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Selective capture of most celiac immunogenic peptides from hydrolyzed gluten proteins

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ABSTRACT

The available immunomethods for gluten quantitation could underestimate or overestimate the net immunoactivity of foods and beverages if the chosen analytical antibody is not specific to the relevant gluten immunogenic peptides (GIP). Accurate detection of the most active GIP is desirable to assess the potential celiac toxicity of food.

We evaluated the capacity of the G12 monoclonal antibody for selectively depleting GIP in samples from two different gluteomes. Samples of hydrolyzed gliadin from wheat and a barley beer were used. The input (starting peptide digest of prolamins), the flow-through (unbound peptides), and the output (captured peptides) were analyzed by G12 and R5 competitive ELISA as well as by stimulation assays of T-cells from celiac patients. Most of the GIP were retained by the G12-agarose and represented the largest part of the immunogenicity of the gluten peptidome.

G12 immunodepletion experiments with hydrolyzed gluten showed that this antibody reacted with those with the highest immunoactivity for celiac patients.

1. Introduction

Celiac disease (CD) is an immune-based reaction to dietary gluten, which affects the small intestine in those with a genetic predisposition (Bernardo & Peña, 2012; Woodward, 2007). Gluten is a collective term for several hundred homologous, alcohol-soluble, seed storage proteins in wheat (gliadins and glutenins), barley (hordeins), rye (secalins), and some oat varieties (avenins), representing up to 80% of total grain proteins (Rosell, Barro, Sousa, & Mena, 2014; Tanner, Colgrave, Blundell, Goswami, & Howitt, 2013). Strict life-long exclusion of gluten is the only treatment for CD resulting in full clinical and histological remission (Barratt, Leeds, & Sanders, 2011).

The difficulty of gluten detection in foods and beverages lies in the huge diversity of food ingredients and the extremely polymorphic mixture of cereal toxic prolamins. A test for the analysis of gluten should react quantitatively with the intact prolamins and glutenins of immunotoxic cereals, as well as with the gluten immunogenic peptides (GIP) in different degrees of hydrolysis remaining after manufacturing (i.e. in beers, baby food, syrups, or cooked food, among others). The analysis of GIP in food and beverages confers safety for celiac consumption. Simplicity, reliability, and economy are the main premises of an analytical method to implement in the food industry, preventing as much as possible an impact on the consumer price of food labeled gluten-free.

Despite the efforts to define relevant gluten epitopes, the characterization of the complete repertoire of peptides involved in the pathogenesis of CD remains a daunting task because of the great heterogeneity of gluten proteins (Camarca, Del Mastro, & Gianfrani, 2012). Studies based on gluten epitopes that are immunoreactive to celiacs have shown that a few highly immunogenic peptides could account for more than 90% of the celiac-specific response (Anderson, Degano, Godkin, Jewell, & Hill, 2000;

Abbreviations: CD, celiac disease; moAb, monoclonal antibody; GIP, gluten immunogenic peptides; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; AAEM, anti-endomysial antibody; AATG, anti-tissue transglutaminase antibody; HLA, human leukocyte antigen; PBMCs, peripheral blood mononuclear cells; IFN-γ, interferon gamma.

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Shan et al., 2002; Tye-Din et al., 2010). Among the CD-immunodominant gluten peptides, the α-gliadin-derived 33-mer (residues 57-89) is regarded as one of the most prominent and investigated (Chen, Hoffmann, Östman, Sandberg, & Olsson, 2011; Fraser et al., 2003; Qiao et al., 2004; Shan et al., 2002; Tye-Din et al., 2010).

In recent years, next-generation monoclonal antibodies (moAbs) specifically raised against immunotoxic peptides have been developed. Mitea et al. (2008) proposed an approach based on 5 moAbs for a comprehensive screening of the presence of harmful gluten and gluten-like peptides and proteins in foods. However, the complexity of the results is too great for routine food testing. Antibodies against the α-gliadin 33-mer, A1 and G12, have proved to be practical tools to detect specifically immunoreactive gluten in food (Comino et al., 2011; Morón, Cebolla, et al., 2008; Morón, Bethune, et al., 2008; Picariello et al., 2015; Real et al., 2014). Although the current R5 Codex Alimentarius type I method theoretically reacts against various toxic peptides contained in gliadins, hordeins, and secalins, it has been demonstrated that it is from 100 to 60,000 times less sensitive in detecting the 33-mer than are A1 or G12 moAb, respectively (Torgler, Síglez, Vilchez, Cebolla, & Sousa, 2011).

To establish the reliability of current immunological techniques for estimating the potential toxicity of food and drinks for celiacs, it is important to know the immunological activity of the measured peptides. In this work, we have evaluated to what extent the specificity of G12 moAb could capture the peptide pools immunogenic for celiacs in hydrolyzed gliadin as well as in a barley beer. We performed immunoaffinity chromatography, using a G12-agarose column and stimulation assays of T-cells isolated from celiac patients.

2. Material and methods

2.1. Simulated gastrointestinal gliadin digestion

To reproduce gastric digestion *in vitro*, PWG gliadin (Prolamin Working Group) was incubated at 37 °C for 60 min in HCl solution (pH 2.0) that contained 0.60 mg pepsin/mL (Sigma). Digests were adjusted to pH 6.0 with phosphate-buffered saline (PBS) and subjected to a simulated duodenal digestion by sequential addition of the pancreatic enzymes trypsin (0.375 mg/mL) and chymotrypsin (0.375 mg/mL) (both from Sigma) at 37 °C for 30 min (Ehren et al., 2009).

2.2. Beer sample and preparation of prolamins from rice flour

Duvel commercial barley beer was used in the study. Beer was degassed and aliquoted for testing. As negative control for assay, the alcohol-soluble protein fractions were extracted from rice (*Oryza sativa*) whole flour. These fractions were subjected to pepsin, trypsin, and chymotrypsin sequential digestion, according to Ehren et al. (2009).

2.3. Peptides, protein standards, and G12 moAb

The 33-mer peptide LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF was supplied by Biomedal S.L. (Seville, Spain).

C-LYTAG-33-mer is the fusion of the C-LYTAG coding sequence of the pALEXb plasmid constructed with the complete sequence of the 33-mer peptide (Morón, Bethune, et al., 2008). C-LYTAG-33-mer was supplied by Biomedal (Seville, Spain).

G12 moAb and its derived horseradish peroxidase (HRP)-conjugated moAb (G12-HRP) were used in this study (Biomedal S.L., Seville, Spain).

2.4. Antibody immobilization

High-density glyoxal 4BCL resin (ABT, Madrid, Spain) was used for the immobilization of the G12 moAb. The size of bead resin ranged from 40 to 180 μm. Antibody (1 mg) was immobilized in 1 mL of glyoxal agarose bead resin. The immobilization procedure was as follows: 1 mL of resin was washed 3 times with distilled water (5 mL); the antibody was diluted in 9 mL of carbonate buffer (CB) at pH 10. The glyoxal agarose beads were added to the ligand solution. The mix was incubated at room temperature (RT) with strong agitation overnight. The reaction was stopped with 10 mg of $NaBH_4$ and the mixture was stirred for 30 min in a fume hood. The suspension was washed thoroughly with distilled water. The antibody-derived resin was stored at 4 °C in PBS with Bronidox as preservative.

2.5. Capture assay

To test the specificity of G12-resin for the α-gliadin 33-mer in protein extracts, 100 μL of the G12-agarose resin was packed in a 0.5 mL spin column. C-LYTAG-33-mer peptide was added and the mixture incubated for 30 min at RT. The column was eluted twice with 100 μL of glycine (0.1 M, pH 2.0) for incubation times of (first) 15 min and (last) 5 min. Elution fractions were neutralized with Tris-HCl (1 M, pH 9.0).

2.6. Immunoprecipitation assay

For immunoprecipitation, beer samples and hydrolyzed gliadin peptides were incubated with G12 moAb covalently coupled to agarose beads. After being gently mixed overnight at RT, the beads were washed sequentially with PBS (pH 7.0), and washed samples were collected as flow-through. Finally, bound peptides were eluted and recovered with arginine (2M, pH 4.3) and neutralized by addition of Tris-HCl (1M, pH 8.0) as output sample.

2.7. Competitive ELISA

Two commercial competitive ELISA kits were used in this study in accordance with the supplier's instructions: G12 GlutenTox ELISA Competitive (Biomedal Diagnostics, Seville, Spain) and R5 ELISA RIDASCREEN kit (Gliadin competitive R-Biopharm AG, Darmstadt, Germany). Rice prolamins were used as negative control, and ELISA solution without prolamins was used as blanks.

2.8. Patients

Eleven patients with active CD were included in this study. The diagnosis of CD was primarily determined by serological screening tests and finally confirmed by biopsy of the small intestine. The mucosal specimens were graded independently according to the Marsh–Oberhuber classification (Marsh, 1992; Oberhuber, Granditsch, & Vogelsang, 1999).

Subjects were prospectively screened for CD using anti-endomysial antibodies (AAEMs), anti-tissue transglutaminase antibodies (AATGs), and CD-specific HLA (human leukocyte antigen) typing (Table 1). Venous blood was taken at the time of index biopsy.

The local Ethics Committee of the Hospital 'Virgen de las Nieves' (Granada, Spain) approved the study protocol. Written consent was obtained from parents or legal guardians of children.

2.9. Peripheral blood mononuclear cells (PBMCs) and cell cultures

PBMCs from eleven patients with active CD who were on a gluten-containing diet were isolated from 6 mL of heparinized