Oral glucocorticoids diminish the efficacy of allergen-specific immunotherapy in experimental feline asthma

Chee-hoon Chang, Leah A. Cohn, Amy E. DeClue, Hong Liu, Carol R. Reinero *

Comparative Internal Medicine Laboratory, Department of Veterinary Medicine and Surgery, College of Veterinary Medicine, University of Missouri, Columbia, MO 65211, USA

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ABSTRACT

Allergen-specific rush immunotherapy (RIT) shows promise in treating asthma; however, pet cats will likely require at least initial concurrent glucocorticoids (GCs) to control serious clinical signs. How the immunosuppressive effects of GCs would impact RIT in cats is unknown. The hypothesis of this study was that oral, but not inhaled GCs will diminish the efficacy of RIT in experimental feline asthma. Cats ($n = 6$/group) were sensitized using Bermuda grass allergen (BGA) and randomized to receive BGA-specific RIT for 9 months with an oral GC (prednisolone 10 mg daily), inhaled GC (fluticasone 220 μg twice daily), or placebo administered for the first 6 months. Bronchoalveolar lavage fluid (BALF) percent eosinophils and other immunological assays were performed.

Eosinophilic airway inflammation was suppressed in all groups at month 6 of RIT (group mean ± SD, $5 ± 2\%$, $13 ± 4\%$, and $7 ± 2\%$ for oral GC, inhaled GC, and placebo, respectively; $P = 0.291$). BALF percent eosinophils significantly increased over time only in oral GC/RIT cats between months 6 and 9 ($P = 0.031$). Placebo/RIT cats had significant decreases over time in BGA-specific serum IgE ($P = 0.031$). Concentration of interleukin (IL)-5 in BALF significantly increased over time in inhaled GC/RIT cats ($P = 0.031$). No significant differences were found between groups at month 6 or over time in each group for BGA-specific lymphocyte blastogenesis, percent blood T regulatory cells, or number of IL-10-producing cells. Given the significant increase of airway eosinophilia over time in RIT cats initially treated with an oral GC, inhaled GCs might be better for dampening eosinophilic inflammation until RIT normalizes the dysregulated immune system.

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Introduction

Feline allergic asthma is most commonly treated with glucocorticoids (GCs) and bronchodilators, although neither addresses the underlying aberrant immune response. Furthermore, GCs are contraindicated with concurrent diseases such as diabetes mellitus. To minimize these systemic effects, inhaled GCs have been applied in asthmatic cats (Cohn et al., 2010; Leemans et al., 2012; Reinero et al., 2005). In place of palliative treatment, allergen-specific immunotherapy (ASIT) has been advocated with the potential to cure allergic asthma.

Using an experimental feline asthma model, we developed a protocol for an injectable abbreviated form of ASIT called rush immunotherapy (RIT) (Reinero et al., 2006b) and compared its safety and efficacy with an adjuvant (Reinero et al., 2008) and with mucosal RIT (Lee-Fowler et al., 2009b). These studies demonstrated promising results in dampening eosinophilic airway inflammation via alterations in allergen-specific immunoglobulins, induction of

hyporesponsive lymphocytes, and modulation of cytokine profiles. Recently we documented that RIT could blunt eosinophilic inflammation even when the allergen(s) used for RIT were unrelated to or were just a partial repertoire of allergen(s) implicated in sensitization (Reinero et al., 2011). These data are especially important in application of RIT in pet cats, because accurate allergen identification can be challenging due to limitations of allergen-specific IgE assays (Lee-Fowler et al., 2009a), concurrent drug therapy (Chang et al., 2011) or intermittent allergenic exposure.

A primary goal of asthma treatment is reduction of airway inflammation, since inflammation contributes to airway hyperresponsiveness and remodeling. In experimental feline asthma, RIT reduces inflammation over several months. Asthmatic pet cats will require concurrent GC therapy until RIT becomes effective. Consequently, determining the impact of GCs on RIT efficacy is prerequisite before RIT can be translated to asthmatic pet cats. The current investigation was designed to determine how the immunosuppressive effects of GCs will impact RIT by simulating the clinical situation where RIT is used with concurrent oral or inhaled GC treatment before weekly RIT becomes a sole treatment. Because inhaled GC has shown less systemic immunosuppressive effects than

* Corresponding author. Tel.: +1 573 882 7821.
  E-mail address: reineroc@missouri.edu (C.R. Reinero).

oral GC (Allen et al., 2003; Reinero et al., 2006a), we hypothesized that oral but not inhaled GC would diminish the efficacy of RIT in experimentally induced asthmatic cats.

Materials and methods

Animals

Eighteen purposely bred domestic cats (11 males, 7 females; 6 months to 1.5 years old) were used and cared for in accordance with the *NIH Guide for the Care and Use of Laboratory Animals* and with approval by the University of Missouri Animal Care and Use Committee (ACUC protocol number: 6912).

Induction of asthma

Naïve cats were sensitized with Bermuda grass allergen (BGA), as previously described (Reinero et al., 2011), receiving weekly BGA aerosol challenges until study completion. Briefly, on day 0, cats received a subcutaneous (SC) injection of 12 μg of BGA/10 mg of alum and 100 ng *Bordetella pertussis* toxin SC; on day 14, intranasal BGA (75 μg of BGA in 200 μL of PBS); and on day 21, 12 μg of BGA/10 mg alum SC. On day 28, sensitization was confirmed with a positive intradermal skin test (IDST). During the next 2 weeks, cats were challenged seven times with nebulized BGA (up to 500 μg, titrated to effect). Following the intensive aerosol challenges, the asthmatic phenotype was confirmed (defined as bronchoalveolar lavage fluid [BALF] % eosinophils >17%).

Treatments

As previously described (Lee-Fowler et al., 2009b), all cats received SC RIT for 9 months. For acute escalation of the allergen dose for RIT, cephalic intravenous (IV) catheters were placed and cats were monitored closely for allergic complications. Subsequently, weekly maintenance SC RIT injections (200 μg of BGA in 1 mL of PBS) were given to all cats. Cats were randomly divided into three groups of six to receive simultaneous treatment with oral GC (10 mg prednisolone daily), inhaled GC (220 μg fluticasone twice daily), or gelatin capsules as placebo for the first 6 months of the study.

Airway sampling

BAL samples were collected using a blind technique at month 6 (M6) and month 9 (M9) as previously described (Reinero et al., 2011). Cytocentrifugation was used to prepare a slide for staining with a modified Wright's stain. A 200 cell differential count was performed. Remaining BALF was centrifuged at 300 g for 10 min and the supernatant harvested and stored at −20 °C until analysis.

Serum BGA-specific IgE

Serum was harvested at M6 and M9 and banked at −20 °C until analysis. An enzyme-linked immunosorbent assay (ELISA) using polyclonal chicken anti-feline IgE antisera (developed and validated in a similar fashion to the previously published protocol using polyclonal rabbit antisera) was conducted to measure serum BGA-specific IgE. Pooled sera from experimentally induced asthmatic cats used in a different study with strong IDST reaction to BGA was used as a positive control; PBS and pooled sera from non-sensitized cats were used as negative controls. The following modifications to the original protocol were made, however, the times of incubation remained the same and all volumes were 100 μL unless noted. Washes were performed between each step. The 96-well plate after initial coating with 1 μg of BGA was blocked with 1% bovine serum albumin (BSA) in PBS–Tween 0.5%, and incubated with each of the following in a step-wise fashion: 1:5 diluted serum samples in duplicate (in PBS–Tween 0.5%/0.5% BSA), 1:15,000 diluted polyclonal chicken anti-feline IgE, 1:20,000 diluted biotinylated donkey anti-chicken antibody (Jackson ImmunoResearch Laboratories), 1:1000 diluted peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratories), and 200 μL of substrate (O-phenylenediamine dihydrochloride, Sigma) for 45 min. A spectrophotometer (SpectraMax Plus 384, Molecular Devices) was used to read the plate at a dual wavelength of 450–650 nm.

The optical density (OD) of each well was measured and the OD of background wells (PBS–Tween 0.5%) subtracted. To normalize the OD value between different plates and days, the mean OD of positive control from all plates run was divided by the OD of positive control of the plate and the number was multiplied by the OD value of each sample on that plate. Consequently, the results of serum BGA specific IgE were recorded as the percentage of a normalized positive control.

BALF interleukin-5

Banked BALF supernatants from M6 and M9 were used to measure the concentration of interleukin (IL)-5 using a commercially available ELISA kit (R&D systems) following the manufacturer's instructions. Samples were run neat and in duplicate. The range of detection was 0.0156–1 ng/mL.

Lymphocyte blastogenesis

Using density gradient centrifugation, peripheral blood mononuclear cells (PBMC) were isolated from whole blood in EDTA and resuspended in complete RPMI (cRPMI; 1640 with 10% FBS, 5 mL 1 M Hepes, 5 mL penicillin–streptomycin–glutamine, and 0.35 μL diluted beta-mercaptoethanol). To a 96-well plate (3595 Costar, Corning), 1×10^5 PBMC were added in triplicate with media alone or BGA (50 μg/mL). The plate was incubated at 37 °C with 5% CO_2 for 6 days (media replaced at 3 days). A commercially available kit for lymphocyte proliferation was used according to instruction (Cell Proliferation ELISA, Roche Diagnostic). The results are represented as a stimulation index (SI) for each cat: $SI_{BGA} = OD_{BGA}/OD$ media alone.

T regulatory cells

Whole blood in EDTA (100 μL) was added to 12×75 polystyrene tubes and red blood cells were lysed using ACK lysis buffer (8.26 g NH_4CL, 1 g $KHCO_3$, 0.037 g Na_2EDTA, in 1 L deionized distilled water, pH 7.2). Cell pellets were added to a 96-well plate (3799 Costar, Corning) and stained for surface markers (CD4 and CD25) and an intracellular marker (FoxP3) with appropriate isotype controls. In brief, anti-feline CD4 (clone 3-4F4, Southern Biotech) and anti-feline CD25 (clone 9F23, provided by Dr. W.A. Tompkins, North Carolina State University) were incubated with cells and FACS buffer (PBS with 3% FBS and 0.09% sodium azide) on ice for 30 min. Using the FoxP3/Transcription Factor Staining Buffer Set (eBioscience), cells were fixed and permeabilized on ice for 30 min and stained with FoxP3 (clone FJK-16, eBioscience) for 60 min.

After washing and resuspension in FACS buffer, the cells were analyzed with a Cyan ADP Flow Cytometer (Becton Dickenson). Lymphocytes were gated on a forward vs. side scatter plot and then on a CD4 vs. CD25 plot. The CD4+CD25+ T cells were then applied to a histogram of FoxP3+, so the CD4+CD25+FoxP3+ lymphocytes could be quantified. To determine % CD4+CD25+FoxP3+ T regulatory cells (Tregs), the quantified numbers were divided by the number of peripheral blood lymphocytes in the forward vs. side scatter gate.

IL-10 ELISPOT

An ELISPOT kit (Feline IL-10 Development Module, R&D Systems) was used according to the manufacturer's instructions with minor modifications. Diluted (1:120) feline IL-10 capture antibody was added at 100 μL to a 96-well filtration plate and incubated overnight at 4 °C. Blocking buffer (200 μL) was added after washing and the plate was incubated at room temperature for 2 h. Next, 5×10^4 PBMC in cRPMI were added in triplicate. Recombinant feline IL-10 and sterile media were used as positive and negative controls, respectively. The plate was incubated at 37 °C with 5% CO_2 for 2 days and 100 μL of diluted (1:120) detection antibody were added after washing with subsequent overnight incubation at 4 °C. After washing, ELISPOT Blue Color Module (R&D System) was added and the plate was rinsed with deionized water and dried. Spots in each well were quantitatively measured using a CTL Immunospot Analyzer (Cellular Technology).

Statistical analysis

A commercially available software program (SigmaStat, Systat Software) was used. Given the small sample size, non-parametric statistical tests were used. For each parameter (% BALF eosinophils, BGA-specific serum IgE, BALF IL-5, proliferating lymphocytes, % Tregs, IL-10 producing cells), Kruskal–Wallis one-way analysis of variance on Ranks was used to compare the data among groups at M6. Additionally, to compare the data over time between M6 and M9 in each group, a Wilcoxon signed rank test was performed. $P < 0.05$ was considered statistically significant.

Results

Percent BALF eosinophils

At M6 of RIT while still receiving concurrent oral or inhaled GC or placebo, similar suppression of eosinophilic airway inflammation was shown in all groups (oral GC 0–14%; inhaled GC 3–29%; placebo 1–12%; Table 1) with no significant difference between groups ($P = 0.291$; Fig. 1). However, after discontinuing oral or inhaled medications, a significant increase in % BALF eosinophils over