

Transglutaminase-mediated cross-linking of a peptic fraction of ω -5 gliadin enhances IgE reactivity in wheat-dependent, exercise-induced anaphylaxis

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Background: Patients with wheat-dependent, exercise-induced anaphylaxis (WDEIA) experience recurrent anaphylactic reactions when exercising after ingestion of wheat products. We have identified ω -5 gliadin (Tri a 19) as a major allergen in WDEIA, but the role of exercise in eliciting the symptoms remains obscure.

Objective: The aim was to examine whether tissue transglutaminase (tTG)-mediated cross-linking could be involved in modulating the IgE-binding ability and in vivo reactivity of digested ω -5 gliadin peptides in WDEIA.

Methods: Purified ω -5 gliadin was digested with pepsin or with pepsin and trypsin and treated with tTG. The binding of IgE antibodies in pooled sera from 10 patients with WDEIA was studied by means of immunoblotting before and after tTG treatment of the digested peptides. The peptides derived from pepsin digestion were separated by means of gel-filtration chromatography, and IgE reactivity of 4 different peptide fractions was studied by immunoblotting before and after tTG treatment. The fraction showing the greatest degree of cross-linking by tTG was further studied by means of IgE ELISA, ELISA inhibition, and skin prick testing.

Results: The IgE-binding ability of ω -5 gliadin was retained after pepsin and pepsin-trypsin digestion. tTG treatment of the whole peptic digest formed large peptide complexes, with molecular weights ranging from 40 to greater than 200 kd. These cross-linked aggregates bound IgE antibodies in immunoblotting more intensely than untreated, pepsin-digested, or pepsin-trypsin-digested ω -5 gliadin. A gel-filtration fraction of the whole peptic digest corresponding to the highest peak of the chromatogram and showing the greatest degree of tTG-mediated cross-linking showed an increase in serum IgE reactivity in ELISA after tTG treatment, as well as a shift

of reactivity to cross-linked complexes. In the 20 patients with WDEIA, the mean skin prick test wheal elicited by this tTG-treated peptic fraction was 77% larger ($P < .001$) than that elicited by the untreated peptic fraction and 56% larger ($P < .01$) than that elicited by intact ω -5 gliadin.

Conclusions: ω -5 Gliadin-derived peptides are cross-linked by tTG, which causes a marked increase in IgE binding both in vitro and in vivo. Activation of tTG during exercise in the intestinal mucosa of patients with WDEIA could lead to the formation of large allergen complexes capable of eliciting anaphylactic reactions. (J Allergy Clin Immunol 2003;111:1386-92.)

Key words: Anaphylaxis, exercise, gliadin, IgE, transglutaminases, wheat hypersensitivity

Ingestion of a specific food before physical exercise triggers anaphylactic reactions in patients with food-dependent, exercise-induced anaphylaxis (FDEIA).¹ This severe form of allergy is regarded as a subset of classic exercise-induced anaphylaxis, for which exercise is the only precipitating factor.² The symptoms in FDEIA, including pruritus, urticaria, angioedema, dyspnea, hypotension, collapse, and shock, are indistinguishable from those in typical IgE-mediated anaphylaxis.^{3,4} Patients with FDEIA usually have IgE antibodies to the offending food, yet food ingestion alone fails to elicit symptoms.¹ Although various foods, such as celery, shellfish, peanut, tree nuts, and tomato, can trigger FDEIA,^{1,4-6} one of the most frequent causes is wheat.^{4,5,7,8}

To our knowledge, wheat is the only food triggering FDEIA, in which allergens have been characterized at the molecular level. IgE antibodies in patients with wheat-dependent, exercise-induced anaphylaxis (WDEIA) are directed against the ethanol-soluble storage proteins of the wheat grain, known as gliadins.^{3,8-10} We have identified ω -5 gliadin (Tri a 19) as a major allergen in these patients and demonstrated the presence of cross-reactive allergens in rye and barley.^{3,11}

Dietary gliadins, as all ingested food proteins, are exposed to gastrointestinal digestive enzymes (pepsin and trypsin) before absorption through the gut epithelium. Tissue transglutaminase (tTG) is an enzyme localized beneath this single-layered epithelium, and gliadins are among its few known substrates.¹² tTG can modify proteins by means of calcium-dependent deamidation or cross-linking of specific glutamine residues. Cross-linking involves an acyl transfer reaction between a peptide-

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Abbreviations used

FDEIA: Food-dependent, exercise-induced anaphylaxis
TBS: Tris-buffered saline
tTG: Tissue transglutaminase
WDEIA: Wheat-dependent, exercise-induced anaphylaxis

bound glutamine and lysine, resulting in the formation of an irreversible isopeptide bond protein cross-link.¹³ In addition to forming gliadin-gliadin complexes, tTG can incorporate itself into high-molecular-weight complexes with gliadins.¹² ω -5 Gliadin contains many potential target sites for tTG because the glutamine content of this allergen is exceptionally high (ie, >50%).¹⁴

Under normal physiologic conditions, tTG exists as a latent intracellular enzyme that is inactive in regard to protein cross-linking.¹⁵ However, the enzyme is released and activated under stress-like conditions, such as inflammation and oxidative stress.^{16,17} Because exercise-induced alterations in the immune and neuroendocrine systems are similar to those induced by many physical stressors (eg, trauma, sepsis, and surgery), physical exercise has been introduced as a model of the stress response.¹⁸

Both exercise and specific IgE antibodies are needed to provoke symptoms in WDEIA. The reason for this dual trigger is, however, poorly understood. We hypothesize that tTG could be activated during exercise in patients with WDEIA and that tTG-mediated cross-linking could enhance the IgE-binding ability of ingested ω -5 gliadin peptides.

METHODS

Patients

Twenty patients with WDEIA (7 female and 13 male patients; mean age, 43 years; range, 25-70 years) took part in the study. All patients had convincing clinical histories of FDEIA after ingestion of wheat products and positive skin prick test reactions to wheat (1:10 wt/vol 0.9% NaCl), gliadin (1 mg/mL in 20% ethanol; crude, Sigma Chemical Co, St Louis, Mo), and purified ω -5 gliadin (50 μ g/mL in 20% ethanol-PBS) prepared as previously described.³ ω -5 Gliadin-specific IgE levels ranged from 0.1 to 2.1 AU (ELISA absorbance units at 405 nm; mean, 0.77 AU). Avoidance of dietary gluten (ie, wheat, rye, and barley) has kept the patients symptom free. The control group for the skin prick tests consisted of one adult (age 47 years) with exercise-induced anaphylaxis not related to food ingestion and 3 patients with immediate ingested wheat allergy not related to exercise: one child (age 9 years) and 2 adults (age 19 and 23 years) with wheat allergy persisting from childhood. The study was approved by the Ethical Committee of the Helsinki University Hospital.

Purification of ω -5 gliadin, digestion, and treatment with tTG

ω -5 Gliadin (*Triticum aestivum*; cultivar, Tjalve) was purified by means of reversed-phase HPLC as previously described,³ evaporated in a vacuum centrifuge, and dissolved in 0.1 mol/L HCl (pH 1.8) containing 5% dimethyl sulfoxide. Pepsin (P6687, Sigma) was added at an enzyme/protein ratio of 1:100, and the mixture was incubated for 2 hours at 37°C. Before trypsin digestion or tTG treatment, the samples were evaporated and washed twice with deionized water. Trypsin

(T7418, Sigma) was added at an enzyme/protein ratio of 1:100 in 0.1 mol/L Tris-HCl (pH 8), and the mixture was incubated for 4 hours at 37°C. The reaction was stopped by heating at 96°C for 10 minutes. The peptic and peptic-tryptic digests of ω -5 gliadin were treated with increasing amounts (enzyme/protein ratios of 1:3, 1:1, and 3:1) of tTG (guinea pig liver transglutaminase, T5398, Sigma) for 2 hours at 37°C in 0.1 mol/L Tris-HCl (pH 8) containing 2 mmol/L CaCl_2 .

Fractionation with gel-filtration HPLC

The peptic digest of ω -5 gliadin was fractionated by means of gel-filtration HPLC on a Superdex 75 HR 10/30 column (Amersham Pharmacia Biotech, Uppsala, Sweden) at a flow rate of 0.4 mL/min in 20% acetonitrile containing 0.1% trifluoroacetic acid. Monitoring was done at 214 nm, and the fractions were collected, pooled, and examined by means of immunoblotting (see below) before and after tTG treatment.

The gel-filtration fraction (fraction B) of the whole peptic digest corresponding to the highest peak of the chromatogram was most reactive to tTG treatment in immunoblotting. Of fraction B, 10 μ g was subjected to HPLC gel filtration on a Superdex 200 HR10/30 column (Amersham Pharmacia Biotech) before and after tTG treatment (30 μ g, enzyme/protein ratio of 3:1). In addition, 30 μ g of tTG was fractionated on the same column. Tris-buffered saline (TBS; pH 7.4) was used at a flow rate of 0.4 mL/min. The fractions were collected and used as solid-phase antigens in IgE ELISA.

IgE immunoblotting

Immunoblotting was performed as previously described to assess IgE reactivity and tTG-mediated cross-linking of the digests.¹¹ Briefly, 3 μ g of intact or digested (whole peptic and peptic-tryptic digests, 4 peptic gel-filtration fractions) ω -5 gliadin or the same amount treated with tTG was dissolved in 15 μ L of reducing buffer. After heating at 96°C for 2 minutes, the samples were loaded on 11%, 12.8%, or 14% SDS polyacrylamide gels and electrophoresed at 200 V for 40 minutes on a Mini Protean II device (Bio-Rad, Hercules, Calif). The proteins were transferred from the gels to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, Mass) by means of electroblotting for 1 hour at 40 V and 1 hour at 100 V. The membranes were blocked with 5% nonfat milk powder (Valio, Helsinki, Finland) in TBS (pH 7.4) and incubated overnight with pooled sera from 10 patients with WDEIA (diluted 1:10 in 5% milk-TBS). The membranes were incubated for 30 minutes with biotinylated goat anti-human IgE (diluted 1:1000 in 5% milk-TBS; Vector, Burlingame, Calif) and then for 30 minutes with alkaline phosphatase conjugated to streptavidin (diluted 1:24,000 in TBS; Zymed, San Francisco, Calif). Alkaline phosphatase color development buffer (Bio-Rad) was added and rinsed after 10 minutes with tap water.

ELISA and ELISA inhibition

ELISA was performed as described earlier.³ An equal volume (50 μ L) of each fraction derived from gel filtration (Superdex 200) of tTG or peptic fraction B before and after tTG treatment was used for coating microtiter plates. The wells were postcoated with 1% human serum albumin in 50 mmol/L carbonate buffer, and 100 μ L of pooled sera from 10 patients with WDEIA (diluted 1:10) was added. Biotinylated goat anti-human IgE (diluted 1:1000, Vector) was added, followed by streptavidin-conjugated alkaline phosphatase (diluted 1:1000, Zymed) and the color substrate solution containing paranitrophenyl phosphate (104, Sigma). The optical density was read at 405 nm with an automated ELISA reader (Titertek Multiscan; Eflab, Turku, Finland).

In ELISA inhibition microtiter plates were coated at a concentration of 2 μ g/mL with intact ω -5 gliadin or peptic fraction B treat-

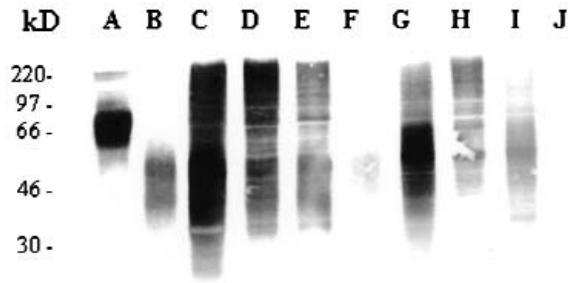


FIG 1. IgE immunoblotting of pooled patient sera ($n = 10$) to 3 μ g of intact, pepsin-digested, and pepsin-trypsin-digested ω -5 gliadin treated with 9, 3, and 1 μ g of tTG. *Lane A*, Intact ω -5 gliadin; *lane B*, pepsin digest; *lane C*, pepsin digest plus tTG (3:1); *lane D*, pepsin digest plus tTG (1:1); *lane E*, pepsin digest plus tTG (1:3); *lane F*, pepsin-tryptic digest; *lane G*, pepsin-tryptic digest plus tTG (3:1); *lane H*, pepsin-tryptic digest plus tTG (1:1); *lane I*, pepsin-tryptic digest plus tTG (1:3); *lane J*, 9 μ g of tTG.

ed with tTG. Individual and pooled sera from 10 patients with WDEIA were preincubated for 1 hour with serial 10-fold dilutions of ω -5 gliadin or tTG-treated peptic fraction B before applying them to the microtiter plate. The inhibitor concentrations, ranging from 0.005 to 50 μ g/mL, corresponded to the protein concentrations before tTG treatment. All other steps were performed as described above for ELISA.

Skin prick testing

The skin prick test extracts were prepared by dissolving 5 μ g of untreated ω -5 gliadin, the whole peptic digest, the peptic-tryptic digest, or peptic fraction B from gel filtration in 100 μ L of 20% ethanol in 0.1 mol/L Tris-HCl (pH 8; concentration, 50 μ g/mL). An equal amount of each antigen was treated with tTG (15 μ g, enzyme/protein ratio of 3:1), and the volume was adjusted to 100 μ L in 20% ethanol in 0.1 mol/L Tris-HCl. Histamine dihydrochloride (10 mg/mL; ALK, Copenhagen, Denmark) was used as a positive control, and tTG (150 μ g/mL in 20% ethanol-Tris-HCl), 20% ethanol-Tris-HCl, and physiologic saline solution were used as negative controls.

RESULTS

tTG treatment of digested ω -5 gliadin forms high-molecular-weight complexes showing strong IgE binding in immunoblotting

In immunoblotting pooled sera from 10 patients with WDEIA showed clear IgE binding to the peptides derived from pepsin digestion of ω -5 gliadin (Fig 1). Subsequent tTG treatment of the whole peptic digest formed large peptide aggregates, with molecular weights ranging from 40 to over 200 kD. These cross-linked aggregates showed more intense IgE binding in immunoblotting than did the peptic digest or intact ω -5 gliadin. Successive pepsin and trypsin digestion fractionated ω -5 gliadin into smaller peptides that were cross-linked by tTG to a lesser extent than the peptic digest and showed weaker IgE binding in immunoblotting (Fig 1).

The peptides in the whole peptic digest were further characterized by means of gel-filtration HPLC (Fig 2, A). Of the 4 fractions (fractions A-D) studied with immunoblotting (Fig 2, B), the first 3 were cross-linked

by tTG (Fig 2, C). The second fraction (fraction B), corresponding to the highest peak of the gel-filtration chromatogram and thus to the largest amount of protein, showed the greatest degree of cross-linking by tTG and the most intense IgE binding in immunoblotting.

tTG treatment of peptic fraction B increases IgE binding in ELISA

The most reactive peptic fraction B and an equivalent amount of the fraction treated with tTG were subjected again to gel-filtration HPLC to examine the complexes formed after tTG treatment. Fractions, separated according to size, were collected, and their IgE-binding abilities were compared in ELISA. tTG treatment created a protein peak that eluted earlier in the chromatogram and was not present in the untreated peptic fraction B (Fig 3, A). In ELISA IgE from pooled patient sera ($n = 10$) showed clear binding to this tTG-derived protein peak, as well as higher IgE values to all fractions after tTG treatment (Fig 3, B). None of the patient sera showed IgE binding to tTG or any of its gel-filtration fractions.

ω -5 Gliadin inhibits more than 90% of serum IgE binding to the tTG-treated peptic fraction B

In ELISA inhibition intact ω -5 gliadin (0.005-50 μ g/mL) inhibited in a dose-dependent manner up to 90% to 95% of patient serum ($n = 10$) IgE binding to the tTG-treated peptic fraction B and vice versa (Fig 4). The inhibition curve was almost identical when IgE binding to solid-phase ω -5 gliadin was inhibited with ω -5 gliadin itself.

tTG treatment enhances in vivo reactivity in skin prick testing

In 18 of the 20 patients with WDEIA, the tTG-treated peptic fraction B caused a markedly larger skin prick test reaction than untreated peptic fraction B (Fig 5, A). In the 20 patients the mean wheal area of the tTG-treated peptic fraction B (53 mm²) was 77% larger ($P < .001$, Wilcoxon matched pairs test) than that of untreated peptic fraction B (30 mm²) and 56% larger ($P < .01$, Wilcoxon matched pairs test) than that of intact ω -5 gliadin (34 mm²; Fig 5, B). Compared with the reaction caused by histamine (19 mm²), ω -5 gliadin caused, on average, a 1.8-fold larger reaction, peptic fraction B a 1.6-fold larger reaction, and tTG-treated peptic fraction B a 2.8-fold larger reaction. The mean wheal areas of the whole peptic digest (30 mm²) and the peptic-tryptic digest (30 mm²) increased slightly after tTG treatment (34 and 36 mm², respectively), but the differences were statistically insignificant (data not shown). Skin prick test responses to tTG were negative in all patients and control subjects. The 3 control subjects with ingested wheat allergy not related to exercise had positive reactions to intact ω -5 gliadin (mean wheal area 20 mm²) but had negative reactions to untreated and tTG-treated peptic fraction B. Skin prick test responses were completely negative in the patient with exercise-induced anaphylaxis not related to food ingestion.

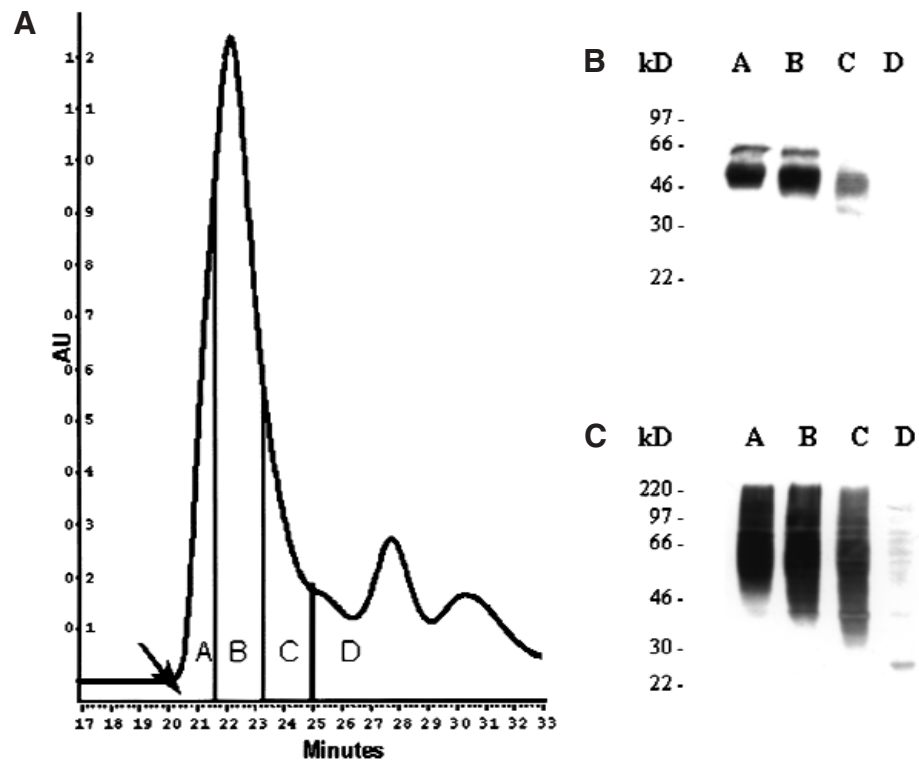


FIG 2. A, Gel-filtration HPLC (Superdex 75) chromatogram at 214 nm of the whole peptic digest of ω -5 gliadin. The arrow indicates the approximate elution position of intact ω -5 gliadin. B, IgE binding in immunoblotting of pooled patient sera to 3 μ g of gel-filtration fractions A through D of the whole peptic digest of ω -5 gliadin. C, IgE binding in immunoblotting of pooled patient sera to 3 μ g of the same fractions A through D after tTG treatment.

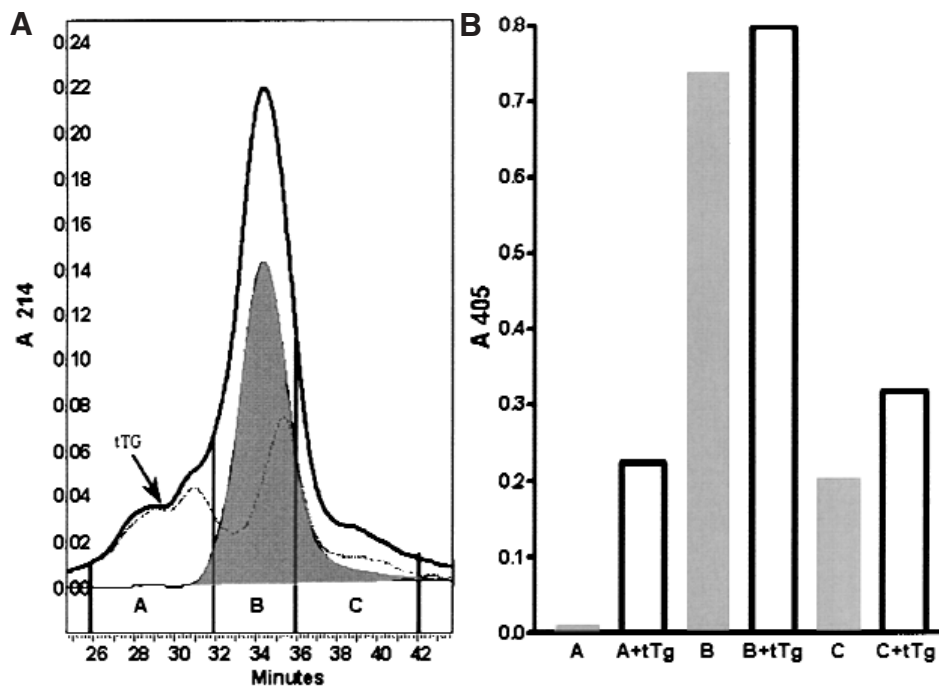


FIG 3. A, Gel-filtration HPLC (Superdex 200) chromatogram at 214 nm of the peptic fraction B of ω -5 gliadin before (gray fill) and after (no fill) tTG treatment and of tTG only (dotted line). The arrow indicates the approximate elution position of tTG at 78 kD. B, IgE antibody levels in ELISA (absorbance units) to the 3 peptide pools (A-C) of the peptic fraction B of ω -5 gliadin before (shaded bars) and after (open bars) tTG treatment in pooled patient sera.

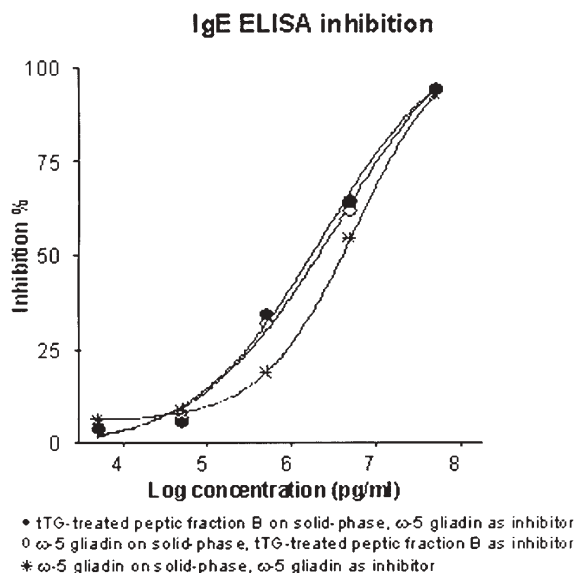


FIG 4. Inhibition of IgE binding from pooled patient sera to solid-phase ω -5 gliadin by tTG-treated peptic fraction B (open circles), and ω -5 gliadin (stars) and to tTG-treated peptic fraction B on solid phase by ω -5 gliadin (filled circles).

DISCUSSION

We show that the IgE-binding ability of ω -5 gliadin, a major allergen in WDEIA, is retained after pepsin and trypsin digestion and that digested peptides are cross-linked by tTG into high-molecular-weight complexes. Cross-linking of a dominant peptic fraction of ω -5 gliadin considerably increases IgE binding both in vitro and in vivo in patients with WDEIA. We hypothesize that tTG activation during physical exercise could create large allergen complexes capable of eliciting anaphylactic reactions in sensitized individuals.

Immunoblotting experiments, as well as gel-filtration experiments, demonstrated that tTG treatment of digested ω -5 gliadin forms high-molecular-weight complexes showing strong IgE binding in immunoblotting assays, ELISAs, and skin prick tests. The detailed structures of these complexes were not studied, but they are likely to consist of ω -5 gliadin peptides cross-linked to each other but also to tTG itself.¹² The finding that intact ω -5 gliadin inhibited serum IgE binding to these complexes and vice versa suggests that the IgE-binding epitopes in ω -5 gliadin are conserved during digestion and that tTG does not create neoepitopes. Although the actual number of IgE-binding epitopes in the digested peptides seems unaltered after tTG treatment, covalent incorporation of these peptides into highly stable aggregates alters their spatial arrangement, which could explain the significantly enhanced skin prick test responses. Namely, large complexes of IgE-binding peptides irreversibly bound to a carrier-like protein could cross-link IgE molecules on cutaneous mast cells more effectively than the same amount of epitopes present on individual peptides.

tTG belongs to the family of transglutaminase enzymes that modify proteins by inserting isopeptide bonds between polypeptide chains, forming highly stable protein cross-links.^{13,19} Unlike other transglutaminases, tTG can also hydrolyze ATP and guanosine triphosphate and act as a signal-transducing G-protein.²⁰ tTG has been implicated in diverse biologic processes, including cell growth and differentiation, tissue repair, receptor-mediated endocytosis, and the induction of apoptosis, but its precise physiologic function remains unknown.^{13,19} Inappropriate tTG activation is related to various disease processes, including neurodegenerative diseases, tissue fibrosis, atherosclerosis, and cataract formation.¹⁷ The role of tTG is well established in celiac disease. In this inflammatory disease of the small intestine, tTG-catalyzed deamidation of ingested gliadins enhances their recognition by gut-derived T cells,^{12,21} and tTG itself is a target for autoantibody formation.²²

Under physiologic conditions, tTG exists in an inactive guanosine triphosphate-binding state but might function as a signal-transducing G-protein.^{15,20} Increased calcium levels (eg, after cellular damage) induce a conformational change that activates the cross-linking function of tTG.^{23,24} Increased cross-linking activity has been observed under stress conditions, including inflammation, infection, and metabolic and oxidative stress.^{16,17} Induction of the acute-phase response in mice by LPS led to an increase in transglutaminase activity.²⁵ In addition, many molecules, such as the cytokines IL-6 and TNF- α ,^{25,26} retinoids,²⁷ glucocorticoid hormones,²⁸ and reactive oxygen species,²⁹ can induce tTG expression and increase its activity.

Although no direct evidence of tTG activation in response to exercise exists, many factors released during exercise are known to activate this stress-responsive enzyme. Physical exercise elicits a subclinical inflammatory response manifested by increases in the pro-inflammatory cytokines TNF- α and IL-1 β , followed by a dramatic increase in the inflammation-responsive cytokine IL-6.^{30,31} The contracting skeletal muscle produces IL-6 in large amounts during exercise, and it is released into the circulation to a greater extent than any other cytokine.^{32,33} In addition, exercise increases plasma concentrations of glucocorticoid stress hormones, including cortisol, growth hormone, and catecholamines.³¹ Moreover, intense physical exercise has the potential to increase production of reactive oxygen species and lead to oxidative stress.^{34,35} Strenuous exercise reduces splanchnic blood flow up to 50%,^{36,37} which might lead to gastric mucosal ischemia and increased intestinal permeability.^{38,39} Taken together, tTG can be activated by TNF- α , IL-6, glucocorticoid hormones, and reactive oxygen species, all of which are detectable in an exercising individual. Furthermore, severe or prolonged exercise might cause intestinal ischemia and cellular damage. It is thus reasonable to speculate that the cross-linking function of tTG could be activated during physical exercise.

Several earlier studies have addressed the role of exercise in provoking symptoms of FDEIA. These studies

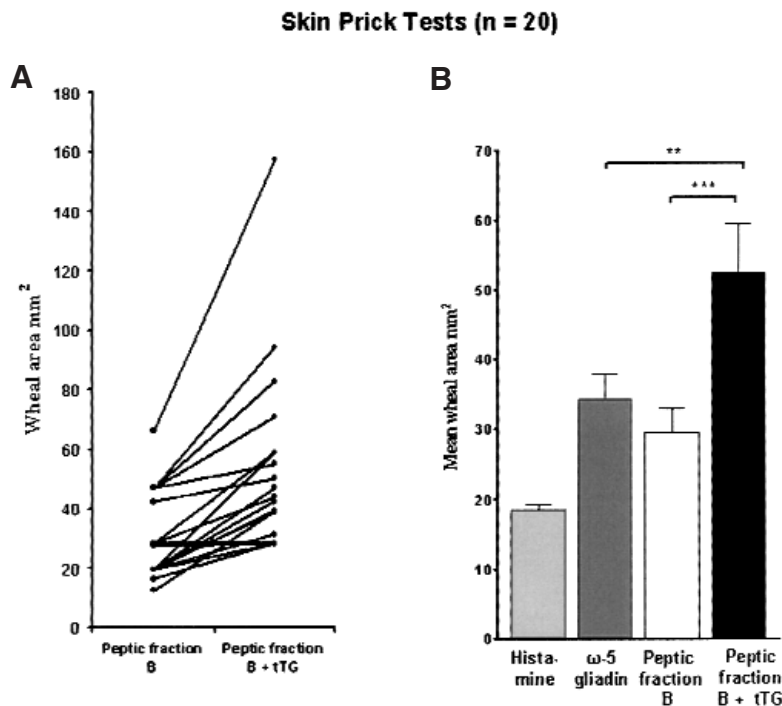


FIG 5. A, Skin prick test results in 20 patients with WDEIA to peptic fraction B of ω -5 gliadin before and after tTG treatment. B, Mean skin prick test wheal areas to histamine, ω -5 gliadin, peptic fraction B, and tTG-treated peptic fraction B in the 20 patients. The bars represent the means, and the error bars represent the SEMs. ** $P < .01$ and *** $P < .001$, Wilcoxon matched pairs test.

have hypothesized that exercise might increase intestinal allergen absorption, provoke abnormal responses of the autonomic nervous system, or lower the threshold for IgE-mediated mast cell degranulation.^{40,41} Our previous study demonstrated high basophil histamine release in patients with WDEIA in response to gliadin stimulation,¹⁰ but evidence on lower mast cell degranulation thresholds during exercise or increased intestinal allergen absorption in WDEIA is lacking. Interestingly, 2 recent reports demonstrated that aspirin potentiated wheat-induced symptoms during exercise provocation tests and that combined wheat and aspirin ingestion was able to trigger symptoms in patients with WDEIA, even without exercise.^{42,43} Earlier in vitro studies have shown that nonsteroidal anti-inflammatory drugs, such as aspirin, augment allergen-induced histamine release from human mast cells and basophils.⁴⁴ On the other hand, aspirin is known to induce cellular injury to the gastrointestinal mucosa,⁴⁵ which could possibly contribute to the activation of tTG. Allergen absorption might also increase because aspirin intake during prolonged exercise causes a significant increase in gastrointestinal permeability.⁴⁵ Nevertheless, aspirin intake was not a contributing factor in any of our 20 patients with WDEIA.

Here we examined whether gastrointestinal digestive enzymes and tTG modulate the IgE-binding ability of ω -5 gliadin in patients with WDEIA. We demonstrate that pepsin digestion of ω -5 gliadin forms peptides that are cross-linked by tTG into high-molecular-weight com-

plexes showing strong binding of serum IgE antibodies. Cross-linking of a dominant peptic digest fraction brings about a dramatic increase in skin prick test reactivity. Our findings, together with recent data on tTG activation and exercise immunology, support the hypothesis that activation of tTG during exercise could create large allergen complexes capable of eliciting anaphylactic reactions in patients with WDEIA. Further in vivo studies demonstrating exercise-induced activation of tTG and the presence of gliadin-tTG complexes in the circulation or intestinal mucosa of patients with WDEIA are needed to confirm our hypothesis. Future work will also elucidate whether tTG can cross-link other food allergens capable of eliciting FDEIA.

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REFERENCES

- Kidd JM 3rd, Cohen SH, Sosman AJ, Fink JN. Food-dependent exercise-induced anaphylaxis. *J Allergy Clin Immunol* 1983;71:407-11.
- Sheffer AL, Austen KF. Exercise-induced anaphylaxis. *J Allergy Clin Immunol* 1980;66:106-11.
- Palosuo K, Alenius H, Varjonen E, Koivuluhta M, Mikkola J, Keskinen H, et al. A novel wheat gliadin as a cause of exercise-induced anaphylaxis. *J Allergy Clin Immunol* 1999;103:912-7.
- Romano A, Di Fonso M, Giuffreda F, Papa G, Artesani MC, Viola M, et al. Food-dependent exercise-induced anaphylaxis: clinical and laboratory findings in 54 subjects. *Int Arch Allergy Immunol* 2001;125:264-72.
- Guinnepain M-T, Eloit C, Raffard M, Brunet-Moret M-J, Rassemont R, Laurent J. Exercise-induced anaphylaxis: useful screening of food sensitization. *Ann Allergy Asthma Immunol* 1996;77:491-6.

6. Shadick NA, Liang MH, Partridge AJ, Bingham C, Wright E, Fossel AH, et al. The natural history of exercise-induced anaphylaxis: survey results from a 10-year follow-up study. *J Allergy Clin Immunol* 1999;104:123-7.
7. Kushimoto H, Aoki T. Masked type I wheat allergy. Relation to exercise-induced anaphylaxis. *Arch Dermatol* 1985;121:355-60.
8. Varjonen E, Vainio E, Kalimo K. Life-threatening, recurrent anaphylaxis caused by allergy to gliadin and exercise. *Clin Exp Allergy* 1997;27:162-6.
9. Morita E, Kameyoshi Y, Mihara S, Hiragun T, Yamamoto S. γ -Gliadin: a presumptive allergen causing wheat-dependent exercise-induced anaphylaxis. *Br J Dermatol* 2001;145:182-4.
10. Lehto M, Palosuo K, Varjonen E, Majuri ML, Andersson U, Reunala T, et al. Humoral and cellular responses to gliadin in wheat-dependent, exercise-induced anaphylaxis. *Clin Exp Allergy* 2003;33:90-5.
11. Palosuo K, Alenius H, Varjonen E, Kalkkinen N, Reunala T. Rye γ -70 and γ -35 secalins and barley γ -3 hordein cross-react with ω -5 gliadin, a major allergen in wheat-dependent, exercise-induced anaphylaxis. *Clin Exp Allergy* 2001;31:466-73.
12. Molberg O, Mcadam SN, Korner R, Quarsten H, Kristiansen C, Madsen L, et al. Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease. *Nat Med* 1998;4:713-7.
13. Chen JS, Mehta K. Tissue transglutaminase: an enzyme with a split personality. *Int J Biochem Cell Biol* 1999;31:817-36.
14. Kasarda D, Autran J, Lew E, Nimmo C, Shewry P. N-terminal amino acid sequences of ω -gliadins and ω -secalins: implications for the evolution of prolamin genes. *Biochim Biophys Acta* 1983;747:138-50.
15. Smethurst PA, Griffin M. Measurement of tissue transglutaminase activity in a permeabilized cell system: its regulation by Ca^{2+} and nucleotides. *Biochem J* 1996;313:803-8.
16. Korner G, Deutsch VR, Vlodavsky I, Eldor A. Effects of ionizing irradiation on endothelial cell transglutaminase. *FEBS Lett* 1993;330:41-5.
17. Kim SY, Jeitner TM, Steinert PM. Transglutaminases in disease. *Neurochem Int* 2002;40:85-103.
18. Hoffman-Goetz L, Pedersen BK. Exercise and the immune system: a model of the stress response? *Immunol Today* 1994;15:382-7.
19. Aeschlimann D, Thomazy V. Protein crosslinking in assembly and remodeling of extracellular matrices: the role of transglutaminases. *Connect Tissue Res* 2000;41:1-27.
20. Im MJ, Russell MA, Feng JF. Transglutaminase II: a new class of GTP-binding protein with new biological functions. *Cell Signal* 1997;9:477-82.
21. Anderson RP, Degano P, Godkin AJ, Jewell DP, Hill AV. In vivo antigen challenge in celiac disease identifies a single transglutaminase-modified peptide as the dominant A-gliadin T-cell epitope. *Nat Med* 2000;6:337-42.
22. Dieterich W, Ehnis T, Bauer M, Donner P, Volta U, Riecken EO, et al. Identification of tissue transglutaminase as the autoantigen of celiac disease. *Nat Med* 1997;3:797-801.
23. Di Venere A, Rossi A, De Matteis F, Rosato N, Agro AF, Mei G. Opposite effects of Ca^{2+} and GTP binding on tissue transglutaminase tertiary structure. *J Biol Chem* 2000;275:3915-21.
24. Liu S, Cerione RA, Clardy J. Structural basis for the guanine nucleotide-binding activity of tissue transglutaminase and its regulation of transamidation activity. *Proc Natl Acad Sci U S A* 2002;99:2743-7.
25. Suto N, Ikura K, Sasaki R. Expression induced by interleukin-6 of tissue-type transglutaminase in human hepatoblastoma HepG2 cells. *J Biol Chem* 1993;268:7469-73.
26. Kuncio GS, Tsyganskaya M, Zhu J, Liu SL, Nagy L, Thomazy V, et al. TNF- α modulates expression of the tissue transglutaminase gene in liver cells. *Am J Physiol* 1998;274:G240-5.
27. Davies PJ, Murtaugh MP, Moore WT, Johnson GS, Lucas D. Retinoic acid-induced expression of tissue transglutaminase in human promyelocytic leukemia (HL-60) cells. *J Biol Chem* 1985;260:5166-74.
28. Johnson TS, Scholfield CI, Parry J, Griffin M. Induction of tissue transglutaminase by dexamethasone: its correlation to receptor number and transglutaminase-mediated cell death in a series of malignant hamster fibrosarcomas. *Biochem J* 1998;331:105-12.
29. Mirza A, Liu SL, Frizell E, Zhu J, Maddukuri S, Martinez J, et al. A role for tissue transglutaminase in hepatic injury and fibrogenesis, and its regulation by NF- κ B. *Am J Physiol* 1997;272:G281-8.
30. Ostrowski K, Rohde T, Asp S, Schjerling P, Pedersen BK. Pro- and anti-inflammatory cytokine balance in strenuous exercise in humans. *J Physiol* 1999;515:287-91.
31. Pedersen BK, Hoffman-Goetz L. Exercise and the immune system: regulation, integration, and adaptation. *Physiol Rev* 2000;80:1055-81.
32. Steensberg A, van Hall G, Osada T, Sacchetti M, Saltin B, Pedersen BK. Production of interleukin-6 in contracting human skeletal muscles can account for the exercise-induced increase in plasma interleukin-6. *J Physiol* 2000;529:237-42.
33. Pedersen BK, Steensberg A, Schjerling P. Muscle-derived interleukin-6: possible biological effects. *J Physiol* 2001;536:329-37.
34. Vider J, Lehtmaa J, Kullisaar T, Vihalemm T, Zilmer K, Kairane C, et al. Acute immune response in respect to exercise-induced oxidative stress. *Pathophysiology* 2001;7:263-70.
35. Cooper CE, Vollaard NB, Choueiri T, Wilson MT. Exercise, free radicals and oxidative stress. *Biochem Soc Trans* 2002;30:280-5.
36. Qamar MI, Read AE. Effects of exercise on mesenteric blood flow in man. *Gut* 1987;28:583-7.
37. Osada T, Katsumura T, Hamaoka T, Inoue S, Esaki K, Sakamoto A, et al. Reduced blood flow in abdominal viscera measured by Doppler ultrasound during one-legged knee extension. *J Appl Physiol* 1999;86:709-19.
38. Otte JA, Oostveen E, Geelkerken RH, Groeneveld AB, Kolkman JJ. Exercise induces gastric ischemia in healthy volunteers: a tonometry study. *J Appl Physiol* 2001;91:866-71.
39. Pals KL, Chang RT, Ryan AJ, Gisolfi CV. Effect of running intensity on intestinal permeability. *J Appl Physiol* 1997;82:571-6.
40. Kivity S, Sneh E, Greif J, Topilsky M, Mekori YA. The effect of food and exercise on the skin response to compound 48/80 in patients with food-associated exercise-induced urticaria-angioedema. *J Allergy Clin Immunol* 1988;81:1155-8.
41. Fukutomi O, Kondo N, Agata H, Shinoda S, Shinbara M, Orii T. Abnormal responses of the autonomic nervous system in food-dependent exercise-induced anaphylaxis. *Ann Allergy* 1992;68:438-45.
42. Harada S, Horikawa T, Ashida M, Kamo T, Nishioka E, Ichihashi M. Aspirin enhances the induction of type I allergic symptoms when combined with food and exercise in patients with food-dependent exercise-induced anaphylaxis. *Br J Dermatol* 2001;145:336-9.
43. Aihara M, Miyazawa M, Osuna H, Tsubaki K, Ikebe T, Aihara Y, et al. Food-dependent exercise-induced anaphylaxis: influence of concurrent aspirin administration on skin testing and provocation. *Br J Dermatol* 2002;146:466-72.
44. Wojnar RJ, Hearn T, Starkweather S. Augmentation of allergic histamine release from human leukocytes by nonsteroidal anti-inflammatory-analgesic agents. *J Allergy Clin Immunol* 1980;66:37-45.
45. Lambert GP, Broussard LJ, Mason BL, Mauermann WJ, Gisolfi CV. Gastrointestinal permeability during exercise: effects of aspirin and energy-containing beverages. *J Appl Physiol* 2001;90:2075-80.