

Can clinical response to cyclosporin in chronic severe asthma be predicted by an *in vitro* T-lymphocyte proliferation assay?

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Can clinical response to cyclosporin in chronic severe asthma be predicted by an in vitro T-lymphocyte proliferation assay? A.G. Alexander, N.C. Barnes, A.B. Kay, C.J. Corrigan. ©ERS Journals Ltd 1996.

ABSTRACT: This study tests the hypothesis that the clinical response to cyclosporin therapy of patients with chronic severe asthma is related to the sensitivity of their T-lymphocytes to the antiproliferative effects of cyclosporin *in vitro*. In a previous study, we observed such a relationship with glucocorticoids and the same lectin-driven proliferation assay was used in the present study.

Peripheral blood mononuclear cells were obtained from 33 patients participating in a cross-over trial of oral cyclosporin therapy during both cyclosporin and placebo treatment periods, and cultured in the presence of phytohaemagglutinin and serial dilutions of cyclosporin and dexamethasone. Proliferation was measured by tritiated thymidine uptake.

Both cyclosporin and dexamethasone inhibited T-lymphocyte proliferation in a concentration-dependent manner *in vitro* at concentrations encompassing those achieved in peripheral blood during therapy *in vivo*. T-lymphocytes from the asthmatic patients showed a range of sensitivity to the antiproliferative effects of cyclosporin, but this could not be correlated with improvements in peak expiratory flow rate (PEFR) or forced expiratory volume in one second (FEV₁) during cyclosporin therapy as compared with placebo.

In contrast to previous observations with glucocorticoids, this *in vitro* T-lymphocyte proliferation assay is not predictive of clinical response to cyclosporin therapy in chronic severe asthmatics.

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Glucocorticoids, by inhaler and when necessary systemically, are the mainstay of treatment for asthma [1], but a subset of patients respond poorly or not at all to glucocorticoid therapy [2, 3]. In a previous study, we classified a group of patients with chronic severe asthma as clinically glucocorticoid sensitive or resistant on the basis of changes in lung function after oral prednisolone therapy [3]. In a lectin-driven assay, dexamethasone at a therapeutic concentration (10^{-7} mol·L⁻¹) inhibited the proliferation [3] of, and cytokine elaboration [4] by, peripheral blood T-lymphocytes from the sensitive, but not the resistant patients. In contrast, cyclosporin at a therapeutic concentration (500 ng·mL⁻¹) significantly inhibited both proliferation of and cytokine production by T-lymphocytes from the resistant as well as sensitive patients [4]. Taking the sensitive and resistant patients together, the clinical response of the patients to glucocorticoid therapy correlated with the degree of inhibition of their peripheral blood T-lymphocytes by glucocorticoids *in vitro* [3].

In a recent double-blind, cross-over trial, we showed that cyclosporin therapy for 12 weeks resulted in a mean increase in peak expiratory flow rate (PEFR) of 12% as compared with placebo therapy ($p < 0.004$) in patients with oral glucocorticoid-dependent chronic severe asthma [5]. A spectrum of clinical response was observed, with some patients showing an increase in PEFR of greater

than 50% as compared with placebo, whilst others did not improve at all.

By analogy with glucocorticoids, we sought to obtain evidence, prospectively, that any clinical improvement of the asthmatic patients with cyclosporin therapy might be related to T-lymphocyte inhibition. We hypothesized that peripheral blood T-lymphocytes from these patients show a range of sensitivity to the antiproliferative effects of cyclosporin *in vitro*, and that the degree of inhibition of T-lymphocyte proliferation can be correlated with the extent of the clinical response to oral cyclosporin therapy. It might then be possible to use the T-lymphocyte proliferation assay to identify, prospectively, patients likely to respond clinically to cyclosporin therapy. We also wished to investigate our previous observation that the sensitivity of T-lymphocytes to inhibition by cyclosporin and dexamethasone are not linearly related, and whether the antiproliferative effects of the two drugs *in vitro* were influenced by oral administration of cyclosporin *in vivo*.

Methods

Patients

The clinical trial investigating the efficacy of cyclosporin therapy in asthma has been described in detail

Table 1. – Baseline (pretrial) patient characteristics, treatment and respiratory function data

Sex M/F	13/20
Age yrs*	49 (21–64)
Duration of asthma yrs*	27 (5–54)
Skin prick test positive# n	20
IgE IU·mL ⁻¹ *	146 (6–1250)
Continuous oral prednisolone yrs*	9.3 (0.3–25)
Prednisolone dosage mg·day ⁻¹ *	8.5 (5–20)
Inhaled glucocorticoid usage µg·day ⁻¹ +	1665 (90)
Oral theophylline usage n	23
PEFR L·min ⁻¹ +	239 (19.0)
% pred	54
FEV ₁ L ⁺	1.73 (0.14)
% pred	60
VC L ⁺	3.09 (0.21)
% pred	83

*: data presented as mean and range in parenthesis; +: data presented as mean and SEM in parenthesis; #: defined as one or more positive skin-prick tests to a range of 12 common aero-allergens in the presence of a positive histamine control and a negative vehicle control. M: male; F: female; IgE: immunoglobulin E; PEFR: peak expiratory flow rate; FEV₁: forced expiratory volume in one second; VC: vital capacity; % pred: percentage of predicted value.

previously [5]. Briefly, documented chronic asthmatics aged 18–65 yrs who had a forced expiratory volume in one second (FEV₁) and/or PEFR below 75% of the predicted value and >20% reversibility to β₂-agonist were considered for inclusion in the trial if they required long-term maintenance treatment with 5–20 mg oral prednisolone daily in addition to maximal, tolerated and effective other therapy, including high-dose inhaled glucocorticoids (table 1).

Written informed consent was obtained from each patient and the study was approved by the Ethics Committee of the Royal Brompton National Heart and Lung Hospitals.

Study design

After a 4 week run-in period, 33 subjects were randomized to receive cyclosporin or matching placebo for 12 weeks in addition to their usual treatment. Following a 2 week wash-out period, subjects crossed over to placebo or cyclosporin medication for a further 12 weeks. The cross-over design allows direct intrasubject comparisons, with each patient acting as his/her own control. For this reason, all treatment except inhaled bronchodilator usage was kept constant during the trial, although during exacerbations oral prednisolone dosage was increased if required. Exacerbations were defined as worsening of asthma symptoms in association with decreased PEFR, and were treated by increasing the prednisolone dosage. In view of the previously described effects both of disease exacerbation and oral prednisolone therapy on T-lymphocyte activation *in vivo* [6, 7], patients whose prednisolone dosage had been increased during a disease exacerbation and at the time of blood sampling were receiving prednisolone at a greater dosage than maintenance were excluded from the study.

Patients attended the clinic weekly for the first 4 weeks of each treatment period and, thereafter, every 2 weeks. Cyclosporin was started at 5 mg·kg⁻¹ daily and,

thereafter, dosage was adjusted to achieve whole blood concentrations of 100–200 ng·mL⁻¹, measured using a specific monoclonal radioimmunoassay (Cyclo-Trac SP; Incstar Corp., Stillwater MN, USA). The mean concentrations of all patients during weeks 9–12 of cyclosporin therapy was 151.7 (SEM 7.2) ng·mL⁻¹ [5].

Materials

Stock solutions were prepared as follows and the same batches used for all experiments. Appropriate dilutions were made up in RPMI-1640 (Gibco, Paisley, Scotland) containing 2 mmol·L⁻¹ L-glutamine for individual experiments.

Phytohaemagglutinin (PHA) (Sigma, Poole, UK) was prepared at a concentration of 100 µg·mL⁻¹ in RPMI-1640 and filtered sterile. This was stored in aliquots at -20°C and was added to cultured cells at a final concentration of 5 µg·mL⁻¹.

Dexamethasone (Sigma) was dissolved in ethanol to a concentration of 2×10⁻⁴ mol·L⁻¹ and stored at -20°C. In use, RPMI-1640 was added to produce a final stock concentration of 2×10⁻⁶ mol·L⁻¹.

Cyclosporin oily suspension (100 mg·mL⁻¹) (Sandoz Pharmaceuticals) was dispersed with thorough mixing in RPMI-1640 to a concentration of 10 µg·mL⁻¹ and stored in aliquots at -20°C. Cyclosporin oily suspension vehicle (Sandoz Pharmaceuticals) was diluted in the same way.

Serial dilutions of drugs were made at the time of each experiment by dissolving the stock solutions in RPMI-1640 supplemented with 2 mmol·L⁻¹ L-glutamine. Solutions were filtered sterile through a 0.22 µm pore size filter as appropriate.

Peripheral blood mononuclear cell isolation and culture

Fifty millilitres of peripheral venous blood were obtained from each patient at the same time of day at a clinic visit during the last 4 weeks both of the cyclosporin and placebo treatment periods, a mean of 17.0 (SEM 0.44) h after the previous cyclosporin (or placebo) dose. The blood was added to preservative-free heparin in a sterile fashion and then mixed with an equal volume of RPMI-1640 medium. Thirty millilitre aliquots were layered onto 20 mL aliquots of Ficoll-Paque (Pharmacia, Uppsala, Sweden) in 50 mL sterile conical tubes (Falcon, Becton Dickinson, Cowley, UK). After centrifugation at 400×g for 20 min at 20°C, peripheral blood mononuclear cells (PBMCs) were removed from the plasma/Ficoll interface with gentle suction, transferred to sterile polystyrene universal containers, and washed three times with RPMI-1640. Cell manipulations were performed at 4°C.

PBMCs were resuspended in RPMI-1640 supplemented with 2 mmol·L⁻¹ L-glutamine at 4×10⁶ cells·mL⁻¹. Seventy five microlitre aliquots of this cell suspension, with or without added PHA, were added to 75 µL aliquots of serially diluted cyclosporin and dexamethasone solutions or medium control in triplicate in sterile 96-well round-bottomed culture plates (Cel-cult; Sterilin, Hounslow, UK). The total volume in each well was 150 µL, with final concentrations of the cell suspension of 2×10⁶ cells·mL⁻¹, PHA of 5 µg·mL⁻¹, and drug concentrations in the range 25–5,000 ng·mL⁻¹ for cyclosporin and 10⁻⁹–10⁻⁶ mol·L⁻¹ for dexamethasone.

Culture plates were incubated for 72 h in a humidified atmosphere with 5% carbon dioxide. T-lymphocyte proliferation was measured by uptake of tritiated methylthymidine. Sterile tritiated thymidine solution (Amersham, Amersham, UK) ($24.6 \text{ kBq}\cdot\text{well}^{-1}$ in a volume of $10 \mu\text{L}$) was added to cell culture wells for the last 6–8 h of the 72 h incubation period. After incubation, cells were harvested onto glass-fibre filter paper with a cell harvester apparatus (Ilacon, Tonbridge, UK), and the incorporated radiolabel was counted with a beta-spectrometer. After the geometric mean of the triplicate counts was calculated, results were expressed as percentages of incorporated counts in the presence of drugs as compared with the medium only (positive) control. Dose-response curves were constructed for suppression of T-lymphocyte proliferation by cyclosporin and by dexamethasone.

Statistical analysis

Analysis of the clinical trial has been reported previously [5], but briefly, daily morning prebronchodilator PEFR data for each patient were summarized as a mean for the last 4 weeks of each treatment period. Clinical improvement in mean PEFR was determined as percentage difference between cyclosporin therapy and placebo, *i.e.* $((\text{cyclosporin}/\text{placebo}) \times 100) - 100$.

Dose-response data for the antiproliferative effects of cyclosporin and dexamethasone on T-lymphocytes from the asthmatic patients are expressed as the mean (SEM) percentages of the proliferation observed in the presence of the medium control for each treatment period (fig. 1). These data conformed to a Gaussian distribution at each drug concentration.

In vitro concentrations of $250 \text{ ng}\cdot\text{mL}^{-1}$ cyclosporin and $10^{-7} \text{ mol}\cdot\text{L}^{-1}$ dexamethasone were used in analysis of the antiproliferative effects of these drugs on PBMC

in vitro as they most closely represent the therapeutic concentrations achieved in these patients *in vivo* during cyclosporin [5] and glucocorticoid [3] therapy, respectively. Between-drug and between-treatment comparisons were made using the paired t-test. Subgroup comparisons of atopic status and of theophylline usage were made using the two sample t-test. Probability values for correlation coefficients were calculated using Pearson's test. A two-tailed probability of 5% or less was considered statistically significant. Analysis was performed using the Minitab statistical software package (State College, Pennsylvania, USA).

Results

Thirty patients completed the clinical trial [5], one failing to cross over to cyclosporin therapy and two to placebo. Four patients were excluded during placebo and three during cyclosporin therapy since at the proposed time of blood sampling they were receiving prednisolone at increased dosage during a disease exacerbation (see Methods). Cell culture was unsuccessful for one patient during placebo and three during cyclosporin therapy, and for one patient during placebo therapy no data for dexamethasone inhibition were available. Data are, therefore, available for analysis for 26 patients during placebo and 26 patients during cyclosporin therapy. For between-treatment comparisons, paired data from both treatment periods were available for cyclosporin for 19 patients and for both drugs for 18 patients.

Addition of cyclosporin and dexamethasone to PHA-stimulated peripheral blood T-lymphocytes from the 26 asthmatic patients described above in the concentration ranges $25\text{--}5,000 \text{ ng}\cdot\text{mL}^{-1}$ ($2.1 \times 10^{-8}\text{--}4.2 \times 10^{-6} \text{ mol}\cdot\text{L}^{-1}$) and $10^{-9}\text{--}10^{-6} \text{ mol}\cdot\text{L}^{-1}$, respectively, resulted in dose-dependent suppression of proliferation (fig. 1). No proliferation was observed in the absence of PHA. Viability

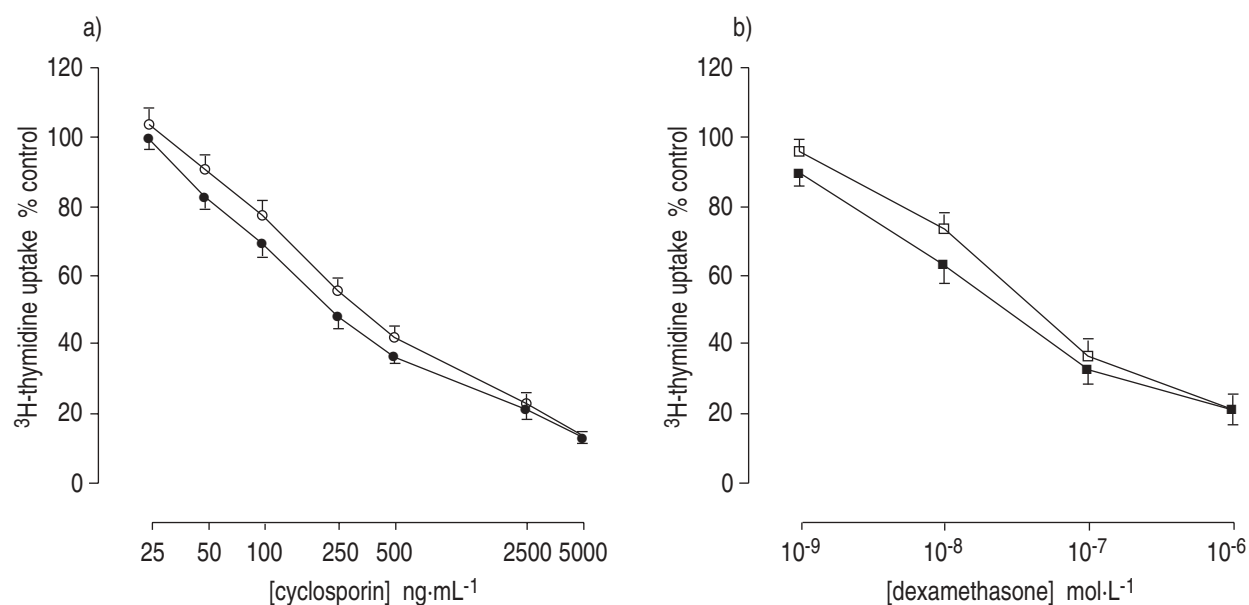


Fig. 1. — Dose-response curves for mean inhibition by: a) cyclosporin; and b) dexamethasone of *in vitro* PHA-induced proliferation of peripheral blood T-lymphocytes from 26 patients with chronic severe asthma during therapy with placebo (open symbols) and cyclosporin (closed symbols). Uptake of ^3H -thymidine (counts per minute) is expressed as a percentage of that measured in the presence of the medium (no drug) control. Vertical bars represent the SEM. PHA: phytohaemagglutinin.

studies with trypan blue exclusion showed no toxic effect of either drug at any concentration employed. The ethanol and cyclosporin vehicles showed no significant effect on lymphocyte proliferation at concentrations used in the lowest drug dilutions.

T-lymphocytes from the asthmatic patients showed a wide range of sensitivity to the antiproliferative effects of both cyclosporin at a concentration of 250 ng·mL⁻¹ (fig. 2a) and dexamethasone at 10⁻⁷ mol·L⁻¹ (fig. 2b) *in vitro*. Although PBMCs from some of the asthmatic patients showed considerable variability in their susceptibility to the antiproliferative effects of cyclosporin *in vitro* when receiving cyclosporin as compared with placebo therapy (fig. 2a), there was no significant difference between the mean percentage inhibition of proliferation in each treatment period when the patients were considered as a group (p=0.73). Similarly, no difference was observed with dexamethasone (fig. 2b) (p=0.71), suggesting that oral cyclosporin therapy did not influence, in a consistent manner, the sensitivity of PBMCs to the antiproliferative effect of either cyclosporin or dexamethasone *in vitro*. There were no significant differences between the antiproliferative effects of either cyclosporin or dexamethasone when atopic patients were compared with nonatopic patients, or according to oral theophylline usage (data not shown).

The sensitivity of PHA-activated peripheral blood T-lymphocytes from the asthmatic patients during placebo therapy to the antiproliferative effects of cyclosporin at an *in vitro* concentration of 250 ng·mL⁻¹, expressed as a percentage of the proliferation observed in the presence of medium control, did not correlate with the clinical response of the patients to cyclosporin therapy, as measured [5] by percentage increases in either morning prebronchodilator PEFr (p=0.47) or FEV₁ (p=0.14) during cyclosporin therapy as compared with placebo.

No correlation was observed between the antiproliferative effects of cyclosporin at 250 ng·mL⁻¹ and dexamethasone at 10⁻⁷ mol·L⁻¹ on T-lymphocytes from the asthmatic patients obtained during the placebo treatment period.

Discussion

In this study, we have demonstrated a range of sensitivity of PHA-activated peripheral blood T-lymphocytes from patients with glucocorticoid-dependent chronic severe asthma to the antiproliferative effects of cyclosporin *in vitro*. This is in line with our previous studies showing a range of sensitivity of T-lymphocytes to the antiproliferative effects of cyclosporin in other groups of patients with chronic severe asthma [4, 8]. Although these patients also showed a range of improvement in lung function when receiving cyclosporin therapy as compared with placebo, no relationship was observed between this clinical response and the sensitivity of the patients' T-lymphocytes to inhibition by cyclosporin *in vitro*. This proliferation assay is, therefore, not predictive of the degree of clinical response to oral cyclosporin therapy *in vivo*.

T-lymphocyte sensitivity both to glucocorticoids and cyclosporin in a similar assay was shown to correlate with outcome of renal allograft rejection and survival in patients with chronic renal failure [9]. In our clinical trial of cyclosporin therapy in asthma [5], no associations were observed between clinical response and variables such as age, atopic status, baseline lung function, or duration of disease. An *in vitro* assay to identify, prospectively, patients more (or less) likely to respond clinically to cyclosporin therapy might, therefore, have been a useful screening test and assessment of its possible value was the main objective of this study. These findings are in contrast to our previous study [3] of glucocorticoids, which suggested that asthmatic patients clinically refractory to oral prednisolone therapy *in vivo* have peripheral blood T-lymphocytes which are relatively resistant to the antiproliferative effects of dexamethasone *in vitro*. As the patients in the present study were receiving long-term oral prednisolone, it was not possible to measure their changes in lung function following a 2 week course of prednisolone, a test which we previously used to define clinical glucocorticoid sensitivity or resistance [3]. Although some individuals who showed clinical

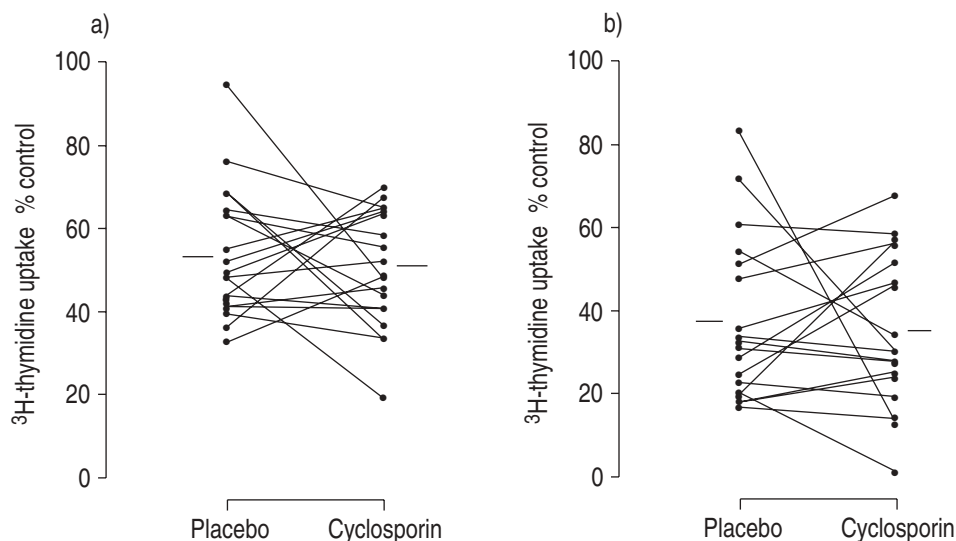


Fig. 2. — Comparison of the inhibition of PHA-induced proliferation of peripheral blood T-lymphocytes from patients with chronic severe asthma by: a) cyclosporin (250 ng·mL⁻¹; n=19); and b) dexamethasone (10⁻⁷ mol·L⁻¹; n=18) while receiving placebo and cyclosporin therapy (see text). ³H-thymidine uptake (counts per minute) is expressed as a percentage of that measured in the presence of medium (no drug) control. Horizontal bars represent means. PHA: phytohaemagglutinin.

improvement during cyclosporin therapy had peripheral blood T-lymphocytes which were relatively resistant to the antiproliferative effects of dexamethasone *in vitro*, suggesting that they might have been clinically resistant to glucocorticoid therapy, we were unable to confirm this and, thus determine whether asthmatic patients clinically resistant to glucocorticoids might be clinically sensitive to cyclosporin therapy.

A number of points may be relevant to the observed absence of an association between the *in vivo* effects of cyclosporin in ameliorating asthma and its *in vitro* effects on T-lymphocyte function in this assay.

Firstly, although two subsequent controlled clinical trials have shown cyclosporin to be efficacious in similar groups of patients [10, 11], the mechanism by which cyclosporin exerts its antiasthma effect is unknown. It is possible that inhibitory actions on other inflammatory cells, such as mast cells [12] and eosinophils [13], are involved. However, cyclosporin is believed to exert its immunosuppressive action principally by inhibiting T-lymphocyte activation [14, 15], and we have recently shown that the clinical response of the same group of patients to cyclosporin therapy was associated with a reduction in their serum concentrations of soluble interleukin-2 receptor [16], a marker of T-lymphocyte activation.

Secondly, since the mechanism of any action of cyclosporin on T-lymphocytes which results in an antiasthma effect is unknown, it is possible to hypothesize that modulation of cytokine elaboration or function by cyclosporin may be more relevant than inhibition of T-lymphocyte proliferation in this regard, and that cytokine production is not necessarily related to cell proliferation. Inhibition by cyclosporin of production by T-lymphocytes of cytokines relevant to asthma pathogenesis may, therefore, be a more appropriate predictor of its clinical efficacy than a proliferation assay. However, we had previously shown, using the same proliferation assay, that *in vitro* sensitivity to glucocorticoids was related to clinical response to prednisolone [3], and as the aim of the present study was to investigate whether the same relationship existed with cyclosporin, we used the same assay protocol.

Thirdly, a possible relationship may have been obscured by the following confounding factors. The patients were receiving variable dosages of oral prednisolone and their T-lymphocytes showed a wide range of sensitivity to the antiproliferative effects of glucocorticoids *in vitro* (fig. 2b). Although, as in a previous study [4], we failed to demonstrate a direct relationship between the antiproliferative effects of glucocorticoids and cyclosporin on peripheral blood T-lymphocytes from these asthmatic patients, this does not exclude the possibility of some degree of interaction of their antiasthma effects *in vivo*. This factor may be relevant, despite the fact that we excluded from the study those patients receiving dosages of prednisolone higher than their maintenance dosage at the time of blood sampling during disease exacerbations.

In addition, *in vitro* T-lymphocyte sensitivity to antiproliferative drugs may be subject to short-term variability. In the case of glucocorticoid sensitivity, we previously showed that our *in vitro* assay is highly reproducible [3, 8], both in terms of the coefficient of variability of repeated assays on the same subjects and also in the longer

term, if the patients are retested after 4–6 months. Furthermore, T-lymphocytes from asthmatic patients were shown to be consistently sensitive or resistant to dexamethasone *in vitro*. We have similarly analysed the variability of repeated measurements of the inhibition of PHA-induced T-lymphocyte proliferation by cyclosporin *in vitro* in a group of seven normal subjects (data not shown). Paired measurements of the cyclosporin concentration inhibiting 50% of the proliferation observed in the absence of drug (IC₅₀) were made at intervals of 7–14 days and the cyclosporin log₁₀ IC₅₀ compared, as described for dexamethasone [8]. The coefficient of variability of the logarithms of the differences in IC₅₀ values measured on the two occasions was 10.5%. Thus, the repeatability of the proliferative assay itself, when not potentially confounded by the effects of superadded therapy, is high. In this regard, it should be noted that the paired measurements shown in figure 2a do not necessarily reflect the inherent repeatability of the assay, since they were made in the presence and absence of cyclosporin therapy.

Although concomitant cyclosporin therapy of the asthmatic patients as a group did not result in any statistically significant change in the susceptibility of their peripheral blood T-lymphocytes both to cyclosporin and dexamethasone inhibition *in vitro* (in contrast to observations [17] on the inhibition by cyclosporin of mediator release from basophils *ex-vivo*), it may still have influenced these paired measurements within individuals. Similarly, it is possible that concomitant, variable oral prednisolone therapy may have influenced T-lymphocyte sensitivity to cyclosporin inhibition *in vitro* in certain individuals. Nevertheless, although there may be some interaction of the activities of cyclosporin and glucocorticoids in ameliorating asthma, our data suggest that this is apparently not evident in terms of any consistent antiproliferative effect *in vitro* on peripheral blood T-lymphocytes.

Finally, the lack of predictive value of the *in vitro* assay may reflect the fact that inhibitory effects of cyclosporin on T-lymphocytes within the bronchial mucosa, which might be more relevant to the improvement of clinical asthma, differ from those on peripheral blood T-lymphocytes. This possibility may be clarified further in future, if preparations of cyclosporin suitable for topical application to the bronchial mucosa become available. Indeed, it may then be possible to develop a short-term clinical predictive test for the antiasthma efficacy of cyclosporin, similar to that which we developed for glucocorticoids [3]. At present, in the case of orally administered cyclosporin, data from our existing trial [5] showed that the clinical response to cyclosporin therapy was not rapid, but progressive over the 12 week duration of the trial. Thus, any clinical trial of oral cyclosporin therapy might have to be conducted over a period of at least 12 weeks, limiting its usefulness as a "screening test". Furthermore, it was shown that clinical responsiveness to cyclosporin therapy could not be predicted by an exhaustive range of clinical variables, including age, gender, atopic status, baseline lung function and disease duration. It seems likely, therefore, that reliable tests for the antiasthma efficacy of cyclosporin must await more relevant *in vitro* tests and/or the possibility of assessment of topical therapy, which might produce more clear-cut

and rapid clinical responses, with the additional advantage of a higher benefit/risk ratio.

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