

Evaluation of biomarkers in bronchoalveolar lavage fluid for discrimination between asthma and chronic bronchitis in cats

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Objective—To compare concentrations of interleukin (IL)-4, interferon (IFN)- γ , tumor necrosis factor (TNF)- α , and total nitric oxide (NO) metabolites in bronchoalveolar lavage fluid (BALF) for discrimination between asthma and chronic bronchitis in cats.

Animals—97 cats.

Procedures—Cats screened with cytologic examination of BALF included 13 client-owned cats with naturally developing asthma, 8 client-owned cats with chronic bronchitis, 23 research cats with experimentally induced asthma, 33 research cats with experimentally induced nonseptic suppurative inflammation of the airways, and 20 healthy control cats. Banked unconcentrated BALF supernatant samples were assayed for concentrations of IL-4, IFN- γ , TNF- α , and total NO metabolites.

Results—Concentrations of IL-4 and IFN- γ in BALF were less than the limits of detection for most cats, precluding statistical analysis. No significant differences were detected among groups for TNF- α concentrations. Concentrations of total NO metabolites were significantly higher in cats with clinical chronic bronchitis, compared with research cats with nonseptic suppurative inflammation or research cats with asthma.

Conclusions and Clinical Relevance—There were no significant differences in tested biomarkers between cats with asthma and healthy control cats. None of the measured cytokines or NO metabolites were useful for discriminating between cats with naturally developing asthma and those with chronic bronchitis. (*Am J Vet Res* 2010;71:583–591)

Allergic asthma and chronic bronchitis are inflammatory diseases affecting the lower airways of cats. In the veterinary literature, the terminology used to describe these 2 conditions is confusing and includes bronchial asthma, asthmatic bronchitis, allergic bronchitis, feline asthma syndrome, chronic bronchitis, chronic asthmatic bronchitis, chronic bronchitis with emphysema, idiopathic small airway disease, feline obstructive lung disease, feline lower airway disease, and feline bronchial disease.^{1–5} It is postulated that asthma in cats is allergic in etiology and is similar to human allergic asthma, caused by a Th2-lymphocyte-driven hypersensitivity reaction to inhaled aeroallergens.^{2,6,7} Cytokines produced by Th2 cells lead to eosinophilic airway inflammation, airway hyperreactivity, and airway remodeling (permanent architectural changes of the lung); these immunopathologic changes have been characterized in cats on the basis of allergens identified in

ABBREVIATIONS

BALF	Bronchoalveolar lavage fluid
CLIN ASM	Naturally developing clinical asthma
CLIN CB	Naturally developing chronic bronchitis
EXP ASM	Experimentally induced asthma
IFN	Interferon
IL	Interleukin
NO	Nitric oxide
RES SUPP	Research cats with experimentally induced nonseptic suppurative inflammation
Th	T-helper
TNF	Tumor necrosis factor

client-owned cats with naturally developing asthma.⁶ The cause of chronic bronchitis is less well understood and is thought to be secondary to a previous insult (eg, infection or inhaled irritants) that damages the airways. Chronic bronchitis is characterized cytologically by inflammation with nondegenerate neutrophils.⁸ Because chronic allergic asthma can damage the airways, leading to variable neutrophilic inflammation, there is also overlap between these diseases (termed chronic asthmatic bronchitis).⁵

It is difficult to discriminate between asthma and chronic bronchitis on the basis of clinical signs alone because both can induce coughing and wheezing. Although asthma can also be associated with episodic respiratory distress that is at least in part reversible with

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bronchodilators, not all asthmatic cats have clinically important bronchospasm. Additionally, in rare instances, cats with chronic bronchitis may progress to development of emphysema and have expiratory distress minimally responsive to bronchodilators. Diagnostic tests used in practice to attempt to differentiate these conditions include thoracic radiography and cytologic analysis of BALF. Thoracic radiography may reveal normal findings in as many as 23% of cats,¹ and other radiographic abnormalities (eg, bronchial and bronchointerstitial patterns) are frequently found in cats with asthma and those with chronic bronchitis.³ Results of cytologic examination of BALF are also problematic in discriminating between these diseases because the types of inflammatory cells are affected by the timing of exposure to aeroallergens or other inhaled irritants, concurrent secondary bacterial infections, and effects of immunosuppressive drugs or diseases.

Biomarkers—endogenously synthesized biochemical compounds used in the diagnosis, management, and prognostication of disease—have not yet been evaluated in cats with naturally occurring lower airway disease. Because of the difficulty of discriminating asthma from chronic bronchitis in cats via traditional diagnostic tests, it is worthwhile to evaluate the use of inflammatory mediators as potential biomarkers. In humans with asthma, increases in concentrations of IL-4, TNF- α , and NO metabolites or exhaled NO have been found, prompting interest into further investigation of the role of these inflammatory mediators in asthma in cats.^{9–11} Conversely, other studies^{11,12} have found that the Th1 cytokine IFN- γ is reduced in naturally developing and experimental asthma and therefore contributes to the Th2-Th1 imbalance characteristic of allergic disease. Although TNF- α has been implicated in the pathogenesis of chronic bronchitis¹³ and increased exhaled NO in humans with chronic bronchitis,¹⁴ it is not clear if there are differences in the magnitude of increase of these mediators in BALF between humans with asthma and those with chronic bronchitis. The purpose of the study reported here was to compare concentrations of IL-4, IFN- γ , TNF- α , and total NO metabolites in BALF supernatant of cats with naturally developing clinical asthma (CLIN ASM group), cats with naturally developing chronic bronchitis (CLIN CB group), research cats with experimentally induced allergic asthma (EXP ASM group), research cats with experimentally induced nonseptic suppurative inflammation (RES SUPP group), and healthy control cats (control group). We hypothesized that cats with allergic asthma (clinical and experimental) would have increased IL-4 concentrations and an increased ratio of IL-4 to IFN- γ , compared with cats with naturally occurring chronic bronchitis, research cats with nonseptic suppurative inflammation, and healthy control cats. Additionally, we expected cats with inflammatory airway disease (RES SUPP, EXP ASM, CLIN ASM, and CLIN CB groups) to have higher concentrations of TNF- α and NO metabolites than cats in the control group.

Materials and Methods

Client-owned cats—All cats that underwent BALF collection at the Veterinary Medical Teaching Hospital,

College of Veterinary Medicine, University of Missouri, between February 2006 and September 2008 were eligible for inclusion in the study. Cats were included in the study if they had a diagnosis of asthma or chronic bronchitis based on clinical signs of lower airway disease (presence of coughing, wheezing, or episodic respiratory distress), thoracic radiographic findings interpreted by a board-certified radiologist either as normal or as supportive of bronchial disease (ie, bronchial or bronchointerstitial pattern), and BALF cytologic findings of increased eosinophils, neutrophils, or both (compared with typical findings in healthy cats). Cats were excluded if cytologic findings included evidence of septic suppurative inflammation (with or without positive bacteriologic culture results) or neoplastic cells. Because healthy cats can have as many as 2,000 CFUs of bacteria/mL of BALF, cultures with < 2,000 CFUs/mL were not interpreted as evidence of active infection.¹⁵ If > 2,000 CFUs/mL was obtained via direct culture but no evidence of intracellular or extracellular bacteria was seen and neutrophils were nondegenerate and low in number, contamination of the upper portion of the airway was considered likely and those cats were not excluded from study. The study was performed in a retrospective fashion, with ancillary diagnostic tests at the discretion of the attending veterinarian. Relevant diagnostic tests reported here included a CBC, heartworm antibody or antigen ELISA, thoracic radiography, and bacterial culture of BALF. For the purposes of this study, in client-owned cats, a primary diagnosis of asthma (CLIN ASM group) was made if there were $\geq 17\%$ eosinophils and a diagnosis of chronic bronchitis (CLIN CB group) was made if there were $\geq 7\%$ neutrophils in BALF. Cats with $\geq 17\%$ eosinophils and $\geq 7\%$ neutrophils were grouped with the asthmatic cats (ie, they were assumed to have chronic asthmatic bronchitis). We did not exclude cats receiving glucocorticoids if they had cytologic findings consistent with inflammation of the airways.

Research cats—Cats from a research colony at the University of Missouri from which BALF had been collected and stored were eligible for inclusion in the study. Results of cytologic findings were retrospectively reviewed to obtain samples for the control group ($n = 20$; eosinophils < 17%; neutrophils < 7%), RES SUPP group (33; eosinophils < 17%; neutrophils $\geq 7\%$), and EXP ASM group⁶ (23; eosinophils $\geq 17\%$). Most ($n = 26$) cats included in the RES SUPP group were research cats with naturally developing suppurative inflammation; the remainder of the cats (7) had allergen-induced neutrophilic inflammation. Although naturally developing mild chronic bronchitis has been described in research cats,⁸ unlike in that study, we did not serially monitor those cats to determine if the inflammation was chronic, and they served only as control animals with neutrophilic airway inflammation. All cats were cared for according to the principles outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the University of Missouri Animal Care and Use Committee. Cats were given water and a dry feline maintenance diet ad libitum.

BALF collection—Client-owned cats were anesthetized with propofol and received supplemental

oxygen during or after BALF collection. Collection of BALF was performed with bronchoscopic guidance or with a blind technique at the discretion of the attending clinician. Bronchoscopy was performed with a bronchoscope,^a in which a 10- to 20-mL aliquot of sterile saline (0.9% NaCl) solution was instilled directly through the biopsy port after the bronchoscope was wedged in an airway. Retrieval of saline solution was performed via manual suction. The blind technique was performed by intubating the cats with a cuffed 4.0- to 4.5-mm endotracheal tube and gently advancing an 8F polypropylene or red rubber catheter through the endotracheal tube until resistance was felt. A 10- to 20-mL aliquot of sterile saline solution was instilled into the catheter and retrieved by use of manual suction or vacuum suction into a suction trap (mucus specimen trap with suction catheter).^b The clinical pathology laboratory at the Veterinary Medical Teaching Hospital at the University of Missouri processed the BALF and performed differential cell counts. The remaining BALF was centrifuged at $300 \times g$ for 10 minutes, and the supernatant was harvested and banked at -20°C .

Collection of BALF in research cats was performed by use of ketamine (8 to 10 mg/kg, IV) for anesthesia. A blind technique similar to that performed in client-owned cats, with the following modifications, was performed. After intubation and wedging of the polypropylene or red rubber catheter, a 10- to 20-mL aliquot of PBS solution was instilled into the catheter and retrieved via gentle manual suction. The BALF samples were placed immediately on ice and processed within 2 hours after collection. A cytocentrifuge was used to prepare slides for cytologic evaluation; slides were stained with a modified Wright stain. Differential cell counts were determined by evaluating 200 nucleated cells/slide. The remaining BALF was centrifuged at $300 \times g$ for 10 minutes, and the supernatant was collected and stored at -20°C until further analysis.

Total protein concentration—Total protein concentrations in BALF samples were measured with a commercially available Bradford-type protein assay^c in accordance with the manufacturer's instructions. Standards were run in triplicate, and samples were run in duplicate. The limits of detection of the protein assay were 8 to 500 $\mu\text{g/mL}$.

IL-4 and IFN- γ concentrations—Commercially available feline-specific IL-4 and IFN- γ sandwich ELISA kits^d were used to quantitate BALF IL-4 and IFN- γ concentrations in accordance with the manufacturer's instructions. Standards were run in triplicate, and samples were run in duplicate. The IL-4 and IFN- γ concentrations in thawed unconcentrated BALF were calculated by use of values generated from the standard curve. The range of detection of both cytokines in the ELISA was 0.0625 to 4 ng/mL .

Attempts were made to concentrate cytokines by use of a vacuum concentrator^e in which 1.5 mL of sample was concentrated to a final volume of approximately 200 μL (ie, not to dryness). When PBS solution was spiked with known concentrations of recombinant IL-4^d or IFN- γ ^d and concentrated prior to performing the ELISA, results were not accurate and recovery was poor.

TNF- α activity—Tumor necrosis factor- α activity was determined in thawed BALF supernatant by use of a modification of a cytotoxicity bioassay.^{16,17} Briefly, 3.0×10^5 cells from mouse fibroblast (L929) cells were cultured in modified Eagle medium^f plus 1% horse serum^f and 3 μg of actinomycin D/ mL ^g on a 96-well flat-bottom tissue culture plate.^h Fifty microliters of BALF supernatant was added to the wells in duplicate. After a 20-hour incubation, 3-[4,5-dimethylthiazol-2-yl]-2,5-di-phenyl tetrazolium bromide^g was added and the cells were incubated for an additional 2.5 hours. The formazan crystals were solubilized in 50% dimethylformamideⁱ and 20% SDS.ⁱ Color development after 1 hour was measured at 630 nm. Recombinant feline TNF- α ^j was used to construct a standard curve. The range of detection of this assay was 0.2 to 10 ng/mL . For statistical analysis, samples with activity less than the lower limit of detection of the assay were assigned a concentration of 0.2 ng/mL .

NO metabolites concentrations—Thawed aliquots of BALF supernatant were used for determination of the NO end products nitrite (NO_2^-) and nitrate (NO_3^-) by use of components from a commercially available kit.^k All samples were passed through a 10,000 molecular-weight cutoff filter^{d,l} at $3,000 \times g$ for 30 minutes. Eighty microliters of BALF supernatant was plated in duplicate by use of a 96-well high-binding certified flat-bottom plateⁱ with 10 μL of nitrate reductase and 10 μL of NADPH (enzyme cofactor)/well and incubated for 3 hours at 22°C to reduce nitrate to nitrite. The final concentration of nitrite was measured by use of Griess reagents sulfanilamide and *N*-(1-naphthyl) ethylenediamine to convert the nitrite in BALF to a deep purple azo compound. Absorbance was measured at 540 nm, and the concentration of nitrite was multiplied by 2.5 to obtain nitrate concentration. The range of detection of nitrate for the assay was 2.5 to 87.5 μM .

Statistical analysis—Statistical analyses were performed with commercially available software.^m Data were analyzed for normality by use of a Kolmogorov-Smirnov test. A Kruskal-Wallis ANOVA on ranks test was used to compare the percentages of eosinophils and neutrophils, total nitrate concentration, and TNF- α concentration in BALF among groups. When appropriate, a Dunn multiple comparison method was performed to delineate differences between individual groups. The IL-4-to-IFN- γ ratio was calculated for cats in the EXP ASM group that had detectable concentrations of both cytokines (7/23 cats) by dividing the concentration of IL-4 by the concentration of IFN- γ . Differences were considered significant at $P < 0.05$.

Results

CLIN ASM group—Thirteen cats (8 castrated males, 4 spayed females, and 1 sexually intact male) were identified with naturally developing asthma. Cats had a mean \pm SD age of 7.2 ± 1.1 years and a mean \pm SD weight of 5.3 ± 0.3 kg. Breeds included domestic shorthair ($n = 7$), mixed-breed cat (4), domestic longhair (1), and Ocicat (1).

Clinical signs related to the respiratory tract identified via history or physical examination included

coughing (n = 9 cats), increased bronchovesicular sounds (6), respiratory distress (3), and wheezing (1). When information was available (n = 9), CLIN ASM cats had clinical signs consistent with asthma (eg, coughing, wheezing, and episodic respiratory distress) months or years prior to referral to the Veterinary Medical Teaching Hospital and subsequent BALF collection. In cats that had a CBC performed within 2 months of BALF collection (n = 9), abnormalities related to inflammation included eosinophilia (4), neutrophilia (3), hyperproteinemia (3), and monocytosis (1). Tests for heartworm antibody, antigen ELISA, or both yielded negative results in all CLIN ASM cats tested (n = 8). Medications administered within 2 weeks of BALF collection (n = 6 cats) included prednisone or prednisolone administered orally at 2 mg/kg/d (2), 1.5 mg/kg/d (1), 1 mg/kg/d (1), or 1 mg/kg every other day (1); inhaled fluticasone (220 µg/actuation, q 12 h [1]); orally administered terbutaline (1.25 mg, q 12 h [1]); orally administered cyproheptadine (2 mg, q 12 h [1]); orally administered enrofloxacin (22.7 mg, q 24 h [1]); and orally administered doxycycline (25 mg, q 12 h [1]).

Thoracic radiographs were obtained within 2 months of BALF collection for 12 of 13 cats. Abnormal findings included bronchointerstitial (n = 6 cats) and bronchial (4) lung patterns, hyperinflation of the lungs (1), and atelectasis of the right middle lung lobe (1). Two cats had unremarkable findings. One cat with radiographs obtained > 2 months previously (during a period of severe coughing) had unremarkable findings.

Collection of BALF was performed with a blind technique (n = 10 cats) or bronchoscopic guidance (3). Two cats had bacterial counts > 2,000 CFUs/mL (*Corynebacterium* spp, *Staphylococcus epidermis*, or *Propionibacterium* spp); however, oral contamination or contamination of the upper portion of the airway was thought likely because no intracellular or extracellular bacteria were seen on cytologic examination and the cats only had 1% and 3% neutrophils in BALF, respectively.

CLIN CB group—Eight neutered cats (5 males and 3 females) with a mean ± SD age of 7.9 ± 1.1 years and a mean ± SD weight of 5.2 ± 0.2 kg were identified with naturally developing chronic bronchitis. Breeds included domestic shorthair (n = 3 cats), mixed-breed cat (1), Persian (1), domestic longhair (1), Russian Blue (1), and Himalayan (1).

Clinical signs related to respiratory tract disease determined via history and physical examination included coughing (n = 5 cats), increased bronchovesicular sounds (4), and respiratory distress (1). The cat with expiratory respiratory distress also had evidence of hyperinflation of the lungs and was minimally responsive to orally administered and inhaled bronchodilators (ie, there was suspicion of chronic bronchitis with emphysema). When information was available, cats (n = 7) had clinical signs consistent with chronic bronchitis months or years prior to referral to the Veterinary Medical Teaching Hospital and subsequent BALF collection. Cats that had a CBC performed within 2 months of BALF collection (n = 6 cats) had 2 abnormalities pertaining to inflammation: hyperproteinemia (3) and monocytosis (1). Heartworm antibody and an-

tigen ELISA results were negative for all cats tested (n = 4). Medications administered within 2 weeks of BALF collection (n = 3 cats) included prednisone administered orally at 1 mg/kg every other day (1) and 2.5 mg/kg/d (1) and unknown doses of chlorpheniramine (1) and nebulized enrofloxacin (1).

Thoracic radiographs were obtained within 2 months of BALF collection for cats in the CLIN CB group (n = 7); findings included bronchointerstitial (4) and bronchial (2) patterns, cardiomegaly and pulmonary arterial enlargement (2), and bronchiectasis (1). The 2 cats that had pulmonary arterial enlargement had hypertrophic cardiomyopathy as diagnosed via echocardiography. In 1 cat, thoracic radiographs were obtained that were not available for review by a board-certified radiologist, and the cat was not included in the study.

Collection of BALF was performed in all cats by use of a blind technique (n = 4 cats) or bronchoscopic guidance (4). No cat had bacteriologic culture results that were considered positive (> 2,000 CFUs/mL).

Research cats—All research cats were < 1 year of age when BALF was collected. Weight of the research cats was not routinely recorded at the time of BALF collection. None of the cats in the control and RES SUPP groups had evidence of respiratory tract disease via physical examination or recorded in daily monitoring records. Cats in the EXP ASM group had variable responses to aerosol challenges of allergen, including increased respiratory rate, coughing, and increased bronchovesicular sounds or wheezing. Complete blood counts and thoracic radiography were not performed.

Eosinophils—The percentage of eosinophils in BALF samples was determined (Figure 1). Cats in the EXP ASM and CLIN ASM groups had a significantly ($P < 0.001$) greater percentage of eosinophils, compared with cats in the control, RES SUPP, and CLIN CB groups. There was no significant difference ($P = 0.271$) in percentage of eosinophils between the EXP ASM and CLIN ASM groups. For CLIN ASM cats receiving glucocorticoids and not receiving glucocorticoids, median percentage of eosinophils was 51% (range, 22% to 80%) and 67% (range, 17% to 90%), respectively.

Neutrophils—The percentage of neutrophils in BALF samples was determined (Figure 2). Cats in the CLIN CB group had a significantly ($P < 0.001$) greater

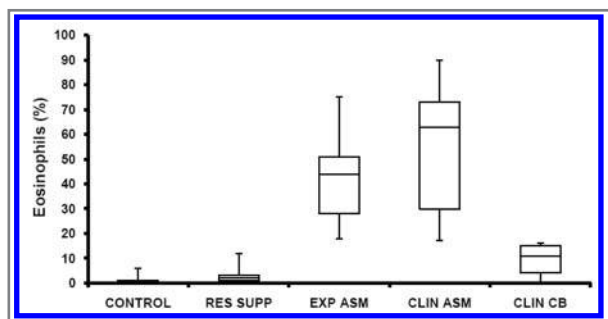


Figure 1—Percentages of eosinophils in BALF samples obtained from cats in various groups. The upper and lower boundaries of each box represent the 75th and 25th percentiles, respectively. The line within the box represents the median value, and the whiskers represent the range.

percentage of neutrophils, compared with cats in the control, EXP ASM, and CLIN ASM groups, and cats in the RES SUPP group had a significantly ($P < 0.001$) greater percentage of neutrophils than did cats in the control and EXP ASM groups. Cats in the CLIN CB group receiving glucocorticoids and not receiving glucocorticoids had a median percentage of neutrophils of 49% (range, 30% to 67%) and 46% (range, 23% to 88%), respectively.

Total protein concentration—Median concentration of total protein in BALF samples was determined in

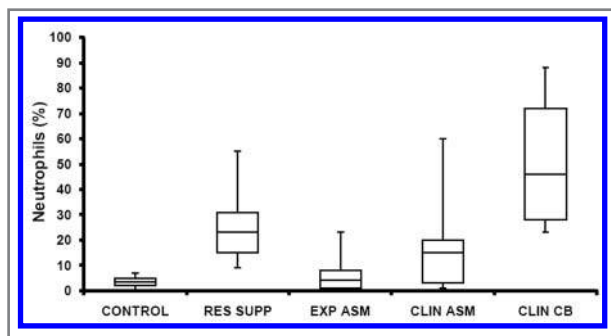


Figure 2—Percentages of neutrophils in BALF samples obtained from cats in various groups. The upper and lower boundaries of each box represent the 75th and 25th percentiles, respectively. The line within the box represents the median value, and the whiskers represent the range.

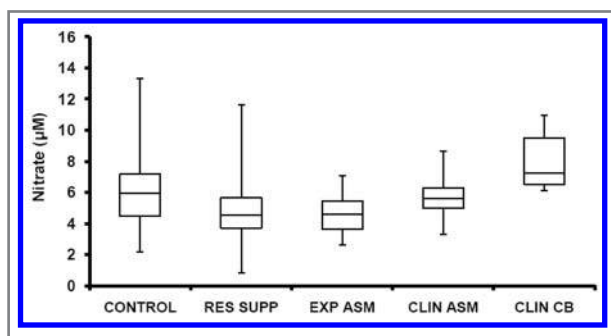


Figure 3—Concentrations of nitrate in BALF samples obtained from cats in various groups. The upper and lower boundaries of each box represent the 75th and 25th percentiles, respectively. The line within the box represents the median value, and the whiskers represent the range.

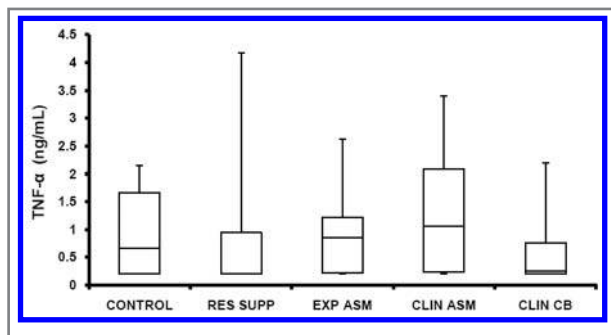


Figure 4—Concentrations of TNF- α in BALF samples obtained from cats in various groups. The upper and lower boundaries of each box represent the 75th and 25th percentiles, respectively. The line within the box represents the median value, and the whiskers represent the range.

the control (140 ng/mL; range, 50 to 616 ng/mL), RES SUPP (179 ng/mL; range, 55 to 926 ng/mL), EXP ASM (145 ng/mL; range, 8 to 401 ng/mL), CLIN ASM (193 ng/mL; range, 50 to 661 ng/mL), and CLIN CB (217 ng/mL; range, 50 to 1,258 ng/mL) groups. There was no significant ($P = 0.167$) difference among groups.

IL-4 and IFN- γ concentrations—Most of the cats had BALF concentrations of IL-4 and IFN- γ that were less than the lower limit of detection for the assays (ie, 0.0625 ng/mL), precluding meaningful statistical analysis. For cats that had detectable concentrations of IL-4 and IFN- γ , the range of cytokine concentrations in BALF samples was determined in the control (IL-4, 0.08 to 0.51 ng/mL; IFN- γ , 0.08 to 0.18 ng/mL), RES SUPP (IL-4, 0.07 to 0.08 ng/mL), EXP ASM (IL-4, 0.07 to 2.12 ng/mL; IFN- γ , 0.06 to 1.09 ng/mL), CLIN ASM (IFN- γ , 0.14 ng/mL), and CLIN CB (IL-4, 0.09 to 0.14 ng/mL) groups. The number of cats in each group that had cytokine concentrations below the lower limit of detection was determined in the control (IL-4, 16/20; IFN- γ , 17/20), RES SUPP (IL-4, 30/33; IFN- γ , 33/33), EXP ASM (IL-4, 11/23; IFN- γ , 15/23), CLIN ASM (IL-4, 13/13; IFN- γ , 12/13), and CLIN CB (IL-4, 5/8; IFN- γ , 8/8) groups. Only a small number of cats in the EXP ASM group (7/23) had detectable concentrations of IL-4 and IFN- γ simultaneously; the mean \pm SD IL-4-to-IFN- γ ratio was 1.97 ± 1.16 .

Interleukin-4 was not detectable in BALF of any cat in the CLIN ASM group, regardless of whether they were receiving glucocorticoids ($n = 6$ cats) or not (7). Interferon- γ was detected in only 1 cat in the CLIN ASM group, and that cat was being treated with glucocorticoids at the time of collection. Interleukin-4 was detectable in only 3 cats in the CLIN CB group; 1 was receiving glucocorticoids, and the other 2 were not. Interferon- γ was not detectable in any cats in the CLIN CB group, regardless of whether they were receiving glucocorticoids ($n = 2$ cats) or not (6).

NO metabolites and TNF- α concentrations—The CLIN CB group had significantly ($P < 0.001$) greater nitrate concentrations in BALF samples, compared with RES SUPP and EXP ASM cats (Figure 3). There was no significant difference in nitrate concentrations among other groups. There was no significant ($P = 0.105$) difference among groups in the concentrations of TNF- α in BALF samples (Figure 4).

Effect of glucocorticoid treatment on biomarkers in CLIN ASM and CLIN CB groups—When pet cats treated with glucocorticoids were removed from the statistical analysis, there was no difference in the overall results of statistical analysis or interpretation of the study.

Discussion

In the present study, none of the potential biomarkers evaluated (IL-4, IFN- γ , TNF- α , and NO metabolites) in BALF were useful for discriminating between client-owned cats with naturally developing asthma and chronic bronchitis. There are several possible reasons for this. Cytokines in the epithelial lining fluid are variably and unpredictably diluted by saline solution in-

stilled during collection of BALF.¹⁸ Although there were no significant differences among groups in BALF total protein concentration (a crude marker of dilution), it is clear that BALF represents substantial dilution of the epithelial lining fluid. Compounding the dilutional effects is the poor sensitivity of available feline-specific cytokine assays. Many of the samples yielded values less than the lower limit of detection of the assays for IL-4, IFN- γ , and TNF- α . Thus, it is possible that if a more sensitive assay were available, differences could be detected among groups. Alternatively, concentrating the cytokines in BALF might enhance detection. Attempts at use of a concentrator did not produce reliable results in the ELISAs by use of saline solution spiked with known concentrations of feline-specific recombinant cytokines, but use of high-molecular-weight cutoff filters is an alternative means of concentration that could be evaluated in future studies. The second possible reason for lack of usefulness of the biomarkers tested in this study was that inflammatory mediators evaluated have minimal roles or no role in the diseases studied. Selection of the cytokines was limited by the availability of reagents for use in cats and the type of banked samples available (ie, acellular BALF supernatant). Although IL-4 and IFN- γ are prototypical Th2 and Th1 cytokines, respectively, that play a role in allergic asthma,^{11,19} IL-5 is a key cytokine that is involved with eosinophil maturation, differentiation, and survival.²⁰ Because eosinophilic airway inflammation is one of the hallmark differences between asthma and chronic bronchitis, analysis for IL-5 would have been logical; however, a feline-specific IL-5 ELISA is not currently available. Another possible reason for the lack of positive results in this study was the fact that in naturally developing disease, there is not a consistent course of inflammatory events. For example, client-owned cats with asthma are likely to be sensitized to different types and quantities of allergens and have variability in timing of exposure. If there was no recent exposure around the time of BALF collection, certain inflammatory mediators might not be increased. When BALF is collected, it is essentially representative of a snapshot in time of the cytokine profile, whereas it is likely that there are continual changes in the expression of the various cytokines. In the cats with experimentally induced asthma, because administration of allergen was standardized with respect to amount and timing, detection of the cytokines of interest was much more likely. Typically, client-owned cats were older (7 years) than research cats (< 1 year). This difference in age may introduce variations in the inflammatory profile among groups because age may alter the immune response. Furthermore, if the client-owned cats had inflammatory cytologic findings, they were not excluded from the study if they were recently or concurrently being administered glucocorticoids; glucocorticoids can affect the inflammatory profile and may have prevented detection of clear differences among biomarkers in different groups.

The purpose of the study was to find discriminatory biomarkers by evaluating possible differences in underlying molecular mechanisms of disease between cats with asthma and those with chronic bronchitis. The veterinary literature has historically focused on

differentiating diseases of the lower portion of the airways of cats on the basis of historical, physical, radiographic, clinicopathologic, and cytologic evaluation^{1,3,15}; however, controversy still exists regarding the exact definitions and criteria for these diseases. Although not clear-cut, there are distinguishing clinical and pathological features that suggest that asthma and chronic bronchitis in cats are not the same disease. For example, episodic airflow limitation and associated expiratory respiratory distress that is at least partially reversible with bronchodilators is a feature of asthma, whereas respiratory distress is uncommon with chronic bronchitis. When cats with chronic bronchitis have evidence of respiratory distress (as did 1 cat in the CLIN CB group), in the authors' opinion, it is usually minimally responsive to bronchodilators both clinically and radiographically (ie, there is radiographic persistence of air trapping). Additionally, because asthma in cats is believed to be allergic in origin,^{2,6,7} eosinophils are a key cell type involved in airway inflammation. Additional support for the idea that cats have allergic asthma is the ability to experimentally replicate the major features of asthma in research cats by use of allergens identified in client-owned cats with naturally developing asthma.⁶ Specifically, cats sensitized to house dust mite or Bermuda grass allergen develop allergen-specific IgE production, airway eosinophilia, an acute-response Th2 cell cytokine profile, airway hyperreactivity in response to allergen challenge, and histologic evidence of airway remodeling. These represent the major features seen in humans with asthma. In contrast, cats with chronic bronchitis develop nonseptic suppurative inflammation.⁸ Increased mucus production and chronic coughing are commonly reported in the veterinary literature as key components of chronic bronchitis in cats, although both are nonspecific and the former is hard to quantitate.

Recent advances in human medicine have focused on the presence of cytokines and inflammatory mediators as a means of developing novel diagnostic tests and therapeutics for diseases of the lower portion of the airways.^{9,10,21} To our knowledge, no effort has been made to identify inflammatory mediators in BALF to use as potential biomarkers to differentiate between allergic asthma and chronic bronchitis. Selection of candidate inflammatory mediators is somewhat dependent on the reagents that are available as well as an understanding of the immunopathologic mechanisms of disease.

Asthma in cats is postulated to be similar to allergic asthma in humans, in which exposure to aeroallergens induces a preferential polarization of the immune response toward Th2 cytokine production (eg, IL-4, IL-5, IL-9, and IL-13) that leads to the hallmark features of allergic asthma, namely, airway inflammation, airway hyperreactivity, and airway remodeling.^{22,23} Moreover, Th2 cytokines are thought to suppress the Th1 immune response and associated cytokines (eg, IFN- γ) that play an important role in the regulation of immune balance.^{24,25} Less is known about the pathogenesis of chronic bronchitis in cats, which is generally accepted to be characterized by mucus hypersecretion and cytologic evidence of nondegenerate neutrophilic inflammation. As in humans, the inflammation arises second-

ary to an insult to the lower airways that is nonspecific and frequently not definitively identified.^{8,13}

The production of IL-4 by Th2 lymphocytes is thought to be critical in development of allergic asthma because it helps Th0 cells (uncommitted T cells) differentiate into Th2 cells, allowing antibody class switching and production of IgE.²⁰ Its central role in asthma has been highlighted in studies²⁶ in IL-4 knock-out mice, which, after sensitization and challenge with ovalbumin, lack both airway eosinophilia and hyperreactivity. Asthmatic children have significantly increased IL-4 concentrations in exhaled breath condensate, compared with healthy children,¹¹ and asthmatic adults have increased IL-4 positive cells in bronchial tissue, compared with adults with chronic bronchitis.²⁷ However, IL-4 is not necessarily detectable in BALF from human asthmatics, even if symptomatic.²⁸ Compatible with the latter study, not a single cat with naturally developing asthma had detectable BALF IL-4 concentrations. This may be explained by the fact that IL-4 appears to play more of a role in the early stages of Th2 development,²⁹ and the cats in the present study likely had chronic disease.

The role for IFN- γ , a Th1 proinflammatory cytokine, is somewhat controversial in allergic asthma, with results of some studies^{11,20,30,31} supporting decreases in this cytokine (presumptively attributable to suppression of the Th1 immune response by IL-4) and results of others^{32,33} indicating an increase in severe chronic or acute asthma. Evaluation of the IL-4-to-IFN- γ ratio allows for comparison of the relative contribution of Th2- and Th1-mediated immunity. In support of this, asthmatic children have increased IL-4 and reduced IFN- γ concentrations in exhaled breath condensate and an increased IL-4-to-IFN- γ ratio, compared with healthy children.¹¹ In the present study, although many cats with experimentally induced asthma had undetectable concentrations of IL-4 or IFN- γ , for the 7 cats in which both cytokines were measurable, the mean IL-4-to-IFN- γ ratio was 1.9, consistent with a Th2-predominant immune response. None of the cats with a primarily neutrophilic airway response (ie, RES SUPP and CLIN CB groups) had detectable concentrations of IFN- γ in BALF.

In humans with asthma, TNF- α contributes to the pathogenesis of disease in various ways, including recruitment of inflammatory cells, airway hyperresponsiveness, and airway remodeling.^{9,20} High concentrations of TNF- α are linked to complications in humans with asthma.¹³ The concentration of TNF- α in human BALF was reported to be higher in patients with severe asthma, compared with that in patients with mild disease, suggesting that the beneficial effects of a TNF- α antagonist may be limited to patients with severe, refractory disease.⁹ However, TNF- α activity is not limited to asthmatics, as indicated by increased TNF- α in the sputum^{20,34} or BALF³⁵ of humans with chronic bronchitis or chronic obstructive inflammatory disease. The lack of significant differences in BALF TNF- α concentration among groups in the present study may suggest that the cats had mild or moderate disease instead of severe disease or that TNF- α is not a critical mediator in these diseases in cats.

Nitric oxide is an important mediator of host defenses via elaboration of reactive nitrogen species; however, excessive production of reactive nitrogen species may also contribute to ongoing airway inflammation in diseases such as asthma and chronic bronchitis.^{14,36,37} Asthmatic children³⁸ and adults¹⁰ have increased NO metabolites in sputum, compared with healthy subjects. Most human studies^{39,40} have evaluated exhaled NO (vs NO metabolites in BALF, as was done in the present study), and there is good evidence that exhaled NO is linked to eosinophilic airway inflammation in asthma. There is also evidence that chronic bronchitis in humans is associated with increased exhaled NO.¹⁴ To the authors' knowledge, metabolites of NO have never been evaluated in the BALF of client-owned cats with naturally developing allergic asthma and chronic bronchitis. On the basis of the human literature, we expected all cats with inflammatory airway disease to have increased total NO metabolites, compared with healthy cats with no cytologic evidence of airway inflammation. We wanted to determine whether there were differences in the magnitude of increased total NO metabolites between cats with asthma and those with chronic bronchitis. Although concentrations of total NO metabolites were increased in the CLIN CB group, compared with those of the RES SUPP and EXP ASM groups, there was no significant difference between the CLIN CB group and the CLIN ASM group. Moreover (and somewhat unexpectedly), there was no significant difference in concentrations of total NO metabolites in the CLIN CB and CLIN ASM groups, compared with concentrations of the control group. Given the small sample size and marked variability in the NO metabolite concentrations, it is likely that the lack of difference was simply because of type II statistical error. Additional study in a larger number of healthy client-owned cats and client-owned cats with naturally developing asthma and chronic bronchitis is warranted.

In the present study, cats with naturally developing asthma (6/13) and chronic bronchitis (2/8) were receiving or had recently (within 2 weeks of BALF collection) received glucocorticoids (orally administered or inhaled). Glucocorticoids represent the mainstay of treatment for cats with asthma and chronic bronchitis because they are considered effective in suppressing the inflammatory response by inhibiting production of various inflammatory mediators.^{8,41,42} Cats were not excluded from the study as long as they had cytologic evidence of inflammation in BALF. Admittedly, glucocorticoids can alter the inflammatory profile in inflammatory airway disease. For example, in mice with chronic asthma, dexamethasone administration decreased expression of IL-4, IFN- γ , and TNF- α ⁴³ but also significantly reduced eosinophilic inflammation in tandem with the decrease in cytokine production. Cats in the CLIN ASM and CLIN CB groups in the present study that were receiving glucocorticoids still had evidence of inflammatory airway disease in BALF samples (mean, 50% eosinophils and 49% neutrophils).

Although there is strong speculation that asthma in cats is allergic in origin and therefore could be amenable to some specific treatments that may alter Th2-cell-driven processes,^{2,6,7,22,23} progress in understanding this naturally developing disease is hampered by a

lack of clear guidelines on differentiating asthma from chronic bronchitis. In fact, there are > 10 terms used to characterize noninfectious lower airway inflammation in cats.^{1-5,15} In the present study, guidelines used were roughly adapted from a review on BALF and defined asthma as a cytologic finding of $\geq 17\%$ eosinophils and chronic bronchitis as a cytologic finding of $\geq 7\%$ neutrophils.⁴⁴ Knowing that asthma is a chronic inflammatory disorder that can damage airway epithelial cells but is still predominantly driven by a hypersensitivity to allergen, we chose to place cats with both $\geq 17\%$ eosinophils and $\geq 7\%$ neutrophils in the asthma group. Neutrophils have been described as important cells involved in the pathogenesis of severe asthma in humans, even when superimposed on the injury incited by eosinophils.^{45,46}

The inflammatory mediators (IL-4, IFN- γ , TNF- α , and NO metabolites) that were investigated as possible biomarkers did not appear to be useful in differentiation between cats with naturally developing allergic asthma and those with chronic bronchitis. However, this study was not without limitations, including the low sensitivity of the assays for detecting IL-4, IFN- γ , and TNF- α in BALF supernatant and the inability to measure other relevant biomarkers because of the lack of feline-specific reagents. Future studies should focus on validating a technique for concentrating BALF supernatant or increasing the sensitivity of the currently available feline-specific cytokine assays. Additionally, future studies with more rigorous inclusion criteria and with non-traditional diagnostic tests (eg, pulmonary mechanics, including methcholine challenge and response to bronchodilator treatment, and evaluation of recent allergen exposure among asthmatic cats by use of intradermal skin testing or serum allergen-specific IgE reactivity) may help to detect BALF biomarkers that can be used to distinguish between cats with asthma and those with chronic bronchitis.

- a. Fujinon EB 110, Wayne, NJ.
- b. Tri-State Hospital Supply Corp, Howell, Mich.
- c. BioRad Laboratories, Hercules, Calif.
- d. R&D Systems, Minneapolis, Minn.
- e. Savant SPP11V SpeedVac, Thermo Electron Corp, Waltham, Mass.
- f. Gibco, Invitrogen Corp, Auckland, New Zealand.
- g. Sigma-Aldrich, St Louis, Mo.
- h. Benton Dickinson & Co, Franklin Lakes, NJ.
- i. Fischer Scientific International, Pittsburgh, Pa.
- j. Animal Health Diagnostic Center, Cornell University, Ithaca, NY.
- k. Cayman Chemical nitrate/nitrite assay kit, Cayman Chemical Co, Ann Arbor, Mich.
- l. Amicon Ultra, Millipore Corp, Billerica, Mass.
- m. SigmaStat, Systat Software Inc, San Jose, Calif.

References

1. Adamama-Moraitou KK, Patsikas MN, Koutinas AF. Feline lower airway disease: a retrospective study of 22 naturally occurring cases from Greece. *J Feline Med Surg* 2004;6:227-233.
2. Corcoran BM, Foster DJ, Fuentes VL. Feline asthma syndrome: a retrospective study of the clinical presentation in 29 cats. *J Small Anim Pract* 1995;36:481-488.
3. Foster SF, Allan GS, Martin P, et al. Twenty-five cases of feline bronchial disease (1995-2000). *J Feline Med Surg* 2004;6:181-188.
4. Moriello KA, Stepien RL, Henik RA, et al. Pilot study: prevalence of positive aeroallergen reactions in 10 cats with small-airway disease without concurrent skin disease. *Vet Dermatol* 2007;18:94-100.
5. Moise NS, Wiedenkiller D, Yeager AE, et al. Clinical, radiographic, and bronchial cytologic features of cats with bronchial disease: 65 cases (1980-1986). *J Am Vet Med Assoc* 1989;194:1467-1473.
6. Norris Reiner CR, Decile KC, Berghaus RD, et al. An experimental model of allergic asthma in cats sensitized to house dust mite or Bermuda grass allergen. *Int Arch Allergy Immunol* 2004;135:117-131.
7. Prost C. Treatment of feline asthma with allergen avoidance and specific immunotherapy: experience with 20 cats. *Rev Fr Allergol Immunol Clin* 2008;48:409-413.
8. Kirschvink N, Leemans J, Delvaux F, et al. Inhaled fluticasone reduces bronchial responsiveness and airway inflammation in cats with mild chronic bronchitis. *J Feline Med Surg* 2006;8:45-54.
9. Brightling C, Berry M, Amrani Y. Targeting TNF-alpha: a novel therapeutic approach for asthma. *J Allergy Clin Immunol* 2008;121:5-10.
10. Jang AS, Choi IS, Lee S, et al. Nitric oxide metabolites in induced sputum: a marker of airway inflammation in asthmatic subjects. *Clin Exp Allergy* 1999;29:1136-1142.
11. Shahid SK, Kharitonov SA, Wilson NM, et al. Increased interleukin-4 and decreased interferon-gamma in exhaled breath condensate of children with asthma. *Am J Respir Crit Care Med* 2002;165:1290-1293.
12. Koch M, Witznath M, Reuter C, et al. Role of local pulmonary IFN-gamma expression in murine allergic airway inflammation. *Am J Respir Cell Mol Biol* 2006;35:211-219.
13. Mukhopadhyay S, Hoidal JR, Mukherjee TK. Role of TNFalpha in pulmonary pathophysiology. *Respir Res* 2006;7:125.
14. Delen FM, Sippel JM, Osborne ML, et al. Increased exhaled nitric oxide in chronic bronchitis: comparison with asthma and COPD. *Chest* 2000;117:695-701.
15. Dye JA, McKiernan BC, Rozanski EA, et al. Bronchopulmonary disease in the cat: historical, physical, radiographic, clinicopathologic, and pulmonary functional evaluation of 24 affected and 15 healthy cats. *J Vet Intern Med* 1996;10:385-400.
16. Baarsch MJ, Wannemuehler MJ, Molitor TW, et al. Detection of tumor necrosis factor alpha from porcine alveolar macrophages using an L929 fibroblast bioassay. *J Immunol Methods* 1991;140:15-22.
17. DeClue AE, Cohn LA, Lechner ES, et al. Effects of subanesthetic doses of ketamine on hemodynamic and immunologic variables in dogs with experimentally induced endotoxemia. *Am J Vet Res* 2008;69:228-232.
18. Dargaville PA, South M, Vervaart P, et al. Validity of markers of dilution in small volume lung lavage. *Am J Respir Crit Care Med* 1999;160:778-784.
19. Barnes PJ. Immunology of asthma and chronic obstructive pulmonary disease. *Nat Rev Immunol* 2008;8:183-192.
20. Barnes PJ. The cytokine network in asthma and chronic obstructive pulmonary disease. *J Clin Invest* 2008;118:3546-3556.
21. Cohn L, Herrick C, Niu N, et al. IL-4 promotes airway eosinophilia by suppressing IFN-gamma production: defining a novel role for IFN-gamma in the regulation of allergic airway inflammation. *J Immunol* 2001;166:2760-2767.
22. Reiner CR, Byerly JR, Berghaus RD, et al. Rush immunotherapy in an experimental model of feline allergic asthma. *Vet Immunol Immunopathol* 2006;110:141-153.
23. Reiner CR, Cohn LA, Delgado C, et al. Adjuvanted rush immunotherapy using CpG oligodeoxynucleotides in experimental feline allergic asthma. *Vet Immunol Immunopathol* 2008;121:241-250.
24. Larche M. Immunoregulation by targeting T cells in the treatment of allergy and asthma. *Curr Opin Immunol* 2006;18:745-750.
25. Romagnani S. The role of lymphocytes in allergic disease. *J Allergy Clin Immunol* 2000;105:399-408.
26. Brusselle GG, Kips JC, Tavernier JH, et al. Attenuation of allergic airway inflammation in IL-4 deficient mice. *Clin Exp Allergy* 1994;24:73-80.
27. Mueller R, Chanez P, Campbell AM, et al. Different cytokine patterns in bronchial biopsies in asthma and chronic bronchitis. *Respir Med* 1996;90:79-85.

28. Broide DH, Paine MM, Firestein GS. Eosinophils express interleukin 5 and granulocyte macrophage-colony-stimulating factor mRNA at sites of allergic inflammation in asthmatics. *J Clin Invest* 1992;90:1414–1424.
29. Barnes PJ, Chung KF, Page CP. Inflammatory mediators of asthma: an update. *Pharmacol Rev* 1998;50:515–596.
30. Hartl D, Griese M, Nicolai T, et al. Pulmonary chemokines and their receptors differentiate children with asthma and chronic cough. *J Allergy Clin Immunol* 2005;115:728–736.
31. Wong CK, Ho CY, Ko FW, et al. Proinflammatory cytokines (IL-17, IL-6, IL-18 and IL-12) and Th cytokines (IFN-gamma, IL-4, IL-10 and IL-13) in patients with allergic asthma. *Clin Exp Immunol* 2001;125:177–183.
32. Marguet C, Dean TP, Warner JO. Soluble intercellular adhesion molecule-1 (sICAM-1) and interferon-gamma in bronchoalveolar lavage fluid from children with airway diseases. *Am J Respir Crit Care Med* 2000;162:1016–1022.
33. Kumar RK, Webb DC, Herbert C, et al. Interferon-gamma as a possible target in chronic asthma. *Inflamm Allergy Drug Targets* 2006;5:253–256.
34. Keatings VM, Collins PD, Scott DM, et al. Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma. *Am J Respir Crit Care Med* 1996;153:530–534.
35. Katsuki M. Neutrophil chemotactic activity in bronchoalveolar lavage fluid recovered from patients with diffuse panbronchiolitis. *Kurume Med J* 1996;43:279–287.
36. Dweik RA, Comhair SA, Gaston B, et al. NO chemical events in the human airway during the immediate and late antigen-induced asthmatic response. *Proc Natl Acad Sci U S A* 2001;98:2622–2627.
37. van der Vliet A, Eiserich JP, Shigenaga MK, et al. Reactive nitrogen species and tyrosine nitration in the respiratory tract: epiphenomena or a pathobiologic mechanism of disease? *Am J Respir Crit Care Med* 1999;160:1–9.
38. Kumar L, Rajput N, Majumdar S. Nitric oxide metabolites in induced sputum: a noninvasive marker of airway inflammation in asthma. *Indian Pediatr* 2005;42:329–337.
39. Payne DN. Nitric oxide in allergic airway inflammation. *Curr Opin Allergy Clin Immunol* 2003;3:133–137.
40. Kharitonov SA, Barnes PJ. Nitric oxide in exhaled air is a new marker of airway inflammation. *Monaldi Arch Chest Dis* 1996;51:533–537.
41. Liu MC, Proud D, Lichtenstein LM, et al. Effects of prednisone on the cellular responses and release of cytokines and mediators after segmental allergen challenge of asthmatic subjects. *J Allergy Clin Immunol* 2001;108:29–38.
42. Reiner CR, Brownlee L, Decile KC, et al. Inhaled flunisolide suppresses the hypothalamic-pituitary-adrenocortical axis, but has minimal systemic immune effects in healthy cats. *J Vet Intern Med* 2006;20:57–64.
43. Herbert C, Hettiaratchi A, Webb DC, et al. Suppression of cytokine expression by roflumilast and dexamethasone in a model of chronic asthma. *Clin Exp Allergy* 2008;38:847–856.
44. Hawkins EC, DeNicola DB, Kuehn NF. Bronchoalveolar lavage in the evaluation of pulmonary disease in the dog and cat. State of the art. *J Vet Intern Med* 1990;4:267–274.
45. Jatakanon A, Uasuf C, Maziak W, et al. Neutrophilic inflammation in severe persistent asthma. *Am J Respir Crit Care Med* 1999;160:1532–1539.
46. Sampson AP. The role of eosinophils and neutrophils in inflammation. *Clin Exp Allergy* 2000;30(suppl 1):22–27.