

*Liver and biliary*

# *In vitro* effect of Cyclosporin A on immunoglobulin production and concanavalin A induced suppression in primary biliary cirrhosis

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**SUMMARY** The *in vitro* effect of Cyclosporin A on the regulation of immunoglobulin production was investigated in 16 patients with primary biliary cirrhosis. A significant improvement in concanavalin A induced suppression of IgG and IgM producing cells was observed after prior incubation of mononuclear cells with 300 ng/ml Cyclosporin A for 30 minutes. No effect was seen on spontaneous or pokeweed mitogen induced immunoglobulin production, nor on con A induced suppression if Cyclosporin A was added after 24 hours. Incubation of mononuclear cells with a variable dose of Cyclosporin A showed an effect only at 250-500 ng/ml. Higher and lower doses had no effect. This dose dependent effect of Cyclosporin A is likely to be related to a differential inhibitory effect on T helper and T suppressor cells and may underlie the clinical benefit being observed in current clinical trials.

Cyclosporin A is being used with considerable therapeutic success in human organ transplantation<sup>1,2</sup> and recently reports have also appeared of its efficacy in conditions that are closely associated with disordered immune responses such as Graves' ophthalmopathy,<sup>3</sup> corticosteroid resistant posterior uveitis,<sup>4</sup> diabetes mellitus,<sup>5</sup> as well as sarcoidosis and Crohn's disease.<sup>6,7</sup> The number of cases so far included in many of these studies is small and attention has focused on clinical efficacy rather than the mode of action. The ratio of helper:suppressor/cytotoxic cells (T4:T8 ratio) has been shown to fall during a pilot study of Cyclosporin A in primary biliary cirrhosis (PBC),<sup>8</sup> but functional studies have only rarely been performed in patients.

In primary biliary cirrhosis, hyperglobulinaemia, non-organ specific auto-antibodies and infiltration of the hepatic parenchyma and portal tracts by mononuclear cells suggest abnormalities of cellular and humoral immunity. In a preliminary study, empirical administration of Cyclosporin A was accompanied by improvement in serum biochemistry, but nephrotoxicity was common and the trial was stopped.<sup>8</sup> Recently we have found it possible to use Cyclosporin A at lower doses, with a reduction in

symptoms and without nephrotoxicity.<sup>9</sup> The purpose of the present study was to investigate the effect of Cyclosporin A on immunoglobulin production and its regulation, as defective suppression of immunoglobulin production has been observed in primary biliary cirrhosis,<sup>10,11</sup> particularly in those with early disease (personal observation).

## Methods

### PATIENTS

The 16 patients (median age 48.6 years, range 53-73 years) investigated, including one man, had histologically proven primary biliary cirrhosis. Five had early disease as assessed histologically (stage II) and 11 late disease (stage III or IV).<sup>12</sup> Three patients were antimitochondrial antibody positive in a titre of less than 1/20. None of the patients was on treatment at the time of the study (Table 1). Six laboratory personnel, all men (median age 28 years, range 26-30 years) served as healthy controls. Previous studies have demonstrated that suppressor cell function is not related to sex or age<sup>13</sup> and this has been confirmed for suppressor cell function in this laboratory for sex and age up to 70 years.<sup>14</sup>

### CELL SEPARATION

Venous blood mixed with dextran (6% w/v) in

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Table 1 Relevant clinical data of the 16 patients with primary biliary cirrhosis (values are given as ranges)

	Women/men	Age	Bilirubin mol/l (normal <17 $\mu$ mol/l)	Serum aminotransferase (normal <45 $\mu$ mol/l)	Antimito- chondrial antibody > 1/20	IgG g/l	IgM g/l
Early disease (Stage II)	5/0	43-66	15-49	40-144	3	6.4-22.6	3.8-13.0
Late disease (Stage III or IV)	10/1	44-47	15-81	24-426	10	9.8-18.9	1.6-8.9

0.15 mol NaCl and heparin (10 U/ml), was suspended at 37°C for 45 minutes to allow sedimentation of red cells. Leucocyte rich plasma was centrifuged on Ficoll-Trisil at 400 g for 20 minutes and the mononuclear cells then depleted of monocytes by incubation in plastic Petri dishes for 45 minutes at 37°C. The non-adherent cells were then washed three times in Hanks balanced salt solution and re-suspended at  $10^6$  cells/ml in 20% heat inactivated fetal calf serum in RPMI 1640 (Gibco) containing 2mM glutamine, 200  $\mu$  penicillin/ml, 100  $\mu$ g streptomycin/ml and 2  $\mu$ g amphotericin B/ml. Peroxidase activity was 3-5%.

#### CELL CULTURE

$2 \times 10^5$  cells peripheral blood mononuclear cells at  $10^6$  cells/ml were cultured in flat-bottomed microplates (Sterilin) in the presence or absence of pokeweed mitogen (Sigma) 20  $\mu$ g/ml. Simultaneously, peripheral blood mononuclear cells at  $2 \times 10^5$  cells/ml were incubated with concanavalin-A 20  $\mu$ g/ml for 24 hours and then washed three times in Hanks balanced salt solution (HBSS) containing 0.3 M  $\alpha$ -methyl-D-mannoside to remove concanavalin A. After further washes in HBSS, these cells were resuspended in 20% fetal calf serum in RPMI 1640 at  $10^6$  cells/ml. Equal numbers of concanavalin A stimulated and unstimulated peripheral blood mononuclear cells were then cultured with pokeweed mitogen at 20  $\mu$ g/ml as outlined above (viability, assessed by Trypan Blue exclusion, exceeded 93% after culture with concanavalin A).

#### HAEMOLYTIC PLAQUE ASSAY

After seven days, peripheral blood mononuclear cells were harvested by centrifugation, washed three times in RPMI 1640 and resuspended at  $10^5$  cells/ml. Viability ranged from 66-96%. Fifty microlitres peripheral blood mononuclear cells was mixed with 50  $\mu$ l 10% sheep red blood cells coated with staphylococcal protein A (Pharmacia), 50  $\mu$ l of an appropriately diluted IgG fraction of rabbit anti-human IgG (or IgM) and 700  $\mu$ l agarose Indubiose (Reactifs IBF) and poured into plastic Petri dishes

and incubated for four hours at 37°C. After developing with guinea pig complement for 2 hours, haemolytic plaques were counted visually in indirect light and the results expressed as the number of IgG (or IgM) producing cells per  $10^6$  viable peripheral blood mononuclear cells. Proliferation of immunoglobulin producing cells has been assessed in the absence of any mitogens to determine the number of cells spontaneously producing immunoglobulin and in the presence of pokeweed mitogen alone. Concanavalin A induced suppression is defined as:

$$1 - \left[ \frac{\text{Number of immunoglobulin producing cells in the presence of concanavalin A and pokeweed mitogen}}{\text{Number of immunoglobulin producing cells in the presence of pokeweed mitogen alone}} \right] \times 100$$

To assess the effect of Cyclosporin A, peripheral blood mononuclear cells were incubated with Cyclosporin A (dissolved in 100% ethanol and then RPMI 1640 to give a final concentration of 300 ng/ml in 0.15% ethanol) for 30' at 37°. After incubation, peripheral blood mononuclear cells were washed in HBSS three times and suspended in RPMI containing 20% heat inactivated fetal calf serum. Mean viability, by Trypan blue exclusion was 98%. As a control, Cyclosporin A solvent (courtesy Sandoz) was dissolved in identical concentrations of ethanol and RPMI 1640. Peripheral blood mononuclear cells from patients and controls were incubated in Cyclosporin A or control media at one of four stages, namely (1) prior to unstimulated culture; (2) prior to culture with pokeweed mitogen; (3) prior to culture with concanavalin A; and (4) after 24h culture with concanavalin A. Subsequently varying doses of Cyclosporin A were used prior to culture with concanavalin A.

#### STATISTICS

For immunoglobulin production, the data are presented as medians (range), while for suppression, the data are presented as means ( $\pm 1$  SD). Comparison has been made using the paired Rank test.

Table 2 Effect of preincubation of peripheral blood mononuclear cells with Cyclosporin A on in vitro immunoglobulin production in primary biliary cirrhosis and normal controls (values expressed as median number of cells (range)/10<sup>6</sup> peripheral blood mononuclear cells producing IgG or IgM)

	IgG Without Cyclosporin A	With Cyclosporin A	IgM Without Cyclosporin A	With Cyclosporin A
<i>Spontaneous Ig production</i>				
Primary biliary cirrhosis (n=10)	292 (109-562)	263 (111-444)	129* (54-217)	162* (56-287)
Normal controls (n=6)	488 (357-805)	499 (326-674)	216 (169-291)	170 (56-278)
<i>Pokeweed mitogen stimulated Ig production</i>				
Primary biliary cirrhosis (n=10)	668 (235-1341)	559 (125-1125)	230 (59-395)	238 (60-425)
Normal controls (n=6)	1688 (795-2658)	1305 (607-3038)	703 (455-1076)	617 (417-949)

\*p=0.05

**Results**

In the absence of mitogen, the number of lymphocytes from patients with primary biliary cirrhosis producing IgG was unaffected by preincubation with Cyclosporin A although the number of lymphocytes producing IgM was slightly higher after incubation with Cyclosporin A (p=0.05). When pokeweed mitogen was added, the number of cells producing IgG or IgM was unaffected by incubation with Cyclosporin A (Table 2). Similarly the number of cells from normal controls producing IgG or IgM, spontaneously or after the addition of pokeweed mitogen, was unaffected by incubation with Cyclosporin A.

**SUPPRESSOR CELL FUNCTION**

Preincubation of peripheral blood mononuclear cells with Cyclosporin A, before activation with concanavalin A, had a marked effect on the suppression of both IgG and IgM producing cells from 10 patients with primary biliary cirrhosis. Per cent suppression of IgG producing cells rose from 35.7±14.0 to 73.7±12.0 (mean±1 SD, p<0.01, Figure 1) while that for IgM producing cells rose from 46.0±13.0 to 76.0±8.0 (p<0.01, Figure 1). In contrast, preincubation of peripheral blood mononuclear cells with Cyclosporin A, before activation with concanavalin A, had no effect on suppression of IgG or IgM producing cells from 6 normal controls (mean % suppression±1 SD with and without Cyclosporin A 71.0±17.0 and

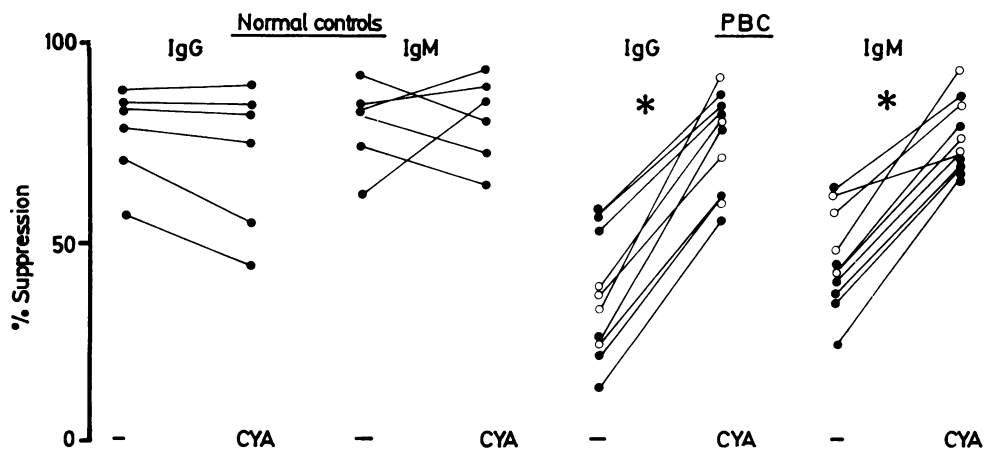


Fig. 1 Effect of incubation peripheral blood mononuclear cells with Cyclosporin A, before concanavalin A activation, on suppressor cell regulation of the number of IgG or IgM producing cells in normal controls and primary biliary cirrhosis. For those with primary biliary cirrhosis closed circles represent those with stage III or IV disease, open circles those with stage I or II disease (\*p<0.01).

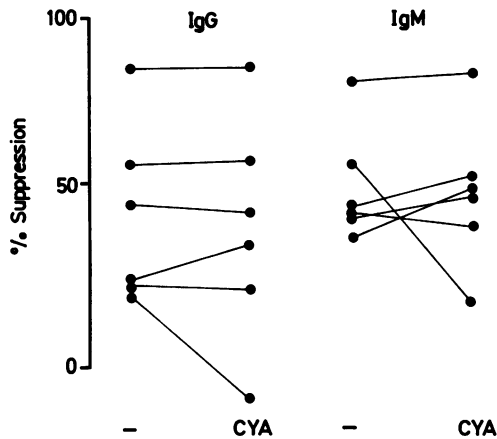


Fig. 2 Effect of incubation of peripheral blood mononuclear cells with Cyclosporin A, after activation with concanavalin A, on suppressor cell regulation of the number of IgG or IgM producing cells in primary biliary cirrhosis.

77.0±11.0 respectively for IgG and 81.0±9.0 and 79.0±10.0 respectively for IgM,  $p=NS$ ).

To determine whether the timing of exposure of peripheral blood mononuclear cells to Cyclosporin A was important, suppression was measured in patients with primary biliary cirrhosis with Cyclosporin A being added after peripheral blood mononuclear cells had been cultured with concanavalin A, but before the addition of pokeweed mitogen. No effect was seen on the suppression of IgG or IgM producing cells (mean %±1 SD, 42.2±22.0 and 41.0±28.0 for IgG and 51.0±13.0 and 50.0±18.0 for IgM in the absence or presence of Cyclosporin A respectively,

$p=NS$ , Fig. 2). To exclude an effect of the Cyclosporin A solvent, suppression was also measured with or without preincubation with the solvent, before exposure to concanavalin A in those with primary biliary cirrhosis. No effect on suppression was seen for either IgG (mean %±1 SD, 37.0±14.0 and 40.6±17.0 in the absence or presence of Cyclosporin A solvent respectively,  $p=NS$ ) or IgM (46.0±13.0 and 42.0±20.0 in the absence or presence of Cyclosporin A solvent respectively,  $p=NS$ ).

#### EFFECT OF VARIED CONCENTRATIONS OF CYCLOSPORIN A ON CONCAVALIN A INDUCED SUPPRESSION

The effect of preincubation with varied concentrations of Cyclosporin A, before concanavalin A activation, on suppression was further investigated in six of the patients with a defect of IgG suppression. The concentrations were 0, 50, 100, 250, 500, 750, and 1000 ng/ml. Insufficient lymphocytes were obtained from any individual to assess the effect at every concentration simultaneously, so that each was studied at alternate concentrations. Viability was unaffected at any concentration (at 50 and 100 ng/ml mean viability 98%, at 250 ng/ml 97%, at 500 ng/ml 96%, and at 1000 ng/ml 98%). No effect was seen on suppression of either IgG or IgM producing cells at 50 or 100 ng/ml Cyclosporin A, while the maximum effect on suppression was observed at 250 ng/ml, declining at higher concentrations (Fig. 3).

#### Discussion

The results of this study show that Cyclosporin A has a highly selective action on peripheral blood mono-

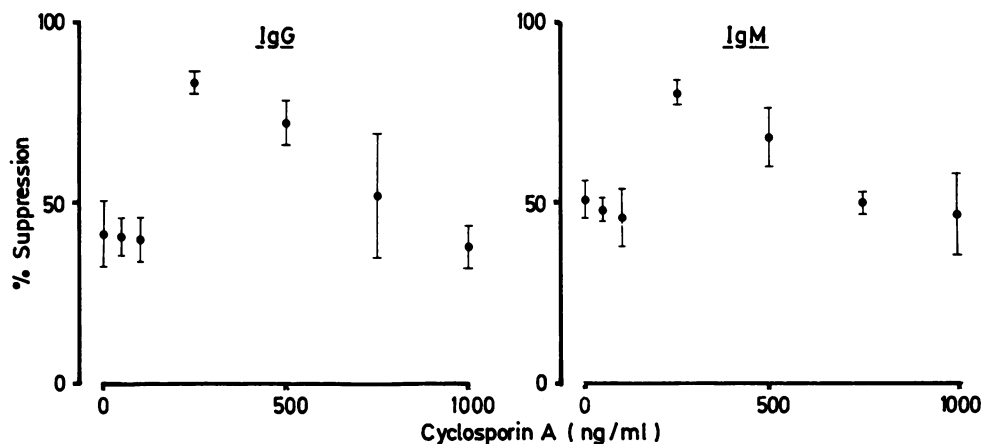


Fig. 3 Effect of incubation of peripheral blood mononuclear cells with various concentrations of Cyclosporin A, before concanavalin A activation, on suppressor cell regulation of the number of IgG or IgM producing cells in primary biliary cirrhosis (see text). Values are given as the mean±1 SD.

nuclear cells from patients with primary biliary cirrhosis and that this is confined to concanavalin A induced suppression of immunoglobulin production while spontaneous and pokeweed mitogen stimulated immunoglobulin production were unaffected. The defect of concanavalin A induced suppression is independent of the stage and severity of hepatic inflammation<sup>10</sup> and a similar defect has been identified in healthy first degree relations of patients with primary biliary cirrhosis.<sup>12</sup> Although a precise role for such a defect in the aetiology of primary biliary cirrhosis has not been defined, correction of the defect *in vitro* by Cyclosporin A is intriguing. Suppression, as measured in this study, however, is a balance between helper and suppressor cell function and the effect of Cyclosporin A may be due to a direct inhibitory action on B cells or be mediated by inhibition of concanavalin A activated T helper cells or stimulation of concanavalin A activated suppressor cells.

Although initial studies had suggested that B lymphocytes were resistant to Cyclosporin A<sup>15 16</sup> more recent studies in man and animals have indicated that certain B cells are sensitive.<sup>17</sup> In the mouse a proportion of B cells responsive to T independent antigens (T-I2) are Cyclosporin A sensitive<sup>18</sup> while in man Cyclosporin A modulates the T independent B cell response to anti- $\mu$  and *Staphylococcus aureus* Cowan.<sup>19</sup> The action of Cyclosporin A may be mediated by inhibition of induction of functionally active B cell growth factor receptors, essential for the proliferation of activated B cells.<sup>20</sup> Inhibition of B cells, however, is unlikely to underlie increased suppression seen in the present study as no effect was observed in either spontaneous or pokeweed mitogen stimulated culture. The lack of effect of Cyclosporin A in these cultures suggests that the cells are already fully activated, presumably a consequence of *in vivo* activation, and that they have already differentiated beyond the stage of activation that is sensitive to Cyclosporin A.

Cyclosporin A inhibits the response to several mitogens although concanavalin A is the lectin most sensitive to its action,<sup>21 22</sup> and concanavalin A activated T cells were the most likely target for Cyclosporin A in the present study. Previous studies have shown that Cyclosporin A can inhibit T helper cells<sup>23</sup> while a stimulatory effect on T suppressor cells is less likely. In the mixed leucocyte reaction, Cyclosporin A has been shown to inhibit cytotoxic cells selectively with much less effect on suppressor cells at the same dose.<sup>21 24</sup> Similarly, in a murine model of autoimmune haemolytic anaemia, Cyclosporin A appeared to allow the expression of suppressor cells regulating the production of auto-antibody to murine red cells, but not those regulat-

ing antibody production to rat erythrocytes, the priming antigen.<sup>25</sup> Thus Cyclosporin A exerts a dose dependent differential effect on various limbs of the immune system.

Several studies have suggested that the inhibitory effects of Cyclosporin A are probably directed against the earliest stages of activation. The precise level of inhibition remains to be elucidated, but Cyclosporin A allows expression of the Tac receptor, while inhibiting secretion of IL-1 and IL-2 and inhibiting expression of T9, T10 and DR antigens.<sup>26-30</sup> These observations may be related to inhibition of protein synthesis by unstimulated lymphocytes.<sup>30 31</sup> If, however, activation has already occurred, then Cyclosporin A exerts no effect, which explains why in this study Cyclosporin A was effective only before exposure to concanavalin A.

In this context, the observation that suppression is augmented at a concentration of 250 ng/ml but unaffected or inhibited at concentrations of >500 ng/ml is of interest. A possible explanation may be that Cyclosporin A exerts an inhibitory effect on T helper and T suppressor cells but that T suppressor cells are relatively resistant at lower concentrations—a further example, of the dose dependent differential effect previously observed.

A differential effect on the function of lymphocytes is consistent with the characteristics of uptake of Cyclosporin A by various mononuclear cells. There is no evidence of a specific receptor for Cyclosporin A on lymphocytes but binding characteristics are distinctive. B lymphocytes have both high and low affinity binding sites, while T lymphocytes have only low affinity binding sites and T helper cells bind Cyclosporin A more avidly than T suppressor cells, and binding is complete within 2–15 minutes.<sup>32</sup> The concentration used in the first part of this study is within the recommended range for trough plasma concentrations for patients undergoing organ transplantation. The concentrations at which suppressor cell function was unaffected or inhibited in the present study, 500–1000 ng/ml, are similar, however, to peak concentrations achieved in such patients.

We have previously shown that Cyclosporin A, at doses of 2–4 mg/kg, also corrects suppressor cell function *in vivo* in patients with primary biliary cirrhosis.<sup>33</sup> The clinical usefulness of Cyclosporin A in primary biliary cirrhosis may, however, be limited by its lack of effect on spontaneous immunoglobulin production as well as the narrow range of concentrations over which it inhibits suppressor cell function. Interestingly, the range of activity of Cyclosporin A *in vitro* in primary biliary cirrhosis was identical to that previously described for prednisolone in primary biliary cirrhosis,<sup>10</sup> while a

number of uncontrolled reports have documented improvement in liver function tests after administration of corticosteroids.<sup>8 9 34 35</sup>

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