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Natural variation in avenin epitopes among oat varieties: Implications for celiac disease

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ABSTRACT

Celiac disease (CD) is a chronic inflammatory disease affecting the small intestinal mucosa. The causative agents have been identified as gluten proteins from wheat, barley, and rye, and the only available treatment for CD patients is a lifelong gluten-free diet. Non-gluten containing cereals would be a valuable contribution to the gluten-free diet. In this respect, oats are a good choice. However, commercial lots of oat flakes and flour frequently are contaminated with wheat, barley, and rye, and two studies have reported that some peptides derived from the gluten-like avenin storage proteins of oat can trigger an immune response in some CD patients. In the present study we have initiated the investigation whether all oat varieties contain similar amounts of potentially harmful sequences by biochemical and immunological methods. We confirm that commercial oat preparations are contaminated with other cereals that contain gluten or gluten-like proteins. Moreover, our results demonstrate that contamination-free oat varieties differ in their capacity to stimulate an avenin-sensitive gamma-gliadin specific T cell line derived from a patient with CD, indicative for differences in the two known avenin epitopes among oat varieties, implying that selection and breeding of completely safe oat varieties for all CD patients may be a realistic possibility.

1. Introduction

Celiac disease (CD) is a food intolerance that affects approximately 1% of the population (Maki et al., 2003). Typical symptoms include diarrhea, abdominal distention and pain. Extra-intestinal manifestations like anemia, infertility, growth deficiency and neurological symptoms can also be present (Green, 2005).

CD is an immune mediated disease in which protein fragments from wheat, barley, and rye provoke an inappropriate immune response. It is well established that the disease almost only develops in HLA-DQ2 and/or -DQ8 positive individuals (Mearin et al., 1984; Sollid et al., 1989). HLA-DQ2 and -DQ8 are HLA-class II molecules involved in binding peptides derived from exogenous proteins and "presenting" these peptides to the T cells of the immune system (Lundin et al., 1993). Both HLA-DQ2 and -DQ8 can bind gluten-derived peptides, particularly after enzymatic modification by the enzyme tissue transglutaminase (tTG) (Folk and Chung, 1985), which introduces negative charges in gluten peptides required for efficient binding to the HLA-molecules. Upon binding, the HLA-DQ-gluten peptide complexes can trigger inflammatory T cell responses which ultimately lead to disease (Molberg et al., 1998; van de Wal et al., 1998). As such gluten specific T cells can only be isolated from the small intestine of CD patients, these adaptive immune responses are a critical factor in disease pathogenesis.

Upon withdrawal of gluten, the inflammation subsides and patients can lead a normal life as long as they stick to a lifelong gluten-free diet, thus devoid of any products prepared from wheat, barley, and rye. Food products based on gluten-containing cereals, however, form an important component of the human diet and celiac patients need alternative cereals that substitute this source of fiber and nutrients. One of the possible candidates is oat but this is

Abbreviations: CD, celiac disease; cpm, counts per minute; ELISA, enzyme-linked immunosorbent assay; IMDM, iscove's modified Dulbecco's media; LMW, low molecular weight; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell; RT, room temperature; SDS-PAGE, sodiumdodecyl sulphate polyacrylamide gel electrophoresis; tTG, tissue transglutaminase.

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still controversial as contradictory reports have appeared concerning the safety of oat for CD patients. Several studies have documented that >99% of CD patients can safely consume oat (Kemppainen et al., 2008; Koskinen et al., 2009; Pulido et al., 2009), and on that basis non-contaminated ('pure') oat is now considered as gluten-free in EC-regulation 41/2009.

However, two studies have found CD patients that do not tolerate contamination-free oats: three CD patients developed intestinal inflammation upon oat exposure (Arentz-Hansen et al., 2004) and one developed partial villous atrophy (Lundin et al., 2003). It has also been demonstrated that gluten-reactive T cells from some CD patients can also respond to avenin-derived peptides (Vader et al., 2003a). Also, an avenin specific T cell line has been isolated from the biopsy of a celiac patient which developed villous atrophy during an oat-containing, but otherwise standard gluten-free diet (Arentz-Hansen et al., 2004). These results thus indicate that oat may not be completely harmless to all patients. Furthermore, contamination of oats with other cereals, due to the shared use of equipment for transportation and fabrication for both oat and other cereals, is quite frequent as was previously reported (Garcia et al., 2005; Hernando et al., 2008). Therefore, the toxicity of oat can be due to both contamination and intrinsic toxicity but the result is the same: it leads to uncertainty about introduction of oat in the gluten-free diet, especially in those countries where oat is a not-frequently consumed food product.

The present study is focused on the characterization of this potential intrinsic immunogenicity of a selection of 26 oat varieties using immunological and biochemical methods.

2. Materials and methods

2.1. Oat samples

The grains of 26 oat varieties (1: Ascot, 2: Astor, 3: Charming, 4: Charmoise, 5: Dalguise, 6: Dominik, 7: Fervente, 8: Firth, 9: Freddy, 10: Gambo, 11: Gele van Timmermans, 12: Gerald, 13: Gigant, 14: Leanda, 15: Mansholt III, 16: Markant, 17: Mustang, 18: Ouderwetse Zeeuwse Partij, 19: Panache de Roye, 20: Powys, 21: Sang, 22: Troshaver uit Besel, 23: Valiant, 24: Wodan, 25: Zandster, 26: Zwarte President) were used in this study. All varieties were obtained from CGN (Wageningen, The Netherlands) and the grains were washed with 60% aqueous ethanol and dried overnight to remove any trace of other cereals before grinding in a coffee mill to obtain a fine homogenized powder. As contamination of oats by other cereals is well documented (Garcia et al., 2005; Hernando et al., 2008), we analyzed eight varieties for possible contamination using a sandwich R5 ELISA kit (Ingezim® Gluten, Ingenasa, Spain) and a competition assay based on a specific mAb which recognizes the α20-gliadin epitope and homologous sequences from barley and rye (Spaenij-Dekking et al., 2004). These varieties were Astor, Gele van Timmerman, Mansholt III, Mustang, Panache de Roye, Troshaver uit Besel, Wodan and Zwarte President. All samples were found to be contamination free by both methods (<1.5 mg/kg for the R5 method and <25 μg/kg for the α20-gliadin epitope specific competition assay).

2.2. Preparation of protein fractions from oat varieties

Prolamins were extracted from the oat samples using 60% (v/v) ethanol as described before (Garcia et al., 2005). Trypsin/pepsin digests were prepared as follows: 0.5 g of oat sample was solubilized in 4 mL of 1 mol/L acetic acid and boiled for 15 min. After cooling to room temperature (RT), 2.5 mg of pepsin was added and the mixture was incubated for 4 h at 37 °C. Subsequently the pH was adjusted to 7.8 with NaOH, followed by addition of 5 mg of trypsin. After incubation overnight at 37 °C, the samples were boiled for 15 min. For the next 48 h the samples were dialyzed against water using dialysis tubing with a cutoff of 10 kDa. The dialyzed material was centrifuged and fractionated over a 10 kDa membrane for removal of the enzymes and any remaining insoluble material. For the subsequent experiments, the fractions smaller than 10 kDa were used. A control sample was prepared using a commercially available gliadin preparation. For the T cell assay, the pepsin/trypsin digests were treated with tissue transglutaminase (N-Zyme) as described previously (Stepniak et al., 2005).

2.3. T cell proliferation assays

The presence of T cell stimulatory epitopes in the oat samples was determined using a T cell line isolated from a small intestinal biopsy of a celiac disease patient (Vader et al., 2003a). Proliferation experiments were performed in triplicate in 150 μL Iscove's Modified Dulbecco's Media (IMDM) with 10% normal human serum in 96-well flat-bottom plates using 2×10^4 gluten specific T cells stimulated with 10^5 irradiated (3000 rad) HLA-DQ2 or -DQ8 matched allogenic peripheral blood mononuclear cells (PBMCs) in the presence of or absence of the antigen (4 μg/well). After 2 days, 0.5 μCi/well ^{3}H-thymidine was added to the cultures and after 18–20 h the cells were harvested and the ^{3}H-tymidine incorporation was measured using a liquid scintillation counter (MicroBeta counter, Perkin Elmer).

2.4. Synthetic peptides

Peptides were synthesized by standard Fmoc chemistry on a SyroII peptide synthesizer as described previously (Spaenij-Dekking et al., 2004). The integrity of the peptides was checked by reversed-phase HPLC and mass spectrometry. When required, biotin was introduced in the resin-bound peptides by a 2-h coupling with a 6-fold equimolar preactivating mixture of biotin and benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate.

2.5. Protein analysis by 1D sodiumdodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) and western blotting

To determine the level of T cell stimulatory epitopes, 10 μL of the prolamin extracts were dried in a CHRIST ALPHA freeze-dryer (Salm en Kipp, Breukelen, The Netherlands), resuspended in 20 μL of protein sample buffer [62.5 mM Tris–HCl pH 6.8, 5% (v/v) glycerol, 2% (w/v) SDS, 0.0005% (w/v) bromophenol blue and 5% (v/v) β-mercaptoethanol] and incubated for 5 min at 95 °C in a water bath. After that, the samples were spun down using a centrifuge and 20 μL supernatant was loaded into the wells of a 12.5% (w/v) SDS-PAGE gel. The proteins were visualized either directly using Imperial™ Protein Stain (Pierce, Rockford, IL) or transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). For the Western Blot analysis, the proteins were visualized with monoclonal antibodies (mAbs) specific for stimulatory T cell epitopes from α20-gliadin and Low Molecular Weight (LMW)-glutenin (Mitea et al., 2008a, b; Spaenij-Dekking et al., 2004, 2005).

2.6. Competition assay for the quantitative detection of a T cell stimulatory epitope

The content of a T cell stimulatory epitope involved in celiac disease and present in α20-gliadin was determined using specific Enzyme-Linked ImmunoSorbent Assays (ELISAs). Maxisorb Immunoplates (Nunc, Copenhagen, Denmark) were coated overnight at +4 °C with 100 μL/well of 2–5 μg/mL mAb in Phosphate