

The effect of dexamethasone, cyclosporine, and rapamycin on T-lymphocyte proliferation in vitro: Comparison of cells from patients with glucocorticoid-sensitive and glucocorticoid-resistant chronic asthma

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Inhibition of T-lymphocyte activation may provide a useful approach to the treatment of chronic severe asthma. We compared rapamycin, a novel immunosuppressive drug, with cyclosporine and dexamethasone for its effects in inhibiting proliferation of T lymphocytes from patients with glucocorticoid-resistant and glucocorticoid-sensitive asthma.

Phytohemagglutinin-stimulated peripheral blood T lymphocytes from 11 patients with clinically glucocorticoid-resistant and 8 patients with glucocorticoid-sensitive chronic asthma were tested for sensitivity to these drugs in a highly reproducible proliferation assay. All drugs inhibited proliferation in a dose-dependent manner (10^{-6} to 10^{-10} mol/L). T lymphocytes from the patients with glucocorticoid-resistant asthma were significantly less sensitive ($p < 0.01$) to dexamethasone than those of patients with glucocorticoid-sensitive asthma over a wide concentration range. In contrast, cyclosporine and rapamycin inhibited cells from both patient groups to an equivalent extent. The presence of exogenous interleukin-2 abrogated the inhibitory effect of dexamethasone but not that of cyclosporine or rapamycin, suggesting that dexamethasone may act principally by inhibition of interleukin-2 production, whereas the latter drugs exert distinct or additional inhibitory effects. Stimulation of peripheral blood T lymphocytes with phytohemagglutinin for 24 hours before addition of the drugs abolished the inhibitory effect of dexamethasone and significantly reduced that of cyclosporine. The inhibitory effect of rapamycin was, however, unaltered. These data suggest that dexamethasone and cyclosporine exert their effects only at an early stage of T-lymphocyte activation, whereas rapamycin is able to inhibit lymphoblasts. The fact that the inhibitory mechanisms of these drugs are different might explain why cyclosporine and rapamycin are effective in inhibiting T lymphocytes from both patients with glucocorticoid-sensitive and those with glucocorticoid-resistant asthma. The data further suggest that cyclosporine and rapamycin may be effective for the therapy of glucocorticoid-resistant asthma. (J ALLERGY CLIN IMMUNOL 1994;93:510-19.)

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Over the past few years studies have accumulated in support of a role for T lymphocytes in the pathogenesis of asthma. Activated T lymphocytes,

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Abbreviations used

FEV₁: Forced expiratory volume in 1 second

IC₅₀: Fifty percent inhibition of cellular proliferation observed in the absence of drug

IL: Interleukin

PBMCs: Peripheral blood mononuclear cells

PHA: Phytohemagglutinin

through their release of specific cytokines, may be at least partly responsible for the accumulation and activation of eosinophils in the bronchial

mucosa, a cardinal feature of asthma. Activated (CD25-positive) lymphocytes have been identified in the bronchial mucosa, bronchoalveolar lavage fluid, and peripheral blood of patients with asthma in elevated numbers as compared with control subjects.¹⁻³ In various studies on patients with asthma the degree of T-lymphocyte activation has been shown to correlate with eosinophilia and disease severity.³⁻⁵ Furthermore, peripheral blood T-lymphocyte activation was shown to be reduced after glucocorticoid therapy in patients with acute severe asthma to a degree that correlated with the extent of clinical improvement.⁵ Finally, cells both in the bronchial mucosa and bronchoalveolar lavage fluid of patients with asthma have been shown to synthesize messenger RNA encoding cytokines, which are relevant to eosinophil accumulation and activation.^{6, 7}

Glucocorticoids form the mainstay of current asthma therapy. There exists, however, a subset of patients with asthma who respond poorly or not at all to glucocorticoid therapy.⁸ Recent studies of patients with glucocorticoid-resistant asthma have shown that clinical resistance to therapy was associated with a relative unresponsiveness of T lymphocytes from these patients to the inhibitory effects of therapeutic concentrations of glucocorticoids *in vitro*.⁹⁻¹¹ This suggests that T-lymphocyte inhibition may be one mechanism of action of glucocorticoids in asthma and also that alternative drugs that inhibit T-lymphocyte function may be effective in asthma therapy. Characterization of other anti-T-lymphocyte drugs for their possible therapeutic benefits in asthma is therefore of great interest, particularly for those patients who are poorly responsive to glucocorticoids.

We hypothesized that the glucocorticoid dexamethasone, the undecapeptide cyclosporine and macrolide rapamycin, which have all been shown to inhibit T-lymphocyte proliferation *in vitro* might exert their inhibitory effects by different mechanisms and consequently, that T lymphocytes from patients with glucocorticoid-resistant asthma, which are refractory to glucocorticoid inhibition *in vitro*, might be sensitive to inhibition by cyclosporine and rapamycin. We therefore compared the inhibitory effects of dexamethasone, cyclosporine, and rapamycin on the proliferation of lectin-activated T lymphocytes isolated from patients with glucocorticoid-sensitive and glucocorticoid-resistant asthma *in vitro*. In addition, we tested the reversibility of this inhibition by exogenous interleukin-2 (IL-2) to determine to what extent the action of these drugs may be accounted for by inhibition of IL-2 synthesis.

Finally, we examined the effects of these drugs on T lymphocytes activated by lectin 24 hours before drug exposure to determine whether the drugs exert an inhibitory effect on T lymphocytes at an early phase of activation or on lymphoblasts committed to proliferation.

METHODS

Patient selection

Patients with moderate to severe asthma (baseline forced expiratory volume in 1 second [FEV₁] less than 70% of the predicted value despite regular high-dose inhaled glucocorticoid therapy) were characterized clinically as sensitive or resistant to oral prednisolone therapy according to their FEV₁ response to orally administered prednisolone.^{8, 9} Baseline FEV₁ (before bronchodilator administration) was measured in all patients with a dry bellows spirometer (Vitalograph, Buckingham, U.K.). The patients were then prescribed oral prednisolone 20 mg daily, and FEV₁ (before bronchodilator administration) was remeasured again after 7 days at the same time of day. If the FEV₁ had not improved by more than 15% of baseline, patients were prescribed prednisolone 40 mg daily for an additional 7 days. Consumption of tablets was verified by the attending physician. Patients who showed an increase in prebronchodilator FEV₁ of 15% or more from baseline after this 14-day course of oral glucocorticoid therapy were classified as sensitive; the remainder were classified as resistant. Eight sensitive and 11 resistant patients were recruited for the present study (Tables I and II). Informed consent was obtained from all patients before participation in the study, which was approved by the Ethical Committees of the Royal Brompton and Western General Hospitals.

All patients were afebrile throughout the study, had normal chest x-ray films, and did not have any other chronic disease. In selecting patients, strict criteria were invoked for the diagnosis of asthma and to exclude patients with irreversible airway obstruction. Thus all patients were required to demonstrate reversibility of at least 20% in their peak expiratory flow rate or FEV₁ either spontaneously or after administration of inhaled β_2 -agonist (nebulized salbutamol 5 mg). Smokers were excluded.

Materials

Stock solutions were prepared as follows: appropriate dilutions were made up in RPMI-1640 (Gibco, Paisley, Scotland) containing 2 mmol/L L-glutamine for individual experiments. The same batches of stock solutions were used for all experiments.

Phytohemagglutinin (PHA) (Sigma, Poole, England) was prepared at a concentration of 100 mg/ml in RPMI-1640 and sterile-filtered. This was stored in aliquots at -20° and was added to cultured cells at a final concentration of 5 μ g/ml.

Human IL-2 (recombinant) (British Biotechnology, Oxford, England) was reconstituted in 1 ml of RPMI-

TABLE I. Demographic characteristics of glucocorticoid-sensitive patients and their FEV₁ response to oral prednisolone

Age (yr)	Sex	Duration of asthma (yr)	Therapy*	FEV ₁ (% predicted)		
				Before†	After†	Percent change‡
68	F	48	Bec 2000 µg	43.9	94.4	102.5
45	M	21	Bec 2000 µg	39.4	51.5	30.8
			Theo 900 mg			
55	M	32	Bud 1600 µg	53.1	78.1	47.1
57	F	20	Bec 2000 µg	40.0	52.0	30.0
			Theo 450 mg			
53	F	28	Bec 1500 µg	44.0	64.0	45.5
			Theo 675 mg			
36	F	31	Bec 1500 µg	38.7	61.3	58.3
70	F	48	Bec 2000 µg	65.0	100.0	53.8
51	F	14	Bec 1000 µg	52.2	68.7	31.7

Bec, Beclomethasone dipropionate; Theo, theophylline-based preparation; Bud, budesonide.

*Therapy (inhaled or oral) at the time of the trial of oral prednisolone (see Methods section). All patients were receiving inhaled β_2 -agonists. Doses represent total daily doses.

†Prebronchodilator FEV₁ (percent of predicted value) before and after trial of oral prednisolone therapy (see Methods section).

‡Change in FEV₁ after oral prednisolone therapy expressed as a percentage of the baseline value.

1640 containing 0.1% bovine serum albumin to produce a stock solution of 10,000 U/ml, then aliquoted and stored at -80°C . In use, appropriate dilutions were prepared in RPMI-1640 containing 0.1% wt/vol bovine serum albumin.

Disodium dexamethasone (Sigma) was dissolved directly in RPMI-1640 at a concentration of 10^{-4} mol/L.

Cyclosporine oily suspension (100 mg/ml) (a kind gift from Sandoz Pharmaceuticals) was diluted in ethanol, then in RPMI-1640 to a final concentration of 10^{-4} mol/L.

Rapamycin (a kind gift from Wyeth-Ayerst Research) was dissolved in ethanol at a concentration of 20 mg/ml and stored in glass vials in aliquots. In use, RPMI-1640 was added to produce a final stock concentration of 10^{-4} mol/L.

Serial 10-fold dilutions of drugs were made at the time of each experiment by dissolving the stock solutions in RPMI-1640 supplemented with 2 mmol/L L-glutamine. Solutions were sterile-filtered through a 0.22 µm pore size filter as appropriate.

Peripheral blood mononuclear cell isolation and culture

Fifty milliliters of peripheral venous blood was taken from each of the 19 patients with asthma at a time point remote from the trial of oral glucocorticoid therapy and added to preservative-free heparin in a sterile fashion. Blood was mixed with an equal volume of RPMI-1640 medium. Thirty-milliliter aliquots were layered onto 20 ml aliquots of Ficoll-Paque (Pharmacia, Uppsala, Sweden) in 50 ml sterile conical tubes (Falcon, Becton Dickinson, Cowley, England). After centrifuging at 400 g for 20 minutes 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 twice with RPMI-1640.

PBMCs isolated as described above were resuspended in RPMI-1640 supplemented with 2 mmol/L L-glutamine at 4×10^6 /ml. Cells were cultured in triplicate in sterile 96-well round-bottomed culture plates (Cel-cult, Sterilin, Hounslow, England). The precise culture conditions are described below.

Culture plates were incubated for 48 hours in a humidified atmosphere with 5% carbon dioxide. Cell proliferation was measured by uptake of tritiated methylthymidine. Sterile tritiated thymidine solution (Amersham, Amersham, England) (24.6 kBq/well in a volume of 10 µl) was added to cell culture wells for the last 6 to 8 hours of the 48-hour incubation period. After incubation, cells were harvested onto glass-fiber filter paper with a cell harvester apparatus (Skatron, Lier, Norway), and the incorporated radiolabel was counted with a beta-spectrometer. After the geometric mean of triplicate counts was calculated, results were expressed as the percentage inhibition of incorporated counts in the presence of the drugs as compared with the positive (no drug) control.

Measurement of the effects of dexamethasone, cyclosporine, and rapamycin on the proliferative response of T lymphocytes

PBMCs were resuspended in RPMI-1640 with 2 mmol/L L-glutamine at 4×10^6 /ml. Eighty-microliter aliquots of this cell suspension with or without added PHA were added to 20 µl aliquots of serially diluted drug solutions or medium control in triplicate in sterile 96-well culture plates as described above. Sixty microliters of medium was added to produce a total volume of 160 µl and final concentrations of the cell suspension

TABLE II. Demographic characteristics of glucocorticoid-resistant patients and their FEV₁ response to oral prednisolone

Age (yr)	Sex	Duration of asthma (yr)	Therapy*	FEV ₁ (% predicted)		
				Before†	After†	Percent change‡
53	F	22	Bec 2000 µg Theo 450 mg	40.9	45.5	11.1
49	M	19	Bec 2000 µg	55.6	55.6	0.0
70	F	57	Bud 1600 µg	45.0	45.0	0.0
56	M	32	Bud 1600 µg	41.2	35.3	-14.3
48	M	18	Ipra 2000 µg Bud 1600 µg	38.7	35.5	-8.3
62	M	14	Theo 400 mg Bud 1600 µg	60.0	66.1	10.1
56	F	55	Theo 900 mg Ipra 2000 µg Bud 1600 µg	22.5	24.0	6.7
62	M	10	Theo 450 mg Bec 2000 µg	33.3	33.9	1.8
62	M	8	Bec 1000 µg	56.5	61.3	8.6
70	M	6	Bec 1500 µg	53.6	58.9	10.0
49	F	6	Bec 1000 µg	63.2	68.0	7.6

Bec, Beclomethasone dipropionate; Theo, theophylline-based preparation; Bud, budesonide; Ipra, ipratropium bromide.

*Therapy (inhaled or oral) at the time of the trial of oral prednisolone (see Methods section). All patients were receiving inhaled β_2 -agonists. Doses represent total daily doses.

†Prebronchodilator FEV₁ (percent of predicted value) before and after trial of oral prednisolone therapy (see Methods section).

‡Change in FEV₁ after oral prednisolone therapy expressed as a percentage of the baseline value.

of 2×10^6 cell/ml, PHA of 5 µg/ml, and drug solutions as shown in the Results section. Culture plates were incubated for 48 hours, and T-lymphocyte proliferation was measured as described above.

Measurement of the effect of exogenous IL-2

Preliminary dose-response studies with different concentrations of IL-2 (0.1 to 100.0 U/ml) showed that optimal enhancement of T-lymphocyte proliferation in the presence of PHA occurred at 10 U/ml (data not shown). PBMCs were resuspended in RPMI-1640 containing 2 mmol/L L-glutamine at 4×10^6 cells/ml. Eighty microliters of PBMC suspension with and without PHA (5 µg/ml) and 20 µl of serially diluted drug solutions or medium control were placed in wells in triplicate. Twenty-microliter aliquots of IL-2 stock solution and 40 µl of RPMI-1640 were added to each well to produce a total volume of 160 µl and a final concentration of IL-2 of 10 U/ml with cell numbers, PHA concentrations, and drug concentrations as before. Culture plates were incubated for 48 hours, and T-lymphocyte proliferation was measured as described above.

Preincubation of PBMCs with PHA before drug addition

PBMCs isolated and resuspended as described above were cultured at 2.5×10^6 cells/ml with or without PHA (5 µg/ml) in a volume of 120 µl. After 24 hours, 20 µl of serially diluted drug solutions and 20 µl of

IL-2 stock solution were added to the cell cultures as before. After an additional 48 hours of incubation, cellular proliferation was measured as described above.

Statistical methods

Pooled data from groups of patients are expressed as means $\pm$ SEM percentage inhibition of proliferation. Significance levels were calculated with the Mann-Whitney U test. The *p* values refer to the differences between the inhibitory effects of dexamethasone or T lymphocytes from patients with glucocorticoid-resistant and glucocorticoid-sensitive asthma (Fig. 1), the differences between the effects of the various drugs in the presence and absence of exogenous IL-2 (Fig. 2), and the differences between the effects of the various drugs added at the time of initiation of T-lymphocyte activation and 24 hours later (Fig. 3).

RESULTS

Inhibition of proliferation of T lymphocytes from patients with glucocorticoid-sensitive and glucocorticoid-resistant asthma

Addition of dexamethasone, cyclosporine, and rapamycin to cultured peripheral blood T lymphocytes from both patients with glucocorticoid-sensitive asthma and those with glucocorticoid-resistant asthma at a concentration range of 10^{-10} to 10^{-6} mol/L resulted in a dose-dependent suppres-

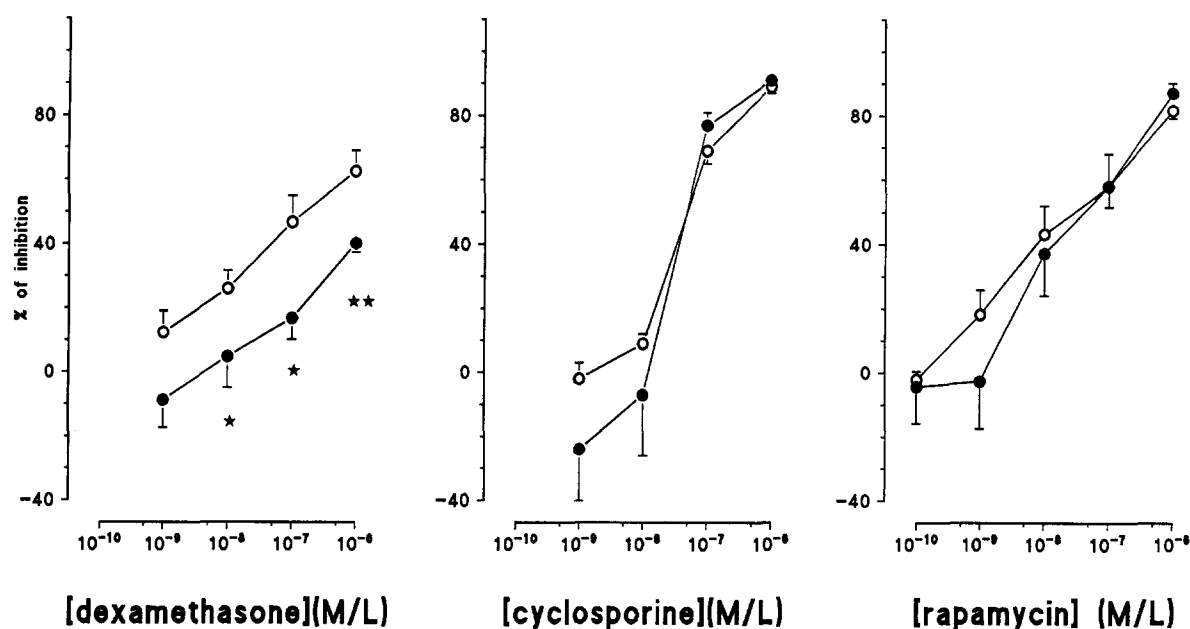


FIG. 1. The effect of dexamethasone, cyclosporine, and rapamycin on PHA-induced proliferation of T lymphocytes from patients with glucocorticoid-resistant (solid circles; $n = 11$) and those with glucocorticoid-sensitive (open circles; $n = 8$) asthma. Results are expressed as percentage inhibition of proliferation as compared with medium (no drug) control. * $p < 0.05$; ** $p < 0.01$; bars represent the standard error of the mean.

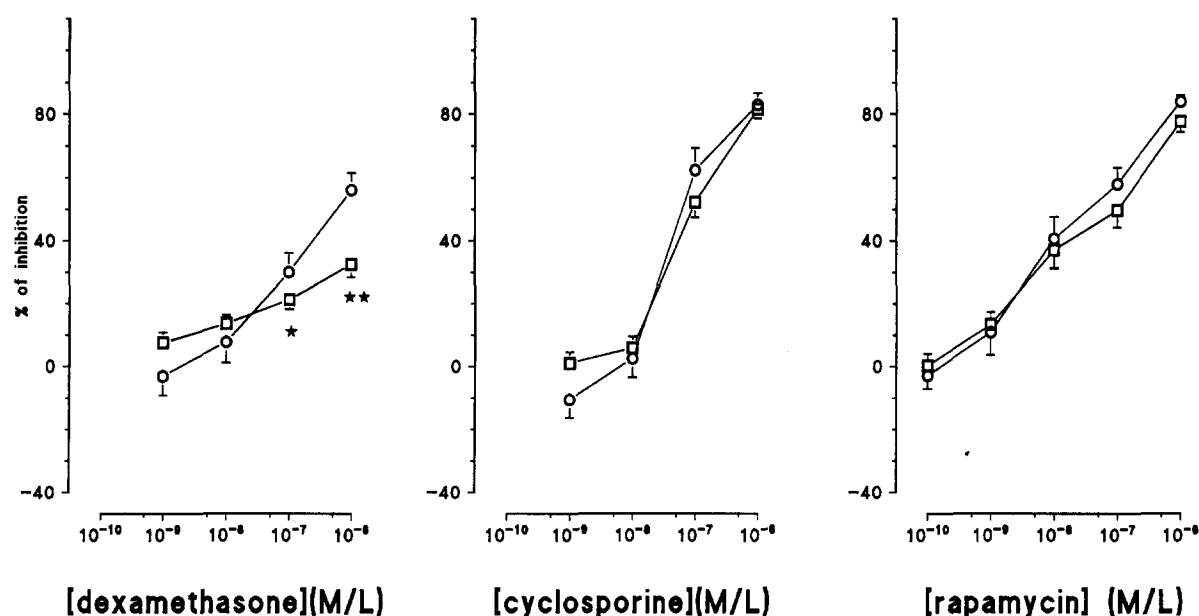


FIG. 2. Influence of exogenous IL-2 on the inhibitory effects of dexamethasone, cyclosporine and rapamycin. PBMCs were cultured for 48 hours with PHA and drug solutions or medium control in the presence of IL-2 (10 U/ml) (squares) or medium control (circles). Results are expressed as percentage inhibition of proliferation in the presence and absence of IL-2 as compared with medium (no drug) control. * $p < 0.05$; ** $p < 0.01$; bars represent the standard error of the mean ($n = 19$).

sion of PHA-induced proliferation (Fig. 1). No proliferation was observed in the absence of PHA, whether or not drugs were present (data not shown).

Ethanol and cyclosporine vehicle dilution control experiments showed no effect on lymphocyte proliferation at the concentrations used in the lowest drug dilutions (data not shown). Viability

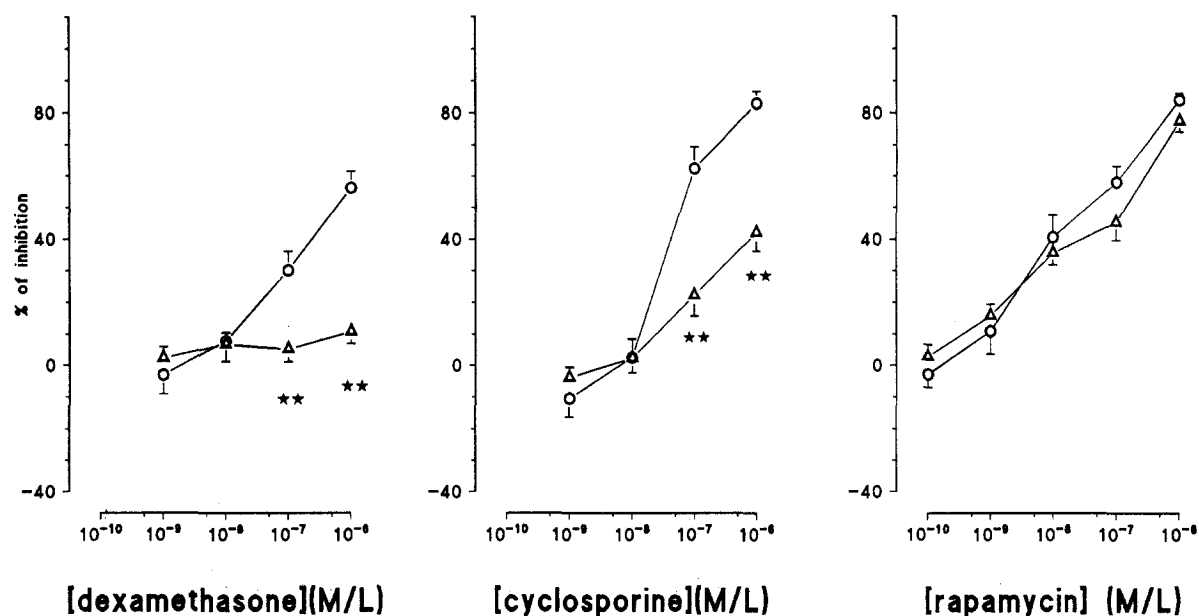


FIG. 3. The effect of preincubation of PBMCs with PHA on inhibition of proliferation of dexamethasone, cyclosporine, and rapamycin. Drug solutions were added to cultured PBMCs at the time of stimulation with PHA (circles) or 24 hours later (triangles). Proliferation was measured after an additional 48 hours of incubation. Results are expressed as percentage inhibition of proliferation as compared with medium (no drug) control. * $p < 0.05$; ** $p < 0.01$; bars represent the standard error of the mean ($n = 19$).

TABLE III. Short-term repeatability of dexamethasone IC_{50} measurements in three subjects

	IC_{50} * $\log_{10}(\text{mol/L})$	Mean IC_{50} $\log_{10}(\text{mol/L})$	Coefficient of variation†
Patient No. 1			
Experiment 1	-5.9		
Experiment 2	-6.1		
Experiment 3	-6.3	-6.1	3.1%
Patient No. 2			
Experiment 1	-6.3		
Experiment 2	-6.8		
Experiment 3	-6.8	-6.6	4.35%
Patient No. 3			
Experiment 1	-5.6		
Experiment 2	-6.1		
Experiment 3	-6.1	-5.93	4.85%

Dose-response curves were constructed for the inhibitory effect of dexamethasone on PHA-induced proliferation of T lymphocytes from the peripheral blood of three subjects on three occasions (experiments 1, 2, and 3), spanning a period of up to 2 months.

* IC_{50} value (that concentration of dexamethasone inhibiting 50% of the proliferation observed in the absence of drug).

†Coefficient of variation of the three measurements (standard deviation divided by the mean of the three measurements expressed as a percentage).

studies with trypan blue dye exclusion showed no toxic effect of any of the drugs at any concentrations used (data not shown).

T lymphocytes from the patients with clinically glucocorticoid-resistant asthma were significantly less sensitive to dexamethasone compared with those of sensitive patients ($p < 0.01$ at 10^{-6} mol/L; $p < 0.05$ at 10^{-7} mol/L and at 10^{-8} mol/L (Fig.

1). Indeed, the lowest concentration of dexamethasone used (10^{-9} mol/L) caused a small but significant enhancement of proliferation of cells from the resistant patients. In contrast, cyclosporine and rapamycin inhibited cells from both patient groups to an equivalent extent (Fig. 1). Rapamycin was more potent than cyclosporine, however, because after pooling data from the

TABLE IV. Longer term variability of peripheral blood T-lymphocyte sensitivity to dexamethasone in six patients with glucocorticoid-resistant asthma

	Percent inhibition*		
	Mean	SEM	<i>p</i> Value†
First measurement			
Dexamethasone 10 ⁻⁶ mol/L	46.88	2.13	NS
Dexamethasone 10 ⁻⁷ mol/L	24.38	4.14	NS
Dexamethasone 10 ⁻⁸ mol/L	12.78	3.00	NS
Dexamethasone 10 ⁻⁹ mol/L	-0.23	4.82	NS
Second measurement			
Dexamethasone 10 ⁻⁶ mol/L	48.62	4.03	
Dexamethasone 10 ⁻⁷ mol/L	22.85	5.12	
Dexamethasone 10 ⁻⁸ mol/L	7.88	3.08	
Dexamethasone 10 ⁻⁹ mol/L	0.72	4.24	

NS, Not significant.

PHA-stimulated peripheral blood T lymphocytes obtained from six patients with glucocorticoid-resistant asthma on two occasions (first and second measurements), separated by 4 to 6 months, were cultured in the presence of varying concentrations of dexamethasone and medium control.

*Percentage inhibition of proliferation as compared with medium control expressed as the mean and standard error of the mean (SEM) (*n* = 6).

†Mann-Whitney U test comparison of first and second measurements.

sensitive and resistant patients, although both drugs significantly (*p* < 0.01) inhibited proliferation at 10⁻⁶ and 10⁻⁷ mol/L as compared with control samples in the absence of any drug, only rapamycin significantly inhibited proliferation at 10⁻⁸ mol/L (mean, 30%; *p* < 0.01).

Effect of exogenous IL-2

Addition of exogenous IL-2 to the lectin-stimulated T-lymphocyte cultures at the time of activation significantly reduced the inhibitory effect of dexamethasone at therapeutically relevant concentrations (*p* < 0.01 at 10⁻⁶ mol/L; *p* < 0.05 at 10⁻⁷ mol/L) (Fig. 2). In contrast, the inhibitory effects of cyclosporine and rapamycin were not affected (Fig. 2). There was no difference in the response of T lymphocytes from patients with glucocorticoid-resistant asthma and those with glucocorticoid-sensitive asthma in terms of this effect of exogenous IL-2 (data not shown). The results shown in Fig. 2 therefore represent the means ± SEM of the pooled data from the patients with glucocorticoid-resistant and glucocorticoid-sensitive asthma (*n* = 19).

Resistance of lymphoblasts (24 hours after PHA stimulation) to the effect of immunosuppressive drugs

Stimulation of peripheral blood T lymphocytes with PHA for 24 hours before addition of the

drugs abolished the inhibitory effect of dexamethasone and significantly reduced that of cyclosporine (*p* < 0.01 at 10⁻⁷ and 10⁻⁶ mol/L; Fig. 3). In contrast, the inhibitory effect of rapamycin was not reduced at any concentration. Again, these effects were not significantly different on T lymphocytes from both patients with glucocorticoid-sensitive asthma and those with glucocorticoid-resistant asthma. The pooled data from all 19 patients are shown in Fig. 3.

Reproducibility of in vitro T-lymphocyte proliferation assays

To test the short-term reproducibility of the inhibitory effect of dexamethasone on PHA-induced proliferation of peripheral blood T lymphocytes, blood samples were obtained from three subjects on three separate occasions, spanning a period of up to 2 months. Dose-response curves for the inhibitory effect of dexamethasone were constructed, and from these curves the concentrations of dexamethasone causing 50% inhibition of the cellular proliferation observed in the absence of drug (IC₅₀) were calculated. In all three subjects the coefficient of variation of the log₁₀ (IC₅₀) concentrations did not exceed 4.85% (Table III). To test longer term variability in the sensitivity of T lymphocytes from patients with asthma to inhibition by dexamethasone in vitro, blood samples were obtained from six patients with glucocorti-

coid-resistant asthma on two occasions separated by 4 to 6 months. PHA-stimulated peripheral blood T lymphocytes were cultured in the presence of varying concentrations of dexamethasone as before. As shown in Table IV, the degree of inhibition of cellular proliferation in the presence of each concentration of dexamethasone did not differ significantly on the two sampling occasions.

DISCUSSION

This study confirms the findings of several others,⁹⁻¹² showing that peripheral blood T lymphocytes from patients with asthma that is clinically resistant to oral glucocorticoid therapy are relatively resistant to the antiproliferative effects of glucocorticoids *in vitro*. These observations suggest that glucocorticoids may be therapeutically effective in asthma at least partly through their inhibition of T-lymphocyte function and that clinical glucocorticoid resistance in asthma may arise in part from a relative insensitivity of T lymphocytes to the inhibitory effects of these drugs.

Two important questions relating to the *in vitro* studies on these patients concern (1) whether they are reproducible in the short term and (2) whether the sensitivity of peripheral blood T lymphocytes to dexamethasone inhibition in individual patients varies with time. The data in Tables III and IV indicate that our *in vitro* assay is indeed highly reproducible in terms of the coefficient of variation of repeated assays on the same subjects and also in the longer term if patients are retested over a period of time. The latter finding was also noted in one of our previous studies.¹⁰

It has been demonstrated in previous studies^{9, 11} that the absorption of orally administered prednisone, its metabolism to prednisolone, and subsequent elimination do not differ in patients with glucocorticoid-sensitive asthma and those with glucocorticoid-resistant asthma. It would appear therefore that variability in the clinical response of these patients cannot be attributed to differences in glucocorticoid bioavailability.

Another question of interest is whether glucocorticoid resistance arises as a consequence of increasing glucocorticoid therapy or whether it is an inherent characteristic of individual patients. In the present study the total duration of disease in the sensitive and resistant patients did not differ significantly (Tables I and II). Although we were unable to precisely determine the total amounts of glucocorticoids given orally to the patients in each group over the total course of

their disease because of the long durations of disease, variations in therapeutic preparations over the years, and unavailability of previous hospital records, we have no reason to suppose that the amount of medication given to the patients in each group differed markedly. Similar observations were made in the study of even larger numbers of patients.⁸

The concentrations of the drugs used in the *in vitro* studies were designed to encompass the therapeutic blood concentrations of these drugs *in vivo*. In the case of glucocorticoids, we previously reported⁹ that after a single standardized dose of oral prednisone (40 mg per 1.73 m² body surface area), plasma concentrations of prednisolone in a group of patients with glucocorticoid-sensitive asthma rose to a mean of 469.2 ng/ml (1.3×10^{-6} mol/L) at 2 hours and then declined to 216.5 ng/ml (6.0×10^{-7} mol/L) at 6 hours after dosing. Similar concentrations were achieved in a group of resistant patients. Even allowing for the different potencies of prednisolone and dexamethasone, these concentrations fall well within the range of those used in the *in vitro* studies when the different sensitivities of the cells from the sensitive and resistant patients were apparent. In addition, in a trial of the clinical efficacy of cyclosporine in patients with chronic glucocorticoid-dependent asthma,¹³ we reported that improvement in lung function in these patients was associated with doses of cyclosporine resulting in a mean whole blood trough concentration of 151 ng/ml (1.3×10^{-7} mol/L). We are not aware of any existing studies that have addressed the appropriate therapeutic concentration of rapamycin in the treatment of human asthma. It was, however, demonstrated in an animal cardiac allograft model¹⁴ that whole blood concentrations of rapamycin that resulted in maximal efficacy with minimal toxicity ranged from 10 to 60 µg/L (1.1 to 6.7×10^{-8} mol/L).

One of the above studies¹⁰ showed that peripheral blood T lymphocytes from patients with resistant asthma were more sensitive to the anti-T-lymphocyte drug cyclosporine as compared with dexamethasone at therapeutic concentrations, whereas a second study¹¹ showed that the effect of methylprednisolone on T lymphocytes from patients with resistant asthma was enhanced in the presence of the macrolide troleandomycin. We have confirmed the observation that proliferation of T lymphocytes from both patients with sensitive and resistant asthma is inhibited by therapeutic concentrations of cyclosporine to an equivalent

degree, and we have demonstrated in addition a similar effect of the novel macrolide rapamycin. In addition, we have shown that rapamycin has a more potent inhibitory effect on T-lymphocyte proliferation as compared with cyclosporine, although the apparent potency of rapamycin in inhibiting PHA-stimulated T cells in vitro was shown to depend on the PHA concentration.¹⁵

One possible explanation for this differing sensitivity of T lymphocytes to glucocorticoids, cyclosporine, and macrolides may be that these drugs inhibit T lymphocytes by distinct mechanisms. This led us to investigate further the inhibitory mechanisms of these drugs on activated T lymphocytes. First, we addressed the importance of the inhibition of IL-2 production by these immunosuppressive drugs because this cytokine plays a key role in T-lymphocyte proliferation and it has been suggested that overproduction of IL-2 may be associated with glucocorticoid resistance.¹⁶ Both dexamethasone and cyclosporine have been reported to inhibit the production of IL-2 by activated T lymphocytes,¹⁷⁻²¹ whereas rapamycin appears to have little or no effect on IL-2 synthesis.^{22, 23} We observed that exogenous IL-2 added to cultures of lectin-activated T lymphocytes from both patients with sensitive and resistant asthma abrogated most of the inhibitory effect of dexamethasone. This effect was observed consistently in all of the patients, although the mean differences shown in Fig. 2 are rendered less striking by the fact that the data from the sensitive and resistant patients have been pooled and the degree of inhibition of proliferation by dexamethasone in the resistant patients was not initially large. These observations suggest that the principal mechanism by which glucocorticoids inhibit T-lymphocyte proliferation is through their reduction of IL-2 synthesis. Cyclosporine and rapamycin, on the other hand, retained their ability to inhibit proliferation even in the presence of exogenous IL-2, suggesting that these drugs exert distinct or additional inhibitory effects on T lymphocytes, as has been suggested in other studies.²⁴⁻²⁶

We then investigated the effects of these drugs on T lymphocytes activated 24 hours before drug exposure to determine whether they would inhibit proliferative events subsequent to the synthesis of IL-2 and its interaction with the IL-2 receptor. In concordance with previous studies,^{23, 26} we found that dexamethasone was ineffective in inhibiting lymphoblasts, and the inhibitory effect of cyclosporine was also markedly reduced, suggesting

that these drugs inhibit only early events in the T-lymphocyte proliferative cycle. On the other hand, rapamycin inhibited lymphoblasts and freshly activated cells to an equivalent degree, suggesting that this drug exerts its inhibitory effect on cells that are already committed to proliferation. This confirms results of other studies suggesting that cyclosporine and rapamycin inhibit T lymphocytes by distinct mechanisms²³ and that rapamycin potentially inhibits the response of T lymphocytes to IL-2.²⁶ It is of considerable interest that T lymphoblasts from both patients with glucocorticoid-sensitive asthma and those with glucocorticoid-resistant asthma were sensitive to rapamycin to an equivalent degree, implying that rapamycin may inhibit ongoing, as well as de novo, T-lymphocyte activation in both patients with glucocorticoid-sensitive asthma and those with glucocorticoid-resistant asthma.

In summary, we have confirmed that T lymphocytes from patients with glucocorticoid-resistant asthma are refractory to the inhibitory effects of therapeutic concentrations of glucocorticoid in vitro, and we have shown that cyclosporine and rapamycin at therapeutic concentrations are effective in inhibiting these glucocorticoid resistant cells, probably because they exert their inhibitory effects on different facets of the activation process. These observations in turn suggest that anti-T-lymphocyte drugs such as cyclosporine and rapamycin, used alone or in combination, may be effective for the therapy of glucocorticoid-resistant asthma. As mentioned above, it has recently been shown that cyclosporine therapy improved lung function and reduced the frequency of disease exacerbations in a group of patients with severe, glucocorticoid-dependent asthma.¹³ Further clinical studies are needed to assess other drugs as rapamycin for their efficacy and safety in the treatment of asthma, especially for patients with glucocorticoid-refractory asthma.

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