect (Guggenheim *et al.*, 1978). The *in vitro* experiments disclosed further information on the mode of action of CS-A. Figures 1 and 2 show that CS-A reacting with spleen cells *in vivo* provoked a strong suppression *in vitro* when CS-A cells were stimulated with Con A and PHA in the presence of control serum. Using CS-A-containing serum instead of control serum the suppressive effect was only moderately enhanced. Nevertheless, CS-A serum efficiently diminished the response to Con A of spleen cells from control rats (Fig. 1, Con A 0·5 μg). In considering these findings one has to bear in mind that culture conditions using 1% or 3% serum only poorly mimic the *in vivo* potential of the respective sera.

Previously, evidence was presented that CS-A impairs T-cell functions by interfering with an early stage of mitogenic triggering (Borel *et al.*, 1977). But it is not yet known whether suppressor cells are affected by CS-A. In our hands, CS-A had the following effects: (i) a shift of the peak response to lower concentrations of Con A (Fig. 1) and PHA (unpublished results), (ii) a lower yield of T cells after filtration through Ig anti-IgG columns, and (iii) an enhanced response to Con A of the passing cells from CS-A treated rats compared with those from control animals (Table 3). Based on these observations, we concluded that CS-A provoked

a change of the cell-surface characteristics, resulting in a shift of the peak response of the remaining reactive cells, and a loss of cells on the Degalan columns. The latter might be due to the expression of Fc receptors, a property which prevents lymphocytes from passing through the column under the conditions used (Hertel-Wulff & Rubin, 1976). Thus, it is evident that CS-A acts on T-cell subsets by clonal deletion and that unaffected cells can be enriched, e.g. by column filtration methods.

With regard to the role of suppressor cells, we have no direct evidence that CS-A might have acted by activating this regulatory cell type, nor can we exclude the possibility that this very cell population was retained on the Ig anti-IgG column by their Fc receptors (Hertel-Wulff & Rubin, 1976). This also would have led to an enhanced response of passing T cells from CS-A-treated rats. This question deserves further attention.

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