

Letter to the Editor

Tissue transglutaminase generates deamidated epitopes on gluten, increasing reactivity with hydrolyzed wheat protein-sensitized IgE

To the Editor:

Exercise-induced systemic allergy after wheat ingestion experienced by “Cha no Shizuku” facial soap users has become a serious problem in Japan.^{1,2} The soap contained the acid-hydrolyzed wheat protein (HWP) Glupearl 19S (Glp19S), and soap sensitization can be diagnosed by using Glp19S-specific IgE measurement, a skin prick test (SPT) for Glp19S, or both. HWP-sensitized subjects are generally less responsive to wheat extract on SPTs, although they exhibit obvious clinical symptoms after wheat ingestion and some exercise.¹ However, it is unclear why HWP-sensitized subjects exhibit systemic allergic reactions after ingestion of normal wheat that does not contain HWP.

We recently developed a cell-based IgE reactivity (mast cell-activating) assay that measures IgE crosslinking-induced luciferase expression (EXiLE).^{3,4} This new method demonstrated that the IgE reactivity of the HWP-sensitized subjects to acid-hydrolyzed gluten differed from that of patients with pediatric wheat food allergy (PedWA).⁵ HWP IgE was less reactive to native gluten but strongly reactive to hydrolyzed gluten, whereas IgE from patients with PedWA reacted to both. Binding of IgE of the HWP-sensitized subjects to gluten increased markedly after 30 minutes of 0.1 N hydrochloric acid treatment at 100°C, indicating neopeptide generation on the gluten molecules. These results suggest that orally ingested gluten might be metabolized *in vivo* to acquire cross-reactivity with the industrially generated acid HWP Glp19S.

We therefore compared the IgE reactivities of HWP-sensitized subjects and patients with PedWA using EXiLE assays with gluten treated with gastrointestinal enzymes, such as pepsin, pancreatin, and tissue transglutaminase (tTG). Gluten was treated with these enzymes according to the method of Palosuo et al.,⁶ with some modifications (see the **Methods** section in this article's Online Repository at www.jacionline.org). Clinical profiles of the study subjects are summarized in **Table E1** in this article's Online Repository at www.jacionline.org. Three (60%) of the HWP-sensitized subjects had negative SPT responses to wheat, although all presented with systemic allergic reactions after wheat ingestion. Humanized rat RS-ATL8 mast cells³⁻⁵ were sensitized with 1:100 dilutions of the subjects' sera overnight and stimulated for 3 hours with the treated gluten and Glp19S (Katayama Chemical Industries, Osaka, Japan) suspended in minimum essential medium supplemented with 10% FCS.

Pepsin and pancreatin digestion did not increase the reaction of the gluten with IgEs from either of the subject groups (**Fig 1**). HWP IgE showed low and no responses to intact and digested gluten, respectively, but IgE from patients with PedWA reacted strongly to both. Surprisingly, however, tTG treatment dramatically increased the reactivity of HWP IgE to intact and digested gluten without affecting the response of IgE from patients with PedWA. IgE immunoblotting also showed strong binding of HWP IgE to high-molecular-weight (HMW) components of tTG-treated gluten (tTG-Glu), with a characteristic smear pattern similar to that of Glp19S binding (see **Fig E1** in this article's Online Repository at www.jacionline.org). Although

the IgE of this patient (patient 2; gluten IgE, 36.2 UA/mL) recognized not only HMW components but also a 12- to 13-kDa undigested protein, which was considered to be an α -amylase inhibitor, she was less responsive to natural gluten, as revealed by using the EXiLE test (**Fig 1, A**). Considering that the EXiLE test can distinguish between binding and cross-linking of IgE with the antigen,^{3,4} it appears reasonable to conclude that the IgE-responsive antigens were the HMW components rather than the 12- to 13-kDa proteins. These results indicate that tTG treatment generates neopeptides on gluten molecules (even after digestion) in HWP-sensitized subjects, and these epitopes have sufficient affinity to cross-link IgE and induce mast cell activation.

Next, we performed inhibition ELISAs by using serum samples from selected patients and antigen-coated polystyrene beads (see the **Methods** section in this article's Online Repository). Antigen-specific IgE was adsorbed to antigen-coated beads, and the remaining IgE in the supernatant was measured by using ELISA. When Glp19S was coated on an ELISA microplate, the binding of HWP IgE was strongly inhibited (71% to 92%) by the Glp19S-coated beads and moderately inhibited (41% to 63%) by the tTG-Glu-coated beads; however, untreated gluten-coated beads were less effective (<27%) at inhibiting the binding (**Fig 2, A**). In contrast, all the beads exhibited strong inhibition (68% to 108%) with IgE from patients with PedWA. However, when tTG-Glu was coated on the plates, the gluten- and tTG-Glu-coated beads inhibited the binding of HWP IgE moderately (38% to 46%) and strongly (78% to 88%), respectively (**Fig 2, B**). Glp19S-coated beads used in HWP-sensitized subjects and gluten/tTG-Glu/Glp19S-coated beads used in patients with PedWA (**Fig 2, B**) exhibited inhibition similar to that seen in **Fig 2, A**. These results indicate that tTG treatment of gluten generates IgE epitopes that cross-react with Glp19S, although tTG-Glu at least partially retains gluten-like structures. These results also suggest that IgE from patients with PedWA binds to tTG-insensitive structures in gluten, which are distinct from HWP IgE epitopes.

Many celiac disease studies have shown that digestion-resistant peptide fragments of wheat gluten can access the lamina propria and be deamidated by tTG.⁷ However, it is also well known that gluten is deamidated during hydrolysis under acidic and heating conditions.⁸ Therefore we performed limited proteolysis of gluten, tTG-Glu, and Glp19S with the endoproteinase Glu-C, which cleaves at glutamic acid residues in peptides (see the **Methods** section in this article's Online Repository). tTG treatment induced a mobility shift of gluten with a smear pattern, which was completely eliminated by Glu-C treatment (see **Fig E2** in this article's Online Repository at www.jacionline.org). An increase in negatively charged glutamyl residues because of deamidation of glutamine residues might induce conformational changes in gluten, causing the observed mobility shift in SDS-PAGE.⁸ The Glu-C-resistant components in the gluten and tTG-Glu were similar, suggesting that part of tTG-Glu remains unaffected by tTG treatment.

In Japan more than 1800 subjects have been confirmed as being sensitized to Glp19S-containing “Cha no Shizuku” soap as of February 2013. After recall of the soap by the distributor, gluten- and Glp19S-specific IgE levels have decreased in the sera of most

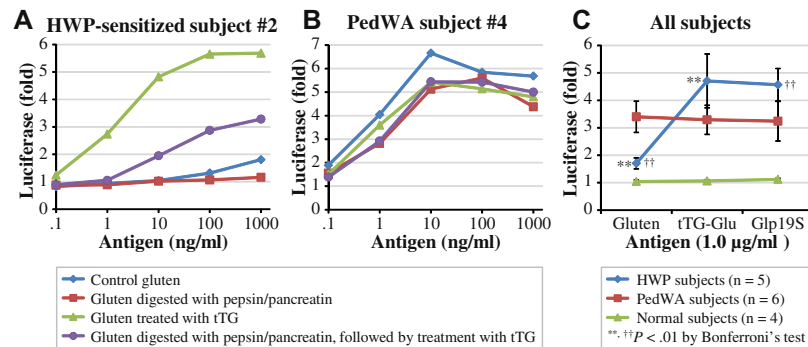


FIG 1. Typical EXiLE responses of serum IgE from HWP-sensitized subject (A) and patient with PedWA (B) to various treated gluts and EXiLE responses of all subjects (HWP-sensitized subjects, patients with PedWA, and healthy subjects) presented in Table E1 to gluten, gluten treated with tTG (tTG-Glu), and GIp19S (C). Values are presented as mean $\pm$ SEMs. **, †† P < .01, n-way ANOVA and the *post hoc* test with the Bonferroni correction.

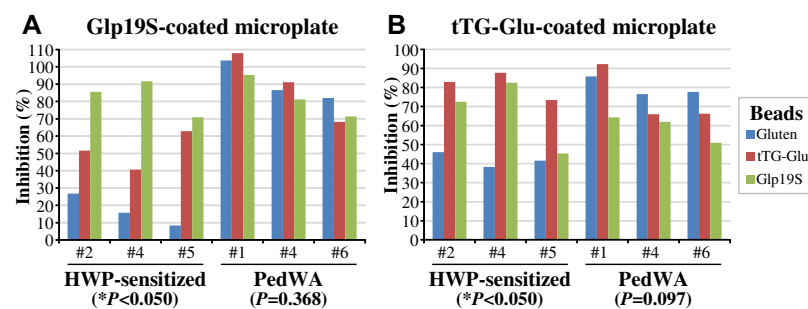


FIG 2. IgE ELISA inhibition with antigen-coated beads. Diluted sera from HWP-sensitized subjects and patients with PedWA were treated with antigen-coated beads for 2 hours, followed by centrifugation. The GIp19S-specific (A) or tTG-Glu-specific (B) IgE remaining in the supernatants was measured by using ELISA. Data are means of triplicate measurements and are shown as percentages of binding inhibition. P values of the Friedman test are shown.

patients.⁹ However, many patients continue to experience allergic symptoms elicited by wheat ingestion, even after having negative serum gluten-specific IgE levels. We propose that patients with high GIp19S-specific IgE levels should avoid wheat ingestion, even if their gluten-specific IgE levels have decreased, because orally ingested wheat gluten can be deamidated by tTG after digestion, resulting in the generation of IgE epitopes that cross-react with GIp19S.

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METHODS

Treatment with gastrointestinal enzymes

Gluten treatment with porcine digestive enzymes and guinea pig transglutaminase was performed according to the method of Palosuo et al,^{E1} with minor modifications. In brief, gluten (Sigma, St Louis, Mo) was suspended in 0.1 mol/L Tris-HCl (pH 8.0) at 4 mg/mL and digested with addition of porcine pepsin (Sigma) at an enzyme/gluten ratio of 1:100 (wt/wt) in 0.2 mg/mL NaCl (adjusted to pH 1.2 with HCl) at 37°C for 1 hour with rotation and tapping. Subsequently, the pH was adjusted to 8.0 with Na₂CO₃, and porcine pancreatin (Sigma) was added at an enzyme/gluten ratio of 1:100 (wt/wt) and incubated at 37°C for 1 hour with rotation and tapping. The reaction was halted by heating at 90°C for 5 minutes. Guinea pig tTG (Sigma) was added at an enzyme/gluten ratio of 1:10 (wt/wt) and incubated at 37°C overnight with rotation and tapping. A potent increase in luciferase expression (>4-fold at 1 µg/mL) was observed after a 30-minute incubation of gluten with tTG in the case of HWP-sensitized subject 2 and increased in a time-dependent manner (data not shown).

Inhibition ELISA with antigen-coated beads

Gluten, tTG-Glu, and Glp19S were coated on 0.5-µm Fluoresbrite microsphere beads (Polysciences, Warrington, Pa), according to the manufacturer's protocol. Negative control beads were coated with BSA. The subject's serum was diluted 1:50 with 0.1% BSA in PBS to 350 µL, and antigen-coated beads were added at an amount equivalent to 7.5 µg protein per tube and incubated at room temperature for 2 hours with rotation and tapping. Antigen-bound IgE was removed by means of centrifugation, and the supernatant was collected. The binding of IgE was detected with ELISA microplates coated with 0.1 µg of tTG-Glu or Glp19S in carbonate/

bicarbonate buffer (pH 9.6), incubated at 4°C overnight, and blocked with 0.1% casein in PBS at room temperature for 1 hour. A total of 50 µL of serum supernatant was added to each well and incubated at 4°C overnight. IgE binding was measured by using a colorimetric assay with horseradish peroxidase, and the percentage of inhibition was calculated by using the following formula:

$$\text{Inhibition (\%)} = 100 \times \frac{ABS_{BSA} - ABS_{Sample}}{ABS_{BSA} - ABS_{NoSerum}},$$

where ABS_{BSA} , ABS_{Sample} , and $ABS_{NoSerum}$ were the degree of chromogenesis (450 nm) with the control BSA bead-treated serum, with antigen-coated bead-treated serum, and without serum, respectively.

Glu-C endoprotease treatment

A total of 200 µg each of gluten, tTG-Glu, and Glp19S was pretreated with 25 mmol/L NH₄HCO₃, 0.8 mol/L urea, and 5 mmol/L dithiothreitol at 60°C for 20 minutes. After a 15-minute incubation with 15 mmol/L iodoacetamide at room temperature, Glu-C (Promega, Madison, Wis) was added at an enzyme/gluten ratio of 1:50 (wt/wt), and the reaction mixture was incubated at 37°C overnight with rotation and tapping. Under these conditions, Glu-C preferentially cleaves the C-terminal side of glutamic acid residues in a peptide.

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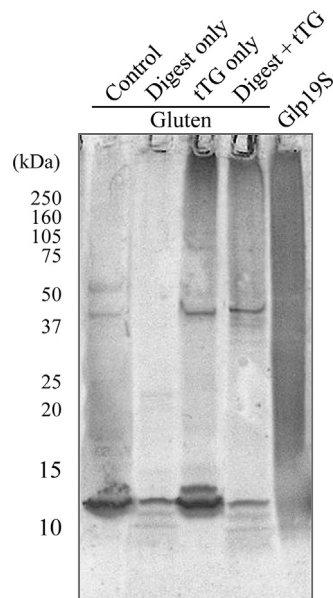


FIG E1. Immunoblot of treated glutens with IgE from HWP-sensitized subjects. Gluten was digested with pepsin and pancreatin, followed by treatment with tTG. The glutens were resolved on SDS-PAGE and blotted with the serum of subject 2 on a polyvinylidene fluoride membrane. tTG treatment induced strong IgE binding to HMW components with a smear pattern similar to that with Glp19S, even after digestion, whereas digestion alone showed almost no effect.

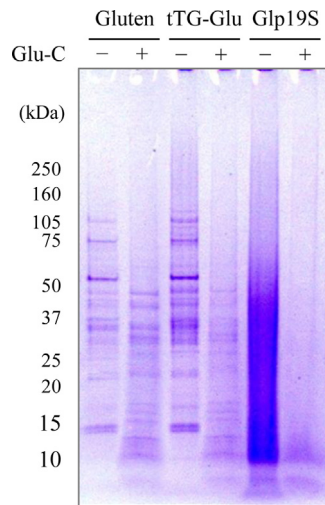


FIG E2. Deamidation of gluten with tTG. Gluten, tTG-Glu, and Glp19S were digested with Glu-C endoproteinase. The mobility shift of tTG-Glu and Glp19S with a smear pattern can be explained by the increased negative charge of glutamic acid residues as a result of deamidation of glutamine residues. The mobility shift was completely eliminated with Glu-C treatment.

TABLE E1. Serologic and clinical features of enrolled wheat allergic subjects

Patient ID	Age (y)/sex	ImmunoCAP (UA/mL)			Total IgE (IU/mL)	SPT (wheal diameter in mm)					“Cha no Shizuku” facial soap experience	Clinical features after wheat ingestion (diagnosed by...)
		Wheat	Gluten	ω-5 gliadin		0.01% Glp19S	0.1% Glp19S	Wheat	Bread	Positive control		
HWP-sensitized subjects												
1	55/F	4.38	6.15	0.34	123	3	9	0	0	3	Yes	WDEIA (history)
2	41/F	21.8	36.2	7.01	285	5	8	7	5	7	Yes	WDEIA (history)
3	51/F	2.28	3.65	0.34	210	5	ND	3	2	4	Yes	WDEIA (history)
4	42/F	23.4	31.4	2.06	4,050	3	ND	0	0	4	Yes	WDEIA (history)
5	23/F	1.79	3.12	0.34	1,510	3	9	0	0	6	Yes	WDEIA (history)
Patients with PedWA												
1	5/M	100	ND	ND	800	ND	ND	ND	ND	ND	No	FA (history)
2	1/F	92.8	ND	ND	2,900	ND	ND	ND	ND	ND	No	FA (history)
3	2/M	100	ND	ND	23,221	ND	ND	ND	ND	ND	No	FA (history)
4	3/M	(Class 4)	66.6	0.4	512	ND	ND	ND	ND	ND	No	FA with anaphylaxis (history)
5	1/F	17.6	17.6	ND	191	ND	ND	ND	ND	ND	No	FA (OFC)
6	7/M	11.4	20	0.49	ND	ND	ND	ND	ND	ND	No	FA (OFC)
Healthy control subjects												
1	24/F	ND	ND	ND	22	ND	ND	ND	ND	ND	No	None
2	61/F	ND	ND	ND	37	ND	ND	ND	ND	ND	No	None
3	55/F	ND	ND	ND	17	ND	ND	ND	ND	ND	No	None
4	55/M	ND	ND	ND	17	ND	ND	ND	ND	ND	No	None

For SPTs, Glp19S (a gift from Katayama Chemical Industries, Osaka, Japan) and commercial wheat and bread allergen extracts (Torii Pharmaceutical, Tokyo, Japan) were used. As positive controls, 10 mg/mL histamine dihydrochloride was used.

FA, Food allergy; ND, not done; OFC, oral food challenge; WDEIA, wheat-dependent exercise-induced anaphylaxis.