RESEARCH ARTICLE

Significant differences in coeliac immunotoxicity of barley varieties

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Scope: The only treatment available for coeliac disease (CD) is a strict diet in which the intake of wheat, barley, rye, or oats is avoided. Barley is a major cereal crop, grown mainly for its use in brewing, and it has high nutritional value. The identification of varieties with a reduced toxicity profile may contribute to improve the diet, the quality of life and health of CD patients.
Methods and results: Searching for harmless barleys, we investigated accessions of malting and wild barley, used for developing new cultivated cereals. The CD toxicity profile of barleys was screened using G12 antibody and cell proliferation and IFN-γ release from peripheral blood mononuclear cells and intestinal biopsies from CD patients. We found a direct correlation between the reactivity with G12 and the immunogenicity of the different barleys.
Conclusion: The malting barleys were less immunogenic, with reduced levels of toxic gluten, and were possibly less harmful to CD patients. Our findings could raise the prospect of breeding barley species with low levels of harmful gluten, and the attractive goal of developing nontoxic barley cultivars, always taking into account the Codex standard for foods for special dietary use for persons intolerant to gluten.

Keywords:
Barley / Coeliac disease / Interferon-gamma / Monoclonal antibody / 33-mer

1 Introduction

Barley is an ancient cereal, which upon domestication began to evolve from a food grain to a feed and malting grain. Barley food remains important in different cultures around the world, particularly in Asia and northern Africa, and there is renewed interest worldwide in barley food because of its nutritional value [1, 2]. It is used in soups, stews, and barley bread, but its main use is for the production of beers (malt) and distilled beverages. Barley grain provides low-fat, complex carbohydrates (mainly starch) for energy, relatively well-balanced protein to meet amino acid requirements, minerals, vitamins (especially vitamin E) and other antioxidants (primarily polyphenolics), and insoluble and soluble fibre with general (rapid food passage in the colon) and specific health benefits [1].

This cereal, along with others such as wheat, rye, and oats, are not well tolerated in genetically predisposed individuals who suffer from coeliac disease (CD) [3–5]. The major CD-predisposing genes are located in the class II human histocompatibility leucocyte antigen (HLA) region, namely the HLA-DQ2 and/or DQ8 genotypes found in at least 98% of

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Abbreviations: AAEM, antiendomysial antibody; **AATG**, antitransglutaminase antibody; **CD**, coeliac disease; **CR**, cross-reactivity; **33EPs**, 33-mer equivalent epitopes; **IFN-γ**, interferon-gamma; **GF**, gluten-free; **GFD**, gluten-free diet; **IC50**, concentration of the antigen giving a 50% reduction of the peak signal in the ELISA; **HRP**, horseradish peroxidise; **moAb**, monoclonal antibody; **PBMC**, peripheral blood mononuclear cell; **RT**, room temperature; **tTG**, tissue transglutaminase

patients [6–8]. The disease is characterized by inflammatory response to ingested gluten proteins present in the cereals mentioned above. The ingestion of gluten by patients with CD leads to a cascade of inflammatory reactions, and eventually to the hallmark small intestinal lesion, whose most important consequence is a decrease in nutrient absorption [9], characterized by CD4+T cells activation, increase number of intraepithelial lymphocytes with partial to total villus atrophy [10].

A common feature of gluten-derived epitopes is the presence of multiple proline and glutamine residues. The high content of proline residues makes the gluten peptides resistant to degradation by digestive proteases. At the same time, glutamine residues in these peptides are selectively deamidated by tissue transglutaminase (tTG). The modified peptides are able to bind to HLA-DQ2/DQ8, which stimulate CD4+ T-helper 1 (Th1) cells in the lamina propria. These T cells become activated upon recognition of gluten peptides and produce many different cytokines, of which interferon-gamma (IFN-γ) predominates, resulting in an inflammatory response in the small intestine that leads in flattening of the mucosa [3, 9, 11–13]. tTG is the target of an autoimmune humoral response to endomysium. It results in the production of intestinal and circulating antibodies predominantly of the immunoglobulin (Ig) A isotype. The enzymatic deamidation by tTG of specific glutamine peptides makes deamidated gluten peptides more antigenic than native gluten peptides.

The 33-mer peptide derived from α-2 gliadin is one of the most highly antigenic peptides identified to date [14, 15]. This peptide was identified as the primary initiator of the inflammatory response to gluten in CD patients, being resistant to breakdown by all gastric, pancreatic, and intestinal brush border membrane endoproteases [14]. Sequence alignment showed that homologues of the 33-mer peptide are found in wheat gliadins, barley hordeins, and rye secalins, all of which are toxic cereals in the CD diet.

The only treatment available to CD patients is a strict diet free of gluten from wheat, barley, rye, and some varieties of oats. However, this diet is very complex to follow as gluten is a ubiquitous additive in most sectors of the prepared-food industry. For this reason, dietary transgressions are relatively frequent among CD patients, who there remain exposed to a high morbidity risk. At the same time, a gluten-free diet (GFD) may be detrimental to gut health as it leads to reductions in beneficial gut bacteria populations and the ability of faecal residues to stimulate the host's immunity [16]. As a result, novel strategies are being proposed to find new therapies or to reduce toxic gluten epitopes from cereal grains [9, 17–21]. An alternative possibility, investigated in this work is the identification of new varieties of cereals with a reduced immunotoxicity profile, which may contribute to improving the quality and variety of food for the coeliac diet. In oats, immunological methods revealed that there may be some varieties with no known apparent immunotoxicity for coeliac patients [5]. Currently, it is unknown whether all barleys are equally harmful for CD patients. Comparison between cultivated and wild barley varieties will identify new sources of variation in relation to CD. In contrast to the genetic complexity of bread-wheat and oats, which have three genomes, barley is diploid ($2n = 14$), with a high degree of inbreeding. Moreover, barley is easy for cross-breeding and can be cultivated in a wide range of climatic conditions [22]. Recently, about 86% of the barley genes have been assigned to individual chromosome arms using a novel approach that incorporated chromosome sorting, next-generation sequencing, array hybridization, and systematic exploitation of conserved synteny with model grasses [23]. These features make barley (*Hordeum vulgare* L.) a system much simpler than wheat for genetic studies [24].

Recent reports on CD recommend updating the concept of "gluten detection" to "potential relative immunotoxicity of gluten" for the safety of coeliac patients. In earlier works, two moAbs (G12 and A1) against the main immunogenic epitope of the α-2 gliadin, 33-mer peptide, were obtained [25, 26]. These antibodies presented a high specificity with a high degree of sensitivity, precision, and reproducibility against prolamines of cereals toxic to coeliac patients. Our results suggested that the reactivity of these moAbs was correlated with the potential immunotoxicity of the dietary grains from which the proteins were extracted [25, 27]. Studies carried out with different oat varieties proved that the reactivity of these antibodies with cereal storage proteins of different cultivars of oats was correlated with the immunotoxicity of the dietary grains [5]. In the present study, we have extended our previous work, assessing the affinity of the anti-gliadin 33-mer G12 moAb against different lines of barley to know the potential toxicity of each one. We found that barley immunotoxicity was associated with the barley variety used. Marked differences in IFN-γ release were found when PBMCs and intestinal biopsy culture were exposed to the different barley varieties. Interestingly, we showed low levels of 33-mer equivalent epitopes (33EPs) in certain cultivars of malting barley. The incorporation of barley with low CD toxicity could extend the variety of foods and beverages that can be tolerated by CD patients. Results reported here may enable the selection of barley varieties with low toxicity levels for foods and breeding/malting programs.

2 Materials and methods

2.1 Plant material

Plants used in this study were from the germplasm collection of the Instituto de Agricultura Sostenible (CSIC, Córdoba, Spain), and included *H. chilense* accessions H1, H16, H57, H75, H208, and H303 ($2n = 2x = 14$; $H^{ch}H^{ch}$), *H. vulgare* varieties H106 and GP ($2n = 2x = 14$; HH). Rice (*Oryza sativa* subsp. *Japonica*, J. Sendra variety), durum wheat (*Triticum durum*, Don Pedro variety), and commercial gliadin (Sigma, St. Louis, Missouri, USA) were used as controls.

2.2 33-mer peptide and anti-33-mer moAb

The 33-mer peptide LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF was supplied by Biomedal S.L. (Sevilla, Spain). G12 moAb and its derived horseradish peroxidase (HRP)-conjugated moAb (G12-HRP) were used in this study. Both proteins were provided by Biomedal S.L. (Sevilla, Spain). G12 and G12-HRP moAbs concentrations are the same as reported previously [25].

2.3 DNA extractions and PCR amplification

Extraction of DNA and amplification by PCR were carried out according to Comino et al. [5]. Oligonucleotides used in this work were provided by Biomedal S.L. (Sevilla, Spain) and are listed in Supporting Information Table S1.

2.4 Extraction of prolamines from cereal samples

The steps in the process for extraction of the cereal prolamines were the following: the grains were ground in a mortar and the flour was mixed in 60% (v/v) ethanol and stirred for 1 h at room temperature (RT). The suspension was centrifuged at 13 000 × *g* for 10 min, and the supernatant was recovered. The concentration of proteins was measured by the Bradford method [28]. Commercial gliadin was prepared in 60% (v/v) ethanol at a concentration of 1 mg/mL.

2.5 MALDI-TOF MS analysis

MALDI-TOF MS analysis was performed as reported previously by Hernando et al. [29]. To 5 μL of the prolamine extracts obtained after treatment with 60% (v/v) ethanol (detailed in Section 2.4) were added 2 μL 50 mM octyl-β-D-glucopyranoside detergent and 25 μL of saturated sinapinic acid in 30% (v/v) aqueous acetonitrile containing 0.1% (v/v) TFA used as a matrix solution. The matrix-sample mixture was dried in a Speed-Vac centrifuge (15 min) and the residue was dissolved in 6 μL of 60% aqueous ethanol containing 0.1% TFA. A 2-μL volume of sample-matrix mixture was placed on a 100-sample stainless-steel probe and dried at RT for 5 min.

Samples were measured on a PE Biosystems MALDI-TOF Voyager DE-PRO instrument in the standard configuration. Mass spectra were recorded in the linear positive mode at an acceleration voltage of 25 kV with a grid voltage of 93%, 0.25% guide wire, and 700 ns delay time by accumulating 200 spectra of single laser shots under threshold irradiance. The equipment was externally calibrated using singly and doubly charged signals of BSA with a molecular mass of 66.43 kDa and 33.21 kDa, respectively.

2.6 Western blotting

Two micrograms of the protein obtained after 60% ethanol treatment of the barley samples was diluted 1:1 (v/v) in SDS-PAGE denaturing buffer (containing 2-mercaptoethanol) and denatured by boiling at 100°C for three times (5 min each). SDS-PAGE was prepared with 12.5% acrylamide, and the gels were run. Proteins in the gel were transferred to a polyvinylidene fluoride membrane. The membranes were incubated overnight in 20 mL of blocking buffer (TBS buffer supplemented with 5% nonfat dry milk) and then exposed to G12 moAb. After washing, anti-mouse IgG-phosphatase antibody (Sigma) was added.

2.7 Competitive ELISA

Maxisorp microtitre plates (Nunc, Roskilde, Denmark) were coated with gliadin solution (5 μg/mL, initially prepared in 60% etanol, detailed in Section 2.4) in 0.1 M of Na_2CO_3-$NaHCO_3$ (pH 9.6) and incubated overnight at 4°C. The plates were washed with PBS-Tween 20 buffer and blocked with blocking solution (PBS-5% nonfat dry milk) for 1 h at RT. Serial dilutions were made of gliadin and/or 33-mer peptide, and prolamines extracted from the grains of barley and rice in PBS-BSA 3%, to each of which was added G12-HRP antibody solution. The samples were preincubated for 2 h at RT with gentle stirring, and then added to the wells. After 30 min of incubation at RT, the plates were washed, and substrate solution (TMB, Sigma) was added. After 30 min of incubation at RT in the dark, the reaction was stopped with 1 M sulphuric acid, and the absorbance at 450 nm was measured (microplate reader UVM340, Asys Hitech GmbH, Eugendorf, Austria).

2.8 Peptic-trypsic-chymotrypsin digestion and deamidation

Prolamines were subjected to peptic, trypsic, and chymotrypsin sequential digestion according to Comino et al. [30]. Samples were incubated at 37°C for 60 min in HCl solution (pH 2.0) that contained 0.60 mg pepsin/mL (Sigma) [31]. These digests were adjusted to pH 6.0 with sodium phosphate buffer and subjected to a 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. Samples were heat-treated at 100°C for at least 5 min to inactivate the enzymes. Prolamine digests were freeze-dried, lyophilized, and stored at −20°C until used.

Deamidation with guinea-pig liver tTG (Sigma) was performed in the presence of 2 mM $CaCl_2$, at 37°C for 4 h [32,33].

2.9 Patients

The study was approved by the ethics committee of the "Virgen de las Nieves" Hospital, Granada (Spain), and informed

Table 1. Coeliac patients' characteristics

Case	Sex	Age	Duodenal histology	AAEM	AATG	HLA-DQ[a)]	HLA-DR[a)]
1	F	9	Marsh 3b	+	139	0201–0202	3–7
2	F	5	Marsh 3b	+	102	0201–0604	3–11
3	M	13	Marsh 3b	−	2	0202–0301	7–11
4	F	3	Marsh 3a	+	20	0201–0202	3–7
5	M	6	Marsh 3b	+	252	0201–0202	3–7
6	F	2	Marsh 3b	+	15	0302–0301	4–4
7	F	4	Marsh 3b	+	28	0201–0501	1–3
8	F	7	Marsh 2	+	111	0201–0202	3–7
9	M	5	Marsh 3b	+	118	0201–0202	3-7
10	M	10	Marsh 3b	+	165	0202–0301	7-11

AAEM, antiendomysial antibody; AATG, antitransglutaminase antibody expressed as U/mL; HLA, human leucocyte antigen.
a) CD is associated with specific HLA class II alleles—that is, DQA1*0501/DQB1*0201 (DQ2) present on a haplotype with DRB1*03 or associated with DRB1*05/07 and DQA1*0301/DQB1*0302 (DQ8) present on a haplotype with DRB1*04 [51, 52].

consent was obtained. Ten coeliac patients (six females/four males, 2–13 years old) with active disease were included in this study (Table 1). Five healthy patients were considered as the control group (three females/two males, 6–12 years old).

2.10 Serological and histological analysis

The diagnosis of CD was established in the patients by serological screening tests accompanied by biopsy of the small intestine and confirmation of a clinical response to gluten elimination from the diet. Subjects were prospectively screened for CD using anti-endomysial antibodies (AAEMs), anti-gliadin antibodies, tissue transglutaminase antibodies, and CD-specific HLA typing (HLA-DQ). Biopsy specimens of small intestine were obtained by gastrointestinal endoscopy. The small-intestine biopsy specimens were classified according to the criteria of Marsh [34] (Table 1). Venous blood was taken at the time of the index biopsy. The healthy control patients were serologically negative for AAEMs, anti-gliadin antibodies, and antitransglutaminase antibodies (AATGs). Similarly, at the time of collection of the samples to establish the diagnosis of CD, peripheral blood, and small intestinal mucosa biopsy were also collected for this study.

2.11 Cell proliferation analysis and IFN-γ production

PBMCs from patients with active CD ($n = 10$) on gluten-containing diet were isolated from 6 mL heparinized blood by Histopaque gradient centrifugation, and cultured at a density of 1×10^6 cells/mL in RPMI-1640 culture medium. The cells were cultured (37°C, 5% CO_2) on 6-well plates. After 48 h, PBMCs were incubated with prolamine digests (reconstituted in 60% ethanol and incubated up 50 μg/mL in culture medium). All cultures were performed in duplicate for each antigen concentration. Cell proliferation was determined after 48 h of incubation using the ELISA 5-bromo-2-deoxyuridine cell proliferation test (Millipore Chemicon, Temecula, California, USA). The stimulation index (SI) value was calculated by dividing (the mean absorbance of duplicate assays for each prolamin digest for 10 subjects at 450 nm after stimulation)/10 by (the mean absorbance of cells exposed to the culture medium alone (negative control))/10. The proliferation of PBMCs exposed to the different prolamine digests was expressed as the mean fluorescent intensity 48 h after exposure.

Supernatants from PBMC cultures were collected and stored at −80°C for IFN-γ determination using a commercial ELISA kit (Thermo Scientific, Madrid, Spain) in accordance with the manufacturer's instructions. Standards were run on each plate. Assay sensitivity was less than 2 pg/mL.

2.12 Small-intestinal mucosal biopsy organ culture

The culturing of small-intestine mucosal biopsies derived ex vivo from CD patients was originally introduced by Browning and Trier [35]. Briefly, biopsies (ten coeliac biopsies and five control biopsies) are positioned villous side up on a nylon mesh gauze (Millipore Chemicon) and placed in the central well of a 2-mL tissue culture dish. The biopsies can be kept viable in the organ culture system for 48 h to guarantee the quality of mucosal morphology after a culture. The prolamine digests are added to the culture supernatant, and the incubation continues for 24 h.

2.13 Statistical analysis

2.13.1 Anti-gliadin 33-mer ELISA

Two separate assays were performed, each with three repetitions. Prolamine/33-mer peptide curves were obtained by plotting percentage of maximum absorbance against logarithm of antigen concentration. The software package Sigma Plot 9.0 (Systat Software, Inc., Point Richmond, CA, USA) was used to calculate the IC50. The IC50 is defined as the

concentration of the line that reduces the peak absorbance by 50% in the assay. The cross-reactivity (CR) was calculated as follows: (IC50 of the antigen for which the moAb was raised/IC50 of each antigen assayed) × 100.

2.13.2 Cell proliferation and IFN-γ assays

Each experiment was carried out in duplicate on separate days. Data are expressed as mean ± SD. All statistical analysis was performed with the STATGRAPHICS program. The differences between groups were examined by one-factor analysis of variance. When it was statistically significant, secondary Bonferroni-corrected *t*-tests were applied among groups. A statistical probability of $p < 0.05$ was considered significant.

3 Results and discussion

3.1 Patterns of prolamines of cultivated and wild barley varieties

The genus *Hordeum* contains several species, of which *H. vulgare* (HH) is the most widely used in the food industry. *H. chilense* ($H^{ch}H^{ch}$) is a wild relative of *H. vulgare*, used in the generation of a new cereal crop, named tritordeum, by crossing with durum wheat, *T. turgidum* (AABB) [36]. This new crop exhibits traits of interest for bread making and the malting food industry [37]. In this work, two accessions of malting barley (*H. vulgare)* and six accessions of the wild barley *H. chilense* were studied in order to assess the natural variation of these barley varieties in immunotoxicity for CD patients.

To determine the purity of the barley samples, a visual examination of each barley grain sample and a molecular analysis by PCR were carried out to identify a potential amplifiable DNA of oats, rye, and wheat in barley samples, as described by Comino et al. [5]. In barley samples, positive results were obtained when the barley-specific primers were used. However, no amplified DNA products were obtained with the PCR specific for oats, rye, and wheat in these samples. In the present experiment rice and wheat samples, and their specific primers, were used as controls. The results indicated the purity of the barley samples used in this work, as no DNA amplifications of oats, rye, or wheat, or mixtures of these cereals, were found in the barley samples (Fig. 1).

The MALDI-TOF MS technology is useful to identify a great number of prolamine subunits of wheat and other cereals [38, 39]. Here, we used it to analyze the hordeins contained in barley, as well as to identify and characterize the barley varieties (see an example of the spectra of the prolamine fractions of *H. vulgare* cv H106 and GP, as well as *H. chilense* cv H303 and H1 in Fig. 2). The hordein spectra revealed major differences between barley species. The number of hordein peaks was higher in the wild barley than in the malting varieties. In addition, the range of molecular weights of prolamines in

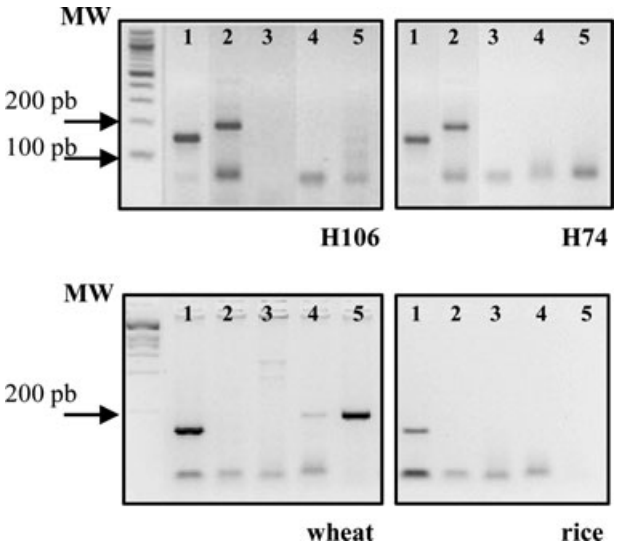


Figure 1. Determination of the presence of DNA in varieties of barley, rice, and wheat by PCR. An ethidium-bromide-stained agarose gel with barley, oat, rye and wheat PCR amplification products of two representative barley samples, H106 and H74 (cultivated and wild-type seeds, respectively), and other cereals (rice, and wheat). MW: DNA molecular weight marker (100-bp ladder). Positive controls: DNA of these cereals amplified with 18S primers. The corresponding primers used were the following: 1. 18 S, 2. ω-hordein (barley), 3. ω-avenin (oats), 4. ω-secalin (rye), and 5. ω-gliadin (wheat). A representative example from three biological replicates performed is shown.

the wild lines was wider (27–49 kDa) than in the cultivated varieties (31–44 kDa). However, the majority of the *H. chilense* hordeins had smaller molecular masses, ranging between 27 kDa and 33 kDa, in accord with previous observations that hordein sequences from *H. chilense* were shorter than those of *H. vulgare* [40]. The gliadins of wheat, and their homologues in barley, were characterized by the presence of a central domain, in which a short peptide was repeated a certain number of times, determining the final length of the protein. Although prolamines from *H. vulgare* and *H. chilense* shared the same repetitive motif [40, 41], the number of repetitions was lower in *H. chilense* than in *H. vulgare*, so that most of the hordeins from *H. chilense* were shorter than those from *H. vulgare* [40, 42]. These differences in the spectra could be related to functional properties or toxicity of barley lines with regard to CD.

3.2 Immunological quantitative assessment for toxic gluten detection in barley hordeins by anti-gliadin 33-mer moAb

The reactivity of the G12 moAb against the different barley lines was determined by competitive ELISA and Western blot analysis. In order to quantify the affinity, the IC50 and the CR were determined for each line. Barley lines assayed showed different affinity for the G12 moAb, which may correlate with

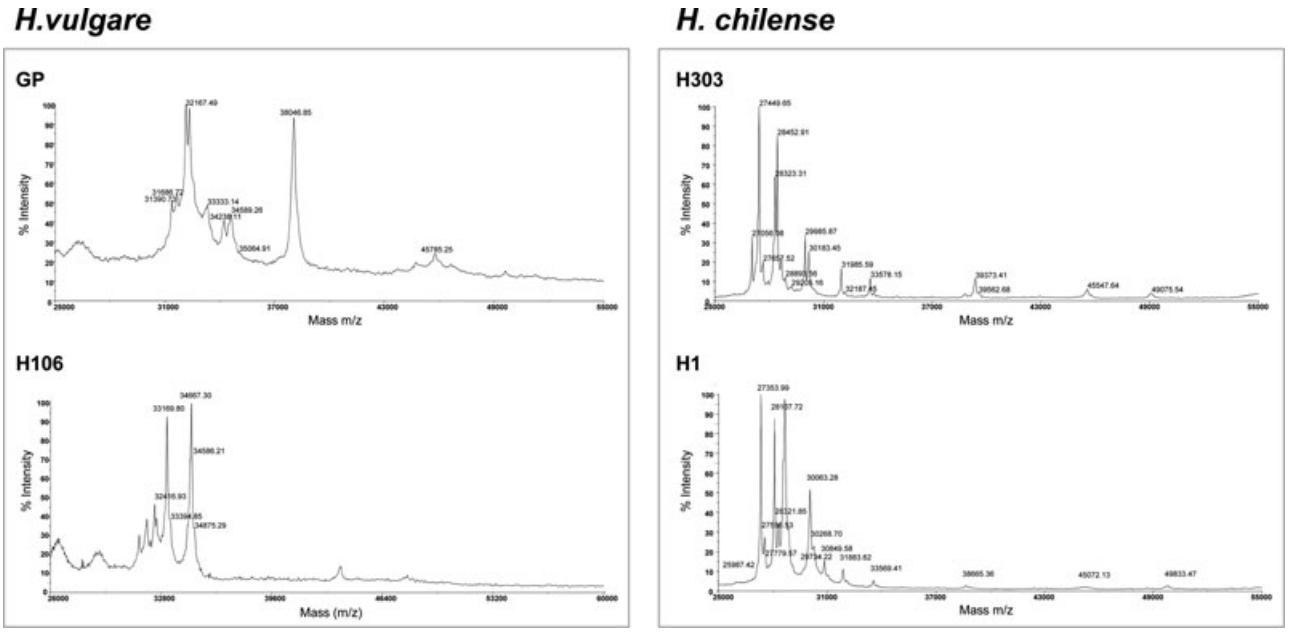


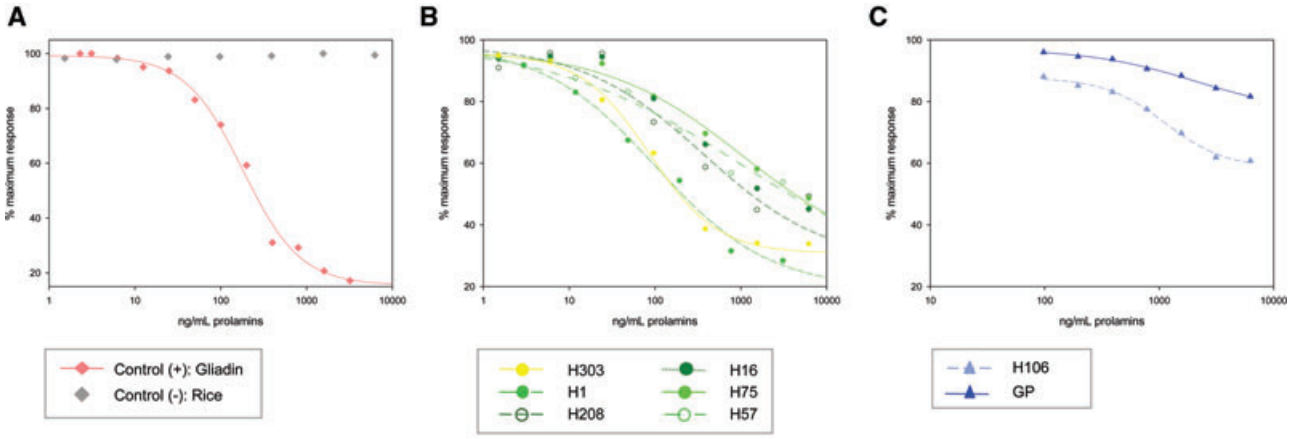
Figure 2. Hordein pattern of wild and malting barley accessions MALDI-TOF mass spectra of *H. chilense* (lines H303 and H1) and *H. vulgare* (lines GP and H106).

their potential toxicity for coeliac patients. We found a group formed by the wild varieties H303, H1, H208, H16, H75, and H57 for which the antibody presented a high affinity (Fig. 3A, B, and D). Another group, which included cultivated H106 and GP varieties, showed a sharp decrease in the sensitivity of the anti-33-mer G12 (Fig. 3C and D). The gluten of line H303 presented the greatest reactivity for G12 antibody, which might suggest that this line was potentially the most immunotoxic for coeliac patients. H1 also showed a high reactivity for the anti-33-mer antibody (Fig. 3B and D), with a CR of 83.4%. Both H1 and H303 lines showed an IC50 lower than that of commercial gliadin (IC50 181.99 ng/mL). H208 and H16, with a cross-reactivity of 23.3 and 23.1%, respectively, were recognized by the G12 moAb, but with a lower sensitivity. Although lines H75 and H57 were recognized by the antibody, the affinity decreased, with a lower cross-reactivity (about 8%). In the case of the lines H106 and GP, the affinity of the G12 antibody decreased markedly, such that the IC50 values were 13-fold and 25-fold higher than that of the most reactive wild line H303. Therefore, the GP and H106 barley lines are possibly less harmful to coeliac patients than the aforementioned ones. These results were confirmed by G12 moAb immunoblotting with the representative lines H1, H208, H16, and GP (Fig. 3E). H1 was the most reactive, followed by H208 and H16. However, the reactivity and the bands obtained were much lower for GP. The Western assay was consistent with those previously obtained by anti-gliadin 33-mer ELISA. These results showed a large difference between lines of *H. vulgare* and *H. chilense*, with the lines of wild barley being the most reactive. Moreover, differences in the potential immunotoxicity were found between lines of the same barley species.

Historically, immunological assays had shown that there was a large variation in the wheat accessions [43]. Diploid and tetraploid-hulled wheat species *T. monococcum* and *T. turgidum* subsp. *dicoccum*, respectively had been found to be low in noxious prolamines [44], but nothing was known about the toxic gluten variations in other cereals such as barley. We found a relationship between the species of barley used and its CD immunotoxicity. Lines from *H. chilense* generally showed higher immunoreactivity than lines from *H. vulgare*. In addition, PCR showed that the immunotoxic differences were not due to possible contamination with other toxic cereals. Our findings suggested that the contrast in immunotoxic potential exhibited between the lines of *H. chilense* and *H. vulgare* was due to variation in their storage protein compositions.

3.3 Determining the immunotoxic potential of different lines of barley by evaluation of immunogenic epitopes, cell proliferation, and IFN-γ release

A 33-amino-acid peptide from wheat α-2 gliadin (residues 57-89) was the immunodominant antigen for CD. This 33-mer fragment is naturally formed by digestion with gastric and intestinal proteases, it binds to DQ2 after deamidation by tTG, and it contains a cluster of 6 T-cell epitopes [15]. On the basis of these observations, we assumed that the potential relative immunotoxicity of gluten correlated to the effective concentration of 33EPs. To test the concentration of 33EPs present in the different barley accessions, one sample was chosen from each of the groups previously identified: H303, as the



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Barley lines	IC50 (ng/mL)	CR (%)
H303	85.40	100
H1	102.35	83.44
Gliadin	181.99	46.93
H208	366.71	23.29
H16	369.84	23.09
H75	1050.96	8.13
H57	1062.85	8.03
H106	1122.13	7.61
GP	2150.58	3.97
Rice	N.A.	N.A.

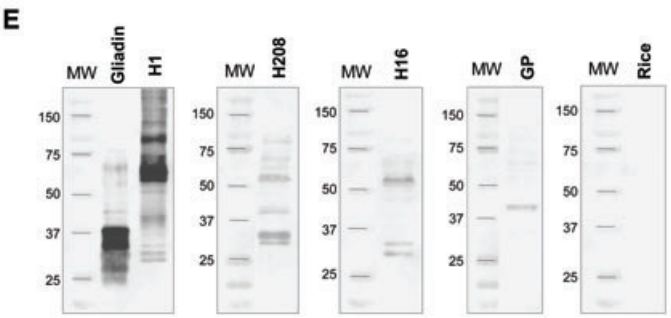

Figure 3. Relative affinity of the anti-gliadin 33-mer moAb for different barley lines, gliadin, and rice. (A, B, C, and D) Competitive ELISA using G12-HRP antibody to determine the relative affinity of this antibody for the different lines of barley. Two assays were performed, with three replicates of each. Gliadin and rice were used as positive and negative control, respectively. (E) Western blot analysis of toxic fractions of gliadin and different prolamines extracted from the grains of barley and rice. Analysis was carried out with 2 μg of prolamine. Membranes were immunologically analysed using moAb G12. MW, molecular weight (kDa). IC50 is defined as the concentration of the line that reduces the peak absorbance by 50% in the assay. The cross-reactivity (CR) was calculated as follows: (IC50 of the antigen for which the moAb was raised/IC50 of each antigen assayed) × 100.

most reactive variety; and GP and H106, as less toxic lines. These accessions were subjected to digestion with gastric and pancreatic enzymes (see Materials and Methods) and tested by the quantitative ELISA method using a standard curve of gliadin 33-mer (Fig. 4A). All the barley lines showed detectable levels of 33EPs. However, in the case of H303 *H. chilense*, the 33EP concentration was 5148.1 ng/mL, it was about tenfold more reactive than the GP and H106 accessions. The study of native and digested prolamines did not reflect significant differences in the 33EP levels for the same barley line.

Stimulatory activity of the different barley lines was assessed by cell proliferation and IFN-γ response from peripheral blood cells isolated from patients with biopsy-proven active CD (see Table 1). The ability of hordein from different barley varieties (H303, GP, and H106) to induce a noxious immune response was studied in comparison with wheat gliadin as positive control and rice prolamine as negative control. Samples were subjected to peptic, trypsic, and chymotrypsin sequential digestion and to deamidation with tTG. As expected, PBMCs from healthy subjects failed to proliferate after exposure to prolamine digests from the different barleys. However, the results of cell proliferation from coeliac PBMCs clearly showed that H303 induced a strong proliferative response (SI = 2.2 ± 0.4), even higher than that of the positive control, gliadin (SI = 1.65 ± 0.3). We found a significant decrease in cell proliferation in the cultures incubated with the cultivated varieties H106 and GP (1.35 ± 0.2 and 1.1 ± 0.2, respectively; $p < 0.05$ with respect to H303).

All barley accessions stimulated the secretion of the proinflammatory T-cell specific cytokine, IFN-γ, but with large differences between them (Fig. 4B and C). The highest immunostimulatory capacity was found in H303 lines of *H. chilense* (18.5 ± 1.5 pg/mL), with values higher even than for the positive control, wheat gliadin (13.8 ± 1.4 pg/mL). The exposure with H106 and GP resulted in a lower IFN-γ response (9.2 ± 1.1 pg/mL and 7.3 ± 1.1 pg/mL, respectively) with respect to H303 ($p < 0.001$). These results clearly showed that

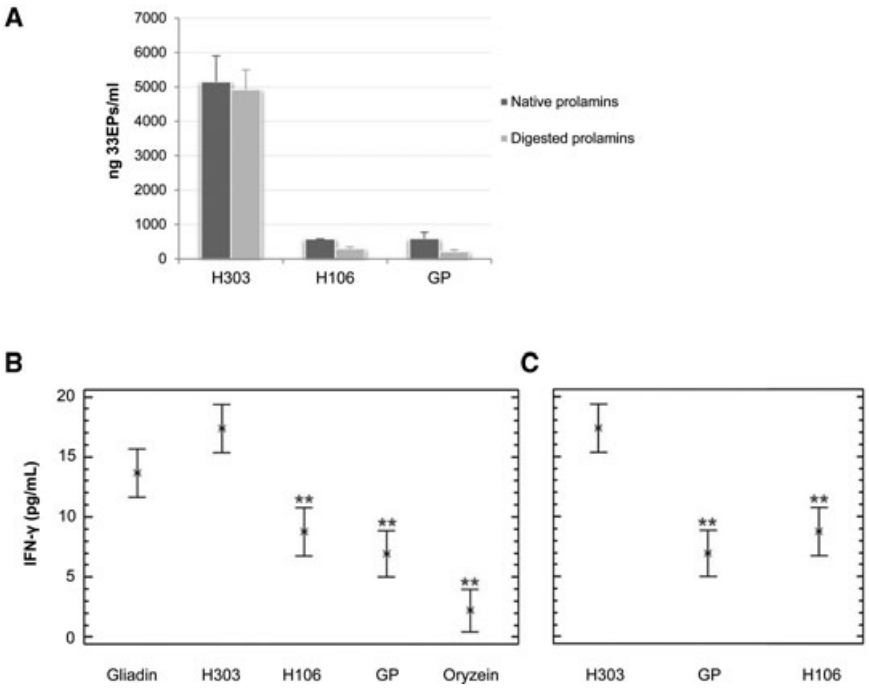

Figure 4. Immunogenic pattern of different barley accessions for coeliac patients. (A) Concentration of 33EPs (ng/mL) in barley varieties by G12-HRP moAb competitive ELISA. The concentration of 33-mer peptide was determined by comparison with a synthetic 33-mer standard curve. The results are shown as mean ± SD of triplicate assays. (B and C) IFN-γ release by PBMCs with prolamine digests from barley lines. The results are expressed as mean ± SD of duplicated cultures (n = 10). Gliadin and rice prolamines were used as the positive and negative control, respectively. Significance with respect to (B) gliadin and (C) H303. $^{**}p < 0.005$.

H303 displayed the highest activity, and was potentially the most immunogenic.

In CD, the histological lesion is associated with a predominant Th1 cell response. Lamina propria T lymphocytes isolated from the intestine of patients with active CD released IFN-γ in response to gluten stimulation, and neutralisation of endogenous IFN-γ prevented the gliadin-mediated morphological changes in an ex vivo organ culture of treated CD biopsies [45, 46]. Consequently, the ability of digests from the barley varieties to induce IFN-γ production was tested in intestinal mucosal lymphocytes derived from patients with untreated coeliac disease and from normal donors. We studied a wild variety, H303, and a cultivated variety, H106. IFN-γ release from mucosal lymphocytes was measured after the exposure of coeliac and control biopsies to barley peptides. Increased levels of IFN-γ were found in the culture supernatants (Fig. 5). After exposure to prolamine digests from both wild and malting barleys, mucosal lymphocytes from normal donors failed to proliferate. The differences in PBMC response observed between the barley varieties were paralleled by similar differences in IFN-γ production from mucosal biopsy. Mucosal lymphocytes exposed to the prolamine digests of variety H303 led to IFN-γ release as high as 21 ± 1.4 pg/mL, whereas those exposed to the prolamine digests from H106 exhibited a decrease in the levels of IFN-γ (13 ± 1.4 pg/mL).

Cell proliferation and IFN-γ release in the culture medium were measured as indices of immune activation. Our data showed that H303 displayed in vitro activities related to CD pathogenesis. These results suggest that varieties belonging to barley species can differ from each other in the amount of CD-stimulatory epitopes in their prolamine sequences.

Our findings confirmed that there are considerable differences between barley varieties. *H. chilense* H303 was highly enriched in 33EPs and showed the greatest pathogenic cell response by measurement of cell proliferation and IFN-γ

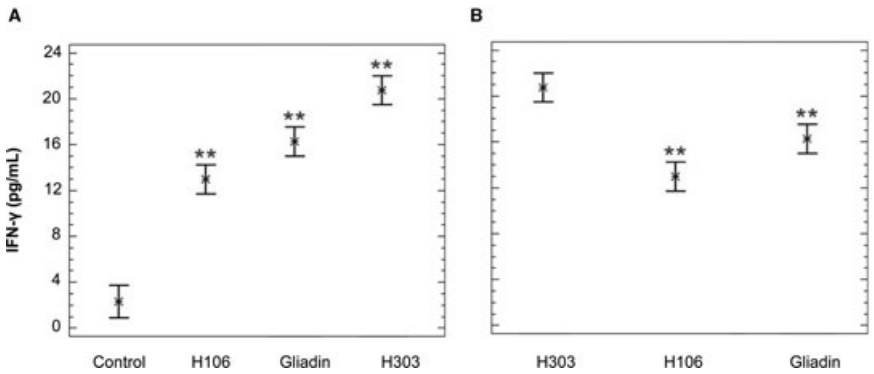

Figure 5. IFN-γ production by small intestine biopsy organ culture from different barley varieties. The results are expressed as mean ± SD of duplicated cultures (n = 10). (A) Significant with respect to healthy controls, (B) significant with respect to H303. $^{**}p < 0.005$.

release (above that of the positive control, gliadin). The detection of 33EP levels and cell immunological activation confirmed those previously obtained by anti-gliadin 33-mer assays. As we demonstrated in a previous work [20], a direct correlation of the reactivity with G12 moAb and the immunogenicity of the prolamines was observed by measuring cell proliferation and IFN-γ release.

CD immunotoxicity studies have focused on wheat gluten [47, 48]; however, little is known about the effect of other toxic cereals such as barley [49]. Moreover, importance has been given to wheat gluten studies because wheat is the most-frequently consumed cereal in Western diets. However, barley use remains important in Asia and northern Africa, and there is renewed interest worldwide in barley food because of its nutritional value [1]. The USA Food and Drug Administration recently issued a health benefit endorsement for barley, based on the effects of β-glucan in lowering blood cholesterol (http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/2005/ucm108543.htm). The high content in fibre and/or other components also has a satiety effect, which can positively affect weight control, as well as speed up the passage of food in the colon.

The identification of barley varieties with a reduced toxicity profile may contribute to enriching a GFD and improving the nutritional quality and health of CD patients. In this work, we examined in vitro analysis that may enable identification, selection, or production of malting barleys with low levels of noxious gluten proteins and which can be used for the production of food that is GF or with low gluten. In particular, these findings could also be useful in the beer industry: some brewing products that have low gluten content could be favoured as raw material for the safety of final products. Some authors have demonstrated that, after the filtration process, beer is colloidally stabilized by removal of proteins, resulting in <0.11% of the original content of gluten [50]. Therefore, barley with low levels of harmful gluten could lead to beers with gluten levels suitable for coeliac patients.

These results showed that there was variability in the immunogenicity of the different barleys. Hence, it might be possible to get barley varieties with low prolamine content that could render processed products below the thresholds acknowledged as harmful for CD patients (Commission Regulation (EC) No 41/2009 (http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:016:0003:0005:EN:PDF). The incorporation of wild germplasm in breeding programs is a common practice to broaden the genetic base of cultivated species. However, caution must be exercised not to increase the toxicity of cultivated varieties, as—in the case of barley—the wild varieties seem to be more toxic than the cultivated. Barley varieties with decreased immunotoxicity described in this work might be included in breeding programs aimed at developing barley varieties suitable for CD patients. Moreover, the immunological assays described could be used for the development of high throughput tools to evaluate the toxicity of storage proteins and thereby accelerate the selection process in the breeding programs.

4 Concluding remarks

This is the first time that a comparison has been made of the differences in levels of immunotoxicity for CD patients between cultivated and wild barley varieties. In the present study, we have shown that cultivated barley has lower levels of toxic gluten. Our findings could raise the prospect of breeding barley species with low levels of harmful gluten, and the attractive goal of developing low-toxic barley cultivars. This work proposes the basis for the development of new barley varieties useful for the preparation of food with reduced levels of gluten. It could be useful as a starting point for developing new varieties by crosses with those that present less immunotoxicity.

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