Immunogenicity of monococcum wheat in celiac patients

Carmen Gianfrani, Mariatonia Maglio, Vera Rotondi Aufiero, Alessandra Camarca, Immacolata Voecca, Gaetano Iaquinotto, Nicola Giardullo, Norberto Pogna, Riccardo Troncone, Salvatore Auricchio, and Giuseppe Mazzarella

ABSTRACT

Background: Research is intense to find wheat of low or null toxicity for patients with celiac disease (CD). Among candidates, there are diploid wheat species.

Objective: We compared the immunological properties of 2 lines of diploid monococcum wheat (Triticum monococcum ssp. monococ- cum), Monlis and ID331, with those of common wheat (Triticum aestivum).

Design: Interferon-γ production and the proliferation of intestinal gliadin-specific T cells and clones were measured as evidence of T cell activation by peptic and tryptic (PT) digests of gliadins from 2 monococcum lines. Furthermore, organ cultures of jejunal biopsies from 28 CD patients were set up to assess the effects of PT gliadin on innate and adaptive immune response by using immunohistochemistry.

Results: Monlis and ID331 induced interferon-γ production and proliferation in celiac mucosal T cells. In organ cultures, Monlis PT digest induced a significant increase of IL-15 epithelial expression and crypt enterocyte proliferation, whereas ID331 had no effect. Both monococcum lines caused intraepithelial T cell infiltration and lamina propria T cell activation.

Conclusions: Our data show that the monococcum lines Monlis and ID331 activate the CD T cell response and suggest that these lines are toxic for celiac patients. However, ID331 is likely to be less effective in inducing CD because of its inability to activate the innate immune pathways. Am J Clin Nutr 2012;96:1339–45.

INTRODUCTION

Celiac disease (CD)

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INTRODUCTION

Celiac disease (CD) is an immune-mediated disease that is triggered by the ingestion of wheat gliadins and related prolamin proteins from other toxic cereals, such as barley and rye. An immune response against these cereal-derived proteins is mediated by the innate and adaptive immune branches. The adaptive response starts when the gluten peptides are presented by the human leukocyte antigen (HLA)-DQ2/8 molecules of specialized antigen-presenting cells to CD4+ T cells (1) or by HLA class I molecules to CD8+ T cells (2). More specifically, several gluten peptides were identified to activate proinflammatory T cells that release interferon-γ (3), which is a dominant cytokine with a key role in tissue damage (4).

It has been shown that gluten also activates a stress-like immune response mediated by lymphokine-activated killer cells of the innate immune system and a marked proliferation of crypt enterocytes (5–7). IL-15 expressed by enterocytes is a major mediator of this innate immune response so that it is most likely that the toxicity of cereal prolamins for CD patients is due to the presence of both peptides that are able to activate T cell responses and sequences that induce a stress-innate proliferative response of mucosal epithelial cells. Therefore, a cereal suitable for a CD diet should be poor of both classes of peptides. Research is intense to find wheat varieties with low or null toxicity for both the treatment and prevention of CD. Diploid wheat species are among the suitable candidates for their low capability to activate intestinal T cell responses in CD patients (8, 9). Compared with tetraploid and hexaploid wheats commonly used in the making of bread and pasta, the ancient diploid Triticum monococcum ssp. monocococum wheat showed a marked reduction, or even a lack, of toxicity in vitro cellular assays, which suggested their potential use as new dietary opportunities for CD patients (10–12).

More recently, an extensive genetic analysis of α-gliadin transcripts from either diploid (T. monococcum) and hexaploid wheat (Triticum aestivum) accessions showed a high-sequence heterogeneity and considerable differences in the number of T cell–stimulatory peptides in wheat species. This study provided evidence of the existence of natural peptide variants of known α-gliadin epitopes contained in some diploid wheat accessions, in which a single amino acid substitution was sufficient to completely abolish recognition by cognate T cells (13). However, additional studies are needed before the view is accepted that products from monococcum wheat are less toxic or nontoxic for CD patients. Furthermore, the existence of several accessions of diploid monococcum wheat (T. monococcum ssp. monocococum) cv. Sagittario; PT-ID331, PT gliadin digest from Triticum monococcum cv. Sagittario; PT-ID331, PT gliadin digest from Triticum monococcum ID331; PT-Monlis, PT gliadin digest from Triticum monococcum Monlis; TCC, T cell clone; TG2, tissue transglutaminase.

this ancient wheat with different gluten protein compositions also raised the question as to whether all varieties might be equally toxic for CD patients. The aim of this study was to investigate the immunological properties of 2 T. monococcum lines, Monlis and ID331, in view of their possible use in CD patients.

SUBJECTS AND METHODS

Patients

Twenty-eight CD patients who underwent small intestinal biopsies were enrolled for an organ-culture study. Seventeen patients (median age: 6 y; range: 2.5–10.5 y) presented villous atrophy [type M3c according to the classification of Marsh as modified by Oberhuber et al (14)] and high serum concentrations of anti–tissue transglutaminase (TG2) antibodies. The other 11 patients (median age: 32 y; range: 18–57 y) consumed a gluten-free diet for ≥2 y and had normal intestinal mucosa and normal serum concentrations of anti-TG2 antibodies.

Intestinal T cell lines (iTCLs) were generated from duodenal biopsies of 7 CD patients (3 untreated and 4 treated; mean age: 24 y; range: 6–42 y). All enrolled patients were HLA-DQ2 positive as assessed by using HLA typing. The enrollment of subjects whose biopsies were used to generate iTCLs and to set up organ-culture studies began in February 2008. The patients or their parents gave consent to participate in the study. The protocol of the study was approved by the Ethical Committee of the University of Naples Federico II (protocol n. 230/05).

Gliadin extract and peptide preparation

The fractionation of storage protein from monococcum wheat flour by acidic polyacrylamide gel electrophoresis at pH 3.1 and SDS-PAGE did not reveal any contamination with durum or common wheat prolamins. The alcohol-soluble protein fraction (gliadin) was extracted from whole flour of Monlis and ID331 and from hexaploid wheat (T. aestivum cv. Sagittario), according to the Osborne procedure with modification and then digested with pepsin and trypsin as previously described (15). Peptic and tryptic (PT)–gliadin digests are indicated as PT-Monlis (T. monococcum Monlis), PT-ID331 (T. monococcum ID331), and PTG (T. aestivum). Deamidation of PT gliadins was performed in 0.125 M HCl that contained 1 mmol/L calcium chloride, 10 mmol/L dithiothreitol, and 0.2 μg/μL TG2 (Sigma-Aldrich) at pH 8.5 for 4 h.

Immunogenicity assay on intestinal T cells

Endoscopic mucosal explants were digested with collagenase A as previously described (16). Long-term T cell lines were established by using 2–3 stimulations of intestinal cells with autologous mononuclear cells and deamidated PTG and, thereafter, with phytohemagglutinin and feeder cells. iTCLs resulted in more than 90% of the CD3+CD4+ phenotype. Cells derived from the iTCL (CD230204) were cloned by limiting the dilution as previously described (16). Both iTCLs and T cell clones (TCCs) were assayed for a response to deamidated gliadin digests by the detection of interferon-γ production and cell proliferation (16). All antigens were assayed in duplicate and in ≥3 independent experiments for each iTCL or TCC.

Organ culture of small intestinal biopsies

Organ cultures of duodenal biopsy specimens obtained from 17 untreated and 11 treated celiac patients were performed as previously reported (17). Biopsy specimens were cultured in the presence of medium alone or PT-prolamin digests (500 μg/mL). In the experiments aimed at the evaluation of the proliferation of crypt epithelial cells, bromodeoxyuridine (10 μM; Sigma-Aldrich) was added to the culture medium of small intestinal specimens from untreated celiac patients. After 24 h of culture, the tissues were embedded in optimal cutting temperature compound (Tissue-Tek; Sakura Finetek). Five-micrometer cryostat sections were prepared for immunohistochemical stainings.

IL-15 expression

IL-15 expression was immunohistochemically assessed in small intestinal fragments from treated CD patients after an in vitro challenge for 24 h with PTG, PT-Monlis, PT-ID331, and medium alone. Cryostat sections were treated as previously reported (18). The staining of epithelial and crypt cells that expressed IL-15 was graded as no signal (−) = 1, weak (+) = 2, moderate (+++) = 3, or strong (++++) = 4, as previously reported (18). IL-15 staining of lamina propria mononuclear cells (LPMCs) was separately scored on a scale from 1 (no signal) to 4 (very strong signal) on the basis of the density and intensity of staining of positive cells. Five sections from each sample were blindly evaluated by 2 investigators. Images shown were acquired by using a light microscope (Axioskop2 Plus; Zeiss).

Cryop epithelial cell proliferation tests

Crypt epithelial cell proliferation was assessed in cultured small intestinal biopsies from untreated CD patients by evaluating bromodeoxyuridine incorporation. Cryostat sections were examined by using double immunofluorescence as previously reported (19). Stained sections were observed by using a confocal microscope (LSM 510; Zeiss MicroImaging Inc). More than 500 cytokeratin-positive epithelial cells were counted in each sample, and the number of bromodeoxyuridine-positive cells was expressed as a percentage of the total number of cytokeratin-positive cells.

Intraepithelial lymphocyte infiltration and activation of lamina propria cells

Intraepithelial CD3+ lymphocytes and lamina propria mononuclear CD25+ cells were detected in cultured small intestinal samples of treated CD patients as previously described (17). After the staining, slides were analyzed blindly by 2 observers by using a light microscope (Axioskop2 Plus; Zeiss). The density of cells that expressed CD3 in the intraepithelial compartment was evaluated per millimeter of epithelium, whereas the number of CD25+ cells was counted within a total area of 1 mm2 lamina propria. Activated T cells (CD3+CD25+) were detected with immunofluorescence by using confocal microscopy (Leica SP), as previously described (17). Lamina propria CD3+CD25+ cells were counted as a percentage of total CD3+ cells.

Statistical analysis

Student’s 2-tailed t test (Statistical Package for Social Sciences version 15.0; SPSS Inc) was used to compare specimens
exposed to PTG or PT digests of the 2 monococcum wheats with those exposed to medium alone. \( P < 0.05 \) was considered statistically significant.

**RESULTS**

**Gliadin from both monococcum lines Monlis and ID331 activate immune response in celiac iTCLs**

We have investigated the immune stimulatory properties of Monlis and ID331 varieties on iTCLs, which were generated from 7 DQ2-positive celiac individuals (20). These iTCLs were raised against PT-gliadin digests from common wheat and had a heterogeneous profile of gliadin-peptide recognition (20). All PT gliadins were assayed after deamidation with TG2 because it has been reported that this enzymatic treatment highly increases the T cell stimulatory activity of gluten peptides (21). As shown in Figure 1A, both Monlis and ID331 PT gliadins strongly stimulated T cells from celiac intestinal mucosa to produce interferon-\( \gamma \) (\( P < 0.005 \) and \( P < 0.002 \), respectively) compared with the use of medium alone. Gliadins from the monococcum Monlis were also able to induce a proliferative response (\( P < 0.05 \)), whereas the cell proliferation in response to PT-ID331, although increased, did not reach significance compared with that of medium alone (\( P = 0.072 \)) (Figure 1B).

We also compared gliadins from hexaploid and diploid AA wheats in their ability to stimulate a TCC specific for the DQ2-\( \alpha \)-I/III epitopes [new nomenclature: DQ2.5-glia-\( \alpha \)-1a/DQ2.5-glia-\( \alpha \)-1b (22)], included in the dominant 33-mer of hexaploid \( \alpha \)-gliadin. As shown in Figure 1C, overlapping stimulatory profiles were obtained, which confirmed the presence of DQ2.5-glia-\( \alpha \)-1a/DQ2.5-glia-\( \alpha \)-1b or peptide analogs in the prolamin fraction from both monococcum lines.

**Gliadins from Monlis but not ID331 induced IL-15 expression in intestinal mucosa of treated CD**

High levels of IL-15 have been reported in intestinal mucosa of celiac patients in the active phase of disease (23) and in clinical remission (18). With the use of immunohistochemistry, we investigated IL-15 expression in the epithelium and lamina propria of treated biopsies after 24 h of an in vitro challenge with PTG, PT-Monlis, or PT-ID331. In biopsies cultured with PTG, we observed a significant increase of IL-15 expression in villous (\( P < 0.0001 \)) and crypt (\( P < 0.0001 \)) epithelium as well as in LPMCs (\( P < 0.001 \)) in comparison with in samples cultured with medium alone (Figure 2, A–C). Similarly, a marked increase of IL-15 was observed in the epithelium of villi (\( P < 0.0001 \)) (Figure 2, A and D) and crypts (\( P < 0.01 \)) (Figure 2, B and D) after treatment with PT-Monlis; the IL-15 staining also increased in LPMCs of biopsies cultured with PT-Monlis, although the increment did not reach significance (\( P = 0.063 \)) (Figure 2C). Conversely, no differences of IL-15 expression were noted in biopsy specimens cultured with PT-ID331 in comparison with in medium alone, in any compartment analyzed (villous and crypt epithelium as well as lamina propria) (Figure 2, A–D), which indicated that ID331 failed to stimulate IL-15 production or expression in CD mucosa.

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**FIGURE 1.** Immune stimulatory properties of diploid wheats PT-Monlis and PT-ID331 on CD mucosa T cells. A and B: PT gliadins (50 \( \mu \)g/mL) from Monlis, ID331, and the common wheat cv. Sagittario stimulated intestinal T cell lines raised against hexaploid gliadins as indicated by increased IFN-\( \gamma \) production (A) and cell proliferation (B). IFN-\( \gamma \) concentrations were detected in cell supernatant fluid by using a standard sandwich ELISA, whereas cell proliferation was detected by using 3-H thymidine DNA incorporation. Dashes indicate mean values in all tested patients, and dotted lines denote themean of response to medium alone obtained in all patients. Statistical significance was calculated by comparing responses to medium-alone values. Unpaired Student’s \( t \) test: *\( P < 0.05 \), **\( P < 0.005 \), ***\( P < 0.0001 \). C: Dose-response curves of a T cell clone specific for the immunodominant \( \alpha \)-gliadin epitopes DQ2-\( \alpha \)-I/III stimulated with deamidated PT gliadins from common wheat cv. Sagittario and diploid monococcum wheat lines Monlis and ID331. Values are means of duplicates and are representative of 3 separate experiments. IFN-\( \gamma \), interferon-\( \gamma \); PT, peptic and tryptic; PT-ID331, PT gliadin digest from *Triticum monococcum* ID331; PT-Monlis, PT gliadin digest from *T. monococcum* Monlis.
Gliadins from Monlis but not ID331 induced proliferation of crypt enterocytes in CD biopsies

Because gliadin extracts from hexaploid wheat are able to induce the proliferation of crypt epithelial cells in celiac intestinal mucosa (18), we investigated whether PT-Monlis and PT-ID331 displayed similar proliferative properties in intestinal fragments of atrophic CD patients in vitro cultured for 24 h. Consistent with previous finding (18), the hexaploid PTG induced a bromodeoxyuridine incorporation in 30.5 ± 6.7% of epithelial crypt cells compared with 16.0 ± 6.3% of epithelial cells in fragments cultured in the presence of medium alone (P, 0.001) (Figure 3). A similar proliferative rate was observed in PT-Monlis–cultured biopsies compared with that of fragments cultured with only medium (P, 0.001). By contrast, no significant differences were noted in biopsy specimens cultured with PT-ID331 (P = 0.6) compared with medium alone (Figure 3). This finding showed that gliadins from monococcum wheat Monlis and common wheat Sagittario induced the proliferation of crypt epithelial cells in CD patients. Conversely, monococcum wheat ID331 lacked this capability.

Gliadins from monococcum wheat lines Monlis and ID331 induced intraepithelial T cell infiltration and activation in treated CD biopsies

A significant increase of intraepithelial CD3+ lymphocytes was seen in biopsies of treated CD patients cultured for 24 h in the presence of PTG (mean ± SD: 31 ± 15; P < 0.001), PT-Monlis (29 ± 10; P < 0.001), and PT-ID331(29 ± 14; P < 0.01) compared with those cultured in medium alone (14 ± 7) (Figure 4A). Similarly, all tested PT digests increased the number of CD25+ cells in lamina propria; the mean ± SD of CD25+ cells/mm² were 73 ± 40, 43 ± 26, and 47 ± 21 for PTG (P < 0.001), PT-Monlis (P < 0.01), and PT-ID331 (P < 0.01), respectively, compared with those cultured in medium alone (10 ± 5) (Figure 4B). We also showed a significant increase (P < 0.05) of lamina propria CD3+CD25+–activated T cells in CD biopsies challenged in vitro with PTG (10.4 ± 3.3), PT-Monlis (8.5 ± 2.6), and PT-ID331 (8.2 ± 2.5), compared with medium alone (2.8 ± 0.9) (Table 1).
IMMUNOGENICITY OF TRITICUM MONOCOCCUM IN CELIAC

DISCUSSION

The identification of nontoxic or, more likely, less toxic cereals for celiac patients, especially in wheat species, represents an important goal that could open interesting perspectives including the prevention of CD in at-risk individuals. To achieve this ambitious goal, studies have explored the presence of T cell–stimulatory gluten peptides in ancient diploid wheat cultivars with respect to phylogenetically more recent varieties (8, 9). These studies have demonstrated variability, from low to high, in the induction of T cell responses, which was independent of the different genetic ploidy asset or background of the accessions of the wheat species analyzed. Also, a marked reduction of the content of α-gliadin immunogenic peptide in ancient diploid AA (8, 9) and BB/SS species have been shown (9). In contrast, it is becoming clearer that the toxicity of gluten is not only restricted to the ability to activate adaptive immune responses. Gluten contains peptides that activate the innate branch of the immune system (5–7) and induce the proliferation of crypt enterocytes with marked mucosal tissue remodeling (19). However, very little is known about the capability of diploid wheats to stimulate innate immune pathways in celiac mucosa.

In the current study, we have screened the immune toxicity of 2 accessions of diploid wheat, Monlis and ID331, by using in vitro or ex vivo assays as CD patient–derived gliadin reactive T cell lines (16) and the organ culture of CD mucosa (17, 18). These procedures allowed us to monitor the toxicity of cereals by investigating both adaptive and innate immune responses that occur in CD intestinal mucosa. We chose to study these 2 diploid wheat varieties because they are extensively cultivated in some areas of Central Italy. In particular, Monlis is a registered cultivar that is commercially grown in ~300 ha, whereas ID331 is cultivated in <10 ha in Central Italy and Sardinia island, and monococcum wheat pasta is produced and distributed in several countries including the US market.

Our findings unequivocally showed that gliadins from both monococcum lines have the ability to stimulate celiac mucosal polyclonal T cell lines with a magnitude of responses comparable to common wheat gliadins, as indicated by interferon-γ production and cell proliferation. To further confirm this finding, we tested the capability of diploid gliadins to stimulate TCCs specific for hexaploid DQ2-α-I/III epitopes [new nomenclature: DQ2.5-glia-α1a/ DQ2.5-glia-α1b (22)], included in the dominant 33-mer peptide. Overlapping stimulatory profiles were obtained, which confirmed the presence of DQ2.5-glia-α1a/DQ2.5-glia-α1b in gliadin fractions from both monococcum lines.

In the past decade, much evidence has highlighted the central role of innate immunity in the induction of tissue damage in CD mucosa. In the current study, we showed that PTG and PT-Monlis increased IL-15 in the epithelium of duodenal fragments of CD patients who consumed a gluten-free diet after 24 h of culture. Conversely, we did not observe any IL-15 increase in biopsies cultured with PT-ID331. Direct evidence of the activation of the innate immune system by gliadins and, in particular, peptide p31-43 has been obtained in organ-culture experiments (6, 7). Most of the events of innate immune activation seem to be mediated by the IL-15 that is produced in response to p31-43 by both epithelial cells and dendritic cells (6, 7). Our results suggest that Monlis activates innate immunity through IL-15 production, whereas ID331 seems to be unable to elicit this kind of immune response.

Increased crypt cell proliferation is as an early alteration of CD mucosa that leads to crypt hyperplasia. More recently, we reported that the crypt epithelial cell proliferation induced by gliadin peptides in intestinal mucosa of CD patients is epidermal growth factor and IL-15 dependent (18, 24). In this study, we showed that whole-gliadin digest from common wheat cv. Sagittario and from the diploid wheat Monlis increased the proliferation of crypt epithelial cells in cultured intestinal mucosa from CD patients with villous atrophy. In contrast, gliadin extracts from diploid wheat ID331 did not increase crypt epithelial cell proliferation in untreated CD mucosa. On the basis of recent findings that showed an IL-15–dependent proliferation of crypt epithelial cells induced by gliadin (24), the inability of ID331 to induce a crypt epithelial cells proliferation may be substantially explained by the lack or limited IL-15 production in ID331 organ-cultured biopsies.

### TABLE 1

<table>
<thead>
<tr>
<th>CD patients (n = 8)</th>
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<tr>
<td>Medium</td>
<td>2.8 ± 0.9</td>
</tr>
<tr>
<td>PTG</td>
<td>10.4 ± 3.3*</td>
</tr>
<tr>
<td>PT-Monlis</td>
<td>8.5 ± 2.6*</td>
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<tr>
<td>PT-ID331</td>
<td>8.2 ± 2.5*</td>
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*All values are means ± SDs. Compared with medium alone (unpaired Student’s t test): *P < 0.05, PTG, PT gliadin digest from *Triticum aestivum*; PT-ID331, PT gliadin digest from *Triticum monococcum* ID331; PT-Monlis, PT gliadin digest from *T. monococcum* Monlis.
The activation of mucosal inflammation signs indicated that gliadin extracts from Monlis and ID331 significantly enhanced the number of activated (CD25+\textsuperscript{*}) mononuclear cells in the lamina propria, which was similar to the value obtained with hexaploid gliadin. Furthermore, we showed an increase of CD3\textsuperscript{+} cells in activated CD25\textsuperscript{*} lamina propria cells, which suggested that these 2 monococcum lines were able to activate mucosal T-cell-mediated immunity in the same extension of \textit{T. aestivum}. Another phenomenon we have shown to consistently occur in treated CD mucosa in response to hexaploid gliadins is the infiltration of the intraepithelial density of CD3\textsuperscript{+} cells (2, 17, 18). The treatment of celiac mucosal explants with PT gliadin from Monlis and ID331 resulted in a marked increase of the intraepithelial density of CD3\textsuperscript{+} cells to an extent comparable with PT gliadins from common wheat. This massive infiltration in the intestinal epithelium compartment by T lymphocytes is a hallmark of the active CD lesion, but the mechanism that induces this phenomenon still remains to be clarified (1).

Together, our results suggest that both lines of \textit{T. monococcum}, Monlis, and ID331 are virtually unsafe for CD patients. Nevertheless, in our system, Monlis, seemed to be able to activate both innate and adaptive immune responses, whereas ID331 seemed to activate only an adaptive immune response. In this context, Monlis is rather unusual because it lacks \textit{w}-gliadins in its prolamin pattern, which is a rare (<2\%) condition in the dozens of monococcum wheat accessions investigated thus far. Monlis and monococcum lines devoid of \textit{w}-gliadins, but not ID331, and normal monococcum lines proved to be comparable to common wheat cultivars in driving in vitro agglutination of K562(S) cells (25).

In conclusion, our findings do not support the use of old wheat crops for the diet of individuals with CD. Notwithstanding, the reduced ability of some diploid wheat lines, such as ID331, to in vitro activate the innate immune response in CD mucosa could render these cultivars less active in inducing CD. More studies are required to explore if a monococccus-based diet could result in a reduced incidence of CD.

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The authors' responsibilities were as follows—CG, RT, and SA: contributed to the study design, data analysis, and critical revision and drafting of the manuscript; AC and IV: conducted the immunogenicity assay on intestinal T cells; MM: analyzed and interpreted immunohistochemical analyses; None of the authors had a personal or financial conflict of interest.

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