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Anti-schistosomal activity of cyclosporin A: studies on murine spleen cells and the influence of a cyclosporin antagonist on resistance to infection

L. R. BRANNAN*, L. H. CHAPPELL, J. WOO & A. W. THOMSON *Parasitology Unit, Department of Zoology and Immunopathology Laboratory, Department of Pathology, University of Aberdeen, Aberdeen*

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SUMMARY

Mice were treated with 5-day courses of cyclosporin A (CsA) around the time of infection with *Schistosoma mansoni*. Recovery of lung-stage worms 4–8 days post-infection (p.i.) was substantially reduced (80%) and no sexually mature adults were recovered from the hepatic portal system at 7 weeks p.i. Flow cytometric analysis of spleen cells from CsA-treated animals during the period of maximal parasite attrition revealed transient reductions in $CD3^+$ and $CD4^+$ cells and in the $CD4^+:CD8^+$ ratio compared with drug vehicle-treated, infected controls. No significant numerical changes in B cells, macrophages or eosinophils were detected relative to vehicle-treated infected mice. Transfer of spleen cells from CsA-treated donors 8 days after infection failed to confer increased resistance to *S. mansoni* infection on untreated recipients. Moreover, concomitant administration of CsA and an inducer of interleukin-2 production (ADA-202-718) did not interfere with the anti-schistosomal effect of CsA. Despite our incomplete understanding of the *in vivo* properties of CsA and reports of its paradoxical effects on immune responses, these new data indicate that the influence of CsA in schistosomiasis is unlikely to be mediated by modulation of host cell mediated immunity. This contrasts with certain other anti-parasitic effects of CsA which appear to be mediated by an action on T cells.

INTRODUCTION

The immunosuppressive properties of cyclosporin A (CsA) are consistent with the capacity of the drug to interfere with cytokine gene expression and interleukin production by $CD4^+$ (helper) T cells *in vitro* (Hess, Tutschka & Santos, 1982; Thomson *et al.*, 1983a; Granelli-Piperno, Keane & Steinman, 1988) and with the clonal expansion of $CD8^+$ (suppressor) T cells (Hess, Esa & Colombani, 1988). Most of what is known, however, about the mechanism of action of CsA, is based on *in vitro* observations, and much concerning its influence on immunological events *in vivo* remains to be elucidated. Thus, in addition to numerous publications describing the suppression of cell-mediated immunity by CsA, there are reports of T-helper cell priming (Klaus & Kunkl, 1983), clonal expansion of antigen-activated T cells (Chisholm & Bevan, 1988) and the inhibition of T-suppressor cell activity (Braida & Knop, 1986; Schiltknecht & Ada, 1986) in animals receiving immunosuppressive doses of the drug. Moreover, the augmentation of delayed-type-hypersensitivity reactions has been described in CsA-treated rodents following drug withdrawal (Thomson *et al.*, 1983b; Kaibara *et al.*, 1983).

In addition to its immunomodulatory properties, CsA (at non-toxic dosage) is a potent anti-parasitic agent (Thomson, Smith & Chappell, 1986b) and exhibits protective effects in rodents against *Schistosoma sp.*, *Plasmodium sp.*, *Leishmania major*, *Dipetalonema viteae* and other species (Chappell and Thomson, 1988). In recent studies, we and others have described the prophylactic and therapeutic effects of CsA against *S. mansoni* in mice and rats (Bout, Deslee & Capron, 1986; Chappell *et al.*, 1987). This anthelmintic action of CsA is not understood but could, conceivably, be related to its immunomodulatory activity.

In this study, we present new data on the influence of CsA on host spleen cell populations during the early course of *S. mansoni* infection, when the drug exerts its most potent anthelmintic activity (Chappell *et al.*, 1987). In addition, we have investigated, for the first time, the anti-schistosomal property of CsA in mice treated concomitantly with a cytokine inducer, ADA-202-718 (ADA), which has recently been shown

* Present address: The Wellcome Laboratories for Experimental Parasitology, University of Glasgow, Bearsden Road, Glasgow G61 1QH, U.K.

Abbreviations: ADA, ADA-202-718; CsA, cyclosporin A; DTH, delayed-type hypersensitivity; FCS, fetal calf serum; i.p., intraperitoneally; PBS, phosphate-buffered saline; p.i., post infection; s.c., subcutaneously; SRBC, sheep red blood cells.

Correspondence: Dr A. W. Thomson, Immunopathology Laboratory, Dept. of Pathology, University of Aberdeen, Aberdeen AB9 2ZD, U.K.

to antagonize the effects of CsA on IL-2 production and cell-mediated immunity (Hiestand & Stasser, 1985;Woo & Thomson, 1989).

MATERIALS AND METHODS

Mice

Male MF1 mice (8–10 weeks old) were used throughout. They were bred in the Zoology Department, University of Aberdeen, maintained in a temperature-controlled environment and received Oxoid rat and mouse breeding diet with tap water *ad libitum*.

Parasite infection and recovery

The Wellcome strain of *S. mansoni* was maintained in laboratory-reared *Biomphalaria glabrata* and in male MF1 mice. Infection of mice was accomplished by the abdominal ring method (Smithers & Terry, 1965) after nembutal (Sagatal, May & Baker, Dagenham, Essex) anaesthesia: mice were exposed to either 10^3 (for lung stage recovery) or 10^2 (all other experiments) freshly liberated cercariae for 2 hr. Lung-stage schistosomula were obtained (as described by Smithers & Gammage, 1979) following perfusion of the lungs with medium 199 (Gibco, Paisley, Renfrewshire) containing heparin (20 IU/ml). The tissue was finely chopped and incubated for 18–20 hr at 37° in the same medium, supplemented with 5% newborn calf serum (Gibco). After removal of tissue debris using a grade O scintered glass filter, parasites in the filtrate were sedimented by centrifugation, resuspended and counted under a dissecting microscope. Adult schistosomes were harvested at 7 weeks post-infection (p.i.) by aortic perfusion of the hepatic portal system with ice-cold citrate saline (1·5% sodium citrate in 0·9% sodium chloride). They were counted, sexed and their dry weights recorded on a Mettler M3 microbalance. Mouse body weights and fresh liver and spleen weights were also recorded.

Drugs

Cyclosporin A (CsA) and ADA-202-718 (ADA) (a gift from Dr P. C. Hiestand) were provided by Sandoz Ltd, Basel, Switzerland. Each was obtained as a white powder and dissolved in absolute ethanol, to which was added nine parts olive oil (Boots PLC, Nottingham, Notts). After thorough mixing, the drug solution was administered (0·1 ml) either subcutaneously (s.c.; CsA) on the dorsal midline of the back or intraperitoneally (i.p.; ADA). Fresh solution was prepared for each experiment and stored in the dark at 4° for no more than 5 days. CsA (25 or 50 mg/kg) was administered on Days -2–$+2$ relative to infection or (for DTH experiments) on Days 0–3 following immunization. ADA (1 mg/kg) was injected concomitantly with CsA.

Delayed-type hypersensitivity responses

Mice were immunized with sheep red blood cells (SRBC) obtained from whole blood in Alsever's solution (Difco Laboratories, West Molesey, Surrey). The SRBC were washed three times in ice-cold Dulbecco 'A' phosphate-buffered saline (PBS), pH 7·2, and injected (10^6) in PBS (0·2 ml; 27G needle) via a lateral tail vein. Animals were challenged, 96 hr after immunization, with 10^8 SRBC in 50 μL PBS under the right hind footpad, the left footpad receiving PBS alone. Reactions were assessed 24 hr 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, Schluechtern, FRG). All measurements were conducted by the same individual and results expressed as specific increases in footpad thickness (10^{-1} mm, mean $\pm$ 1 SD).

Preparation of spleen cell suspensions and cell transfer

Animals were killed by cervical dislocation and spleens removed. Cell suspensions were prepared from individual animals as previously described (McIntosh *et al.*, 1986) and erythrocytes lysed by incubation in 0·83% (w/v) Tris-buffered NH_4Cl (pH 7·2) for 10 min at 37°. Following two washes in ice-cold PBS, cells were resuspended in RPMI-1640 (Gibco), supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cell concentration and viability were estimated by trypan blue (0·2% w/v) dye exclusion, using an improved Neubauer haemocytometer.

Immunofluorescence staining and flow cytometric analysis

The concentration of spleen cells was adjusted to 2×10^7/ml using RPMI-1640, supplemented as described above. An indirect immunofluorescence method was employed to demonstrate cell phenotypes, using the appropriate primary rat IgG2b anti-mouse monoclonal antibodies: anti-Thy-1 (all T cells); anti-L3T4 ($CD4^+$ helper-inducer T cells); anti-Ly2 ($CD8^+$ suppressor-cytotoxic T cells); anti-Mϕ (M1/700.15) and anti-kappa (B cells, OX20), Sera-Lab, Crawley Down, Sussex. On receipt, each antibody was diluted 1:10 in PBS, containing 1% w/v bovine serum albumin (BSA; Sigma Chemical Company Ltd, Poole, Dorset.) and kept at $-70°$. Immediately before use, the antibodies were further diluted to the following: anti-Thy-1, 1:200; anti-L3T4, 1:200; anti-Ly 2, 1:200; anti-Mϕ, 1:500 and anti-Kappa, 1:500, in PBS with 10% heat-inactivated normal rabbit serum (Scottish Antibody Production Unit, Carluke, Lanarkshire.) The secondary antibody employed was FITC-conjugated rabbit IgG anti-rat IgG (H+L, ICN Biomedicals Ltd, High Wycombe, Bucks), which was preincubated for 90 min at 4° in PBS containing 10% normal mouse serum. Fifty microlitres of cell suspension were mixed with an equal volume of primary antibody for 1 hr at room temperature. After two washes in PBS, the cells were resuspended in 100 μl of secondary antibody (1:50) and incubated for 30 min at 4°. After two further washes, the cells were suspended in 1 ml cold PBS and maintained on ice until analysis. Controls consisted of omission of the primary layer rat antibodies and replacement of those by normal rat Igs. The percentage of cells expressing specific cell surface markers was analysed using an 'EPICS C' flow cytometer (Coulter Electronics, Luton, Beds) as described previously (Webster & Thomson, 1988). Five thousand cells were counted for each sample.

Quantification of eosinophils

Eosinophils in spleen cell suspensions were enumerated as previously described in detail (Thomson *et al.*, 1986a).

Statistics

The significances of differences between means was calculated using the non-parametric Mann–Whitney *U*-test (for DTH results) or Student's *t*-test for all other experiments.

Table 1. Effect of CsA on recovery of lung stage and sexually mature *S. mansoni*

Day after infection (No. of mice)	No. cercariae /mouse	No. of worms recovered			
		Drug vehicle	(%)	CsA	(%)
Lung stage					
Day 4 (3)	10^3	148±27	(14·8)	7±3**	(0·7)
Day 6 (6)	10^3	233±87	(23·3)	25±19**	(2·5)
Day 8 (6)	10^3	204±93	(20·4)	47±33*	(4·7)
Sexually mature					
Day 49 (16)	10^2	43±9	(43·0)	0**	(0)

Results are numbers of parasites (mean±1 SD). Symbols indicate the significance of differences from drug vehicle-treated controls.
* $P<0·01$; ** $P<0·001$.

Table 2. Influence of CsA on T cells in *S. mansoni*-infected mice

Day after infection: treatment	Positive cells (× 10^{-7})		
	Thy-1$^+$ (CD3$^+$)	L3T4$^+$ (CD4$^+$)	Lyt-2$^+$ (CD8$^+$)
Control (uninfected; no CsA)	2·50±0·70	1·94±0·32	0·75±0·22
Day 4			
Drug vehicle	2·59±0·54	1·54±0·38	0·69±0·17
CsA	1·77±0·86	0·99±0·52	0·50±0·23
Day 6			
Drug vehicle	2·65±0·32	1·29±0·21	0·72±0·10
CsA	1·61±0·68*	0·82±0·35†	0·57±0·22
Day 8			
Drug vehicle	3·39±0·70‡§	2·05±0·53‡	0·98±0·39
CsA	2·61±0·50*§	1·93±0·48¶**	0·54±0·17*

Results are means±1 SD obtained from groups of six mice.

Symbols indicate the significances of differences from corresponding drug vehicle-treated controls (*$P<0·05$; †$P<0·01$) or between members of similarly treated groups at different times; Days 4 and 6 (no significant differences); Days 6 and 8 (‡$P<0·05$; ¶$P<0·01$); days 4 and 8 (§$P<0·05$; **$P<0·01$).

RESULTS

Anti-schistosomal activity of CsA

The influence of CsA administered around the time of infection on the numbers of lung stage and adult parasites recovered from *S. mansoni*-infected mice is illustrated in Table 1. It is clear that CsA exerted its most pronounced anti-schistosomal activity early during the course of infection. The reduction in worm numbers recovered on Days 4–8 was not due simply to a delay in parasites reaching the lung, since no evidence of this was obtained by sampling groups of CsA-treated infected mice later during the course of infection (between days 8 and 49, data not shown).

Table 3. Worm recovery from *S. mansoni*-infected mice given spleen cells from untreated or CsA-treated infected donors

Treatment of spleen cell donors	No. of recipients (*n*)	Mean worm number		
		Male	Female	Total
No. cell transfer	(6)	26·5±8·9	14·3±5·5	42·5±8·7
Uninfected; drug vehicle	(7)	32·8±6·0	19·3±6·1	52·2±7·8
Uninfected; CsA	(6)	32·0±9·8	18·8±5·9	50·8±13·6
Infected; drug vehicle	(5)	29·4±10·3	17·8±7·2	47·4±14·9
Infected; CsA	(5)	24·0±14·8	13·6±8·1	37·6±20·2

Results are means±1 SD. There were no significant differences between any of the groups in terms of worm recovery (above) or liver, spleen or dry worm weights (data not shown).

Uninfected or infected donor mice received drug vehicle or CsA (50 mg/kg) on Days −11−−7 (inclusive) relative to infection on Day −9 with 10^2 cercariae. Eights days later (Day 0), 5×10^7 viable spleen cells were transferred intravenously into naive recipients, which were infected simultaneously with 10^2 cercariae. Spleen cell recipients were killed 7 weeks after infection to provide the above data.

Table 4. Effects of CsA and the cytokine inducer ADA-202-718 on delayed hypersensitivity reactions

Treatment	No. of mice (*n*)	DTH reaction (mm^{-1})	% of control
Drug vehicle control	(11)	4·64±1·53	100
CsA (25 mg/kg)	(6)	1·52±0·73*	33
ADA-202-718 (1 mg/kg)	(12)	6·63±1·56†	143
CsA+ADA-202-718	(6)	3·23±0·13‡	72

Results are means±1 SD. Symbols indicate significances of differences from drug-vehicle treated controls (*$P<0·05$); (†$P<0·01$) or from corresponding CsA-treated group (‡ $P<0·01$).

Influence of CsA on T cells

To ascertain whether the dramatic reduction in numbers of schistosomules reaching the lung was accompanied by changes in lymphocyte, macrophage or eosinophil populations, numbers of these cells were quantified in spleens of CsA-treated mice from Days 4–8 p.i. (Table 2). In infected, drug vehicle-treated mice, there was a progressive (30%) increase in pan T (CD3$^+$) cells between Days 4 and 8 ($P<0·05$) and compared with uninfected controls ($P<0·05$); although mean numbers of CD4$^+$ and CD8$^+$ cells also rose over the same period, the increases were statistically significant only for CD4$^+$ cells between days 6 and 8. In CsA-treated animals, there was a transient reduction in each T-cell population on Days 4 and 6 p.i., compared with control groups (drug vehicle-treated, infected mice and uninfected untreated controls); normal values of L3T4 (CD4)$^+$ cells, however, were restored in the CsA group by Day 8, at which time CD3$^+$ (pan T) and CD8$^+$ cell numbers were significantly lower than in infected, drug-treated controls.

Determination of the CD4$^+$: CD8$^+$ ratio revealed no significant changes compared with normal mice in the infection only

Table 5. Worm recovery from *S. mansoni*-infected mice treated with CsA and the cytokine inducer, ADA-202-718

Treatment	Organ weight (% body weight)		Mean worm number	
	Liver	Spleen	Male	Female
Control (uninfected; no CsA)	5·0±0·3	0·3±0·1	0	0
Drug vehicle	8·6±0·7	1·5±0·2	10·4±3·4	9·8±2·1
ADA	8·2±1·3	1·8±1·2	14·2±4·4	10·0±3·4
CsA	4·5±0·2*	0·3±0·0*	0·2±0·4*	0·4±0·6*
CsA+ADA	4·9±0·4*	0·3±0·0*	1·0±1·2*	1·8±0·5*

Results are means±1 SD. obtained from groups of five mice. Symbols indicate significances of differences from drug-vehicle treated controls (* $P<0{\cdot}01$).

CsA (25 mg/kg s.c.) or/and ADA (1 mg/kg i.p.) were administered on days, −2, −1, 0, +1, +2 relative to infection with 10^2 cercariae on Day 0. Animals were killed on Day 47 after infection to provide the above data.

control group (data not shown), whilst in the CsA-treated group, a significant increase was detected between Day 4 ($CD4^+:CD8^+=2{\cdot}0\pm0{\cdot}7$) and Day 8 ($3{\cdot}9\pm1{\cdot}9$; $P<0{\cdot}02$). The Day 8 value, however, did not differ significantly between the two experimental groups.

Influence of CsA on B cells, macrophages and eosinophils

Absolute numbers of splenic B cells (normal value $3{\cdot}54\pm0{\cdot}92\times10^{-7}$) increased significantly ($P<0{\cdot}05$) in both the drug vehicle (55%) and CsA-treated (47%) groups between Days 4 and 8 (data not shown). There was, however, no significant difference between the groups. No changes in macrophage or eosinophil numbers were detected.

Spleen cell transfer

As shown in Table 3, the transfer of spleen cells from CsA-treated infected (8 days previously) or uninfected donors did not affect the capacity of naive recipients to resist *S. mansoni* infection, as determined by worm numbers recovered 7 weeks after infection.

Influence of CsA and ADA on delayed-type hypersensitivity

The effect of CsA and ADA on DTH reactions is shown in Table 4. Whilst ADA alone significantly augmented DTH, its administration also caused significant alleviation of the potent immunosuppressive activity of CsA.

Influence of ADA on resistance to infection

Administration of ADA alone from Days −2 to +2 relative to infection did not influence the hepatosplenomegaly or numbers of worms recovered from *S. mansoni*-infected mice. Moreover, when given together with CsA it failed to affect significantly the resistance to infection conferred by CsA (Table 5).

DISCUSSION

In this study, we have investigated quantitative changes in T-cell populations, B cells, macrophages and eosinophils accompanying the markedly enhanced resistance to *S. mansoni* infection induced by CsA in the mouse. Our analysis of spleen cells from CsA-treated, infected animals and their capacity if any, to confer protection against *S. mansoni* when adoptively transferred to naive recipients, was conducted during the early period after infection ('lung stage') during which time the parasite was most vulnerable to the influence of the drug (Chappell *et al.*, 1987). By antagonizing the influence of CsA on cell-mediated immunity using the interleukin inducer ADA, we have examined further whether the anti-schistosomal action of the drug may be mediated immunologically.

Several conclusions may be drawn from our results. First, the lung recovery data confirm that there were heavy parasite losses within the first 8 days of infection. That this CsA-induced attrition was not due simply to a delay in parasites reaching the lung has been confirmed by the harvest of worms at later time points and by tracking the fate of ^{35}Se-selenomethionine-labelled cercariae in CsA-treated hosts (L. R. Brannan, D. J. McLaren, G. Munro, L. H. Chappell & A. W. Thomson, manuscript submitted for publication). Second, the 5-day course of CsA around the time of infection led to a transient reduction (Day 6) in pan T cells and $CD4^+$ cells, although the $CD4^+:CD8^+$ ratio was not affected significantly compared to drug vehicle-treated infected controls. No other significant numerical changes in spleen cell populations relative to controls were observed. Third, spleen cells from animals protected against schistosomiasis failed to confer increased resistance on naive recipients. Fourth, doses of ADA which antagonized the effect of CsA on cell-mediated immunity failed to reverse its powerful antiparasitic activity.

Although the mechanism(s) whereby CsA mediates its antiparasitic effects are poorly understood, it has been suggested that the drug may modulate complex events during the induction of anti-parasite immune responses. Thus a 3-day course of CsA treatment not only eliminates primary *S. mansoni* infections in mice, but also induces long-term protection against reinfection (Bout, Deslee & Capron, 1984). Similarly, CsA may allow protective cell-mediated immunity to *Leishmania major* to develop in mice (Behforouz *et al.*, 1986). Furthermore, an influence of low dose CsA on $CD4^+$ T cells has also been suggested by the capacity of the drug to inhibit murine cerebral malaria (Grau, Gretener & Lambert, 1987).

A previous quantitative analysis of spleen cells in *Leishmania tropica*-infected mice revealed that CsA (50 mg/kg) markedly suppressed infection-associated increases in T lymphocytes, particularly the Lyt-1^+ population ($CD5^+$ T cells and some B cells) (Solbach *et al.*, 1986). The authors concluded that CsA interfered with the accumulation/clonal expansion of antigen-reactive T cells. These findings are in agreement with the CsA-induced reduction in $L3T4^+$ ($CD4^+$) T cells observed in this study, a recent investigation of T cells in CsA-treated *S. mansoni*-infected rats (Thomson & Chappell, 1988) and with the apparent sparing of $CD8^+$ T cells reported in other experimental animal systems (Kupiec-Weglinski *et al.*, 1984). Whilst diminution of T-cell-mediated responses by CsA, including the proliferation of macrophages (the parasite environment), may be instrumental in the inhibition of experimental cutaneous

leishmaniasis, it is difficult to implicate a link between the depression of T cells following CsA administration in the present investigation, and an immune-targeted protective mechanism against schistosomiasis. Indeed, both the failure of splenocytes from CsA-treated, infected donors to confer increased resistance on naive recipients and the inability of ADA to reverse the protective effect of CsA renders such an association unlikely. Moreover, CsA has been shown to be effective against schistosomiasis in congenitally athymic hosts (Bout *et al.*, 1986) and both we and others have reported that non-immunosuppressive cyclosporin derivatives exhibit anti-schistosomal activity *in vivo* (Chappell *et al.*, 1987). Whilst in this study no effect of CsA on B-cell or macrophage numbers was recorded, Grau *et al.* (1987) observed no influence of low dose CsA on T-dependent polyclonal B-cell activation, malaria-associated hypergammaglobulinaemia or the specific humoral response to malaria antigens.

Although these observations reinforce the view that the anti-schistosomal activity of CsA is not mediated immunologically, there are, nevertheless, a number of poorly explained examples of augmentation of cell-mediated or humoral (IgE) immunity following short courses of CsA in rodent models. These include syngeneic graft-versus-host reactivity in rats (Glazier *et al.*, 1983), potentiation of DTH (Thomson *et al.*, 1983b), interference with tolerance induction (Webster & Thomson, 1988) and up-regulation of (non-parasite) antigen-specific IgE production in mice (Chen, 1988). Until the mechanisms underlying these phenomena are elucidated, a possible contributory role of the host immune system to the anti-schistosomal activity of CsA can not be entirely discounted. On the basis of evidence presently available, however, it appears that the anti-schistosomal actions of CsA, unlike its effect in leishmaniasis, may not be mediated via the immune system.

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