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Effects of cyclosporin A administered into the airways against antigen-induced airway inflammation and hyperreactivity in the rat

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Abstract

The immunosuppressant cyclosporin A given orally has anti-asthma properties but carries an undesirable risk of systemic effects. We administered cyclosporin A to Brown Norway rats either orally (p.o.) or topically by intratracheal (i.t.) instillation into the airways before inhaled antigen. Cyclosporin A suppressed the antigen-induced accumulation of activated ($CD25^+$) $CD4^+$ T lymphocytes and eosinophils in the lung, interleukin-5 mRNA expression in lung tissue and airway hyperreactivity. Intratracheal cyclosporin A suppressed cell accumulation at a 10-fold lower dose than that required orally. Minimum effective doses were 3 mg kg^{-1} i.t. and 30 mg kg^{-1} p.o. Intratracheal administration reduced the plasma concentration and systemic exposure compared with an equieffective oral dose, but the reduction (4–5-fold) was not as large as anticipated. Our data suggests that although topical administration to asthmatics would provide some potential for an improved safety margin, it may not offer any major advantage over existing oral therapy. However, the data clearly demonstrate that a novel immunosuppressant with similar anti-inflammatory properties but reduced potential for systemic effects would offer an attractive therapy for severe asthma.

Keywords: Asthma; Airway inflammation; T lymphocyte; Eosinophil; Cyclosporin A; (Rat)

1. Introduction

Cyclosporin A is a cyclic peptide with potent immunosuppressive properties. Its actions result primarily from inhibition of signal transduction pathways in activated T lymphocytes that control transcription of a number of genes including those for key pro-inflammatory cytokines and the interleukin-2 receptor (Calderon et al., 1992; Weiderrecht et al., 1993). Thus, cyclosporin A suppresses T lymphocyte proliferation mediated by interleukin-2 and inhibits inflammatory events mediated by T lymphocyte-derived cytokines. Indeed, this drug has become a standard immunosuppressant for the prevention of allograft rejection and the treatment of a number of autoimmune diseases (Calderon et al., 1992; Weiderrecht et al., 1993).

The evidence of a central role for T lymphocytes in asthma pathogenesis has led to clinical and preclinical investigations with oral cyclosporin A. Clinical investigations revealed improved lung function and reduced disease exacerbations in severe asthmatics treated with oral cyclosporin A (Alexander et al., 1992; Lock et al., 1996; Sihra et al., 1997). These results are encouraging, but the long-term use of orally administered cyclosporin A is limited by the risk of adverse systemic effects such as nephrotoxicity.

However, there is data in the lung transplant literature which demonstrates that when cyclosporin A is administered directly into the lung, the efficacy achieved is considerably greater than that achieved after oral administration (Iacono et al., 1997; Keenan et al., 1995). Therefore, direct administration into the lungs may offer an enhanced anti-inflammatory profile in the asthmatic lung with reduced systemic exposure and subsequent side effects.

The antigen-challenged Brown Norway rat provides a model of airway inflammation and hyperreactivity that is characterised by an increased number of activated (interleukin-2 receptor-expressing, $CD25^+$) T lymphocytes in lung tissue, increased expression of interleukin-5 mRNA within the tissue and an accompanying airway eosinophilia (Underwood et al., 1997). Thus, this model shares impor-

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tant features with asthmatic airway inflammation in humans. We have now used the model to compare the topical anti-inflammatory efficacy of cyclosporin A after intratracheal instillation into the airways with its oral efficacy, and have determined the systemic exposures achieved after administering pharmacologically effective doses by each route. We have also investigated whether topically administered cyclosporin A is able to prevent antigen-induced interleukin-5 gene expression and the development of airway hyperreactivity in this model. The aim of our study was to determine whether topical administration of cyclosporin A into the airways could offer an alternative route of administration with improved efficacy and safety for the treatment of asthma.

2. Materials and methods

2.1. Animals

Brown Norway (male) rats were used for airway inflammation and lung function studies. Sprague–Dawley rats (male) were used for pharmacokinetic studies since physiological parameters that affect drug disposition and metabolism in this strain are well characterised (Davies and Morris, 1993). All rats were purchased from Harlan-Olac (Bicester, UK) at 7–9 weeks of age and housed for 2 weeks before being used for experimental protocols. Food and water were supplied ad libitum. UK Home Office guidelines for animal welfare based on the Animals (Scientific Procedures) Act 1986 were strictly observed.

2.2. Sensitization, treatment and challenge regimens

Brown Norway rats were sensitized by injection on experiment days 0, 12 and 21 with antigen (ovalbumin, 100 μg) administered together with aluminium hydroxide adjuvant (100 mg) in saline (1 ml, intraperitoneal, i.p.). Unsensitized animals received vehicle alone at the same time points. On any one day between days 30 and 36, rats were placed in a 72-l chamber and challenged by exposure for 30 min to an aerosol of ovalbumin generated from a 10 mg ml^{-1} solution by a nebulizer (deVilbiss Ultraneb, deVilbiss Healthcare, London, UK). Control (unchallenged) animals were similarly exposed to an aerosol of saline.

Twenty-four hours and 1 h before antigen challenge, rats were dosed with cyclosporin A or vehicle administered either orally (p.o.) or by intratracheal (i.t.) instillation. Suspensions of cyclosporin A were prepared by grinding and sonicating the solid drug in 0.5% methyl cellulose/0.2% polysorbate 80 in either water (for p.o. dosing) or saline (for i.t. dosing). Dose volume was 1 ml kg^{-1} (p.o.) or 0.5 ml kg^{-1} (i.t.). To allow intratracheal dosing, rats were anaesthetized by inhalation of halothane (4% in O_2 for 3–5 min) and inclined head up at an angle of 45°. A blunt-ended dosing needle was gently inserted through the larynx into the trachea as described previously (Underwood and Raeburn, 1996). Untreated (unchallenged and challenged) control rats were not anaesthetized or dosed. Treatment groups contained 6–8 animals.

2.3. Cell recovery and counts

2.3.1. Cell recovery from the airway lumen

Twenty-four hours after antigen challenge, rats were euthanised with sodium pentobarbitone (200 mg kg^{-1}, i.p.) and the trachea was cannulated. Cell influx into the airway lumen was quantified by counting cells recovered in bronchoalveolar lavage fluid. Bronchoalveolar lavage was performed by flushing the airways with two aliquots (each 10 ml kg^{-1}) of RPMI 1640 medium containing 10% foetal bovine serum delivered through the tracheal cannula. The two recovered aliquots were pooled and centrifuged ($800 \times g$, 10 min), the supernatant removed and the cells resuspended in 1 ml of RPMI 1640 medium/10% foetal bovine serum.

2.3.2. Cell recovery from lung tissue

Immediately after bronchoalveolar lavage, the thorax was opened and the lungs and heart were removed en bloc. A cannula was inserted through the right ventricle to allow the pulmonary vasculature to be flushed at low pressure with RPMI 1640 medium/10% foetal bovine serum to remove the blood pool of cells. The right lung lobes with associated main stem bronchus were removed and cut into 0.5-mm pieces. To disaggregate cells, 400 mg of homogenous lung tissue were incubated (37°C) with agitation in 45-ml RPMI 1640 medium/10% foetal bovine serum containing collagenase (20 U ml^{-1} for 2 h, then 60 U ml^{-1} for 1 h). The recovered cells were filtered (mesh size 70 μm), washed and resuspended in 1 ml RPMI 1640 medium/10% foetal bovine serum.

2.3.3. Cell counts

Counts of total number of leukocytes recovered in bronchoalveolar lavage fluid and lung tissue digest were made using an automated cell counter (Argos Cobas, Roche Diagnostic Systems, France). Cytocentrifuge preparations of bronchoalveolar lavage fluid and lung tissue digest were stained with Wright–Giemsa stain (Underwood and Raeburn, 1996). Differential cell counts were made using light microscopy and standard morphological criteria were used to identify eosinophils, neutrophils, monocytes/macrophages and lymphocytes. The numbers of lymphocytes in bronchoalveolar lavage fluid were too low to accurately count.

T lymphocytes recovered from lung tissue digest were quantified by flow cytometry. Cells (1×10^5) were incubated (30 min, 4°C) with saturating concentrations of

fluorophore-labelled monoclonal antibodies against CD2 (phycoerythrin-labelled MRC OX34), a pan-T cell marker expressed on approximately 80% of T cells in the rat lung (Strickland et al., 1996), CD4 (fluorescein isothiocyanate-labelled W3/25) and CD25 (phycoerythrin-labelled MRC OX39), a marker of the interleukin-2 receptor used as an indicator of cell activation. Following incubation, erythrocytes were removed with fluorescence-activated cell sorting (FACS) lyzing solution and the remaining cells were fixed (Immunoprep). Unlabelled cells and matched immunoglobulin G isotype controls were used to control autofluorescence and non-specific binding, respectively. Flow cytometry was performed using an EPICS XL flow cytometer and software (Coulter Electronics, UK). Preliminary gating was achieved with forward- and side-scatter characteristics to define the total lymphocyte population. T cells within the total lymphocyte population were then defined by $CD2^+$ fluoresence and the $CD4^+$ cells were defined within this population. The degree of $CD4^+$ T cell activation was determined by counting the number of $CD25^+$ cells after further gating on the $CD4^+$ population.

2.4. Semiquantitative reverse transcription and polymerase chain reaction (RT-PCR) for interleukin-5 mRNA in lung tissue

2.4.1. RNA extraction

Expression of mRNA in lung tissue was determined in a separate group of Brown Norway rats. Twenty-four hours after antigen challenge, rats were euthanised with sodium pentobarbitone (200 mg kg^{-1}, i.p.). The thorax was opened and the lungs and heart were removed en bloc. A cannula was inserted through the right ventricle to allow the pulmonary vasculature to be flushed at low pressure with Hank's balanced salt solution (4°C) to remove the blood pool of cells. The right lung lobes were then removed, immediately frozen in liquid nitrogen and stored at −80°C until required for mRNA assay.

RNA was extracted from the lung tissue using a commercial extraction kit (RNeasy, Qiagen, UK) and following the manufacturers protocol. Briefly, lung tissue was homogenized and lysed. The lysate was applied to a spin column to allow the RNA to bind to a silica gel membrane. The bound RNA was washed and then eluted. The RNA yield was determined using ultraviolet spectrophotometry (260 nm) and the quality confirmed by agarose gel electrophoresis (as below) to check for degradation.

2.4.2. RT-PCR

RNA (4 μg) was reverse transcribed to complementary deoxyribonucleic acid (cDNA) in 40-μl reverse transcriptase buffer (50 mM Tris–HCl pH 8.0, 50 mM KCl, 5 mM $MgCl_2$) containing RNA Guard (40 U), dithiothrietol (10 mM), oligo dT (5 μg), 0.5 mM each deoxynucleoside triphosphate (dNTP) and avian myeloblastosis virus (AMV) reverse transcriptase (20 U). Tubes were incubated at 20°C for 10 min, then 37°C for 60 min. Reverse transcriptase was then inactivated by incubation at 72°C for 10 min.

PCR was carried out to amplify target fragments of interleukin-5 and β-actin (a control gene) cDNA. PCR mixtures consisted of 5 μl of the cDNA solution in 40 μl of polymerase buffer (10 mM Tris–HCl pH 9.0, 50 mM KCl, 2.5 mM $MgCl_2$) containing 1.25 μM of each primer pair for a particular target sequence, 0.25 mM of each dNTP and 2 U Taq polymerase. The mixture was overlaid with mineral oil. PCR was carried out in a thermal cycler (GeneAmp 2400, Perkin Elmer, UK). Each cycle consisted of three steps: denature (94°C for 30 s or 4 min for the first cycle), primer anneal (60°C for 30 s), primer extension (72°C for 30 s). Preliminary PCR runs were carried out to determine the numbers of cycles necessary to ensure linear amplification of each target fragment (data not shown). From these results it was determined that the number of cycles needed was 20 for β-actin and 30 for interleukin-5. Negative controls (PCR mixtures without reverse transcribed RNA) and positive controls (a standard cDNA sample) were included in all PCR runs.PCR products together with molecular size markers (100 base-pair DNA ladder) were separated by electrophoresis through agarose gels (2% in Tris/boric acid/EDTA buffer containing 5 μg ml^{-1} of ethidium bromide to stain PCR products and markers). Bands of each target fragment were visualized by ultraviolet transillumination and scanned on a densitometer. Integrated optical densities (OD × mm) of the bands were calculated by image analysis software (Imagemaster, Pharmacia Biotech, Sweden).

2.5. Assessment of airway reactivity

Airway reactivity was determined in a separate group of Brown Norway rats that were not used for cell recovery or RNA extraction. Rats were anaesthetized (sodium pentobarbitone, 60 mg kg^{-1}, i.p.) and the trachea was cannulated to allow mechanical ventilation with a respiration pump (model 683 rodent ventilator, Harvard, UK) operating at 80 strokes min^{-1} with a stroke volume of 10 ml kg^{-1}. Airway resistance was measured as described previously (Raeburn et al., 1992). Airway resistance (R_{aw}), the resistance to air flow through the airways, was computed by a respiratory mechanics analyzer and software (Pulmonary Mechanics System 300, Mumed, UK) from breath-by-breath measurements of tracheal airflow and transpulmonary pressure.

After allowing 20 min for the preparation to stabilize, aerosols of acetylcholine were generated by an ultrasonic nebulizer (deVilbiss Pulmosonic, deVilbiss Healthcare, UK) connected into the inspiratory line from the respiration pump as described previously (Underwood and Raeburn, 1996). Aerosols (10, 20, 40 and 100 mM) were

generated for 5 s at 3.5-min intervals and the resulting bronchoconstrictions were recorded as increases in R_{aw}.

2.6. Pharmacokinetic profile

Male Sprague Dawley rats (8–10 weeks old) were anaesthetised with isoflurane and the jugular vein was isolated and cannulated with silastic tubing (Sani-Tech, UK, internal diameter 0.025 in., external diameter 0.047 in.). The animals were allowed to recover and 36–48 h after surgery, the rats were dosed with cyclosporin A (30 mg kg^{-1} p.o. or 3 mg kg^{-1} i.t or i.v.) as described above. These doses afforded a comparable degree of protection against antigen-induced airway inflammation. Blood samples were taken from the jugular vein after 5, 15 and 30 min, and 1, 2, 3, 4, 6, 8, 12 and 24 h. Plasma was isolated by centrifugation and stored at −20°C until analysis. Cyclosporin A concentrations were measured using LC-MS.MS (Sciex API III Plus MS.MS in turbo-ionspray MRM (+ve ion mode). Cyclosporin A was separated using 20 × 2 mm C18 (3 μM) column at 70°C with a mobile phase of water (pH 3, adjusted with glacial acetic acid)/methanol/acetonitrile (20:10:70) at a flow rate of 0.1 ml min^{-1} and a back pressure of 900 psi. The limit of detection of this methodology was 3 ng ml^{-1}. Bioavailability values were determined by comparison against plasma concentrations achieved after intravenous bolus injection. Each treatment group contained four animals.

2.7. Data analysis

Cell numbers in bronchoalveolar lavage fluid are expressed as cells ml^{-1}. Cell numbers in lung tissue are expressed as cells mg^{-1}. Results are presented as group mean ± S.E.M. RT-PCR data (integrated optical densities) are presented as scatter plots, with mean values. Dose–response curves to acetylcholine were constructed with each data point representing the group mean ± S.E.M. For each group, the provocation concentration of acetylcholine causing an increase in R_{aw} of 100 cm H_2O l^{-1} s^{-1} (PC_{100}), an increase on the steepest part of the dose–response curve, was calculated by regression analysis. PC_{100} data are presented as mean ± S.E.M.

The maximum drug concentrations achieved in plasma (C_{max}) after oral or intratracheal administration are each presented as group mean ± S.E.M. The times when maximum drug concentrations in plasma were achieved (T_{max}) after oral or intratracheal administration are presented as the range within each group. The total systemic drug exposure from time of dosing to complete elimination was calculated as the area under the curve for drug concentration plotted against time ($AUC_{0-\infty}$) and is presented as group mean ± S.E.M. The fraction of the drug dose that was absorbed after oral or intratracheal administration (absolute bioavailability, F) was calculated by comparing the group mean plasma level achieved with that after intravenous administration of the same dose.

In all analyses, the statistical significance of the differences between group results was determined using the Kruskal Wallis multiple comparison test for non-parametric data followed by Dunn's post-test. $P < 0.05$ was accepted as significant.

2.8. Materials

Cyclosporin A was synthesized by Rhône-Poulenc Rorer. Other materials were purchased from Sigma (Poole, UK) except for: aluminium hydroxide from Prolabo (Fontenay, France); sodium pentobarbitone (Sagatal) from Rhône Mérieux (Harlow, UK); RPMI 1640 medium, Hank's balanced salt solution and foetal bovine serum from Gibco (Paisley, UK); fluorophore-labelled monoclonal antibodies for flow cytometry from Serotec (Kidlington, UK); Immunoprep from Coulter Electronics (UK); FACS lysing solution from Becton-Dickinson (London, UK); reagents for RNA isolation from Qiagen (Dorking, UK); dithiothrietol, RNA Guard and reagents for RT-PCR from Pharmacia Biotech (St. Albans, UK); PCR primers from R&D Systems (Abingdon, UK). The primer sequences (published previously, Noble et al., 1993) were: interleukin-5, 5′TGCTTCTGTGCTTGAACGTTCTAAC3′ and 5′TTCTCTTTTTGTCCGTCAATGTATTTC3′, which amplify a 298 base-pair cDNA fragment; β-actin, 5′AGAAGAGCTATGAGCTGCCTGACG3′ and 5′CTTCTGCATCCTGTCAGCCTACG3′, which amplify a 236 base-pair cDNA fragment.

3. Results

3.1. Cell accumulation in lung tissue

Sensitization with antigen had no significant effect on the numbers of $CD2^+$, $CD4^+$ or $CD25^+$ lymphocytes or eosinophils recovered from lung tissue, or the number of eosinophils recovered from the airway lumen by bronchoalveolar lavage (data for unsensitized animals are not shown). Twenty-four hours after antigen challenge, there was a significant accumulation of $CD2^+$ and $CD4^+$ lymphocytes in the lung tissue (Fig. 1). There was also a significant increase in the number of activated ($CD25^+$) $CD4^+$ T lymphocytes (Fig. 1). Antigen challenge also caused a significant accumulation of eosinophils in the tissue and airway lumen (Fig. 2).

In antigen-challenged rats, oral or intratracheal administration of cyclosporin A vehicle had no significant effect on the numbers of lymphocytes or eosinophils (Figs. 1 and 2). When administered orally, cyclosporin A (30 and 100

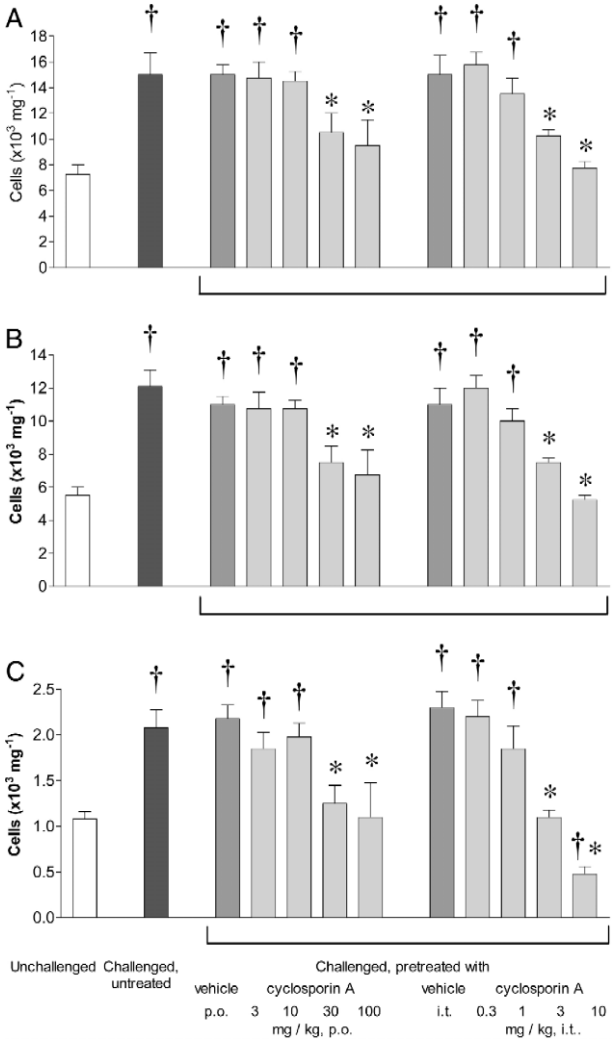

Fig. 1. The effects of cyclosporin A on antigen-induced $CD2^+$ (A), $CD4^+$ (B) and $CD4^+CD25^+$ (C) T lymphocyte accumulation in the lung tissue of Brown Norway rats. Cyclosporin A or vehicles were administered either orally (p.o.) or by intratracheal (i.t.) instillation into the airways 24 and 1 h before challenge with inhaled antigen. Cells were disaggregated from lung tissue 24 h after challenge. Group size was six to eight. Results represent mean ± S.E.M. †$P < 0.05$ compared with unchallenged group; *$P < 0.05$ compared with challenged, vehicle-treated group.

mg kg^{-1}) caused a statistically significant inhibition of the antigen-induced accumulation of $CD2^+$, $CD4^+$ and $CD4^+CD25^+$ lymphocytes in lung tissue (Fig. 1). A dose-dependent inhibition of $CD2^+$, $CD4^+$ and $CD4^+CD25^+$ lymphocyte accumulation was also observed after intratracheal administration of cyclosporin A (3 and 10 mg kg^{-1}) (Fig. 1). The highest intratracheal dose of cyclosporin A (10 mg kg^{-1}) reduced the number of $CD4^+CD25^+$ T lymphocytes to below the number seen in unchallenged control animals.

Oral administration of cyclosporin A (30 and 100 mg kg^{-1}) inhibited the antigen-induced accumulation of eosinophils in lung tissue and the influx of eosinophils into the airway lumen (Fig. 2). Intratracheal administration of cyclosporin A (3 and 10 mg kg^{-1}) also caused a significant reduction of eosinophil accumulation in the tissue and lumen (Fig. 2). Both routes of administration resulted in a complete inhibition of the antigen-induced increase in eosinophil numbers in lung tissue, while only a partial inhibition of eosinophil accumulation in the airway lumen was observed.

3.2. Expression of interleukin-5 mRNA in lung tissue

Sensitization with antigen had no significant effect on expression within lung tissue of mRNA for interleukin-5 or the control protein β-actin (Fig. 3). Twenty-four hours after antigen challenge there was a significant increase in expression of mRNA for interleukin-5, but not β-actin. Intratracheal instillation of cyclosporin A (10 mg kg^{-1}), but not vehicle, significantly inhibited the increased expression of mRNA for interleukin-5 (Fig. 3).

3.3. Airway reactivity

There was no significant difference in basal R_{aw} between any of the treatment groups (Table 1). Inhaled acetylcholine caused a dose-related bronchoconstriction

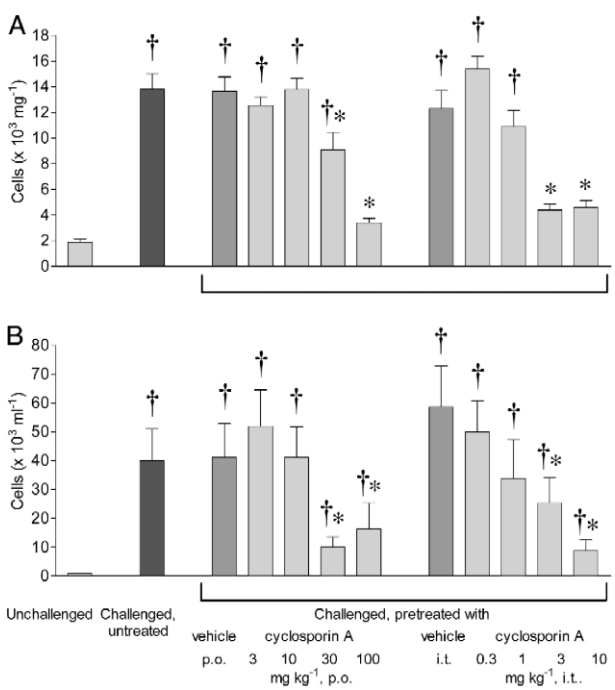

Fig. 2. The effects of cyclosporin A on antigen-induced eosinophil accumulation in the lung tissue (A) and airway lumen (B) of Brown Norway rats. Cyclosporin A or vehicles were administered either orally (p.o.) or by intratracheal (i.t.) instillation into the airways 24 and 1 h before challenge with inhaled antigen. Cells were disaggregated from lung tissue or recovered from the airway lumen by bronchoalveolar lavage 24 h after challenge. Group size was 6–8. Results represent mean ± S.E.M. †$P < 0.05$ compared with unchallenged group; *$P < 0.05$ compared with challenged, vehicle-treated group.

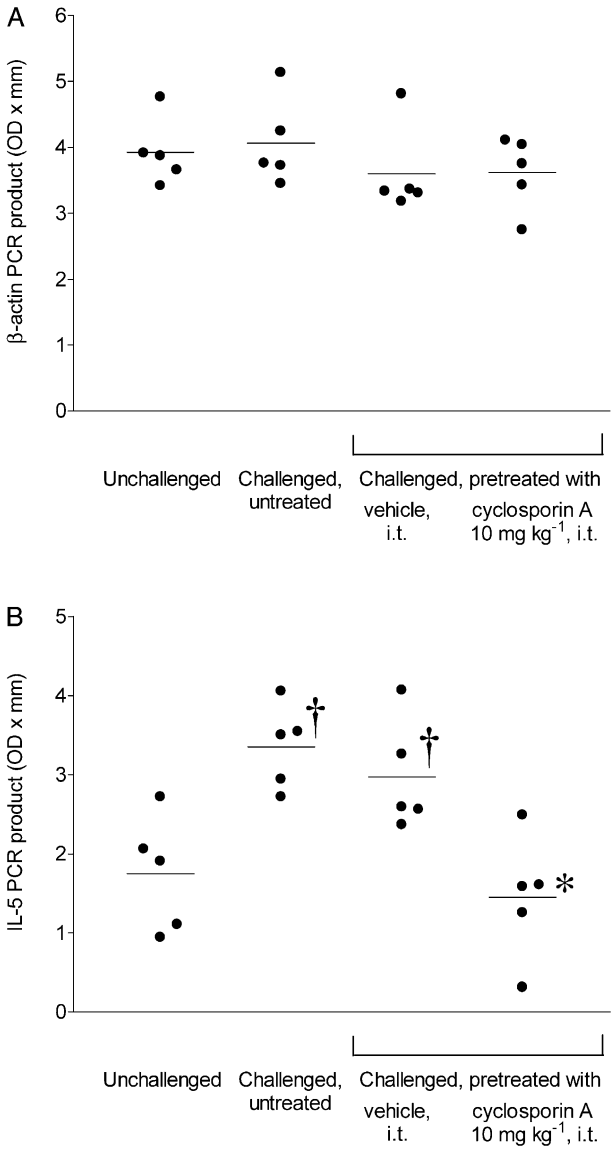

Fig. 3. The effects of cyclosporin A on expression of (a) β-actin and (b) interleukin-5 mRNA in lung tissue of Brown Norway rats after antigen challenge. Cyclosporin A (10 mg kg^{-1}) or vehicle were administered by intratracheal (i.t.) instillation 24 and 1 h before challenge with inhaled antigen. RNA was extracted from lung tissue 24 h after challenge and mRNA measured by RT-PCR. PCR products, stained with ethidium bromide, were separated by agarose gel electrophoresis, visualised by u.v. transillumination, and scanned. Integrated optical densities (OD × mm) were calculated by image analysis software. Group size was 5. Horizontal lines represent group means. †$P < 0.05$ compared with unchallenged group; *$P < 0.05$ compared with challenged, vehicle-treated group.

that peaked within 100 s. In antigen-challenged rats, the acetylcholine dose–response curves was shifted significantly to the left and the PC_{100} was reduced significantly (Table 1; Fig. 4). These findings demonstrate that 24 h after antigen challenge, the airways were hyperreactive. Pretreatment with cyclosporin A (10 mg kg^{-1}, i.t.), but not with lower doses or vehicle, prevented the antigen-induced airway hyperreactivity (Table 1; Fig. 4).

Table 1
The effect of cyclosporin A on antigen-induced airway hyperreactivity to inhaled acetylcholine in the anaesthetized Brown Norway rat
Cyclosporin A or vehicle were administered by intratracheal (i.t.) instillation into the airways 24 and 1 h before challenge with inhaled antigen. Airway reactivity to inhaled acetylcholine was measured 24 h after challenge. PC_{100} values were calculated from acetylcholine dose–response curves by logarithmic regression analysis.

Treatment	Basal R_{aw} (cm H_2O l^{-1} s^{-1})	Acetylcholine PC_{100} (mM)
Unchallenged, untreated	93 ± 11	81 ± 9
Challenged, untreated	82 ± 10	39 ± 12[a]
Challenged, vehicle, i.t.	96 ± 12	24 ± 12[a]
Challenged, cyclosporin A, 0.3 mg kg^{-1}, i.t.	87 ± 7	34 ± 5[a]
Challenged, cyclosporin A, 1 mg kg^{-1}, i.t.	87 ± 8	30 ± 5[a]
Challenged, cyclosporin A, 3 mg kg^{-1}, i.t.	91 ± 10	43 ± 12[a]
Challenged, cyclosporin A, 10 mg kg^{-1}, i.t.	94 ± 6	65 ± 13[b]

[a] $P < 0.05$ compared with unchallenged group.
[b] $P < 0.05$ compared with challenged, vehicle-treated group.

3.4. Pharmacokinetic profile

When administered orally at an anti-inflammatory effective dose (30 mg kg^{-1}), cyclosporin A had a C_{max} of 1.9 ± 0.2 μg ml^{-1}, T_{max} of 4–8 h, $AUC_{0-\infty}$ of 41.0 ± 5.5 μg ml^{-1} h^{-1} and a bioavailability (F) of 30% (Table 2). When administered intratracheally (3 mg kg^{-1}) cyclosporin A exhibited a C_{max} of 0.5 ± 0.1 μg ml^{-1}, T_{max}

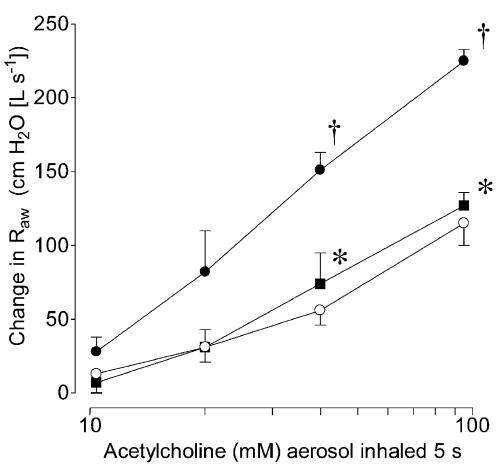

Fig. 4. The effect of cyclosporin A on antigen-induced airway hyperreactivity to inhaled acetylcholine in the anaesthetized Brown Norway rat. Cyclosporin A (10 mg kg^{-1}) or vehicle were administered by intratracheal (i.t.) instillation 24 and 1 h before challenge with inhaled antigen. Airway reactivity to inhaled acetylcholine aerosol was measured 24 h after challenge. Open circles: unchallenged; solid circles: challenged, vehicle-treated; solid squares: challenged, pre-treated with cyclosporin A (10 mg kg^{-1}, i.t.). Group size was six. †$P < 0.05$ compared with unchallenged group; *$P < 0.05$ compared with challenged, vehicle-treated group.

Table 2
Pharmacokinetic data for cyclosporin A
Cyclosporin A was administered either orally (p.o.) or by intratracheal (i.t.) instillation into the airways at doses shown to afford a comparable degree of protection against antigen-induced airway inflammation. Group size was four. Plasma concentrations were determined at intervals until 24 h after dosing.

Dose	C_{max} ($\mu g\ ml^{-1}$)	T_{max} (h)	$AUC_{0-\infty}$ ($\mu g\ ml^{-1}\ h^{-1}$)	F (%)
30 mg kg^{-1}, p.o.	1.9 ± 0.2	4–8	41.0 ± 5.5	30
3 mg kg^{-1}, i.t.	0.5 ± 0.1	0.5–4	7.3 ± 3.1	54
3 mg kg^{-1}, i.v.	–	–	13.5 ± 1.1	–

C_{max}: maximum drug concentration achieved in plasma.

T_{max}: time after dosing when maximum drug concentration in plasma was achieved.

($AUC_{0-\infty}$): total systemic drug exposure from time of dosing to complete elimination.

F: absolute bioavailabilty (fraction of the drug dose that was absorbed).

of 0.5–4 h, $AUC_{0-\infty}$ of 7.3 ± 3.1 $\mu g\ ml^{-1}\ h^{-1}$ and a bioavailability of 54% (Table 2).

4. Discussion

Challenge with inhaled ovalbumin caused an accumulation of eosinophils and total ($CD2^+$), helper ($CD4^+$) and activated (interleukin-2 receptor expressing, $CD25^+$) helper T lymphocytes in the lung tissue of sensitized Brown Norway rats. In addition, there was an increase in the expression of interleukin-5 mRNA in the lung tissue after antigen challenge. Antigen challenge also caused a significant increase in eosinophil numbers recovered by bronchoalveolar lavage. These changes mimic the increased numbers of $CD4^+CD25^+$ T lymphocytes and eosinophils that are characteristics of airway inflammation in human asthmatics (Bradley et al., 1991; Laitinen and Laitinen, 1994; Robinson et al., 1993) and that are similarly accompanied by an increase in the numbers of cells that express interleukin-5 mRNA (Fukuda et al., 1994; Robinson et al., 1993).

In our study, a marked anti-inflammatory effect was observed with cyclosporin A when it was administered either intratracheally or orally. A significant inhibition of eosinophil accumulation in both bronchoalveolar lavage fluid and lung tissue was observed after treatment with cyclosporin A by both routes of administration. Our data confirms that of other groups that have similarly reported the effectiveness of cyclosporin A against antigen-induced airway eosinophilia in the rat (Ceyhan et al., 1998; Elwood et al., 1992; Huang et al., 1999). Importantly, our results demonstrate that the minimum effective dose of intratracheal cyclosporin A is 10-fold lower than that required after oral administration. This is comparable with data reported by Morley (1992) in the guinea pig, where a 10-fold lower aerosolised dose of cyclosporin A was required to obtain similar efficacy to that achieved after oral administration.

Clinical efficacy with cyclosporin A has been reported in corticosteroid-dependent severe asthmatics (Alexander et al., 1992; Lock et al., 1996; Sihra et al., 1997). These beneficial effects were accompanied by a decrease in the serum levels of soluble interleukin-2 receptor and interleukin-2 receptor-bearing $CD4^+$ T lymphocytes (Alexander et al., 1995; Mori et al., 1995; Rolfe et al., 1997). These findings support the hypothesis that the benefits afforded by cyclosporin A were mediated by inhibition of T lymphocyte activation. In animal models, other groups have reported an effect of cyclosporin A on lymphocyte numbers (Elwood et al., 1992; Huang et al., 1999) but there are no previous reports of an effect of cyclosporin A on the antigen-induced accumulation of activated T lymphocytes in the lung. The data in our study clearly demonstrates for the first time that cyclosporin A caused a complete inhibition of the antigen-induced accumulation of total, helper ($CD4^+$) and activated ($CD25^+$) T lymphocytes in the lung tissue. This supports the hypothesis that cyclosporin A is inhibiting lung eosinophilia by suppressing T lymphocyte activation.

Interleukin-5 promotes eosinophil differentiation, recruitment, activation and survival (Corrigan, 1996) and has been implicated in mediating airway eosinophilia in human asthma. Indeed, the number of eosinophils in asthmatic airways shows a positive correlation with the number of $CD4^+CD25^+$ T lymphocytes, the number of cells that express mRNA for interleukin-5 and the concentration of interleukin-5 in bronchoalveolar lavage fluid (Bradley et al., 1991; Robinson et al., 1993; Sur et al., 1995; Walker et al., 1991). Although eosinophils, mast cells and some other cell types within human asthmatic airways may also transcribe the interleukin-5 gene (Bradding et al., 1994; Lamkhioued et al., 1996), there is evidence that mRNA for this cytokine is expressed predominantly by T lymphocytes (Kay et al., 1995).

At an intratracheal dose that markedly and significantly inhibited antigen-induced T lymphocyte and eosinophil accumulation, cyclosporin A suppressed the increased expression of interleukin-5 mRNA in the rat lung. Our findings suggest that abrogated interleukin-5-mediated eosinophil chemotaxis may contribute to the protective effects of cyclosporin A against airway eosinophilia in the rat model, although it is likely that the production of other pro-eosinophilic cytokines and chemokines from T lymphocytes and other cell types is also reduced. In addition to inhibiting the recruitment and activation of cell types such as $CD4^+$ T lymphocytes that express the interleukin-5 gene, it may be that cyclosporin A also suppressed gene processing. Indeed, cyclosporin A has been shown to reduce expression of interleukin-5 mRNA in human T lymphocytes and interleukin-5 secretion from these cells in

vitro (Schmidt et al., 1994; Mori et al., 1995; Rolfe et al., 1997).

In the clinic, cyclosporin A has been reported to modulate airway hyperreactivity (Fukuda et al., 1995). Studies of the effects of cyclosporin A in the rat have shown that suppression of antigen-induced airways eosinophilia is not accompanied by protection against the development of airway hyperreactivity if the compound is administered orally (Elwood et al., 1992; Huang et al., 1999). In contrast, our study demonstrated that an intratracheal dose of cyclosporin A that completely inhibited eosinophilia in lung tissue was also able to completely prevent airway hyperreactivity. Similarly, inhaled cyclosporin A has been shown to protect against antigen-induced airway hyperreactivity in the guinea pig (Arima et al., 1994). These differing effects of cyclosporin A on hypereactivity after oral or intratracheal administration are interesting and suggest that topical application might achieve localised suppression of cell activity that is necessary to efficiently protect against these changes in lung function.

The clinical and experimental efficacy of cyclosporin A is encouraging, but the clinical utility of cyclosporin A is limited by unwanted systemic effects, especially nephrotoxicity (Calderon et al., 1992). Local administration of cyclosporin A to the lung should limit systemic exposure and subsequent adverse effects and may even enhance anti-inflammatory efficacy. Indeed, a clinical study has now shown that systemic exposure of cyclosporin A after inhalation of a single 20-mg dose is much lower than that achieved after administration of the 3-mg oral dose that has been shown to be efficacious for treating asthma (Rohatagi et al., 2000), although relative anti-asthma effects were not assessed in this study. Efficacy in our study was achieved after administration of an intratracheal dose, which was 10-fold lower than that required to achieve efficacy after oral administration. Furthermore, intratracheal administration of cyclosporin A was more effective at inhibiting accumulation of activated T lymphocytes. Following intratracheal administration, the antigen-induced accumulation of activated T lymphocytes was reduced to below basal levels, while oral administration simply inhibited the antigen-induced increase.

Although the minimum effective intratracheal dose was 10-fold lower than the minimum effective oral dose, the systemic exposure following intratracheal administration was not 10-fold lower than that achieved after oral administration. Only a 4–5-fold reduction in systemic exposure was achieved after intratracheal administration. The absorption or clearance of cyclosporin A is apparently greater after intratracheal vs. oral administration. This increased absorption or clearance after local administration to the rat lung has been reported for cyclosporin A by other groups (Luke et al., 1990; Taljanski et al., 1997). However, unlike the present study, the systemic levels achieved after local administration were not evaluated at a dose that induces efficacy. Analysis of the data obtained in our study suggests that although there is a reduction in systemic exposure following local administration, it is not as large as one would anticipate after administration of a tenth of the oral dose of cyclosporin A.

In conclusion, this study has demonstrated that cyclosporin A, delivered into the airways, markedly inhibits the antigen-induced accumulation of eosinophils and T lymphocytes (including activated $CD4^+$ T lymphocytes) in the rat lung. Furthermore, a reduction in antigen-induced expression of interleukin-5 mRNA in the lung tissue and airway hyperreactivity were also observed. The minimum effective dose after local administration into the airways was 10-fold lower than that required orally. The peak plasma concentration and total systemic exposure of cyclosporin A after an effective intratracheal dose were 26% and 19% of the values reached after administration of an equieffective oral dose. However, this reduction in systemic exposure following local administration was not as large as one would anticipate after administration of a tenth of the oral dose of cyclosporin A. Although local administration of cyclosporin A does reduce systemic exposure and would provide a potential for an improved safety margin, the smaller than expected reduction suggests that local administration may not offer a major advantage over existing oral therapy. However, the data clearly demonstrate that if an agent can be developed, which modulates these inflammatory axis and does not induce systemic toxicity, it would be an attractive therapy for severe asthmatics.

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