

Research article

The tripeptide feG ameliorates systemic inflammatory responses to rat intestinal anaphylaxis

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Abstract

Background: Food allergies are generally associated with gastrointestinal upset, but in many patients systemic reactions occur. However, the systemic effects of food allergies are poorly understood in experimental animals, which also offer the opportunity to explore the actions of anti-allergic drugs. The tripeptide D-phenylalanine-D-glutamate-Glycine (feG), which potentially alleviates the symptoms of systemic anaphylactic reactions, was tested to determine if it also reduced systemic inflammatory responses provoked by a gastric allergic reaction.

Results: Optimal inhibition of intestinal anaphylaxis was obtained when 100 µg/kg of feG was given 20 min before the rats were challenged with antigen. The increase in total circulating neutrophils and accumulation of neutrophils in the heart, developing 3 h and 24 h, respectively, after antigen challenge were reduced by both feG and dexamethasone. Both anti-inflammatory agents reduced the increase in vascular permeability induced by antigen in the intestine and the peripheral skin (pinna), albeit with different time courses. Dexamethasone prevented increases in vascular permeability when given 12 h before antigen challenge, whereas feG was effective when given 20 min before ingestion of antigen. The tripeptide prevented the anaphylaxis induced up regulation of specific antibody binding of a cell adhesion molecule related to neutrophil activation, namely CD49d (α4 integrin).

Conclusions: Aside from showing that intestinal anaphylaxis produces significant systemic inflammatory responses in non-intestinal tissues, our results indicate that the tripeptide feG is a potent inhibitor of extra-gastrointestinal allergic reactions preventing both acute (30 min) and chronic (3 h or greater) inflammatory responses.

Background

Type 1 or IgE-mediated allergic reactions play an important role in food sensitivity reactions [1]. These reactions affect gut motility [2–4], absorption and secretion by the intestinal epithelium [5–7], and enteric as well as central nervous system function [8–11]. Systemic anaphylactic re-

actions, initiated outside the gastrointestinal tract, have pronounced impact on its integrity and function [12,13], but few studies have determined the impact of intestinal anaphylaxis on other tissues and organs.

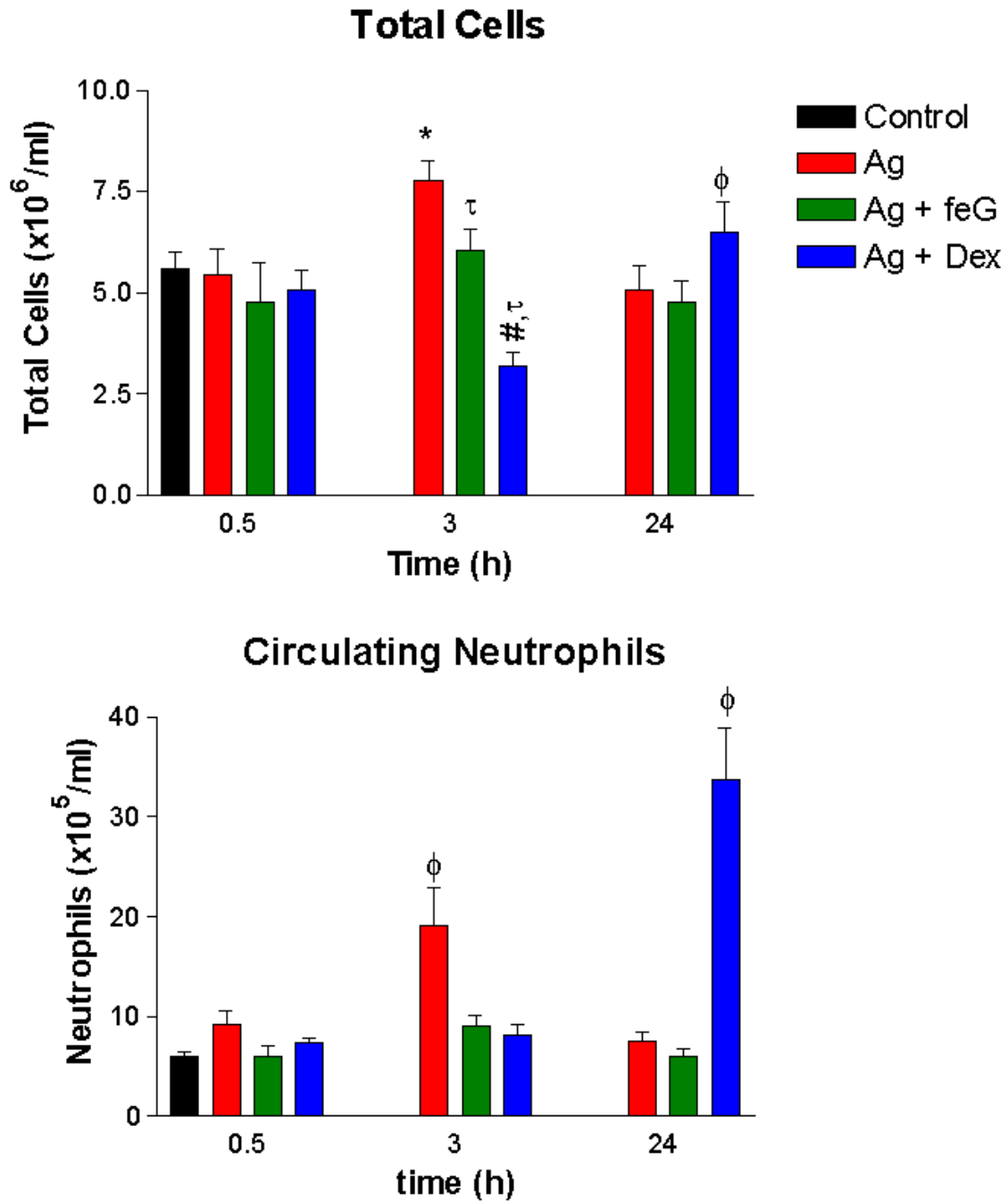


Figure 1
White blood cells after intestinal anaphylaxis. Time course of total cells (A) and neutrophils (B) in heart blood of ovalbumin sensitized rats receiving either saline (Control), antigen p.o. (Ag), feG (100 µg/kg, p.o.) and antigen (feG + Ag), or dexamethasone (4 mg/kg; i.p.) & antigen (Dex + Ag). Number of animals = 4 to 6. Significance (P < 0.05): * greater than Control; τ less than Ag at time indicated; # less than Control; φ greater than Ag and Ag + feG at times indicated.

In experimental animals minor and infrequent respiratory and cardiovascular effects after oral challenge with antigen in sensitized Brown Norway rats were reported [14]. However, systemic anaphylactic reactions involving the cutaneous, respiratory and cardiovascular systems following ingestion of food are fairly common in humans as they occur with 10–30% of sensitized individuals [15–18]. In our own studies, where the presence of diarrhea was used as a clinical sign of intestinal anaphylaxis in orally challenged ovalbumin sensitized Hooded-Lister rats [19–21], frequent piloerection was observed. This observation led us to suspect that systemic effects of intestinal anaphylaxis may be subtler than expected, and we decided to examine the hematological and vascular permeability changes associated with intestinal anaphylaxis. In these earlier studies we described protective effects of a heptapeptide, submandibular gland peptide-T (SGP-T) and its associated fragments, the tripeptide FEG and its D-isomeric form feG, on gastrointestinal function with orally administered antigen [19,20,22].

The present investigations examine systemic effects of oral antigen challenge of ovalbumin sensitized Hooded-Lister rats. The circulating neutrophilia, increases in vascular permeability, neutrophil accumulation in the heart and activation of cell adhesion molecules caused by oral ingestion of antigen were significantly reduced by the tripeptide – feG.

Results

Blood cell counts

Changes in total white blood cell counts were only seen at 3 h after antigen challenge (Figure 1A). A significant increase was observed in rats receiving only antigen ($7.8 \pm 0.5 \times 10^6$ cells/mL), relative to sensitized control rats ($5.6 \pm 0.4 \times 10^6$ cells/mL). This increase was prevented by the treatment with feG ($6.0 \pm 0.55 \times 10^6$ cells/mL). The rats treated with dexamethasone experienced a sharp drop in their total white blood cell counts ($3.2 \pm 0.4 \times 10^6$ cells/mL) at 3 h after antigen challenge. At this time a significant increase in circulating neutrophils was seen in the OA-challenged animals (Figure 1B). This increase in circulating neutrophils was prevented by treatment with feG and dexamethasone, although at 24 h neutrophil number increased more than five fold from $6.0 \pm 0.5 (\times 10^5)$ cells/mL to $33.5 \pm 5.2 (\times 10^5)$ cells/mL in the dexamethasone, but not feG, treated rats. No changes were seen in the number of circulating lymphocytes at any of the time points sampled (not shown).

Hematocrit

Intragastric challenge with antigen did not modify hematocrit, relative to unchallenged sensitized rats ($40.4 \pm 0.8\%$), at any of the time points tested. However, rats treated with feG experienced a small decrease in hematocrit at 0.5 h after antigen challenge ($39.1 \pm 0.5\%$).

In contrast, dexamethasone treated rats had significantly higher hematocrit values, than the other 3 groups of animals, at 0.5 h ($45.6 \pm 0.4\%$) and 24 h ($45.5 \pm 1.8\%$), but not at 3 h ($40.2 \pm 0.6\%$) post-challenge.

Bone marrow counts

The effect of intestinal anaphylaxis and the treatment with feG on bone marrow counts was examined at 3 h after antigen challenge. In control-sensitized rats the bone marrow was $13.6 \pm 1.2 (\times 10^6)$ cells, and this was not significantly altered by either antigen alone $11.0 \pm 1.4 (\times 10^6)$ cells) or in feG-treated challenged rats $11.1 \pm 1.0 (\times 10^6)$ cells).

Vascular permeability changes

The acute (30 min) changes in vascular permeability induced by antigen in the pinna, jejunum and duodenum was prevented by feG, but not by dexamethasone, when these compounds were given 20 min before antigen challenge (Table 1). On the other hand dexamethasone prevented the changes in vascular permeability provoked by antigen when given 12 h before initiation of the allergic reaction, whereas this prior treatment with feG was ineffective. Antigen challenge alone or in drug treated rats did not affect vascular permeability in the heart or the trachea (not shown).

Leukocyte infiltration into the heart

Leukocyte infiltration into the heart, measured with the MPO assay, increased ~75% in OA challenged animals (0.69 ± 0.09 units/mg tissue) relative to controls (0.40 ± 0.05 units/mg tissue) at 24 h after antigen challenge (Figure 2). Treatment with feG or dexamethasone at the time of antigen challenge prevented this accumulation of neutrophils in the myocardium. Interestingly, at 3 h after antigen challenge the MPO content of the heart decreased, relative to control animals, in both feG and dexamethasone treated rats. Only at 0.5 h were significant changes in tissue MPO content of the lung seen, with feG treated rats exhibiting a decrease from 1.01 ± 0.08 units/mg tissue to 0.79 ± 0.08 units/mg tissue. On the other hand, at 24 h after antigen challenge dexamethasone treatment resulted in a significant increase in MPO activity to 1.55 ± 0.03 units/mg of lung tissue.

Cell adhesion marker expression

To investigate a relationship between the effects of feG on CD11b and CD49d expression, these antigens were measured on the surface of peritoneal neutrophils isolated from sensitized rats 24 h after exposure to antigen and treatment with feG or the saline vehicle. The inhibitory effect of feG, seen on basal expression of CD11b was not observed in rats that had received an antigen challenge 24 h previously (Figure 3). CD49d was not modified by sen-

Table 1: Anaphylaxis induced vascular permeability. Effects of feG and dexamethasone (Dex) on vascular permeability changes induced by oral challenge with OA (100 mg/kg). The animals were treated with feG or Dex at 30 min or 12 h before antigen challenge.

Tissue	No Antigen		Antigen			
	Control	Saline	feG -30 min	Dex-30 min	feG -12 h	Dex -12 h
Pinna	2.4 ± 0.2 ^a	3.2 ± 0.2*	2.1 ± 0.5#	3.3 ± 0.4*	2.9 ± 0.3	2.2 ± 0.3#
Jejunum	16.6 ± 0.9	32.2 ± 4.3*	15.8 ± 0.8#	ND	20.5 ± 2.3	17.6 ± 2.8#
Ileum	19.0 ± 1.4	27.8 ± 3.4*	16.3 ± 1.3#	22.8 ± 0.8	23.7 ± 1.9	17.2 ± 2.3#

a = Values presented as mean ± SEM. N values = 4. Significance: P < 0.05; * greater than Control; # less than Saline (antigen only).

sitization or antigen challenge, but feG treatment significantly reduced the expression of this adhesion molecule in OA-exposed animals to values that were 50% of those measured in rats receiving antigen only.

Interstitial cells in intestinal smooth muscle

Twenty-four hours after the administration of oral antigen a decreased number of cells expressed the CD18 marker in the smooth muscle of the jejunum (Table 2). This reduction was partially reversed in animals receiving feG. Two morphological distinct leukocytes that stained for ED9 (LPS receptor) could be identified. One type was round and displayed the typical nucleus structure of neutrophils, and the other type had the stellate shape of interstitial macrophages. The number of macrophage-like leukocytes binding ED9 antibody was not affected by antigen challenge, whereas there was a 70% decrease in the number of ED9⁺ neutrophils. This decrease was significantly reduced by feG.

Discussion

Aside from the general malaise and gastrointestinal discomfort that food allergic individuals experience, pronounced respiratory and cardiovascular effects resulting in serious discomfort and requiring medical intervention are uncommon, but when they occur a medical emergency may ensue [15,17]. As with humans, severe systemic reactions in animal models of food allergy are uncommon, although in a rat model, the orally sensitized and -challenged Brown Norway rats, some animals exhibited minor respiratory and cardiovascular changes [14]. Oral antigen challenge of the adjuvant-primed intraperitoneally sensitized Hooded-Lister rats also produces both intestinal and systemic reactions [3,4]. These two animal models of sensitization can produce an IgE-dependent intestinal allergic reaction yielding an anaphylaxis-dependent increase in intestinal permeability [5,14]. A distinguishing feature of the Hooded-Lister rat model is

diarrhea; a clinical response that has not been reported in other animal models of intestinal allergy.

Our results indicate that gastrointestinal anaphylaxis results in significant systemic effects. Changes in vascular permeability occurred with 0.5 h, peripheral blood neutrophilia appeared at 3 h, and significant accumulation of neutrophils in the heart was seen at 24 h after antigen challenge. Several factors could contribute to the extra-intestinal manifestations of intestinal allergic reactions, including the vascular leak of antigen [23] activation of peripheral mast cells [14], appearance of intestinal mast cell mediators in the blood [21], and/or a neurogenic reflex [9]. The absence of an increased vascular leak in the trachea following gastric administration of antigen (Table 2), contrasts with the effects of intravenous [24] or airways [25,26] application of the sensitizing allergen. These differences may relate to the species used, as pulmonary allergic reactions have never been reported for Hooded-Lister rats. As seen in other species of rats, even with intravenous challenge with antigen [24,13], we found no vascular leak into the heart with an anaphylactic reaction.

Although anaphylactic reactions induced by intraperitoneal [27] or intravenous [28] administration of antigen are generally accompanied by marked and rapid hemoconcentration, the intestinal hypersensitivity reaction did not produce significant modification of hematocrit, even with the observed increases in vascular permeability. This absence of hemoconcentration indicates a much less severe systemic anaphylaxis following oral challenge with antigen than occurs when the antigen is applied directly into the circulation. Nonetheless, the delayed (3 h) blood neutrophilia (Figure 1) seen after oral antigen also indicates that intestinal anaphylaxis has hematological consequences. Circulating neutrophilia may be a hallmark of anaphylactic reactions as they are also observed in mon-

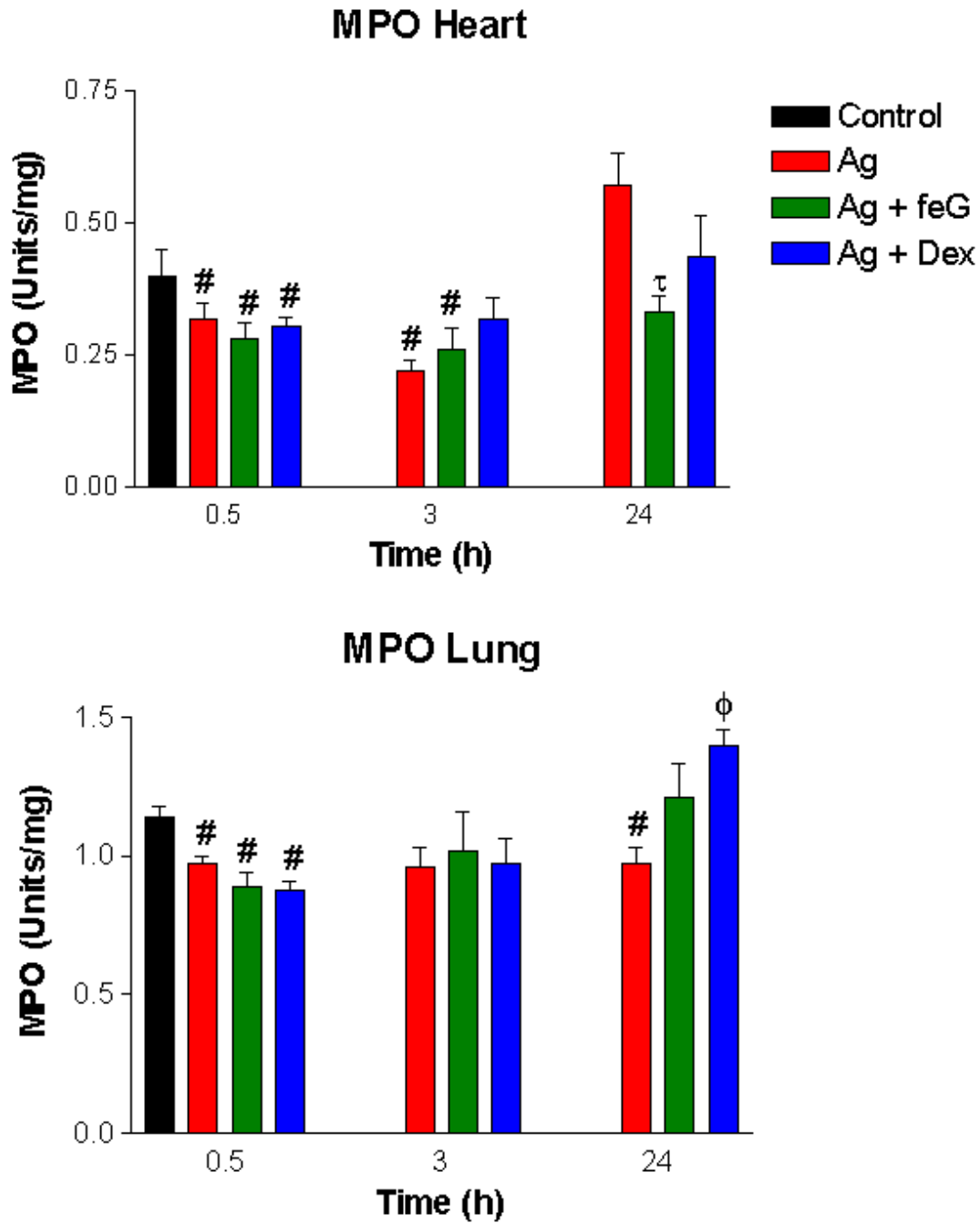


Figure 2
Neutrophil accumulation in heart and lungs. Time course of myeloperoxidase content of the heart (A) and lungs (B) of ovalbumin sensitized rats receiving either saline (Control), antigen p.o. (Ag), feG (100 μ g/kg, p.o.) and antigen (feG + Ag), or dexamethasone (4 mg/kg; i.p.) & antigen (Dex + Ag). Number of animals = 4 to 6. Significance ($P < 0.05$): # less than Control; τ less than Ag at time indicated; ϕ greater than Ag and Ag + feG at times indicated.

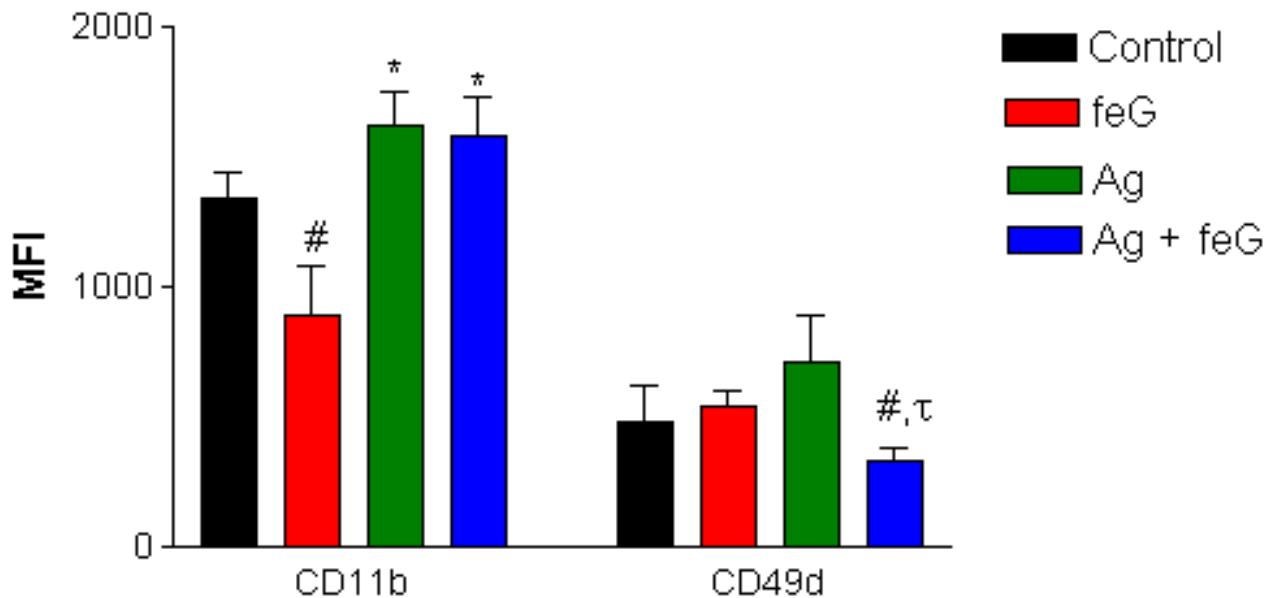


Figure 3
Cell adhesion molecules on peritoneal neutrophils. Changes in mean fluorescence intensity (MFI) for CD11b and CD49d on peritoneal neutrophils in sensitized rats that were either untreated (Control), received feG alone (feG), only antigen (Ag) or both antigen and feG (Ag + feG). Significance: ($P < 0.05$): # less than Control; * greater than Control; τ less than Ag at time indicated.

keys [31], and in rats using different methods of sensitization and routes of antigen administration [29,30]. A notable exception is the neutropenia seen in guinea pigs [32].

Anaphylaxis has important effects on neutrophils as exemplified by their early (3 h) loss, but late (24 h) increase in the heart (Figure 2), and decrease in intestinal smooth muscle (Table 1). This early loss of neutrophils from the heart could represent an allergy-mediated degranulation of the tissue resident neutrophils, and subsequent degradation of the enzyme, whereas the late-phase neutrophilia (24 h) reflects a net migration of cells into the myocardium consequent to action of release of mediators of anaphylaxis. The reduced number of ED9⁺ neutrophils in intestinal muscle was also seen with rats receiving intraperitoneal lipopolysaccharide [33], and may represent a loss of the ED9 cell marker with a phenotypic change to the interstitial neutrophils [34] following the inflammatory insult. Although direct injury to the heart, such as occurs with ischemia-reperfusion injury [35] and other forms of cardiomyopathy [36], are accompanied by pronounced neutrophil influx into the damaged tissue, the current report is the first description of anaphylaxis induced accumulation of neutrophils in the heart. Left ventricular contraction is compromised with systemic

anaphylaxis [37], and our results suggest that mild anaphylactic reactions, as may occur with a gastrointestinal reaction, could have deleterious, and long term, effects on the heart. The accumulation of neutrophils in the myocardium may in part be reduced by the peptide feG consequent to decreased expression of $\alpha 4$ integrin (CD49d) from neutrophils (Figure 3), since the adherence of neutrophils to cardiac tissue is mediated primarily by $\alpha 4\beta 1$ integrin [38,39].

This action on CD49d indicates that a reduction in cell adhesion by interfering with integrins may be an important component of feG's anti-inflammatory effects directly upon inflammatory leukocytes. The prevention, by feG, of the decrease in resident intestinal neutrophils, blood neutrophilia and vascular leak concord with this proposed mechanism since these three processes are integrin dependent [40–42]. In several respects feG is distinguished from anti-inflammatory steroids, especially with regards to a shorter onset and duration of action, and an absence of some of the pro-inflammatory effects, such as circulating and pulmonary neutrophilia. These differences in anti-inflammatory actions between feG and dexamethasone probably relate to the time delay of 4 h to 12 h associated with the steroids actions as a transcription inhibitor

[43], which, however, eventually results in reduced integrin expression on leukocytes [44].

Food allergy in humans frequently results in digestive, cutaneous, respiratory and cardiovascular reactions [15–18], however detection of allergen-induced cardiac inflammation in humans would be difficult, and to our knowledge food allergen induced cardiac inflammation in humans has never been reported. However, co-inflammatory insults, such as food allergy, that cause activation of cardiac resident mast cells or enhance inflammatory cell infiltration should be avoided in patients with an ongoing inflammation of the heart, i.e. myocarditis, dilated cardiomyopathy, ischemic cardiac injury and unstable angina [35,36,45,46]. The utility of feG in treating allergen-induced inflammations in humans has not been tested, and if such a use was contemplated the peptide would probably be more useful in reducing the inflammation that develops during the later-phases of the allergic reactions as seen herein and other studies [19,33].

Conclusions

Unlike asthmatic reactions, for which several therapeutic agents are available, the continuous use of anti-anaphylactic drugs to manage food allergies is a therapeutic option that neither widely practiced nor promoted. The present study suggests that another component to the management of food allergies is to reduce the severity of systemic effects. In this regard the tripeptide feG was found to reduce the early and delayed systemic anaphylaxis provoked by oral antigen and this peptide could conceivably be taken after exposure to oral antigen and thereby limit the severity of delayed systemic reactions.

Materials and Methods

Animals and surgical procedures

All experiments described in this study were approved by The University of Calgary, Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care. Male Hooded-Lister rats, weighing 150–170 g, were raised at the Life and Environmental Sciences Animal Resource Centre, The University of Calgary. The rats were housed under controlled lighting conditions (lights on from 7:00 H to 19:00 H), and provided with food and water *ad libitum*. All surgical procedures were performed with the animal under halothane anesthesia.

Sensitization to antigen and determination of antibody titer

The rats were sensitized by intraperitoneal injection of 10 µg of the antigen chicken ovalbumin (OA) (Grade V, Sigma Chemical Co., St. Louis, MO) and 10 mg of aluminum hydroxide (Al(OH)₃) adjuvant in saline. This sensitization protocol results in an IgE-dependent anaphylaxis [47], and blood anti-OA IgE antibody titer was measured

by passive cutaneous anaphylaxis 10 days after sensitization [3] using a small aliquot of blood (500 µl) obtained by cardiac puncture. The serum was then diluted serially (1:8 to 1:64) and 300 µl of each sample was injected into the shaved back of Sprague-Dawley rats. After 48 h, the rats were challenged with an intracardiac injection of a solution containing 2.5 mg of Evan's blue and 5 mg of OA in a total volume of 2.5 mL. Evan's blue extravasation was determined at the injection site, and a serum anti-OA antibody titer of 1:64 was required for an animal to be included in the experiments.

Antigen challenge and drug treatments

Four different groups of sensitized rats received one of the following treatments: (1) control animals received 0.9% saline only; (2) OA-challenged rats received 100 mg/kg of OA in 0.9% saline; (3) feG (Core Laboratories, Queen's University, Kingston, ON) treated rats received 100 µg/kg of the peptide and antigen; and (4) dexamethasone treated rats received 4 mg/kg of Azium (Schering, Point Claire, QC) intraperitoneally.

Circulating leukocyte and bone marrow cell counts

Under halothane anesthesia whole blood (200 µl) was drawn by cardiac puncture at 0.5, 3 and 24 h after antigen challenge. Total leukocyte counts were determined using a Unopette microcollection system (Becton Dickinson, Franklin Lakes, NJ) and Hylite hemocytometer (Hauser Scientific, Boulder, CO). Another sample was used to prepare blood smears for microscopic leukocyte differentiation using a Giemsa stain. The hematocrit was determined with a microcapillary centrifuge.

The animals were killed with an overdose of sodium pentobarbital (MTC Pharmaceuticals, Cambridge, ON). The right femur bone marrow was eluted with 10 mL of phosphate buffered saline (PBS) and leukocyte counts were determined using a hemocytometer. The PBS had the following composition (mM): NaCl (137), KCl (2.7), Na₂HPO₄ (8.1), KH₂PO₄ (1.47), CaCl₂ (1.19), MgCl₂ (0.54), glucose (7.5), pH (7.4).

Measurement of plasma extravasation

The rats were anesthetized with halothane and injected with Evans Blue (20 mg/kg; Sigma) in 0.9% saline via cardiac puncture, and 10 min later they were orally challenged with OA and the drugs feG and dexamethasone were administered. Two other groups of rats were treated with feG or dexamethasone 12 hours before the OA challenge. Thirty minutes later, and under halothane anesthesia, the rats were perfused with 1 L/kg of PBS through the left ventricle. Pieces of heart, trachea, pinna, jejunum, and ileum tissue were excised, weighed, and placed in 1 mL of formamide (Sigma) for 72 h at 20°C. The optical density of the formamide was then read at 620 nm with a Beck-

Table 2: Activation markers on interstitial cells. Effects of feG on staining of interstitial leukocytes in the jejunal muscle for CD18 and ED9 antigens.

	CD18	ED9-Macro	ED9-Neut
Saline – control	7.4 ± 1.7 ^a	9.4 ± 2.6	13.1 ± 3.1
Antigen	3.3 ± 0.5*	13.5 ± 1.7	3.9 ± 0.7*
Antigen & feG	5.6 ± 1.2	11.6 ± 0.6	9.1 ± 1.1*#

a = Values presented as mean ± SEM. N values = 4. Significance: P < 0.05; * less than saline; # greater than antigen

man DU-50 spectrophotometer (Beckman Coulter Canada Inc., Mississauga, ON) and the amount of Evan's Blue present was calculated from a standard curve.

Myeloperoxidase measurements

As a specific enzymatic marker of neutrophil infiltration into the tissue, myeloperoxidase (MPO) activity was determined in myocardial tissue and in lungs according previously described procedures [48]. Tissues (100–200 mg), excised from anesthetized rats were homogenized (Polytron homogenizer; Daigger, Vernon Hills, IL) in 0.5% hexadecyltrimethyl ammonium bromide (Sigma) dissolved in 50 mM potassium phosphate buffer at pH 6.0 for 1 min. Homogenates were then centrifuged at 4°C for 15 min at 1000 g. The supernatant was collected and reacted with 0.167 mg/mL o-dianisidine dihydrochloride (Sigma) and 0.005% H₂O₂ in 50 mM phosphate buffer at pH 6.0. The change in absorbance was measured spectrophotometrically at 460 nm. One unit of MPO activity is defined as the quantity of enzyme degrading 1 μmol of peroxide/min at 25°C.

Intestinal histochemistry

Eighteen hours after allergen-challenged or saline treatment mid-jejunal tissues were removed and whole mounted with pins and fixed in paraformaldehyde for 20 min at ~150% of resting tissue length and ~250% of resting tissue diameter. After fixing, the tissues were stripped of their mucosa, and histochemistry was performed on the muscularis [38]. The intestinal tissues were incubated for 24 h at 4°C with either ED9 or CD18 primary antibody (Cedarlane, Hornby, ON) at a 1:100 dilution followed by three 5-min washes in PBS. The tissues were then incubated with a labeled secondary antibody (goat anti-mouse coupled to biotin) at 4°C overnight. After washing 3 times for 5 min with PBS buffer, avidin coupled horseradish peroxidase was added for 30 min at room temperature. Following further washes diaminobenzadine (DAB – Peroxidase Substrate Kit; Vector Laboratories, Burlingame, CA) was added until desired staining was attained. The intestinal tissues were then dry mounted on chrom alum-coated slides, cover slipped and viewed at ×200 magnifi-

cation. Counting was performed blind and the number of labeled cells determined in four random fields of each specimen.

Leukocyte preparation and use

Blood was collected by cardiac puncture into a 10 mL syringe from rats anesthetized with halothane. The blood was diluted with approximately 30 mL of PMN buffer (PBS buffer without calcium) in a 50 mL polypropylene centrifuge tube, and spun at 300 g for 15 min at 20°C. After the buffer was carefully decanted from the pellet, the red blood cells were lysed (0.15 M NH₄Cl) for 3 min at room temperature. After a second spin at 200 g for 10 min at 20°C, the supernatant was discarded and the pellet washed with PMN buffer and spun again 200 g for 10 min at 20°C. The supernatant was then discarded and the cells resuspended in 1 mL of PMN buffer, and total cell number determined using Trypan Blue exclusion on a hemocytometer. Cell differentials were determined on cell smears stained with May-Grünwald-Giemsa stain. Lymphocytes were 68.7% of the circulating white blood cells, neutrophils accounted for 20.4% and monocytes were 11.0%.

To isolate peritoneal leukocytes 10 mL of 0.9% sterile saline was injected intraperitoneally into halothane anesthetized rats and the abdomen massaged gently. After a clean laparotomy the peritoneal fluid (7 to 10 mL) was recovered and placed into a 50 mL polypropylene tube and diluted to 30 mL with PMN buffer. The cells were then prepared as described for blood leukocytes. Of the peritoneal cells 21.5% were neutrophils and 46.5% were macrophages.

Statistical analysis

Results were expressed as mean ± SEM and n is the number of animals. Statistical significance of differences between means was determined at p < 0.05, using, where appropriate, one-way analysis of variance (ANOVA) with the Student t-test applied for comparison of differences between two means.

Abbreviations

CD11b, α M integrin; CD49d, α 4 integrin; feG, D-phenylalanine-D-glutamate-Glycine; MPO, myeloperoxidase; PBS, phosphate buffered saline; SGP-T, submandibular gland peptide-T

Authors' contributions

Turesin F participated in the design of the study, managed the animals and carried out the cell counts and myeloperoxidase measurements. Sadr A performed the vascular permeability experiments. Davison JS participated in study design. Ronald Mathison conceived the study, participated in its coordination and drafted the manuscript. All authors read and approved the manuscript.

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