Celiac disease : towards new therapeutic modalities

Mitea, D.C.

Chapter 4

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.

INTRODUCTION

Celiac disease (CD) is a food intolerance that affects approximately 1% of the population (10). Typical symptoms include diarrhea, abdominal distention and pain. Extra-intestinal manifestations like anemia, infertility, growth deficiency and neurological symptoms can also be present (4).

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 (11, 19). 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 (9). Both HLA-DQ2 and -DQ8 can bind gluten-derived peptides, particularly after enzymatic modification by the enzyme tissue transglutaminase (tTG) (2), 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 (14, 28). 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 subdues 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 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 (6, 7, 15), 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 (1) and one developed partial villous atrophy (8). It has also been demonstrated that gluten-reactive T cells from some CD patients can also respond to avenin-derived peptides (24). 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 (1). 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 (3, 5). 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.

MATERIALS AND METHODS

Oat samples

The grains of twenty-six 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 over-night 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 (3, 5), 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 (20). 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).

Preparation of protein fractions from oat varieties

Prolamins were extracted from the oat samples using 60% (v/v) ethanol as described before (3). 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 minutes. After cooling to room temperature (RT) 2.5 mg of pepsin was added and the mixture was incubated for 4 hours 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 minutes. For the next 48 hours 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 commercial available gliadin preparation. For the T-cell assay the pepsin/trypsin digests were treated with tissue transglutaminase (N-Zyme) as described previously (22).

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 (24). 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 $2x10^4$ 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 hours the cells were harvested and the ^{3}H-tymidine incorporation was measured using a liquid scintillation counter (MicroBeta counter, Perkin Elmer).

Synthetic peptides

Peptides were synthesized by standard Fmoc chemistry on a SyroII peptide synthesizer as described previously (20). 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.

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 95ºC in a water bath. After that, the samples were spin 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 (12, 20, 21).

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 Buffered Saline (PBS; 154 mmol/L NaCl and 1.4 mmol/L NaH_2PO_4/Na_2HPO_4; pH 7.5). The plates were washed (5 times) with 0.02% (v/v) Tween-20 in PBS and the residual binding sites on the plates were blocked for 30 min at RT with 150 µL/well of 2% (w/v) skim milk powder (Fluka, Zwijndrecht, The Netherlands) in PBS. After a washing step, the plates were incubated for 1 h at RT with 50 µL/well of different dilutions of the prolamin extracts with 0.1%

(v/w) Tween-20/0.2% (w/v) skim milk powder in PBS, mixed with another 50 µL/well of the biotinylated indicator peptide at a concentration of 0.002-10 µg/mL, also with 0.1% (v/w) Tween-20/0.2% (w/v) skim milk powder in PBS. After this step, the plates were washed and incubated for 30 min at RT with an excess of streptavidin-conjugated horseradish peroxidase (Sigma Aldrich, Zwijndrecht, The Netherlands) diluted with 0.2% (w/v) skim milk powder in PBS. After a washing step, bound peroxidase was visualized by incubation for 30 min, at RT and darkness, with 100 µL/well of a solution of 3,3',5,5'-tetramethylbenzidine (TMB; Sigma Aldrich). The color reaction was stopped by the addition of 100 µL/well of 2 M H_2SO_4. Finally, absorbance at 450 nm was read on a multiscan plate reader (Wallac, Turku, Finland). For quantification, the standard curve was made using a synthetic peptide containing the immuno-stimulatory celiac disease epitope, in a concentration range from 1 µg/mL to 1 ng/mL. The assays were repeated at least twice.

Direct binding assay

Direct binding assays were performed in a similar way as the competition assays for the quantitative detection of T-cell stimulatory epitopes (a20-gliadin and LMW-glutenin). Maxisorb Immunoplates (Nunc, Copenhagen, Denmark) were coated overnight at +4°C with 100 µL/well of the different peptides in a concentration range between 0.1 and 10 µg/mL in Phosphate Buffered Saline (PBS; 154 mmol/L NaCl and 1.4 mmol/L NaH_2PO_4/Na_2HPO_4; pH 7.5). The plates were washed (5 times) with 0.02% (v/v) Tween-20 in PBS and the residual binding sites on the plates were blocked for 30 min at RT with 150 µL/well of 2% (w/v) skim milk powder (Fluka, Zwijndrecht, The Netherlands) in PBS. After a washing step, the plates were incubated for 1 h at RT with 100 µL/well of the different mAbs at a concentration of 1.5 µg/mL with 0.1% (v/w) Tween-20/0.2% (w/v) skim milk powder in PBS. After this step, the plates were washed and incubated for 30 min at RT with an excess of rat-anti-mouse horseradish peroxidase conjugated polyclonal antibodies (Sigma Aldrich) diluted with 0.2% (w/v) skim milk powder in PBS.

In gel digestion and characterization of proteins by mass spectrometry

The desired gel bands, isolated from an Imperial™ (Pierce, Rockford, IL) stained gel, were digested with chymotrypsin using the Proteineer DP digestion robot (Bruker, Bremen, Germany). The protocol supplied by the manufacturer was followed. Digested proteins were analyzed by mass spectrometry as described previously (23). Searches were performed in the UniProt kB database by using FASTA alignment as described previously (23).

RESULTS

A gamma-gliadin specific T cell line differentially responds to oat samples

Previously we have shown that a gamma-gliadin specific T cell line isolated from a small intestinal biopsy of a child with CD was reactive with avenin-derived peptides (24). We now used this T cell line to test for the presence of such peptides in the selection of oat samples. For this purpose pepsin/trypsin digests were prepared and treated with tTG. As controls we included four avenin-derived peptides, two of which were shown to stimulate the T cell line (24). Subsequently these samples were tested for T cell stimulatory capacity in a T cell proliferation assay. The results demonstrate that the majority of oat samples contain epitopes that can stimulate the gamma-gliadin specific T cell line, largely similar to the stimulation by the control avenin peptides but less strong compared to stimulation with gliadin. However, several samples were found to hardly induce T cell proliferation, indicating that some varieties appear to contain a substantial lower amount of T cell stimulatory avenin peptides (Fig. 1).

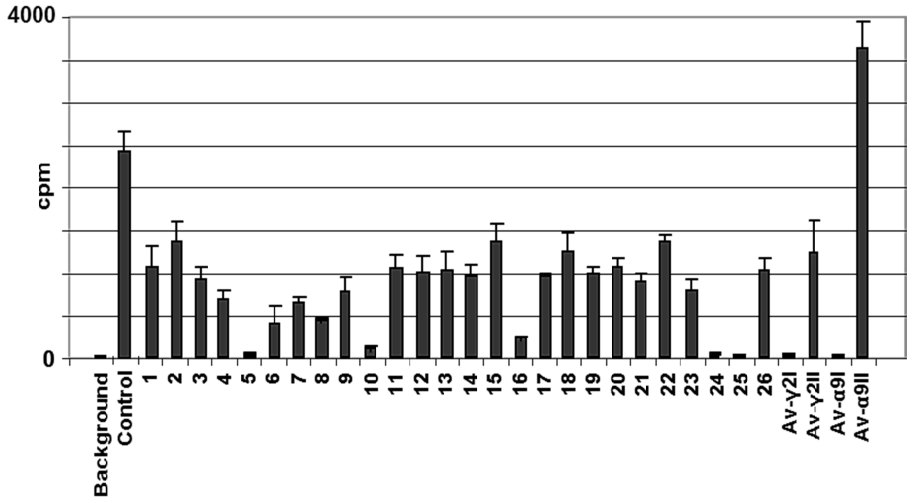

Figure 1. Proliferation assay using a gliadin specific T cell line known to recognize avenin epitopes.

Proliferation of a gamma-gliadin specific T cell line, in the presence of peptic/tryptic digests of 26 oat varieties and 4 synthetic peptides containing avenin epitopes. All samples were first treated with tTG and after measuring protein concentration identical amounts of the digests were used. The proliferative responses were measured by thymidine (^{3}H) incorporation. Background: proliferation in the absence of samples, control: proliferation in the presence of gliadin extracts, cpm: counts per minute. The experiment shown is representative of three independent experiments.

Anti-LMW-glutenin antibody cross-reacts with oat protein extracts

mAbs can be useful tools to screen for the presence of gluten or gluten-like molecules in cereals. In a previous study we have characterized the reactivity of several of such antibodies against wheat, barley, rye and oats. In addition we have generated a mAb for a LMW-glutenin derived peptide. This antibody specifically detects the sequence PFSQ, a sequence that shares homology with PFVQ which has a two-amino acid overlap with the avenin-derived T cell stimulatory peptides (24). We tested if this antibody would be useful for the detection of potentially harmful sequences in oat. First we determined if the antibody reacted with synthetic avenin peptides and observed binding of the antibody to the 4 avenin peptides tested but not to a control peptide lacking the PFVQ sequence (Fig. 2). Moreover, none of these peptides were recognized by the α20-gliadin specific mAb (results not shown). Subsequently we tested the reactivity of the antibody in a competition assay and in Western Blot analysis of the oat preparations. Strong reactivity was observed in the competition assay (results not shown) and in the Western Blot analysis (Fig. 3). To demonstrate that these detected proteins indeed correspond to the avenins, gel slices containing the detected bands were excised, treated with chymotrypsin and the resulting fragments were characterized by tandem mass spectrometry in combination with data base searches. This revealed that the detected bands contained two known avenin epitopes, both containing the PFVQ sequence: QQPFVQQQQPFVQQ and QYQPYPEQQQPFVQ. Together these results indicate that the mAb is able to detect avenin sequences that may contain an avenin epitope.

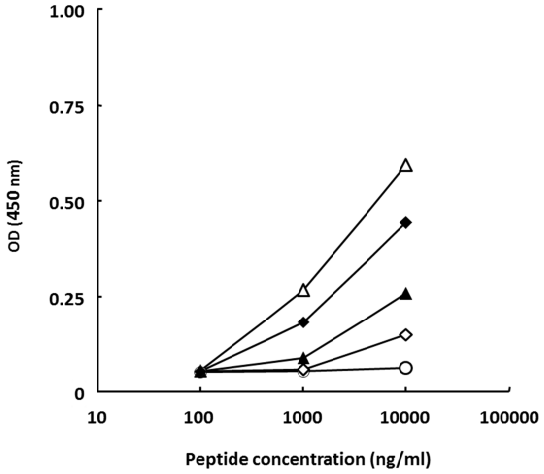

Figure 2. Reactivity of the anti-LMW-glutenin mAb against synthetic peptides containing avenin epitopes.

The reactivity of the anti-LMW-glutenin mAb against peptides possessing 4 known avenin epitopes was tested in direct binding assays. (△) Av-γ2I QQPFVQQQQPFVQ, (▲) Av-γ2II QQPFVQQQQPFVQQ, (◇) Av-α9I QYQPYPEQQEPFVQ, (◆) Av-α9II QYQPYPEQQQPFVQ and (○) control peptide QPGQGQPGYYPTSPQB. OD: optical density.

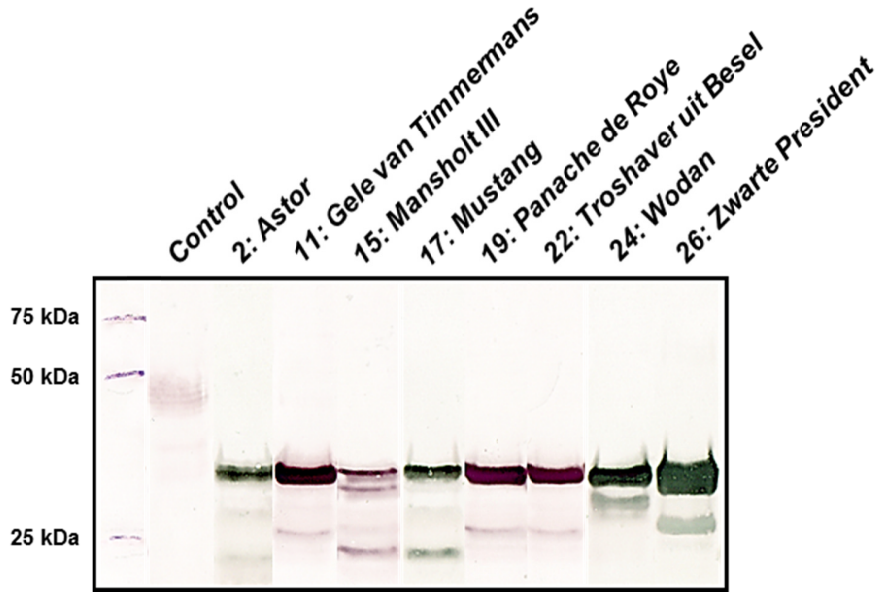

Figure 3. Western Blot analysis of 8 oat varieties.

Ethanol extracts were performed as described and same amount of proteins were separated on 1D-gel electrophoresis followed by Western Blotting and staining with an antibody recognizing the Glt-156 LMW epitope. The stained bands were isolated from the gel and digested for mass spectrometric analysis. Control: Rice baby food spiked with 1% (w/w) wheat baby food.

DISCUSSION

Since CD patients do not tolerate wheat, barley and rye, alternatives to substitute for these commonly used cereals are desirable. In this respect oat has been proven to be a good candidate, especially in the Nordic countries during the last decade (6), but the introduction of oat in the gluten-free diet, especially in those countries where oat is not commonly consumed, is still controversial as oat contains gluten-like avenins that are known to contain a few peptides that can stimulate T cells isolated from small intestinal biopsies of CD patients. Nonetheless, many reports have shown that CD patients can tolerate an oat containing gluten-free diet (6, 7, 15). There may, however, be a logical explanation for these seemingly contradictory findings: oat is phylogenetically more distantly related to wheat than barley and rye and this is reflected in several key differences between the avenins of oat and the gluten and gluten-like molecules in the other cereals. First, the amount of avenins in oat grains is substantially lower than that of gliadin in wheat (10% of the total protein content in oat, compared to 40-50% in wheat), as most storage proteins in oat are globulins. Second, avenins contain

less proline, the amino acid that contributes especially to the resistance of gluten to degradation in the gastrointestinal tract and that is crucial for the specific modification by the enzyme tTG that is linked to gluten toxicity (24). Hence, the potential of releasing stimulatory peptides is much lower. Together this means that oat contains much fewer sequences that are harmful for CD patients, and these are less abundant and more easily degraded in the gastrointestinal tract. This combination likely contributes significantly to the observed tolerance of CD patients to oat. It also fits in the "threshold model" in which a certain amount of exposure to immunogenic gluten peptides is tolerated while higher exposure leads to disease (24). Recent clinical data indicate that the addition of oat to a gluten-free diet can even result in more rapid intestinal improvement (Markku Mäki, personal communication, 2010).

A specific problem with oat is contamination (3, 5). Also in the present study did we observe that commercially grinded oat flours were mostly contaminated with other cereals (data not shown). Such contaminated oat is not considered safe for consumption by CD patients. This result underlines the importance of establishing a contamination-free oat chain for the production of suitable products for CD patients, which has been realized in Scandinavian countries and The Netherlands.

For the determination of the relative immunogenicity of the oat samples we made use of a gamma-gliadin reactive T cell line that was previously shown to also respond to two avenin peptides (24). Such gluten-reactive T cells are isolated from the small intestine of celiac patients and thus are strongly linked with the disease. Such cells can give valuable information on the immunogenicity of a sample. In a series of experiments with this T cell line we observed reproducible differences in the T cell stimulatory capacity of the oat samples, indicative of differences in immunogenicity among 26 oat varieties tested. This confirms and extends the results of Silano and collaborators, who observed differences among four oat varieties (17). A mAb originally raised against a LMW-glutenin peptide reacted with the oat preparations in Western Blot analysis, and mass spectrometric analysis demonstrated that the protein bands stained by the antibody contained, amongst others, two known immunogenic avenin peptides with sequence QQPFVQQQQPFVQQ and QYQPYPEQQQPFVQ.

The oat varieties gave different signals with the LMW-glutenin antibody and the gamma-gliadin reactive T cell line. For example, variety Wodan (#24), which showed a strong reaction against the LMW-glutenin antibody, was also found to hardly induce T cell proliferation. By contrast, variety Mansholt III (#15), which gave a faint Western Blot reaction, proved to be very active in stimulating the gamma-gliadin specific T cell line. These observations could be explained by the different recognition patterns of both systems as the LMW-glutenin antibody reacted with the four avenin peptides tested while the gamma-gliadin specific T cell line only responded to two of them. Since the T cells are isolated from patients, their reactivity pattern probably best reflects the situation *in vivo*.

In conclusion, our results show that most non-contaminated oat varieties contain avenin epitopes that are potentially harmful for a minority of the CD patient population. Similarly to the situation in wheat (16, 21, 26, 27, 29), not all oat varieties display the same immunogenic profile, suggesting that the selection and breeding of oat varieties that have a lower risk profile or have no risk at all for CD patients may be realistic.

A first step will be to clone and sequence avenin genes and cDNAs from oat varieties to analyze the presence of avenins with and without the epitopes. The T cell line will be useful to detect differences in the immunogenic potential of the oat material (flakes, flour, etc) derived from selected varieties. However, as true safety of cereals can only be ascertained when consumption by patients does not lead to clinical symptoms, further research is needed to determine the clinical relevance of our results.

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