THE EFFECTS OF AN ANTISERITONERGIC DRUG AND ANTIHISTAMINE IN AN EXPERIMENTAL MODEL OF FELINE ASTHMA

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

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and hereby certify that, in their opinion, it is worthy of acceptance.

Carol Reinero

Leah Cohn

Charles Brown

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LIST OF ABBREVIATIONS

AA	*Ascaris suum*
BAL	Bronchoalveolar Lavage
BALF	Bronchoalveolar Lavage Fluid
BGA	Bermuda Grass Allergen
CD4	Cluster of Differentiation 4
CD40	Cluster of Differentiation 40
C_{max}	Plasma Peak Concentration
EEM	Excitation-Emission-Matrix
ELISA	Enzyme-linked Immunosorbent Assay
ETAC	Early Treatment of the Atopic Child
GM-CSF	Granulocyte-macrophage Colony-stimulating Factor
H1	Histamine Receptor 1
HDMA	House Dust Mite Allergen
HPLC	High Performance Liquid Chromatography
ICAM1	Intercellular Adhesion Molecule
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-8	Interleukin-8

IL-10	Interleukin-10
IL-13	Interleukin-13
IL-16	Interleukin-16
INF	Interferon
LTC4	Leukotriene C4
LTD4	Leukotriene D4
LTE4	Leukotriene E4
MHC	Major Histocompatibility Complex
MIF	Macrophage Migration Inhibitory Factor
OD	Optical Density
PARAFAC	Parallel Factor Analysis
PBS	Phosphate Buffered Saline
PO	Per OS
RIT	Rush Immunotherapy
SD	Standard Deviation
$t_{1/2}$	Half Life
Th1	T-helper 1 Cell
Th2	T-helper 2 Cell
TNF	Tumor Necrosis Factor
TP	Total Protein
UV	Ultraviolet

ABSTRACT

Introduction: The use of corticosteroids and bronchodilators has been the mainstay of management of feline asthma. These drugs, although effective, can have undesirable effects with prolonged use in some cats. Cyproheptadine, a serotonin antagonist, has been shown to decrease airway constriction in an *in vitro* model of feline asthma. Low dose (2mg/kg) cyproheptadine decreased airway hyperreactivity in 1/3 of cats in a Bermuda Grass Allergen induced feline asthma model. The use of cyproheptadine has been proposed as a method to decrease the dose of corticosteroid needed in asthmatic cats. Cetirizine is a 2nd generation selective histamine (H1) antagonist that has been used extensively in human medicine mostly for allergic rhinitis but also for the treatment of asthma. Cetirizine has been administered anecdotally in allergic cats. The usefulness of either of these drugs in feline asthma has not been previously described. This thesis addresses three specific aims: 1) to obtain pharmacokinetic information about cetirizine in cats 2) to determine the effects of high dose cyproheptidine and cetirizine on bronchoalveolar lavage fluid (BALF) eosinophil percentages; and 3) to determine the effects of these drugs on serum and BALF immunoglobulin concentrations, cytokine (IL-4 and IL-10) concentrations and plasma and BALF histamine and serotonin levels in experimental feline asthma. We tested the hypotheses 1) a dose of 5 mg of oral cetirizine would be adequately absorbed by the cat and would reach therapeutic levels; and 2) oral cyproheptadine and cetirizine would blunt eosinophilc airway inflammation in cats sensitized to Bermuda Grass Allergen (BGA).

Methods: For specific aim 1, nine healthy, client owned cats were used. Heparinized blood (2 mL) was collected at baseline, and at 0.5, 1, 2, 4, 6, 8, 10 and 24 hours after oral

administration of 5 mg of cetirizine (dose range, 0.59-1.36 mg/kg). No adverse drug effects were observed in any cat. The plasma was separated by centrifugation (1730g x 20 minutes) within 1 hour of collection and samples were banked at -20^{o}C until analysis. A reverse-phase HPLC assay was developed by fortifying feline plasma obtained from client owned blood donors with a pure analytical reference standard. The plasma concentrations were analyzed with a compartmental pharmacokinetic model, using first-order input and one-compartment distribution. For specific aim 2, nine research cats were sensitized to BGA. Cats were randomized to receive 1 week treatments of placebo, cyproheptadine 8mg po BID, or cetirizine 5mg po BID. Each treatment period was followed by a 1 week washout period. Cats were challenged with BGA by aerosol delivery once weekly for 5 minutes for the duration of the study. On day 7 of each treatment period, cats were anesthetized for BALF fluid collection and blood collection. A blind BAL technique was performed by gently passing a 7 Fr polypropylene catheter through the endotracheal tube until resistance was met. A 200 cell count was performed on the BALF and % eosinophils was determined. ELISAs were performed to evaluate serum and BALF immunoglobulin, IL-4 and IL-10 concentrations and plasma and BALF histamine levels. Plasma and BALF serotonin was measured using a fluorometric method.

Results: Specific Aim 1-The mean terminal half-life of cetirizine was 10.7 (+/- 4.1) hrs and mean peak plasma concentration was 3.8 (+/- 1.5) mcg/mL. The volume of distribution and clearance (both corrected for absorption) were 0.256 (+/- 0.09) L/kg, and 0.295 (+/- 0.086) mL/kg/min, respectively, suggesting a small volume of distribution and low clearance. Mean plasma concentrations were maintained above 0.85 mcg/mL for 24

hours. Specific Aim 2- No significant difference between treatment groups was found with respect percent BALF eosinophils (p=0.196; Mean ± SD: 40.4 ± 22.4% for placebo, 31.2 ± 19.9% for cetirizine and 26.5 ± 15.6% for cyproheptadine). No significant differences were found between treatment groups for the other measured immunologic variables.

Conclusions: These results indicate that a single dose of approximately 1 mg/kg orally in cats was well-tolerated and produced high plasma concentrations compared to what has been reported in humans. The half-life is long enough to maintain plasma concentrations with once-daily dosing. Oral administration of cyproheptadine and cetirizine in an experimental model of feline asthma did not decrease airway eosinophilia or alter other immune variables. This study does not support the use of either cyproheptadine or cetirizine as monotherapy for the treatment of eosinophilic airway inflammation in feline asthma.

Chapter 1
Literature Review

Asthma is one of the most common respiratory diseases in the cat affecting between 1-5% of the population.[1] It was first described in the cat by Hill in 1906.[2] Since that time, our understanding of this disease process has advanced substantially but our armament of therapeutics is still limited. This review will discuss the speculated pathophysiology of feline allergic asthma, describe what is known about the naturally occurring disease, discuss different experimental models of asthma in the cat and finally review current therapeutics.

Pathophysiology:

Feline asthma is believed to be an allergic disease of the lower airways. Classic changes found in the airways of asthmatic cats include bronchial inflammation, smooth muscle constriction, epithelial edema and mucous gland hypertrophy.[1] These changes are likely the result of mediators released during the asthmatic response. In people, it is well established that allergen induced activation of CD4+ T-helper 2 (Th2) cells is the driving force behind these immunopathologic changes.[3]

Inflammation does not usually occur in the respiratory tree secondary to exposure to foreign protein. There is limited access of foreign protein to the respiratory epithelium due to mechanisms such as the mucous layer and tight junctions.[3] Most of the time, even when the allergens are able to penetrate, the production of IgG or IgA occurs which will ultimately neutralize the allergen leading to no clinical signs.[4] When an individual is predisposed to an exaggerated Th2 response, it will produce IgE which leads to a type I

hypersensitivity reaction. These individuals are considered atopic due to their increased production of IgE. The origin of atopy is complex and includes genetic and environmental factors.[5]

Human studies have found that when an atopic individual is exposed to inhaled allergen, the allergen is taken up by dendritic cells in the airway mucosa and processed via the MHC class II pathway. The dendritic cell then migrates to the local lymph nodes where it presents the allergen to naive T-helper cells. Exposure to locally produced IL-4 causes the naïve T-helper cells to mature into Th2 cells. The Th2 cells produce the cytokines IL-4 and IL-13. These cytokines along with co-stimulation from CD40 cause a class switch within the allergen specific B-cells allowing for production of IgE. This newly formed IgE then attaches to the Fcε receptor on local mast cells and awaits additional exposure to allergen.[5,6]

Upon exposure to additional allergen, the IgE molecules on the surface of the mast cells will bind the allergen. Allergen binding of the IgE molecules causes cross-linking of the IgE molecules allowing for release of mast cell granules. It has been shown in multiple species that granules released by the mast cells contain substances such as proteases, histamine, serotonin and eosinophil chemotactic factor. The release of these factors occurs within minutes of allergen exposure. The cross-linking of IgE also leads to activation of phospholipase A and protein kinases which lead to formation of leukotrienes, prostaglandins and promote formation of additional cytokines. Because formation of these molecules takes a number of hours, this is called the late phase response.[5]

The activated Th2 cells also produce IL-5 upon exposure to allergen. IL-5 is responsible for recruitment and mobilization of eosinophils to the airways as well as activation of the eosinophils. Activation of the eosinophils allows release of several mediators including major basic protein, eosinophil cationic protein, eosinophil peroxidase, TNF-α, GM-CSF, IL-4, IL-5, IL-13, RANTES, eotaxin, and platelet-derived growth factor. These mediators can perpetuate the inflammatory response as well as cause direct damage to the airway epithelium, increase mucous secretion and induce airway hyperreactivity. Activated eosinophils can also cause airway fibrosis and remodeling.[3] The airway epithelium itself has also been found to be important in the migration of eosinophils into the airways of humans. It has been shown that epithelial cells produce several eosinophil chemotactic factors including RANTES, IL-5, and eotaxin 1, 2 and 3.[7] This shows that there is a combination of factors leading to eosinophilic inflammation in asthmatics.

A recent paper found that human chronic asthmatics have a large proportion of CD4+ invariant natural killer T cells along with the typical Th2 cell repertoire.[8] These CD4+ invariant natural killer T cells produce the Th2 cytokine profile and appear to assist in the pathogenesis of allergic asthma.[8] It is unknown if these cells play a similar role in feline asthma.

Two mediators to note are histamine and serotonin. Histamine is a vasoactive amine that causes increase in vascular permeability and vasodilation which contributes to increased cellular infiltration into the airways. Histamine also causes increased mucous production and it can pass though the damaged epithelium and cause increased reactivity of airway smooth muscle and bronchoconstriction.[9] Serotonin causes

bronchoconstriction in cats experimentally.[10] Plasma levels are increased in human asthmatics and are significantly related to asthma severity.[11] There is evidence that serotonin has indirect effects that contribute to airway inflammation, for example by inducing secretion of IL-16 from epithelial cells in a murine model of asthma. IL-16 is a cytokine that recruits and activates Th cells.[12] Serotonin also modulates the release of eotaxin (a chemokine that attracts eosinophils) from human lung fibroblasts.[13]

Naturally Occurring Disease:

Feline asthma tends to affect young to middle aged cats with the Siamese breed being over represented in some studies.[14,15] Cats with asthma commonly present for a cough or an expiratory wheeze. Occasionally an owner will mistake the cough for vomiting or "hacking up hairballs." In severe situations, cats can present with tachypnea and open mouth breathing on an emergency basis. Historical findings may include worsening of symptoms with exercise or environmental changes such as a change of litter or seasonal changes.[15]

On physical exam, the cat may be normal but typically one will notice a pronounced expiratory component to the respiratory cycle caused by lower airway obstruction. The airway obstruction also leads to the classical expiratory wheeze heard on auscultation of the chest. Cats may have tracheal sensitivity on palpation. As noted previously, in an emergent situation these cats can present with open mouth breathing and cyanosis. One case report exists of a cat with a lung lobe torsion secondary to chronic asthma.[16] This is not a common finding in asthmatic cats but should be considered in cases of severe respiratory distress.

Numerous other diseases can present with similar clinical signs therefore, the diagnosis of feline asthma tends to be a diagnosis of exclusion. The diagnostic plan includes a complete blood count, serum chemistry profile and urinalysis; thoracic radiographs, parasitic examinations and culture and cytology of bronchoalveolar lavage fluid. The CBC and chemistry profile are typically nonspecific for respiratory disease but about 20% of cats will have an increased peripheral eosinophil counts[14,15] and a mild hyperproteinemia is found in about 33% of cats due to chronic inflammation.[14] The most common radiographic findings are a bronchial pattern or bronchointerstitial pattern. Asthmatic cats can also have signs of air trapping within the thoracic cavity and atelectasis. The atelectasis is thought to be due to complete airway obstruction caused by bronchoconstriction and mucous plug formation and occurs primarily in the right middle lung lobe.[14,17] It should be noted that completely normal radiographs cannot exclude this disease. Parasitic disease can also cause a cough and eosinophilia so performing a heartworm antigen and antibody test as well as baermann fecal test for *Aelurostrongylus abstrusus* is prudent in these cases. Ultimately, examination of airway cytology is the confirmatory test in these cats. Asthmatic cats will have a predominance of eosinophils within their airways. They may also have a neutrophilic component if there is coexisting chronic bronchitis or a secondary bacterial infection.[14,15,17] It should be noted that normal cats can have a relatively high BALF eosinophil percent compared to other species.[18,19] Therefore, when making the diagnosis of feline asthma all clinical parameters should be taken into account. When a bronchoalveolar lavage is performed, samples should be turned in for bacterial culture. It should be noted that bacterial infection is uncommon in the cat but bacterial contamination of the airways is very common. One study found

positive bacterial cultures in 77% of healthy, asymptomatic cats undergoing BAL.[15] Therefore, antibiotic treatment should be based on both culture and cytologic findings. *Mycoplasma* species have not been cultured out of the lungs of a normal cat and is therefore thought to be a true pathogen when cultured.[20] *Mycoplasma* can cause significant damage to the epithelial lining of the airways. *Mycoplasma* are difficult to culture and in cases of lower airway disease in the cat it is important to specifically culture for these organisms and treat if the culture is positive.[20,21] Histopathology is rarely performed in clinical cases of feline asthma but typical lesions include eosinophilic and neutrophilic bronchial inflammation, smooth muscle hyperplasia, globlet cell hypertrophy, mucous and cellular debris in the airway lumen, epithelial erosion and in severe cases emphysema.[22]

Another diagnostic test used primarily in people is pulmonary function testing which allows the clinician to test for airflow and airway obstruction. Dye et al found that cats with naturally occurring bronchial disease had evidence of airway obstruction but that there was little correlation between severity of disease (determined by a score of historical, physical and radiographic abnormalities) and pulmonary functional measurements.[15] They also noted a response to terbutaline, a bronchodilator, in a proportion of cats indicating that the airway obstruction was reversible in some cases. Finally, they examined airway response to methacholine aerosolization in 7 cats and found that 6 of these cats had increased airway reactivity in response to the drug compared to healthy cats.[15] McKiernan et al described the use of tidal breathing flow-volume loops in healthy cats and cats with bronchial disease.[23] They found that cats with bronchial disease had decreased expiratory flow rates, prolonged expiratory time and

decreased tidal breathing expiratory volume at 0.1 and 0.5 seconds compared to normal cats.[23] These findings are consistent with lower airway obstruction found in asthmatic cats. Pulmonary function testing is not typically used clinically due to the need for anesthesia and specialized equipment.

Feline Asthma Models:

Much of our knowledge about the pathophysiology and treatment of feline asthma has been obtained from studies performed in experimental feline models. There have been several notable models developed over the past 30 years. Each of these models were used to look at the efficacy of possible treatments and helped to elucidate mechanisms of the disease.

Barch et al conducted a study in 1976 sensitizing cats to ovalbumin as a model of anaphylaxis. It was noted that although the cats did have systemic signs, respiratory signs predominated. The cats were given a daily intraperitoneal injection of 5% ovalbumin solution for three days. Forty to sixty days later the cats were anesthetized and challenged with a 1 milliliter intravenous injection of 5% ovalbumin. At that time, pulmonary resistance was measured. They found that 85% of the cats that were sensitized responded with increased pulmonary resistance. Signs displayed by the cats included dyspnea, bronchial wheezes, increased mucous production, cough, cyanosis, hemoptysis and death. The cats also displayed some signs of systemic reaction including vomiting, defecation and urination. Fifteen percent of the cats died as a result of the challenge. Histopathology of the cats that died revealed petechial hemorrhages within the lungs, blood tinged mucous in the airways and air trapping in the peripheral airways. They followed up this experiment by treating the cats with aminophylline, a

methylxanthine, and isoproterenol, a β_1 and β_2 agonist post-challenge. The cats treated with aminophylline showed decreased airway resistance for the 45 minutes that they were monitored. Cats treated with isoproterenol had an immediate decrease in bronchoconstriction that then returned rapidly to post-challenge levels.[24] This study proved that cats could have bronchoconstriction due to allergen challenge and that it could be reversed with medical management. Unfortunately, it was not noted whether the cats had airway inflammation on histopathologic examination.

The second model that provided important information was developed by Padrid et al in 1995.[25] This model is based on exposure to *Ascaris suum* (AA) antigen. These investigators initially gave two intramuscular injections of the allergen two weeks apart and followed that with aerosolized exposure to the allergen three times a week for six weeks. They found that the cats had increased pulmonary resistance post-challenge and an increased number of airway eosinophils. They tested these variables both before and after the 6 week course of aerosoliztion and got similar results. At the end of the study the cats were euthanized and histopathology was performed on their lungs. Lesions noted included mucous and cellular debris in the airways, goblet cell hyperplasia, epithelial erosion and increased smooth muscle thickness.[25]

This group continued to use the AA model in a number of experiments examining different immune variables and treatments for asthma. Initially, they noted that the increased airway resistance due to bronchoconstriction could be reversed with an intravenous dose a terbutaline, a β_2-adrenergic agonist.[25] Another study researched the effects of a serotonin antagonist, cyproheptadine, on strips of tracheal and bronchial smooth muscle from AA sensitized cats.[10] There was decreased contraction of the

muscle after exposure to allergen in samples that were bathed in cyproheptadine compared to samples that were not pretreated with the drug.[10] They also examined the response of tracheal and bronchial smooth muscle to physostigmine, an acetylcholinesterase inhibitor. Findings included increased muscular contraction upon exposure to acetylcholine in sensitized cats compared to controls and decreased contraction of tracheal smooth muscle but not bronchial smooth muscle upon exposure to physostigmine.[26] Finally, this group researched the role of cyclosporine A as a potential treatment for feline asthma in two different studies. First, they used cyclosporine treated cats that were chronically exposed to allergen. They found that the treated cats had decreased airway hyperreactivity, decreased BALF eosinophil count and had no histologic changes consistent with airway remodeling compared to untreated cats.[27] The second study evaluating the effects of cyclosporine examined BALF histamine concentrations following acute antigen challenge.[28] They found that cats treated with cyclosporine have similar BALF histamine concentrations (indicating mast cell degranulation) and evidence of bronchoconstriction compared to controls. They found similar results when they looked at contraction of the tracheal smooth muscle in vitro. They concluded that treatment of asthmatic cats with cyclosporine does not inhibit the acute phase response or mast cell degranulation following antigen challenge.[28]

Recently a model has been developed based on sensitization to Bermuda Grass Allergen (BGA) or House Dust Mite Allergen (HDMA).[29] One of the advantages to this model is that the cats were sensitized to an allergen (BGA or HDMA) to which they can naturally develop an allergic response. The allergens were chosen based on serum IgE testing in cats with naturally occurring disease. The cats were sensitized using a protocol

involving two subcutaneous injections of BGA or HDMA and alum and one intranasal allergen administration. The cats were also aerosolized with allergen for 7 treatments over a 2 week period. It was found that cats sensitized by this protocol had clinical signs consistent with asthma, produced allergen-specific IgE, increased allergen-specific serum and BALF IgG and IgA over time, demonstrated airway hyperreativity after allergen exposure, had increased airway eosinophilia and had lung histology consistent with feline asthma.[29]

This model has since been used to investigate several therapies that have been proposed for asthma. The first study looked at the efficacy of oral prednisone, inhaled flunisolide, zafirlukast (a leukotriene receptor antagonist) and cyproheptadine (a serotonin receptor antagonist) in the treatment of asthma.[30] It found that oral prednisone decreased airway eosinophilic inflammation and decreased serum BGA-specific IgE. The inhaled steroid (flunisolide) was able to decrease airway eosinophilia. Zafirlukast did not significantly change any of the variables measured in the study. It should be noted that 2 out of 6 of the cats treated with cyproheptadine did have decreased airway resistance after treatment.[30] The most recent study investigated the effect of rush immunotherapy (RIT) on sensitized cats.[31] RIT involves administration of increasing concentrations of allergen over a short time period. The goal is to modulate the immune response and minimize the asthmatic phenotype. The study found that cats receiving RIT had diminished airway eosinophil counts, increased serum BGA-specific IgG, and decreased serum BGA-specific IgE concentrations compared to non-treated controls. A shift in the BAL cytokine profile, from a Th2 profile pre-sensitization to a more Th1 profile (increased IFN-γ and IL-10) post-sensitization, was also noted.[31] The Th1

cytokines tend to shift an individual's T helper cells away from the Th2 profile and thus minimize allergic disease.[5] Additionally IL-10 has immunosuppressive effects.[31]

Therapeutics:

Treating the underlying airway inflammation has been the mainstay of treatment for feline asthma. Addition of bronchodilators may be helpful in some situations. There are several novel therapeutics that are being tried in both human and veterinary medicine. This section focuses on corticosteroids, bronchodilators, cyclosporine, leukotriene receptor antagonists, cyproheptadine (an antiseritonergic drug) and cetirizine (an antihistamine).

Corticosteroids have long been the treatment of choice for clearing underlying inflammation. Corticosteroids bind to receptors in a cells' cytosol and then move into the nucleus where they inhibit transcription and translation of inflammatory mediators. Steroids are able to decrease inflammatory cell migration, promote apoptosis and decrease mucous production within the airway.[32] In cats corticosteroids can be given orally, by injection or via a mask inhaler. Despite the success of steroids in decreasing the amount of inflammation, there are still numerous studies showing that we are not adequately decreasing the amount of inflammation in the airways with steroids. For example, in people with severe asthma, it has been found that despite continued high dose corticosteroids these individuals continue to have increased amounts of tissue eosinophils and T-lymphocytes.[33] Steroids have also been ineffective in reducing the amount of airway remodeling in chronic human asthmatics.[3]

Bronchodilators are used either in the emergent situation or as part of the chronic treatment plan in severe asthmatics. There are two classes of these drugs β_2-adrenergic

agonists and methylxanthines. β_2-adrenergic agonists include terbutaline and albuterol. They work by stimulating the β_2-adrenergic receptors within the airways leading to smooth muscle relaxation. Other beneficial effects of these drugs include inhibition of acetylcholine release, stabilization of mast cell membranes, reduction of vascular permeability and promotion of mucociliary clearance.[32] Methylxanthines inhibit phosphodiesterase causing increased cAMP and bronchodilation. Methylxanthines may also inhibit adenosine which is a mediator of bronchoconstriciton.[22] Specific drugs in this class include aminophylline and theophylline. These drugs have been found to relieve bronchoconstriction, stabilize mast cells, increase the rate of ciliary movement and increase the strength of the respiratory muscles.[22,32]

Cyclosporine A exerts its effects by inhibiting the maturation of T-cells and thus causing suppression of the inflammatory cascade that follows. Cyclosporine A also has been shown to decrease mast cell degranulation and secretion of cytokines in vitro. As discussed above, there are conflicting reports about the utility of cyclosporine in the treatment of feline asthma. Both studies that looked at its effectiveness used the *Ascaris suum* model. One found beneficial effects in chronic allergic asthma including decreased airway hyperreactivity, decreased BALF eosinophil numbers and diminished histologic changes compared to untreated controls.[27] The other found that in an acute allergen exposure cyclosporine treatment did not decrease in vivo mast cell degranulation and airway constriction or decrease in vitro smooth muscle constriction. At this time, due to the expense, side effects and controversy regarding its effectiveness cyclosporine is rarely used to treat naturally developing feline asthma.

Leukotriene receptor antagonists have been shown to be effective in the control of moderate to severe cases of human asthma. Cysteinyl leukotrienes (LTC_4, LTD_4, LTE_4) increase mucous production, cause epithelial edema and encourage bronchoconstriction. Due to the efficacy of drugs such as Zyflo, Accolate and Singulair in human asthmatics it has been thought that these drugs may also play a role in the treatment of feline asthma.[21] In humans, urinary leukotriene levels reflect whole body leukotriene levels and have been looked at as a marker of airway inflammation in asthmatics. A study performed in a feline model did not show increased urinary leukotriene levels after sensitization with BGA.[34] In the same cats, it was found that they had lower BALF leukotriene levels compared to pre-sensitization levels despite increased BALF inflammatory cells.[34] That study brings into question the significance of the role of leukotrienes in feline asthma. A second study found little change in immune variables when BGA sensitized cats were treated with zafirlukast, a leukotriene receptor antagonist.[30] Based on the results of these studies, leukotriene receptor antagonists are not recommended as treatment of asthma in cats.

Cyproheptadine hydrochloride is an antagonist of serotonin, and, in the previously described study by Padrid , blocked serotonin receptors on the smooth muscle leading to smooth muscle relaxation.[25] In non-asthmatic research cats, cyproheptadine reversed bronchoconstriction induced by an infusion of serotonin.[35] In cats, cyproheptadine has been used most frequently as an appetite stimulant and is commonly dosed at 2 mg orally twice daily. This drug appears to be safe and well tolerated in cats, with minimal adverse effects.[36] Data obtained from a BGA model of feline asthma using oral cyproheptadine at a dose of 2 mg BID demonstrated a marked decrease in airway hyperreactivity in one-

third of the treated cats but no change in eosinophilic airway inflammation.[30] A pharmacokinetics study of cyproheptadine in cats suggests that some cats may require a much higher dose than the typical 2 mg dose to show beneficial effects.[36] There is evidence that serotonin has indirect effects that contribute to airway inflammation, for example by inducing secretion of IL-16 (a cytokine that recruits and activates Th cells) from epithelial cells in a murine model of asthma[12] or of inducing eotaxin (a chemokine that attracts eosinophils) from human lung fibroblasts.[13] It would be important to know if the release of serotonin locally in the lung of cats also contributes to the inflammation, which is a key pathologic feature of asthma. If cyproheptadine reduces both airway hyperreactivity and airway inflammation, it could be used as monotherapy for treatment of feline asthma. If cyproheptadine primarily blunts bronchoconstriction, an anti-inflammatory medication like prednisone will also need to be administered

Cetirizine, a second generation histamine antagonist, has shown evidence in vitro of inhibiting expression of pro-inflammatory mediators including TNF-alpha, ICAM 1 and super oxide radicles.[37] Histamine 1 (H1) receptor independent effects include inhibiting eosin induced transendotheial migration of eosinophils in vitro[38] and inhibiting macrophage migration inhibitory factor (MIF) and IL-8 production in mouse allergy model.[39] These factors may blunt inflammatory cell infiltration in the airways. Studies in humans show cetirizine causes dose dependent protection against bronchoconstriction in humans affected by mild asthma undergoing histamine challenge.[40,41] Additionally long term use of cetirizine in children at high risk of developing asthma decreased the relative risk of developing asthma.[37] Little is known about the use of this drug in

asthmatic cats but if it can blunt inflammatory cell infiltration and decrease airway hyperreactivity it may be possible to use this drug as a monotherapy for this disease.

This review has discussed what we believe to be the underlying pathologic mechanism of feline asthma and summarized our understanding of the naturally occurring disease. Additionally, a brief discussion of models of feline asthma was included to highlight that much of our current knowledge about feline asthma and its treatments has been based on studies performed in feline models. Finally, a brief discussion was included outlining current available therapeutics. The remainder of this thesis will discuss the use of high dose cyproheptadine and cetirizine for the treatment of asthma in a BGA model of feline asthma.

Chapter 2
Pharmacokinetics of Cetirizine in Healthy Cats

Introduction:

Treatment of allergic disease in cats relies heavily on the use of glucocorticoids. Although this treatment tends be effective for most cats, there are certain situations when it would be desirable to minimize the use of glucocorticoids. For instance, glucocorticoids are relatively contraindicated in cats with active infections, predisposing factors for infection (urinary calculi), diabetes mellitus or heart disease. Cats are more resistant than other species to the negative side effects of glucocorticoids but, adverse effects can be seen including behavioral changes, ravenous appetite, weight gain, excessive bruising and hepatomegaly. Therefore, it is desirable to investigate other drugs effective for allergic disease. A promising treatment (based on its success in people with allergic disease) is cetirizine. Cetirizine (Zyrtec®) is a 2nd generation selective histamine (H1) antagonist that has been used extensively in human medicine for the treatment of asthma, allergic rhinitis and urticaria.[42]

Histamine is a key mediator in the allergic cascade. Allergies are caused by activation of CD4+ T helper 2 (Th2) lymphocytes. These cells are the driving force behind immunopathogenic changes in allergic disease through the actions of the cytokines they secrete. Some important actions of Th2 cytokines include IgE production and recruitment/activation of inflammatory cells. Mast cells are key resident inflammatory cells at mucosal sites that are activated when bound IgE on their surface recognizes cognant allergen. Cross-linked IgE on the surface of the mast cell leads to

degranulation and release of a number of preformed mediators, including histamine. These mediators lead to bronchoconstriction, eosinophil and basophil influx, vasodilation and increased vascular permeability.[5,22]

Histamine is released from mast cells and basophils both from the aforementioned immunologic mechanisms as well as from non-immunologic stimuli such as heat, pressure, platelet activating factor and substance P.[11] The release of histamine and binding of histamine to the H1 receptor leads to inflammatory cell maturation and recruitment, vascular permeability, vasodilatation, mucous secretion and smooth muscle contraction which are ultimately responsible for many of the clinical signs seen in allergic disease.[37]

The second generation antihistamines, including cetirizine, have been shown to minimize many of the effects caused by histamine both in vitro and in vivo. The improvement of clinical signs can be attributed to both antihistamine effects and anti-inflammatory effects independent of blocking of the H1 receptor. Cetirizine has been shown to inhibit the expression of multiple pro-inflammatory mediators including TNF-α, ICAM-1 and superoxide radicals in vitro.[37] An example of the H1-receptor independent effects of cetirizine was demonstrated in a recent study showing that cetirizine inhibits eosin (an eosinophil chemotactic factor) induced transendothelial migration of eosinophils in vitro.[38] Another study revealed that cetirizine inhibited macrophage migration inhibitory factor (MIF) and production of IL-8 in a mouse allergy model.[39] Both of these examples show that cetirizine may help blunt inflammatory cell infiltration common in allergic diseases. In people affected by seasonal allergic rhinitis, it has been found that treatment with cetirizine decreased the number of eosinophils, neutrophils, IL-

8 and improved nasal symptoms during pollen season.[37] The Early Treatment of the Atopic Child (ETAC) was a large, double-blinded, placebo controlled, clinical trial that treated children at high risk of developing asthma with cetirizine. It was found that the relative risk of developing asthma was decreased in treated children.[37] There have also been studies performed showing that cetirizine causes dose-dependent protection against bronchoconstriction in humans affected by mild asthma that undergo a histamine challenge.[40] Additional studies have also supported a role for second generation antihistamines in the treatment of mild to moderate asthma in people (i.e., they consistently improve asthma symptoms), and that they have a corticosteroid-sparing effect.[42-44]

While the veterinary profession has started to use second generation antihistamines in an attempt to minimize the use of glucocorticoids for varying allergic diseases, the doses are anecdotal and not based on scientific studies. When searching the veterinary lay press one can find "suggested" doses for cetirizine that range from 2.5 mg/cat/day to 10 mg/cat twice daily.[45] In people, the recommended dose for adults is 10 mg/day[42]; additionally, the plasma half-life of cetirizine has been found to be 7.0-10.5 hours.[46,47]

Several methods have been developed for detection of cetirizine in human plasma including gas chromatography, high performance liquid chromatography (HPLC) with ultraviolet and mass spectrometric detection.[48] We decided to base our assay on the HPLC method used for human samples because it will allow us to detect low amounts of plasma cetirizine. It is rapid and allows multiple samples to be run in a short amount of time and uses an extraction technique that can be performed easily by trained individuals

using available instrumentation.[49] Currently, no pharmacokinetic studies have been performed in the cat to investigate the oral bioavailability, frequency of administration, or time to achieve steady-state concentration. This information must be obtained before we can objectively evaluate the use of cetirizine in feline allergic diseases, and specifically in feline asthma.

The objective of this study was to determine the pharmacokinetic profile of oral cetirizine in healthy cats in order to ascertain an appropriate dosing regimen for use in feline patients with allergic disease. There were two specific aims. First, we developed an HPLC assay for plasma cetirizine levels based on an assay used in human pharmacokinetics studies (for this aim we collaborated with Dr. Mark Papich at North Carolina State University). We then analyzed multiple timed plasma samples from healthy adult cats given a single oral dose of 5 mg in order to determine the pharmacologic disposition of cetirizine. We hypothesized that a dose of 5 mg of cetirizine orally once daily in cats will reach effective therapeutic concentrations and will be suitable for use in clinically affected cats with allergic disease.

Materials and Methods:

HPLC Assay- A reverse phase high performance liquid chromatography assay using a C-8 column was validated at North Carolina State University. Blank feline donor plasma was spiked with known amounts of a pure analytical reference standard of cetirizine.[a] The samples were run using HPLC technology. The mobile phase was composed of 55% buffer and 45% acetonitrile. Plasma samples were extracted with C-18 solid phase extraction cartridges.[b] UV detection was at 210 nm and the retention time was 3.5-4.0

minutes. Extra blank plasma (i.e., plasma from an untreated healthy cat) was collected from a privately owned cat at the University of Missouri and shipped to NCSU.

Pharmacokinetics Determination:

Species/number of animals— Nine healthy, client owned cats were used for the collection of samples for this project. Breeds included Domestic Shorthair (n=8) and Siamese (n=1). Mean age (+/- SD) was 6.6 +/- 2.8 years (range 3 to 12 years) and mean body weight (+/- SD) was 5.3 +/- 1.4 kg (range 3.7 to 8.5). The owners were asked to read and sign an informed consent prior to enrolling their cat in the study. A thorough physical examination was performed at the start of the collection period. Blood and urine samples were also collected for a packed cell volume, total solids, white blood cell differential, serum biochemical profiles, and urine specific gravity/dipstick examination to evaluate the health status of each cat.

Sample collection—Two milliliters of whole blood were collected into a heparinized vacutainer tube at baseline, and then at 0.5, 1, 2, 4, 6, 8, 10 and 24 hours after administration of 5 mg (approximately 1 mg/kg) of cetirizine (Zyrtec®) orally. The plasma was separated by centrifugation (1730Xg for 20 minutes) within 1 hour of collection and samples were banked at -20^{o}C until time of analysis.

Technique for analysis—Banked plasma samples were analyzed by the HPLC method developed in Dr. Papich's laboratory at NCSU (described above).

Protein Binding- Pooled plasma samples were spiked with cetirizine at two concentrations (2 mcg/ml and 4 mcg/ml). Protein binding was measured using a microcentrifugation system. The percent protein binding was calculated using the following formula:

$$\text{Protein binding (\%)} = \frac{[\text{total}] - [\text{unbound}]}{[\text{total}]} \times 100$$

Data analysis—Pharmacokinetic analysis was performed using a compartmental pharmacokinetic model, using first-order input and one-compartment distribution. Data for each cat was analyzed separately and then averaged to determine the mean and standard deviation for the group. Values were determined for plasma terminal half-life ($t_{1/2}$), plasma peak concentration (C_{max}), volume of distribution and volume of clearance.

Results:

Animals- All animals had normal physical examinations, complete blood counts and serum chemistry profiles. No adverse effects were noted in the cats after administration of the drug.

Pharmacokinetics- The mean terminal half-life of cetirizine was determined to be 10.7 (+/- 4.1) hours. The absorption half-life was 0.54 hours. The volume of distribution and clearance (both corrected for absorption) were 0.256 (+/- 0.09) L/kg, and 0.295 (+/- 0.086) mL/kg/min, respectively. Mean plasma concentrations were maintained above 0.85 mcg/mL for 24 hours. Cetirizine had 97.67% protein binding at a concentration of 2mg/ml and 93.44% protein binding at a concentration of 93.44%.

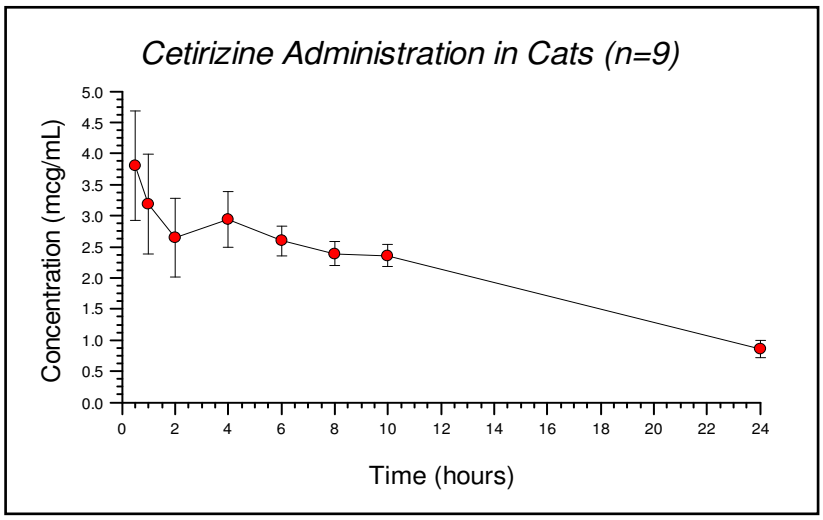

Figure 2-1: Concentration of orally administered cetirizine in cats over time.

Discussion:

The use of cetirizine in veterinary medicine has been limited due to the lack of pharmacokinetic and efficacy studies. The results of our study indicate that orally administered cetirizine has systemic availability and a pharmacokinetic profile allowing for once daily dosing.

The effective free drug concentrations in humans has been reported to be 19.5-27.3ng/ml.[50] Because a therapeutic range for cetirizine is not known in cats, we chose to use the human therapeutic concentration as a basis for comparison for our study. The results of our study show that orally administered cetirizine was well absorbed and produced plasma concentrations higher than the therapeutic plasma concentration reported in people. Additionally, the plasma concentration remained higher than this level for 24 hours. (figure 1-2). We found that similar to people, cetirizine is highly protein bound.

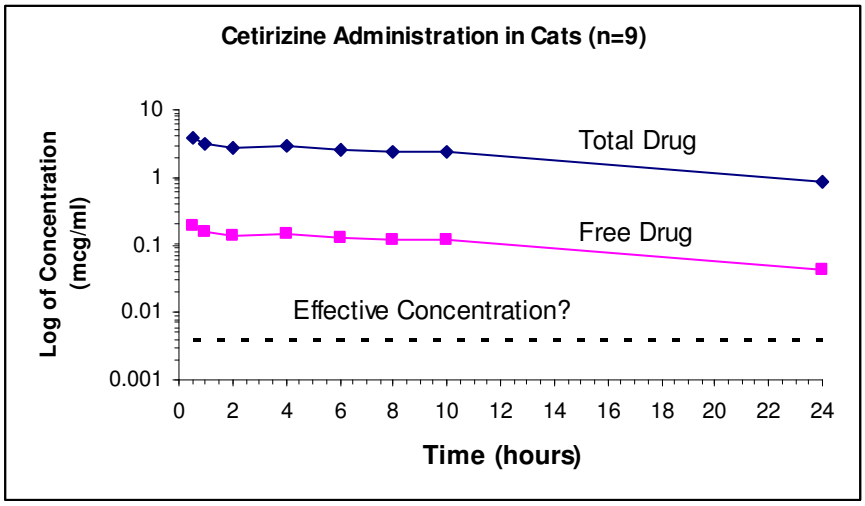

Figure 2-2: Log concentration of cetirizine (mcg/ml) in feline plasma over time. Note that both the total drug and free drug concentrations are higher than the human therapeutic level.

This study was able to determine that once daily oral administration of cetirizine in cats is safe and appears to have good systemic absorption. We did not perform any pharmacodynamic studies in this project as this will be addressed further in chapter 3.

Chapter 3
The Effects of Cyproheptadine and Cetirizine in a Model of Feline Asthma

Introduction:

Feline asthma is believed to be allergic in origin, caused by a T helper 2 lymphocyte (Th2) driven response to inhaled aeroallergens from the environment. The Th2 cells lead to production of allergen-specific IgE by plasma cells. The IgE avidly binds mast cells on the mucosal surface of the airways. Upon re-exposure to specific allergen, two IgE molecules are cross linked on the surface of mast cells and lead to mast cell degranulation. Preformed mediators within mast cells including serotonin, histamine and other inflammatory cell chemotactic factors are released. These mediators lead to bronchoconstriction, vasodilation, increased vascular permeability and an inflammatory cell influx as part of the acute phase asthmatic response.[6,51]

The hallmark features of asthma include eosinophilic airway inflammation, airway hyperreactivity and remodeling (permanent architectural changes in the lung). Airway inflammation is a cardinal feature of asthma, as it can contribute to both airway hyperreactivity and remodeling.[3] Therapies that can blunt the inflammatory cascade may lead to a maximal benefit to patients if they can also diminish bronchoconstriction and airway remodeling. There is no cure for allergic asthma, and current treatments focus on antagonizing or suppressing the inflammatory cascade once it has been well established. Corticosteroids are the mainstay of therapy because they suppress the transcription of genes that form pro-inflammatory mediators. Additionally, bronchodilators are

prescribed for cats that develop life threatening respiratory distress from bronchoconstriction.[14,15]

Long term use or high doses of corticosteroids may be contraindicated in some situations, for example, cats with co-existent endocrinopathies, heart disease or infectious disease. In these situations, other therapeutic options would be desirable. Drugs which attenuate other portions of the inflammatory cascade deserve further evaluation. Since serotonin and histamine have been implicated in the acute phase response, blocking these mediators should have an effect on downstream events, notably the inflammatory cell influx. There is a substantial body of literature evaluating the roles of these mediators in allergic disease.

Serotonin is a common mediator in mast cells and has been determined to cause airway constriction in cats in vivo.[35] In a model of feline asthma, it has been found that tracheal and bronchial smooth muscle constricts when bathed in serotonin in vitro. When these smooth muscle strips were bathed in cyproheptadine, a serotonin antagonist, prior to addition of serotonin, the degree of constriction was attenuated.[10]

While it is well accepted that serotonin has effects on airway hyperreactivity, its effects on inflammation have not been studied in the cat. In other species, there is evidence that serotonin contributes to airway inflammation, although indirectly. For example, in a murine model of asthma, serotonin induced secretion of IL-16 from epithelial cells. IL-16 is a cytokine that recruits and activates Th cells. These Th cells presumptively help drive the asthmatic response.[12] As another example, in human lung fibroblasts, serotonin modulated the release of eotaxin (a chemokine that attracts eosinophils).[13]

Like serotonin, histamine is stored as a preformed mediator in mast cells. Histamine is a specific chemoattractant and activator of human eosinophils.[11] Vasodilation within the airways caused by histamine has also been implicated in inflammatory cell influx. Histamine is also a mediator of bronchoconstriction seen in asthmatics.[9] The role of histamine in naturally occurring feline asthma has not been evaluated to date.

Cyproheptadine, a serotonin antagonist, decreases airway reactivity to serotonin infusion in healthy cats.[35] In an experimental model of feline asthma, cyproheptadine at a commonly used but low dose (2 mg BID) decreased airway hyperreactiivty in a subpopulation of cats, but did not blunt eosinophilic airway inflammation.[30] However, the pharmacokinetics of cyproheptadine suggest that some cats may require substantially higher doses (up to 8mg) to reach therapeutic levels.[36] This higher dose had not been studied in asthmatic cats to date.

Cetirizine, a second generation H1 antagonist, inhibits expression of pro-inflammatory mediators including TNF-alpha, ICAM 1, and superoxide radicals.[37] Another study proved cetirizine inhibits eosin induced transendothelial migration of eosinophils in vitro.[38] In a mouse allergy model, cetirizine inhibited macrophage migration inhibitory factor (MIF) and IL-8 production.[39] These factors may blunt inflammatory cell infiltration in the airways. Additionally, in people affected by mild asthma undergoing histamine challenge, cetirizine causes dose dependent protection against bronchoconstriciton.[40,41]

Previously, a model of feline asthma based on sensitization to Bermuda Grass Allergen was developed.[29] This model mimics naturally occurring feline asthma in a

number of ways, including eosinophilic airway inflammation, increases in allergen-specific IgE and bronchoconstriction after allergen exposure.[29] This model was used in this study to test the hypothesis that cyproheptadine and cetirizine, by blocking serotonin and histamine respectively, would result in decreased eosinophilic airway inflammation. An additional objective of the study was to determine if serotonin or histamine blockade would affect concentrations of blood and BALF serotonin, histamine, IL-4, IL-10 and BGA-specific immunoglobulins.

Materials and Methods:

Experimental Animals—Nine male intact domestic short hair cats, aged 6-9 months, (and weighing 4.4-5.0 kg) were obtained from a specific pathogen free colony from a commercial research animal provider.[c] The cats were cared for according to the principles outlined in the NIH Guide for the Care and Use of Laboratory Animals and the University of Missouri, Animal Care and Use Committee.

All cats had normal physical examinations. The cats were housed as a group in an indoor facility. Water and a dry feline maintenance diet were fed ad libitum. Prior to enrollment in the study, all cats were confirmed to have negative intradermal skin tests for Bermuda Grass Allergen (BGA) and an absence of elevated eosinophils in their bronchoalveolar lavage fluid (BALF; percentage of eosinophils in BALF was <5% in all cats, reference range 16 ± 14%).[19]

Allergen Sensitization- The cats were sensitized to BGA[d] by a previously described protocol.[29] Briefly, the cats were administered 12 μg of BGA in 10 mg alum SC and 10^7 *Bordetella pertussis* organisms IM on day 0. On day 14, 0.2 ml BGA (0.08 mg) was administered IN. On day 21, 12 μg of BGA in 10 mg alum was administered SC.

Parenteral sensitization was confirmed with intradermal skin testing on day 28. Three cats did not adequately respond (i.e., had wheals in response to BGA that were less than 50% of the difference between the diameters of the positive and negative wheals) and were given another injection of 12 μg BGA in 10 mg alum on day 28. Aerosol challenge (0.5 mg BGA in 5 ml PBS/exposure) was administered to unsedated animals in a sealed chamber fitted for an air compressor[e] with a nebulizer. Each cat was exposed to allergen for 15 minutes per exposure for the first 3 treatments. The time was then decreased to 10 minutes per exposure for the remainder of the study. Aerosol exposure was started on day 30 and consisted of every other day treatments for a total of 7 treatments. Two days after allergen challenge BALF percentage eosinophils was measured to confirm the asthmatic phenotype. They were found to be substantially higher then pre-sensitization eosinophil percents (mean ± SD; 56 ± 14 %). To maintain airway sensitivity to allergen, the cats were then aerosolized weekly for the remainder of the study.

Study design— After the cats developed eosinophilic airway inflammation in response to BGA, they were enrolled in the blinded, randomized, placebo controlled, crossover study to evaluate the effects of cetirizine and cyproheptadine. Cats were randomly assigned one of three drug treatments: cyproheptadine[f] (8 mg PO BID), cetirizine[g] (5 mg PO BID) and placebo (flour in a #4 gelatin capsule[h]). Each treatment was administered for one week followed by a one week washout period. Each cat received all three treatments over the course of the study. Samples were collected at baseline (prior to administration of drug) and after one week of drug treatment.

Collection of BALF— Cats were anesthetized for baseline and post-treatment sample collections with ketamine[i] (8-14 mg/kg IV). For BALF collection, the cats were

intubated with cuffed 4.0-4.5 mm endotracheal tubes and a 7-Fr polypropylene catheter was gently inserted until the catheter was wedged into a small airway. A 12 ml aliquot of 0.9% PBS was flushed into the catheter and retrieved with gentle suction. The BALF samples were placed immediately on ice and processed within 2 hours of collection.

A total nucleated cell count on BALF was performed using a hemocytometer. Slides were prepared for cytologic evaluation using a cytocentrifuge and were stained with a modified Wright's stain. Differential cell counts were performed by one investigator (EKS) based on a 200 nucleated cell count/slide. The number of eosinophils was determined by multiplying the percentage of eosinophils times the number of total nucleated cells. The remaining BALF was centrifuged at 300xg for 10 minutes and the supernatant was harvested and stored at -20^{o}C until further analysis.

Collection of blood- Blood samples were collected weekly in EDTA and additive free tubes by jugular venipuncture. The EDTA tubes were placed directly on ice and then centrifuged at 400xg for 10 minutes within 2 hours of collection. Plasma was harvested and stored at -20^{o}C until further analysis. Blood in the additive free tubes was allowed to clot at 24^{o}C and then centrifuged at 1730xg for 20 minutes. The serum was harvested and stored at -20^{o}C until time of analysis.

Plasma and BALF Histamine Concentration- A commercially available histamine competitive ELISA kit[j] was used to quantitate plasma and BALF histamine concentrations, in accordance with the manufacturer's instructions. All standards and unknown samples were assayed in duplicate. The histamine concentration in each sample was calculated by use of values generated from a standard curve. The range of detection of histamine in the ELISA was 2.5-50 ng/ml.

Plasma and BALF Serotonin Concentration- Absorbance and emission spectra were recorded in quartz cuvettes containing the sample in a solution of Tris buffer (plasma) or pure samples (BALF). Absorbance spectra were collected on a Varian Cary Bio 50 scanning diode array spectrometer with 1 nm resolution[k]. Emission spectra were collected on a Varian Cary Eclipse L-format fluorometer[l] in excitation-emission-matrix (EEM) mode. Emission spectra were collected from 275 to 500 nm at 2 nm intervals, with a 2.5 nm band-pass. These spectra were taken using excitation wavelengths from 250 to 400 nm at 5 nm intervals and a 2.5 nm excitation band-pass. Each point was an internal average of eight collections. A set of nine standards with varying non-correlated concentrations of tryptophan and serotonin were taken before each data set was collected. The plasma was interrogated at a concentration of 2ul/ml and 4ul/ml in Tris. The BALF samples were examined without solvent dilution. The sample data was collected, and exported into MATLAB[m] for Parallel Factor Analysis (PARAFAC). Results for plasma serotonin were reported in ug/ml. Results for BALF serotonin were corrected for BALF total protein concentrations obtained using a protein assay.[n] BALF values were reported as:

$$\frac{\text{(concentration unknown sample/TP unknown sample)}}{\text{(concentration positive control sample/TP positive control sample)}}$$

Serum Content of BGA-Specific IgE- We used an ELISA involving a polyclonal rabbit anti-feline IgE antibody previously developed by our group[52] to determine BGA-specific IgE in our samples. Pooled positive control samples were collected from cats previously sensitized to BGA. Results were reported as optical density (OD), and expressed as a percentage of the positive pooled control sample.

Serum and BALF Content of BGA-Specific IgG and IgA- BGA-specific IgG and IgA were measured in serum and BALF samples by an ELISA method previously validated by our group.[4] Pooled serum and BALF samples from BGA sensitized cats from a previous study were used as a positive control. BALF results were normalized to BALF total protein concentrations obtained by use of a protein assay[o]. BALF values were reported as:

$$\frac{\text{(OD unknown sample/TP unknown sample)}}{\text{(OD positive control sample/TP positive control sample)}}$$

Serum and BALF Content of IL-4 and IL-10- Commercially available IL-4[p] and IL-10[q] sandwich ELISA kits was used to quantitate serum and BALF IL-4 and IL-10 concentrations, in accordance with the manufacturer's instructions. All standards and unknown samples were assayed in duplicate. The IL-4 and IL-10 concentrations in each sample were calculated by use of values generated from a standard curve. The range of detection of IL-4 in the ELISA was 0.06-4 ng/ml. The range of detection of IL-10 in the ELISA was 0.125-8 ng/ml.

Statistical Analysis- A commercially available software program[r] was used to perform the Shapiro-Wilk test for normality. Differences between treatments were assessed using a mixed model analysis of variance to adjust for the effects of animals. All variables met criteria suitable for normality assumptions. For all parameters a p-value <0.05 was considered significant.

Results:

BALF Percent Eosinophils- There were no significant differences between treatment groups for the percentage of eosinophils in BALF ($p=0.196$). Mean ± SD for the percent

eosinophils was 40.4% ± 22.4% for placebo, 31.2% ± 19.9% for cetirizine and 26.5% ± 15.6% for cyproheptadine (Figure 3-1). Additionally, no significant difference between treatment groups (p=0.0801), time (p=0.127) or treatment verses time (p=.1301) was found when comparing the treatment versus baseline percentage of eosinophils in BALF. This indicates that the degree of eosinophilic inflammation in response to BGA in the cats did not change over the time of the study and was not affected by the treatment outcome.

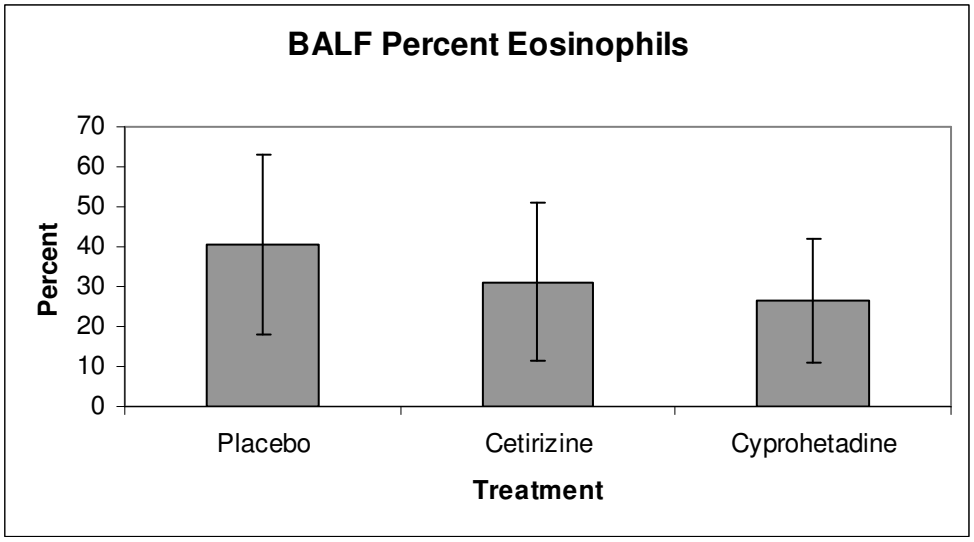

Figure 3-1- Mean ± SD percentage BALF eosinophils detected in BALF of 9 asthmatic cats following treatment with placebo, cetirizine and cyproheptadine.

Plasma and BALF Histamine Concentration- Plasma histamine concentration did not differ significantly between treatment groups (p=0.094). Mean ± SD for the treatment groups were 21.9 ng/ml ± 9.3 ng/ml, 15.8 ng/ml ± 5.7 ng/ml and 25.5 ng/ml ± 10.8 ng/ml for placebo, cetirizine and cyproheptadine respectively (figure 3-2). Histamine concentrations in BALF were found to be below the limit of detection of the ELISA in 60% of our samples, therefore no statistical analysis could be performed.

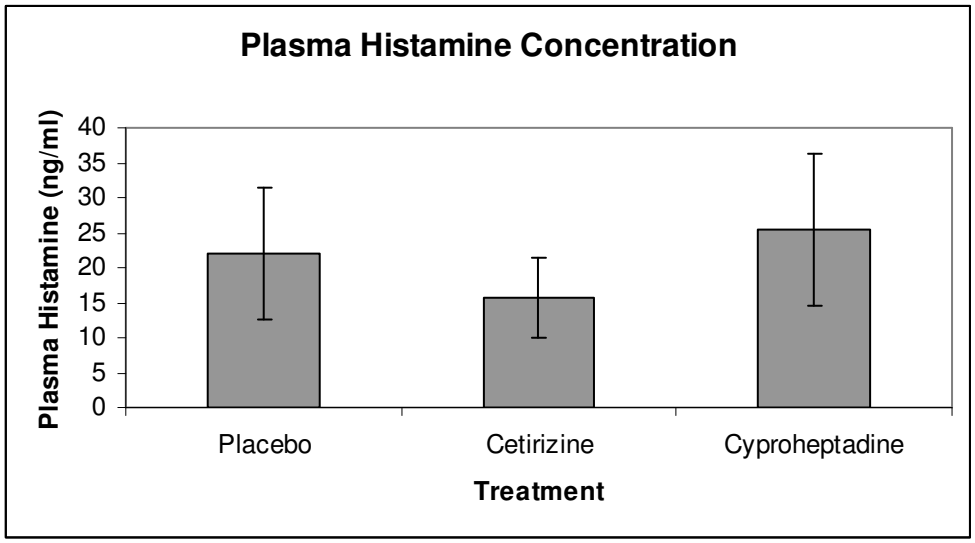

Figure 3-2- Mean ± SD plasma histamine concentration (ng/ml) of asthmatic cats following treatment with placebo, cetirizine and cyproheptadine.

Plasma and BALF Serotonin Concentration- Plasma and BALF serotonin concentrations were not significantly different between treatments (p=0.772, p=0.459 respectively). Mean ± SD for plasma serotonin concentration were 4.03 ug/ml ± 4.96 ug/ml for placebo, 4.9 ug/ml ± 4.28 ug/ml for cetirizine and 5.36 ug/ml ± 5.88 ug/ml for cyproheptadine (Figure 3-3). BALF serotonin:TP ratios were 10.19 ±11.2 for placebo, 18.33 ± 20.58 for cetirizine and 19.69 ± 15.49 for cyproheptadine (Figure 3-4).

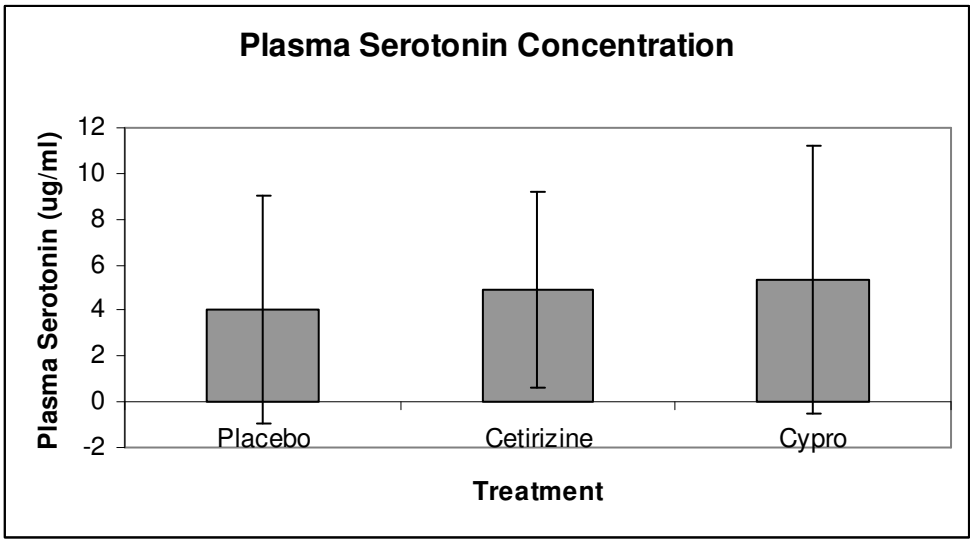

Figure 3-3 - Mean ± SD plasma serotonin concentration (ug/ml) of asthmatic cats following treatment with placebo, cetirizine and cyproheptadine.

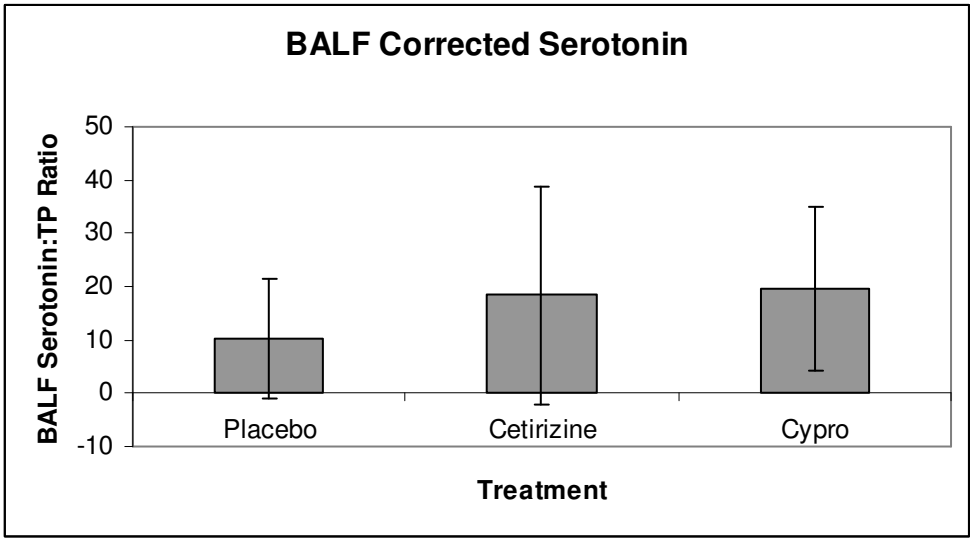

Figure 3-4 - Mean ± SD BALF Serotonin:TP Ratios of asthmatic cats following treatment with placebo, cetirizine and cyproheptadine.

Serum Content of Allergen-Specific IgE- No significant difference was detected between treatment groups for serum BGA-specific IgE (p=0.506). Mean ± SD values (expressed as a percentage of a positive pooled control) for IgE were 143.1% ± 84.7% for

placebo, 125.9% ± 74.5% for cetirizine and 147.1% ± 99.7% for cyproheptadine (Figure 3-5).

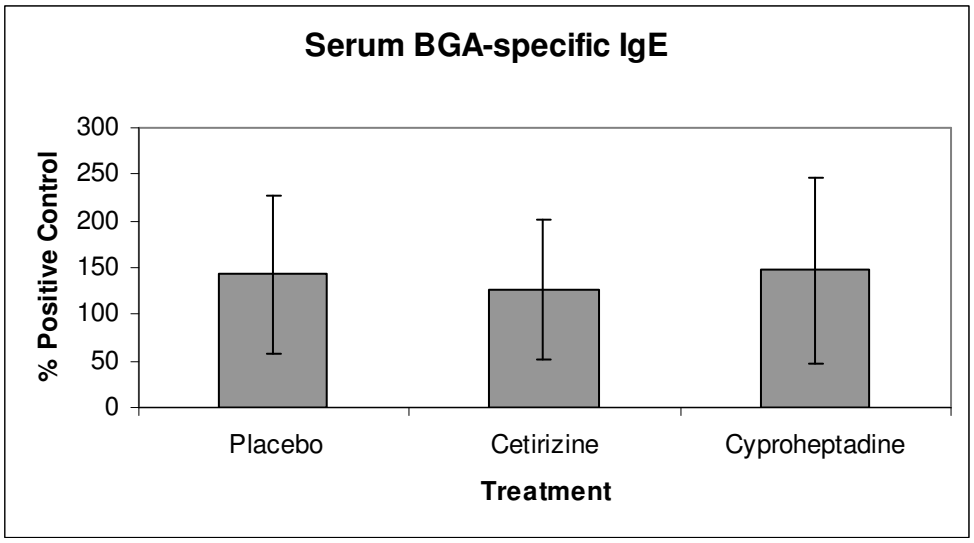

Figure 3-5- Mean ± SD Serum BGA-specific IgE of asthmatic cats following treatment with placebo, cetirizine and cyproheptadine

Serum and BALF Content of Allergen-Specific IgG and IgA- Plasma and BALF BGA-specific IgG and IgA levels did not differ significantly between treatment groups for the nine cats. Mean ± SD for OD values for serum immunoglobulin content and immunoglobulin:TP ratios for the BALF were calculated (Table 3-1).

Treatment	**Serum IgG** (% positive control)	**Serum IgA** (% positive control)	**BALF IgG** (BALF Ig:TP ratio)	**BALF IgA** (BALF Ig:TP ratio)
Placebo	49.88 ± 28.06	5.33 ± 3.9	0.175 ± 0.15	0.15 ± 0.12
Cetirizine	42.11 ± 23.19	7.11 ± 5.51	0.67 ± 0.68	0.55 ± 0.53
Cyproheptadine	44.44 ± 22.13	6.22 ± 4.41	0.35 ± 0.37	0.34 ± 0.35

Table 3-1- Mean ± SD values for serum (% positive control) and BALF content (BALF Ig:TP ratio) of BGA-specific IgG and IgA in 9 asthmatic cats following treatment with Placebo, Cetirizine and Cyproheptadine.

Serum and BALF Content of IL-4 and IL-10- IL-4 and IL-10 concentrations in serum and BALF were found to be below the limit of detection of the ELISA in a majority of our samples, therefore no statistical analysis could be performed.

Discussion:

Traditionally, treatments for feline asthma have focused on glucocorticoids and bronchodilators.[14,15] Until recently little attention was given to discovery of novel therapeutics for feline asthma in vivo. Since the inception of the BGA model of feline asthma, several studies have looked at new therapeutic options.[30,31] The current study evaluated medications that should block inflammatory mediators released toward the end of the asthmatic hypersensitivity cascade. Ideally, drugs used as a monotherapy for asthma should reduce airway inflammation. We decided to evaluate eosinophilic airway inflammation as a marker of drug efficacy since eosinophils have been noted to be essential in the pathogenesis of asthma.[3] While cyproheptadine and cetirizine can block portions of the allergic inflammatory cascade by blocking serotonin and histamine receptors, respectively, they were not effective in dampening eosinophilic airway inflammation in this model of allergic asthma. Additionally, they were unable to alter other immune parameters measured in this study. Because these drugs lacked efficacy in these parameters they can not be recommended as monotherapy for the treatment of feline asthma.

We chose to evaluate cyproheptadine and cetirizine in a model of feline asthma because this model mimics the immunologic and physiologic variables associated with naturally occurring asthma without the inconsistencies of naturally occurring disease. Cats that develop asthma naturally can have a variable course to the disease depending on

the time and duration of exposure to the allergen. By using this model we were able to expose the cats to a set amount of allergen on a weekly basis thereby maintaining a consistent environmental allergen exposure. Additionally, by using a model for this study we were able to eliminate problems associated with owner compliance in administration of medications.

Neither cyproheptadine nor cetirizine showed a substantial decrease in the percentage of BALF eosinophils. We postulated that because serotonin has some pro-inflammatory effects in other species[12,13] this may also be true in the cat. Similarly, histamine is an attractant to human eosinophils[11] and vasodilation induced by histamine has been implicated in airway inflammation.[9] Additionally, cetirizine decreases eosin induced migration of human eosinophils.[38] Because cyproheptadine and cetirizine block serotonin and histamine receptors respectively, we had hoped they would decrease some of these pro-inflammatory properties and thus decrease airway eosinophilia.

The inability of these drugs to significantly blunt eosinophilic airway inflammation could be due to a number of reasons. First, serotonin and histamine may not play a major role in inducing eosinophilic inflammation in feline asthma. A second consideration is that these mediators are released from mast cells well after Th2 cells have released IL-5 which is responsible for the initial eosinophil migration into the airways.[3] The release of these mediators may be too far down the allergic cascade from the initial eosinophil migration for antagonism of their receptors to make a substantial difference. Finally, since serotonin and histamine have been implicated in the acute phase response it is possible that their beneficial acute effects were missed by performing the airway lavage 48 hours after the cats were exposed to allergen. It is impossible to say

from this study whether these drugs blunted the acute phase response, but the late phase response (which in other species is driven by production of cysteinyl leukotrienes and cytokines) was unaffected by these drugs. The late phase response may have had a more powerful overall effect on eosinophilic airway inflammation. It has previously been established that cysteinyl leukotrienes are not important mediators in feline asthma,[30,34] but the role of cytokines in the late phase response is unknown. Importantly, however, even if the cyproheptadine and cetirizine blunted eosinophilic inflammation during the acute phase response, there was still too much eosinophilic inflammation at the time of sampling to consider these medications successful ongoing treatments.

We performed ELISAs to detect BALF and serum BGA-specific IgG and IgA, serum BGA-specific IgE and serum and BALF IL-4 and IL-10 concentrations. We examined these parameters because histamine and serotonin have been implicated in inflammatory cell influx. Both can cause release of IL-16 which causes recruitment of activated T-cells.[11,12] It is possible that these T-cells were responsible for cytokine production ultimately causing B-cells to form immunoglobulins or cause isotype switching. We were interested in seeing if either cyproheptadine or cetirizine by blocking the effects of serotonin and histamine would indirectly alter the formation of immunoglobulins. Additionally, we felt that if inhibition of these mediators resulted in decreased T-cell migration, production of IL-4 and IL-10 may have been altered. We did not find significant changes in immunoglobulin production between our treatment groups suggesting that serotonin and histamine have minimal effects, at most, on the formation of immunoglobulin. Additionally, we were unable to detect IL-4 or IL-10 in a majority

of our samples and are therefore unable to discuss the effects of these drugs on production of these cytokines.

Finally, we examined plasma and BALF serotonin and histamine concentrations. We did not find a change in plasma or BALF serotonin or plasma histamine concentrations between our drug groups. We were unable to interpret our BALF histamine concentrations because a majority of our samples were below the limit of detection of the ELISA. Mitchell et al measured BALF histamine concentrations in cats sensitized to *Ascaris suum.*[28] Mean BALF histamine concentrations reported in that study were below the limit of detection of our ELISA kit so it is not surprising we were unable to collect data for that parameter.[28] Miura et al measured plasma histamine levels in cats sensitized to *Ascaris suum* immediately after allergen challenge.[53] Two populations were tested, cats pretreated with the antihistamine pyrilamine and control cats. Both populations had a significant increase in histamine concentration 5-10 minutes post-antigen challenge compared to baseline, but no significant differences in histamine concentration were noted between groups (35±9.7 ng/ml versus 42.2±11.5 ng/ml, respectively). Treatment with this antihistamine reduced the airway resistance in pretreated cats compared with control cats. This suggests that although plasma levels of histamine were not reduced in the antihistamine treated cats, blockade of histamine led to physiologic effects (i.e., diminishing acute post-antigen challenge bronchoconstriction).[53] Because both cyproheptadine and cetirizine work by competitive inhibition of the receptors,[35,54] we had expected an increase in the concentration of histamine in cetirizine treated cats and an increase in serotonin concentration in cyproheptadine treated cats. Since no change was seen it is possible that either the drugs

didn't bind to a significant amount of receptors or that we missed the acute phase response as noted above by taking samples two days after allergen exposure. The BALF collection two days after allergen challenge was done based historically on having a large body of data that supported substantial eosinophilic inflammation and Th2 cytokine profiles at that time; it is now a standard protocol for the BGA model. While evaluating eosinophilic airway inflammation at that time point is appropriate, in retrospect, it probably would have been better to collect blood samples for mediator concentrations shortly after allergen challenge.

Eosinophilic inflammation is not the only important pathologic change seen allergic asthma. Airway hyperreactivity and airway remodeling are also important in the pathogenesis of this disease. Serotonin has been implicated as an important mediator of airway smooth muscle constriction.[35] Previous studies looking at the effects of cyproheptadine in feline asthma had focused on its effects of alleviating bronchoconstriction.[10] Another study that investigated the effects of cyproheptadine on airway inflammation noted that a proportion of treated cats did have decreased airway hyperreactivity despite a lack of effect of this medication on airway inflammation.[30] A previous study examining the pharmacokinetics of cyproheptadine found that some cats may need a substantially higher dose than was used in that study (up to 8 mg of cyproheptadine for maximal effect).[36] Unfortunately, we were unable to assess airway hyperreactivity for this study. Future studies could investigate the effects of high dose cyproheptadine on this parameter.

In conclusion, high dose cyproheptadine and cetirizine do not appear to blunt eosinophilic inflammation in this experimental model of feline asthma. Additional

studies are warranted to examine the effects of these medications on airway hyperreactivity or for use as a combination therapy for glucocorticoid sparing effects. At this time, we cannot recommend these medications as monotherapy for the treatment of feline asthma.

Chapter 4
Conclusions and Future Directions

This project involved evaluation of the "novel" therapeutics, cyproheptadine and cetirizine, in a feline model of allergic asthma. Cyproheptadine, an antiserotonergic, has shown some evidence of decreasing some of the airway hyerreactivity found in feline asthma.[10,30] Antihistamines such as cetirizine have been shown to decrease inflammatory cell migration in vitro.[38,39]. Other studies have also supported the role of second generation antihistamines, such as cetirizine, in the treatment of mild to moderate asthma in people by improving asthma symptoms and that they have corticosteroid-sparing effects.[42-44] Since serotonin and histamine have been implicated in vasodilation, increased vascular permeability, an inflammatory cell influx and airway smooth muscle constriction, we hypothesized that blockade of these mediators might have effects on the allergic inflammatory cascade and decrease airway inflammation in asthma.[6,51]

Our first major obstacle was determination of appropriate doses of the drugs for study. The pharmacokinetics of cyproheptadine have previously been determined and suggested a dose of 8 mg/cat may be needed in some cats for full effects.[36] No previous pharmacokinetic profile had been determined for cetirizine in this species. Therefore, chapter 2 outlines the pharmacokinetics of cetirizine for appropriate dose selection. We were able to show that in clinically healthy cats a single dose of 5 mg (approximately 1 mg/kg) orally administered cetirizine was absorbed from the gastrointestinal tract well. Because only the unbound portion of drug is considered active we determined the protein binding of this drug and found it to be high, similar to in humans.[46] Over a 24 hour

period plasma free drug concentrations remained higher than effective free drug concentrations reported in people. Additionally, no cats experienced any adverse effects from this drug despite the fact that total drug concentrations were higher than those reported for people. Our study also found that the half life of cetirizine in cats was long enough to allow for once daily dosing. Limitations of this study included the relatively low number of cats (n=9) used and the lack of testing specific populations (ie young and geriatric cats) for adverse events. Additionally, we did not evaluate the therapeutic efficacy of cetirizine in this study as that was our goal for the second part of this thesis.

Next, chapter 3 describes the results of the study evaluating the ability of antiserotonergic (cyproheptadine) and antihistaminic (cetirizine) drugs to diminish the asthmatic phenotype in an experimental model of feline asthma. Naturally occurring feline asthma is one of the most common bronchopulmonary disorders in cats and is believed to be allergic in etiology. It most commonly affects young to middle aged cats.[14,15] Cats with asthma commonly present for a cough or an expiratory wheeze. Cats can present with life threatening respiratory distress and open mouth breathing in severe situations.[15] The diagnostic plan for feline asthma includes a complete blood count, serum chemistry profile and urinalysis; thoracic radiographs, parasitic examinations and culture and cytology of bronchoalveolar lavage fluid. The CBC and chemistry profile tend to be nonspecific for respiratory disease but about 20 % of cats will have a peripheral eosinophilia[14,15] and a mild hyperproteinemia is found in about 33% of asthmatic cats due to chronic inflammation.[14] The most common radiographic findings are a bronchial pattern or bronchointerstitial pattern.[14,17] Ultimately, examination of airway cytology is the confirmatory test in these cats. Asthmatic cats will have a

predominance of eosinophils within their airways. They may also have a neutrophilic component if there is coexisting chronic bronchitis or a secondary bacterial infection.[14,15,17] It should be noted that normal cats reportedly have a relatively high BALF eosinophil percent compared to other species. Some studies claim a healthy cat can have a BAL eosinophil count at high as 16-18%[18,19] Therefore, when making the diagnosis of feline asthma all clinical parameters should be taken into account. Histopathologic lesions include eosinophilic and neutrophilic bronchial inflammation, smooth muscle hyperplasia, globlet cell hypertrophy, mucous and cellular debris in the airway lumen, epithelial erosion and in severe cases emphysema.[22] Histology is rarely done in clinical cases due to the relative ease of diagnosis with less invasive tests noted above.

The hallmark studies investigating naturally occurring feline asthma have evaluated response to treatment by noting improvement of clinical or radiographic signs and rarely, improvement in pulmonary function testing.[14,15] Recent studies using the BGA model have evaluated immunologic and physiologic variables to test drug efficacy.[30,31] One of the greatest advantages of the BGA model is that it uses a natural allergen (BGA) to develop the allergic response in the cats. In the development of an animal model this is extremely important because it allows the model to more closely mimic naturally occurring disease. When this model was first developed, the investigators chose (BGA) based on serum IgE testing in cats with naturally occurring disease.[29] This asthma model routinely produces cats with clinical signs consistent with asthma. These cats produce allergen-specific IgE, increased allergen-specific serum and BALF IgG and IgA over time, demonstrate airway hyperreativity after allergen exposure,

have increased airway eosinophilia and have lung histology consistent with feline asthma.[29]

We chose to evaluate cyproheptadine and cetirizine in a model of feline asthma because we felt a model could mimic the immunologic and physiologic variables associated with naturally occurring asthma without the inconsistencies of naturally occurring disease. In naturally occurring asthma, time and duration of exposure to the allergen can lead to a variable course of the disease. By using a model, we were able to maintain a consistent environmental allergen exposure. Additionally, we were able to eliminate problems associated with owner compliance in administration of medications by avoiding clinical cases.

In this experimental model of feline asthma, neither cyproheptadine nor cetirizne significantly decreased airway eosinophilia. We postulated that because both serotonin and histamine have been implicated in airway inflammation, by blocking these mediators we could decrease airway eosinophilia in asthmatic cats. Because airway inflammation is thought to contribute both to airway hyperreactivity and to airway remodeling, drugs that fail to dampen airway inflammation can not be recommended as monotherapy for asthma.[3]

We decided to evaluate the percent BALF eosinophils as evidence of efficacy for the two drugs since eosinophilic airway inflammation has been reported to be a key pathologic change in asthma. The inability of these drugs to significantly blunt eosinophilic airway inflammation could be due to a number of reasons. First, other mediators besides serotonin and histamine may play a more important role in inducing eosinophilic inflammation in feline asthma. For example, Th2 cells produce IL-5 which is

responsible for eosinophil maturation, differentiation, survival and the initial eosinophil migration into the airways.[3] Second, the release of serotonin and histamine from mast cells is a relatively late event in the allergic inflammatory cascade, occurring well after allergen presentation, activation of Th2 cells with their subsequent cytokine elaboration, and plasma cell allergen-specific IgE production. Therefore, antagonism of their receptors may not make a substantial difference in the overall airway eosinophilia as other inflammatory pathways have already been activated. Finally, since serotonin and histamine have been implicated in the acute phase response it is possible that their maximal beneficial effects were missed by performing the airway lavage 48 hours after the cats were exposed to allergen. It is not possible to say from this study whether these drugs blunted the acute phase response, but the late phase response (which in other species is driven by production of cysteinyl leukotrienes and cytokines) played a more dominant role in establishing eosinophilic airway inflammation. It has previously been demonstrated that cysteinyl leukotrienes are not important mediators in feline asthma,[30,34] but the role of cytokines in the late phase response in cats has not yet been studied. Importantly, however, even if cyproheptadine and cetirizine blunted eosinophilic inflammation during the acute phase response, there was still too much eosinophilic inflammation at the time of sampling to consider these medications successful clinical treatments.

Plasma and BALF serotonin and histamine concentrations were measured in the cats of this study. We did not find a significant difference in plasma or BALF serotonin or in plasma histamine concentrations between our treatment groups. Because both cyproheptadine and cetirizine work by competitive inhibition of serotonin and histamine

receptors, respectively,[35,54] we had postulated an increase in serum and BALF histamine concentrations in cetirizine treated cats and an increase in serum and BALF serotonin concentrations in cyproheptadine treated cats. It is possible that we missed the maximal effect of these medications by taking samples two days after allergen exposure. Collecting BALF two days after allergen challenge was performed based historically on having a large body of data that supported substantial eosinophilic inflammation and Th2 cytokine profiles at that time; it is now a standard protocol for the BGA model.[29] While evaluating eosinophilic airway inflammation at that time point is appropriate, in retrospect, it probably would have been better to collect blood samples for analysis of mediator concentrations shortly after allergen challenge.

In addition to finding that cyproheptadine and cetirizine were ineffective in dampening eosinophilic airway inflammation in this model of allergic asthma, we also noted that they failed to significantly alter any of the other measured immune parameters. Serotonin and histamine have been implicated in vasodilation, increased vascular permeability, an inflammatory cell influx and airway smooth muscle constriction, and therefore it would be reasonable to speculate that blockade of these mediators might have far-reaching effects on other components of the allergic inflammatory cascade.[6,51] The BGA-specific IgE, IgG and IgA content of serum, and BGA-specific IgG and IgA content of BALF were evaluated to determine if blockade of serotonin and histamine would indirectly alter the formation of allergen specific immunoglobulins. We did not find significant differences in immunoglobulin production between our treatment groups suggesting that blockade of serotonin and histamine have insignificant effects on allergen-specific immunoglobulin production. Additionally, we measured the Th2

cytokines IL-4 and IL-10 in serum and BALF. Unfortunately, concentrations of IL-4 and IL-10 in a majority of the samples were not detectable, and therefore no clear conclusion about the impact of cyproheptadine and cetirizine on these cytokines can be made in our model. Because serotonin and histamine are released relatively late in the cascade of inflammatory events in allergic asthma, and because of overlapping, redundant and additive or synergistic actions of various components of the inflammatory cascade, it is not surprising that these drugs failed to produce readily apparent significant effects on immunoglobulin and cytokine concentrations.

Eosinophilic inflammation is not the only important pathologic change seen allergic asthma. Airway hyperreactivity and airway remodeling are also important in the pathogenesis of this disease. Serotonin has been implicated as an important mediator of airway smooth muscle constriction.[35] Previous studies looking at the effects of cyproheptadine in feline asthma had focused on its effects of alleviating bronchoconstriction.[10,35] Another study that looked at the effects of cyproheptadine on airway inflammation in experimental feline asthma noted that one-third of treated cats had decreased airway hyperreactivity despite not significantly reducing airway inflammation.[30] The pharmacokinetics of cyproheptadine in cats suggests that they may need a substantially higher dose of cyproheptadine for maximal effect than was used in that study.[36] We did not assess airway hyperreactivity in the current study, but future studies could look at the effects of high dose cyproheptadine on this parameter.

This paper investigated two drugs which should have blocked two of the mediators involved in the allergic pathway. Although theoretically blocking these mediators should have an effect on certain aspects of asthma, blocking mediators at the

end of the allergic cascade are unlikely to diminish all the detrimental aspects of the pathology of asthma (i.e. eosinophilic airway inflammation, airway hyperreactivity and airway remodeling). Another strategy that has been very successful in human medicine is the drug Omalizumab. This drug is a humanized monoclonal antibody targeting the high-affinity receptor binding site on human immunoglobulin E (IgE). By blocking IgE binding to mast cells Omalizumab is able to prevent the release of mediators from mast cells. This may have a more widespread effect then blocking the mediators after they have been released by the mast cells.[55] Unfortunately for our patients Omalizumab is a humanized monoclonal antibody and therefore it is unlikely to be of use in cats. There is no equivalent feline specific antibody available. Finally, immunomodulation may be the best strategy for a potential cure for asthma. Allergen specific immunotherapy uses subcutaneous injection of small amounts of purified antigen to desensitize the individual to the allergen. The exact mechanism of desensitization is unknown but may involve stimulation of IL-10 and release of transforming growth factor-β from regulatory T-cells.[55] A preliminary study developing a rush immunotherapy protocol showed promise in dampening eosinophilic airway inflammation; however, side effects were common and in a small percentage of cats, severe.[31] Ongoing studies using this model are investigating ways to improve the safety and efficacy of immunotherapy protocols by using adjuvants and by employing mucosal routes of allergen administration.

In conclusion, neither cyproheptadine nor cetirizine significantly blunt eosinophilic inflammation in this experimental model of feline allergic asthma. Additional studies may be warranted to examine the effects of these medications on airway hyperreactivity or for use as a combination therapy for glucocorticoid sparing

effects. At this time, these medications can not be recommended as monotherapy for the treatment of feline asthma.

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[a] Cetirizine HCl, Pfizer, New York, NY.

[b] SPEC-C18AR, Varian, Inc, Palo Alto, CA.

[c] Liberty Research, Waverly, NY

[d] Bermuda grass allergen, 7.41 mg/vial, #XP2D3A, Greer Laboratories Inc, Lenoir, NC.

[e] Easy Air 15, Model PM15P, Precision Medical Inc, Northampton, PA.

[f] Cyproheptadine HCL, IVAX Pharmaceuticals, Inc, Miami, FL.

[g] Zyrtec, Pfizer, New York, NY.

[h] Gelatin capsule, Eli Lilly and Company, Indianapolis, IN.

[i] Ketaset, Fort Dodge, Fort Dodge, IA.

[j] Histamine ELISA kit #409010, Neogen, Lexington, KY.

[k] Cary Bio 50 scanning diode array spectrometer, Varian, Inc, Palo Alto, CA.

[l] Cary Eclipse L-format fluorometer, Varian, Inc, Palo Alto, CA.

[m] MATLAB version 7.1 (R14), The MathWorks, Inc, Natick, MA.

[n] Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA.

[o] Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA.

[p] IL-4 ELISA, kit # DY948, R&D Systems, Minneapolis, MN

[q] IL-10 ELISA kit # DY736, R&D Systems, Minneapolis, MN

[r] SAS version 9.1, SAS Institute Inc., Cary, NC.

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