FOOD COMPOSITION AND ADDITIVES

The Celiac Patient Antibody Response to Conventional and Gluten-Removed Beer

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Enzymatic digestion, or hydrolysis, has been proposed for treating gluten-containing foods and beverages to make them safe for persons with celiac disease (CD). There are no validated testing methods that allow the quantitation of all the hydrolyzed or fermented gluten peptides in foods and beverages that might be harmful to CD patients, making it difficult to assess the safety of hydrolyzed products. This study examines an ELISA-based method to determine whether serum antibody binding of residual peptides in a fermented barley-based product is greater among active-CD patients than a normal control group, using commercial beers as a test case. Sera from 31 active-CD patients and 29 nonceliac control subjects were used to assess the binding of proteins from barley, rice, traditional beer, gluten-free beer, and enzymatically treated (gluten-removed) traditional beer. In the ELISA, none of the subjects' sera bound to proteins in the gluten-free beer. Eleven active-CD patient serum samples demonstrated immunoglobulin A (IgA) or immunoglobulin G (IgG) binding to a barley extract, compared to only one nonceliac control subject. Of the seven active-CD patients who had an IgA binding response to barley, four also responded to traditional beer, and two of these responded to the gluten-removed beer. None of the nonceliac control subjects' sera bound to all three beer samples. Binding of protein fragments in hydrolyzed or fermented foods and beverages by serum from active-CD patients, but not nonceliac control subjects, may indicate the presence of residual peptides that are celiac-specific.

Celiac disease (CD) is an autoimmune reaction initiated by the gluten fraction of the proteins of wheat, rye, barley, and related grains that results in intestinal damage and subsequent pathologies. CD affects 1% or more of the populations of the United States and Europe, affects both children and adults (1), and is increasing in prevalence (2). To date, the only treatment for CD is the avoidance of dietary gluten (3). Dietary avoidance is achieved by careful attention to food labels and consumption of products that are naturally gluten-free or are shown to be gluten-free (defined by regulation as <20 ppm gluten; 4–6) through laboratory testing methods.

Wheat provides approximately 20% of the world's calories (7), so the avoidance of wheat and its related grains (predominantly rye and barley) can result in very limited food choices. Among many proposed solutions to this limitation, researchers have examined enzymatic methods of breaking down the hazardous gluten fraction of these prohibited grains when they are used in food production (as reviewed in refs 8–11). The methods used to date to hydrolyze gluten include extended fermentation with sourdough lactobacilli with or without fungal proteases (12–17), digestion with crude papain or its derivatives (18–20), digestion with prolyl endopeptidases (PEPs) from *Aspergillus niger* (21–24), and digestion with endogenous grain peptidases (25). These digestions theoretically fragment gluten into small peptides that will not initiate an immune reaction, or specifically degrade proline-rich peptides that are known to be toxic in CD.

A 2014 proposed regulation from the U.S. Food and Drug Administration (FDA; 26) would prohibit the use of enzymatic or chemical hydrolysis of gluten to produce gluten-free foods for consumers. The rationale behind this regulation is an absence of validated test methods that can detect residual gluten fragments in foods. For hydrolyzed gluten products to be available and beneficial to the celiac community, their safety must be demonstrated, but current methods for detecting hydrolyzed gluten proteins in food directly are considered insufficient by the FDA.

Without an acceptable assay for detecting and quantitating gluten levels in hydrolyzed foods and beverages, an alternative method to determine the safety of these products is a clinical "challenge" study, in which persons with CD consume the product in known dosages and the effects of this consumption are analyzed. As reviewed by Stoven et al. (10) and Caputo et al. (27), very few clinical studies have been done to test the safety of hydrolyzed gluten in foods. Although some studies have shown clinical improvement in patients consuming hydrolyzed gluten (20, 28), few have included biopsy data, the gold standard for

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the diagnosis of CD (16). The majority of studies have evaluated the theoretical safety of gluten hydrolysates by assessing the size of the remaining protein fragments (17, 22, 29, 30) or by performing ELISA analysis of residual gluten levels (14, 16, 17, 22, 28, 30–35), in vitro analysis (13–15, 18, 21–23, 25, 31, 33, 35–38), or LC-MS (reviewed in ref. 39).

As demonstrated by Greco et al. (16), there is concern that gluten that is not broken down to the amino acid level will still be capable of initiating an immune response. In those studies of hydrolyzed gluten in which two-dimensional electrophoresis or chromatographic data were available alongside ELISA results of <20 ppm, residual glutelins or large peptides (>8 kDa) were detected by electrophoresis or chromatography. The ELISA testing in each of these cases was performed with a gliadin-specific antibody, possibly explaining this discrepancy. There have long been concerns with the use of ELISAs for detecting residual gluten after hydrolysis (40–42), including a lack of accuracy and a lack of clinical correlates.

LC-MS analysis has been used to analyze gluten samples digested with PEPs, which are enzymes that specifically target the amino acid proline as a substrate for hydrolysis. Because the known toxic peptides of gluten are proline-rich, this digestion method is being used in some commercial beers, which are then advertised as gluten-removed. PEP digestion was shown by reversed-phase HPLC and LC-MS to create fragments with an average length of 20.3 amino acid residues, much larger than the 8 or 9 residues needed for recognition by T-cell receptors. These residues induced T-cell proliferation in 6 of 14 polyclonal T-cell lines derived from small intestine biopsy samples of CD patients (43). Other LC-MS studies have focused on the PEP digestion of a specific subset of prolamin-containing peptides to assess the safety of gluten digests (44, 45), finding moderate removal of these fractions. LC-MS methods have some of the same limitations as ELISAs, in that they extrapolate gluten levels from a few select peptides and, although they can detect a large number of peptides simultaneously, they cannot detect all of the potentially toxic peptides implicated in CD, in part because not all the toxic peptides are known (46).

In vivo clinical challenge trials involving small bowel histology would be the gold standard for verifying the safety of foods produced with hydrolyzed gluten (16), but these studies are difficult to initiate and very expensive. The current study uses an alternative biological marker, i.e., recognition by serum antibodies of active-CD patients, as a less-invasive test to assess the safety of a gluten-removed beer. This method may be able to detect both known and unknown immunogenic gluten epitopes, depending on the antibody repertoire of the subjects, in contrast to the single or select few peptides targeted by ELISA or LC-MS. A product that had been sufficiently hydrolyzed should not contain residual protein fragments that are recognized by the serum antibodies of CD patients, and the demonstration of antibody binding would indicate that further product analysis, perhaps through MS studies to identify the recognized peptide fragments and challenge studies to determine their toxicity, is necessary.

The serum antibody repertoire of CD patients is broad but not uniform (47), meaning that each individual with CD may react to different gluten proteins or to the same proteins with differing intensity. However, with an appropriate cutoff based on a normal control group, the expression of anti-gliadin immunoglobulin G (IgG) and IgA in active CD can be greater than 80% sensitive and specific (48–50) for the presence of the disease. Thus, patients with active CD would be expected to be expressing serum antibodies that would recognize residual antigenic proteins found in foods and beverages made from fermented or hydrolyzed gluten-containing grains, if they were present. Examining serum antibody binding of proteins found in food and beverages requires the recruitment of CD patients in an active disease state (i.e., still consuming gluten), because serum antibody titers decline after patients begin a gluten-free diet (51).

The study presented here used serum samples from patients with active CD and from nonceliac control subjects to examine their antibody responses to commercially available beers, including a traditional barley beer, a gluten-free beer made from sorghum, and a gluten-removed beer. Although the sera of the active-CD patients all contained detectable levels of anti-wheat gliadin antibodies, a hallmark of the diagnosis, cultivated barley has been shown to be less immunogenic than wheat (52). Therefore this study used a barley extract to first determine which patients were expressing detectable levels of anti-barley antibodies, and then examined these patients' reactions to the beer samples.

In the production of traditional beer, many proteins are extracted or degraded during fermentation, and others are lost during filtration. In gluten-removed beer, these residual proteins undergo additional enzymatic hydrolysis. A detectable antibody response to the protein fractions of these products would indicate that there are residual proteins that are recognized by those patients who are already expressing antibodies that detect barley, and raise concerns that those residual proteins might be capable of inducing an antibody response in those patients who are predisposed to CD.

Materials

Subject Sera

Serum samples were obtained through the University of Chicago Celiac Disease Center from both pediatric and adult active-CD patients (n = 31) and nonceliac control subjects (n = 29). The demographics of the subjects and the patients, including comorbidities and the results of anti-gliadin and anti-tissue transglutaminase (anti-tTG) antibody testing, are presented in Tables 1 and 2, respectively.

Test Samples

(a) *Commercially available rice flour*.—Fairway Market (New York, NY).

(b) *Commercially available barley flour*.—Fairway Market.

(c) *Commercially available barley beer consisting of malted barley, hops, yeast, and water*.—Fairway Market.

(d) *Commercially available gluten-free beer consisting of sorghum, brown rice, maltodextrin, yucca extract, hops, water, and yeast*.—Fairway Market.

(e) *Commercially available gluten-removed beer consisting of barley malt, rice, hops, yeast, and water*.—Fairway Market.

Assay Reagents and Equipment

(a) *QUANTA Lite Gliadin IgG II ELISA*.—Cat. No. 066704520 (Inova Diagnostics, San Diego, CA).

Table 1. Control subject demographics and results of standard anti-tTG and anti-gliadin tests

	Nonceliac control subject demographics			Antibody test results[a]			
ID	Comorbidities	Age, y	Sex	Anti-gliadin IgA	Anti-gliadin IgG	Anti-tTG IgA	Anti-tTG IgG
C1	None	16	M[b]	1	1	2	1
C2	None	7	F[c]	2	1	3	1
C3	GERD[d]	5	M	2	1	2	1
C4	EE[e]	19	M	<1	2	2	1
C5	None	11	M	2	3	1	1
C6	EoE[f]	4	F	1	<3	2	<1
C7	C, LI[g]	46	M	3	1	3	2
C8	EoE	17	M	3	1	2	1
C9	EoE	10	M	3	4	1	1
C10	None	66	F	3	<1	4	<1
C11	None	19	F	3	1	5	2
C12	None	14	F	2	2	2	3
C13	GERD	19	F	3	1	2	2
C14	None	18	M	1	2	3	1
C15	None	17	M	4	1	5	1
C16	IBD, Cr, A[h]	35	F	2	2	5	3
C17	GERD, H[i]	8	F	1	2	1	1
C18	None	4	M	<1	1	<1	<1
C19	GERD	5	M	2	1	2	1
C20	None	18	F	2	<1	4	1
C21	ADHD, MA, PA[j]	12	M	58	32	4	1
C22	None	20	F	24	<1	2	NA[k]
C23	None	17	F	2	4	2	NA
C24	None	15	F	2	3	4	NA
C25	LI	18	F	3	1	3	NA
C26	Reflux	13	F	2	1	3	NA
C27	None	17	F	5	3	4	NA
C28	None	7	F	1	5	9	NA
C29	None	66	M	5	<1	4	NA

[a] A test result >20 is considered positive.

[b] M = Male.

[c] F = Female.

[d] GERD = Gastroesophageal reflux disease.

[e] EE = Erosive esophagitis.

[f] EoE = Eosinophilic esophagitis.

[g] C = Cancer; LI = lactose intolerance.

[h] IBD = Inflammatory bowel disease; Cr = Crohn's disease; A = anemia.

[i] H = Hypoglycemia.

[j] ADHD = Attention deficit hyperactivity disorder; MA = milk allergy; PA = peanut allergy.

[k] NA = Not analyzed.

Table 2. Active-CD patient demographics and results of standard anti-tTG and anti-gliadin tests

	Active-CD patient demographics			Antibody test results[a]			
ID	Comorbidities	Age, y	Sex	Anti-gliadin IgA	Anti-gliadin IgG	Anti-tTG IgA	Anti-tTG IgG
CD1	D1[b]	12	M[c]	6	11	58	5
CD2	Pan, DQ2[d]	52	F[e]	7	6	155	13
CD3	None	41	M	34	78	45	36
CD4	D1	13	F	>1700	320	1380	24
CD5	Thy[f]	29	F	56	58	134	6
CD7	None	15	F	28	81	27	15
CD8	D1	11	M	106	65	115	39
CD9	GERD, Hy[g]	7	F	41	36	128	18
CD10	None, DQ2	61	F	>100	181	>100	55
CD11	None	9	F	345	330	203	8
CD12	None	14	F	34	39	180	27
CD13	HT, A, DD[h]	50	F	100	24	67	4
CD14	None	34	F	45	27	226	5
CD15	None	43	M	74	39	100	46
CD16	None	21	M	53	110	177	29
CD17	None	17	F	25	22	66	5
CD18	Fr[i]	12	F	300	240	203	16
CD19	None	43	M	147	91	25	5
CD20	DH, O, DQ2, DQB1[j]	50	M	275	93	22	1
CD21	KS[k]	45	M	29	43	19	NA[l]
CD22	None	7	F	185	122	>100	4
CD23	None	17	M	102	39	189	NA
CD24	D1	13	M	84	75	>100	3
CD25	Ost[m]	61	F	26	21	73	1
CD26	None, DQ8	32	M	21	>100	>100	54
CD27	CGD[n]	12	M	7	46	20	3
CD28	ADHD[o]	15	M	6	13	45	1
CD29	None	7	F	16	33	121	14
CD30	ADHD	10	F	9	21	143	28
CD31	JRA[p]	20	F	15	26	125	21

[a] A test result >20 is considered positive.

[b] D1 = Type 1 diabetes.

[c] M = Male.

[d] Pan = Pancreatitis; DQ = HLA DQ genotype.

[e] F = Female.

[f] Thy = Thyroiditis.

[g] GERD = Gastroesophageal reflux disease; Hy = hydronephrosis.

[h] HT = Hashimoto's thyroiditis; A = anemia; DD = vitamin D deficiency.

[i] Fr = Fractures.

[j] DH = Dermatitis herpetiformis; O = obesity.

[k] KS = Kidney stones.

[l] NA = Not analyzed.

[m] Ost = Osteopenia.

[n] CGD = Constitutional growth delay.

[o] ADHD = Attention deficit hyperactivity disorder.

[p] JRA = Juvenile rheumatoid arthritis.

(b) *QUANTA Lite Gliadin IgA II ELISA*.—Cat. No. 066704525 (Inova Diagnostics).

(c) *QUANTA Lite h-tTG IgG ELISA*.—Cat. No. 066708755 (Inova Diagnostics).

(d) *QUANTA Lite h-tTG IgA ELISA*.—Cat. No. 066708760 (Inova Diagnostics).

(e) *EZ Gluten assay*.—ELISA Technologies, Inc. (Gainesville, FL).

(f) *Polyethylene glycol*.—Cat. No. P6667 (Sigma, St. Louis, MO).

(g) *Spectra/Por 3 dialysis tubing with a 3.5 kDa MW cutoff*.—Cat. No. 132725 (Spectrum Laboratories, Inc. Rancho Dominguez, CA).

(h) *0.05 M carbonate/bicarbonate buffer, pH 9.6*.—Cat. No. SRE0034 (Sigma); used at 10X.

(i) *Nunc-Immuno MaxiSorp 96-Well ELISA plates*.—Cat. No. 62409-024 (VWR, Radnor, PA).

(j) *Phosphate-buffered saline (PBS)*.—Cat. No. P5493 (Sigma).

(k) *Bovine serum albumin (BSA)*.—Cat. No. A7906 (Sigma).

(l) *Horseradish peroxidase (HRP)-conjugated anti-human IgA secondary antibody*.—Cat. No. 31417 (Life Technologies, Carlsbad, CA).

(m) *HRP-conjugated anti-human IgG secondary antibody*.—Cat. No. 05-4220 (Life Technologies).

(n) *3,3′,5,5′-Tetramethylbenzidine (TMB) substrate*.—Cat. No. T0440 (Sigma).

(o) *Phosphoric acid (H_3PO_4)*.—Cat. No. 79607 (Sigma).

(p) *Eppendorf 5427R centrifuge*.—Eppendorf North America (Hauppauge, NY).

(q) *NanoDrop Lite spectrophotometer*.—Thermo Fisher Scientific (Ashville, NC).

Methods

Sample Preparation

Rice flour and barley flour were extracted for use as negative and positive controls, respectively. Rice flour was tested for gluten using the EZ Gluten lateral flow assay, an AOAC INTERNATIONAL *Performance Tested Method*^{SM}, and was negative for gluten with this test, which has a lower LOD of 10 ppm. Five gram portions of each flour were mixed with 45 mL 40% ethanol–water (v/v) solution, shown by Skerritt and Hill to be suitable for gluten extraction from raw, cooked, and processed foods (53). The samples were vortex-mixed and then incubated for 30 min at 45°C, with rotation at 500 rpm. After this incubation, 1.5 mL portions of each flour extract were transferred to 1.7 mL centrifuge tubes and centrifuged for 1 min at 10 000 relative centrifugal force using an Eppendorf 5427R centrifuge operating at room temperature. The resulting supernatants were analyzed for protein content using a NanoDrop Lite spectrophotometer, which was blanked on a 40% ethanol-water (v/v) solution, and total protein concentration was measured using the absorbance reading at the 280 nm wavelength.

Two hundred milliliter portions of each beer type were dialyzed against dry polyethylene glycol for 72 h using Spectra/Por 3 dialysis tubing with a 3.5 kDa MW cutoff in order to remove residual polyphenols that might interfere with the ELISAs. The resulting concentrates, ranging from 2 to 4 mL in volume, were diluted in an equal volume of a 40% ethanol–water solution, and analyzed for total protein content using the NanoDrop Lite spectrophotometer, as described above.

ELISAs

The flour extracts, as well as the beer concentrates, were diluted to 10 µg total protein/mL in 0.05 M carbonate/bicarbonate buffer, pH 9.6, and loaded onto 96-well Nunc-Immuno MaxiSorp plates at 100 µL/well. Wells were coated so that each serum sample could be tested in duplicate wells for each of the flour extracts and beer concentrates, as well as in duplicate wells coated only with 0.05 M carbonate/bicarbonate buffer (blanks).

The coated wells were allowed to incubate for 2 h at room temperature. A comparison of identical samples tested after a 2 h room-temperature coating incubation and an overnight coating incubation at 2–8°C showed no appreciable difference in results, so the 2 h coating incubation was used. After the coating incubation, the coating solutions were emptied from the 96-well plate, and all wells were blocked with 200 µL PBS containing 1% BSA. The blocking solution was allowed to incubate for 1 hour at room temperature, and then it was emptied from the wells and the wells were washed one time with 300 µL PBS just before adding the serum samples.

To test the serum IgA and IgG responses, each serum sample was diluted 1:200 with PBS/1% BSA for IgA testing and diluted 1:800 with PBS/0.1% BSA for IgG testing, and 50 µL was added to duplicate blank, flour, and beer wells. The sera were incubated for 2 h at room temperature, and then the wells were washed three times with 300 µL PBS. Fifty microliters HRP-conjugated anti-human IgA secondary antibody diluted 1:10 000 in PBS/1% BSA or 50 µL HRP-conjugated anti-human IgG secondary antibody diluted 1:500 in PBS/0.1% BSA was added to each well and incubated for 1 hour at room temperature, and then the wells were washed six times with 300 µL PBS. Fifty microliters TMB substrate was added to each well and incubated for 20 min at room temperature, and the reaction was stopped by adding 50 µL 25% H_3PO_4/water stop solution to each well. The ELISA plate was read using a 450 nm filter. The optical density (OD) values for each of the duplicate measurements were averaged, and the average values were corrected for nonspecific binding by subtracting the average OD of the uncoated wells from each. Responses to the flour and beer samples were determined to be positive if they were greater than 3 SDs above the OD mean of the nonceliac control group. The mean values calculated for the nonceliac control group included data from subjects C21 and C22 who, although they showed positive responses in standardized anti-gliadin and anti-tTG testing, did not have reactions above background to any of the flour or beer samples. Any samples that gave high OD values on initial testing, indicating a possible positive response, were repeated for confirmation on a separate ELISA plate.

Results

The goal of the current study was to determine whether sera from patients with active CD might be used as a biological marker for residual gluten proteins in processed foods by examining serum reactivity to conventional beer, gluten-free beer, and gluten-removed beer. Rice flour was intended to be used as a negative control, but two active-CD patient samples

gave responses to the rice flour extract. Both of these sera also reacted to barley, raising a concern that the rice was contaminated with gluten that was not detected by the gluten ELISA used before extraction. However, assay control was demonstrated by the gluten-free beer, which showed no responses in either the control or active-CD groups.

Barley flour was used as a positive control, and 11 of 31 active-CD patients demonstrated significant antibody binding to the barley extract. Although there are some identical gluten peptides found in wheat and barley, cultivated barley has been shown to be less immunogenic than wild-type barley (52), and celiac patients have variable responses to different varieties of barley and wheat (54), so these results were not unexpected. In addition, the ELISAs were performed on native proteins and not on proteins deamidated by tTG, which might enhance their immunoreactivity. Because of the similarity of some wheat and barley gluten protein sequences, it was predicted that active-CD patients, but not nonceliac control subjects, would demonstrate binding of a barley flour extract. As shown in Tables 3 and 4, only 1 of 29 nonceliac control subjects (3%) showed detectable IgA or IgG binding to barley, whereas 11 of 31 active-CD patients (35%) had detectable IgA or IgG binding to barley (Chi-square = 10.0427; $P = 0.002$). Only those subjects whose serum antibodies bound to barley would be expected to have an antibody repertoire that might react to residual barley gluten in conventional beer or gluten-removed beer, if any was present. Tables 3 and 4 show the positive OD values for any patients and subjects who gave a positive result for barley.

Four active-CD patients reacted to both barley and the conventional beer sample, compared to none of the nonceliac control subjects (Chi-square= 5.2809; $P = 0.02$). Three active CD patients showed a response to barley and the gluten-removed beer, and again, none of the nonceliac control subjects showed this reaction pattern.

One nonceliac control subject (C8) gave a positive IgG response to the barley flour extract but did not react to the conventional beer or gluten-removed beer (this data point was not used in calculating the mean and SD for the nonceliac control group). This subject had been diagnosed with eosinophilic esophagitis, and his response to barley may suggest that he had an existing allergic response to a component of barley that is either not present in beer or is degraded in the brewing process.

Of the seven active-CD subjects who demonstrated IgA binding to the barley flour control extract, four responded to the conventional beer sample, and two of those responded to the gluten-removed beer sample. Of the six active-CD subjects who demonstrated IgG binding to the barley flour control extract, one responded to the conventional beer sample and another responded to the gluten-removed beer sample.

Table 3. Blank-subtracted OD values (450 nm) for active-CD patients who showed an IgA reaction to barley

	IgA, OD				
ID	Rice	Barley	Beer[a]	GF beer[b]	GR beer[c]
CD4	0.369	1.079	0.349	—	—
CD12	—[d]	0.347	—	—	—
CD13	—	0.385	0.371	—	0.417
CD17	—	0.561	—	—	—
CD18	—	0.442	—	—	—
CD19	—	0.970	0.805	—	0.884
CD20	—	1.007	0.557	—	—

[a] Beer = Commercially available barley beer consisting of malted barley, hops, yeast, and water.

[b] GF beer = Commercially available gluten-free beer consisting of sorghum, brown rice, maltodextrin, yucca extract, hops, water, and yeast.

[c] GR beer = Commercially available gluten-removed beer consisting of barley malt, rice, hops, yeast, and water.

[d] Only OD values above the cutoff of 0.325 are shown.

Table 4. Blank-subtracted OD values (450 nm) for one control subject and six patients who showed an IgG reaction to barley

	IgG, OD				
ID	Rice	Barley	Barley beer[a]	GF beer[b]	GR beer[c]
Nonceliac control subject					
C8	—[d]	1.817	—	—	—
Active-CD patients					
CD4	0.473	0.405	0.556	—	—
CD10	—	0.695	—	—	1.741
CD11	—	0.334	—	—	—
CD16	0.872	0.616	—	—	—
CD20	—	0.5	—	—	—
CD28	—	0.342	—	—	—

[a] Beer = Commercially available barley beer consisting of malted barley, hops, yeast, and water.

[b] GF beer = Commercially available gluten-free beer consisting of sorghum, brown rice, maltodextrin, yucca extract, hops, water, and yeast.

[c] GR beer = Commercially available gluten-removed beer consisting of barley malt, rice, hops, yeast, and water.

[d] Only OD values above the cutoffs (0.200 for rice, 0.325 for barley, 0.350 for Beer, 0.865 for GF beer, and 0.425 for GR beer) are shown.

Discussion

The goal of this study was to examine the use of sera from active-CD patients as a detection tool for residual celiac-reactive proteins in gluten-removed beer. The ELISA method used was able to detect IgA and IgG binding of native proteins from barley flour, conventional beer, and gluten-removed beer. Subjects who demonstrated binding to the barley flour ethanol extract were the primary focus, because they had the potential for an antibody response to the hordein fraction of barley. Although the proteins in the beer test samples were likely not all from gluten, the pattern of reactivity with barley and conventional beer was used to indicate the likelihood of reaction with residual gluten proteins in the gluten-removed beer sample. It is known that the antibody response of CD patients who are not on a gluten-free diet can spread to other nongluten proteins (46), but it is unknown whether the reaction to these nongluten proteins has any pathogenic significance. Further analysis of the proteins detected by the assay used here is needed to determine their potential toxicity.

None of the 29 nonceliac control subjects reacted to all three barley-based samples (barley extract, traditional beer, and gluten-removed beer), whereas 2 of 31 active-CD patients (6.4%) responded to all three samples. Although the patient pool used in this study was drawn from one geographic region, and these data cannot be extrapolated to the larger celiac population, the results indicate that there are residual proteins in the traditional and gluten-removed beer samples that are recognized by a subset of this patient population.

The ELISAs in this study looked at antibody responses to the ethanol-soluble proteins in barley and rice flour and the total proteins larger than 3.5 kDa in the beer samples. Proteins larger than 3.5 kDa will, on average, contain more than 30 amino acids, which is a larger peptide than is needed for antibody recognition. Therefore, it is possible that the dialysis process used to remove polyphenols, which can interfere with ELISAs, also removed small antigenic proteins that may have been detected by the participants' sera. Using only the ethanol-soluble fraction of barley as a positive control also eliminated the detection of responses to barley hordeins that are only soluble in reducing agents. Additional studies are needed to determine the effect of these factors on protein binding.

Known comorbidities showed no correlation with the IgA or IgG reaction patterns, and there was not enough information available on the genetics of the subjects to evaluate any correlation between DQ genotype and reaction patterns. In general, the IgA reaction patterns were more predictable than those for IgG, with only one active-CD patient showing a positive response to rice and none reacting to the gluten-free beer, and with low background levels for all samples. High variability of the IgG reaction patterns in the nonceliac control subjects resulted in varying cutoff values for the IgG data, including the high cutoff for the gluten-free beer. The use of IgA-based assays (anti-gliadin, anti-tTG, or anti-endomysial) is generally recommended in the diagnosis of CD (3), so further work on this method and analysis of detected proteins will focus on IgA responses to the test samples.

Conclusions

The data presented here indicate that testing hydrolyzed food/beverage extracts against active-CD patient sera may allow the detection of residual gluten peptides in those products. Using serum samples from a large population of CD patients provides an opportunity to detect a wide variety of gluten epitopes, which is a benefit over current ELISA and LC-MS methods. With additional studies, patient sera might also be used to identify new epitopes and proteins that are recognized by persons with CD. The current data demonstrate that the active-CD patient sera used in this study did not respond to gluten-free beer made from sorghum and rice, but several did respond to conventional beer, with a subset of these individuals also responding to the gluten-removed beer. Although the absence of a significant humoral response to the proteins in a food or beverage is not absolute proof of their safety, a strong response from CD patients in the absence of equivalent reactions from normal control subjects would suggest that there are residual peptides in the product that may be specifically recognized by persons with CD.

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References

(1) Guandalini, S., & Assiri, A. (2014) *JAMA Pediatr.* **168**, 272–278. doi:10.1001/jamapediatrics.2013.3858
(2) Riddle, M.S., Murray, J.A., & Porter, C.K. (2012) *Am. J. Gastroenterol.* **107**, 1248–1255. doi:10.1038/ajg.2012.130
(3) Rubio-Tapia, A., Hill, I.D., Kelly, C.P., Calderwood, A.H., & Murray, J.A. (2013) *Am. J. Gastroenterol.* **108**, 656–676. doi:10.1038/ajg.2013.79
(4) CODEX (2008) Codex Alimentarius Commission, Food and Agriculture Organization of the United Nations and World Health Organization, Rome, Italy, STAN 118-1979
(5) Commission Regulation No. 41/2009/EC (2009) *Off. J. Eur. Comm.* **L16,** 3–5
(6) U.S. Food and Drug Administration (2013) *Fed. Regist.* **78**, 47154–47179
(7) Wheat Initiative (2015). Strategic Research Agenda, Wheat Initiative Ed., Paris, 52p
(8) M'Hir, S., Ziadi, M., Chammem, N., & Hamdi, M. (2012) *Afr. J. Biotechnol.* **11**, 7323–7330
(9) Gobbetti, M., Rizzello, C.G., Di Cagno, R., & De Angelis, M. (2007) *Food Microbiol*. **24**, 187–196. doi:10.1016/j.fm.2006.07.014
(10) Stoven, S., Murray, J.A., & Marietta, E. (2012) *Clin. Gastroenterol. Hepatol.* **10**, 859–862
(11) Cabrera-Chávez, F., & Calderón de la Barca, A.M. (2010) *J. Cereal Sci.* **52**, 337–341. doi:10.1016/j.jcs.2010.06.020
(12) Di Cagno, R., De Angelis, M., Lavermicocca, P., De Vincenzi, M., Giovannini, C., Faccia, M., & Gobbetti, M. (2002) *Appl. Environ. Microbiol.* **68**, 623–633. doi:10.1128/AEM.68.2.623-633.2002
(13) Curiel, J.A., Coda, R., Limitone, A., Katina, K., Raulio, M., Giuliani, G., Rizzello, C.G., & Gobbetti, M. (2014) *J. Cereal Sci.* **59**, 79–87. doi:10.1016/j.jcs.2013.09.011
(14) di Cagno, R., de Angelis, M., Alfonsi, G., de Vincenzi, M., Silano, M., Vincentini, O., & Gobbetti, M. (2005) *J. Agric. Food Chem.* **53**, 4393–4402. doi:10.1021/jf048341+
(15) De Angelis, M., Rizzello, C.G., Fasano, A., Clemente, M.G., De Simone, C., Silano, M., De Vincenzi, M., Losito, I., & Gobbetti, M. (2006) *Biochim. Biophys. Acta* **1762**, 80–93. doi:10.1016/j.bbadis.2005.09.008
(16) Greco, L., Gobbetti, M., Auricchio, R., Di Mase, R., Landolfo, F., Paparo, F., Di Cagno, R., De Angelis, M., Rizzello, C.G., Cassone, A., Terrone, G., Timpone, L., D'Aniello, M., Maglio, M., Troncone, R., & Auricchio, S. (2011) *Clin. Gastroenterol. Hepatol.* **9**, 24–29. doi:10.1016/j.cgh.2010.09.025
(17) Rizzello, C.G., De Angelis, M., Di Cagno, R., Camarca, A., Silano, M., Losito, I., De Vincenzi, M., De Bari, M.D., Palmisano, F., Maurano, F., Gianfrani, C., & Gobbetti, M. (2007) *Appl. Environ. Microbiol.* **73**, 4499–4507. doi:10.1128/AEM.00260-07
(18) Buddrick, O., Cornell, H.J., & Small, D.M. (2015) *Food Chem.* **170**, 343–347. doi:10.1016/j.foodchem.2014.08.030
(19) Cornell, H.J., Doherty, W., & Stelmasiak, T. (2010) *Amino Acids* **38**, 155–165. doi:10.1007/s00726-008-0223-6
(20) Messer, M., Anderson, C.M., & Hubbard, L. (1964) *Gut* **5**, 295–303. doi:10.1136/gut.5.4.295

(21) Stepniak, D., Spaenij-Dekking, L., Mitea, C., Moester, M., de Ru, A., Baak-Pablo, R., van Veelen, P., Edens, L., & Koning, F. (2006) *Am. J. Physiol. Gastrointest. and Liver Physiol.* **291**, G621–G629
(22) Luoto, S., Jiang, Z., Brinck, O., Sontag-Strohm, T., Kanerva, P., Bruins, M., Edens, L., Salovaara, H., & Loponen, J. (2012) *J. Cereal Sci.* **56**, 504–509. doi:10.1016/j.jcs.2012.06.004
(23) van Zandycke, S. (2013) *The New Brewer* **Nov/Dec**, 79–84
(24) Gass, J., Bethune, M.T., Siegel, M., Spencer, A., & Khosla, C. (2007) *Gastroenterology* **133**, 472–480. doi:10.1053/j.gastro.2007.05.028
(25) Schwalb, T., Wieser, H., & Koehler, P. (2012) *Eur. Food Res. Technol.* **235**, 1161–1170. doi:10.1007/s00217-012-1853-1
(26) U.S. Food and Drug Administration (2014) *Fed. Regist.* **80**, 71990–72006
(27) Caputo, I., Lepretti, M., Martucciello, S., & Esposito, C. (2010) *Enzyme Res*. **2010**, 174354. doi:10.4061/2010/174354
(28) Di Cagno, R., De Angelis, M., Auricchio, S., Greco, L., Clarke, C., De Vincenzi, M., Giovannini, C., D'Archivio, M., Landolfo, F., Parrilli, G., Minervini, F., Arendt, E., & Gobbetti, M. (2004) *Appl. Environ. Microbiol.* **70**, 1088–1096. doi:10.1128/AEM.70.2.1088-1096.2004
(29) De Angelis, M., Cassone, A., Rizzello, C.G., Gagliadi, F., Minervini, F., Calasso, M., Di Cagno, R., Francavilla, R., & Gobbetti, M. (2010) *Appl. Environ. Microbiol.* **76**, 508–518. doi:10.1128/AEM.01630-09
(30) De Angelis, M., Coda, R., Silano, M., Minervini, F., Rizzello, C.G., Di Cagno, R., Vicentini, O., De Vincenzi, M., & Gobbetti, M. (2006) *J. Cereal Sci.* **43**, 301–314. doi:10.1016/j.jcs.2005.12.008
(31) Gerez, C.L., Dallagnol, A., Rollan, G., & Font de Valdez, G. (2012) *Food Microbiol.* **32**, 427–430. doi:10.1016/j.fm.2012.06.007
(32) Walter, T., Wieser, H., & Koehler, P. (2015) *Eur. Food Res. Technol.* **240**, 517–524. doi:10.1007/s00217-014-2350-5
(33) Rizzello, C.G., Curiel, J.A., Nionelli, L., Vincentini, O., Di Cagno, R., Silano, M., Gobbetti, M., & Coda, R. (2014) *Food Microbiol.* **37**, 59–68. doi:10.1016/j.fm.2013.06.017
(34) Guerdrum, L.J., & Bamforth, C.W. (2012) *J. Am. Soc. Brewing Chemists* **70**, 35–38
(35) Loponen, J., Sontag-Strohm, T., Venalainen, J., & Salovaara, H. (2007) *J. Agric. Food Chem.* **55**, 978–984. doi:10.1021/jf062755g
(36) Loponen, J., Kanerva, P., Zhang, C., Sontag-Strohm, T., Salovaara, H., & Ganzle, M.G. (2009) *J. Agric. Food Chem.* **57**, 746–753. doi:10.1021/jf803243w
(37) Di Cagno, R., Barbato, M., Di Camillo, C., Rizzello, C.G., De Angelis, M., Guiliani, G., De Vincenzi, M., Gobbetti, M., & Cucchiara, S. (2010) *J. Pediatr. Gastroenterol. Nutr.* **51**, 777–783
(38) Cabrera-Chávez, F., Islas-Rubio, A.R., Rouzaud-Sández, O., Sotelo-Cruz, N., & Calderón de la Barca, A.M. (2010) *J. Cereal Sci.* **52**, 310–313. doi:10.1016/j.jcs.2010.06.013
(39) Ferranti, P., Mamone, G., Picariello, G., & Addeo, F. (2007) *J. Mass Spectrom.* **42**, 1531–1548. doi:10.1002/jms.1361
(40) Diaz-Amigo, C., & Popping, B. (2013) *J. Agric. Food Chem.* **61**, 5681–5688. doi:10.1021/jf3046736
(41) Rosell, C.M., Barro, F., Sousa, C., & Mena, M.C. (2014) *J. Cereal Sci.* **59**, 354–364. doi:10.1016/j.jcs.2013.10.001
(42) Lester, D.R. (2008) *Plant Methods* **4**, 26–40. doi:10.1186/1746-4811-4-26
(43) Marti, T., Molberg, O., Li, Q., Gray, G., Kholsa, C., & Sollid, L. (2005) *J. Pharmacol. Exp. Ther.* **312**, 19–26. doi:10.1124/jpet.104.073312
(44) Ehren, J., Morón, B., Martin, E., Bethune, M.T., Gray, G.M., & Khosla, C. (2009) *PLoS ONE* **4**, e6313. doi:10.1371/journal.pone.0006313
(45) Shan, L., Marti, T., Sollid, L.M., Gray, G.M., & Khosla, C. (2004) *Biochem. J.* **383**, 311–318. doi:10.1042/BJ20040907
(46) Huebener, S., Tanaka, C.K., Uhde, M., Zone, J.J., Vensel, W.H., Kasarda, D.D., Beams, L., Briani, C., Green, P.H.R., Altenbach, S.B., & Alaedini, A. (2015) *J. Proteome Res.* **14**, 503–511. doi:10.1021/pr500809b
(47) Skerritt, J.H., Johnson, R.B., Hetzel, P.A.S., La Brooy, J.T., Shearman, D.J.C., & Davidson, G.P. (1987) *Clin. Exp. Immunol.* **68**, 189–199
(48) Fälth-Magnusson, K., Jansson, G., Stenhammar, L., & Magnusson, K.-E. (1994) *J. Pediatr. Gastroenterol. Nutr.* **18**, 56–62. doi:10.1097/00005176-199401000-00010
(49) Lerner, A., & Lebenthal, E. (1991) *J. Pediatr. Gastroenterol. Nutr.* **12**, 407–409. doi:10.1097/00005176-199105000-00001
(50) Bruins, M. (2013) *Nutrients* **5**, 4614–4641. doi:10.3390/nu5114614
(51) LaBrooy, J.T., Hohmann, A.W., Davidson, G.P., Hetzel, P.A.S., Johnson, R.B., & Shearman, D.J.C. (1986) *Clin. Exp. Immunol*. **66**, 661–668
(52) Comino, I., Real, A., Gil-Humanes, J., Piston, F., de Lorenzo, L., de Lourdes Moreno, M., Lopez-Casado, M.A., Lorite, P., Cebolla, A., Torres, M.I., Barro, F., & Sousa, C. (2012) *Mol. Nutr. Food Res.* **56**, 1697–1707. doi:10.1002/mnfr.201200358
(53) Skerritt, J.H., & Hill, A.S. (1990) *J. Agric. Food Chem.* **38**, 1771–1778. doi:10.1021/jf00098a029
(54) Konic-Ristic, A., Dodig, D., Krstic, R., Jelic, S., Stankovic, I., Ninkovic, A., Radic, J., Besu, I., Bonaci-Nikolic, B., Jojic, N., Djordjevic, M., Popovic, D., & Juranic, Z. (2009) *BMC Immunol.* **10**, 32–38. doi:10.1186/1471-2172-10-32