Sensitive detection of cereal fractions that are toxic to celiac disease patients by using monoclonal antibodies to a main immunogenic wheat peptide[1–3]

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ABSTRACT

Background: Celiac disease is an immune-mediated enteropathy caused by the ingestion of gluten, a protein fraction found in certain cereals. Immunotoxic gluten peptides that are recalcitrant to degradation of digestive enzymes appear to trigger celiac syndromes. A 33-mer peptide from α-2 gliadin has been identified as a principal contributor to gluten immunotoxicity. A gluten-free diet is the usual first therapy for celiac disease patients; therefore, the characterization and quantification of the toxic portion of the gluten in foodstuffs is crucial to avoid celiac damage.

Objective: We aimed to develop immunologic assays as a novel food analysis tool for measuring cereal fractions that are immunotoxic to celiac disease patients.

Design: The design focused on the production of monoclonal antibodies against the gliadin 33-mer peptide and the development of enzyme-linked immunosorbent assays (ELISAs) and Western blot analysis with the use of novel antibodies.

Results: A sandwich ELISA method showed a detection limit for wheat, barley, and rye of <1 ppm prolamine. However, the method required a sample that was ≥ 1 order of magnitude greater for the detection of low-toxic oats, and there was no signal with the safe cereals maize and rice. A competitive ELISA method was also developed for detection of the toxic peptide in hydrolyzed food, which had a detection limit of <0.5 ppm gliadin.

Conclusions: Both ELISAs designed for use with the toxic gliadin 33-mer peptide suggested a high correlation between the presence of the peptide and the amount of cereal that was toxic to celiac disease patients. The sensitivity was significantly higher than that of equivalent methods recognizing other gluten epitopes. *Am J Clin Nutr* 2008;87:405–14.

KEY WORDS Monoclonal antibodies, 33-mer peptide, gluten, enzyme-linked immunosorbent assays, celiac disease

INTRODUCTION

Celiac disease (CD) is a gluten-sensitive enteropathy that occurs in genetically predisposed persons and that leads to the destruction of the microscopic finger-like projections of the small intestine that are called villi (1, 2). The disease is triggered by the ingestion of the gluten proteins contained in wheat, barley, rye, and, in some cases, oats (3, 4). Screening studies have showed that CD is very common, affecting almost 1% of the world's population (5, 6), primarily adults (7).

The principal toxic components of wheat gluten belong to a family of closely related proline- and glutamine-rich proteins called gliadins. The disease is triggered by the presence of peptides, from the fragmentation of gliadins, that are not digested by human proteases and that are toxic for CD patients. Shan et al (8) have shown by in vitro and in vivo studies in rats and humans that a 33-mer peptide from α-gliadin is stable toward breakdown by all gastric, pancreatic, and intestinal brush border membrane endoproteases. This peptide was identified as the primary initiator of the inflammatory response to gluten in celiac sprue patients (8). The identification of the gliadin residues 57–89, which comprise the 33-mer peptide LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF (8) from α-2 gliadin, showed that highly antigenic gluten epitopes identified to date are located in proline-rich regions of gliadin (9).

Two deletion mutants of α-2 gliadin, one excluding only the 33-mer sequence and the other also deleting other C-terminal residues, were not toxic (8, 10). These results suggested that the 33-mer peptide was a principal contributor to α-2 gliadin immunotoxicity. In addition, homologs of this peptide were found in all food grains (except oats) that are toxic to celiac sprue patients, but they were absent in all nontoxic food grains (8).

Treatment with a gluten-free diet is the usual first therapy for CD patients; however, cereal proteins are a ubiquitous additive in most sectors of the prepared-food industry. Thus, an analytic method that measures the toxicity of foodstuffs for CD patients should identify the presence of potential cereal peptides.

Antibodies that recognize peptides of the gluten fraction currently are on the market (11–14), but they were not specifically

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designed for the toxic peptide. In the present study, in vitro quantification assays were developed that were based on the toxicity of the 33-mer peptide that is recalcitrant to gluten digestion. Monoclonal antibodies (MoAbs) against the toxic 33-mer peptide were obtained that were able to detect, by different configurations of enzyme-linked immunosorbent assays (ELISAs), gliadin concentrations < 1 ng/mL, a concentration lower than the latest Codex Alimentarius recommendation [10 ppm (15)]. They also could detect toxic fractions of gluten present in wheat and other cereals, such as barley, rye, and, with a much lower level of sensitivity, oats; the detection at a lower sensitivity in oats correlates with the lower degree of toxicity of oats to CD patients.

MATERIALS AND METHODS

Materials, strains, and growth conditions

Commercially available flours from cultivars of wheat, barley, rye, oats, maize, and rice were used in the present study. Cultures of *Escherichia coli* REG1 (pALEXb-33-mer) were grown overnight at 37 °C, diluted in Luria broth (16) supplemented with ampicillin (100 μg/mL) and kanamycin (50 μg/mL), and grown at 37 °C to $OD_{600} = 0.8–1$. Cultures were then induced by the addition of 2 mmol salicylate/L and incubated for 5 h at 30 °C.

E. coli cells (M15 strain) were employed as host bacteria for the overproduction of recombinant heat-shock proteins (HSPs) 33-mer-T-HSP70 and 33-mer-X2-HSP70. *E. coli* cultures were grown at 37 °C in Luria broth supplemented with ampicillin (100 $\mu g \cdot mL^{-1}$) and kanamycin (25 $\mu g \cdot mL^{-1}$). Overexpression of recombinant 33-mer-T-HSP70 and 33-mer-X2-HSP70 was induced by the addition of 0.02 mmol isopropyl-β-D-1-thiogalactopyranoside (IPTG)/L and 0.1 mol IPTG/L, respectively, to the cultures in logarithmic growth phase for 90 min.

Preparation of recombinant 33-mer fusions for mice immunization

To obtain MoAbs against the toxic 33-mer peptide, a fragment of DNA codifying for the toxic 33-mer peptide of α-2 gliadin was constructed by synthesis of the following 4 overlapping oligonucleotides: Glia1, 5′-GATCCGCTGCAGTTACAACCGTTTCCGCAGCCACAACTGCCGTATCCG-3′; Glia2, 5′-CAACCACAGTTGCCGTACCCGCAGCCGCAATTACCGTATCCTCAACCG CAACCGTTTTAA-3′; Glia3, 5′-GTACGGCAACTGTGGTTGCGGATACGGCAGTTGTGGCTGCGGAAACGGTTGTAACTGCAGCG-3′; and Glia4, 5′-AGCTTTAAAACGGTTGCGGTTGAGGATACGGTAATTGCGGCTGCGG-3′. These synthetic DNAs were designed according to the typical codon usage of *E. coli* to maximize the expression. The resultant synthetic DNA fragment was assembled by hybridization and cloned into the *Bam*HI-*Hin*dIII–digested pALEXb plasmid (Biomedal SL, Seville, Spain). This plasmid contained the Pm promoter of the Cascade expression system (17, 18) and the C-LYTAG for single-step affinity purification (Biomedal SL). The resultant pALEXb-33-mer plasmid was checked by sequencing and was transformed into the Cascade expression host *E. coli* REG1 (Biomedal SL). The purified product was used for the detection of the 33-mer by the MoAbs.

To produce MoAbs against the 33-mer peptide, a recombinant protein bearing the epitopes contained in the 33-mer peptide was overproduced in *E. coli* and purified to homogeneity for inoculation into mice. Because the 33-mer polypeptide has a low molecular weight (4.04 kDa) and because we needed to analyze the adjuvant capacity of the 2 carrier molecules, the sequence coding for the 33-mer peptide was cloned into the pQE-32 expression vector (Qiagen, Hilden, Germany) that was in-frame-fused to the sequence coding for these carrier molecules. Thus, the sequence coding for the toxic 33-mer peptide was in-frame-fused to that coding for 6 histidines and to those sequences coding for the 2 carrier molecules: recombinant *Trypanosoma cruzi* HSP70 (19) and a specific protein fragment derived from *T. cruzi* HSP70, T-HSP70 (20). Briefly, the 33-mer peptide coding sequence was amplified with polymerase chain reaction using the pALEXb-33-mer plasmid as the DNA template and using the primers Glia 5′ *Bam*HI (5′-GCCAAGGATCCAGCTGCAGTTACAACCG-3′) and Glia 3′ *Kpn*I/*Bam*HI primer (5′-GCTAAGGATCCGGTACCAAAACGGTTGCGGTTG-3′), which included *Bam*HI (5′ primer) and *Kpn*I and *Bam*HI (3′ primer) restriction sites (underlined), respectively; this step allowed the direct cloning of 33-mer coding sequences into different vectors.

To produce the pQE-32–33-mer-T-HSP70 expression construct, which bears the 33-mer coding sequence fused to the T-HSP70 carrier molecule, the 130 nucleotide (nt) was cloned in the pQE-32fII(70) vector (20). To enhance the immune response to the small 33-mer peptide, 2 copies of the 130 nt polymerase chain reaction– amplified fragment were cloned into the pQE-32-HSP70 recombinant plasmid (19). The resultant plasmid was named pQE-32–33-mer-X2-HSP70.

Purification of the recombinant proteins

Recombinant 33-mer-T-HSP70 and 33-mer-X2-HSP70 were purified by Ni^{2+}-affinity chromatography (Qiagen). Recombinant 33-mer-T-HSP70 was extracted from the bacterial pellet in sonication buffer [60 mmol NaCl/L, 10 mmol Na_2HPO_4/L, and 0.05% sodium dodecyl sulfate (SDS) (pH 8)]. The soluble protein extract was adjusted to 0.3 mol NaCl/L and 50 mmol Na_2HPO_4/L (pH 8) and incubated with Ni^{2+}-nickel nitrilo-triacetic acid agarose resin for 2.5 h at room temperature. The resin was subsequently washed twice with 0.3 mol NaCl/L at pH 8 and at pH 7.5. Finally, recombinant 33-mer-T-HSP70 was eluted with 50 mmol phosphate buffer/L, 0.3 mol NaCl/L, 1 mmol phenylmethanesulfonyl fluoride (PMSF)/L, and 0.025% SDS (pH 6).

Recombinant 33-mer-X2-HSP70 was solubilized under mild sonication conditions in 50 mmol phosphate buffer/L, 60 mmol NaCl/L, and 0.025% SDS (pH 8). Soluble extracts were adjusted to reach final concentrations of 300 mmol NaCl/L, 10 mmol β-mercaptoethanol/L, 1 mmol PMSF/L, 5 mmol $MgCl_2$/L, and 10% glycerol and bound to the Ni^{2+}-nickel nitrilo-triacetic acid resin overnight. The resin was washed with the same buffer at pH 8 and then at pH 7.5. The recombinant protein was eluted with 50 mmol phosphate buffer/L, 300 mmol NaCl/L, 1 mmol PMSF/L, 5 mmol $MgCl_2$/L, 10% glycerol, and 0.025% SDS (pH 6) and then extensively dialyzed against the same buffer without SDS.

The degree of purity of the recombinant proteins was evaluated by SDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie blue staining. Moreover, all purified recombinant protein preparations were tested with the a *Limulus* Amebocyte Lysate reaction kit (E-Toxate; Sigma, St Louis, MO), which showed that the endotoxin concentrations were below the detection limit of the kit (<0.1 endotoxin units/mL). Protein concentrations were measured by using the Bradford method.

Purification of recombinant C-LYTAG-33-mer protein was carried out according to the manufacturer's instructions (C-LYTAG Purification System; Biomedal SL). Briefly, the soluble protein extract was adjusted to 20 mmol potassium phosphate buffer/L (pH 7.0), 300 mmol NaCl/L, 0.1% triton X-100, and 5 mmol choline chloride/L and passed through the C-LYTRAP resin. The resin was subsequently washed with the same buffer, and finally the recombinant protein was eluted with 20 mmol potassium phosphate buffer/L (pH 7.0), 300 mmol NaCl/L, 0.1% triton X-100, and 250 mmol choline chloride/L. Protein concentrations were measured by using the Bradford method.

Generation and purification of monoclonal antibodies against gliadin

Two MoAbs against the 33-mer gliadin peptide were generated according to the standard method with some specific modifications. Briefly, 2 groups of BALB/c mice, obtained from IFFA-CREDO (St Germain sur l'Arbesle, France), were subcutaneously immunized twice with the use of a 25-μg dose of 33-mer-T-HSP70 or with purified recombinant 33-mer-X2-HSP70 as immunogen. Two weeks after the last immunization, a third dose of the fusion protein was inoculated intravenously into the mice of each group. All immunizations were carried out without adjuvant. Titers of the antibodies were evaluated against recombinant C-LYTAG-33-mer protein with the use of an ELISA on day 4 after the last immunization. Immunized mice were killed, and the spleens were removed for use as a source of cells for fusion with SP2 myeloma cells. Single spleen cells from immunized mice were fused with the previously prepared myeloma cells and grown on RPMI media supplemented with 20% fetal bovine serum and containing hypoxanthine-aminopterin-thymidine, because hypoxanthine-aminopterin-thymidine–containing medium allows only the fused cells to survive in culture. Fused cells were distributed into 96-well plates containing medium supplemented with aminopterin and containing feeder cells derived from saline peritoneal washes from mice.

The H-G12 and H-A1 hybridomas were selected according to the the specificity and binding affinity that the MoAbs they produced exhibited for the 33-mer peptide as determined by ELISAs using the corresponding supernatant. The H-G12 hybridoma was obtained from B-lymphocytes of mice inoculated with 33-mer-T-HSP70, and the H-A1 hybridoma was obtained from the B-lymphocytes of mice immunized with 33-mer-X2-HSP70. Sixteen-week-old mice were inoculated intraperitoneally with cloned hybridomas, and ascitic fluids were recovered 1 wk later. Then, MoAbs were purified by using fast-protein liquid chromatography and Protein G Sepharose 4 Fast-Flow (Amersham Biosciences, Little Chalfont, United Kingdom) according to standard methods. The final concentration of G12 and A1 MoAbs was 2.05 and 2.34 μg/mL, respectively. The G12 MoAb was conjugated to horseradish peroxidase (HRP) with the use of a conjugation kit for labeling antigen and antibodies (Alpha Diagnostic International, San Antonio, TX).

Preparation of gliadin solution

Gliadin (Sigma Chemical Co) was prepared in 60% (by vol) aqueous ethanol at 1 mg/mL. European gliadin reference (IRMM-480) was supplied by Ingenasa (Madrid, Spain). Trypsin and pepsin digestion of gliadin was provided by Eduardo Arranz (University of Valladolid, Valladolid, Spain).

Preparation of prolamines from wheat, barley, rye, oat, maize, and rice flours

Prolamines from wheat, barley, rye, oat, maize, and rice flours were extracted by mixing 0.125 g flour with 5 mL of 60% (by vol) ethanol in a rotary shaker for 1 h at room temperature. The suspension was then centrifuged at 2500 $\times$ *g* for 10 min at room temperature; the supernatant was separated, and the protein concentration was measured by using the Bradford method. The required concentration of these samples was prepared in 0.02 mol phosphate buffer/L containing bovine serum albumin [(BSA) 5 μg/mL)] before they were used in immunoassays.

Indirect enzyme-linked immunosorbent assay

A Maxisorp microtiter plate (Nunc, Roskilde, Denmark) was coated overnight at 4 °C with 100 μL/well of the purified C-LYTAG-33-mer protein serially diluted with 0.02 mol Na_2CO_3-$NaHCO_3$/L (pH 9.6). After being washed with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (washing buffer), 300 μL blocking buffer (washing buffer supplemented with 5% nonfat dry milk) was added to each well, and the plates were incubated for 1 h at room temperature. Plates were incubated for 1 h at room temperature with the G12 antibody (1:1000 in the blocking solution) or A1 antibody (1:500 in the blocking solution). After washing, anti-mouse IgG-peroxidase antibody produced in goat (Biomedal SL) was added (1:2000 in the blocking solution). Plates were washed again, and the substrate solution (TMB liquid substrate system; Sigma Chemical Co) was added. After a 30-min dark incubation, the reaction was stopped with 1 mol sulfuric acid/L. Absorbance at 450 nm was measured in the microplate reader (UVM340; Asys Hitech GmbH, Eugendorf, Austria).

Sandwich enzyme-linked immunosorbent assay

Maxisorp ELISA plates (Nunc) were coated with 0.5 μg/well of A1 antibody in 0.02 mol Na_2CO_3-$NaHCO_3$/L (pH 9.6) and incubated overnight at 4 °C. After the coating solution was removed, the wells were washed with washing buffer and blocked with blocking solution for 1 h at room temperature. Plates were incubated for 1 h with samples diluted in 0.02 mol phosphate buffer/L containing BSA (5 μg/mL). After the plates were washed, conjugated G12-HRP was added (1:500 in the blocking solution), and the plates were incubated for 1 h at room temperature. Plates were washed again, and the TMB liquid substrate solution was added. After a 30-min dark incubation, the reaction was stopped with 1 mol sulfuric acid/L. Absorbance at 450 nm was measured in the UVM340 microplate reader.

Competitive enzyme-linked immunosorbent assay

Maxisorp ELISA plates (Nunc) were coated with 100 μL/well of Sigma gliadin solution (5 μg/mL) in 0.02 mol Na_2CO_3-$NaHCO_3$/L (pH 9.6) and incubated overnight at 4 °C. Plates were washed with washing buffer and blocked with blocking solution for 1 h at room temperature. Gliadin and samples serially diluted in PBS containing 3% BSA (100 μL) and 100 μL G12-HRP solution (1:20 000 in PBS containing 3% BSA) were incubated for 2 h at room temperature with gentle mixing and then were added to the wells. After a 30-min incubation at room temperature, the plates were washed, and 100 μL TMB liquid substrate solution was added to each well. After a 30-min dark incubation at room temperature, color development was stopped with 1 mol

sulfuric acid/L (100 μL/well), and the absorbance was measured at 450 nm in the UVM340 microplate reader.

Western blot reactivity of the G12 and A1 monoclonal antibodies

For the Western blot of cereal extracts, after one-dimensional SDS-PAGE, proteins were electrotransferred onto polyvinylidene difluoride membranes, incubated directly with G12-HRP, and developed by using the ECL Western Blotting Analysis System immunodetection (Amersham Biosciences).

Statistical analysis

Gliadin standard curves were obtained by plotting the percentage of maximum absorbance against gliadin concentrations (sandwich ELISA) or the logarithm of gliadin concentrations (indirect and competitive ELISAs). The least-squares method was applied To obtain the function describing a linear model (sandwich ELISA), the least-squares method was applied by using EXCEL software (version 2003; Microsoft Corporation, Redmond, WA). SIGMA PLOT software (version 9.0; Systat Software Inc, Point Richmond, CA) was used to calculate the 4 parameters for fitting the sigmoidal curve equation (competitive ELISA). The limit of detection was defined here as reagent blank + 3 SDs of reagent blank. The limit of quantification was defined here as reagent blank + 10 SDs of reagent blank.

Repeatability (interplate variability) was assessed by measuring the standard curve for the same gliadin sample on 2 separate ELISA plates on the same day in parallel experiments. Reproducibility (intraplate variability) was calculated by measuring the standard curve 8 times for the same gliadin sample on a single ELISA plate. The CV was calculated by using the following equation:

$$CV = SD/mean \times 100 \quad (1)$$

RESULTS

Cloning, production, and purification of protein fusions to 33-mer peptide of α-2 gliadin

It has been verified that the 33-mer peptide from α-2 gliadin is a principal contributor to gluten immunotoxicity (8). Thus, the production of MoAbs to the toxic fraction of gluten polypeptides could be of great importance in both research and diagnosis. To raise MoAbs to the 33-mer peptide, mice were immunized with the 33-mer fused to carrier molecules (HSP70 and T-HSP70) because HSP70 from different organisms has proven to be very effective in strengthening the immune response against an antigenic protein to which it is coupled (21–23). Thus, we generated recombinant plasmids that express the 33-mer peptide-HSP70 fusion protein and the 33-mer peptide-T-HSP70 chimeric protein, made up of 33-mer fused to an HSP70 fragment named T-HSP70 (20). To increase the chance of obtaining an antibody with high sensitivity and specificity, we fused 2 copies of the sequence coding for the 33-mer antigen to the longer HSP70 polypeptide.

So that we could have a source of the 33-mer peptide for the further experiments, a fusion of the codifying sequence of the C-LYTAG of the pALEXb plasmid was carried out by one-step affinity purification of recombinant proteins. The Pm promoter of these plasmids is the terminal promoter of the Cascade expression system (17), which very efficiently overexpresses recombinant proteins and which is finely controlled in response to salicylate. The new plasmid construct carrying this fusion, pALEXb-33-mer, was introduced by means of transformation into the Cascade host strain REG1 of *E. coli*, which enabled the overexpression of genes cloned under the Pm promoter. The overexpression of the fusion protein was verified by detection of a salicylate-induced protein of the expected size that appeared as the most expressed protein in SDS-PAGE (**Figure 1**) and in the Western blot with anti-C-LYTAG antibodies (data not shown).

SDS-PAGE analysis of the purified recombinant proteins after the soluble fraction of the total expressed proteins was passed through an Ni^{2+}-affinity chromatography column is shown in Figure 1. In the 33-mer-T-HSP70– and 33-mer-X2-HSP70–labeled lanes, intensely stained bands of ≈32.6 and 82.0 kDa, respectively, were observed. The purity was >95% as assessed by Coomassie blue staining. The E-Toxate reaction showed that the purified recombinant proteins were free of lipopolysaccharide bacterial contaminants.

Obtaining and characterizing the anti-33-mer toxic peptide monoclonal antibodies

Previous studies showed that *T. cruzi* HSP70 has a singular stimulatory effect on spleen and ganglion cells of naive mice (19). Thus, to obtain the anti-33-mer MoAbs, mice were immunized with recombinant 33-mer-T-HSP70 or 33-mer-X2-HSP70. Hybridomas were obtained by fusing spleen cells from the sensitized mice with myeloma cells. Two hybridomas expressing MoAbs of high affinity for the 33-mer peptide were selected and cloned (*see* Materials and methods). MoAbs were

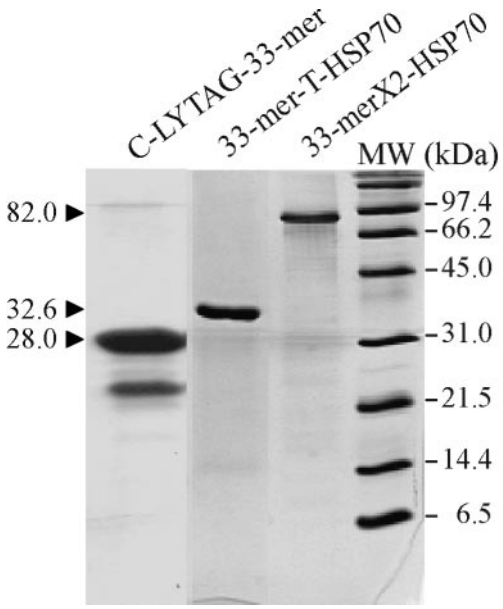

FIGURE 1. Purification of recombinant proteins bearing the 33-mer peptide for production of monoclonal antibodies against gliadin 33-mer peptide. Purified recombinant C-LYTAG-33-mer, 33-mer-T-HSP70 (HSP, heat-shock protein), and 33mer-X2-HSP70 were electrophoresed in 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and stained with Coomassie blue. Lane MW, molecular weight markers (in kDa).

purified from culture supernatants of the various cloned hybridomas and from the ascitic fluid of mice previously inoculated with the cells of cloned hybridomas.

To determine whether the MoAbs against the 33-mer peptide were able to recognize the peptide fused to other peptides, total extracts from bacteria overexpressing C-LYTAG-33-mer protein were resolved in 16% SDS-PAGE and analyzed by Western blot by using the G12 and A1 purified antibodies at 1/32 200 and 1/6400 dilution, respectively (data not shown). A single band of ≈28 kDa was detected in the salicylate-induced fractions. However, the purified MoAbs did not recognize any proteins in the noninduced fractions. This fact and the absence of recognition of any protein of a size similar to endogenous HSP70 were taken as indications that the antibodies were directed solely against the 33-mer peptide.

We also performed an indirect ELISA in which different amounts of the recombinant C-LYTAG-33-mer protein were fixed on the plate, starting from a solution with a concentration of 2 μg/mL, and we carried out serial double dilutions. Both MoAbs showed high sensitivity and specificity against the 33-mer peptide (**Figure 2**). However, the G12 MoAb exhibited an affinity for the toxic peptide 8 times that of the A1 MoAb.

Once the capacity of the antibodies to detect the 33-mer toxic peptide was determined, we conducted further analysis of the G12 MoAb because of its higher sensitivity for the 33-mer sequence. We investigated whether the G12 MoAb could detect the peptide's presence in prolamines from the various cereals. The prolamines from wheat, barley, rye, oats, maize, and rice were extracted with ethanol (60%), and the samples were analyzed by Western blot with the use of the G12 MoAb. First, the minimum detectable amounts of gliadin in commercial wheat were measured in the Western blot assays. Decreasing amounts of commercial wheat gliadin were used, starting from 10 μg and finishing at 0.01 μg (**Figure 3**). The results indicated that the antibody presents cross-reactivity against the prolamines of wheat, barley, and rye, although with different affinities. The sensitivity against avenin was lower than that against gliadin, hordein, and secalin (**Figure 4**). These results agreed with those

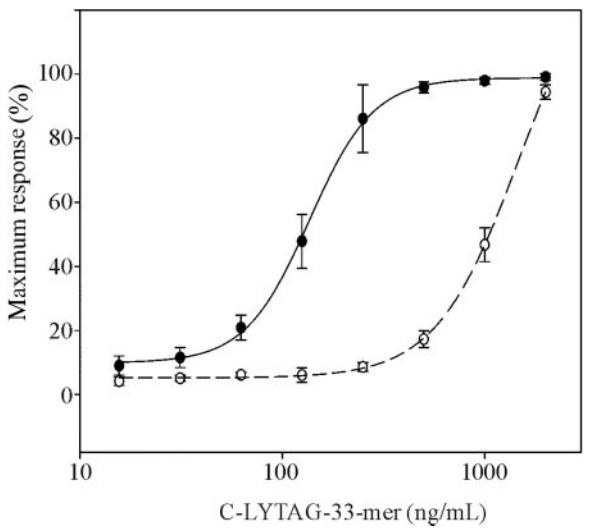

FIGURE 2. Standard curve for detection of C-LYTAG-33-mer by indirect enzyme-linked immunosorbent assay with the use of monoclonal antibodies G12 (●) and A1 (○). Each point of the curve represents the mean ± SD of 4 assays.

FIGURE 3. Western blot analysis of gliadin from Sigma Chemical Co (St Louis, MO) detected by G12 antibody. Lanes 1, 2, 3, and 4: 10, 1.0, 0.1, and 0.01 mg gliadin, respectively.

reported by Shan et al (8) who carried out sequence-alignment searches of the 33-mer gliadin by using BLASTP (Internet: www.ncbi.nlm.nih.gov). They showed that the gliadins (from wheat), hordeins (from barley), and secalins (from rye) contain sequences homologous to those of the 33-mer gliadin. In contrast, food-grain proteins such as avenins (in oats) do not contain sequences homologous to those of 33-mer gliadin, which would explain the results obtained with oats, in which the sensitivity was lower. The slight binding activity observed with oat peptides may be due to the possibility that the potential sequences for antibody recognition are less prevalent in this cereal. None of the samples assayed signaled the presence of the prolamines extracted from rice and maize. Similar results were obtained by indirect ELISA (data not shown). The presence of different profiles of recognition of the G12 MoAb in the various flours makes this antibody a very useful tool for determining the source of prolamine contamination that can affect gluten-free foodstuffs. Subsequently, the conjugation of this antibody to HRP was carried out with the aim of using it as a detection antibody in sandwich and competitive ELISAs.

Development of a sandwich enzyme-linked immunosorbent assay using the G12 and A1 monoclonal antibodies

The sandwich ELISA is the most common enzyme immunoassay formats used in the detection of proteins. It is highly sensitive and is especially useful for the quantification of antigens when their concentration is low, when they are contained in samples with a large amount of contaminating proteins, or both. Thus, we set out to develop a sandwich ELISA enabling high-sensitivity detection of the presence in foodstuffs of gluten fractions that are toxic to CD patients. We used G12 MoAb as the detection antibody and A1 MoAb as the capture antibody; both were developed against the 33-mer peptide. Different amounts of A1 antibody (0.5–2 μg/well) fixed on the plate and different dilutions of G12 antibody (1:250–1:25 000) were tested, and the

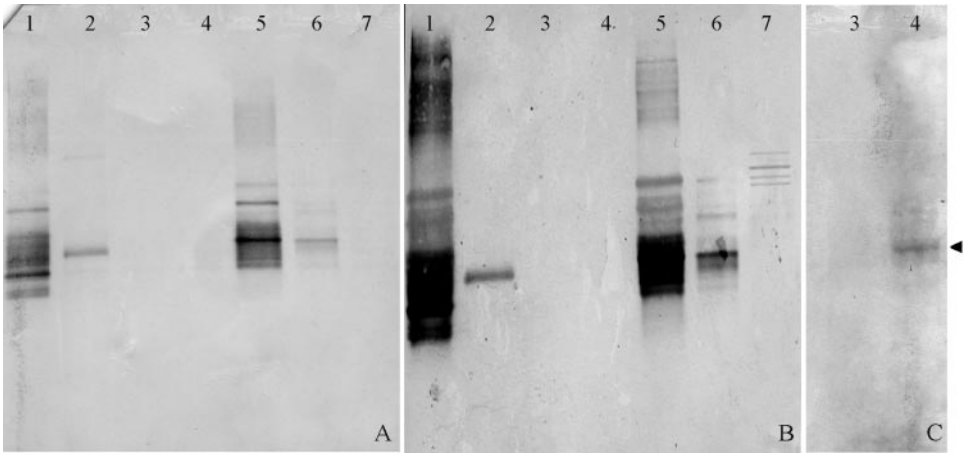

FIGURE 4. Western blot analysis of toxic peptides from gliadin, gluten, and different flour extracts. Lanes 1–7: gliadin (Sigma Chemical Co, St Louis, MO), rye, rice, oats, gluten, wheat, and barley, respectively. Analysis was carried out with 1 (A), 10 (B), and 25 (C) mg extract. Membranes were immunologically detected by using G12 antibody. Arrowhead, avenin signal.

temperature and incubation time were varied. The results enabled us to establish the ideal conditions in which a sandwich ELISA could detect the toxicity of foodstuffs for CD patients (*see* Materials and methods). A standard curve was performed, using gliadin (Sigma Chemical Co) as antigen. It was observed that the method of analysis presented high sensitivity against the analyte assayed (**Figure 5**); the limits of detection and quantification were 0.6 and 1.5 ng/mL, respectively. Taking the minimum working dilution as 1:25, and starting from a sample [obtained by ethanolic (60%) extraction from a foodstuff] at a concentration of 125 mg/5 mL extraction solution, the sandwich ELISA developed would enable the detection of the presence of gliadin in foodstuffs at values as low as 0.06 mg/100 g foodstuff (0.6 ppm gliadin or 1.2 ppm gluten) and would be able to quantify values as low as 0.15 mg gliadin/100 g foodstuff (1.5 ppm gliadin or 3 ppm gluten). In both cases, for the calculation of the gluten content, we have assumed a gliadin-to-glutenin ratio of 1:1.

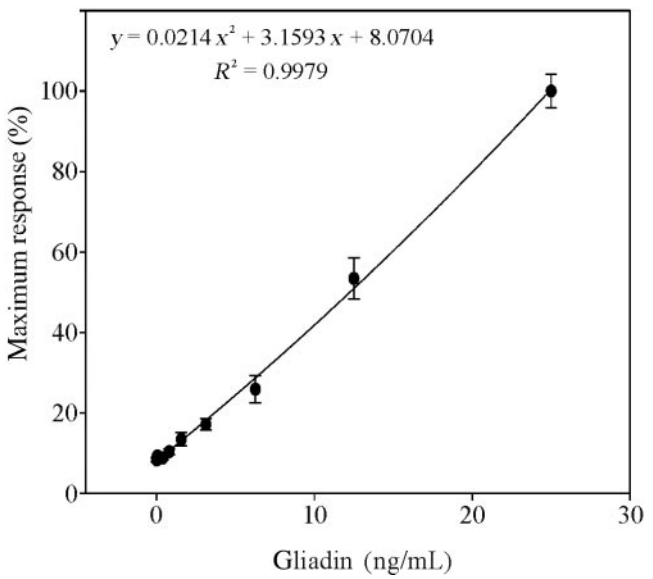

FIGURE 5. Standard curve for quantification of gliadin (Sigma Chemical Co, St Louis, MO) by sandwich enzyme-linked immunosorbent assay. The gliadin was added to the sandwich enzyme-linked immunosorbent assay in different amounts (*see* abcissa). Data points: plotting of concentration values (x) against % maximum response (y). Each point of the curve represents the mean ± SD of 8 assays. The least-squares method was applied to obtain the function describing a linear model.

To determine the reproducibility and repeatability of the sandwich ELISA developed, we calculated the interassay and intraassay CVs for each point of the standard curve (25–1.56 ng/mL). The intraassay CV was 4%–13%, and the interassay CV was 7%–12% (**Table 1**).

Once the capacity of the sandwich ELISA for high-sensitivity recognition of gliadin was established, its capacity to detect the other prolamines that have been shown to be toxic toward CD patients (secalin, hordein, and, to a lesser extent, avenin) was determined. Samples of wheat, barley, rye, oats, rice, and maize were extracted with ethanol (60%) and analyzed by sandwich ELISA. As shown in **Figure 6**, the assay developed enables the detection of gliadin, secalin, and hordein with a detection limit of <1 ng/mL (1 ppm), which is superior even to the detection limit recommended in the latest Codex Alimentarius (ie, 10 ppm). For oats, the sensitivity was lower. The assay proved highly specific for these cereals, because no signal was observed in samples containing prolamines from rice (oryzein) or maize (zein), cereals that are nontoxic to CD patients (Figure 6).

TABLE 1
Intraassay and interassay variation of gliadin standards analyzed by sandwich enzyme-linked immunosorbent assay

	Intraassay (n = 8)		Interassay (n = 2)	
	Maximum response	CV	Maximum response	CV
	%	%	%	%
Gliadin (ng/mL)				
25	100 ± 4.15[1]	4.15	100 ± 7.47	7.47
12.5	53.41 ± 5.10	9.54	51.26 ± 6.28	12.26
6.25	25.85 ± 3.36	12.99	26.98 ± 2.54	9.42
3.12	17.11 ± 1.45	8.45	19.06 ± 1.44	7.56
1.56	13.41 ± 1.66	12.39	15.48 ± 1.51	9.76

[1] $\bar{x}$ ± SD (all such values).

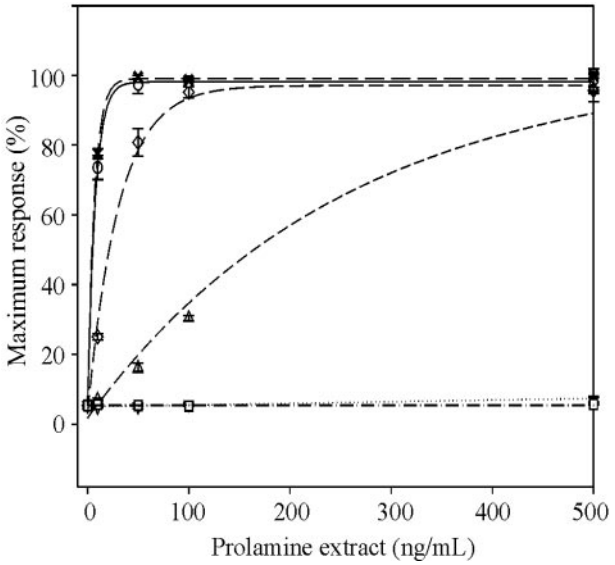

FIGURE 6. Comparative reactivity of prolamins during sandwich enzyme-linked immunosorbent assay performed on aqueous ethanol prolamin extracts from wheat (– ◇ –), barley (⊖), oats (- △ -), rice (·▽·), and maize (–⊡·). Each point of the curve represents the mean ± SD of 3 assays.

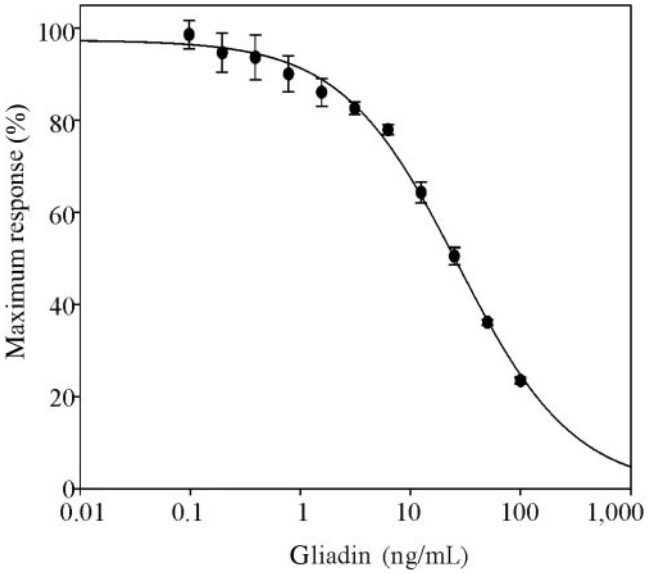

FIGURE 7. Standard curve of the competitive enzyme-linked immunosorbent assay for detection of gliadin (Sigma Chemical Co, St Louis, MO) with the use of monoclonal antibody G12-HRP (HRP, horseradish peroxidase), obtained under optimized conditions. Each point of the curve represents the mean ± SD of 8 assays. The 4-parameter logistic model was used to calculate for fitting the sigmoidal curve with the use of the following equation: % maximum (max) response = minimum (min) + [max − min/1 + $(x/IC50)^{Hill\ slope}$], where max = 97.40, min = 1.88×10^{-7}, the concentration needed to inhibit action, binding, or activity by 50% (IC50) = −26.92, and Hill slope = 0.82.

All of these samples were analyzed by using a gliadin from Sigma Chemical Co. The effect of changing the gliadin was addressed by also using a different gliadin (European reference gliadin; Ingenasa, Madrid, Spain). Values did not differ significantly between the 2 gliadins (data not shown).

Development of a competitive enzyme-linked immunosorbent assay by using the G12 monoclonal antibody

Gluten can be present in solid, liquid, and semi-liquid foodstuffs. During the preparation of many of these foodstuffs, they are subjected to heat or enzymatic processes, which alter the gluten so that it becomes insoluble, completely or partially hydrolyzed, or both. As a result, the values of gluten extracted from foodstuffs processed by heat or hydrolyzed may be underestimated, whatever the method used for the quantification. Therefore, it was necessary to develop a competitive ELISA in which the antigen fixed on the plate and the antigen in solution, which are contained in the sample, compete for union with the capturing antibody, so that a decrease in the signal indicates the presence of antigen in the sample. For the development of this ELISA, various assays were carried out to optimize the conditions, by altering the concentration of gliadin fixed on the plate (1–0.01 μg/well), the dilution of G12-HRP antibody used (1:1000–1:50 000), and the time (15 min to 4 h) and temperature (4 °C, room temperature, or 37 °C) of preincubation and incubation. A gliadin concentration of 0.5 μg/well fixed on the plate and a 1:20 000 dilution of the G12-HRP antibody were found to be optimum (data not shown). Different conditions before and during incubation (temperature and time) were tested; a 2-h preincubation period and a 30-min incubation, both at room temperature, were found to be optimum. The result was a highly sensitive competitive assay with a limit of detection of 0.44 ng/mL and a limit of quantification of 3.95 ng/mL. The gliadin concentration giving a 50% reduction in the maximum signal in the ELISA (ie, IC50) in the standard curve of the competitive assay was determined to be 26.92 ng/mL. The standard curve for the detection of toxic gliadin by competitive ELISA under the established conditions is shown in **Figure 7**, and there is good correlation of the data (R^2 = 0.99).

The repeatability and reproducibility of the method, calculated from various standard curves performed on the same ELISA plate (intraassay) and on different ELISA plates (interassay), respectively, are shown in **Table 2**. For the standards situated between 25 and 1.56 ng gliadin/mL, the intraassay CV was 1.38–3.75%, and the interassay CV was 1.65–10.30% for the same standards.

To determine whether the perfected competitive assay was able to detect small fragments originated by gliadin digestion, we analyzed a sample of gliadin that had been digested with trypsin

TABLE 2
Intraassay and interassay variation of gliadin standards analyzed by competitive enzyme-linked immunosorbent assay

	Intraassay (n = 8)		Interassay (n = 2)	
	Maximum response	CV	Maximum response	CV
	%	%	%	%
Gliadin (ng/mL)				
25	50.48 ± 1.89[1]	3.75	58.33 ± 6.01	10.30
12.5	64.33 ± 2.25	3.49	69.10 ± 2.31	3.35
6.25	77.94 ± 1.08	1.38	81.83 ± 6.19	7.56
3.12	82.59 ± 1.38	1.67	87.22 ± 2.62	3.01
1.56	86.09 ± 3.00	3.49	91.05 ± 1.50	1.65

[1] $\bar{x}$ ± SD (all such values).

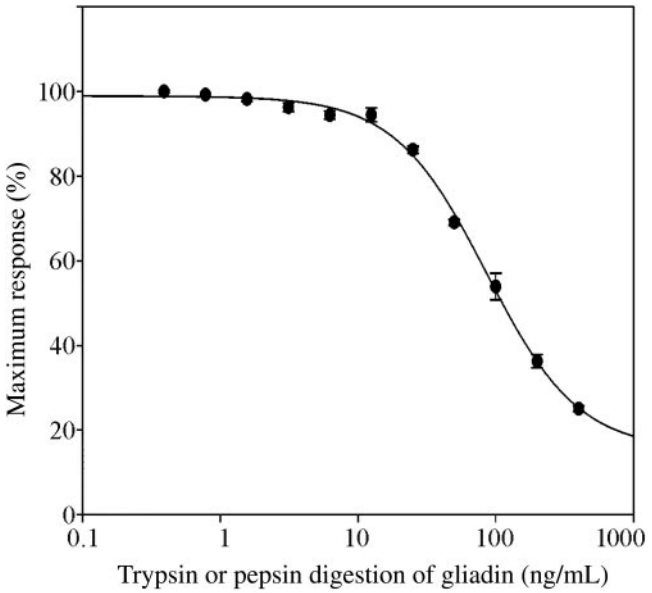

FIGURE 8. Competition assay for detection of trypsin of pepsin digestion of gliadin with the use of monoclonal antibody G12-horseradish peroxidase, obtained under optimized conditions. Each point of the curve represents the mean ± SD of 4 assays. The 4-parameter logistic model was used to calculate for fitting the sigmoidal curve.

and pepsin. We observed that the developed assay could detect the peptides coming from the degradation of gliadin by these enzymes (**Figure 8**). We also analyzed a sample of hydrolyzed baby cereals in which the gliadin had been partially hydrolyzed during processing. These cereals contain a mixture of wheat, barley, rye, oat, and rice flours. The ethanolic extract obtained from the foodstuff was analyzed by using the competitive ELISA. The partially hydrolyzed prolamines present in the sample were able to compete, and the quantitative assay could be performed (data not shown).

DISCUSSION

In the present study, we developed MoAbs recognizing the gliadin 33-mer peptide that served to develop assays to detect gluten fraction in cereals and that could be used in the assessment of relative food toxicity for CD patients. People with this disease cannot tolerate the protein gluten in their diet, and they have a broad range of sensitivity to gluten intake. The usual first therapy for CD is complete dietary exclusion of gluten. The gluten-free diet does not define the relation between the amount of gluten ingested and the seriousness of the clinical symptoms and histologic anomalies in CD patients. Individual variation and clinical heterogeneity in CD patients pose serious problems when attempts are made to identify acceptable threshold values for very small amounts of gluten, whose presence could be allowed in foods that are nominally free of the substance. Because only 10% of gluten seems to be made up of potentially toxic gliadin peptides, it is desirable to quantify the part of the gluten ingested by a CD patient that is really toxic, so that the true toxicity of the gluten present in foods can be more precisely established.

The problem of ascertaining exactly what should be measured in assessments of toxic gluten proteins has long existed (24). The principal toxic components of wheat gluten belong to a family of closely related proline- and glutamine-rich proteins called gliadins (25). Crucial epitopes that make gliadin toxic have been defined (8), and that work shows what triggers CD. It has been verified that the 33-mer peptide from α-2 gliadin is in fact a principal contributor to the protein's immunotoxicity (8).

The most practical method for food analysis is based on immunologic analysis (26). An ideal antibody used for gluten analysis in food not only should be a reliable indicator of the presence of prolamines from cereal species known to be toxic to CD patients but also should recognize the specific intramolecular region responsible for such toxicity. The identification of the exceptionally immunoreactive 33-mer peptide led us to use it to obtain MoAbs that enabled the quantification of toxic fractions of gluten and that, thus, should be a valuable tool for the quality control of gluten-free food. These MoAbs were obtained by fusing the 33-mer toxic peptide with a *T. cruzi* HSP, one of a highly conserved family of stress-response proteins (27). HSPs function primarily as molecular chaperones; they facilitate the folding of other cellular proteins, prevent protein aggregation, or target improperly folded proteins to specific degradative pathways (28). The microbial HSP70 family has acquired special significance in immunity, because its members have been shown to be potent activators of the innate immune system (29). Previous works showed that the addition of HSP70 from *T. cruzi* to different types of antigen enables the induction of rapid stimulation of specific antibodies with a high affinity for the accompanying antigen (22, 30, 31). Therefore, it was used as an efficient means for the induction and production of MoAbs against the 33-mer peptide.

To produce the MoAbs, the toxic peptide was fused to the HSP70 carrier molecule, and mice were immunized separately with recombinant 33-mer-T-HSP70 and 33-mer-X2-HSP70. Hybridomas were obtained by fusing spleen cells from the sensitized mice with myeloma cells. The MoAbs were characterized to establish their specificity against the 33-mer toxic peptide; the G12 antibody showed the highest sensitivity against the 33-mer peptide.

ELISAs based on these MoAbs (G12 and A1) showed a wider specificity for prolamines that are toxic to CD patients, along with a higher degree of sensitivity, accuracy, and reproducibility, than did the other ELISAs. For gluten analysis, we obtained a highly sensitive sandwich ELISA that uses 2 single MoAbs that recognize an allergenic peptide of gluten in gliadins, hordeins, and secalins. In contrast to other MoAbs (13, 14), the G12 MoAb was not as effective at detecting wheat, barley, and rye gluten; this difference in sensitivity was probably a consequence of the number of times that the complete or partial sequence of the 33-mer peptide is present in these cereals. The detection limit was 0.6 ng gliadin/mL, which corresponds to 0.6 ppm gliadin, or one-third of the concentration obtained by other methods described to date (13).

Historically, there has been concern that oats may not be safe for CD patients, either adults or children (32, 33). Some patients have been reported who appear to show both clinical and immunologic responses to a pure oat product (4, 34). A potential advantage of the G12 MoAb is its ability to detect oat avenins directly prepared from oat grains; other ELISAs described in the literature do not have this capacity. Peptide recognition by the G12 MoAb has been confirmed by Western blot, which indicates a pattern not related to other cereal contamination. The detection of oats required much higher concentrations in both the ELISA

and the Western blot than are needed for wheat, barley, and rye gluten. This difference in sensitivity was possibly due to the fact that the sequence that the G12 MoAb recognizes is less represented in avenins than in gliadins, hordeins, and secalins or to the fact that sequences similar to the toxic epitope exist in oats. It could be concluded that the reactivity of G12 with avenins seems to be an advantage, because clinical studies have shown that oats damage the mucosa in a certain proportion of CD patients but are probably safe at moderate concentrations (4). It would be of interest to identify those oat peptides with some celiac immunotoxicity and to determine the affinity for the G12 antibody.

The G12 MoAb proved to be efficient in measuring prolamine concentrations in both native and partially hydrolyzed cereals. The development of a novel competitive assay using this MoAb to a 33-mer peptide recognized in vivo to be toxic to CD patients is also reported. This assay is highly sensitive and reproducible, with a detection limit of 0.44 ppm gliadin. The ELISA systems described here showed high reproducibility and repeatability (<15% for both). The high sensitivity of G12 detection in the ELISAs enables the determination of gluten concentrations in foods as low as 1 ppm, which is far below the 20-ppm threshold proposed by the Codex Alimentarius Commission (15).

CD patients have different sensitivities to gluten and different maximum amounts that they can tolerate. The recommended maximum daily ingestion of gluten was indicated to be <50 mg gluten (35), and our methods detected <1 ppm gluten. An average human secretes gastric juices at a rate of ≈1.2–2.4 L/d. The method reported here could detect concentrations several orders of magnitude below the maximum recommended gluten concentration in the digestive tract (ie, <20 mg/L) of CD patients. Furthermore, the detection of toxic peptides in hydrolyzed foods may be more appropriate for the anti-33-mer antibodies. Other antigliadin antibodies with no specificity for toxic peptides may originate false-negative signals not related to the remaining toxicity of the hydrolyzed food.

The present study establishes a valid method, with respect to accuracy, precision, and reproducibility, for the quantification of toxic fractions of gluten that CD patients cannot tolerate. In addition to the slightly greater sensitivity than is seen for other commercial antibodies [eg, R5 (13)], this method is much more sensitive to the toxic 33-mer gliadin peptide, which could be detected in hydrolyzed food with the competitive ELISA developed, and it can detect certain amounts of oats with potential toxicity. This new assay, based on the directly toxic portion of gluten, has linked the latest scientific knowledge of the mechanism of CD to the demand for food safety in CD patients. These MoAbs against the immunotoxic peptide could be applied in the development of bioassays to measure the effectiveness of enzymatic therapy and more-advanced systems for food analysis (eg, biosensors). The recently suggested role of the deamidated 33-mer as a stimulant of interleukin-15 expression in healthy subjects (36) may also extend the usefulness of the information that the developed G12 immunologic assays provide about the digestibility of foods.

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The authors' responsibilities were as follows—AC and MA-M: cloned, produced, and purified the 33-mer recombinant fusion; MdCT and MCL: cloned, produced, and purified the 33-mer heat-shock protein recombinant fusion proteins and obtained and purified the monoclonal antibodies against the 33-mer peptide; BM, HM, MM, and CS: developed the competitive and sandwich enzyme-linked immunosorbent assays; and BM, AC, MCT, MCL, and CS: analyzed the data, interpreted the results, and wrote and revised the manuscript. None of the authors had a personal or financial conflict of interest.

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