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

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Abstract

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

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and likely reflects the appearance of the antigen in the bloodstream. (J Allergy Clin Immunol 2014;)

Keywords

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 colleagues²² 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 group²³ 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 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

Basophil activation assay

The various stimulation conditions included RPMI medium (ThermoFisher Scientific, Waltham, Mass) containing 10 µg/mL beef thyroglobulin (Sigma-Aldrich, St Louis, Mo),

100 µg/mL cetuximab (ImClone, Bridgewater, NJ), 1 µg/mL cetuximab, 1 µg/mL anti-IgE antibody (Invitrogen Life Technologies, Grand Island, NY), or 2 µmol/L fMLP (Sigma-Aldrich). RPMI medium alone was used for all unstimulated control subjects. Blood was collected into Vacutainer tubes containing acid citrate dextrose buffer (BD, Franklin Lakes, NJ) before oral food challenge and at hourly intervals for up to 6 hours after food consumption. Additionally, before the activation assay itself, all solutions and whole peripheral blood collected before ingestion of mammalian meat were separately incubated for 15 minutes at 37°C to allow for temperature equilibration.

For the basophil activation assay, 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, 350 µL of PBS plus 20 mmol/L EDTA was added to each sample to stop the activation process. For hourly interval time points collected during the food challenge, 3 mL of whole peripheral blood was mixed directly with 350 µL of PBS plus 20 mmol/L EDTA. (Note: no *ex vivo* stimulation was performed on samples collected during a meat challenge.) All samples were spun at 1400 rpm for 10 minutes, with the resulting supernatant manually removed and the remaining cell pellet immediately stained for flow cytometric analysis.

Flow cytometric analysis

Multiple gating strategies were used over the initial mammalian meat food challenges to establish optimal fluorochromes for flow cytometric analysis of whole blood basophils. Although we did perform Ficoll purification of basophils, we did not find that this additional purification step led to appreciable differences in results. Included in the optimization process was assessment of whether differences emerged when collecting peripheral blood through intravenous needle draw, as well as the conditions, protocols, and reagents for measuring mediators. We report activation as the percentage of CD63 cells over baseline. Our analysis includes CD203c as well; however, we found this marker most reliable for *in vitro* assays in which a controlled stimulation occurred for 15 to 30 minutes. CD203c was not a consistent marker of activation across subjects during meat challenges (where samples were taken hourly), and this is likely because of the more rapid nature of CD203c as an activation marker.^{E1} For multicolor 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. Antibodies used were at a final concentration of 1 µg/100 µL and allophycocyanin-conjugated anti-CD63 (MEM-259; BioLegend, San Diego, Calif), Brilliant Violet 421-conjugated anti-CD123 (9F5; BD Biosciences, San Jose, Calif), PerCP-Cy5.5-conjugated anti-HLA-DR (LN3; eBioscience, San Diego, Calif), fluorescein isothiocyanate-conjugated lineage cocktail 1 (anti-CD3, anti-CD14, anti-CD16, anti-CD19, anti-CD20, and anti-CD56; BD Biosciences), allophycocyanin-Cy7-conjugated anti-CD41 (HIP8; BioLegend, San Diego, Calif), and phycoerythrin-conjugated anti-CD203c (97A6; Beckman Coulter, Indianapolis, Ind). Single color compensation controls were created by using anti-mouse immunoglobulin beads (BD Biosciences). Stained cells were washed with FACS buffer (PBS supplemented with 0.5% BSA and 2 mmol/L EDTA). Red blood cells were then lysed by adding FACS Lysing Solution (BD Biosciences) to each sample for 15 minutes. Stained cell suspensions were analyzed with a FACScalibur flow cytometer (BD Biosciences) with a

Cytek DxP10 upgrade (Cytek, Fremont, Calif) and FlowJo software, version 7.6.5 (Tree Star). For all analyses, compensation and gating controls (cells stained with all reagents minus one) were included. A minimum of 1000 Lin¹-HLA-DR⁻CD41⁻CD123⁺ events (ie, basophils) were recorded for each condition or the sample was excluded.

Allergen stimulation generates a distinct subpopulation of basophils that express CD63 with a high density and consequently can be expressed as a percentage of activated CD63⁺ basophils for each condition or time point.^{E2} This percentage was corrected for nonspecific activation by gating the percentage of activated (CD63⁺) basophils in the appropriate negative control or baseline condition at 1% (Figs 2 and E2). Although some authorities have suggested that a percentage of CD63⁺ basophils of 8% to 10% might reflect activation,^{E3} we found that a percentage of at least 15% CD63⁺ cells was more consistently correlated with clinical symptoms. Therefore “basophil activation” was set at greater than 15% CD63⁺.

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Abbreviations used

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

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Key messages

- The delay in reactions to red meat reported by patients with IgE to alpha-gal has been confirmed in observed challenges.
- Basophil activation during the food challenge corresponds to the timing of clinical symptoms.

FIG 1.
Representative urticaria appearing in patient FC-09 at 257 minutes after eating 150 g of pork sausage. The right flank is shown.

FIG 2.

Basophil cell-surface expression of CD63 during mammalian meat challenge of patient FC-09 with IgE to alpha-gal (**A**) and control subjects (**B** and **C**). Whole blood was collected at baseline and hourly during a mammalian meat challenge, as described in the Methods section. CD63 expression peaked at hour 4 (63.1%) in the subject (Fig 2, **A**) and at hour 4 (24.5%) in a control subject (Fig 2, **B**). The control subject in Fig 2, **C**, did not show evidence of basophil activation.

FIG 3.

Basophil CD63 expression at hourly time points in individual patients with positive results for IgE antibody (A) and control subjects (B) over the course of a mammalian meat challenge. $P = .025$ for mean maximal activation of patients (33.4 ± 6.4) versus control subjects (14.7 ± 4.6).

FIG E1.

Gating strategy for the basophil activation assay. Basophils were identified by means of flow cytometry as HLA-DR⁻ lineage 1⁻ and then CD41⁻CD123⁺ cells. Basophil activation was assessed based on expression of CD63 and reported as a percentage of CD63⁺ cells, with baseline set to 1%. *APC*, Allophycocyanin; *FITC*, fluorescein isothiocyanate.

FIG E2.

Representative FACS contour plots from patient FC-02 using whole blood basophils stimulated *in vitro* for 30 minutes with medium (A), 50 µg of beef thyroglobulin (B), 5 µg of cetuximab (C), 5 µg of anti-IgE (D), and 10 µmol/L fMLP (E).

FIG E3.

Representative FACS contour plots from patient FC-02 with IgE to alpha-gal using whole blood basophils stimulated *in vitro* for 4 hours with medium (A), 50 µg of beef thyroglobulin (B), 5 µg of cetuximab (C), 5 µg of anti-IgE (D), and 10 µmol/L fMLP (E).

FIG E4. Patient with IgE to alpha-gal (FC-08) demonstrating baseline forearms and palms (**A**) compared with distinct palmar erythema (**B**) that appeared at 4 hours and 38 minutes after eating 150 g of pork sausage.

FIG E5.

A, Basophil cell-surface expression of CD63 during mammalian meat challenge of patient FC-08 shown in Fig E4. No sample was obtained at 6 hours because of clinical care of the subject. **B** and **C**, Additional photos of hives from patient FC-08.

FIG E6. Basophil cell-surface expression of CD63 (**A**) and supporting photos (**B** and **C**) during mammalian meat challenge of patient FC-03.

FIG E7.

A, Basophil cell-surface expression of CD63 during mammalian meat challenge of patient FC-06. **B**, Patient FC-06 before eating mammalian meat. **C**, Patient FC-06 at 6 hours and 45 minutes after consuming 150 g of mammalian meat.

Patients’ characteristics and mammalian meat food challenge results

TABLE I

Subject ID	Age (y)/sex	History of tick bites	IgE to alpha-gal (IU/mL)	Total IgE (IU/mL)	Alpha-gal/total IgE	Cow's milk IgE (IU/mL)	IgE to casein (IU/mL)	Beef IgE (IU/mL)	Pork IgE (IU/mL)	Symptoms	Time to symptoms	Treatment
FC-01b	54/M	Yes	26.7	757	3.5%	3.9	<0.35	6.8	6.5	Pruritus, urticaria	4:15	AH
FC-02	28/M	Yes	22.4	109	21%	2.3	<0.35	5.5	4.9	Pruritus, urticaria, heartburn	4:22	AH
FC-03*	25/F	Yes	17.6	184	9.5%	3.4	0.6	4.8	4.5	Cough, urticaria, chest tightness, abdominal cramping, hypotension	3:54	AH, prednisone, albuterol, epinephrine
FC-04	55/M	Yes	43.6	78.1	56%	0.6	<0.35	8.3	7.8	None	NA	None
FC-05	32/M	Yes	10.1	27.0	37%	1.1	<0.35	3.0	2.7	“Grumbling stomach”	4:18	None
FC-06	26/M	Yes	9	204	4.4%	0.8	<0.35	2.2	2.1	Pruritus, urticaria	4:52	AH
FC-07*	52/M	Yes	22.6	77.9	29%	1.5	<0.35	3.3	2.9	Pruritus, urticaria	4:40	AH
FC-08	46/F	Yes	30.3	146	21%	1.2	<0.35	3.7	3.0	Palmar erythema, pruritus, urticaria, chills, hypotension	4:38	AH, prednisone, epinephrine
FC-09	48/M	Yes	31.7	143	22%	4.4	<0.35	11.6	11.2	Palmar itching, pruritus, urticaria	3:23	AH, prednisone
FC-10	19/M	Yes	25.7	324	7.9%	1.5	<0.35	9.5	9.8	Heart burn, abdominal cramping	5:04	AH
FC-11*	37/M	Yes	10.3	163	6.3%	2.9	<0.35	5.0	5.1	Palmar and plantar itching	4:09	None
FC-12	51/F	Yes	13.2	143	9.2%	0.6	<0.35	2.9	2.5	Palmar itching, flushing, urticaria	2:45	AH, prednisone

AH, Antihistamine; F, female; M, male; NA, not applicable.

* Subjects who were avoiding cow's milk, limiting dairy intake, or both before food challenge because of associated symptoms.

TABLE II

Control subjects' characteristics and mammalian meat food challenge results

Subject ID	Age (y)/sex	History of tick bites	IgE to alpha-gal (IU/mL)	Total IgE (IU/mL)	Cow's milk IgE (IU/mL)*	Beef IgE (IU/mL)	Pork IgE (IU/mL)	Symptoms (IU/mL) [†]
FC-20	34/F	No	<0.35	83.8	<0.35	<0.35	<0.35	None
FC-21	29/M	Yes	<0.35	252	<0.35	<0.35	<0.35	None
FC-22	28/F	No	<0.35	22.9	<0.35	<0.35	<0.35	None
FC-23	31/F	No	<0.35	42.3	<0.35	<0.35	<0.35	None
FC-24	37/M	Yes	<0.35	<2	<0.35	<0.35	<0.35	None
FC-25	25/F	No	<0.35	107	<0.35	<0.35	<0.35	None
FC-26	44/M	Yes	<0.35	2.9	<0.35	<0.35	<0.35	None
FC-27	27/M	No	<0.35	7.7	<0.35	<0.35	<0.35	None
FC-28	46/F	Yes	<0.35	53.8	<0.35	<0.35	<0.35	None
FC-29	36/M	No	<0.35	11.1	<0.35	<0.35	<0.35	None
FC-30	23/F	Yes	<0.35	127	<0.35	<0.35	<0.35	None
FC-31	39/M	Yes	<0.35	96.1	<0.35	<0.35	<0.35	None
FC-32	22/F	No	<0.35	32.4	<0.35	<0.35	<0.35	None

F, Female; M, male.

* Measurement of IgE levels to casein (milk component) was not performed in subjects with a negative immunoassay result for IgE to cow's milk.

[†] None of the control subjects reported symptoms, and therefore no treatment was administered.

TABLE III

Basophil activation and tryptase measurements during mammalian meat challenge of subjects with and without IgE to alpha-gal

Patient	CD63 activation [*]	Serial tryptase [†]
Subjects with positive results for IgE antibody to alpha-gal		
FC-01b	23% at 3 h	No change
FC-02	17% at 4 h	No change
FC-03	33% at 5 h	4.9/4.7/9.9/10.7/9.6
FC-04//	67% at 5 h	No change
FC-05//	42% at 4 h	No change
FC-06	47% at 5 h	ND
FC-07	None	No change
FC-08	30% at 4 h	4.2/3.8/5.1/18.2/20.1
FC-09	27% at 3 h	6.2/5.9/14.8/18.3 [‡]
FC-10	20% at 6 h	No change
FC-11	None	No change
FC-12	None [§]	ND
Control subject	CD63 activation	Serial tryptase
Subjects with negative results for IgE antibody to alpha-gal		
FC-20	46% at 4 h [*]	No change
FC-21	34% at 4 h	No change
FC-22	None	No change
FC-23	None	No change
FC-24	None	No change
FC-25	None	No change
FC-26	None	No change
FC-27	17% at 5 h	No change
FC-28	None	No change
FC-29	24% at 4 h	No change
FC-30	None	No change
FC-31	43% at 4 h	No change
FC-32	None	No change

ND, Not done.

^{*} Basophil activation was defined as the first time point when the percentage of CD63⁺ basophils was greater than 15%. The actual percentage of activation was noted with the corresponding hourly time point.

[†] Tryptase was expressed as nanograms per milliliter and listed in order of collection (baseline/2 hours/3 hours/4 hours/5 hours); serial mature tryptase levels were considered positive if the level increased greater than 11.4 mg/mL or 2+1.2 times baseline.²⁶

[‡] Patient FC-09 did not have a 5-hour tryptase sample.

[§] The basophil CD63 percentage reached 13% at hour 4.

// Subject considered to have a negative meat challenge result.