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Inhibition of allergen-induced pulmonary inflammation by the tripeptide feG: a mimetic of a neuro-endocrine pathway

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Interactions between the neuro-endocrine system and immune system help maintain health. One interaction involves the superior cervical ganglia (SCG), which regulate the prohormone submandibular rat 1 (SMR1) produced by the submandibular gland (SMG). A peptide derived from SMR1, feG, has anti-inflammatory activity, and modification to D-isomer feG enhances bioactivity. We tested feG as a therapeutic agent for airways inflammation, using rats sensitized by OVA or *Nippostrongylus brasiliensis* (Nb). Treatment with feG but not fdG down-regulated OVA-challenge-induced increases in bronchoalveolar lavage (BAL)-derived macrophages, eosinophils and PMN (neutrophils) by 44%, 69% and 67%, respectively, at 24 h. We found that feG also reduced ICAM-1 on BAL-derived macrophages and eosinophils by 27% and 65%, and L-selectin on PMN by 55% following OVA challenge. Furthermore, feG but not fdG reduced the OVA-induced TNF increase in BAL fluid. We showed that feG also down-regulated both hyper-responsiveness to methacholine (by 27%) and microgranuloma formation in the lung parenchyma. In Nb-challenged rats, feG treatment inhibited *ex vivo* allergen-induced contraction of tracheal smooth muscle by up to 73%. In conclusion, feG, which is a mimetic of a peptide derived from a rat salivary gland prohormone, has anti-inflammatory properties in allergic airways inflammation in Brown-Norway rats. The role of the SCG-SMG neuro-endocrine pathway in allergic asthma and other inflammatory diseases requires additional study.

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1 Introduction

There is evidence that the immune, nervous and endocrine systems are linked and that their interactions play important roles in maintaining health [1]. One such interaction involves the axis between the cervical

sympathetic trunk (CST) and the submandibular gland (SMG) (i.e. the CST–SMG axis). It has long been known that SMG contain immunosuppressive and anti-inflammatory agents [2], and are innervated by CST nerves that originate in the superior cervical ganglia (SCG) or elsewhere. Our initial rat studies on neuro-endocrine interactions showed that severing the link between the SMG and the nervous system, by decentralization or ganglionectomy of the SCG, decreased allergen-induced anaphylaxis and pulmonary inflammation [3]. We showed that these protective effects were mediated by the SMG since sialadenectomy with decentralization ablated the protective effect of decentralization of the SCG [4]. We postulated that the protective effects of decentralization or ganglionectomy were due to removal of sympathetic

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Abbreviations: Ach: Acetylcholine **BAL:** Bronchoalveolar lavage **BALF:** BAL fluid **BN:** Brown-Norway **Eo:** Eosinophil **Ly:** Lymphocyte **Nb:** *Nippostrongylus brasiliensis* **PC₂₀₀:** Provocative concentration 200% **SCG:** Superior cervical ganglia **SD:** Sprague-Dawley **SMG:** Submandibular gland **WE:** Worm-equivalent

inhibitory tone exerted by the SCG on the SMG, leading to increased secretion of SMG-derived mediators that could modulate immune and inflammatory responses [5]. We discovered two biologically active peptides derived from the SMG prohormone submandibular rat 1 (SMR1) [6, 7].

One of these peptides is a seven-amino-acid (aa) fragment of SMR1 that we called SMG peptide-T (SGP-T), on the basis of its SMG origin and its C-terminal threonine (aa 138–144: TDIFEGG). SGP-T occurs naturally in rat SMG and has protective effects against endotoxic shock, allergen-induced intestinal anaphylaxis and disruption of gut-migrating myoelectric complexes [7, 8]. We discovered that the tripeptide FEG, derived from SGP-T (aa 141–143) also has biological activity [9]. FEG has similar effects as SGP-T, but with increased potency. Modification of the first two aa of FEG to the D-enantiomer, feG, conferred oral bioactivity in the inhibition of intestinal anaphylactic reactions [9], and increased potency in an in vitro gut smooth-muscle contractility assay [10]. Furthermore, modification of the second aa from glutamic acid to aspartic acid (fdG) ablated bioactivity of the molecule in this assay [10].

To determine if the anti-anaphylactic activities of feG extend beyond anaphylaxis and the gastrointestinal tract, we explored the effects of feG on airways inflammation and responsiveness in an animal model of allergic asthma. Our study shows that feG but not fdG had significant beneficial effects on several antigen-induced inflammatory responses in rats, including airway hyper-responsiveness, smooth-muscle contraction and adhesion-molecule expression.

2 Results

2.1 Inflammation

OVA challenge of sensitized Brown-Norway (BN) rats induced wheezing in 79% (90 out of 114) of animals. In saline-challenged animals, wheeze was never detected (0 out of 27). The lungs of normal or sensitized/saline-challenged BN rats were subjected to bronchoalveolar lavage (BAL) at 24 h and total cells were counted ($3.9 \pm 0.5 \times 10^6$, $n=31$) (Fig. 1A). Cells derived from BAL of sensitized, saline-challenged rats comprised 90% MΦ, 5% lymphocytes (Ly), 3% PMN (i.e. neutrophils), and 2% eosinophils (Eo) (Fig. 1B, C, D, E). When OVA-sensitized BN rats were challenged with OVA, a 7-fold increase in total BAL cell numbers was observed by 24 h post-allergen challenge ($22.5 \pm 2.1 \times 10^6$, $n=55$) (Fig. 1A). The increases for specific cells were 2.5-fold for MΦ, 6-fold for Ly, 79-fold for PMN, and 54-fold for Eo.

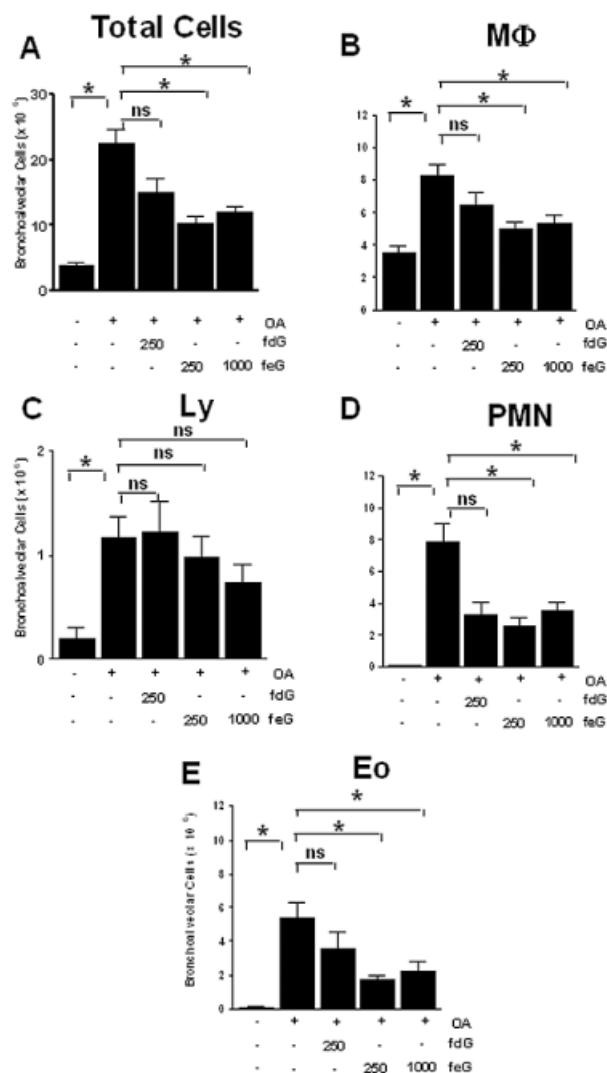


Fig. 1. Effect of oral feG treatment following OVA challenge on inflammatory-cell recruitment into the lungs of sensitized BN rats. The peptide feG (250 or 1000 µg/kg) was administered orally 30 min following OVA challenge (5%, 5 min); OVA is labeled “OA” here. The control peptide fdG was used at 250 µg/kg. (A) Total cell numbers were determined following BAL. From total cell numbers, the proportion of (B) MΦ, (C) Ly, (D) PMN and (E) Eo present in each BAL sample was determined by cytocentrifuge preparations stained with May-Grunwald and Giemsa. Statistical significance was determined using one-way ANOVA followed by Student’s *t*-test (* $p < 0.05$; ns, not significant) ($n=12$ –55). Normal animals $n=31$, OVA-challenged animals $n=55$, fdG control $n=12$, feG 250 µg/kg $n=28$ and feG 1000 µg/kg $n=12$. Error bars represent the SEM.

When feG (250 µg/kg or 1000 µg/kg) was administered orally 30 min following OVA challenge, the cell number derived from BAL, 24 h post challenge, was reduced by

55% and 52%, compared with OVA challenge without feG treatment (Fig. 1A). By contrast, and as found in other assay systems, the control peptide fdG did not significantly affect inflammatory-cell recruitment as measured following BAL (Fig. 1A–E). Sham controls were used in subsequent experiments.

Treatment with feG at 30 min (250 or 1000 $\mu\text{g/kg}$) following OVA challenge reduced BAL-derived M Φ by 40%, or 44% (Fig. 1B), Eo by 69%, or 59% (Fig. 1E), and PMN by 67%, or 56% (Fig. 1D), respectively. The feG did not significantly affect Ly numbers in the airways following OVA challenge (Fig. 1C). This inactivity of feG on Ly is consistent with our previous work showing that decentralization or ganglionectomy of the SCG had no effect on Ly recruitment to the lungs [4].

2.2 Lung histology

As described previously [11], we found a small number of foci of microgranulomatous inflammation in the normal BN lung. These foci were bronchocentric and composed mainly of histiocytes and Eo. Edema and hemorrhage were not evident in the unchallenged animals (Fig. 2B).

Twenty-four hours following OVA challenge (Fig. 2C and D) ($n=3$), a marked increase in interstitial, alveolar and perivascular inflammation was observed compared with lungs of unchallenged animals (Fig. 2A and B). Furthermore, edema, focal parenchymal hemorrhage and microgranulomata numbers were increased throughout the tissue as compared with normal lungs (Fig. 2A and B). These microgranulomata were larger than in normal rats,

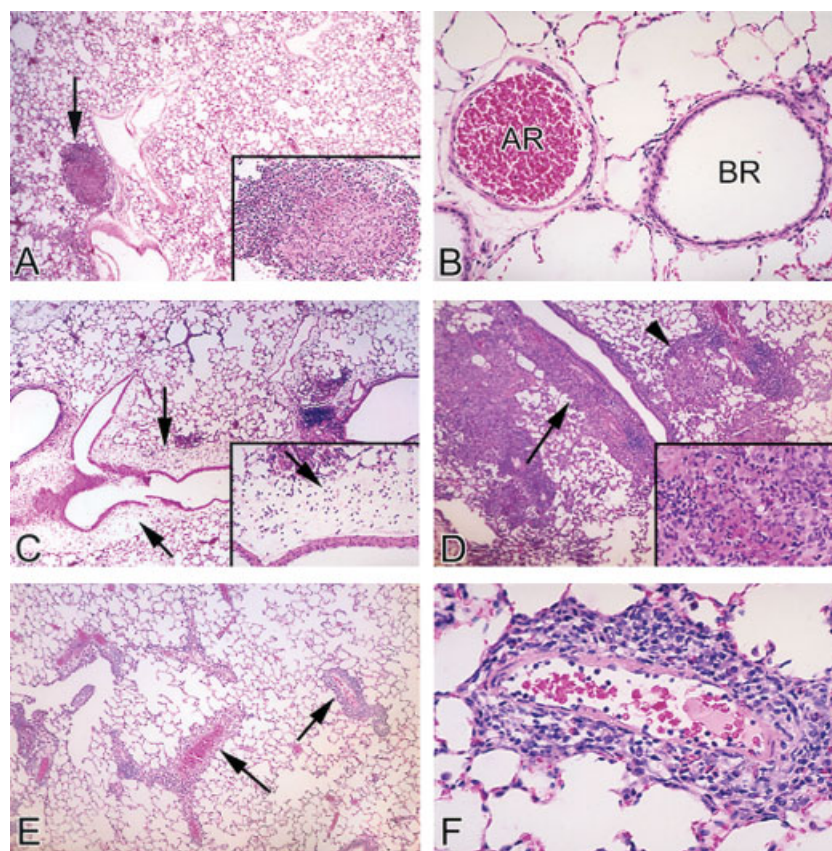


Fig. 2. Formalin-fixed, paraffin-embedded and H&E-stained BN rat lung sections from normal rats (A and B), OVA-challenged rats (C and D), and rats given feG orally (1000 $\mu\text{g/kg}$) then challenged with OVA (E and F). In normal rats, sparse bronchocentric microgranulomata can be seen (panel A, arrow and inset). Lung sections are otherwise free of inflammation and edema (B), with artery (AR) and bronchiole (BR) visible. In OVA-challenged rats, severe edema (panel C, arrow and inset), and widespread perivascular and peribronchial inflammation can be seen (panel D, arrow). Microgranulomatous inflammation was increased (panel D, arrowhead and inset). Microgranulomata were composed of histiocytes, PMN and Eo. In feG-treated rats, inflammation was perivascular (panel E, arrows), but inflammatory cells were still present in large numbers within this “cuff” (panel F). Pictures shown are representative of two separate experiments performed.

and involved alveolar spaces, resulting in a patchy inflammation (Fig. 2D). Treatment with feG (1000 $\mu\text{g/kg}$) ($n=3$) 30 min prior to OVA challenge appeared to diminish the magnitude of this patchy inflammation and parenchymal hemorrhage (Fig. 2E). Perivascular inflammation was present in OVA-challenged animals not given feG treatment (Fig. 2C). Histological changes observed in the OVA-challenged groups (feG-treated and -untreated) were more severe than in the unchallenged control group. However, OVA-challenged but untreated animals could not be reliably separated from the OVA-challenged feG-treated group on the basis of histomorphology alone.

2.3 Measurement of airway hyper-responsiveness

In sensitized BN rats receiving sham feG treatment and saline challenge ("normals"), the mean PC_{200} (provocative concentration 200%) was 12.0 ± 1.1 mg/ml ($n=7$) (Fig. 3). Animals receiving sham feG treatment and OVA challenge had a mean PC_{200} of 8.6 ± 0.7 mg/ml ($n=24$). Treatment with feG (1000 $\mu\text{g/kg}$) of rats 30 min prior to OVA challenge significantly increased the amount of methacholine required to induce PC_{200} to 11.7 ± 0.8 mg/ml ($n=8$), which was not significantly different from normal animals' PC_{200} . Treatment with feG alone had no effect on the response to methacholine (15.2 ± 2.0 mg/ml) ($n=9$) compared to saline-challenged animals.

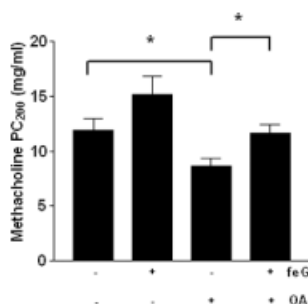


Fig. 3. Effect of oral feG on OVA-induced airway hyper-responsiveness to inhaled methacholine in BN rats. Sensitized rats were treated orally with feG (1000 $\mu\text{g/kg}$) or saline (0.9%), 30 min prior to OVA challenge (5%, 5 min); OVA is labeled "OA" here. Twenty-four hours following OVA challenge, a baseline airway-resistance value was established using 0.9% saline challenge (30 s). The animal was then challenged with sequential doubling concentrations of methacholine (0.5–32.0 mg/ml), allowing a recuperation period between each dose. The 200% increase in airway resistance (PC_{200}) was determined using linear regression. Statistical significance between each treatment was determined using the Kruskal Wallis rank sum test followed by Dunn's multiple comparison post test ($*p < 0.05$). Normal animals $n=7$, OVA-challenged animals $n=24$, feG treatments $n=9$ –21. Error bars represent the SEM.

2.4 Smooth-muscle contraction

To ensure that tissues were healthy, only tracheas that were responsive to acetylcholine (ACh) were used in these experiments. Under the conditions tested, tracheas from BN rats were unresponsive to intraluminally administered OVA. Therefore, to test the effects of feG on tracheal smooth-muscle responsiveness to allergen, we used *Nippostrongylus brasiliensis* (Nb)-infected Sprague-Dawley (SD) rats [12]. Allergen challenge of rats sensitized with Nb antigen yields IgE-mediated inflammatory responses in the lung [13], which are similar to the responses obtained with OVA sensitization. Tracheas from unsensitized SD rats were responsive to ACh, but unresponsive to lumenally administered Ag (Fig. 4). Tracheas from sensitized rats responded to both ACh as well as lumenally administered Ag at 0.1, 1 and 10 worm-equivalents (WE)/ml by contracting to 5%, 25%, and 34% of maximal ACh contraction, respectively (Fig. 4). When feG (1000 $\mu\text{g/kg}$) was administered 30 min before the trachea was removed from the animal, there was a 73% and 52% inhibition of smooth-muscle contraction to Ag at 1 and 10 WE/ml respectively, as compared with Nb-challenged rats not receiving feG. Interestingly, if feG was administered to the tracheas in

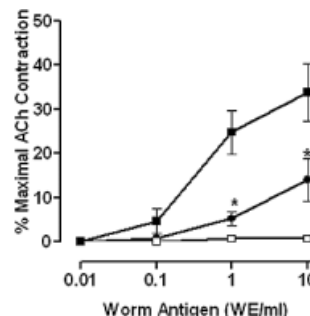


Fig. 4. Effect of oral feG on Nb-induced tracheal smooth-muscle contraction in SD rats. Here, feG (1000 $\mu\text{g/kg}$) was administered orally 30 min prior to euthanasia. The trachea was removed immediately following euthanasia and connected to an organ bath. Following appropriate washout and equilibration periods, an ACh dose-response curve was performed to obtain the maximal contraction value. Following additional washout, cumulative doses of Nb Ag (0.01, 0.1, 1, 10 WE/ml) were administered sequentially following the maximal contraction of the previous dose. Ag-induced contraction is expressed as percent of maximal contraction induced by ACh. Open squares: Ag-challenged trachea from unsensitized rats. Closed squares: Ag-challenged trachea from sensitized rats. Closed circles: Ag-challenged trachea from feG-treated (1000 $\mu\text{g/kg}$ orally, *in vivo*), sensitized rats. Statistical significance was determined using one-way ANOVA followed by Student's *t*-test ($*p > 0.05$) ($n=4$ –6). Error bars represent the SEM.

vitro 30 min prior to Nb challenge, smooth-muscle contraction was not inhibited (not shown).

2.5 TNF in levels in BAL fluid

TNF levels in BAL fluid (BALF) of normal BN rats were 121 ± 20 pg/ml ($n=31$) (Fig. 5). Twenty-four hours following challenge with OVA, TNF levels increased to 418 ± 53 pg/ml ($n=23$). Oral administration of feG (250 or 1000 μ g/kg), 30 min following OVA challenge, significantly reduced TNF levels to 233 ± 47 pg/ml ($n=12$) and 165 ± 21 pg/ml ($n=9$), respectively. Administration of fdG (250 μ g/kg) 30 min following OVA challenge did not significantly alter BALF TNF levels (274 ± 68 pg/ml) ($n=7$).

2.6 Flow cytometry

There was no significant effect of feG on VCAM-1 (CD49d) expression in M Φ , PMN or Eo following OVA challenge (Fig. 6A, B and C). Twenty-four hours following challenge, ICAM-1 (CD54) expression on M Φ and Eo increased to 79% and 68% respectively compared with normal rats (21% and 24%) (Fig. 6D, F and K). Treatment with feG (250 or 1000 μ g/kg) 30 min after Ag challenge significantly down-regulated the number of CD54⁺ M Φ (56% or 58%) and Eo (34% or 24%) (Fig. 6D, F and K), but had no significant effect on the number of CD54⁺ PMN (Fig. 6E).

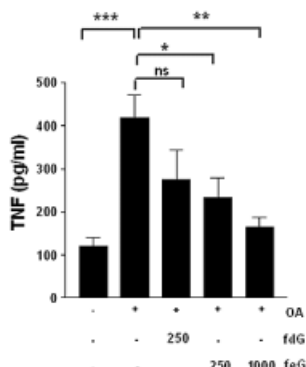


Fig. 5. Effect of feG on TNF protein levels in BALF of BN rats. Here, feG (250 μ g/kg or 1000 μ g/kg) or fdG (250 μ g/kg) was administered orally 30 min OVA challenge (5%, 5 min); OVA is labeled "OA" here. The lung was washed once with 5 ml PBS, and BALF was depleted of cells by centrifugation at $150 \times g$ for 10 min and analyzed for TNF content by ELISA. Statistical significance was determined using one-way ANOVA followed by Student's *t*-test ($p < 0.05$, $**p < 0.01$, $***p < 0.001$). Normal animals $n=31$, OVA-challenged animals $n=23$, feG treatments $n=9$ –12 and fdG treatment $n=7$. Error bars represent the SEM.

Twenty-four hours following OVA challenge, the proportion of BAL-derived PMN with surface expression of L-selectin (CD62L) was significantly decreased (47%), compared with unchallenged animals (77%) (Fig. 6H and J). Interestingly, oral feG treatment (1000 μ g/kg) 30 min after Ag challenge significantly decreased (by 56%) PMN surface expression of CD62L as compared with OVA-challenged BN rats receiving saline treatment. (Fig. 6H and J). Neither OVA nor feG had any significant effect on CD62L expression on M Φ or Eo (Fig. 6G and I).

3 Discussion

We showed that feG had several beneficial effects on allergic airways inflammation. These effects may be linked to integrins and selectins involved in leukocyte transmigration from the endothelium to the airways. BAL-derived M Φ can express higher levels of integrins than their peripheral blood counterparts do [14]. Moreover, BAL-derived Eo from asthmatic patients have elevated levels of integrins compared with blood Eo from the same patients [15]. The increased expression of adhesion molecules on the surface of BAL-derived leukocytes may be important in cell communication and activation, and account for functional differences between BAL-derived and peripheral blood cells. Entry of PMN into inflamed tissue requires L- and P-selectin activity [16], and increased CD62L shedding has been suggested to have inhibitory effects on PMN adhesion and transmigration across endothelium, thus reducing PMN recruitment [17]. Thus, down-regulation of CD62L on PMN by feG may have a role in reducing PMN numbers in the lungs of OVA-challenged rats. Because PMN depend heavily on CD62L for rolling, feG-enhanced shedding of this molecule may prevent them from establishing firm adhesion with the endothelium, leading to decreased recruitment.

Other studies have suggested that airway PMN recruitment occurs via ICAM-1–selectin interactions [18, 19]. Our data however, show no inhibition of ICAM-1 expression on PMN by feG (Fig. 6E). Furthermore, feG had no effect on CD18 ($\beta 2$ integrin) surface expression on PMN (data not shown); CD18 is an important adhesion molecule in PMN recruitment in Fc-receptor-mediated lung injury [20].

Although Eo and M Φ also require selectins for transmigration across endothelium, recruitment of these cells was inhibited by feG administered either pre or post OVA challenge. A recent study showed that feG blocks CD49d-mediated leukocyte adhesion in the heart [21]. Our data suggest that, in contrast to PMN, Eo and M Φ rely on ICAM-1 for recruitment to the airways. For example, ICAM-1 is critically important for the Ag-

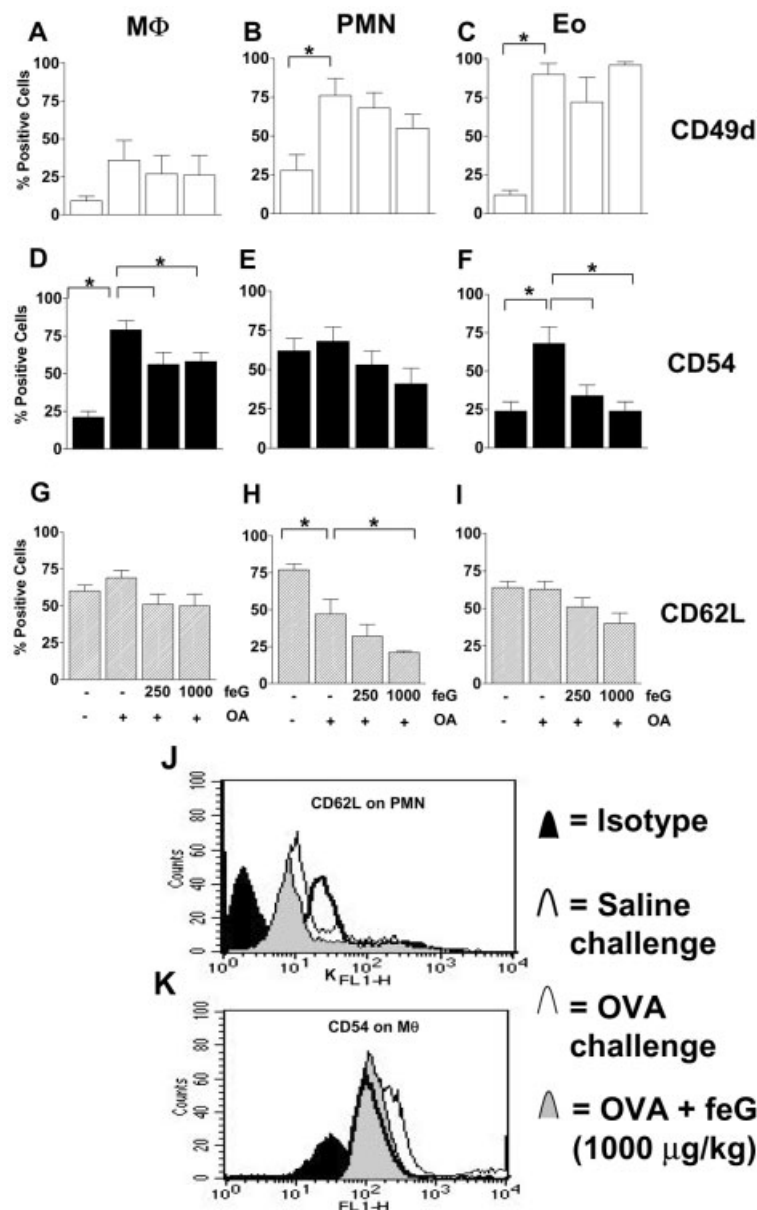


Fig. 6. Effect of feG (1000 µg/kg) on surface expression of adhesion molecules in MΦ, PMN and Eo in BN rats. Cells were labeled with FITC-conjugated antibodies to CD49d (open bars), ICAM-1 (closed bars) or CD62L (hatched bars). For each antibody, the percentage of positive MΦ (A, D, G), PMN (B, E, H), and Eo (C, F, I) was determined. A representative histogram is shown for fluorescence intensity versus cell number for CD62L on PMN (J) and CD54 on MΦ (K). The fluorescence intensity for CD54 expression on Eo is not shown because it showed a similar trend as in MΦ. Statistical significance was determined using one-way ANOVA followed by Student's *t*-test (**p* < 0.05) (*n* = 3–11). Error bars represent the SEM.

specific recruitment of Eo, but not PMN, to the lung [22]. Blocking ICAM-1 in mice following allergen challenge reduces infiltration of MΦ and Eo into the airways by 50% and 70%, respectively [23]. We have shown that feG reduces ICAM-1 expression on both Eo and MΦ, which may help explain the reduced numbers of these cells in lungs of feG-treated animals.

Histological analysis showed that alveolar and interstitial inflammation was reduced by feG in OVA-challenged animals, but perivascular inflammation was similar to that observed in OVA-challenged animals not receiving feG (Fig. 2C). This resulted in a clearly observable “cuffing” effect of leukocytes around blood vessels in feG-treated animals (Fig. 2E and F). From this, we hypothesize that

leukocytes in feG-treated animals migrate through the endothelium upon antigen challenge of the animal, but that their progress past this point is impaired in some way. Studies have shown that in addition to β_1 and β_2 integrins, leukocytes employ integrins such as $\alpha_v\beta_3$ or $\alpha_4\beta_7$, which bind extracellular matrix (ECM) proteins, to move through the ECM toward the airways [24, 25]. Thus feG may down-regulate expression of ECM-binding integrins on leukocytes, but this hypothesis still remains unexplored.

In BN rats, OVA challenge induces early bronchoconstriction and subsequently inflammation and airway hyper-responsiveness similar to that seen in human asthma [26]. These animals develop significant airway hyper-responsiveness to inhaled methacholine in response to OVA challenge. Hyper-responsiveness has been associated with increased BAL-derived CD4⁺ T cell numbers [27], increased inflammatory-cell influx [28], and increased Eo-associated IL-5 levels in BALF [29]. ICAM-1 is important in generation of hyper-responsiveness, which can be attenuated by treatment with anti-ICAM-1 antibody [30]. Reduced ICAM-1 expression on Eo (Fig. 6F) may be involved in the restoration of baseline hyper-responsiveness observed in feG-treated animals (Fig. 3).

The inhibition of Ag-induced smooth-muscle contraction by feG *in vivo* was not observed when feG was administered *in vitro* at equivalent doses (not shown). One possible explanation for the differences between *in vivo* and *in vitro* treatment is that feG triggers an inhibitory nonadrenergic-noncholinergic (i-NANC) neurogenic feedback loop *in vivo*, acting indirectly by triggering NO release which relaxes the smooth muscle as previously reported [31]. Removal of the trachea from the animal prior to feG treatment may preclude this NO activity, but this hypothesis remains to be investigated.

The down-regulation of TNF levels in BALF may be a key mechanism of action of feG, reducing smooth-muscle hyper-responsiveness and cell recruitment following OVA challenge. High levels of TNF are known to promote leukocyte adhesion and transmigration by up-regulating ICAM-1 and VCAM-1 on pulmonary microvascular endothelium [32]. Reduction of TNF levels by feG (Fig. 5) suggests that this tripeptide modulates NF- κ B activity, a well-known activator of TNF, IL-5 and eotaxin production, but this remains to be investigated.

Since feG is a derivative of the naturally occurring FEG peptide found in the SMR1 prohormone, the relationship between the nervous system, the SMG and the immune system, and the regulation of systemic inflammatory responses merits further investigation. Whether this axis

is compromised in individuals with asthma or other inflammatory conditions is an interesting postulate that should be tested. Further studies on potential targets and mechanism of action of feG are required to strengthen the potential use of feG as a therapeutic agent for allergic asthma.

4 Materials and methods

4.1 Animals

Male BN rats of the Ssn substrain (225 to 250 g) were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN, USA) and maintained in filter-top cages behind a virus-antibody-free barrier at the animal facility of the University of Alberta. They were kept on a 12 h light/dark (0700–1900) cycle, and food and water were provided *ad libitum*. Male SD rats (250 to 300 g) were obtained from Charles River (St-Constant, Quebec, Canada) and were housed in a separate room in the same facility as BN rats. This work was approved by the University of Alberta animal ethics committee in accordance with guidelines of the Canadian Council for Animal Care.

4.2 Sensitization

4.2.1 OVA

For OVA-sensitization, we tested several different protocols and selected the optimal protocol based upon IgE antibody titer using passive cutaneous anaphylaxis. Briefly, BN rats were sensitized with a 1-ml i.p. injection of 0.9% saline containing 10 μ g OVA (Sigma, St-Louis, MO, USA), 150 mg water-soluble Al(OH)₃ (ICN, Aurora OH, USA) and 50 ng purified Bordetella pertussis toxin (Sigma).

4.2.2 *Nippostrongylus brasiliensis*

Sensitization of SD rats to Nb was done by a subcutaneous injection of 3000 L3 larvae in the nape of the neck as previously described, and Nb antigen in WE/ml was prepared as previously described [13].

4.3 OVA challenge

Twenty-one days following sensitization, BN rats were challenged with a 5 min, 5% OVA aerosol in 0.9% sterile, endotoxin-free saline, using a Hudson 880 micromist nebulizer (Hudson RCI, Temecula, CA, USA). One to four rats at a time were challenged in a cage identical to their housing cage, but with a small hole in the side to introduce the aerosol. A positive response to OVA challenge was assessed by an audible wheeze in the upper airways, which

seems to correlate with onset of allergic inflammation in the BN rat (Table 1). As compared to saline-challenged controls, rats that did not wheeze following OVA challenge developed no significant inflammation in their airways 24 h following challenge, whereas rats that did wheeze exhibited significant inflammation (Table 1). Thus, subsequently, wheezing was used as an indicator of a positive response to OVA challenge, and only animals that wheezed following OVA challenge were used in our study. The proportion of OVA-challenged rats that wheezed was 79% (90 out of 114). Control rats were either left unchallenged, or challenged with a 5-min aerosol of 0.9% sterile endotoxin-free saline. Saline-challenged rats never wheezed following challenge (0 out of 27). Saline was tested regularly for endotoxin by the E-Toxate endotoxin detection kit (Sigma) to ensure LPS-free conditions.

4.4 feG administration

The feG that we used was synthesized and its composition verified by amino-acid analysis at the Core Laboratories, Queens University, (Kingston, Ontario Canada). Preliminary experiments (not shown) indicated that feG gave similar results at 1, 10, 250 or 1000 µg/kg but was reproducibility better with the two highest doses administered. Thus, either 250 or 1000 µg/kg of feG or 250 µg/kg fdG control in 0.9% sterile, endotoxin-free saline (500 µl total volume) was administered by gavage via a 15 cm PE 160 tube (Fisher Scientific, Nepean ON, Canada), attached to a 1 ml syringe with a 18G 1½" needle. In the same fashion, control animals received oral administration of equivalent volumes of 0.9% sterile, endotoxin-free saline. The fdG peptide was administered in the same fashion at a single dose of 250 µg/kg.

4.5 Inflammation

Twenty-one days post-sensitization, animals were placed under light anesthesia for feG administration by i.p. injection of 0.1 ml xylazine (20 mg/ml) and 0.1 ml ketamine (100 mg/ml). Animals were either left unchallenged, or challenged with

OVA or 0.9% saline as described above. Lung inflammation was assessed after BAL 24 h following challenge, as previously described [13].

Differential staining for MΦ, Ly, PMN and Eo was then performed on 5000 cells/sample, prepared by cytocentrifugation on a Shandon Cytospin 2 (Fisher Scientific) at 40×g for 2 min in PBS containing 20% FCS. Slides were air-dried overnight, stained with Protocol Hema 3 solutions (Fisher Scientific), dried and mounted with a coverslip using 20 µl Permount (Fisher Scientific). Differential cell counts were performed in a blinded fashion by counting 200 cells on each slide, and the total number of each BAL-derived cell type was determined from cytospin counts and total cell number in BAL-derived samples. Statistical significance was determined by one-way ANOVA followed by Student's t-test (significance level $p < 0.05$).

4.6 Measurement of airway hyper-responsiveness

Twenty-one days post-sensitization, rats were given either oral feG treatment (1000 µg/kg) or sham (saline) treatment under light anesthesia as described above, and then challenged with OVA (5%, 5 min). Twenty-four hours following challenge, rats were anesthetized with i.p. injection of ethyl carbamate (Sigma) (1.5 mg/g body weight), and airway hyper-responsiveness to methacholine was determined as previously described [26].

Commercially available software (RHT infoDat Inc. Montreal, PQ, Canada) was used to obtain airway resistance (R_{aw}) and lung elastance (E_l). Throughout the experiment, the aerosol chamber was ventilated with a 2 l/min : 1 l/min ratio of laboratory air : O₂ except during the challenge phase of the experiment. Challenge consisted of sequential 30-s challenges with doubling doses of methacholine from 0.5 mg/ml to 32 mg/ml (Sigma) using a Hudson 880 Micromist nebulizer (Hudson RCI) with an airflow of 8 l/min into the aerosol chamber. R_{aw} measurements were taken every minute between each dose of methacholine, until a peak R_{aw} value

Table 1. Correlation between wheeze and BAL-derived inflammatory-cell infiltrate following OVA challenge^{a)}

Cells	Saline challenge	OVA challenge	
		Wheeze	No wheeze
MΦ ($\times 10^6$)	4.6±0.6	7.9±0.6*	2.8±0.7 ^{ns}
Ly ($\times 10^6$)	0.2±0.1	1.2±0.2*	0.2±0.1 ^{ns}
PMN ($\times 10^6$)	0.03±0.03	6.0±0.9*	0.7±0.7 ^{ns}
Eo ($\times 10^6$)	0.02±0.01	5.2±0.8*	1.1±1.0 ^{ns}
Total cells ($\times 10^6$)	4.9±0.5	20.3±1.8***	4.8±1.5 ^{ns}

^{a)} Wheeze was tested 5 min following challenge with OVA or saline controls; it was found that the latter animals never wheezed. Inflammatory-cell infiltrates were measured after 24 h ($n=5-25$). Compared with saline challenge: * $p < 0.05$; *** $p < 0.001$; ^{ns} not significant.

was obtained for each dose. A dose-response regression curve using these peak R_{aw} values was constructed, and the methacholine value that gave a 200% increase in R_{aw} was extrapolated from the regression line and termed the PC_{200} . Statistical analysis was performed using the non-parametric Kruskal Wallis rank sum test followed by Dunn's multiple comparison post test (significance level $p < 0.05$).

4.7 Tracheal smooth-muscle contraction

To test whether feG would affect Ag-induced isometric smooth-muscle contraction, both BN rats sensitized to OVA and SD rats sensitized to Nb were used. Briefly, 30 min following oral feG treatment (1000 $\mu\text{g/kg}$), animals were anesthetized with xylazine/ketamine cocktail as described above, and a section of trachea consisting of 13 cartilaginous rings was dissected free of connective tissue, maintaining constant contact with 37°C Krebs's buffer (118.1 mM/l NaCl, 4.7 mM/l KCl, 2.5 mM/l CaCl_2 , 1.2 mM/l MgSO_4 , 25.0 mM/l NaHCO_3 , 1.2 mM/l KH_2PO_4 , 8.3 mM/l glucose). For perfusion, of tracheal tissue, the trachea was tied to fixed points in the organ bath, thus completing a Krebs's perfusion loop.

To measure isometric smooth-muscle contraction, two small hooks were attached longitudinally to the trachea across two cartilaginous rings. One hook was affixed to the bottom of the bath, and the other to a strain gauge connected to a signal amplifier and computer for data acquisition as previously described [33]. Tracheas were perfused with Krebs's buffer for 90 min to allow acclimatization, and then sequential doses of the cholinergic agonist Ach ($-\log M$: 8.0–2.5) were added to the luminal space without washout to generate a cumulative standard curve of smooth-muscle contraction, which we used to standardize our Ag-induced contraction in the same trachea. A washout phase of 45 min was then performed, allowing the tracheas to return to baseline before addition of Ag. Either OVA (0.01 to 10 mg/ml) or Nb Ag (0.1, 1 and 10 WE/ml) were introduced into the luminal space in a cumulative fashion (no washout). The Ag used depended on the sensitization (either OVA or Nb rats). Smooth-muscle contractions were measured and converted to percentage of maximal contraction induced by Ach. Statistical significance was assessed using one-way ANOVA followed by Student's *t*-test (significance level $p < 0.05$).

4.8 Lung histology

Twenty-one days following sensitization, animals were treated with feG (1000 $\mu\text{g/kg}$) and challenged with OVA as described above. Animals were then euthanized with a 1:1 mixture of ketamine (100 mg/ml) and xylazine (20 mg/ml), and whole lungs were inflated with 5 ml of 10% formalin (Fisher Scientific). The trachea was ligated to prevent formalin from escaping; lungs were excised by midline sternotomy and placed intact in 10% formalin. Tissue

preparation, staining and analysis were performed by the University of Alberta Hospital's Department of Laboratory Medicine and Pathology. Briefly, whole lungs were processed, paraffin-embedded, and 4- μm coronal sections were taken. Sections were stained with hematoxylin and eosin (H&E) or Giemsa stains. Sections from two separate experiments, 6 months apart were analyzed by a lung pathologist who was blinded to all treatment information. For each lung section, alveolar and interstitial edema, hemorrhage, vascular congestion, and inflammation were assessed. The inflammation was also assessed for location (airway lumen, airway wall, alveolar, interstitial and perivascular) and cell type.

4.9 TNF levels in BALF

For each animal, a 5-ml washing with PBS was performed, and recovered fluid was depleted of cells by centrifugation at $150 \times g$ for 10 min. Cell-free BALF was frozen at -70°C until assay. TNF levels were assessed by ELISA (Biosource International, Camarillo, CA, USA).

4.10 Immunofluorescent staining and flow cytometry

Cells recovered from BAL were washed in PBS and spun at $200 \times g$ for 5 min. Supernatant was discarded and cells were resuspended in 5% formalin (Fisher Scientific) and incubated for 5 min at room temperature, shaking several times during the incubation. Ice-cold 0.1% BSA (Sigma) in PBS (10 ml) was added and tubes inverted once. Cells were then spun at $250 \times g$ for 10 min and resuspended in PBS with 5% skimmed milk and 0.1% BSA (blocking solution) and incubated in darkness overnight at 4°C . Following overnight incubation, 2 ml PBS was added and cells spun at $200 \times g$ for 5 min, resuspended in 500–1000 μl of blocking solution and incubated on ice in darkness for 15 min with Rat Fc block® (BD Pharmingen, Mississauga, ON, Canada) diluted 1:200. Antibody preparations were made by adding 5–10 μl of the desired FITC-labeled antibody to a 12×75 mm polystyrene tube (Falcon/VWR/Canlab, Mississauga, Canada).

Cells (100 μl) were added to the tube containing the antibody and incubated for 1 h on ice in darkness. Following incubation, excess antibody was washed off with 1 ml of PBS and cells were resuspended in 200 μl PBS. All antibodies were run in parallel with their isotype control to assess nonspecific binding. Analysis of the different cell types following BAL was performed as previously described [34]. FITC-labeled anti-CD49d (clone Mr α 4-1), anti-CD54 (clone 1A29), and anti-CD62L (clone HRL1) antibodies for flow cytometry were purchased from BD Biosciences (BD Pharmingen). FITC-labeled isotype controls were purchased from BD Biosciences. Statistical analysis was determined using one-way ANOVA followed by Student's *t*-test (significance level $p < 0.05$).

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