Delayed clinical and *ex vivo* response to mammalian meat in patients with IgE to galactose-alpha-1,3-galactose

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Background: In 2009, we reported a novel form of delayed anaphylaxis to red meat related to serum IgE antibodies to the oligosaccharide galactose-alpha-1,3-galactose (alpha-gal). Although patients were remarkably consistent in their description of a 3- to 6-hour delay between eating mammalian meat and the appearance of symptoms, this delay has not been demonstrated under observed studies.

Objectives: We sought to formally document the time course of clinical symptoms after the ingestion of mammalian meat in subjects with IgE to alpha-gal and to monitor *ex vivo* for the appearance of markers of an allergic reaction.

Methods: Open food challenges were performed with mammalian meat in 12 subjects with a history of severe urticarial reactions 3 to 6 hours after eating beef, pork, or lamb, as well as in 13 control subjects. Blood samples were taken hourly during each challenge.

Results: Ten of 12 subjects with IgE to alpha-gal had clinical evidence of a reaction during the food challenge (vs none of the control subjects, $P < .001$). The reactions occurred 3 to 7 hours after the initial ingestion of mammalian meat and ranged from urticaria to anaphylaxis. Tryptase levels were positive in 3 challenges. Basophil activation, as measured by increased expression of CD63, correlated with the appearance of clinical symptoms.

Conclusion: The results presented provide clear evidence of an IgE-mediated food allergy that occurs several hours after ingestion of the inciting allergen. Moreover, here we report that *in vivo* basophil activation during a food challenge occurs in the same time frame as clinical symptoms and likely reflects the appearance of the antigen in the bloodstream. (J Allergy Clin Immunol 2014;134:108-15.)

Key words: *Anaphylaxis, alpha-gal, basophil, mammalian meat, food allergy*

Shortly after the approval of cetuximab in 2005, it became clear that a significant number of patients were experiencing severe hypersensitivity reactions during their first infusion of this mAb.[1] Detailed investigation of serum antibodies by our group established that these reactions were occurring in patients who had pre-existing IgE antibodies specific for glycosylation products on the Fab fragment of the mAb.[2] The relevant oligosaccharide is galactose-alpha-1,3-galactose (alpha-gal), which is a blood group substance of nonprimate mammals.[3,4] Since that time, IgE antibodies to alpha-gal have been associated with a novel form of food allergy that appears to occur in some patients after tick bites.[2,5-8] Specifically, patients with IgE to alpha-gal reported that they had generalized urticaria, angioedema, or anaphylaxis and that this occurred 3 to 6 hours after eating beef, pork, or lamb.[6,7] Although we are now aware of more than 2000 persons on at least 4 continents who report delayed allergic reactions to mammalian meat or cow's milk–containing products (most of whom have documented IgE to alpha-gal), direct observation of the delayed reactions has not been confirmed.[9-16]

Here we report detailed observations on the appearance of symptoms after food challenges with mammalian meat in subjects with IgE to alpha-gal and present results showing that clinical symptoms do not appear until at least 3 hours after the consumption of beef or pork. These studies also included 13 control subjects with negative results for IgE to alpha-gal who underwent mammalian meat food challenges and did not have symptoms.

Although tryptase levels (presumed to be from mast cells) largely do not increase in patients with food-induced anaphylaxis, basophils are postulated to be involved in allergic reactions to food, and specific markers of activation have been identified.[17-21] Dr Shreffler and colleagues[22] have shown that regulation of the basophil activation markers CD63 and CD203c corresponds to peanut sensitization and that activation is decreased with oral immunotherapy. Using subjects allergic to insect venom, Dr Saini's group[23] showed that in an intentional sting challenge model there was an increase in these same activation markers.

We report here a detailed clinical investigation of delayed allergic symptoms after consumption of mammalian meat in subjects with IgE to alpha-gal. The results provide direct evidence

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Abbreviations used
Alpha-gal: Galactose-alpha-1,3-galactose
FACS: Fluorescence-activated cell sorting
fMLP: Formyl-methionyl-leucyl-phenylalanine

for a delayed, IgE-mediated food allergy. We also sought to assess basophil CD63 levels *ex vivo* during the food challenges, and these results imply there is a delay in the entrance of the relevant form of antigen into the circulation.

METHODS

Patients and control subjects

The studies reported here were approved by the University of Virginia Human Investigation Committee, and informed consent was obtained from all subjects. Patients presenting to the clinic in Charlottesville with delayed urticarial reactions to mammalian meat and with positive titers of IgE antibody to alpha-gal (n = 12) were enrolled, as well as control subjects with negative titers of IgE antibody to alpha-gal and no history of reactions to mammalian meat (n = 13). For safety reasons, subjects who reported a history of anaphylactic reactions were not eligible to participate in the food challenge, and therefore we specifically selected patients who reported symptoms, such as hives, flushing, rhinitis/nasal congestion, or abdominal cramping. In addition, we only investigated patients who had not sought urgent care for treatment of a reaction and had no history of coronary disease, no major chronic illness, and no contraindication to receiving epinephrine.

Mammalian meat food challenge

Mammalian meat challenges were performed in the allergy clinic of the University of Virginia Hospital or, in one case, at Duke University Medical Center. Participants were invited to return for a meat challenge based on the results of serum screening (sIgE to alpha-gal >1.0), severity of symptoms at prior reaction, and appropriate medical history (eg, not currently taking β-adrenergic receptor blocker medication and no medical contraindication to epinephrine). Subjects were asked to avoid oral antihistamines and leukotriene modifier therapy for 7 days before the challenge. Vital signs and intravenous access were obtained, and subjects consumed 150 g of mammalian meat under observation (roughly equivalent to 2-3 sausage patties). One of the initial challenges in a subject with IgE to alpha-gal was performed with beef; however, this produced a more severe reaction, and pork sausage was used thereafter. In 150 g of pork sausage there were 300 calories, 24 g of fat, 18 g of protein, and 660 mg of sodium. Subjects were monitored for reactions, including a change in vital signs, after the food challenge. Blood was sampled immediately before and then hourly after the dose of meat was eaten. All subjects were monitored for 2 hours after the treatment of clinical symptoms and were only released when vital signs were stable. Subjects were allowed to consume water, but no other food or vigorous exercise was permitted during the duration of the food challenge.

Basophil activation studies *in vitro* and *ex vivo*

The basophil activation assay was adapted from one previously described.[24] For full details, see the Methods section in this article's Online Repository at www.jacionline.com. For the initial basophil activation experiments, 1 mL of warmed whole peripheral blood was mixed with 1 mL of warmed stimulus medium and incubated for 30 minutes, 1 hour, 2 hours, and 4 hours at 37°C. Afterward, EDTA was added to stop the activation process. For the meat challenges, blood was collected into tubes containing acid citrate dextrose before beginning the challenge and at hourly intervals for up to 6 hours after food consumption. Each of these samples of whole peripheral blood was promptly (within 5 minutes) mixed with EDTA stop buffer. (Note: no *ex vivo* stimulation was performed on samples collected during a meat challenge.) All samples were spun at 1400 rpm, the resulting supernatant was removed, and the cell pellet was immediately stained for flow cytometric analysis. Basophil activation was assessed by means of flow cytometry (see Figs E1-E3 in this article's Online Repository at www.jacionline.com for explanation of the gating strategy) and considered to be positive when more than 15% of basophils expressed CD63 (see the Methods section in this article's Online Repository for rationale).

Flow cytometric analysis

For multicolor fluorescence-activated cell sorting (FACS) analysis, specific mAbs were directly added to the stimulated whole peripheral blood samples and incubated for 30 minutes in the dark at 4°C. Stained cells were washed in PBS with 0.5% BSA plus 2 mmol/L EDTA, and red blood cells were lysed. Stained cell suspensions were analyzed with a FACScalibur flow cytometer (BD Biosciences, San Jose, Calif) with a Cytek DxP10 upgrade (Cytek, Fremont, Calif) and FlowJo software, version 7.6.5 (Tree Star, Ashland, Ore). For all analyses, compensation and gating controls were included. Basophils were identified as lineage1$^-$HLA-DR$^-$CD41$^-$CD123$^+$ (see Fig E1), and a minimum of 1000 events (ie, basophils) were recorded for each condition or the sample was excluded.

Immunoassays

Total and specific IgE antibodies were measured by using the commercially available ImmunoCAP system (Phadia US, Portage, Mich). The assays were performed with the ImmunoCAP 250 instrument, and results were expressed as international units per milliliter, where the international unit both for specific and total IgE is approximately 2.4 ng. For specific assays, the standard cutoff point for a positive reaction was 0.35 IU/mL. The streptavidin CAP technique was used to measure IgE levels to alpha-gal, in which approximately 1 μg of biotinylated antigen was added to each CAP.[25] Serum tryptase levels were measured with a commercially available fluoroenzyme immunoassay (Phadia US), according to the manufacturer's instructions.

Statistical analyses

Statistics were performed with the Fisher exact test, and a 2-sided *P* value of less than .05 was considered to indicate statistical significance. Results are presented as means ± SEMs. Statistical analyses were performed with SPSS, version 18.0 (SPSS, Chicago, Ill) and GraphPad Prism, version 6 (GraphPad Software, La Jolla, Calif).

RESULTS

Mammalian meat food challenges

As part of our ongoing studies of anaphylaxis, urticaria, and angioedema related to IgE antibody to alpha-gal, we identified 12 patients with histories of delayed urticaria after eating mammalian meat who agreed to undergo food challenge testing (Table I). Each of the 12 patients had IgE to alpha-gal, as well as beef, pork, and cow's milk (Table I), but none reported symptoms consistent with anaphylaxis or a reaction more severe than generalized hives after natural exposure. All of the patients were actively avoiding mammalian meat, and 3 of them, including the patient with a positive immunoassay result for IgE to the casein component of cow's milk, were also avoiding cow's milk and limiting dairy because of associated symptoms. These 3 patients specifically reported that dairy, in particular ice cream, produced heartburn, abdominal cramping, and even diarrhea 3 to 4 hours after consumption. Because of the prolonged delay between eating mammalian meat and the appearance of symptoms, the procedure was performed as an open food challenge with 150 g of pork sausage consumed as a single dose. Ten of 12 patients with IgE to alpha-gal had positive open food challenge results with mammalian meat ($P < .001$ vs control subjects), and each reaction began at least 150 minutes after