Concentrations of cysteinyl leukotrienes in urine and bronchoalveolar lavage fluid of cats with experimentally induced asthma

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Objective—To evaluate changes in cysteinyl leukotriene (LT) concentrations in urine and bronchoalveolar lavage fluid (BALF) in cats with experimentally induced asthma.

Animals—19 cats with experimentally induced asthma and 5 control cats.

Procedure—Cats were sensitized to Bermuda grass or house dust mite allergen, and phenotypic features of asthma were confirmed with intradermal skin testing, evaluation of BALF eosinophil percentages, and pulmonary function testing. A competitive ELISA kit for LTC4, LTD4, and LTE4 was used for quantitative analysis of LTs. Urinary creatinine concentrations and BALF total protein (TP) concentrations were measured, and urinary LT-to-creatinine ratios and BALF LT-to-TP ratios were calculated.

Results—Mean urinary LT-to-creatinine ratios did not differ significantly between control cats and allergen-sensitized cats before or after sensitization and challenge exposure with saline (0.9% NaCl) solution or allergen, respectively. In BALF, the mean LT-to-TP ratio of control cats did not differ significantly before or after sensitization and challenge exposure with saline. Asthmatic cats had BALF LT-to-TP ratios that were significantly lower than control cats at all time points, whereas ratios for asthmatic cats did not differ significantly among the various time points.

Conclusions and Clinical Relevance—Although LTs were readily detectable in urine, no significant increases in urinary LT concentrations were detected after challenge in allergen-sensitized cats. Spot testing of urinary LT concentrations appears to have no clinical benefit for use in monitoring the inflammatory asthmatic state in cats. The possibility that cysteinyl LTs bind effectively to their target receptors in BALF and, thus, decrease free LT concentrations deserves further study. (*Am J Vet Res* 2003;64:1449–1453)

Allergic asthma is a Th2-cell-driven immune response against inhalant allergens, resulting in the production of allergen-specific IgE antibodies.[1] These antibodies bind to the high-affinity IgE receptor (ie, FcεRI) on mast cells, are cross-linked by allergens, and trigger degranulation of mast cells as well as synthesis of other inflammatory mediators. The phenotypic expression of asthma includes the hallmark responses of bronchoconstriction, eosinophilic inflammation of airways, and remodeling of airways.[2,3] Cysteinyl **leukotrienes (LTs)**, which include LTC4, LTD4, and LTE4, are metabolites that result from 5-lipoxygenation of arachidonic acid found in cell membranes, and they orchestrate many of the biological events that take place during asthma.[4,5] In addition to being produced by mast cells, cysteinyl LTs are also synthesized by eosinophils, basophils, alveolar macrophages, T lymphocytes, and the bronchial epithelium.[4,6] Cysteinyl LTs contribute to bronchoconstriction, increased vascular permeability, influx of inflammatory cells, hypersecretion of mucus, and proliferation of smooth muscle. In human asthmatics and in various other animals with experimentally induced asthma, analysis of samples of urine and **bronchoalveolar lavage fluid (BALF)** allows investigation of the generation of these mediators in the airways.

Cats naturally develop a syndrome of asthma that is remarkably similar to the disease in humans, including bronchoconstriction, eosinophilic inflammation of the central airways, and remodeling of the airways.[7-9] Experimentally induced asthma in cats serves a dual role by helping investigators study the immunopathogenesis of disease in humans and cats.[7,10,11,a] The role of LTs in the pathogenesis of asthma in cats has not been extensively evaluated.

Invasive techniques (eg, collection of tracheobronchial fluid or BALF, biopsy of bronchial mucosa, or lung biopsy) are usually required to provide specimens for direct analysis of immunologic or inflammatory mediators produced by the airways or pulmonary parenchyma. In humans, LTs can be measured in a number of specimens including BALF, plasma, nasal secretions, sputum, and urine.[4,12] In the study reported

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here, we evaluated changes in concentrations of cysteinyl LTs in urine and BALF at multiple time points after sensitization to an allergen and challenge exposure in cats with experimentally induced asthma.

Materials and Methods

Animals—Twenty-four (11 males and 13 females) mixed-breed cats that were 8 months to 3 years old were obtained from a research colony of cats maintained at the University of California, Davis for use in the study. The cats were part of a larger ongoing study on asthma immunopathogenesis. For all 24 cats, results of physical examinations were unremarkable, and results were negative for FeLV and FIV tests. Prior to entry into the study, cats underwent **intradermal skin testing (IDST)** to ensure that they had not been sensitized to the allergens used in the study (ie, **house-dust mite allergen** [**HDMA**; *Dermatophagoides farinae*][b] or **Bermuda grass allergen** [**BGA**][c]). Immediately after IDST, baseline samples of urine and BALF were collected, and pulmonary function testing was performed by use of methacholine. Five cats served as control animals; they were administered PBS solution in place of an allergen during the sensitization procedure. The remaining 19 cats were administered HDMA or BGA to induce an asthmatic state.

IDST—Cats were sedated by administration of ketamine hydrochloride (5 mg/kg, IM), and hair was clipped from the lateral portion of the thorax. Intradermal injections (volume, 0.1 mL) were administered. Compounds injected were histamine phosphate (1:100,000), which served as a positive-control sample; sterile saline (0.9% NaCl) solution, which served as a negative-control sample; and HDMA (0.49 mg/mL) or BGA (0.84 mg/mL). Cats were judged to have had a positive skin reaction when the diameter of the wheal formed in response to injection of HDMA or BGA was greater than or equal to the value halfway between the diameter of the wheals produced by the positive- and negative-control samples.[13] The width and length of each wheal were measured 20 minutes after injection of the compounds.

Evaluation of airway hyperresponsiveness—Cats were anesthetized by administration of propofol (induction, 6 mg/kg, IV; maintenance, 0.2 to 0.6 mg/kg/min, IV, as a constant-rate infusion). After induction, each cat was intubated with a cuffed 4.5-mm endotracheal tube. Cats were placed in sternal recumbency, and the endotracheal tube was attached to a blow-by delivery system set to deliver 100% O_2 at a rate of 2 L/min. Cats were allowed to breathe spontaneously throughout the remainder of the testing. Change in outflow exhausted from this system, which indicated inspiratory and expiratory flow, was recorded by use of a pneumotachometer[d] attached to a pressure transducer.[e] Transpulmonary pressure was measured by use of a differential pressure transducer,[f] with 1 port of the transducer attached to a water-filled cannula placed in the esophagus at the level of the midthorax and the other port of the transducer attached to a side port of the pneumotachometer. These analogue signals were sent to an analogue-to-digital data acquisition system.[g]

Methacholine, a short-acting analogue of acetylcholine that is resistant to acetylcholinesterase, was used for bronchoprovocation experiments. Aerosol was generated with a low-volume nebulizer[h] and delivered to the blow-by system at a rate of 2 L/min. After nebulization of sterile saline solution for 1 minute, measurements of baseline pulmonary resistance were collected for 4 minutes. Challenge exposure to methacholine was performed by use of a dose-response format. Increasing half-logarithmic doses of methacholine were administered for 1 minute, starting at a dose of 0.0625 mg/mL. After delivery of each dose of methacholine, data were collected for 4 minutes. The dose-response experiment was terminated when airway resistance increased to 200% of the value obtained after challenge exposure with the saline solution or arterial blood saturation decreased to ≤ 75%.

Collection of urine and BALF samples—Urine (5 to 6 mL) was collected from each cat via antepubic cystocentesis. Samples of BALF were collected after airway hyperresponsiveness was evaluated. A 7-F polypropylene catheter was inserted through the endotracheal tube with gentle pressure until resistance was felt as the catheter became wedged in a small airway. Three 10-mL aliquots of warmed sterile saline solution were sequentially lavaged through the catheter and retrieved by use of suction into a suction trap. Samples of BALF were placed on ice until analysis; all BALF samples were analyzed within 2 hours after collection. Cytologic preparations were made by use of a cytocentrifuge; preparations were stained with modified Wright stain. Differential cell counts were determined by evaluating 200 cells/slide.

Aliquots of urine and BALF were immediately added to tubes that contained an LT synthesis inhibitor[i]; tubes were gently mixed and placed on ice. Samples were centrifuged at 300 × *g* for 20 minutes, and the supernatant was harvested and stored frozen at –20°C until analysis. All samples were analyzed within 6 months after collection.

Allergen sensitization—Cats were sensitized by parenteral administration of an allergen and subsequently challenge exposed to that allergen via an aerosol, in accordance with a protocol described elsewhere.[a] Cats were sensitized by administration of BGA (16 cats) or HDMA (3); PBS solution was administered in place of an allergen for the 5 cats in the control group. Briefly, cats were administered an allergen (12 µg of HDMA or BGA in 10 mg of alum, SC) and *Bordetella pertussis* (10^5 organisms, IM) on day 0, 0.2 mL of allergen (HDMA, 0.49 mg/mL; or BGA, 0.84 mg/mL), intranasal, on day 14, and 12 µg of HDMA or BGA in 10 mg of alum, SC, on day 21. Intradermal skin testing was used to confirm sensitization to the specific allergen on day 28. Aerosol challenge exposure was initiated on day 28 and consisted of 3 exposures/wk for 2 weeks (ie, days 28, 31, 35, 38, 42, and 45), then 1 exposure/mo for the subsequent 5 months (ie, months 2 through 6). Challenge exposure of the aerosolized allergen (0.5 mg of allergen/exposure) was administered to unsedated cats in a sealed chamber fitted for an air compressor[j] with a nebulizer. Each cat was exposed to the aerosolized allergen for 10 min/exposure.

Collection of samples—Samples of urine and BALF were collected from the 5 control cats at baseline (day 0) and after sensitization and aerosol challenge exposure (day 45) accomplished by use of saline solution in place of an allergen. Urine samples were collected at the same time points (ie, days 0 and 45) from 13 cats sensitized and challenge exposed with BGA. For the remaining 6 allergen-treated cats (3 sensitized to BGA and 3 sensitized to HDMA), samples of urine were collected on day 0, after sensitization with the respective allergen but before aerosol challenge exposure (day 28), after the 3-times-weekly aerosol challenge exposures with the respective allergen (day 45), and after termination of the monthly allergen challenge exposures (6 months). On day 45 and at 6 months, urine samples were collected 36 to 48 hours after aerosol challenge exposure. Additionally, BALF was collected from varying numbers of cats sensitized to BGA at various time points (1, 4, 6, 8, and 48 hours) after allergen challenge exposure via aerosol administration (5 to 10 cats at each time point).

Assay of cysteinyl LT concentrations—A commercially available competitive ELISA kit[k] with monoclonal antisera that recognized LTC4, LTD4, and LTE4 was used for quantitative analysis of urinary LTs, in accordance with the manu-

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facturer's instructions. All standards were assayed in triplicate; undiluted unknown samples were assayed in duplicate. The LT concentration in samples was calculated by the use of values generated from a standard curve. Final concentrations of the standards were 0, 0.04, 0.10, 0.20, 0.80, 1, and 2 ng/mL. Urinary creatinine concentrations were measured by use of a modified kinetic Jaffe method,[14] and urinary LT-to-creatinine ratios were calculated. Total protein concentration in BALF was measured by use of a commercially available protein assay,[l] and BALF LT-to-total protein ratios were calculated.

To ensure that the commercial ELISA would be able to detect feline cysteinyl LTs, urine samples obtained from 24 cats were analyzed for LT concentration prior to the study. Results of assay of those urine samples without adjustment for urinary creatinine concentrations yielded a range of 0.2 to 2.5 ng/mL (mean ± SD, 1.03 ± 0.64 ng/mL). Specimens of BALF from 8 of these cats were used to determine whether cysteinyl LTs could be detected in BALF, and results of assay of those BALF specimens without adjustment for total protein concentration yielded a range of 0.02 to 0.17 ng/mL (mean ± SD, 0.06 ± 0.05 ng/mL).

Statistical analysis—A 2-tailed paired *t* test was used to compare mean urinary LT-to-creatinine and BALF LT-to-total protein ratios measured before sensitization with those that were measured after sensitization and challenge exposure. The BALF LT-to-total protein ratios for various time points after allergen challenge exposure were compared by use of a univariate ANOVA. The Tukey multiple comparison procedure was used for post hoc testing to limit the overall type-I error rate to 5%. Values of $P < 0.05$ were considered significant.

Results

Confirmation of asthmatic phenotype—After sensitization with saline solution, control cats had no evidence of allergen-specific IgE fixed to mast cells as indicated by negative results for IDST (day 28), no increase in mean percentage of eosinophils in BALF (day 45), and only a slight increase in airway resistance in response to bronchoprovocation with methacholine (day 45; **Table 1**). In comparison, cats sensitized with HDMA or BGA had an asthmatic phenotype on the basis of positive results for IDST (day 28), a marked increase in mean percentage of eosinophils in BALF (increased to 41% on day 45), and a noticeable increase in airway resistance (ie, 5.3-fold decrease in the dose of methacholine required to increase airway resistance by 200% on day 45).

Table 1—Results of intradermal skin testing (IDST), mean ± SE eosinophil percentage in bronchoalveolar lavage fluid (BALF), and airway resistance for 5 control cats before and after sensitization treatments with saline (0.9% NaCl) solution and 16 cats with induced asthma before and after sensitization with Bermuda grass allergen

Group	Variable	Before sensitization	After sensitization
Control cats			
	IDST	Negative	Negative
	Eosinophils in BALF (%)	7 ± 3	1 ± 1
	Airway resistance	NA	2.0 ± 0.5*
Asthma-induced cats			
	IDST	Negative	Positive
	Eosinophils in BALF (%)	8 ± 1	41 ± 3
	Airway resistance	NA	5.3 ± 0.8*

*Value represents the increase in airway resistance (ie, fold-decrease in the dose of methacholine required to induce a 200% increase in airway resistance), compared with baseline values.
NA = Not applicable.

Urinary LT-to-creatinine ratios—We did not detect significant differences for control cats in the mean ± SD urinary LT-to-creatinine ratios before ($2.8 \times 10^{-7} \pm 9.38 \times 10^{-8}$) or after ($2.1 \times 10^{-7} \pm 8.1 \times 10^{-8}$) sensitization and challenge exposure with saline solution. In 6 cats sensitized with allergen (3 cats sensitized to HDMA and 3 cats sensitized to BGA), group mean urinary LT-to-creatinine ratios were measured during several phases of sensitization and challenge exposure. Mean values for these ratios were $1.5 \times 10^{-7} \pm 3.7 \times 10^{-8}$ (before sensitization), $1.6 \times 10^{-7} \pm 1.1 \times 10^{-8}$ (after sensitization but before aerosol challenge exposure), $1.3 \times 10^{-7} \pm 2.4 \times 10^{-8}$ (after initial aerosol challenge exposure), and $8.7 \times 10^{-8} \pm 1.2 \times 10^{-8}$ (6 months after allergen sensitization and challenge exposure). However, significant differences were not detected between the group mean urinary LT-to-creatinine values before sensitization, compared with values for any of the time points after sensitization and challenge exposure. In the remaining 13 asthmatic cats (all of which were sensitized to BGA), significant differences were not detected between the mean urinary LT-to-creatinine ratio before sensitization ($2.7 \times 10^{-7} \pm 1.4 \times 10^{-7}$) and after sensitization and challenge exposure ($3.1 \times 10^{-7} \pm 5.1 \times 10^{-7}$).

LT-to-total protein ratios in BALF—In control cats, mean ± SD ratio of LT-to-total protein ratio at baseline (ie, prior to sensitization with saline solution) was $1.0 \times 10^{-7} \pm 9.8 \times 10^{-8}$, compared with a value of $4.5 \times 10^{-8} \pm 2.7 \times 10^{-8}$ after sensitization and challenge exposure with saline solution; values did not differ significantly. In asthmatic cats, BALF was collected at various time points (1, 4, 6, 8, and 48 hours) after allergen challenge exposure. With the exception of the time points at 4 and 6 hours after challenge exposure, BALF was collected from the cats on different days but always with > 1 month between collections. The mean BALF LT-to-total protein ratios were $6.3 \times 10^{-10} \pm 8.3 \times 10^{-10}$, $1.4 \times 10^{-10} \pm 3.0 \times 10^{-10}$, $6.0 \times 10^{-9} \pm 9.5 \times 10^{-9}$, $6.9 \times 10^{-9} \pm 1.3 \times 10^{-9}$, and $1.0 \times 10^{-9} \pm 2.2 \times 10^{-9}$ at 1, 4, 6, 8, and 48 hours after challenge exposure, respectively.

A univariate ANOVA was used to compare mean BALF LT-to-total protein ratios of asthmatic cats at 1, 4, 6, 8, and 48 hours after challenge exposure with BALF LT-to-total protein ratios of control cats at those same time points. Asthmatic cats had ratios that were significantly lower at all time points, compared with ratios for the control cats, whereas ratios for the asthmatic cats did not differ significantly among the various time points. Mean total protein concentrations for the control group did not differ significantly for concentrations of the asthmatic cats at any of the time points.

Discussion

In the study reported here, cats sensitized to an allergen had an asthmatic phenotype, as documented by positive results for IDST, increases in the mean percentage of eosinophils in BALF, and a change in airway

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resistance in response to methacholine (5.3-fold decrease in the amount of methacholine needed to induce the increase). In contrast, control cats that received saline solution instead of allergen did not have positive results for IDST or increases in mean percentage of eosinophils in BALF and had only a minimal response to methacholine during pulmonary function testing. Documentation of the inflammation and hyperreactive airways was important to simultaneously investigate the role of cysteinyl LTs in these cats with experimentally induced asthma.

Leukotrienes are formed from arachidonic acid in the membranes of inflammatory cells and bronchial epithelial cells.[6] Within the lungs, cysteinyl LTs impair mucociliary clearance, increase mucus secretion, alter vascular permeability, and enhance airway hyperreactivity.[5,6,15] In humans without renal or hepatic dysfunction, urinary cysteinyl LT measurements accurately reflect endogenous whole-body cysteinyl LT production.[5] Because urine collection is easy to perform, is relatively noninvasive, and reflects overall LT production in the body, several investigators have evaluated the diagnostic use of urinary LTs as a marker of airway inflammation in human asthmatics.[5,15,16] A search for a simple, noninvasive means to monitor the severity of the airway response in allergic animals prompted evaluation of urinary LTs in the cats with experimentally induced asthma reported here.

In this study, we did not detect significant increases in urinary LT-to-creatinine ratios after sensitization, compared with ratios determined before sensitization. There are several possibilities for this.

First, cysteinyl LTs may not be important mediators in asthma in cats. Consistent with this study are the results of a study[m] in which investigators evaluated the urinary LT-to-creatinine ratio in client-owned cats with naturally developing bronchial disease; no increase in the urinary LT-to-creatinine ratio was detected in that study. Additionally, researchers who conducted an in vitro study[11] using another method to induce asthma in cats (cats were sensitized to *Ascaris suum*) revealed that a selective 5-lipoxygenase inhibitor did not have an effect on maximal contraction of tracheal and bronchial smooth muscle obtained from allergen-sensitized and challenge-exposed cats.

Second, we did not empty the bladder of cats in our study prior to challenge exposure with the allergen, so a dilutional effect may have been evident after sensitization. Third, cats may predominantly excrete LTs by another organ, such as the liver, instead of the kidneys. Contrary to the situation in humans,[5] high transient increases in airway concentrations of LTs in cats may not be reflected by increases in whole-body turnover and, ultimately, urinary excretion.[5] Fourth, there may have been decreased free cysteinyl LTs (ie, not bound to LT receptors) locally in the airways that were not reflected by measuring urinary excretion of these mediators. Finally, the time of peak excretion of urinary cysteinyl LTs may have been missed by collection of samples at only a single time point after allergen sensitization. Future studies could be performed to evaluate urinary LT-to-creatinine ratios at multiple time points in cats whose bladders are manually emptied at the time of allergen challenge exposure. However, a urinary marker of airway inflammation that would be applicable clinically in cats with naturally developing asthma is appealing. In clinical patients, the exact time of allergen exposure is rarely known, and a spot test performed on samples collected at the time of initial examination at a veterinary hospital would be most practical. In our study, a spot test did not appear to have clinical applicability. Given our lack of correlation between allergen exposure and an increase in urinary LT concentrations, we chose to evaluate cysteinyl LTs in BALF.

Although direct measurement of LTs in BALF is likely to be more indicative of their local role in asthma, 1 disadvantage is the invasiveness of this procedure, which would preclude frequent serial monitoring.[6] There also are concerns about the interpretation of data for LT concentrations in BALF obtained from humans because of the nonuniform retrieval of saline solution instilled into the airways.[17] This can be circumvented by determining ratios for cysteinyl LTs to a marker substance (eg, total protein) to indicate the degree of dilution. Leukotriene concentrations in BALF at the time of sample collection also reflect production of these inflammatory mediators during only a fraction of the period during which they are produced.[15,18] Finally, collection of BALF, especially when performed repeatedly, may cause ex vivo generation of LTs via activation of inflammatory cells.[5] Addition of an LT-synthesis inhibitor, such as nordihydroguaiaretic acid, to BALF samples can stop ex vivo production of additional cysteinyl LTs.

Despite inflammatory cells and evidence of airway hyperreactivity in the BALF, we did not detect significant increases in BALF LT-to-total protein ratios in asthmatic cats at any time point after challenge exposure to an allergen. In fact, the opposite was seen, with significant decreases in the ratios at all time points evaluated. It is possible that this decrease is statistically significant but not biologically important. Alternatively, the decrease in BALF LT-to-total protein ratios could reflect binding of cysteinyl LTs to their target receptors, decreasing the amount available for measurement in the BALF.

It is unlikely that the failure to find a pattern of increased cysteinyl LT production in cats similar to that found in human asthmatics can be attributed to the commercial test kit for 2 reasons. First, measurable amounts of cysteinyl LTs were detected in urine and BALF samples of the cats. Leukotrienes are fairly conserved across species, making it less likely that a monoclonal antibody produced in 1 species would not recognize the mediator in another species. Second, the test kit has been evaluated for cross-reactivity to other lipid mediators and was found to have minimal cross-reactivity to LTA4 (2%), LTB4 (< 1%), and 11 other arachidonic acid metabolites (< 0.01%); thus, the possibility of cats having a unique pathway of metabolism that interferes with the ELISA is unlikely.

Effects of LTs are dependent on the concentration and location of the mediators as well as the types, distribution, and number of LT receptors in the lungs.[6,19] Leukotriene antagonists appear to play an important

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role in the treatment of many human asthmatics, but their clinical role in the management of asthma in cats remains to be determined. In the study reported here, we were not able to detect significant increases in LT concentrations in urine or BALF. Interestingly, the BALF LT-to-total protein ratios decreased at all time points evaluated after allergen challenge exposure, compared with values determined before sensitization. The possibility that binding of cysteinyl LTs to their target receptors effectively decreased their concentration in BALF deserves further study. Moreover, elucidating the local role of cysteinyl LTs in asthmatic cats has important implications for determining effective therapeutic interventions in cats with this common respiratory tract condition.

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[a]Norris C, Gershwin L, Schelegle E, et al. Experimental model of asthma in cats sensitized to house dust mite or Bermuda grass allergen (abstr). *Am J Resp Crit Care Med* 2001;163:A602.

[b]House–dust mite allergen, 20,000 pnu/ml, Greer Laboratories Inc, Lenoir, NC.

[c]Bermuda grass allergen, 20,000 pnu/ml, Greer Laboratories Inc, Lenoir, NC.

[d]Series 8300, Hans Rudolph Inc, Kansas City, Mo.

[e]Model MP45, Validyne Engineering, Northridge, Calif.

[f]Model DP15-26, Validyne Engineering, Northridge, Calif.

[g]Po-Ne-Mah, Gould Instrument Systems, Valley View, Ohio.

[h]Miniheart, Westmed Inc, Tucson, Ariz.

[i]Nordihydroguaiaretic acid, Sigma Chemical Co, St Louis, Mo.

[j]Easy Air 15, Model PM15P, Precision Medical Inc, Northampton, Pa.

[k]Leukotriene C4/D4/E4 ELISA kit #406410, Neogen Corp, Lexington, Ky.

[l]Bio-Rad Protein Assay Concentrate #500-0006, Bio-Rad Laboratories, Hercules, Calif.

[m]Mellema M, Gershwin L, Norris C. Urinary leukotriene levels in cats with allergic bronchitis (abstr), in *Proceedings*. Am Coll Vet Intern Med Forum 1998;724.

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