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Differential effects of cyclosporine A after acute antigen challenge in sensitized cats *in vivo* and *ex vivo*

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1 We determined the effect of cyclosporine A (CsA) treatment on mast cell degranulation and lung resistance (R_L) *in vivo*, and tracheal smooth muscle (TSM) contraction *ex vivo* after antigen challenge in sensitized cats. We also determined the direct effects of addition of CsA to the tissue bath on antigen-induced responses of TSM *in vitro*.

2 Cats ($n=10$) were sensitized by i.m. injection of *Ascaris suum* antigen (AA); 5 cats (CsA+) received CsA twice daily for 2 weeks before acute antigen challenge in doses sufficient to suppress interleukin-2 secretion from feline peripheral blood mononuclear cells *ex vivo*.

3 Lung resistance increased comparably within 10 min of exposure to AA ($P<0.03$). Histamine content in bronchoalveolar lavage fluid from both groups increased comparably within 30 min of antigen challenge, from undetectable levels to 542 ± 74 pg ml^{-1} post AA for CsA+ and from 74 ± 19 pg ml^{-1} at baseline, to 970 ± 180 pg ml^{-1} post AA CsA− ($P<0.05$; $P=$NS vs CsA+).

4 In excised TSM, active tension elicited by exposure to AA *in vitro* was 107 ± 38% KCl in the CsA+ group vs 144 ± 56% KCl in the CsA− group ($P=$NS). However, contraction of TSM ($n=4$) harvested from both groups was abolished or greatly diminished after AA challenge when tissues were pre-incubated with 1 μM CsA *in vitro* (8 ± 8% KCl, $P<0.05$ vs CsA+ and CsA−). This was associated with inhibited release of 5-hydroxytryptamine into the organ bath fluid of tissues treated with CsA *in vitro* only.

5 We demonstrated that CsA treatment *in vivo* does not inhibit the early phase asthmatic response or mast cell degranulation following antigen challenge in sensitized cats. Additionally, the effects of CsA on mast cell function *ex vivo* do not reflect lack of effects of CsA on mast cell function *in vivo* in this animal model of atopic asthma.

Keywords: Airway reactivity; mast cells; tracheal smooth muscle; asthma

Introduction

Atopic asthma is a chronic inflammatory airway disease characterized clinically by cough, wheeze and spontaneous airflow limitation. Mast cells long have been recognized to participate in this early phase response to antigen, in part by releasing pre-formed mediators such as histamine, and by synthesis and secretion of leukotriene (LT) C_4, substances capable of causing profound bronchoconstriction and airway wall oedema (Schwartz & Huff, 1991; Galli, 1993). Human lung mast cells also secrete cytokines including interleukin-1,3,4,5,6, tumour necrosis factor-α and granulocyte macrophage-colony stimulating factor and so may contribute additionally to the late phase recuritment and activation of inflammatory cells into airways (Plaut *et al*., 1989; Gordon *et al*., 1990; Bradding *et al*., 1992; 1994).

Cyclosporine A (CsA) is a cyclic undecapeptide metabolite extracted from the fungus, *Tolypocladium inflatum*, that inhibits T cell activation and also inhibits mast cell degranulation and release of pre-formed mediators and secretion of cytokines *in vitro* (Faulds *et al*., 1993; Padrid *et al*., 1996; Padrid, 1996). Recognition of the potential role of activated mast cells and T cells in causing and or propagating the asthmatic state, and the *in vitro* effect of CsA to inhibit mast cell responses had led to the clinical use of CsA to treat patients with severe asthma (Szczeklik *et al*., 1991; Alexander *et al*., 1992; Lock *et al*., 1996). We previously have showed that *Ascaris suum* (AA)-sensitized and acutely challenged cats develop both early and late phase asthmatic responses (Padrid *et al*., 1995b). However, AA-sensitized cats treated with CsA *in vivo* before antigen challenge do not develop late phase responses (Padrid *et al*., 1996).

The objective of the present study was to examine the effect of CsA on the early phase bronchoconstriction responses in AA-sensitized cats *in vivo*. We measured AA-stimulated changes in lung resistance and release of histamine into airway lumen. We also harvested tracheal smooth muscle (TSM) and measured mast cell-dependent, AA-induced TSM contraction, and release of mast cell derived 5-hydroxytryptamine (5-HT; Padrid *et al*., 1995a) into the organ bath fluid. Antigen-elicited contraction and release of 5-HT were compared to the response of TSM strips pre-treated *in vitro* with a non-cytotoxic dose of CsA that inhibits mast cell release of pre-formed mediators *in vitro* (Draberova, 1990; Ezeamuzie & Assem, 1990; Hatfield & Roehm, 1992). We confirmed that *in vitro*, CsA incubation of TSM from sensitized cats greatly inhibits contraction of airway smooth muscle and release of preformed 5-HT in response to antigen challenge through mast cell inhibition. However, cats treated *in vivo* with CsA and acutely exposed to antigen continue to demonstrate mast cell degranulation and airway narrowing. Additionally, TSM harvested from these same cats develop mast cell-dependent contraction and release of 5-HT following *in vitro* acute antigen exposure. These findings demonstrate that CsA

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treatment *in vivo* of antigen sensitized cats does not inhibit mast cell degranulation within airway smooth muscle. Hence, CsA inhibits late phase responses by a mechanism that does not require inhibition of early phase mast cell degranulation in feline asthma.

Methods

General

Male ($n=13$) and female ($n=6$) adult cats of mixed breed weighing 3.5–4.5 kg were screened initially to exclude infection with feline leukaemia virus and feline immunodeficiency virus. A complete physical examination, complete blood count, serum chemistries, faecal examination for parasites, and chest radiographs were obtained initially to document absence of disease. All animals used in these studies of the acute phase response are those for which the late phase response has been characterized in a previous communication (Padrid *et al*., 1996). There was no overlap in the experimental protocols between studies.

All studies were performed with the animals under deep anaesthesia. When studies were performed using paralytic agents, heart rate was continuously monitored. Supplemental anaesthetic was given if there was any suggestion of discomfort, e.g. an increase in heart rate. All studies were conducted in accordance with the PHS Policy of Humane Care and Use of Laboratory Animals and the NIH Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training.

Skin testing

To document lack of prior sensitization, all animals underwent intradermal skin testing with 0.05 ml of 1000 PNU ml^{-1} *Ascaris suum* antigen (AA) while lightly anaesthetized with ketamine HCl (5–10 mg kg^{-1} BW, i.m.). Histamine (1 : 100,000) served as a positive control, while sterile saline served as negative control. A palpable or visual reaction to intradermal AA that was $\geqslant 1:2$ that of the positive histamine control was interpreted as a positive test (Muller *et al*., 1983). Animals testing positive to AA before sensitization were excluded from the study. Skin testing was repeated 2 weeks following the second intramuscular injection of AA plus ajuvant (see below) to document the development of sensitivity to AA.

Sensitization protocol

Titer Max adjuvant (0.25 ml) was mixed with 0.125 ml aqueous AA (0.1%) until an emulsion was formed. An additional 0.125 ml AA was added until the entire mixture was again emulsified; 0.05 ml of this emulsion (33 μg) was injected into each hind leg of the experimental animals. This procedure was repeated again 14 days later.

Anaesthesia and ventilation

Cats were fasted for 12 h before being anaesthetized. An i.v. catheter (23 gauge) was placed in the foreleg through which sodium thaimylal (10–15 mg kg^{-1}, i.v.) was infused to induce anaesthesia. Animals were intubated with a 4.5 mm internal diameter cuffed tube shortened to 12–14 cm length. Intravenous lactated ringers solution was infused at 5–10 ml kg^{-1} h^{-1} for the duration of the anaesthetic period. Depth of anaesthesia was judged by response to toe pinch. Animals were placed on heating pads to maintain core temperature at 37°C. Ventilation was set initially at 10–15 ml kg^{-1} and 25 breaths min^{-1}. Arterial blood gas tensions were determined by sampling blood from the femoral artery, and tidal volume was adjusted to maintain P_{aCO_2} at 32–38 mmHg. Supplemental oxygen was given to maintain baseline P_{aO_2} at 100–150 mmHg. After a deep plane of anaesthesia was induced, animals were paralyzed by slow i.v. injection of vecuronium bromide (0.1 mg kg^{-1}) to inhibit spontaneous respiration. Vecuronium was specifically chosen as the paralytic agent because it has minimal effects to stimulate mast cell degranulation (Moss, 1995; Naguib *et al*., 1995). To ensure that adequate anaesthesia levels were maintained, supplemental doses of anaesthetic (3–5 mg kg^{-1}, i.v.) were given at 15–30 min intervals. Heart rate and rhythm were monitored continuously by electrocardiography. If heart rate increased by 5–7% in response to any noxious stimulus, supplemental anaesthesia was provided. At the completion of measurements of airway reactivity to ACh, paralysis was reversed with neostigmine (0.03 mg kg^{-1}, i.v.).

Measurement of lung resistance

In animals anaesthetized as above, a 12 g × 2" catheter (Terumo Medical Corp, Elkton, MD) was passed through the 7–8 intercostal space and positioned in the left pleural cavity. Transpulmonary pressure (P_{tp}) was measured with a differential pressure transducer (Validyne DP 45-14). One end of the catheter was connected to a side port of the endotracheal tube, and the other end was connected to the pleural catheter. Airflow ($\dot{V}$) was measured with a pneumotachograph (Fleisch 00) in series with the endotracheal tube and connected to a second differential pressure transducer (Validyne DP 45-24). Tidal volume (V_T) was determined by electronic integration of the flow signal. Lung resistance (R_L) was determined at 50% of V_T by the isovolume method of von Neergard and Wirz (1972) and calculated breath by breath by a commercially available computerized pulmonary mechanics analyser (Lake Shore Technologies, Chicago, IL). Data were recorded continuously on a personal computer, stored on hard disk and displayed on a standard monitor.

Bronchoscopy and bronchoalveolar lavage (BAL)

Bronchoscopy and BAL were performed by use of an adaptation of a method we have described previously (Padrid *et al*., 1991). Briefly, the bronchoscope (Pentax 10H) was passed through a Y shaped adapter (Sontek Medical) connected to the endotracheal tube to allow mechanical ventilation to continue during bronchoscopy. The bronchoscope was wedged into a segmental branch of the right caudal lung lobe. Fifty millilitres of 0.9% sterile saline solution in 5 equal, 10 ml aliquots (previously warmed to 37°C) were sequentially lavaged through the biopsy channel of the bronchscope and immediately retrieved by gentle hand sunction. The lavage samples were measured for volume and then pooled and filtered through a single layer of sterile gauze to remove gross mucus. This procedure was performed at the same time (within 30 min) after antigen challenge in all cats.

Quantification of histamine content in bronchoalveolar lavage fluid

Standard (known) and experimental samples (100 μl) were added to tubes containing an acylating reagent and acylation buffer (50 μl). Acylation was allowed to precede for 30 min at

room temperature. An aliquot (50 μl) of acylated standard or sample and histamine-acetylcholinesterase conjugate (200 μl) was added to anti-acylated-histamine antibody-coated microwells of a 96-well microtitre plate (Delaage *et al*., 1988). The microplates were incubated for 18 h at 4°C. The unbound contents were washed from the wells and substrate (200 μl) was added. The enzymatic conversion of substrate by bound histamine-conjugated acetylcholinesterase proceeded for 20 min in the dark at room temperature. Absorbance of the reaction product was monitored at 405 nm. The concentration of histamine in the unknown samples was quantified by interpolation from a standard curve generated in parallel. The assay is sensitive to 8 pg ml^{-1}.

Quantification of 5-HT concentration in smooth muscle organ baths

Standards (known) and experimental samples (100 μl) were added to tubes containing an acylating reagent and acylation buffer (50 μl). Acylation was allowed to proceed for 30 min at room temperature. An aliquot of acylated standard or sample and 5-HT acetylcholinesterase conjugate was added to anti-5-HT antibody-coated microwells of a 96-well microtitre plate. Following a 3 h incubation at room temperature with shaking, unbound contents were washed from the wells and substrate (200 μl) was added. The enzymatic conversion of substrate by bound 5-HT-conjugated acetylcholinesterase proceeded for 20 min in the dark at room temperature. Absorbance of the reaction product was monitored at 405 nm. The concentration of 5-HT in the unknown samples was quantified by interpolation from a standard curve generated in parallel. The assay is sensitive to concentrations $\geqslant$0.05 nM.

Experimental protocols

Before sensitization with AA, animals underwent baseline studies of airway reactivity, bronchoscopy and BAL 3–5 days after skin testing to avoid the potential effects of ketamine on bronchomotor tone (Hirschman *et al*., 1974). Two weeks after the second injection of AA and adjuvant, cats underwent skin testing as previously described. If sensitization was documented, a period of 3–5 days again was allowed to pass before further studies were performed (see below).

Experimental groups

Ten of 15 cats injected with AA and adjuvant developed positive skin test reactions and were considered sensitized. Five sensitized cats were assigned randomly to the experimental group that was antigen-challenged and treated with CsA (CsA+). The *in vivo* effects of CsA treatment in these cats on late phase asthmatic responses and following chronic AA challenge have been described previously (Padrid *et al*., 1996). The remaining 5 sensitized cats served as positive controls and were antigen challenged but not given CsA (CsA−). Four additional cats served as a negative control group.

Administration of cyclosporine

Cyclosporine was administered initially to the CsA+ group at a dose of 10 mg kg^{-1} orally, twice daily. Oral dosing was adjusted to maintain blood levels between 500–1000 ng ml^{-1}. The drug was placed in gelatin capsules and mixed with cat food before administration. Ingestion of the CsA capsules by the experimental animals was witnessed each time. The dose of CsA used in these studies is not toxic to the feline species and has been shown previously to inhibit interleukin (IL-2) production from activated peripheral blood mononuclear cells in these animals (Latimer *et al*., 1986; Gregory *et al*., 1992). Animals serving as positive controls received gelatin capsules filled with the appropriate vehicle (olive oil).

Measurement of cyclosporine concentrations

Cyclosporine concentrations were measured by radioimmunoassay in the clinical radioimmunoassay laboratory at the University of Chicago. Whole blood trough concentrations of CsA were monitored twice weekly just preceding the morning administration of CsA.

Acute antigen challenge

Acute antigen challenge was performed in all cats at a time point when CsA blood levels were consistently $>$500 ng ml^{-1}. All cats were anaesthetized and treated as described, and sufficient time was allowed to obtain a stable baseline value for R_L; this usually occurred within 15 min. *Ascaris suum* antigen (0.01%) was nebulized through the endotracheal tube for 1 min. Immediately following nebulization, R_L was recorded continuously for 30–60 min, or until R_L returned to baseline. All cats were allowed to recover from anaesthesia.

Chronic antigen challenge

Forty-eight hours after bronchoscopy, all sensitized animals were individually placed for the first time into a sealed, pre-oxygenated nebulization chamber and exposed for a maximum of 5 min to 0.01% AA while awake and spontaneously breathing. Animals showing signs of distress before 5 min were immediately removed from the chamber. This procedure was repeated in these same cats 3 times per week for the next 6 weeks. Three days after the final exposure to AA, all cats were killed by an injection of saturated potassium solution while deeply anaesthetized.

Preparation of ex vivo *tissues*

Tracheae were excised and placed in Krebs-Henseleit (K-H) solution of the following composition (in mM): NaCl 115, $NaHCO_3$ 25, KH_2PO_4 1.38, KCl 2.51, $MgSO_4.7H_2O$ 2.46, $CaCl_2$ 1.91 and dextrose 11.2. The solution was gassed continuously with a mixture of 95% O_2 : 5% CO_2 to maintain a pH of 7.40 at 37°C.

Strips of epithelium-denuded feline TSM were dissected as previously described (Padrid *et al*., 1995a). Immediately following dissection, TSM was palced in 15 ml glass tissue baths containing gassed K-H perfusate. One end of each strip was attached with a loop of 000 braided silk suture to a rigidly held glass rod within the bath. The upper end was fastened by silk suture to a force displacement transducer. The force transducer was mounted on a rack and pinion so that the muscle preparation could be stretched to optimal length and held isometrically for the duration of the experiment. Force signals were recorded on a polygraph chart recorder.

Each muscle preparation was contracted periodically over 60–90 min with 63 mM KCl-substituted K-H (Mitchell *et al*., 1989). After a contraction had been elicited, each muscle was washed at least 3 times over 30 min with fresh K-H until active tension returned to baseline. During this period, L_{max}, the optimal length at which maximal and reproducible isometric contraction is elicited, was determined. All

subsequent experiments were performed at this resting length.

Following equilibration, TSM preparations from CsA− ($n=5$) and CsA+ ($n=5$) cats were exposed to AA (250 μl, 0.01%). In 4 additional preparations (2 from the CsA+, 2 from the CsA− group), CsA was added at a concentration of 10^{-6} M and allowed to incubate for 30 min before addition of AA. The peak contraction of each tissue was measured and recorded, and data were normalized as a % of the response of each tissue to the third contraction elicited by 63 mM KCl (% KCl) (Mitchell *et al*., 1989). Active force of contraction was calculated by subtracting the total force achieved after addition of antigen from the initial resting tension.

In 4 experiments from the CsA− group, 1 ml of organ bath perfusate was withdrawn and frozen at −70°C just before addition of antigen. An additional aliquot from the same organ baths was removed after addition of antigen within 10 min of peak TSM contraction. These experiments were repeated in four organ baths for TSM from cats treated with CsA *in vivo* (CsA+), and four additional TSM organ baths (2 from CsA− and 2 from CsA+ cats) pretreated with CsA *in vitro*.

Preparation of reagents

Soluble *Ascaris suum* containing 187,000 PNU ml^{-1} was diluted with saline and used at a concentration of 0.01%. Cyclosporine A was dissolved in 80% ethanol and used at a concentration of 1 μM.

Drugs and suppliers

The following drugs were used (suppliers in parentheses). Thiamylal sodium (Parke Davis, Morris Plains NJ), vecuronium bromide (Organon Inc, West Orange NJ), *Ascaris suum* (Greer Laboratories Inc, Lenoir NC), Titer Max (CytRx, Atlanta GA), ethyl alcohol, RPMI, FCS, (Sigma Chemical Co, St. Louis MO), histamine phosphate (Center Laboratories, Port Washington NY), neostigmine methylsulphate (Elkins-Sinn Inc, Cherry Hill NJ) and cyclosporine A (Sandoz Corp Rahway NJ).

Data analysis

Differences in R_L in response to AA at 10 min, histamine content of BAL fluid 5-HT content in organ bath fluid and TSM responses *ex vivo* were compared by Student's *t* test. Paired comparisons were made where appropriate. Differences between groups were evaluated with a 2 sample Student's *t* test (Zar, 1984). All data are expressed as mean ± s.e. Statistical significance was assumed when $P \leqslant 0.05$.

Results

All animals in the treatment group received the full course of CsA treatment. All cats underwent daily physical examination and weekly blood withdrawal for determination of renal and hepatic function. No significant side effects were seen in any of the treated animals. Whole blood trough concentrations ranged from 220–1400 ng ml^{-1} during the first week the drug was administered. Thereafter, trough concentrations were maintained at 500–850 ng ml^{-1} in all cats by weekly or semi-weekly adjustment of drug dosage. We have shown previously in these specific animals that this concentration of CsA causes suppression of IL-2 secretion from stimulated peripheral blood mononuclear cells *in vitro* (Padrid *et al*., 1996).

Antigen challenge in CsA− cats caused an increase in R_L from 28 ± 1.5 cmH$_2$O L^{-1} s^{-1} to 48 ± 6.1 cmH$_2$O L^{-1} s^{-1} within 10 min of exposure to AA ($P<0.03$) and was comparable to the increase in R_L from 20 ± 1.8 cmH$_2$O L^{-1} s^{-1} to 44 ± 9.5 cmH$_2$O L^{-1} s^{-1} in the CsA+ group ($P=0.05$) (Figure 1).

Histamine content of BAL fluid from both CsA+ and CsA− cats was increased following antigen challenge. For CsA+ cats, histamine was not detected in BAL fluid before AA challenge and equaled 542 ± 74 pg ml^{-1} post AA. For CsA− cats, BAL concentrations of histamine increased from 74 ± 19 pg ml^{-1} at baseline, to 970 ± 180 pg ml^{-1} post AA ($P<0.05$). There was no difference in the change in AA-stimulated histamine content in BAL fluid between CsA+ and CsA− groups ($P=$ NS). There was no comparable increase in histamine in BAL fluid from non-sensitized cats after sham (saline) challenge (6 ± 3 pg ml^{-1} post sham challenge vs 55 ± 26 pg ml^{-1} baseline ($P=$ NS)).

Tracheal smooth muscle was harvested from cats in all groups and challenged *in vitro* with AA. Antigen challenge of TSM from cats treated with CsA *in vivo* caused contraction that was equivalent to contraction of TSM from cats not treated with CsA *in vivo*. However, when TSM from CsA− or CsA+ cats was incubated *in vitro* for 30 min with CsA (1 μM) and then exposed to AA, contraction was abolished in 3 of 4 preparations and was minimal in the remaining tissue ($P<0.05$ vs CsA− and CsA+; Figure 2). The vehicle used to dilute CsA had no significant effect on antigen-induced contraction of sensitized tissues, and CsA plus vehicle did not significantly inhibit acetylcholine-induced smooth muscle contraction (Figure 3).

5-HT was not detected in the perfusate from any sample before the addition of antigen. Ten minutes after addition of antigen, the 5-HT concentration in the perfusate bathing tissues that were not exposed to CsA *in vitro* was 12.0 ± 4.0 nM (CsA−) and 9.0 ± 2.0 nM (CsA+). In contrast, 5-HT was detected in low concentrations and in only 2 of 4 samples (1.0 ± 0.4 nM) in the perfusate of tissues pretreated with CsA *in vitro* ($P<0.04$ vs CsA−, $P<0.01$ vs CsA+).

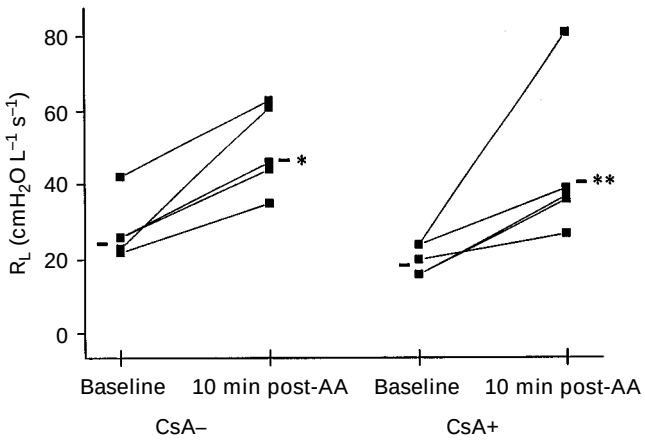

Figure 1 Airway response after acute exposure to nebulized *Ascaris* antigen (AA) in sensitized cats. Cats were *Ascaris suum* sensitized and chronically airway challenged, and treated *in vivo* with either Cyclosporine A (CsA+) or vehicle (CsA−). Lung resistance (R_L) increased within 10 min of exposure to AA in all cats. *$P<0.03$; **$P<0.05$ vs baseline.

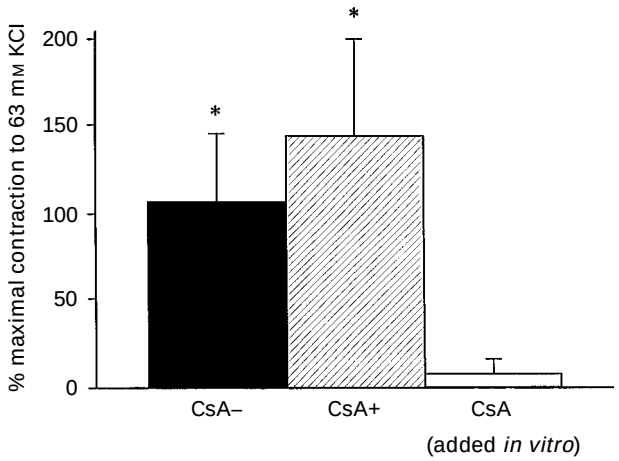

Figure 2 Type-I immediate hypersensitivity reaction of tracheal smooth muscle (TSM) from sensitized cats. Cats were *Ascaris suum* sensitized and chronically antigen challenged and treated *in vivo* with either cyclosporine A (CsA+) or vehicle (CsA−). Some TSM strips were incubated *in vitro* with CsA before exposure to *Ascaris* antigen. Incubation *in vitro* with CsA abolished or greatly attenuated contraction (expressed for each TSM preparation as % of the maximal contraction elicited by 63 mM KCl exposure). $*P<0.05$ vs CsA added *in vitro* group.

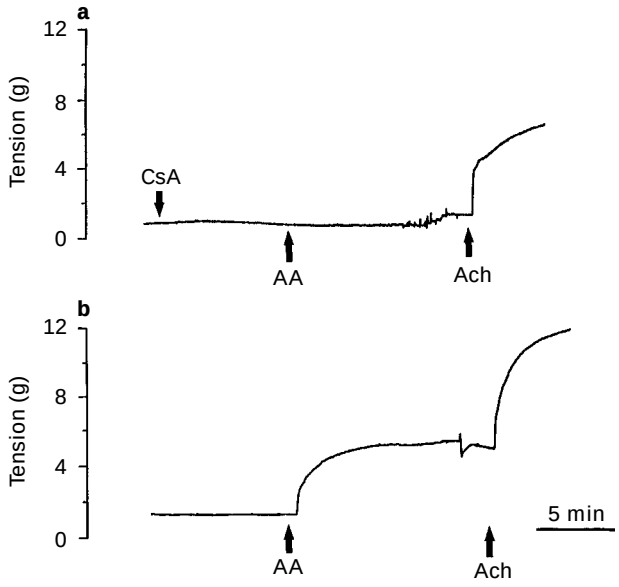

Figure 3 Tracheal smooth muscle (TSM) responses from sensitized cats to *ex vivo* antigen challenge. (a) Representative tracing demonstrating contraction to *Ascaris* antigen (AA) and acetylcholine (ACh) in a sensitized TSM strip pretreated *in vitro* with cyclosporine A (CsA). (b) Representative tracing demonstrating contraction to antigen and acetylcholine in sensitized tissues not pretreated *in vitro* with cyclosporine A. Antigen-activated contraction was abolished in TSM pretreated with CsA while the response to acetylcholine persisted.

Discussion

The objective of this study was to determine the effect of cyclosporine A on early phase bronchoconstrictor responses in a feline model of asthma (Padrid *et al*., 1995b). We examined CsA effects on mast cell degranulation and the subsequent development of airway narrowing *in vivo* and TSM contraction *ex vivo* after antigen challenge in AA-sensitized cats. Cats were selected because this species develops asthma idiopathically (Moise *et al*., 1989) and with chronic antigen exposure (Padrid *et al*., 1995b), and because CsA treatment inhibits late phase asthmatic responses in these animals (Padrid *et al*., 1996). We found that R_L of AA-sensitized and CsA treated cats increased after acute antigen challenge and was similar to sensitized cats not treated with CsA (Figure 1). Increased R_L was associated in both groups (CsA+ and CsA−) with a significant increase in histamine content in BAL fluid, indicating substantial mast cell degranulation. Additionally, antigen challenge of TSM from both groups caused comparable contraction and 5-HT release *in vitro* (Figure 2).

Previous studies in animals have resulted in conflicting results regarding the inhibitory effect of CsA treatment on mast cell degranulation *in vivo*. For example, in a mouse model of cutaneous sensitivity, CsA inhibited the late phase T cell-dependent response to antigen but did not inhibit the early phase response, which is dependent in rodents on the release of 5-HT from mast cells (Geba *et al*., 1991). Additionally, sensitized guinea-pigs treated with CsA have early phase asthmatic responses after antigen challenge (Akutsu *et al*., 1990; Arima *et al*., 1991; Fukuda *et al*., 1991). However, in those studies, CsA concentrations were not measured, the efficacy of the CsA dose was not independently established, and independent markers of airway mast cell degranulation (including histamine concentrations in BAL fluid) were not obtained.

We have confirmed the previous finding that sensitized animals treated with CsA develop early phase bronchoconstriction following antigen challenge (Akutsu *et al*., 1990; Arima *et al*., 1991; Fukuda *et al*., 1991). In the present study, we extended those studies to suggest mast cell degranulation is not inhibited by pretreatment with CsA *in vivo*. Previous *in vitro* studies of CsA on mast cell function have suggested that pretreatment with CsA inhibits degranulation of sensitized mast cells after challenge with antigen (Draberova, 1990; Ezeamuzie & Assem, 1990; Hatfield & Roehm, 1992). Interestingly, we were able to reproduce the inhibitory effect of CsA on mast cell degranulation in airway smooth preparations when the tissues were incubated with CsA *in vitro* before antigen challenge (Figure 2). In preliminary studies, we found that vehicle alone did not inhibit antigen induced contraction of sensitized feline airway smooth muscle and that vehicle plus CsA did not inhibit acetylcholine-induced smooth muscle contraction (Figure 3). The inhibitory effects of CsA on mast cell cytokine secretion *in vitro* have also been shown to be selective and not due to cytotoxicity (Hatfield & Roehm, 1992). Thus, we believe that the action of CsA applied *in vitro* to inhibit antigen-induced contraction and 5-HT release in feline airway smooth reflects a specific *in vitro* inhibitory effect of CsA on mast cell degranulation and release of pre-formed mediators.

Possible mechanisms for this differential effect of CsA *in vivo* and *in vitro* include (1) the concentration of CsA in serum *in vivo* in the cat may not reflect tissue levels, and, *in vitro*, potentially greater concentration of CsA may be able to inhibit mast cell degranulation and (2) mast cells of the extra-pulmonary airways may not be as susceptible to antigen challenge as the mast cells found throughout the lower airways and lung periphery. Although the whole blood trough concentrations of CsA achieved *in vivo* approximate the concentration used *in vitro* (1 μM), it is possible that the concentrations of CsA achieved within airways *in vivo* are lower. However, we note that this dose of CsA was effective in inhibiting late phase responses in these same animals (Padrid *et al*., 1996). Although it has been suggested that the smooth muscle of the trachea is representative of smooth muscle down

to the 7th generation of bronchi (Stephens, 1987), the availability of mast cells for the presentation of antigen is not known. In fact, Stephens (1987) demonstrated for cleanly dissected canine TSM, with total removal of serosal and mucosal layers including epithelium, that mast cells persist in the smooth muscle. We used the same dissection technique in this study of feline TSM. Furthermore, we have demonstrated previously in a ragweed-sensitized canine model of allergic bronchospasm that TSM exposed to sensitizing antigen contracted; this contraction was blocked by antihistamine indicating release of mediator from mast cells in the 'pure' smooth muscle preparation (Mitchell *et al*., 1986). These TSM strips had been dissected from dogs that had been sensitized and challenged *in vivo*, again, similar to our feline model.

We chose a single high concentration of antigen to challenge feline TSM preparations *in vitro*. This concentration was selected (1) to maximize the contractile response in terms of tension development by creating a large concentration gradient for the antigen, (2) to reduce the time for all mast cells to degranulate, again, to ensure maximal response, and (3) to ensure a clear demonstration of the inhibitory effects of CsA on these contractions. As demonstrated in Figure 2, in the presence of this high concentration of antigen, the Schultz-Dale reaction could be inhibited totally by 1 μM CsA. Furthermore, it is typical in this species, as in others in which the airway smooth muscle contracts in response to antigen challenge through degranulation of mast cells inherent in the tissue, that these Schultz-Dale contractions are an all or none phenomena (Mitchell *et al*., 1986). The response can be elicited only once per tissue. Lesser concentrations of antigen would eventually cause a Schultz-Dale contraction, but with a much slower onset, and the response would be submaximal due to the slow release of mediators from mast cell to mast cell. Therefore, we used this single high concentration of antigen to challenge our tissues *ex vivo*.

We chose to assess the effect of CsA on acute mast cell degranulation and subsequent physiological responses *in vivo* and *in vitro*. Because we did not examine isolated mast cells directly in the present study, there are some limitations to the interpretation of these data. However, we believe that our data strongly suggest that feline sensitized airway mast cells degranulated in response to antigen challenge *in vivo*. First, we found an equivalent increase in histamine content in BAL fluid after antigen challenge from sensitized cats treated with CsA or sham-treated with vehicle. Additionally, we found that antigen-challenged feline airway smooth muscle from cats treated with CsA *in vivo* contracted and released 5-HT (a mast cell derived mediator in the feline species) similarly to tissues harvested from non-CsA treated cats. Thus, we conclude that CsA given *in vivo* does not inhibit mast cell degranulation *in vivo* or *in vitro* in the feline system we examined.

Recent studies have suggested an important role for early phase mast cell activation in the development of late phase asthmatic responses (Kung *et al*., 1995; Underwood *et al*., 1997), although conflicting results have also been obtained (Galli, 1997). Because of the inhibitory effect on T lymphocytes and also the presumed inhibitory effects on mast cells, CsA has been used to treat patients with corticosteroid-dependent asthma (Szczeklik *et al*., 1991; Alexander *et al*., 1992; Lock *et al*., 1996). Our data demonstrate that the previously described inhibitory effect of CsA on mast cells *in vitro* does not reflect the effect of CsA on mast cells *in vivo* during the early phase bronchoconstrictor response in cats. These data are consistent with a recent study on the effects of CsA treatment in humans with asthma. In this study, CsA therapy resulted in an inhibition of the late phase response, but had no effect on the early phase response in patients exposed to antigen (Sihra *et al*., 1997). Thus, in this animal model of atopic asthma, CsA inhibits late phase asthmatic responses by a mechanism that does not require inhibition of early phase mast cell degranulation.

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