

feG-COOH blunts eosinophilic airway inflammation in a feline model of allergic asthma

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Abstract

Objective and design This study investigated if feG-COOH would decrease allergen-induced airway inflammation.

Materials or subjects Seven adult cats sensitised to Bermuda grass allergen (BGA) to induce an asthmatic phenotype.

Treatment Cats were randomized to receive either feG-COOH (1 mg/kg, PO) or placebo (saline 1 ml, PO) immediately prior to BGA aerosol challenge in a cross-over design.

Methods Bronchoalveolar lavage fluid (BALF) was collected and airway inflammatory response assessed via inflammatory cell number and type; IL-4, IFN- γ and nitric oxide metabolite concentrations. A paired *t* test was used to compare parameters with a *P* < 0.05 considered significant.

Results The BALF eosinophil percentage was significantly lower in asthmatic cats treated with feG compared with placebo (placebo, 35.3 $\pm$ 12.2%; feG, 22.4 $\pm$ 8.6%; *P* = 0.002). Treatment with feG did not result in a significant change in any other parameter measured.

Conclusions These data indicate that a single dose of feG-COOH partially attenuates eosinophilic airway inflammation in experimental feline asthma.

Keywords Cat · Immunomodulator · Allergy · Cytokine · Bronchoalveolar lavage

Introduction

Feline asthma was first described in 1904 [1]; since then recognition of this disorder in cats has increased. Now considered one of the most common respiratory diseases in the cat, asthma has been estimated to affect between 1 and 5% of the pet cat population [2]. Feline asthma is thought to be allergic in origin, induced by inhalation of allergens that activate CD4 + T helper (Th2) cells [3, 4]. Aeroallergens processed by antigen presenting cells in the lungs promote naïve Th0 cells to polarize towards a Th2 phenotype and away from a Th1 phenotype. Interleukin 4 (IL-4) is a key Th2 cytokine, whereas interferon gamma (IFN- γ) is a hallmark Th1 cytokine; they each have a counter regulatory function on the other. In asthma, the elaboration of Th2 cytokines lead to chronic eosinophilic airway inflammation, intermittent reversible airway obstruction in response to allergenic stimulation, airway hyperreactivity in response to non-allergenic triggers, and airway remodeling [4–7]. Nitric oxide contributes to pulmonary inflammation in allergic asthma by formation of reactive nitrogen species [8]. Proinflammatory influences in poorly controlled asthma can lead to irreversible changes to pulmonary function and architecture. Treatment failures may be because of poor compliance if there are undesirable side effects (as can be seen with glucocorticoids) making it vital that new,

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efficacious therapies with minimal to no appreciable side effects are developed.

The immune, nervous and endocrine systems communicate with each other to maintain a healthy state [9]. The cervical sympathetic trunk-submandibular gland axis of neuroendocrine immunology has been previously investigated for its role in allergic inflammatory disease leading to identification of an important peptide, feG [10]. Studies using the salivary gland tripeptide feG in an experimental model of asthma in rats demonstrated blunted allergen-induced airway inflammation [10, 11]. Prior to using this therapy in pet cats, evaluation of feG in an experimental model of feline asthma [4] is warranted. Therefore, the objective of this study was to evaluate the safety and efficacy of a single dose of feG-COOH in a feline model of asthma induced by Bermuda grass allergen (BGA). We hypothesized that feG would decrease BGA-induced eosinophilic airway inflammation by altering local cytokine and inflammatory mediator production. Specifically, the decrease in bronchoalveolar lavage fluid (BALF) eosinophil percentage would be accompanied by a decreased IL-4:IFN- γ ratio and nitric oxide metabolites (total nitrate) concentrations.

Materials and methods

Animals

Seven healthy adult specific pathogen free cats (Feline Asthma Colony, University of Missouri, Columbia, MO) were cared for according to the principles outlined in the NIH Guide for the Care and Use of Laboratory Animals and all procedures were approved by the University of Missouri Animal Care and Use Committee. The cats were maintained with a commercially available maintenance cat food and water ad libitum and were fasted for 12 h prior to any anesthetic administration. Cats were aged between 0.9 and 1.1 years (1.01 ± 0.13 years) and weighed between 3.0 and 4.5 kg (3.77 ± 0.5 kg). Prior to induction of the asthmatic phenotype, cats were confirmed to have negative intradermal skin tests to Bermuda grass allergen (BGA; Greer Laboratories, Lenoir, NC) and lacked eosinophilic airway inflammation determined by collection of BALF (BALF; eosinophils < 5% in all cats).

Induction of the asthma phenotype

Cats were sensitised BGA to induce an asthmatic phenotype using a protocol previously established in our laboratory [4]. Briefly, cats were administered 12 μ g of BGA in 10 mg of alum s.c. and 10^7 *Bordetella pertussis* organisms (to induce IgE antibody isotype switching) i.m.

on day 0; 80 μ g of BGA in 200 μ l PBS intranasally on day 14; and 12 μ g of BGA in 10 mg of alum, s.c., on day 21. Detection of wheals in response to BGA (positive result) during intradermal skin testing on day 28 was used to confirm sensitisation. After parenteral sensitisation, aerosol allergen challenge exposure was conducted on awake, spontaneously breathing cats in a sealed chamber. An air compressor attached to a nebulizer was used to aerosolize the allergen solution (200 μ g of BGA dissolved in phosphate-buffered saline solution delivered for 10 min/treatment). Allergen aerosol challenges were performed three times/week for 2–4 weeks with the duration of delivery of the aerosols adjusted in each cat based on the presence of clinical signs (cough, increased respiratory rate, or overt respiratory distress). At the first onset of clinical signs, the allergen aerosol was discontinued and an albuterol metered dose inhalant administered through a spacer was given as necessary. Aerosol challenges were administered weekly or as described below (see “[Treatments](#)”) for the remainder of the study and the duration of aerosol treatments were maintained either at 10 min/treatment or until clinical signs became apparent. The cats in this study had been sensitised to BGA and received weekly allergen challenges for at least 6 months prior to initiation of the treatments. The asthmatic phenotype was confirmed prior to initiation of treatments by collecting BALF. Based on our previous experience having sensitised over 100 cats as well as reference ranges for clinically normal cats [12], we defined an asthmatic phenotype as a 24-h post-allergen challenge BALF eosinophil percent > 16% [3, 12–14].

Treatments

This study was conducted in a randomized, blinded, crossover design with each cat acting as its own control. Asthmatic cats were randomized to receive either feG-COOH (1 mg/kg diluted into 1 ml 0.9% NaCl solution, PO) or placebo (1 ml 0.9% NaCl solution, PO) as a single dose immediately prior to BGA aerosol challenge. Treatments were administered by an investigator blinded to the animal's treatment group assignment. There was a 10-day washout period before crossover to the other treatment.

Safety

After each treatment, the cats were observed for 30 min and then daily for 2 days. Any change in behavior or physical examination was recorded.

Sample collection

Collection of BALF was performed 24 h after challenge using a previously described technique [4]. Briefly, the cats

were anesthetized with ketamine (5–10 mg/kg, IV to effect) (Ketaset, Fort Dodge Animal Health, Ft. Dodge, IA) and intubated. A sterile 8 Fr polypropylene catheter was advanced through the endotracheal tube and lodged in a bronchus. Warmed sterile saline (15 ml) was infused through the polypropylene catheter and aspirated back with gentle manual suction. The BALF was then placed in a sterile 15 ml plastic conical tube on ice. Supernatant was immediately harvested from a portion of the BALF using a centrifuge (300 g for 6 min), and banked at -20°C until analysis. The remaining volume of BALF was used within 1 h for cytological analysis.

BALF analyses

All assays were conducted by an investigator blinded to the cat's treatment. Total BALF cell count was determined in triplicate using a Coulter counter. The mean of these three measurements was used for data analysis. Differential cell counts were performed by counting 300 cells on a Wright's stained cytospin preparation and expressing the relative ratio of each cell type as a percentage.

Banked aliquots of BALF fluid stored at -20°C were used unconcentrated to determine total protein, IL-4 and IFN- γ concentrations, and nitrate concentration. Total protein, IL-4, IFN- γ and nitrate concentrations were determined in duplicate.

Total protein concentration was determined using a Bradford type protein assay (BioRad Laboratories, Hercules, CA). The limits of detection of this assay are between 8 and 80 $\mu\text{g/ml}$; samples were diluted when necessary so they would fall on the standard curve. Interleukin-4 and IFN- γ concentrations were using commercially available feline-specific ELISA kits (R&D Systems, Minneapolis, MN). The limits of detection for these assays are 62.5–4000 pg/ml. Interleukin-4 to IFN- γ ratio was determined by dividing the concentration of IL-4 (ng/ml) by the concentration of IFN- γ (ng/ml). Similarly, the IL-4 and IFN- γ to total protein ratios were determined by dividing the concentration of IL-4 (ng/ml) or IFN- γ (ng/ml) by the concentration of total protein (ng/ml).

Nitric oxide metabolites in the BALF were assessed by determining the total nitrate concentration using a commercially available nitrate/nitrite colorimetric assay kit (Cayman Chemical, Ann Arbor, MI). Prior to analysis, BALF was centrifuged at 3,000 g for 30 min through a 10 K molecular weight cut-off filter (Millipore Corporation, Billerica, MA). Samples were plated at 80 μl per well in duplicate, and after addition of nitrate reductase cofactor preparation and nitrate reductase enzyme preparation, were allowed to sit for 3 h at 24°C . Griess reagents 1 and 2 were added (50 μl each) to the wells and the plates were read at an absorbance of 540 nm. The limits of

detection for this assay are 2.5–35 μM . The ratio of nitric oxide to total protein was determined by dividing the nitric oxide concentration ($\mu\text{g/ml}$) by the concentration of total protein ($\mu\text{g/ml}$).

Data analysis

Statistical analyses were performed using commercially available software (SigmaStat, Systat Software Inc., Chicago, IL). The results of each of the parameters were compared between treatments using a paired *t* test if the data were normally distributed. For data that were not normally distributed, a Wilcoxon-signed rank test was used. Data were reported as mean $\pm$ SD unless otherwise indicated. A *P* value of < 0.05 was considered statistically significant.

Results

Safety

No adverse clinical effects were noted after administration of oral feG or placebo in any cat.

BALF cell counts

Treatment with feG partially blunted eosinophilic airway inflammation as evidenced by a significantly lower group mean BALF eosinophil percent in the feG-treated cats as compared to the cats receiving placebo (Fig. 1a) ($P = 0.002$). Although the BALF eosinophil percent was significantly lower after feG treatment than placebo, only 1/7 cats had post-feG treatment eosinophil percent within the reference interval for non-asthmatic cats ($\leq 16\%$); however, this cat had the mildest eosinophilic airway inflammation of all the cats (19% after administration of placebo). The group mean total BALF cell count (placebo, $6.3 \pm 5.6 \times 10^5$ cells/ml; feG, $7.2 \pm 5.1 \times 10^5$ cells/ml; $P = 0.80$) and neutrophil percent (placebo, $5.4 \pm 4.5\%$; feG, $7.7 \pm 5.4\%$; $P = 0.42$) were not statistically different between the treatments.

BALF total protein

Group mean TP concentrations were not significantly different between the treatment groups (placebo, 127.1 ± 137.2 ; feG 115 ± 41.7 $\mu\text{g/ml}$; $P = 0.085$).

BALF IL-4, IFN- γ concentrations

Treatment with feG did not significantly alter group mean IL-4 (placebo, 810 ± 75 pg/ml; feG 700 ± 540 pg/ml; $P = 0.98$) or IFN- γ (placebo, 550 ± 480 pg/ml; feG,

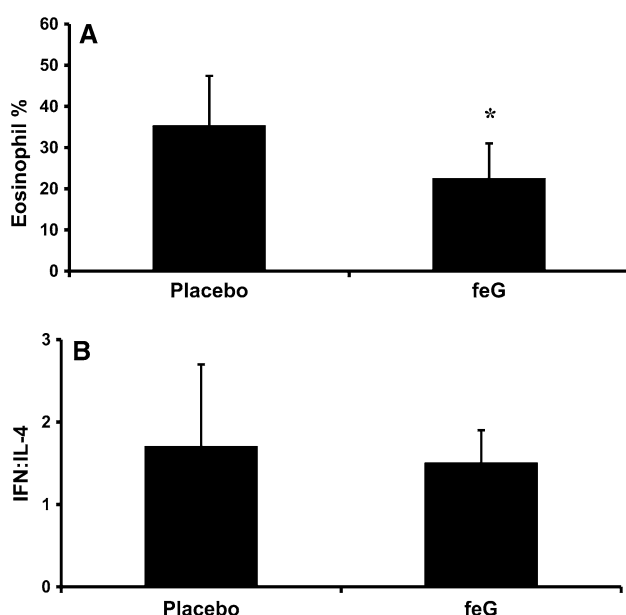


Fig. 1 Group mean $\pm$ SD BALF eosinophil percent (a) and BALF IL-4 to IFN- γ ratio (b) in cats that were administered either placebo or feG. Asterisk indicates $P < 0.05$

630 ± 480 pg/ml; $P = 0.58$) concentrations. Similarly, after normalizing the cytokines to TP to adjust for the effects of dilution the ratio of BALF IL-4 to TP (placebo, 0.015 ± 0.02 ; feG, 0.006 ± 0.004 ; $P = 0.4$) or IFN- γ to TP (placebo, 0.009 ± 0.01 ; feG, 0.005 ± 0.004 ; $P = 0.54$) was not significantly different between groups. The comparison between the group mean ratios of IL-4 to IFN- γ in the BALF fluid did not reflect a significant treatment effect (Fig. 1b) ($P = 0.67$).

BALF nitrate

The group mean BALF nitrate concentration (placebo, 4.6 ± 0.9 μ M; feG 5.4 ± 0.9 μ M; $P = 0.07$) was not statistically different between cats treated with feG and cats administered placebo. Data for BALF nitrate to TP ratio were not normally distributed and thus were analyzed using a Wilcoxon-signed rank test. There was no significant difference in BALF nitrate to TP ratio (placebo, median 4.3, first quartile 1.8, third quartile 1.9; feG, median 2.8, first quartile 1.9, third quartile 4.5 μ M; $P = 0.813$) between the treatments.

Discussion

A single dose of the salivary gland tripeptide feG-COOH administered prior to allergen challenge in cats with experimentally induced asthma partially blunted eosinophilic airway inflammation after 24 h. Eosinophilic airway

inflammation is a hallmark feature of asthma in cats and humans and is a target for therapy because it contributes to airway hyperreactivity and remodeling [15]. Despite the significant attenuation of eosinophilic airway inflammation, complete resolution of eosinophilic inflammation was not achieved. However, the purpose of this pilot study was to evaluate feG-COOH in a pre-clinical animal model that is used for translational research for novel therapeutics in cats and humans to determine if this compound might hold promise if used in long-term therapy. Studies are currently underway to evaluate the effect of regular use of feG-COOH in cats with experimental asthma. Since it is difficult to clinically predict exposure to an asthma trigger, it is critical to be able to assess how well feG will control eosinophilic inflammation when administered after allergen challenge.

The mechanism by which feG, a peptide derived from the prohormone submandibular rat 1 (SMR1) and modified to enhance bioactivity [11], ameliorates eosinophilic airway inflammation has yet to be fully elucidated. Previous studies have demonstrated a role for the neuroimmunoregulatory network in allergic disease [16, 17]. Peptides released from the salivary gland in response to nervous input from the cervical sympathetic trunk were recognized as having immunosuppressive and anti-inflammatory properties [18]. In an allergic model of pulmonary inflammation and anaphylaxis in rats, up-regulation of salivary peptide secretion (i.e., increased endogenous SMR1) via denervation resulted in marked attenuation of allergen-induced pulmonary inflammation, while sialadenectomy (i.e., removal of endogenous SMR1) abrogated this protective effect [16, 17]. In rats sensitised to ovalbumin, a single oral dose of feG significantly reduced eosinophilic airway inflammation 24 h after allergen challenge [10, 17]. The rapid effect of feG on airway inflammation was postulated to be due to blockade of the influx of inflammatory cells from the blood into the lungs. In support of this, administration of feG has been shown to down-regulate allergen-induced eosinophil expression of intracellular adhesion molecule-1 (ICAM-1), a surface receptor necessary for eosinophil movement from the blood into the airway, after allergen challenge in rats [10]. Owing to the current lack of availability of reagents for identification of cell adhesion molecules on eosinophils in cats, the focus of this study was on cytokines and inflammatory mediators involved in the allergic cascade.

Feline-specific cytokine ELISAs for IL-4 and IFN- γ are now commercially available and were used to assess local cytokine production in BALF supernatant [19]. Twenty four hours after allergen challenge, we could find no significant differences in BALF IL-4 or IFN- γ concentrations (or their ratios to TP to adjust for dilutional effects of BALF) between feG and placebo treatments. Moreover, we

could not appreciate a tip in the balance of the Th2:Th1 cytokine profiles to explain the significantly lower eosinophil percent in feG-treated cats. There are several reasons that the eosinophil percent was significantly decreased with feG treatment even though significant alternations in the Th2 and Th1 balance, as measured by IL-4 and IFN- γ , were not observed in this study. It is possible that feG does alter production of IL-4 and IFN- γ , but long-term therapy is necessary to induce a statistically measurable change. Also, since these inflammatory mediators were only sampled at one time point, the immunomodulating effect of feG on IL-4 and IFN- γ may have been missed. However, feG may have no effect on IL-4 or IFN- γ and instead alters eosinophil recruitment through a different mechanism. Other Th2 cytokines known to be involved in eosinophilic airway inflammation in human asthmatics include IL-3, IL-5, and GM-CSF. It is possible that these cytokines may have been affected by feG and could explain the decreased BALF eosinophilia; however, further studies must be performed to prove or disprove that.

Nitric oxide is an important biological mediator in the lung, modulating pulmonary vascular tone, nonadrenergic non-cholinergic (NANC) transmission, and airway inflammation. Evaluation of NO is particularly interesting in our feline model when considering the striking similarity to humans in the NANC inhibitory nervous control over airway caliber [20–22]. In both species, there are at least two mediators of the NANC inhibitory system which are nitric oxide and VIP [23–26]. Clinically, measurement of exhaled NO as a marker for airway inflammation has been useful in the management of asthma in humans [27]. Although measurement of exhaled NO is rapid and simple, it is expensive and requires elaborate equipment. Measurement of sputum nitrate has been shown to have excellent correlation with airway inflammation in asthmatic children [28]. Collection of induced sputum is not possible in cats, so evaluation of nitric oxide metabolites in BALF was performed. In the current study, nitric oxide metabolites in BALF were not significantly different between feG and placebo treatments. However, as stated above, this was only a pilot study evaluating the effects of a single dose of feG. Future studies can focus on the kinetics of NO metabolites with long-term feG therapy.

In conclusion, the use of a single oral dose of feG-COOH immediately prior to allergen challenge in cats with experimentally induced asthma partially attenuated eosinophilic inflammation in airways when evaluated 24 h later. These results support that feG-COOH could hold promise for treatment of asthma and provide the foundation for future studies to evaluate the long-term efficacy of feG-COOH during allergic asthma and the mechanisms by which this tripeptide alters the pathologic allergic inflammatory airway response. However, these data only indicate

a partial blunting of eosinophilic inflammation 24 h after allergen challenge. Since asthma in humans is a chronic condition in which it is unlikely that therapy can be taken just prior to allergen exposure, investigation is necessary to evaluate if longer term use of feG-COOH can blunt chronic airway inflammation.

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