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Laminin γ-1 and collagen α-1 (VI) chain are galactose-α-1, 3-galactose–bound allergens in beef

H. Takahashi[1], Y. Chinuki[1], A. Tanaka[2] & E. Morita[1]

[1]Department of Dermatology, Shimane University Faculty of Medicine, Shimane; [2]Thermo Fisher Scientific K.K., Tokyo, Japan

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Correspondence
Hitoshi Takahashi, Department of Dermatology, Shimane University Faculty of Medicine, Enya 89-1, Izumo 693-8501, Shimane, Japan.
Tel.: +81-853-20-2210
Fax: +81-853-21-8317
E-mail: tkh4@med.shimane-u.ac.jp

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Abstract

Background: Sensitization to the carbohydrate galactose-α-1,3-galactose (α-Gal) has been reported in patients with beef allergy. However, the proteins responsible for this allergy have not yet been identified. This study aimed to identify beef proteins that predominantly react with serum IgE in Japanese patients with beef allergy.

Methods: Sera were collected from 29 patients with beef allergy who had allergic reaction(s) such as urticaria, abdominal pain, vomiting, and anaphylactic shock after ingestion of beef and pork; the sera tested positive for IgE against beef and pork. IgE-binding proteins were detected by immunoblotting sera from the patients and identified using a combination of two-dimensional gel electrophoresis and peptide mass fingerprinting techniques. The involvement of carbohydrate in the binding of IgE to allergens was examined by periodate treatment and an inhibition assay with cetuximab by immunoblotting. Specific IgE binding to cetuximab was measured using the CAP-fluorescent enzyme immunoassay.

Results: Two IgE-binding proteins (240 kDa and 140 kDa) were detected in beef extract and identified as laminin γ-1 and the collagen α-1 (VI) chain from *Bos taurus*, respectively. Periodate treatment or the inhibition assay resulted in the loss of IgE binding to these proteins. Immunoblotting with anti-α-Gal antibody revealed the presence of α-Gal on the 240- and 140-kDa beef proteins. The amount of IgE bound to cetuximab was significantly correlated with that to beef in the patients with beef allergy.

Conclusion: The carbohydrate moiety (α-Gal) on laminin γ-1 and collagen α-1 (VI) chain are possibly common IgE-reactive proteins in the Japanese patients with beef allergy.

Meat accounts for 3% of the causative foods of immediate-type food allergies in the Japanese population (1). An allergic reaction to mammalian meat, referred to as beef allergy or red meat allergy, develops after the ingestion of mammalian meat such as beef and pork, but not chicken or fish. Investigations of allergens causing a meat allergy have mainly focused on cross-reactive allergens in cow's milk in patients with atopic dermatitis who were sensitized to beef; bovine serum albumin (BSA), bovine immunoglobulin G (IgG), and bovine actin have been identified as the cross-reactive allergens (2–8).

Chung et al. (9) reported that the carbohydrate galactose-α-1,3-galactose (α-Gal), which is present in the heavy chain of cetuximab (a chimeric monoclonal antibody against epidermal growth factor receptor), is a major allergen causing hypersensitivity reaction after the first cetuximab infusion. In addition, they showed that the distribution of IgE antibodies to α-Gal was highly variable depending on the region in the United States (9). Based on this finding, a series of studies have clarified that patients with cetuximab hypersensitivity have a red meat allergy, and α-Gal in red meat is responsible for red meat allergy (10–16). However, red meat proteins bearing α-Gal have not yet been identified.

We screened IgE-binding proteins in red meat (beef and pork) using sera from Japanese patients with beef allergy and identified laminin γ-1 and the collagen α-1 (VI) chain as major allergens possessing an α-Gal moiety.

Materials and methods

Patients and sera

Sera were obtained from 29 patients with beef allergy; sera were stored at −20°C until analysis. Beef allergy was diagnosed by the presence of clinical episode(s) of allergic reaction such as urticaria, abdominal pain, vomiting, and anaphylactic shock within 8 h after ingesting mammalian meat, and positive serum-specific IgE tests of beef and pork. Table 1 lists the clinical features of the patients. Serum-specific IgE values were measured by a CAP-fluorescent enzyme immunoassay (CAP-FEIA; ImmunoCAP®; Thermo Fisher Scientific, Uppsala, Sweden). This study was approved by the Ethics Committee of the Shimane University Faculty of Medicine (approval No. 469).

Preparation of meat proteins

Meat (beef, pork, and chicken) was homogenized in a 10× volume of ice-cold phosphate-buffered saline (PBS) containing a protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). The homogenate was centrifuged at 20 000 ***g*** for 15 min at 4°C, and the supernatant was collected as water-soluble proteins. The pellet was solubilized in 2% sodium dodecyl sulfate (SDS) aqueous solution with constant mixing at 95°C for 5 min and centrifuged at 20 000 ***g*** for 15 min at 4°C, and the supernatant was collected as water-insoluble proteins. Protein concentrations were determined using a protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Immunoblot analysis

For IgE immunoblotting, meat proteins were separated by 7.5% SDS–polyacrylamide gel electrophoresis (PAGE) (25 μg/lane) under reducing conditions and subsequently transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore, Billerica, MA, USA). The PVDF membrane was incubated with a blocking solution (20 mM Tris–HCl, pH 8.0, 0.15 M NaCl, 0.1% Tween-20, and 0.6% polyvinylpyrrolidone) for 1 h and further incubated with patient sera diluted to 10% with blocking solution for 16 h at 28°C. After washing the PVDF membranes with TBST (20 mM Tris–HCl, pH 8.0, 0.15 M NaCl, 0.1% Tween-20) three times, bound IgE was visualized on RX-U Fuji medical X-ray film (FUJIFILM Co., Tokyo, Japan) using horseradish peroxidase (HRP)-conjugated goat anti-human IgE antibody (Kirkegaard & Perry Laboratories, Inc., KPL, Gaithersburg, MD, USA) and the ECL prime kit (GE Healthcare UK Ltd, Buckinghamshire, UK). Total protein in the gel was stained with Coomassie Brilliant Blue (CBB).

For α-Gal detection, mouse anti-α-Gal monoclonal antibody (M86 clone; Enzo Life Sciences, Inc., Farmingdale, NY, USA) and HRP-conjugated anti-mouse IgM (μ) antibody (KPL) were used. The chemiluminescent signal was detected as described above.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

To obtain the allergen-rich fraction, water-soluble beef proteins were precipitated with ammonium sulfate at concentrations of 0–20%, 20–40%, 40–60%, and 60–80% at 4°C. Each precipitate was dissolved in PBS, and the suspension was dialyzed twice in PBS at 4°C for 16 h. IgE immunoblot analysis with patient sera revealed that the precipitate obtained with 20–40% ammonium sulfate contained IgE-reactive proteins with relative molecular masses of 240 kDa and 140 kDa.

The allergen-rich fraction was cleaned up with a Ready-Prep 2-D Cleanup kit (Bio-Rad) for isofocusing electrophoresis, and the resultant precipitate was dissolved in a buffer containing 60 mM Tris–HCl (pH 8.8), 6 M urea, 1 M thiourea, 3% CHAPS, and 1% TritonX-100. Residual particles were removed by ultracentrifugation at 200 000 ***g*** for 20 min at 4°C. Protein concentration was determined using an RC DC protein assay kit (Bio-Rad).

2D-PAGE was performed according to the manufacturer's instructions. First-dimension protein separation (40 μg) was carried out in agarGEL (pH 3–8, ϕ 2.5 × 75 mm; ATTO corp., Tokyo, Japan) at 300 V for 3.5 h. The agarGel was subsequently used for second-dimension SDS-PAGE on a 7.5% acrylamide gel under reducing conditions. For IgE immunoblot analysis, proteins separated by 2D-PAGE were electrophoretically transferred to PVDF membranes and further incubated with 10% patient sera. Bound IgE was detected as described above.

Identification of beef allergens

Protein spots were manually excised from the CBB-stained gel, subjected to reductive alkylation by iodoacetamide, and digested with trypsin gold (Promega, Madison, WI, USA) using ProteaseMAX™ Surfactant (Promega) following the manufacturer's instructions. Peptides were crystallized with α-cyano-4-hydroxycinnamic acid on a stainless steel plate. The mass spectra of peptides were recorded in the reflectron mode of matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS; AB SCIEX TOF/TOF 5800 system, Framingham, MA, USA). The mass spectrometer was calibrated with angiotensin II, P14R, and adrenocorticotropic hormone fragments 18–39 (Sigma, St. Louis, MO, USA). Data processing was carried out using Data Explorer software ver. 4.10 (Applied Biosystems/MDS Analytical Technologies, Foster City, CA, USA). For peptide mass fingerprinting (PMF) and the MS/MS ion search, the generated mass lists were searched against the protein data bank of the National Center for Biological Information (NCBI) using the data base search engine MASCOT (Matrix Science, London, UK) after excluding peak lists of known contaminants such as keratin and trypsin. The peptide mass tolerance and fragment mass tolerance were set at ±100 ppm and ±0.3 Da, respectively.

Table 1 Clinical features of 29 patients with beef allergy

Case No.	Age (year)	Gender	Symptom(s)*	Causative food(s)	Time to reaction (h)**	CAP-FEIA (kUa/l)			
						Beef	Pork	Chicken	Cetuximab
1	69	M	U, A	Beef innards and alcohol	3	7.83	6.95	<0.35	48.8
2	71	F	U	Beef meat	1	3.29	0.77	<0.35	26.3
3	69	F	U	Beef meat	2	39.1	32.6	<0.35	60.7
			U, V, AS	Beef and pork meat	2				
			U, AS	Pork meat	1.5				
4	58	F	U	Beef and pork meat	Unknown	1.83	1.28	n.d.***	16.8
			U	Beef meat	1.5				
			U	Beef innards	3				
			U	Beef and pork meat	0.5				
5	47	F	U, D	Beef meat	Unknown	3.35	3.65	<0.35	8.07
6	70	M	U	Beef and pork meat	Unknown	20.7	15.3	<0.35	75.5
7	59	M	U	Beef, pork, and alcohol	6	26.9	23.7	n.d.	86.4
8	68	M	U	Pork meat	5	26.1	21.3	<0.35	60.1
9	78	M	U	Boar meat	Unknown	2.62	1.23	<0.35	n.d.
10	70	F	U	Beef meat	1	1.93	1.72	<0.35	4.03
11	65	M	U	Beef and pork meat	Unknown	30	20.2	<0.35	122
12	68	F	A	Beef and pork meat	Unknown	5.64	4.10	<0.35	65.8
			U, A, D	Beef innards	Unknown				
13	64	M	U	Beef and pork meat	Unknown	2.23	1.94	<0.35	7.27
14	53	F	U, AS	Beef and pork meat	4	0.94	2.64	<0.35	31.0
			U, D	Beef and pork meat	4				
			A, D	Pork meat	7				
15	57	M	U	Chinese noodles	4	32.2	11.9	<0.35	67.6
			U	Pork meat and alcohol	2				
			U, V, A	Beef innards and alcohol	2.5				
16	70	F	D	Beef meat	Unknown	12.4	9.12	<0.35	75.3
17	86	M	U	Beef and pork meat	Unknown	6.27	6.28	n.d.	10.0
18	65	M	U	Raccoon dog meat	2	2.7	1.69	<0.35	30.0
			U	Beef meat	2				
			U	Boar meat	4.5				
			U	Horse meat	Unknown				
			U	Boar meat	1.5				
19	67	M	U	Beef meat	1–1.5	8.49	8.17	<0.35	13.8
			U	Beef meat	Unknown				
20	60	F	U	Beef meat	Unknown	18.7	6.07	<0.35	89.7
			U	Beef meat	Unknown				
21	66	M	U	Beef innards	Unknown	1.86	1.29	<0.35	2.99
			U, D	Beef meat	Unknown				
			U	Pork meat	4				
			U	Beef and pork meat	Unknown				
			U	Pork meat	Unknown				
22	55	F	U	Beef meat and alcohol	5.5	31.2	14.5	<0.35	257
			U	Beef meat and alcohol	7.5				
23	64	F	U	Beef and pork	Unknown	0.94	0.69	<0.35	18.1
			U	Pork meat	Unknown				
24	64	F	U	Beef meat	4	16.4	7.44	<0.35	54.3
25	81	M	U	Beef meat	Unknown	6.37	5.32	<0.35	10.0
			U	Beef and pork meat	Unknown				
			U	Pork meat	1				
26	76	M	U	Beef meat	Unknown	6.41	3.72	<0.35	20.3

Table 1 (Continued)

Case No.	Age (year)	Gender	Symptom(s)*	Causative food(s)	Time to reaction (h)**	CAP-FEIA (kUa/l) Beef	Pork	Chicken	Cetuximab
27	78	M	U	Beef, pork, mutton, chicken meat and alcohol	Unknown	6.38	3.16	<0.35	n.d.
28	59	M	U	Beef liver and chicken meat	Unknown	58.4	n.d.	n.d.	103
29	57	M	U	Beef and alcohol	2	19.5	13.0	n.d.	84.1

*Symptom(s), U; urticaria, A; abdominal pain, V; vomiting, D; diarrhea, AS; anaphylactic shock.
**Time to reaction, time (h) taken for allergic symptom(s) to develop after eating causative food(s).
***n.d., not determined.

Sequence analysis

A homology search of proteins identified from the amino acid sequences was performed with BLASTp on the NCBI nonredundant protein sequence database. A conserved domain search was also performed on the NCBI platform.

Periodate treatment

Water-soluble beef proteins were separated on 7.5% SDS-PAGE under reducing conditions and electrophoretically transferred to a PVDF membrane. Membranes were incubated with a solution containing 50 mM sodium acetate (pH 4.5) and 20 mM sodium periodate for 1 h in the dark and further incubated with patient sera after washing with TBST. Bound IgE was detected as described above.

Inhibition assay by immunoblotting

Serum was diluted to 10% with blocking solution and incubated with different amounts of cetuximab (10, 100 μg) as an inhibitor, or PBS as control for 1 h at 37°C. The pre-incubated serum was reacted with water-soluble beef proteins electroblotted onto the PVDF membrane for 16 h at 28°C. Bound IgE was detected as described above.

Preparation of cetuximab CAP-FEIA

To prepare biotin-labeled cetuximab, cetuximab solution was dialyzed with PBS using the HiTrap desalting column (5 ml, GE Healthcare). The resultant solution, containing 3.3 mg of cetuximab, was labeled with 53.2 mmol biotin using the EZ-link sulfo-NHS-LC biotin reagent (Thermo Fisher Scientific, Waltham, MA, USA) for 30 min at 28°C. Subsequently, unreacted biotin was removed by passing through the HiTrap desalting column. Protein concentrations were determined using the RC DC protein assay kit (Bio-Rad). Cetuximab biotinylation was confirmed by dot blot analysis using HRP-conjugated streptavidin and the ECL kit. To construct cetuximab CAP-FEIA, 5 μg of biotinylated cetuximab was bound to the streptavidin CAP-FEIA system (9, 17). The IgE value to cetuximab was compared with that to beef using the Spearman's rank order correlation test. Statistical analysis was performed with SPSS software, version 20 (IBM Corp., Armonk, NY, USA). A two-sided P value of less than 0.05 was considered to indicate statistical significance.

Results

IgE immunoblot analysis of meat proteins

Serum IgE binding to water-soluble beef proteins was examined by immunoblotting in 29 patients with beef allergy. Multiple bands were detected in all patients, as shown in Fig. 1A, and two bands were commonly seen at 240 kDa and 140 kDa. When serum IgE binding to the water-soluble and water-insoluble proteins of pork and chicken were analyzed by immunoblotting using sera from four patients (case Nos 1, 3, 5, and 7), the 240-kDa band was detected commonly with the water-soluble proteins of pork, but not with those of chicken (Fig. 1B). In contrast, the 140-kDa band in water-soluble proteins of pork was detected only in the serum of patient No. 3. The 140-kDa band seen in the water-insoluble proteins of beef and pork was nonspecific because this band was also seen in sera of healthy subjects.

Identification of the 240-kDa and 140-kDa allergens as laminin γ-1 and the collagen α-1 (VI) chain

On 2D-PAGE, two dominant IgE-binding spots were detected using sera from two patients (case Nos 1 and 3) at 240 kDa and pI 5 (spot 1) and at 140 kDa and pI 5.5 (spot 2) (Fig. 2A). The position of each spot was identical in the sera of the patients tested. IgE-binding spots were not detected in the sera of two healthy subjects (data not shown). The 240-kDa and 140-kDa protein spots were analyzed with PMF and MS/MS ion search using MALDI-TOF MS. MASCOT scores were significant in both PMF and MS/MS analyses (Table 2), and spots 1 (240 kDa) and 2 (140 kDa) were identified as laminin γ-1 and the collagen α-1 (VI) chain from *Bos taurus*, respectively. Other spots detected in the low molecular area by immunoblotting could not be identified because these proteins were not separated clearly (Fig. 2B).

Homology analysis showed that laminin γ-1 from *B. taurus* has significant homology with those from *Sus scrofa*, human, and *Gallus gallus* (Table 3). The collagen α-1 (VI) chain from *B. taurus* also has significant homology with those from human and *G. gallus* (Table 3). The homology between

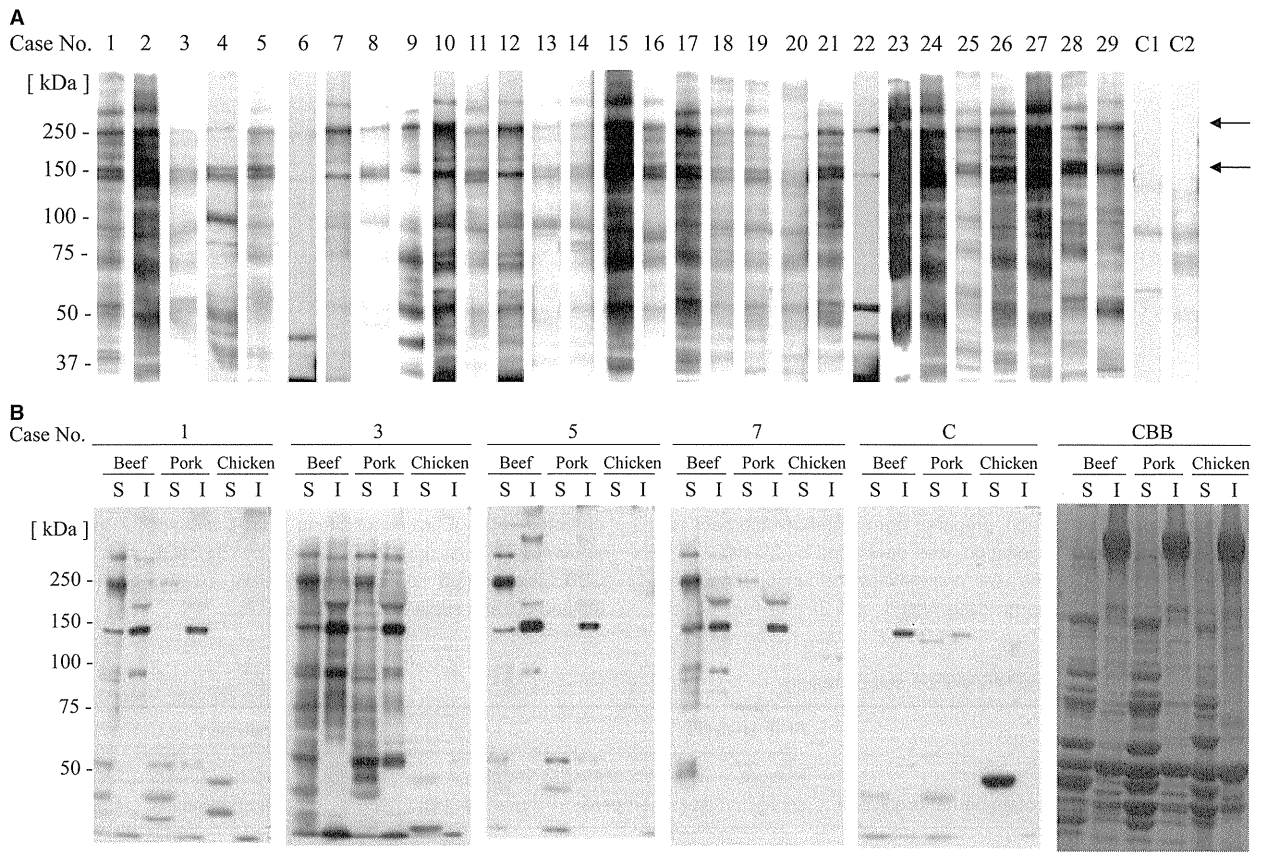

Figure 1 IgE immunoblotting of meat proteins. (A) IgE binding to water-soluble beef proteins was analyzed by immunoblotting using sera from all patients (case Nos 1–29). As a negative control, sera of healthy subjects were used (C1 and C2). Arrows indicate the 240-kDa and 140-kDa proteins. (B) Water-soluble proteins (S) and water-insoluble proteins (I) of beef, pork, and chicken were separated by SDS-PAGE. IgE-binding proteins were detected using patient sera (case Nos 1, 3, 5, and 7). As a negative control, sera from healthy subjects were used (C). Total protein was stained with Coomassie Brilliant Blue (CBB).

laminin γ-1 and the collagen α-1 (VI) chain from *B. taurus* was low, and identity score and similarity were 30% (in a 43-amino acid overlap) and 69% (in a 43-amino acid overlap), respectively. Furthermore, the domain structure was not identical between laminin γ-1 and the collagen α-1 (VI) chain from *B. taurus*.

Determination of glycosylation in laminin γ-1 and the collagen α-1 (VI) chain

Because laminin γ-1 and the collagen α-1 (VI) chain are known to be glycoproteins (18–20), water-soluble beef proteins separated by SDS-PAGE were immunoblotted with anti-α-Gal monoclonal antibody. Proteins bearing α-Gal were detected at 240 kDa and 140 kDa, corresponding to the positions of laminin γ-1 and the collagen α-1 (VI) chain (Fig. 3A). To investigate whether IgE binding to laminin γ-1 and the collagen α-1 (VI) chain was directed against carbohydrate, PVDF-blotted water-soluble beef proteins were treated with periodate (Fig. 3B; + lanes) before the addition of sera from patients with beef allergy (case Nos 1, 3, 5, 7, and 9). Periodate treatment caused the disappearance of the 240-kDa and 140-kDa IgE-binding bands (Fig. 3B).

To confirm that IgE bound to laminin γ-1 and the collagen α-1 (VI) chain through α-Gal, an IgE inhibition test was performed with cetuximab as the inhibitor, which is known to have α-Gal on its heavy chain. Pre-incubation of patients' sera with cetuximab resulted in the complete disappearance of IgE binding to the 240-kDa and 140-kDa bands (Fig. 3C). In addition, all patients with a beef allergy had IgEs that reacted with cetuximab (Table 1), and their binding values to cetuximab were well correlated with those to beef ($r = 0.77$, $P < 0.001$; Fig. 3D).

Discussion

In this study, we identified laminin γ-1 and the collagen α-1 (VI) chain from *B. taurus* as beef allergens and revealed that α-Gal in these allergens was responsible for IgE binding. As values of IgE reacting with beef were well correlated with those of that with cetuximab, α-Gal is possibly the major IgE-binding determinant in the patients studied. In addition,

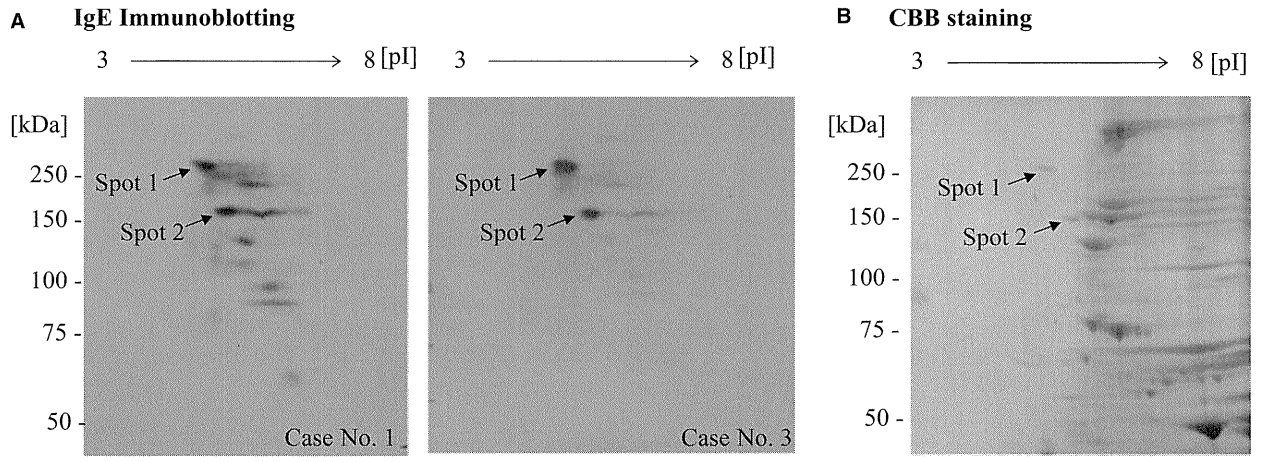

Figure 2 IgE immunoblotting of 2D-PAGE-resolved beef proteins. (A) The allergen-rich fraction, prepared from water-soluble beef proteins by ammonium precipitation, was developed by 2D-PAGE. The binding of IgE to proteins was analyzed by immunoblotting using the sera from two patients (case Nos 1 and 3) and two healthy subjects after 2D-PAGE. Two dominant IgE-binding proteins were detected with IgE immunoblotting (spot 1 and spot 2, indicated by arrows). (B) Total protein developed by 2D-PAGE was stained by CBB. The protein spots were manually excised from the CBB-stained gel and subjected to MS analysis.

Table 2 PMF analysis and MS/MS ion search results

			PMF analysis			MS/MS ion analysis		
Spot No.	Accession number	Protein name (organism)	Mascot score	Matched peaks/ searched peaks	Sequence coverage (%)	Mascot score	Matched peaks/ searched peaks	Sequence coverage (%)
1	XP_611689	Laminin γ-1 (*Bos taurus*)	82	16/45	11	101	4/52	3
2	DAA32939	Collagen α-1 (VI) chain (*B. taurus*)	100	9/11	9	58	3/15	9

Table 3 Homology to laminin γ-1 and the collagen α-1 (VI) chain from *Bos taurus*

	Laminin γ-1				Collagen α-1 (VI) chain			
Organism	Identity (%)*	Similarity (%)*	Accession number	Amino acids	Identity (%)**	Similarity (%)**	Accession number	Amino acids
Sus scrofa	96	97	NP_001258644	1608	Unknown	Unknown	Not recorded	Unknown
Human	94	97	NP_002284.3	1609	91	95	NP_001839	1028
Gallus gallus	79	89	XP_001234659	1603	70	80	NP_990438	1019

*Homology to laminin γ-1 from *B. taurus* (accession number, XP_611689).
**Homology to collagen α-1 (VI) chain from *B. taurus* (accession number, DAA32939).

IgE from patient sera reacted to pork proteins, but not to chicken proteins, which is consistent with the patient's history of allergic episodes.

Whereas all patients' sera reacted commonly with the 240-kDa and 140-kDa proteins, multiple bands were detected in the low molecular area of the patients' sera (Fig. 1A). These bands were not recognized by anti-α-Gal antibody (Fig. 3A), but were diminished by peroxidase treatment and cetuximab treatment (Fig. 3B,C). We speculate that these are carbohydrate moieties mimicking α-Gal. These results indicate that laminin γ-1 and the collagen α-1 (VI) chain are the predominant IgE-reactive proteins, whereas other proteins might be involved in causing beef allergy. BSA, bovine IgG, and actin have been reported as allergens in patients with atopic dermatitis who experienced allergic reactions after consuming cow's milk and meat such as beef or pork (5, 8, 21). In the present study, IgE-reactive bands corresponding to bovine IgG (50 kDa) and actin (42 kDa) were observed in several patients upon IgE immunoblotting (Fig. 1), suggesting that bovine IgG may also be involved in the IgE-mediated allergic reactions seen after the ingestion of beef in these patients because the presence of α-Gal in bovine IgG has been reported (22).

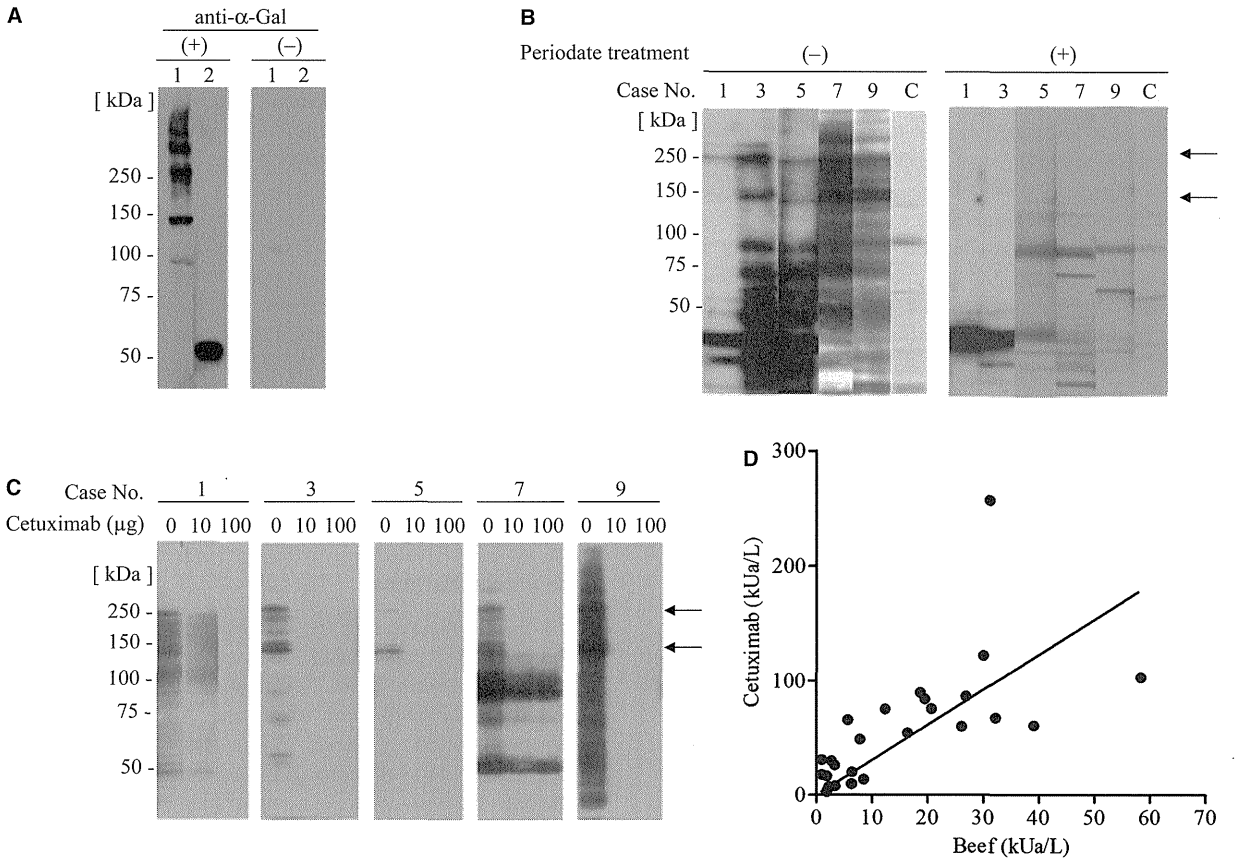

Figure 3 IgE reactivity to α-Gal on allergens. (A) Water-soluble beef proteins (lane 1) and cetuximab (lane 2) were separated by SDS-PAGE. The carbohydrate α-Gal was detected by immunoblotting using anti-α-Gal antibody (anti-α-Gal) (+). (B) Inhibition of IgE binding to beef proteins by periodate treatment. PVDF-blotted water-soluble beef proteins were incubated with a solution containing periodate (+) or without periodate (−), and were then reacted with patient sera (Case Nos. 1, 3, 5, 7, and 9). The serum of healthy subject was used as a negative control (C). IgE-binding proteins were visualized by IgE immunoblotting. Arrows indicate bands corresponding to the position of laminin γ-1 and the collagen α-1 (VI) chain from *Bos taurus*. (C) Water-soluble beef proteins (25 μg) were separated by SDS-PAGE and transferred to a PVDF membrane. Sera of patients (case Nos 1, 3, 5, 7, and 9) were preincubated with cetuximab (10 and 100 μg). IgE-binding proteins were detected by IgE immunoblotting. Arrows indicate bands corresponding to the position of laminin γ-1 and the collagen α-1 (VI) chain. (D) Specific IgE values to beef and cetuximab were measured in patient sera ($n = 27$) by CAP-FEIA. IgE values specific to beef and cetuximab are plotted on the x- and y-axes, respectively.

The IgE in patients' sera reacted with laminin γ-1 and the collagen α-1 (VI) chain and was inhibited with cetuximab; however, their homology is low, indicating that α-Gal on both proteins is the IgE determinant. These data are supported by the fact that IgE from patient sera did not recognize human laminin γ-1 (data not shown), although human laminin γ-1 has high amino acid sequence homology to laminin γ-1 from *B. taurus*. These data are explained by the fact that the gene encoding α-1,3-galactosyl transferase to synthesize α-Gal on the carbohydrate chain is not functional in humans, apes, and old-world monkeys; therefore, these species cannot produce α-Gal (23).

The actual molecular size of laminin γ-1 (240 kDa) was much larger than the estimated molecular size from its amino acid sequence (140 kDa), indicating heavy glycosylation with N-linked oligosaccharides, including α-Gal (24), which is consistent with a previous report by Maruyama et al. (25), indicating that bovine and pig laminin contain α-Gal. The collagen α-1 (VI) chain is one of the three subunits (α-1, α-2, and α-3) that comprise a heterotrimer glycoprotein (20, 26). Our results are consistent with the fact that collagen α-1 (VI) is highly glycosylated and exists mainly in the basement membrane (27), although the linking of α-Gal to the collagen α-1 (VI) chain has not been reported. It is known that bovine collagen α-1 (IV) contains α-Gal (28), but collagen α-1 (IV) was not detected in this study, which may be due to lower collagen α-1 (IV) content in our meat preparation.

According to EST profiles in the NCBI UniGene database, each gene encoding laminin γ-1, the collagen α-1 (VI) chain, and α-1,3-galactosyl transferase is expressed in the kidney, liver, muscle, and intestine, suggesting that almost all organs derived from *B. taurus* are allergenic. These data also explain

the clinical finding that some patients with beef allergy had an allergic reaction upon ingestion of grilled beef intestine.

Commins et al. described that characteristic features of red meat allergy are adult onset and have a delayed (3–5 h) appearance of the allergic reaction to mammalian meats (12). These features are consistent with our patients; 21 of 29 patients were over 60 years of age, and 10 of the 20 patients reacted to mammalian meats with a delay. We do not clarify the reason of this characteristic features in this study. Although some patients had an allergic reaction within 2 h, we speculate that the time to the reaction depends on the condition of the patients, mainly the condition of gastrointestinal tract, and not on the specific features of the patient. Similar observations were reported by Morisset et al. (14), where some patients developed an anaphylactic reaction within 2 h after ingesting meat or kidney.

A possible relationship of exposure to tick bites and adult-onset red meat allergy has been reported in the United States and Europe (29, 30). The report from the United States shows that the distribution of patients with Rocky Mountain spotted fever matches those of patients with red meat allergy who have specific IgEs against α-Gal (29); thus, bites from adult or larval ticks of the species *Amblyomma americanum* are suspected. Recently, Hamsten et al. (30) have reported that Swedish patients with red meat allergy have an IgE response to α-Gal, and the α-Gal epitope is present in the gastrointestinal tract of *Ixodes ricinus*, a common European tick. In Japan, Japanese spotted fever and Tsutsugamushi disease are well known as being due to tick infestation and are distributed widely. In this study, however, patient clinical information regarding tick bites was insufficient to examine the association between tick bites and beef allergy.

In conclusion, the carbohydrate moiety (α-Gal) on laminin γ-1 and the collagen α-1 (VI) chain are possibly common IgE-reactive proteins in the Japanese patients with beef allergy. Although the involvement of ticks in beef allergy has not been established in Japan, clinicians need to be aware of a possible association between tick bites and beef allergy.

Acknowledgments

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Author contributions

All authors contributed to the manuscript. Hitoshi Takahashi performed most experiments and wrote the article. Yuko Chinuki and Eishin Morita evaluated the clinical findings, collected serum, and made diagnoses. Akira Tanaka measured the amount of serum-specific IgE values by CAP-FEIA. Eishin Morita thoroughly supervised the work and participated in writing of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest for the work presented in this study.

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ORIGINAL ARTICLE

Serum Gliadin Monitoring Extracts Patients with False Negative Results in Challenge Tests for the Diagnosis of Wheat-Dependent Exercise-Induced Anaphylaxis

Kunie Kohno[1], Hiroaki Matsuo[2], Hitoshi Takahashi[1], Hiroyuki Niihara[1], Yuko Chinuki[1], Sakae Kaneko[1], Tsutomu Honjoh[3], Tatsuya Horikawa[4], Shoji Mihara[5] and Eishin Morita[1]

ABSTRACT

Background: Challenge testing with wheat plus exercise and/or aspirin is a gold standard for the diagnosis of wheat-dependent exercise-induced anaphylaxis (WDEIA); however, the test may often yield false-negative results. Our previous study suggested that an increase in serum wheat gliadin levels is required to induce allergic symptoms in patients with WDEIA. Based on this knowledge, we sought to extract the patients with false negative results in the challenge tests of WDEIA.

Methods: Thirty-six patients with suspected WDEIA were enrolled. First, group categorizations—Group I, challenge tests were positive; Group II, challenge tests were negative and serum gliadin were undetectable; Group III, challenge tests were negative and serum gliadin were detectable—were given according to the results of wheat plus exercise and/or aspirin challenge testing and serum gliadin levels. Second, diagnoses were made using retests and/or dietary management in Group II and III.

Results: Positive results for wheat plus exercise and/or aspirin challenge tests gave a diagnosis of definite WDEIA in 17 of 36 patients (Group I). Of the remaining 19 challenge negative patients, serum gliadin was undetectable in ten patients (Group II). Of the ten patients (Group II), three of them were diagnosed as definite WDEIA by retesting and six of them were diagnosed as probable WDEIA using a wheat elimination diet, whereas one patient was non-WDEIA. In the rest of the nine challenge negative patients, serum gliadin was detectable (Group III). No allergic episodes with a normal diet provided a diagnosis of non-WDEIA in seven of the nine patients, whereas the remaining two patients were probable WDEIA or had another food allergy because of repeated episodes.

Conclusions: Our study revealed that serum gliadin monitoring during challenge testing is useful.

KEY WORDS

challenge test, serum gliadin monitoring, wheat-dependent exercise-induced anaphylaxis, ω-5 gliadin

[1]Department of Dermatology, Faculty of Medicine, Shimane University, Shimane, [2]Department of Pathophysiology and Therapeutics, [5]Department of Dermatology, Programs for Biomedical Research, Division of Molecular Medical Science, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, [3]Morinaga Institute of Biological Science Inc., Kanagawa and [4]Department of Dermatology, Nishi-Kobe Medical Center, Hyogo, Japan.

Authors' contributions: KK, HM and EM conceived the experiments. KK performed the experiments, and analyzed the data together with HT, HM. Recruitment of patients and the challenge testing were performed by EM, HN, SK, YC, TH, SM and EM. TH prepared the serum gliadin detection kit. KK, HM and EM co-wrote the paper. All authors discussed the results and commented on the manuscript.

Conflict of interest: TH is employed by Morinaga Institute of Biological Science Inc.. The rest of the authors have no conflict of interest.

Correspondence: Kunie Kohno, Department of Dermatology, Faculty of Medicine, Shimane University, 89–1 Enya-cho, Izumo, Shimane 693–8501, Japan.
Email: nine_kk@med.shimane-u.ac.jp

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ABBREVIATIONS
FDEIA, food-dependent exercise-induced anaphylaxis; HMW-glutenin, high-molecular-weight glutenin subunit; NSAIDs, non-steroidal anti-inflammatory drugs; WDEIA, wheat-dependent exercise-induced anaphylaxis.

INTRODUCTION

Food-dependent exercise-induced anaphylaxis (FDEIA) is a severe form of food allergy, in which symptoms are elicited by exercise in combination with ingestion of the culprit food.[1-3] Other factors that elicit allergic symptoms in addition to exercise include non-steroidal anti-inflammatory drugs (NSAIDs), alcohol, patient's general conditions and atmospheric conditions.[3-5] In Japanese patients, the most frequent cause of FDEIA is wheat, which is found in almost 60% of culprit foods.[3,6] In contrast, vegetables are the most frequent culprit food in European cases of FDEIA.[7]

Diagnosis of FDEIA is made based on detailed interviews, allergy skin testing, detection of serum allergen-specific IgE, and challenge testing. In the case of wheat-dependent exercise-induced anaphylaxis (WDEIA), skin testing with commercial wheat extract is not always reliable because the wheat extract consists largely of water-soluble proteins and only a trace amount of water-insoluble proteins (gluten) that are the major cause of WDEIA.[8] A specific IgE test with recombinant ω-5 gliadin is highly reliable and now widely used to identify the patients with WDEIA. However, the test did not positively identify approximately 20% of cases.[9-13] The exercise challenge test combined with wheat ingestion is the most definitive test to diagnose WDEIA, if the test elicits allergic symptoms. However, WDEIA, cannot be excluded when the challenge test is negative, because the challenge tests are not always successful. The success of a test is highly dependent on the amount of wheat, intensity of exercise and the condition of the patients.[3,14] Therefore, a negative test may turn out to be a false-negative result.

In a previous study, we found that gliadin concentrations markedly increased in the sera of patients with WDEIA in parallel with allergic symptoms during positive challenge testing.[15] We also demonstrated that aspirin, a well-documented trigger of WDEIA even in patients without a history of exacerbation due to NSAIDs,[5,6] had an ability to increase the serum concentration of gliadin when taken before wheat ingestion.[16] These observations indicated that both exercise and aspirin intake cause an absorption of non-digested gliadin from the intestine into the blood circulation. In addition, we found that the increase in serum gliadin also occurred in healthy subjects.[15,16] Taken together, we consider that the amount of allergen-absorbed via the intestine is critical for eliciting the allergic symptoms of WDEIA seen after wheat ingestion.

Herein, we propose a new index to determine whether there was a sufficient challenge to cause gliadin absorption, by combining wheat plus exercise and/or aspirin challenge testing with serum gliadin monitoring.

METHODS

SUBJECTS

Thirty-six patients (17 female and 19 male; mean age, 34.9 years; range, 9-71 years), who experienced allergic symptoms after ingesting wheat-containing foods in combination with exercise and/or NSAIDs were enrolled in this study. Table 1 lists the patient characteristics, clinical histories, total IgE and specific IgE levels for wheat, gluten, ω-5 gliadin and high-molecular-weight glutenin subunit (HMW-glutenin). Twenty-nine of the 36 patients experienced more than three allergic episodes (patients 1-18, 20-25, 27-29, 33 and 35), two patients had two episodes (patient 26 and 34) and five had one episode (patients 19, 30-32, and 36). Clinical histories included urticaria, generalized erythema, palpebral edema, sneezing, laryngeal edema, dyspnea, nausea and anaphylaxis. All patients provided informed consent prior to participation in the study. Approval for the study was obtained from the Ethics Committee of the Faculty of Medicine at Shimane University (No. 121).

MEASUREMENT OF SERUM ALLERGEN-SPECIFIC IgE

IgE antibodies specific for wheat, gluten, ω-5 gliadin and HMW-glutenin were detected using the CAP-fluorescent-enzyme immunoassay (ImmunoCAP, Phadia, Uppsala, Sweden) as previously described.[10,11] Values >0.35 kU_A/L were judged to be positive in this study.

CHALLENGE TESTS

Challenge tests for WDEIA were performed as previously described.[15] The tests involved wheat ingestion, exercise, pretreatment with aspirin or a combination of these challenges on a separated day (Fig. 1). Briefly, the wheat products included noodles, bread or pasta containing 60-135 g of wheat flour. Thirty minutes after wheat ingestion, exercise was performed using a treadmill for 15-20 min according to the standard Bruce protocol. Aspirin (500 mg) was administrated 30 min prior to wheat ingestion. A positive test was determined if allergic symptoms such as wheals and/or anaphylaxis were observed. During the challenge tests, 9 mL of blood was drawn at each time point via an indwelling needle. Patients with

Table 1 Patient characteristics and clinical and laboratory data

Group	Patient No.	Age (years)	Gender	Episodes	Symptoms during episodes	Total IgE (kIU/L)	Specific IgE (kUA/L)			
							Wheat	Gluten	ω-5 gliadin	HMW-glutenin
Group I (n = 17)	1	32	M	>3	Anaphylaxis, Urticaria	146	<0.35	<0.35	5.35	<0.35
	2	61	M	>3	Anaphylaxis, Systemic urticaria	45	1.76	1.55	9.08	<0.35
	3	36	F	>3	Anaphylaxis, Urticaria	201	0.42	0.65	12.3	<0.35
	4	16	M	>3	Anaphylaxis, Systemic urticaria	80	<0.35	<0.35	3.72	<0.35
	5	44	M	>3	Anaphylaxis, Systemic urticaria	150	<0.35	<0.35	5.45	<0.35
	6	52	F	>3	Anaphylaxis, Urticaria, Dyspnea	260	0.37	0.66	5.15	<0.35
	7	15	M	>3	Dyspnea, Urticaria	590	<0.35	2.10	<0.35	<0.35
	8	17	M	>3	Anaphylaxis, Urticaria	602	1.34	0.52	13.1	<0.35
	9	43	F	>3	Anaphylaxis, Systemic urticaria	105	<0.35	<0.35	2.14	<0.35
	10	25	F	>3	Anaphylaxis, Systemic urticaria	1007	1.32	2.84	16.3	<0.35
	11	19	M	>3	Anaphylaxis, Wheezing, urticaria	109	<0.35	<0.35	<0.35	3.53
	12	38	F	>3	Anaphylaxis, Systemic urticaria	660	0.66	1.52	4.22	<0.35
	13	23	M	>3	Dyspnea, Generalized erythema	212	15.3	17.3	<0.35	<0.35
	14	47	F	>3	Anaphylaxis, Urticaria	107	<0.35	<0.35	2.15	-
	15	57	F	>3	Anaphylaxis, Systemic urticaria	292	<0.35	0.51	5.96	<0.35
	16	58	M	>3	Anaphylaxis, Systematic urticaria	22	<0.35	0.51	2.20	<0.35
	17	17	M	>3	Anaphylaxis, Systematic urticaria	2220	13.1	<0.35	<0.35	<0.35
Group II (n = 10)	18	34	F	>3	Anaphylaxis, Generalized erythema	770	0.76	0.90	14.1	<0.35
	19	65	F	1	Systemic urticaria, Dyspnea	1190	0.78	0.90	15.5	<0.35
	20	12	M	>3	Anaphylaxis, Systemic urticaria	126	1.21	<0.35	<0.35	1.36
	21	34	M	>3	Anaphylaxis, Systemic urticaria	5420	4.26	13.5	74.9	<0.35
	22	14	M	>3	Palpebral edema, Systemic urticaria	36	<0.35	<0.35	<0.35	0.42
	23	48	M	>3	Anaphylaxis, Urticaria	2028	<0.35	<0.35	1.63	<0.35
	24	9	M	>3	Anaphylaxis, Systemic urticaria	343	<0.35	<0.35	<0.35	1.72
	25	18	F	>3	Systemic urticaria	20022	11.7	1.06	0.49	0.39
	26	24	F	2	Urticaria	434	<0.35	0.83	5.72	<0.35
	27	24	F	>3	Impetiginous eczema	10001	0.95	<0.35	<0.35	<0.35
Group III (n = 9)	28	40	F	>3	Anaphylaxis, Urticaria, Nausea	111	<0.35	<0.35	2.44	<0.35
	29	28	F	>3	Palpebral and laryngeal edema, Sneeze, Dyspnea	360	0.94	0.44	<0.35	<0.35
	30	28	F	1	Systemic urticaria	278	1.19	1.23	<0.35	<0.35
	31	18	M	1	Dyspnea, Palpebral edema	749	0.50	<0.35	<0.35	<0.35
	32	71	F	1	Anaphylaxis, Systemic urticaria	412	2.17	0.55	<0.35	<0.35
	33	64	M	>3	Anaphylaxis	541	<0.35	<0.35	0.36	<0.35
	34	18	M	2	Anaphylaxis, Systemic urticaria	1699	<0.35	<0.35	<0.35	<0.35
	35	60	M	>3	Anaphylaxis, Systemic urticaria	186	<0.35	<0.35	<0.35	<0.35
	36	48	F	1	Anaphylaxis, Systemic urticaria	160	<0.35	<0.35	<0.35	<0.35

Gender: M; male, F; female.

Anaphylaxis: a condition showing symptoms caused by insufficient blood circulation.

-, not done.

negative challenge test results were divided into either an undetectable (<15 pg/ml) (Group II) or a detectable (≥15 pg/m) (Group III) according to their serum gliadin concentration.

SERUM GLIADIN MONITORING

Serum gliadin concentrations were determined using an ELISA kit for wheat gliadin (Morinaga Institute of Biological Science, Yokohama, Japan) as previously described.[15] Briefly, 0.25 ml of serum was mixed with 0.05 ml deionized water and 0.7 ml ethanol and the mixture was vortexed for 1 min. The mixture was then centrifuged at 20,000 g for 20 min at room temperature. The gliadin-containing supernatant was decanted into a new tube, and evaporated in a vacuum centrifuge evaporator (VEC-260, Iwaki Glass Co.,

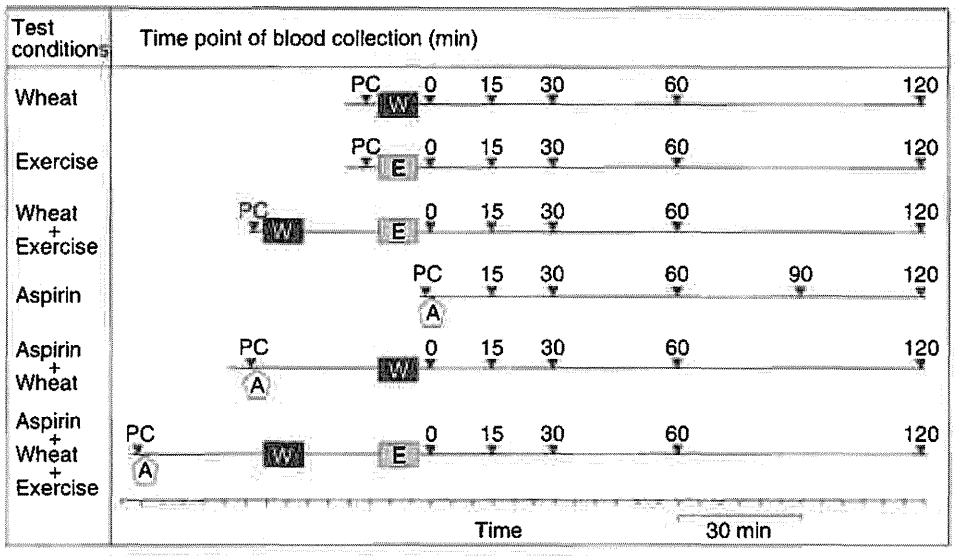

Fig. 1 Challenge test conditions and time-course of blood sampling. Test conditions are shown in the left-hand column and time points for blood collection in the right-hand column. PC, pre-challenge; W in a black square indicates wheat ingestion; E in a gray square indicates exercise; A in a white pentagon indicates aspirin ingestion; the black inverted triangle indicates point of blood sampling.

Ltd., Chiba, Japan). The dried residue was dissolved in 0.05 ml of the sample dilution buffer included in the kit and the gliadin concentration measured according to the manufacturer's instructions. The gliadin concentration in each sample was calculated using a gliadin standard (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan). All measurements were performed in duplicate. The detection limit of serum gliadin level was 15 pg/mL.

DIAGNOSTIC PROCEDURE

The patients were divided into three groups after challenge testing combined with serum gliadin monitoring as illustrated in Figure 2. Patients who were evaluated as having a positive result in the challenge tests were included in the group of definite WDEIA (Group I). Patients with negative challenge test results were divided into either an undetectable (Group II) or a detectable (Group III) according to their serum gliadin concentration. The Group II patients were considered insufficiently challenged and were required to undergo retesting. Patient with positive retests were considered as definite WDEIA. Patients with a negative retest, with undetectable serum gliadin, were considered as a failure of retesting and followed with a diet management. Those who declined retests also followed with a diet management. The diagnostic endpoint of the diet management was the clinical outcomes during a 1-year follow-up of wheat elimination diet or exercise-restriction after their meals. If the patient did not experience symptoms during the 1-year follow-up, a diagnosis of probable WDEIA was given. If a patient in this group had recurrent symptoms without wheat plus exercise and/or NSAIDs, a diagnosis of non-WDEIA was given. The Group III patients maintained a normal diet for a year, and during this time their allergic symptoms were carefully monitored. If a patient in this group had no symptoms during the year, a diagnosis of non-WDEIA was given. If a patient in this group had recurrent symptoms with wheat plus exercise and/or NSAIDs, a diagnosis of probable WDEIA or other food allergy was considered.

RESULTS

CHALLENGE TESTS AND SERUM GLIADIN MONITORING

All patients participated in the challenge testing and serum gliadin was monitored throughout the challenge test. The results of the challenge testing and the maximal serum gliadin concentrations are summarized in Table 2. All 36 patients had a negative reaction to a single challenge with wheat, exercise or aspirin. Of the 36 patients, 17 had a positive reaction with either wheat plus exercise, aspirin plus wheat or aspirin plus wheat plus exercise. However, 19 patients had a negative reaction even in the combination challenge of wheat and exercised and/or aspirin.

Representative patterns of serum gliadin kinetics are shown in Figure 3. Figure 3A shows the kinetics of patient 1 who had a positive challenge test with wheat and exercise. A similar pattern was seen for another seven patients (patients 2-7 and 16). Figure 3B shows the kinetics of patient 11, who had a positive

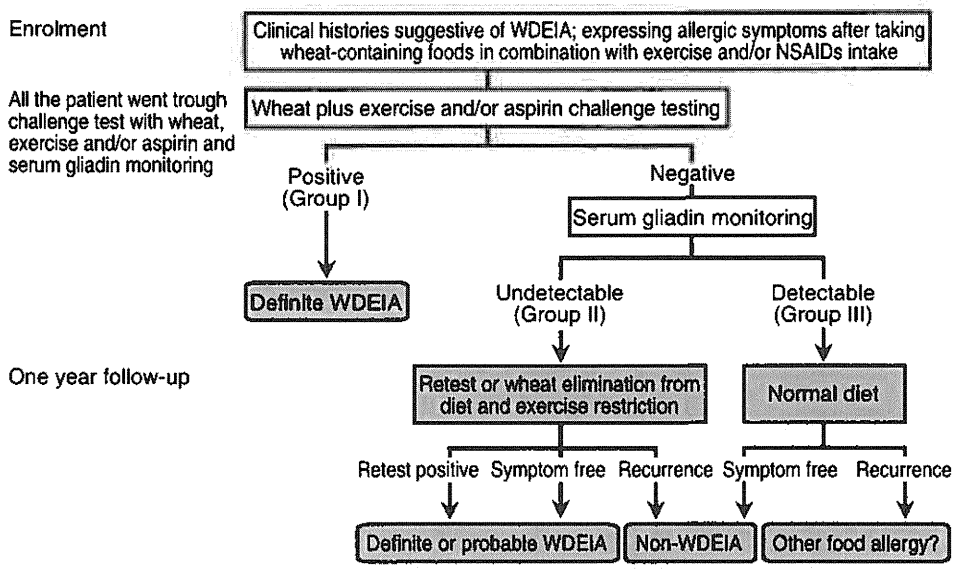

Fig. 2 Diagnostic procedure for WDEIA. Black-framed rectangles indicate criteria for the group categorization; black-framed gray rectangles indicate the follow-up for the diagnosis; rounded gray squares indicate the diagnosis.

challenge test with aspirin and wheat. A similar pattern was seen for another seven patients (patients 6-10, 12 and 16). Figure 3C shows the kinetics of patient 13, who developed allergic symptoms after a triple challenge. This pattern was also observed for patient 14-17. Neither symptoms nor an increase in serum gliadin concentrations were seen under any of the test conditions in ten patients (patients 18-27, Fig. 3D). Serum gliadin was detected in patient 31 even after a negative challenge test with aspirin and wheat (Fig. 3E). A similar pattern was observed for patients 28 and 30-35. In patients 29 and 36, serum gliadin was detected without allergic symptoms after a triple challenge. In the 17 patients with negative challenge testing (patient 18-34), serum gliadin was undetectable in 10 patients (patient 18-27) but was detectable in seven patients (patient 28-36).

CATEGORIZATION OF PATIENTS BY DIAGNOSTIC PROCEDURE

According to the diagnostic procedure, patients were categorized into three groups (Fig. 2). A diagnosis of definite WDEIA was made in 17 patients and they were categorized into Group I (patients 1-17). Those who were negative to wheat plus exercise and/or aspirin challenge with undetectable serum gliadin were categorized into Group II (patients 18-27). Those who had negative challenge testing with detectable serum gliadin concentration were categorized into Group III (patients 28-36).

ELIMINATION DIET TREATMENT AND FINAL DIAGNOSES IN THE GROUP II

Patients in Group II were proceeded to either retest and/or follow a wheat elimination diet. Table 3 summarizes the results. Three patients (patient 18-20) were found to be positive by retesting, whereas patient 21 had negative test. Patient 21 then followed a further elimination diet and experienced recurrence of urticaria with accidental ingestion of wheat product and exercise. Patient 22 visited the emergency room with anaphylactic shock after accidental ingestion of curry, and had a serum gliadin level of 137 pg/mL. The other four patients (patient 23-26) had no allergic symptoms after following a wheat-free diet for more than 1 year, resulting in a diagnosis of probable WDEIA. Patient 27 experienced recurrent symptoms despite wheat elimination and was diagnosed as non-WDEIA. In this procedure, definite WDEIA was diagnosed in three patients, probable WDEIA was diagnosed in six patients, and non-WDEIA was diagnosed in one patient.

NORMAL DIET TREATMENT AND FINAL DIAGNOSIS IN THE GROUP III

A final diagnosis of non-WDEIA was given for 7/9 patients (patients 30-36) in Group III who experienced no symptoms after a normal diet for more than 1 year. Patient 28, who experienced repetitive episodes after a combination of wheat and exercise, was given a final diagnosis of probable WDEIA. In patient 28, challenge testing and serum gliadin monitoring excluded WDEIA. Because she has ω-5 gliadin specific IgE (2.44 kUA/L), which is higher than the cutoff

Table 2 Challenge test and serum gliadin results during testing

Group	Patient No.	Wheat		Exercise		Wheat + Exercise		Aspirin		Aspirin + Wheat		Aspirin + Wheat + Exercise		Retest (based on the episode)	
		Result	Gliadin	Result	Gliadin	Result	Gliadin	Result	Gliadin	Result	Gliadin	Result	Gliadin	Result	Gliadin
Group I (n = 17)	1	N	ud	N	ud	P	127	-	-	-	-	-	-	-	-
	2	N	ud	N	ud	P	216	-	-	-	*	*	*	*	*
	3	N	ud	N	ud	P	15	N	-	N	*	*	*	*	*
	4	N	-	N	-	P	27	-	-	-	-	-	*	*	*
	5	N	-	N	-	P	65	N	-	N	-	P	-	*	*
	6	N	ud	N	ud	P	17	N	-	P	43	-	*	*	*
	7	N	16	N	ud	P	96	N	-	P	15	*	*	*	*
	8	N	ud	N	ud	N	18	N	ud	P	18	*	*	*	*
	9	N	30	-	-	-	-	N	-	P	132	-	-	-	-
	10	N	25	-	-	-	-	N	ud	P	76	-	-	*	*
	11	N	18	-	-	-	-	N	ud	P	107	-	-	*	*
	12	N	ud	-	-	-	-	-	-	P	200	-	-	-	-
	13	N	ud	N	ud	N	ud	N	ud	N	ud	P	18	*	*
	14	N	ud	-	-	N	ud	N	-	N	ud	P	19	*	*
	15	N	-	-	-	N	ud	-	-	-	-	P	91	*	*
	16	N	-	N	ud	P	53	N	-	P	52	P	122	*	*
	17	N	-	N	-	N	-	-	-	-	-	P	333	-	-
Group II (n = 10)	18	N	ud	-	-	-	-	-	-	N	ud	-	-	P	18
	19	N	ud	-	-	N	ud	N	-	N	-	N	ud	P	ud
	20	N	ud	-	-	N	ud	-	-	-	-	N	ud	P	62
	21	N	ud	-	-	-	-	-	-	N	ud	-	-	N	ud
	22	N	ud	-	-	-	-	N	ud	N	ud	*	*	*	-
	23	N	ud	-	-	N	ud	-	-	N	ud	*	*	*	*
	24	N	ud	N	ud	N	ud	-	-	-	-	*	*	*	*
	25	N	ud	-	-	-	-	-	-	N	ud	*	*	*	*
	26	N	ud	N	ud	N	ud	-	-	-	-	*	*	*	*
	27	N	-	-	-	-	-	-	-	N	ud	*	*	*	*
Group III (n = 9)	28	N	ud	N	ud	N	ud	-	-	N	20	-	*	*	*
	29	N	ud	N	ud	N	ud	-	-	-	-	N	29	*	*
	30	N	ud	-	-	N	ud	-	-	N	133	-	*	*	*
	31	N	ud	-	-	-	-	N	ud	N	61	-	*	*	*
	32	N	35	-	-	-	-	-	-	N	70	-	-	-	-
	33	N	ud	-	-	-	-	N	-	N	31	-	-	*	*
	34	N	-	-	-	-	-	N	-	N	181	-	-	*	*
	35	N	-	-	-	-	-	-	-	N	234	-	-	*	*
	36	N	-	-	-	-	-	-	-	-	-	N	255	*	*

Gliadin, serum gliadin concentration (pg/mL); P, positive; N, negative; ud, under the detection limit; -, not done.

Patient 18 showed urticaria when exercising after ingesting a sweet bun. Patient 19 showed urticaria after a combination of bread, aspirin, rebamipide, itopride, and exercise (in accordance with her medical history), although neither rebamipide nor itopride induced symptoms individually. Patient 20 showed urticaria after ingesting bread and exercise. Patient 21 was retested with additional condition; sweet bun with cream was ingested instead of noodles, neither serum gliadin nor symptom were induced.

value we have previously reported, she was considered as probable WDEIA. Although patient 29 must be retested within additional conditions, we could not get the patients cooperation to do so. Patient 29 did not have specific IgEs to ω-5 gliadin nor HMW-glutenin. Thus there is still the possibility that patient has another food allergy.

DISCUSSION

In the present study, we provided evidence that se-

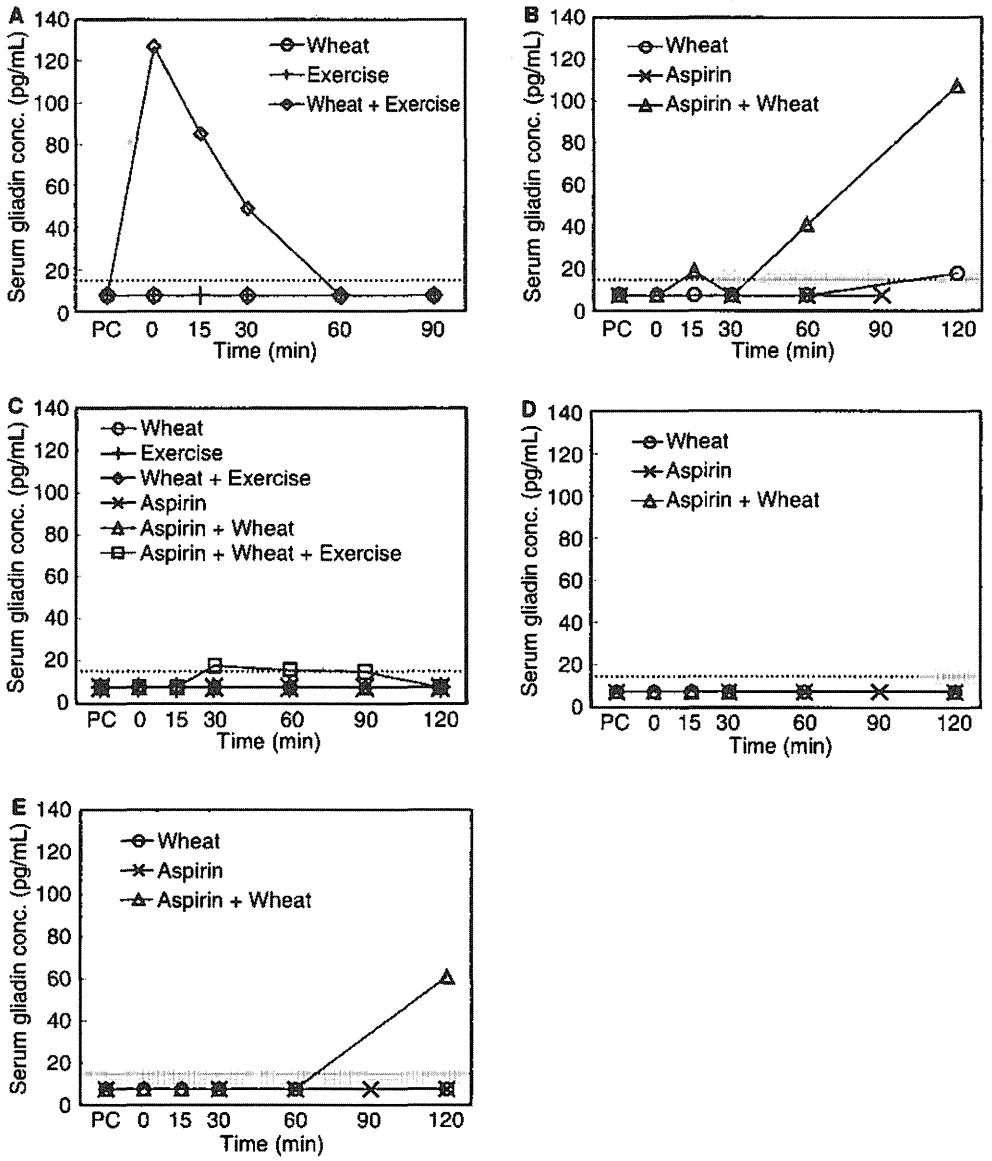

Fig. 3 Time course of serum gliadin kinetics. **A**, **B**, **C**, **D** and **E** show patterns in patients 1, 11, 13, 21 and 31, respectively. Circle, cross, diamond, x, triangle and square indicate challenge with wheat, exercise, wheat + exercise, aspirin, aspirin + wheat and aspirin + wheat + exercise, respectively. The dotted line indicates the detection limit (<15 pg/mL). PC, pre-challenge; conc., concentration.

rum gliadin monitoring is a good tool to use in checking the efficacy of challenge tests for the diagnosis of WDEIA, since a lack of increased serum gliadin can prove an insufficient challenge test. We focused on an extraction of WDEIA-suspected patients who had negative results of the challenge test, because it was believed that an elevation of serum gliadin is required to develop allergic symptoms in WDEIA. As with our previous study, increased serum gliadin levels were observed in the patients with positive challenge testing, supporting research that gliadin levels in the sera correlate with allergic symptoms in WDEIA.[15] The patients who had shown negative challenge testing were divided into two groups based on their serum gliadin levels; patients who had been tested by an efficient challenge with increased serum gliadin level (Group III) and patients who had not been tested efficiently without serum gliadin-increase (Group II). The false-negative challenge performed in Group II was confirmed by the retesting or wheat-elimination diet and the result showed that 9 out of 10 Group II patients were definite/probable WDEIA, whereas the

Table 3 Results of retests or wheat elimination diet of Group II

Patient No.	Retests	Wheat elimination diet	Final diagnosis
18	Positive	Symptom-free	Definite WDEIA
19	Positive	Symptom-free	Definite WDEIA
20	Positive	Symptom-free	Definite WDEIA
21	Negative	Recurrent allergic symptoms were observed during exercise after ingestion of wheat containing meal and exercise.	Probable WDEIA
22	ND	Anaphylaxis was observed after accidental exposure of wheat and exercise.	Probable WDEIA
23	ND	Symptom-free	Probable WDEIA
24	ND	Symptom-free	Probable WDEIA
25	ND	Symptom-free	Probable WDEIA
26	ND	Symptom-free	Probable WDEIA
27	ND	Symptoms were induced in spite of wheat eliminations.	Non-WDEIA

result of normal diet treatment showed that 7 out of 9 Group III patients were non-WDEIA. Thus, the detection of serum gliadin and classification of patients by its level during the challenge testing is of importance for interpreting the negative challenge test result.

The cutoff line between Group II and III were divided according to their serum gliadin level as being undetectable or detectable (15 pg/mL of cutoff value). Although the cutoff line might be debatable, we consider that this is the limitation of our current methodology.

Previously we observed that the absorption of wheat gliadin from the intestine varies according to the individual.[16] This might be because of individual differences in intestinal sensitivity to exercise or aspirin. In the present study, we detected gliadins using anti-gliadin antibodies that bound to several types of gliadin such as α/β-gliadin, γ-gliadin, and ω-gliadin. Tanaka *et al.* recently reported that ω-5 gliadin is absorbed from the gastrointestinal tract more effectively than other wheat components in a mouse model.[17] The same observation was obtained using an *in vitro* caco-2 cell line model.[18] Thus, the specific detection of ω-5 gliadin seemed to provide effective details for the diagnosis of WDEIA. From a practical application standpoint, further study is required to decide the optimum time to measure the serum gliadin and to improve the complicated measurement method.

Retesting with a wheat product other than noodles, was succeeded in three of four patients (patients 18-20). Patients 18 and 20 induced urticaria following a retest that used bread instead of noodles. The difference of challenge test between noodles and bread may be due to amount of gluten contained in the same weight of wheat flour. Usually, wheat flour for making bread contains more gluten than that for making noodles.[14]

A high serum gliadin level (137 pg/mL) was detected during an allergic reaction induced by accidental ingestion of wheat products in patient 22. Serum gliadin was not detected during the negative-challenge tests (Table 2), indicating that this patient was challenged with an insufficient amount of wheat product or needed secondary factors. The lack of allergen absorption in the gastrointestinal tract may explain the failure of challenge tests.

In this study, the diagnosis of WDEIA was made in 27 patients, including seven with probable WDEIA. Of these 27 patients, 19 patients were challenged with wheat plus exercise, and eight had a positive test (Table 2). This shows a positive-induction rate of 42.1% for the wheat plus exercise test. The induction rate of the aspirin plus wheat test was the same as that for the wheat plus exercise test (8/19; 42.1%). However, of the eight patients challenged with aspirin plus wheat plus exercise, six had a positive test (75.0% positive induction rate), indicating an additive effect of aspirin plus exercise. Exercise is known to increase intestinal permeability.[19] Aspirin also increases gastrointestinal permeability[20] and increases serum gliadin levels in a dose-dependent fashion when taken in combination with wheat.[16] The combined effects of NSAIDs plus exercise promote greater gastrointestinal permeability to high-molecular-weight substances.[21,22] The mechanism of NSAID-enhanced gastrointestinal permeability is thought to involve decreased prostaglandin synthesis mediated via the inhibition of cyclooxygenase. This is supported by studies showing that co-administration of prostaglandin E_1 suppresses both allergic symptoms and serum gliadin levels during challenge tests involving aspirin and wheat.[23,24]

Recently, two cases of WDEIA were reportedly induced by aspirin, but not exercise.[25] In our challenge tests, patients 7 and 8 did not show symptoms, despite their serum gliadin concentrations increased after ingesting wheat alone or in combination with exercise. However, symptoms were induced when challenged with aspirin plus wheat, with similar levels of gliadin. These results suggest that co-administration with aspirin may mediate mast cell activation in addi-

tion to increasing gastrointestinal tract permeability.

As we reported previously, HMW-glutenin showed additional diagnostic effectiveness when combined with ω-5 gliadin, because patients who have specific IgE to HMW-glutenin do not have specific IgE to ω-5 gliadin. From the 36 patients in the study, 24/27 (88.8%) of those with WDEIA, and 1/8 (12.5%) of those with non-WDEIA, were positive against ω-5 gliadin- and/or HMW-glutenin-specific IgE tests, respectively. The limitation of the ω-5 gliadin-specific IgE test has been demonstrated by another group.[13] In fact, three patients (patients 7, 13 and 17) with definite WDEIA in the present study did not have IgE against ω-5 gliadin/HMW-glutenin. Patient 33 had specific IgE against ω-5 gliadin (0.36 kUA/L); however, he was diagnosed as non-WDEIA. We have recently shown that the specific IgE test to ω-5 gliadin can produce false positive results in 1.4% of Japanese adults, and that could be the case with patient 33.[26]

In conclusion, serum gliadin monitoring during challenge testing for WDEIA is helpful to extract the patients with false negative results. However further study is needed for the practical application of this method.

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