

Specific IgE Determination to Epitope Peptides of ω -5 Gliadin and High Molecular Weight Glutenin Subunit Is a Useful Tool for Diagnosis of Wheat-Dependent Exercise-Induced Anaphylaxis¹

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Wheat ω -5 gliadin and a high m.w. glutenin subunit (HMW-glutenin) have been reported as major allergens in wheat-dependent exercise-induced anaphylaxis. A simultaneous detection of specific IgE to epitope sequences of both proteins is considered to be a reliable method for diagnosis of wheat-dependent exercise-induced anaphylaxis. However, the IgE-binding epitope of HMW-glutenin remains unknown. The aim of this study was to determine the IgE-binding epitopes of HMW-glutenin to establish a useful system of identifying patients with wheat-dependent exercise-induced anaphylaxis. For determination of IgE-binding epitopes of HMW-glutenin overlapping peptides were synthesized and reactivities of IgE Abs in the sera of patients to those peptides were analyzed. Three IgE-binding epitopes, QQPGQ, QQPGQGQQ, and QQSGQGQ, were identified within primary sequence of HMW-glutenin. Epitope peptides, which include IgE-binding sequences of ω -5 gliadin and a HMW-glutenin, were synthesized and peptide-specific IgE Abs were measured by CAP-System fluorescent enzyme immunoassay. Twenty-nine of 30 patients with wheat-dependent exercise-induced anaphylaxis had specific IgE Abs to these epitope peptides. None of the 25 sera from healthy subjects reacted to both epitope peptides. Twenty-five patients with atopic dermatitis who had specific IgE to wheat and/or gluten had very low or nonexistent levels of epitope peptide-specific IgE Abs. These results indicated that measurement of IgE levels specific to epitope peptides of ω -5 gliadin and HMW-glutenin is useful as an *in vitro* diagnostic method for the assessment of patients with wheat-dependent exercise-induced anaphylaxis. *The Journal of Immunology*, 2005, 175: 8116–8122.

Food-dependent exercise-induced anaphylaxis (FDEIA)³ is a distinct form of a common food allergy induced by the combination of causative food ingestion, physical exercise, and/or aspirin intake (1–3). Symptoms of FDEIA are typically generalized urticaria and systemic allergic reactions such as anaphylactic shock. Many kinds of food such as shrimp (4), shellfish (5), hazelnut (6), buckwheat (7), corn (8), orange (9), and apple (10) have been reported as offending agents for FDEIA. In Japanese patients with FDEIA, the most frequently causative food is wheat (11).

In our previous study, we identified wheat ω -5 type gliadin, which is a component of water/salt-insoluble protein (gluten), as a major allergen in patients with wheat-dependent exercise-induced anaphylaxis (WDEIA) and defined seven sequential IgE-binding epitopes, QQIPQQQ, QQFPQQQ, QQSPQQQ, QQSPEQQ, QQLPQQQ, QQYPQQQ, and PYPP, in an ω -5 gliadin sequence by using synthetic overlapping peptides (12, 13). In addition, our results indicated that there is another minor subgroup of patients

who have IgE Abs that mainly reacts to a high m.w. glutenin subunit (HMW-glutenin) in gluten.

To diagnose WDEIA, we typically perform an exercise challenge test combined with wheat ingestion for patients who have episodes of anaphylaxis after wheat intake. The challenge test is unsafe for patients because anaphylactic shock was sometimes provoked during the test. Therefore, an *in vitro* diagnostic method predicting the development of symptoms by wheat and exercise challenge is necessary for patients with WDEIA. Recent investigations have demonstrated that quantification of food-specific IgE is a useful test not only for diagnosis of food allergies such as egg, milk, peanut, and fish allergies (14, 15) but also for predicting the development of tolerance in milk and egg allergies (16). Radioallergosorbent tests (RAST) for wheat protein and wheat gluten are commercially available for the diagnosis of wheat allergy; however, this test is not satisfactory to diagnose WDEIA because of its low sensitivity and low specificity. Our previous data indicated that a simultaneous measurement of both ω -5 gliadin and HMW-glutenin-specific IgE Abs was more sensitive and specific to diagnose WDEIA than that of gluten-specific IgE (13). This is in accord with the previously reported data that most patients with WDEIA have IgE Abs to ω -5 gliadin (17). Available recombinant food allergens are increasing, and they have been used for diagnosis, research, and therapy of food allergies. Recombinant ω -5 gliadin and HMW-glutenin have been expressed in *Escherichia coli*, yet neither as a full-length protein (18, 19). Beyer et al. (20) have proposed recently that in a peanut allergy a measurement of IgE-binding epitope peptide-specific IgE Abs is a more useful tool to diagnose symptomatic allergic reaction than that of peanut-specific IgE. In the present study, to develop a new useful *in vitro* method for diagnosis of WDEIA, we identified sequential IgE-binding epitopes of HMW-glutenin and then evaluated usefulness

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³ Abbreviations used in this paper: FDEIA, food-dependent exercise-induced anaphylaxis; WDEIA, wheat-dependent exercise-induced anaphylaxis; HMW-glutenin, high m.w. glutenin subunit; RAST, radioallergosorbent test; FEIA, fluorescent enzyme immunoassay.

of measurement of IgE Abs specific to combined epitope peptides of ω -5 gliadin and HMW-glutenin.

Materials and Methods

Subjects

Thirty patients with WDEIA (13 females, 17 males, mean age: 32.3 years, range: 10–75 years, mean total IgE: 987.7 IU/ml, range: 21.9–14,723) with recurrent episodes of anaphylaxis and a positive provocation test after wheat ingestion took part in this study. Twenty-five healthy subjects (10 females, 15 males, mean age: 33.4 years, range: 18–55 years, mean total IgE: 162.2 IU/ml, range: 7.2–500) and 25 patients with atopic dermatitis (11 females, 14 males, mean age: 24.8 years, range: 5–49 years, mean total IgE: 7,931.8 IU/ml, range: 277–21,699) with wheat and/or gluten-specific IgE Abs in their serum served as controls. Detailed interviews were conducted to determine that the healthy subjects and atopic dermatitis patients had experienced no episode of allergic reaction after ingestion of wheat products. Sera were collected from the subjects and stored at -80°C until use. Specific IgE Abs for wheat and gluten in the sera were determined using the CAP-System fluorescent enzyme immunoassay (FEIA) (Pharmacia Diagnostics).

Dot blotting, SDS-PAGE, and Western blotting

Specific IgE Abs in the sera for α -gliadin, β -gliadin, γ -gliadin, ω -5 gliadin, ω -1,2 gliadin, HMW-glutenin, and a low m.w. glutenin subunit purified from wheat flour were detected, and spot intensities were measured using the method described previously (13).

SDS-PAGE was performed with 12.5% acrylamide gel, and fractionated proteins were then visualized by Coomassie brilliant blue staining. For Western blotting, the fractionated protein was electrophoretically transferred to a polyvinylidene difluoride membrane (Immobilon) and blocked with 5% skim milk in TBST (50 mM Tris-buffered saline and 1% Tween 20 (pH 7.4)). The membrane was washed with TBST three times and then probed with 10% patient's serum. After washing with TBST, the membrane was incubated with HRP-conjugated rat anti-human IgE mAbs (BioSource International). To detect human IgE binding, ECL Plus Western blotting detection reagents (Amersham Biosciences) was used. The resulting light was detected on autoradiography film.

Peptide synthesis on the SPOTs membrane and IgE-binding assay

Based on the amino acid sequence of the HMW-glutenin (GenBank accession no. X12928), the individual peptides were synthesized on a SPOTs membrane (Sigma-Genosys) described previously (13). The SPOTs membrane was blocked overnight at 4°C with blocking buffer (Sigma-Genosys). The membrane was washed with TBST three times for 10 min at room temperature and then incubated overnight at 4°C with 10% patient's serum. The bound IgE Abs were detected as described above. The SPOTs membrane was used repeatedly after the following procedure. The membrane was washed with water, *N,N*-dimethylformamide (Wako Pure Chemical), regeneration buffer A (8 M urea and 10% SDS), regeneration buffer B (6 M guanidine hydrochloride), regeneration buffer C (10% (v/v) acetic acid and 50% (v/v) ethanol), and then rinsed with methanol twice for 10 min at room temperature.

IgE-binding inhibition assay of combined epitope peptides

The epitope peptides, KPQQSPQQFPQQIPQQQ (peptide A) and PTSPQSGQGQPGQGQQ (peptide B), were synthesized to a purity of

at least 80% (Thermo Electron). Sera from patients with WDEIA were then preincubated with or without synthetic epitope peptide A or B (100 $\mu\text{g}/\text{ml}$) for 1 h at room temperature. Remaining serum IgE binding to each epitope peptide on the SPOTs membrane was then measured as described above.

Measurement of peptide-specific IgE Abs

Epitope peptide-specific IgE levels in the sera were determined using Pharmacia CAP-System FEIA (Pharmacia Diagnostics).

Results

Immunoblotting for purified wheat gliadins and glutenins

The sera of 30 patients with WDEIA were analyzed for the reaction to purified α -gliadin, β -gliadin, γ -gliadin, ω -1,2 gliadin, ω -5 gliadin, a low m.w. glutenin subunit, and HMW-glutenin. The strongest reaction was observed with ω -5 gliadin, HMW-glutenin, and ω -1,2 gliadin in 23, 6, and 1 of the 30 patients, respectively. Sera of these six patients whose IgE Abs reacted to HMW-glutenin predominantly were used for epitope mapping. Table I shows the result of the dot-blotting analysis of the patients whose IgE Abs in the sera reacted to HMW-glutenin predominantly.

HMW-glutenins are encoded at the *Glu-1* loci on chromosomes 1A, 1B, and 1D, and each locus consists of two genes encoding an x-type and a y-type subunit (21). However, common wheat contains three to five subunits because some of the genes are silent (22). Therefore, we determined which type of HMW-glutenin is the major IgE-binding allergen for the six patients by Western blot experiment with purified protein. The IgE Abs in the sera of all patients reacted to both x-type and y-type HMW-glutenin subunits (Fig. 1).

Multiple IgE-binding epitopes in the HMW-glutenin

Because the amino acid sequences of HMW-glutenin x-type subunit and y-type subunit are very similar and the content of x-type subunit is higher than that of y-type in common wheat flour (22), we used the x-type HMW-glutenin for determination of IgE binding epitope.

Several studies of IgE-binding epitopes of wheat gliadins and glutenins have shown that the length of the epitope sequences is <7 aa (13, 23, 24). Therefore, to determine the regions recognized by the serum IgE Abs of patients with WDEIA, overlapping peptides 14 aa long and offset by 7 aa were synthesized using the SPOTs method based on the amino acid sequence of HMW-glutenin (Fig. 2). Individual sera from six patients were used to map IgE-binding regions in the amino acid sequence of HMW-glutenin. Fig. 3 summarizes the IgE-binding peptides of each of the six patients. Several peptides were reacted by rat anti-human IgE mAbs directly as shown in Fig. 3. Therefore, these peptides were neglected. Many IgE-binding peptides were detected; however, most of these positive sequences were overlapped among the

Table I. Results of dot blot analysis

Patients	Total IgE (IU/mL)	Gluten RAST (kUa/L)	Reactivity (spot intensity)						
			α^a	β	γ	ω -5	ω -1,2	HMW	LMW
1 ^b	345	0.79	83	0	18	333	0	1,218	159
2 ^b	1,779	5.34	63	9	6	176	77	1,586	154
3 ^b	150	<0.35	3	3	1	12	67	171	14
4 ^b	14,723	3.33	460	1,170	646	981	76	1,227	183
5	3,034	1.09	16	8	5	28	25	936	47
6	976	5.95	34	14	7	199	319	1,128	504

^a α , α -gliadin, β , β -gliadin; γ , γ -gliadin; ω -5, ω -5 gliadin; ω -1,2, ω -1,2 gliadin; HMW, high M.W. glutenin subunit; LMW, low M.W. glutenin subunit.

^bPatients were reported previously (12, 13).

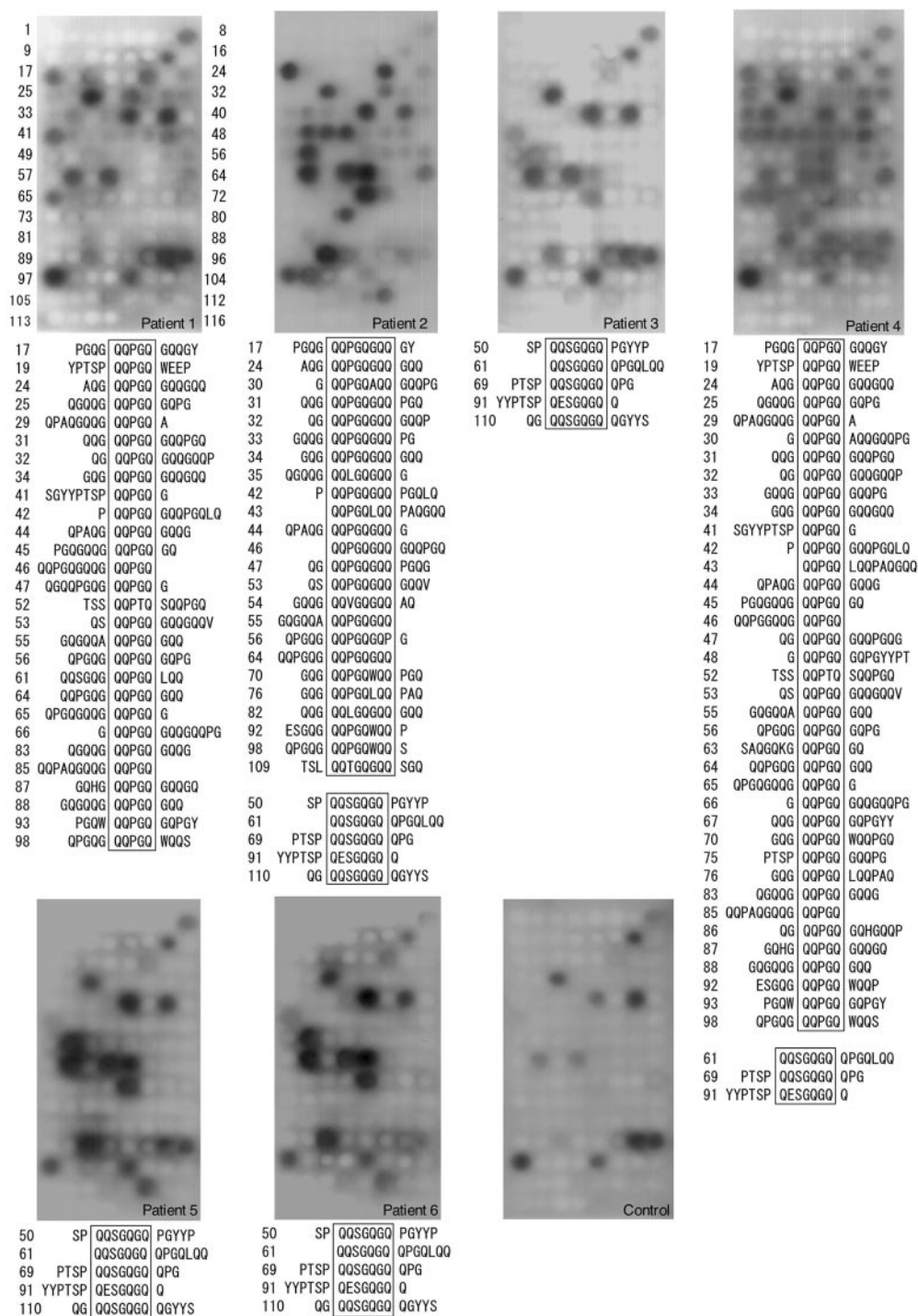


FIGURE 3. Analysis of IgE reactivity to overlapping peptides by using sera from six patients with WDEIA and alignment of IgE-bound peptide sequences. The assumed epitope regions are boxed. Control: without patients' sera.

tool that can identify patients with WDEIA using the synthetic epitope peptides of ω -5 gliadin and HMW-glutenin.

Patients with WDEIA had been divided into two groups by means of reactivity of serum IgE to wheat proteins: one is ω -5 gliadin-reacting group (~80% of patients) and the other is HMW-glutenin-reacting group (12, 13). We had already identified the amino acid sequence of QXXPQQQ (X = any amino acid) as a major IgE-binding epitope of ω -5 gliadin. However, epitopes of HMW-glutenin have not been identified. In the present study, we analyzed the IgE-binding epitope sequences by means of peptide array of HMW-glutenin with the six patients' sera. The epitope

sequences of HMW-glutenin were QSGQGQ, QQPQG, and QPGQGQ and different from the IgE epitopes of gliadins and low m.w. glutenin subunits as reported previously (23, 24). We further determined that the critical amino acids in the three epitopes were positions 1–5(QQPQG) or 1–4(QPGQ) for QQPQG, 1–5(QQPQG) and 7(Q) for QPGQGQ, and 1(Q), 3(S), 4(G), 5(Q), and 7(Q) for QSGQGQ, suggesting that QPGQXQX and QXSGQXQ are major epitope sequences of HMW-glutenin. As shown in Fig. 2, the QPGQXQX and QXSGQXQ sequences are found in HMW-glutenin x-type amino acid sequence at 8 and 37 sites, respectively. These epitopes also appear

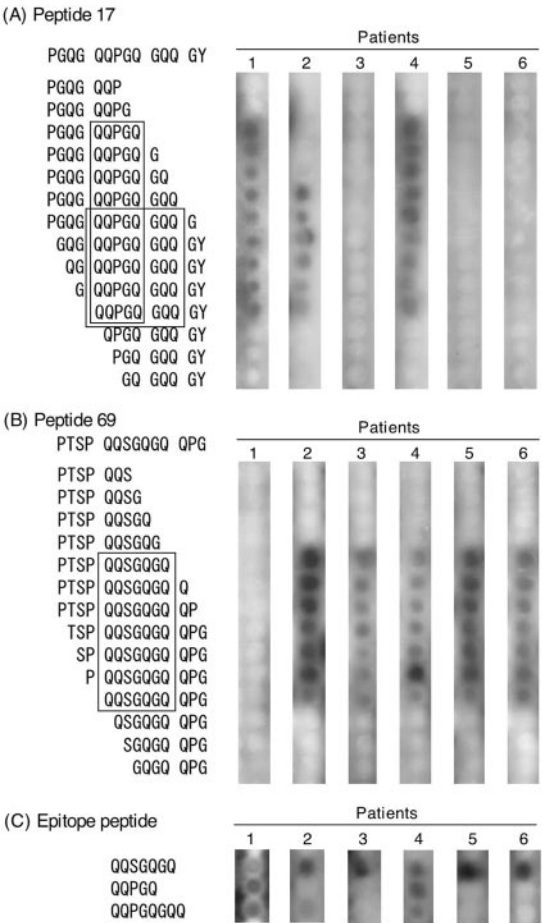


FIGURE 4. Identification of core epitope sequences of synthetic peptides. Fine mapping data of peptide 17 (A) and peptide 69 (B) were shown in the six patients with WDEIA. C, Reactivity of the patients' sera to the three identified epitope peptides. The boxed area indicates the core epitope sequence.

frequently in the amino acid sequence of HMW-glutenin y-type, of which IgE Abs in the sera of patients reacted in the Western blot experiment (Fig. 1). In addition, serum IgE binding to x- and y-type HMW-glutenin, as shown in Fig. 1, was almost completely inhibited by combined epitope peptide B in all patients (data not shown). These results suggest that x- and y-type HMW-glutenin

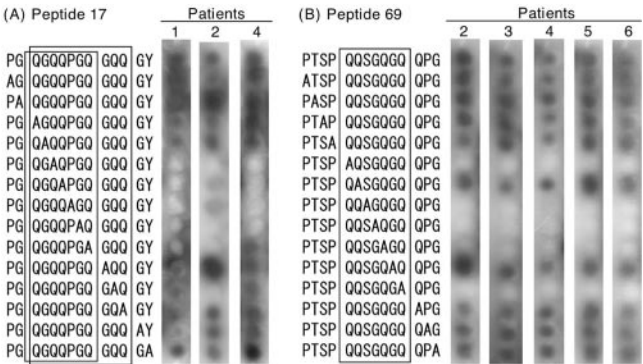


FIGURE 5. Critical amino acids were analyzed in three epitopes, QQPFGQ, QQPFGQGQ, and QQSGQGQ, in HMW-glutenin. The peptides, including epitope sequences, were synthesized with alanine residue substituted for 1 aa at each position based on the sequence of peptide 17 (A) and peptide 69 (B). The boxed area indicates the epitope sequence.

Table II. IgE-binding epitope sequences for patients with WDEIA^a

Patients	Epitope Sequences
1	<u>QQPFGQ</u>
2	<u>QQPFGQGQ</u>
3	<u>QQSGQGQ</u>
4	<u>QQPFGQ</u>
5	<u>QQSGQGQ</u>
6	<u>QQSGQGQ</u>

^a The underline indicates critical amino acids for IgE binding.

share epitopes, and all important allergenic epitopes in the HMW-glutenins were detected even though overlapping peptides with an offset of 7 aa were used in this study.

The CAP-System FEIA is an accurate and standardized procedure of determination of allergen-specific IgE and now widely used for the diagnosis of food allergy. The use of food-specific IgE concentrations in predicting clinical reactivity has been reported in egg, milk, peanut, and fish allergies (15). The measurement of wheat or gluten-specific IgE is available for diagnosis of WDEIA in the CAP-System FEIA. Wheat gluten contains about 5% of ω -5 gliadin and 9% of HMW-glutenin; however, 20% of patients with definite WDEIA have negative CAP-RAST to gluten. In addition, as shown in this study, a number of the patients with atopic dermatitis have positive CAP-RAST scores for gluten, although the patients have no episode of type I allergic reactions after ingestion of wheat products. Therefore, the measurement of gluten-specific IgE is not always satisfactory for diagnosis of WDEIA. We have suggested that the measurement of specific IgE to ω -5 gliadin and HMW-glutenin instead of gluten is useful to diagnose WDEIA (13). Recently, recombinant food allergens have been produced and tried to apply for diagnosis in many food allergies (25, 26). Recombinant proteins of ω -5 gliadin and HMW-glutenin have not been available in enough quality and quantity. Hence, in this study, we synthesized the peptides containing major IgE-binding epitopes of those proteins and applied them to CAP-System FEIA to determine the specific IgE to those epitope peptides. As we suspected, 97% (29 of 30) of the patients with WDEIA were found to be positive in IgE specific for synthetic epitope peptides of ω -5 gliadin and HMW-glutenin, whereas gluten-specific IgE test was positive in only 80% of patients with WDEIA. In addition, specific IgE values in epitope peptide-specific CAP-System FEIA were much higher than that in the gluten-specific test in most patients with WDEIA, indicating higher sensitivity with epitope-specific system. This is due to higher content of epitope peptides used in the CAP-System FEIA. Ten of 25 patients with atopic dermatitis who had no obvious allergic reactions after ingestion of wheat products had positive CAP-RAST (>0.34 kUa/L) for epitope peptides, indicating low specificity of the test. However, when the

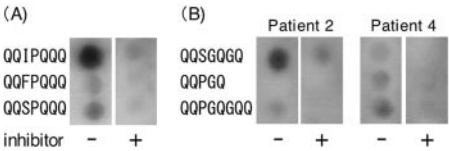


FIGURE 6. Inhibition of IgE binding to individual epitope peptides with combined epitope peptides. A, Serum from a patient with specific IgE Abs to three epitope peptides of ω -5 gliadin was preincubated with or without peptide A (KPQQSPQQQFPQQQIPQQQ) for 1 h at room temperature. B, Sera from two patients, patients 2 and 4, were preincubated with or without peptide B (PTSPQQSGQGQQPFGQGQ). Remaining serum IgE binding to individual epitope peptides on the SPOTs membrane was detected chemiluminescently.

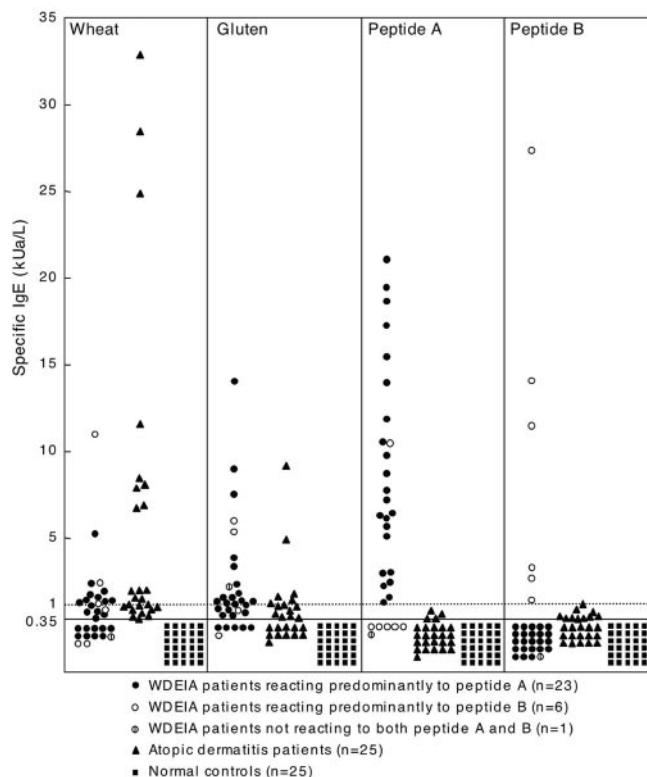


FIGURE 7. Specific IgE levels to gluten and combined epitope peptides of ω -5 gliadin and HMW-glutenin. Peptide A, KPQQSPQQFPQQ-QIPQQ: epitope peptide of ω -5 gliadin; peptide B, PTSPQQS-QGQQPGQQGQQ: epitope peptide of HMW-glutenin.

cutoff value for positive was set at 1.0 kUa/L, sensitivity and specificity of the epitope peptide-specific CAP-RAST were satisfactory because sensitivity and specificity of the test would reach to 97% (29 of 30) and 98% (1 of 50), respectively.

There are several clinical forms of IgE-mediated allergy to wheat such as, baker's asthma, common food allergy to wheat, WDEIA, and contact urticaria to wheat flour. Proteins in the water/salt-soluble fraction, such as α -amylase inhibitor, peroxidase, glyceraldehyde-3-phosphate dehydrogenase, serpin, and triose-phosphate isomerase, have been identified to be major allergens in patients with baker's asthma (27–29). α -Gliadin, γ -gliadin, and ω -5 gliadin in water/salt-insoluble fraction have been identified as allergens in WDEIA (17, 30, 31). However, in patients with classical wheat allergy, the allergens responsible for symptoms have not been defined because serum IgE reacted to both water/salt-soluble and -insoluble proteins (32–36). As just described, it is considered that causative allergens are variable among the clinical forms (37, 38). Recent studies have shown that ω -5 gliadin is a good candidate as a diagnostic tool for not only WDEIA but also immediate allergy to wheat (39–41). These data suggest that specific IgE determination to epitope peptides of ω -5 gliadin may be useful to diagnose immediate type allergy to wheat as well as WDEIA.

WDEIA is a life-threatening disease, but the only reliable therapy is a strict elimination of wheat products from dietary foods. It is not easy to eliminate wheat as wheat flour is used in many kinds of cooking, so patients with wheat allergy desire hypoallergenic wheat products. Immunotherapy using recombinant-mutated allergen was found to be successful for a peanut allergy in an animal model (42). In combination with the major IgE-binding epitopes of ω -5 gliadin, the elucidation of major IgE-binding epitopes on

HMW-glutenin in this study may provide a useful tool for developing hypoallergenic foods as well as an immunotherapy for patients with WDEIA.

In conclusion, our results strongly indicate that determination of specific IgE to synthetic epitope peptide of wheat ω -5 gliadin and HMW-glutenin is useful to diagnose WDEIA. Further studies are required to determine the optimal cutoff values for specific IgE Abs to epitope peptides for predicting clinical outcome in patients with WDEIA.

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Disclosures

The authors have no financial conflict of interest.

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