A 10-residue peptide from durum wheat promotes a shift from a Th1-type response toward a Th2-type response in celiac disease\textsuperscript{1,2}

Marco Silano, Rita Di Benedetto, Francesca Maiialetti, Alessandro De Vincenzi, Roberta Calcuttara, Antonello Trecca, and Massimo De Vincenzi

**ABSTRACT**

**Background:** Celiac disease (CD) is a Th1-driven autoimmune permanent enteropathy that is triggered by dietary gluten. Molecules able to shift the immune response from a Th1- to a Th2-type response have been suggested as therapeutic agents for Th1 autoimmune diseases.

**Objective:** We sought to investigate the possibility that a decapptide from durum wheat (p10mer, QQPQDAVQPF), which was previously shown to prevent the activation of celiac peripheral lymphocytes, may promote a shift from a Th1- to a Th2-type immune response in gluten-specific intestinal T cells of CD patients.

**Design:** Intestinal T lymphocyte lines derived from 8 children with CD were incubated with gliadin peptides both alone and simultaneously with p10mer. Cell proliferation and the production of interferon-γ and interleukin-10 by these T cells were measured.

**Results:** The incubation of celiac intestinal T cells with deamidated gliadin peptides resulted in a significant (\(P < 0.008\)) increase in cell proliferation and interferon-γ release, whereas the simultaneous exposure to p10mer totally abolished the cell proliferation and cytokine release. Moreover, incubation with p10mer maintained an elevated release of interleukin-10, whereas exposure of the cells to culture medium only did not. The replacement of the residues of aspartic acid in position 5 or those of alanine in position 6 in the sequence of p10mer resulted in peptides with no activity in the activation experiments.

**Conclusion:** In vitro, p10mer showed the ability to shift the pathogenic immune response of a CD patient from a Th1- to a Th2-type response. *Am J Clin Nutr* 2008;87:415–23.

**KEY WORDS** Th1-to-Th2 shift, gluten-specific T cells, celiac disease

**INTRODUCTION**

Celiac disease (CD) is an autoimmune permanent enteropathy that is triggered in susceptible persons by the ingestion of gluten, a storage protein fraction present in wheat grain, and by similar proteins of rye and barley (1, 2).

Approximately 96% of CD patients express the HLA molecule DQ2, and the remaining mostly express the less common haplotype DQ8 (3). All of the intestinal gluten-sensitive T cell clones are DQ restricted, which reflects the critical role of this molecule in the pathogenesis of CD. After being bound to DQ, certain deamidated gluten peptides are recognized by interferon-γ (IFN-γ)–producing T cells, and that fact drives the inflammatory response that causes the histologic features of celiac intestinal mucosa (4, 5). Gluten consists of different classes of proteins: the α-gliadins, which are recognized by T lymphocytes from almost all celiac patients, and β-, ω-, and γ-gliadins and glutenins, which apparently are recognized by T cells from a smaller number of patients (6–8).

The only known treatment for CD is the life-long exclusion of gluten-containing food from the diet. Compliance with a gluten-free diet is difficult because of the wide distribution and consumption of cereal-based foods, but strict adherence is necessary to reduce mortality and morbidity (9–11). Consequently, alternative strategies for dealing with the harmful effects of a gluten in persons with CD are being pursued.

One possibility may consist of modifying the gluten sequences that act as epitopes in CD to abolish their immunogenic ability (12). The development of wheat cultivars that do not contain gluten proteins with harmful sequences has also been proposed (13).

An alternative therapeutic option, described in this report, may rely on a shift of the mucosal immune response from a Th1-type inflammatory response to a Th2-type suppressor response. It has been shown that CD is a Th1-polarized condition (14, 15). Molecules able to induce a Th1-to-Th2 shift have been suggested as therapeutic agents for some Th1-driven diseases (16–19). Glutamic acid decarboxylase–derived peptide 524–543 reduces diabetes in nonobese diabetic mice by increasing the production of Th2-associated interleukins, IL-4 and IL-10, and reducing the production of the Th1-related IFN-γ and IL-2 (16). Isoprenoids and lovastatin modulate the onset of experimental autoimmune encephalomyelitis, which blocks the Th1-differentiation of T cells (17). In addition, insulin exerts a beneficial effect during sepsis, generating a greater number of Th2 cells (18). Finally, Salvati et al (19) reported that recombinant human IL-10 suppresses gliadin-induced T cell activation.

We previously described a decapptide (p10mer, sequence QQPQDAVQPF) from the alcohol-soluble protein fraction of...
durum wheat that prevents the activation of peripheral celiac lymphocytes by gliadin peptides (20). In the present study, we focused on the production of 2 key cytokines, IFN-γ and IL-10, by celiac small-intestine CD4+ cells after the exposure of these cells to gliadin peptides and p10mer. Our purpose in this was to investigate the possibility that the p10mer sequence can promote a shift from a Th1-type toward a Th2-type pathogenic immune response in CD patients. We assumed that T cell proliferation and IFN-γ production would provide a measure of Th-1 and that IL-10 would provide a measure of Th2.

SUBJECTS AND METHODS

Antigens

Gliadin preparation and peptic-tryptic digestion

The alcohol-soluble protein fraction from whole cereal flour of bread wheat (Triticum aestivum, variety S Pastore) was extracted and subjected to peptic-tryptic digestion (PTgli), as previously described (21). Gliadin preparations were assayed for endotoxin by using a reagent kit (QCL-100; BioWhittaker, Walkersville, MD); they were found to have endotoxin concentrations of <0.5 enzymatic units/mL. The p10mer and pα-2 (62–75) peptides and variants G5 and G6

The sequence of p10mer (MW: 1157 D) was identified in the alcohol-soluble protein fraction of durum wheat (Triticum durum, variety Adanello) by De Vincenzi et al (22). pα-2 has been shown to be a major immunogenic epitope of aspartic acid at position 5 and alanine at position 6, respectively, in the sequence of p10mer.

The p10 mer and pα-2 peptides and G5 and G6 were synthesized (Primm Company, Milan, Italy) by the solid-phase method (model 431A; Applied Biosystems, Foster City, CA) and purified up to 99% with the use of reverse-phase HPLC (5020 system; Varian Inc, Walnut Creek, CA). The details and sequences of the peptides are shown in Table 1.

Transglutaminase deamidation

An ordered and specific deamidation of gliadin peptides by tissue transglutaminase (tTG) is usually necessary for binding to major histocompatibility complex proteins (DQ2/DQ8) of antigen-presenting cells and subsequent recognition by T cells (25, 26). Consequently, 400 μg PTgli, pα-2, p10mer, G5, or G6 was incubated for 4 h at 37 °C in a solution consisting of 100 μg guinea pig liver/mL (tTG) and 2 mmol CaCl2/L. All reagents were purchased from Sigma Chemical Co (St Louis, MO). The decapetide p10mer is not likely to be deamidated by tTG, because it does not have glutamine residues in the key positions that make glutamine susceptible to the action of tTG (27). However, p10mer was subjected to the transglutaminase to maintain consistency in the experiments.

Patients

Eight children with CD (5 F, 3 M; mean age at enrollment: 7.9 y; range: 4.1–15.2 y) were included in the study. All of the children showed symptoms and signs strongly suggestive of CD. Small bowel mucosa specimens were taken during the children’s diagnostic endoscopic examination when all were consuming gluten-containing food. All patients had a positive titer of antitransglutaminase antibody, and all were DQ2 positive.

Written informed consent was obtained from the parents of the patients. The study was approved by the Ethics Committee of the Istituto Superiore di Sanità (CE-ISS-05/111).

DQ2 determination

The HLA-DQA1 and -DQB1 genotypes of all subjects were determined by using peripheral blood DNA and polymerase chain reaction with sequence-specific primer mixes (20).

Establishment of gluten-specific polyclonal small-intestine T cell lines

Methods were based on those described by Molberg et al (28). Tissue taken from the small intestine of CD patients for biopsy was first oriented on a metal grid in a tissue culture plate (Falcon, Franklin Lakes, NJ) and then incubated with 3 mL of a solution of 5 mg PTgli/mL RPMI medium for 24 h (Gibco, Invitrogen, Carlsbad, CA) for 24 h. The tissue for biopsy then digested with collagenase A (Sigma Chemical Co) in phosphate-buffered saline solution (1 mg/mL) for 1 h at 37 °C, chopped with a scalpel, and passed through a 70-μm cell filter (Falcon; Sial Company, Rome, Italy) to release T cells. The collected cells were cultured in RPMI medium with 10% heat-inactivated heterologous plasma plus 1 × 10^6 autologous peripheral blood mononuclear cells/mL and 10 U human recombinant IL-2 (Peprotech, London, United Kingdom)/mL. Cells were restimulated every 7 d with deamidated PTgli (200 μg/mL), feeder cells, and IL-2.

T cell assays

The antigens tested were tTG deamidated PTgli (2 mg/mL, 1 mg/mL, 500 μg/mL, 250 μg/mL, and 125 μg/mL), pα-2 (10 μg/mL), both alone and in combination with p10mer (10 μg/mL), and decapetides pG5 (10 μg/mL) and pG6 (10 μg/mL), both alone and in combination with PTgli (10 mg/mL). Culture medium alone was the negative control.

Antigens were incubated overnight with the autologous peripheral blood mononuclear cells acting as antigen-presenting cells (5 × 10^6) in 96-well, U-bottom plates in a total volume of 100 μL complete RPMI medium/well before the addition of T cells (5 × 10^5). Each cell line was tested separately, and the results are expressed as the mean ± SE of all the values obtained.

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<td>Details of peptides</td>
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<td>Gliadin peptides (PTgli)</td>
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Plasmocin (2.5 µg/mL; Invitrogen) was added to all stages of cell culture to prevent mycoplasma infection.

T cell proliferation was measured after 24, 48, 72, and 96 h by using the 5-bromo-2-deoxyuridine (BrdU) cell proliferation test (Chemicon International, Temecula, CA) according to the manufacturer’s instructions. IFN-γ and IL-10 concentrations in culture medium were measured at 24, 48, 72, and 96 h by commercial enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturer’s instructions (Biosource, Camarillo, CA). Standards were run on each plate. Samples from all 8 patients were run at the same time in any given experiment with the various antigens.

Statistical analysis

Each experiment was carried out in triplicate on 3 different days. Standards were run for each different plate. Data are expressed as the mean ± SE of the results for T cells from each of the 8 patients. Multivariate repeated-measures analysis of variance was used to test the time × group interaction. When that interaction was significant, the differences between groups at a given time-point were examined by one-factor ANOVA; if the P value was significant, a Bonferroni-corrected Student’s t test was used to compare the individual means, with significance set at P < 0.008. All statistical analyses were performed with SPSS software (version 13.0; SPSS Inc, Chicago, IL).

RESULTS

T cell lines

The small-intestine specimens were challenged overnight with PTgli that had been treated with tTG (dPTgli). T cells were cultured, and the lines were tested with dPTgli after 7 d. A reactive gluten-specific polyclonal cell line was obtained for each of the 8 patients, and the mean stimulation index (SI) was 6.1 ± 0.3.

T cell proliferation

To test the immunomodulatory effect of p10mer on the activity of celiac T cells, the cells were exposed to dPTgli, deamidated pep-2 (dpα-2) alone, and dpα-2 simultaneously with tTG-treated p10mer (dp10mer). The mucosal T cell proliferation was measured and is reported as a measure of the activation of the Th1 immune response (Figure 1). Cell proliferation was measured by the incorporation of BrdU, an analogue of thymidine, in the T cell DNA and was detected by using an immunocolorimetric assay. Figure 1 shows the T cell proliferation, expressed as the mean SI, of the T cell lines obtained from the 8 CD patients enrolled, after the exposure to dPTgli and dpα-2, both alone and simultaneously with the dp10mer. Whereas the incubation with dPTgli increased cell proliferation to an SI of 3.9 ± 0.16 at day 4, the simultaneous incubation with dp10mer resulted in an SI of 1.05 ± 0.09 at 96 h, which did not differ significantly from that of T cells incubated with culture medium only (SI = 0.96 ± 0.07; P < 0.01 for time × group interaction).

In the experiments represented in Figure 1A, dPTgli was used at a concentration of 500 µg/mL, and dp10mer was used at a concentration of 10 µg/mL (ratio 50:1). Various concentrations of dPTgli (2 and 1 mg/mL and 500, 250, and 125 µg/mL) were tested, whereas the concentration of dp10mer was kept constant at 10 µg/mL. The concentration of 500 µg/mL was found to be the highest concentration of dPTgli that would suppress T cell stimulation to the approximate level of the control when the p10mer concentration was 10 µg/mL (data not shown).

The pep-2 was found to induce an increment of SI in T cells that was below the cutoff for positivity arbitrarily set by Ellis et al (29); the time × group interaction was not significant (Figure
Cytokine profile

IFN-γ is the main cytokine involved in the Th-1 inflammatory response of CD. IFN-γ release in the culture medium from celiac mucosal lymphocytes was measured by using a commercial ELISA kit 24–96 h after the exposure of celiac mucosal T lymphocytes to deamidated gliadin peptides and dp10mer, both separately and simultaneously. The mean concentrations of IFN-γ released by the T cell lines from all 8 patients in response to the various peptide preparations are shown in Figure 2. The incubation with dPTgli alone resulted in a significant (P < 0.001) increase in IFN-γ release (122 ± 3.7 pg/mL at 96 h), whereas the simultaneous exposure to dp10mer totally abolished it (71.6 ± 2.9 pg/mL at 96 h compared with 70.9 ± 5.7 pg/mL for the negative control; P < 0.001 for time × group interaction). Combination treatment with dpα-2 and dp10mer induced a significantly (P < 0.001) lower mean value of IFN-γ release from the celiac mucosal lymphocytes than was seen with dpα-2 alone, but the release was very similar to that of the negative control (102 ± 2.4, 66.7 ± 3.5, and 57.9 ± 6.1 pg/mL, respectively; P < 0.001 for time × group interaction).

IL-10 is a down-regulatory Th2-associated cytokine, and its presence results in long-lasting antigen-specific unresponsiveness. The mean concentration of IL-10 in culture medium from the cell lines from all of the children was measured by using a commercial ELISA kit 24–96 h after the exposure of celiac mucosal T lymphocytes to deamidated gliadin peptides and dp10mer, both separately and simultaneously.

IL-10 is produced by CD4+ T cells when they are activated to control the immune response and prevent it from expanding excessively. The differences in its release at 24 h after the various treatments are apparently related to this effect (Figure 3A). When lymphocytes were exposed to medium alone, no immune activation of lymphocytes occurred, and the IL-10 production remained low at 24 h. But when dPTgli, both alone or simultaneously with dp10mer, was present in culture medium, IL-10 production increased, presumably to modulate the immune response. The exposure of mucosal gluten-specific T cells to dpPTgli failed to maintain over the course of 96 h the IL-10 concentration that we found was initially elevated in the culture medium (26.1 ± 1.12 pg/mL after 24 h compared with 20.1 ± 1.01 pg/mL at 96 h; P < 0.001 for time × group interaction). In contrast, the simultaneous treatment of T cells with dpPTgli and dp10mer increased the release of IL-10 over the course of time (24.6 ± 1.16 pg/mL after 24 h compared with 31.4 ± 0.83 pg/mL at 96 h; P < 0.001). It is interesting that the individual exposure of T lymphocytes to dp10mer alone also resulted in an increasing release of IL-10 over the incubation time (15.4 ± 0.96 pg/mL at 24 h compared with 28.1 ± 0.95 pg/mL at 96 h; P < 0.001 for time × group interaction) (Figure 3A).

The ability of dp10mer to maintain elevated release of IL-10 in the culture medium was detectable also when celiac T cells were challenged with dpα-2 (Figure 3B). After 24–96 h of incubation of T cells with the known immunogenic peptide dpα-2 alone, the IL-10 concentration decreased (from 25.3 ± 1.14 to 18.5 ± 1.15 pg/mL; P < 0.001 for time × group interaction), whereas the presence of dp10mer increased the IL-10 concentration over the time course, whether alone (15.4 ± 0.79 pg/mL at 24 h compared with 28.2 ± 0.75 pg/mL at 96 h; P < 0.001) or in combination with dpα-2 (16.6 ± 0.80 pg/mL at 24 h compared with 26.6 ± 0.87 pg/mL at 96 h; P < 0.001 for time × group interaction).

To evaluate the ability of p10mer to promote the Th1-to-Th2 shift in celiac mucosal T cells, the ratio between the mean IFN-γ and IL-10 concentrations (IFN-γ:IL-10) in culture medium after the different treatments was measured (Figure 4). The IFN-γ:IL-10 is an index of Th1 predominance in the immune response. This ratio measured at 24 h did not differ significantly between T lymphocytes exposed to dPTgli alone and those exposed to dPTgli and dp10mer simultaneously. After 96 h of incubation, however, IFN-γ:IL-10 was much higher for T cells exposed to
dPTgli alone than for those exposed to dPTgli and d10mer simultaneously (6.14 ± 0.38 and 2.28 ± 0.16, respectively; *P* < 0.001 for time × group interaction).

**Effect of 2 amino acid substitutions in the p10mer sequence on its immunomodulatory activity**

We assumed (see Discussion) that the activity of p10mer relied on the presence of the residues of aspartic acid and alanine in position 5 and position 6, respectively, in its sequence (20). Therefore, we studied 2 altered peptides: peptide G5 (pG5), in which aspartic residue (D) in position 5 in p10mer sequence was replaced with a glycine (G), and peptide G6 (pG6), in which alanine (A) in position 6 was replaced with G. Glycine was used to obtain the modified peptides because it is not directly involved in the deamidation process and does not affect the binding to HLA DQ2 (30–31). The mean SIs of celiac T cells exposed to deamidated pG5 (dpG5) and pG6 (dpG6) are shown in Figure 5A and B, respectively. The SI at 96 h was 3.68 ± 0.11 for lymphocytes incubated with dPTgli alone and 3.95 ± 0.19 and 4.07 ± 0.17, respectively, for those incubated simultaneously with dpG5 (*P* = 0.3 for time × group interaction; *P* < 0.001 for time and group effects) or dpG6 (*P* < 0.001 for time × group interaction) and p10mer.

Concomitant exposure of mucosal T cells to dPTgli and dpG5 or dpG6 simultaneously did not significantly decrease the IFN-γ release induced by dPTgli alone. However, the effect of dp10mer when combined with the dPTgli (71.6 ± 2.54 pg/mL) was significant (*P* < 0.001 for time × group interaction), as shown in Figure 6.

Both of the modified peptides consistently failed to maintain the IL-10 concentrations at an elevated level over time in the culture medium when incubated with celiac T cells, as shown in Figure 7. The mean IL-10 concentrations at 96 h after the combination treatment with dpG5 and dpG6 did not differ significantly with respect to cells incubated with dPTgli, whereas the value at 96 h of exposure of the T cells to dPTgli and dp10mer (31.4 ± 0.9 pg/mL) is significantly different (*P* < 0.001 for time × group interaction). These results suggest not only that pG5 and pG6 lacked immunomodulatory activity in themselves, but also that they acted as partial agonists of active gliadin peptides.

**DISCUSSION**

Th1-type and Th2-type cells are pivotal in the modulation of the immune response. Each subtype is potentially able to down-regulate the other, which leads to a dynamic balance that adapts...
the immune response. Th1 cells are typically involved in cell-mediated immune response, secreting IFN-γ and tumor necrosis factor-α, which promote an inflammatory response. Th2 cells are important for an antibody-mediated response, secreting IL-4 and IL-10, which are antiinflammatory. Thus, a Th1 immune response drives the inflammation characteristic of several autoimmune diseases, and the Th2 response is characteristic of the allergic diseases (32, 33). Because the shift from a Th1- to a Th2-type response has an inhibitory effect on Th1 differentiation, the enhancement of this effect has been proposed as a therapeutic strategy for autoimmune diseases, such as experimental autoimmune encephalomyelitis, type 1 diabetes, rheumatoid arthritis, and psoriasis (16, 17, 34). It is interesting that the last 3 conditions are frequently associated with CD at a level beyond that expected for chance association.

In CD, the Th1-mediated enteropathy is characterized by intestinal T lymphocytes that primarily IFN-γ but also secrete tumor necrosis factor-α, IL-6, and transforming growth factor-β, when presented with particular deamidated, gluten-derived peptides (14, 15). In contrast, in celiac small bowel mucosa, the production of IL-4 is almost undetectable, and the concentration...
p10mer was shown to shift the celiac immune response toward IL-10 as markers for the Th1 and Th2 processes, respectively, in the culture medium of intestinal T lymphocytes from celiac disease patients (in the culture medium of intestinal T lymphocytes from celiac disease patients (19)).

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The finding that the IL-10 release was up-regulated at 24 h after mucosal gliadin–specific T cells were exposed to gliadin peptides is consistent with the work by Salvati et al (19), who found a greater expression of IL-10 RNA transcript in small-bowel mucosa from untreated CD patients than in mucosa from healthy control subjects and treated CD patients. This may be a compensatory antiinflammatory mechanism, activated initially when gluten-specific T cells are first exposed to antigen. The p10mer may exert its immunomodulatory effect by binding to the antigen-presenting cell and yet interfering with presentation to the T cell receptor and consequently maintaining an elevated release of IL-10 from mucosal T cells. When T cells were presented with gliadin peptides alone, the IL-10 concentrations decreased progressively.

The results of the present work strengthen the evidence from our previous report (20) that the immunomodulatory effect of p10mer is specific to gliadin-activated lymphocytes that are the effector of the celiac pathogenic immune response. In the experiments described in this report, polyclonal gluten–specific intestinal T cell lines were used, and T cell clones restricted to well-defined epitopes were not considered, because we decided initially to investigate the immunomodulatory ability of p10mer toward multiple gluten epitopes. The T cell clones cover only a limited number of specificities, however, and they may not fully represent the repertoire of gluten-reactive T cells within celiac lesions.

The p10mer maintained the immunomodulatory ability toward gliadin peptides up to a 50-fold concentration of dPTgli. The PTgli is a not well defined mixture of digestion-derived and water-soluble peptides that are expected to have various immunogenic activities, so it is not possible at this stage to clearly define the concentration of active gluten peptides toward which p10mer exerts its effect. However, when p10mer was incubated simultaneously with the well-defined synthetic peptide pα-2, it abolished its immunogenic effect at a ratio of 1:1.

The pα-2 was found to induce an increment of SI in T cells that was below the cutoff for positivity set arbitrarily by Ellis et al (29). This may be consistent with the findings by Arentz-Hansen et al (24) that there are differences in gliadin specificity between T cells from adult and pediatric patients and that pediatric biopsies are unable to expand efficiently the T cell clones that are specific for pα-2.

Vader et al (38) designed an algorithm (XXXQ 4 XP 6 Q7 X 2 was found to induce an increment of SI in T cells that was below the cutoff for positivity set arbitrarily by Ellis et al (29). This may be consistent with the findings by Arentz-Hansen et al (24) that there are differences in gliadin specificity between T cells from adult and pediatric patients and that pediatric biopsies are unable to expand efficiently the T cell clones that are specific for pα-2.

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Replacing either the aspartic acid or the alanine residue with a glycine residue resulted in peptides lacking immunomodulatory activity toward deamidated gliadin peptides and perhaps also acting as weak agonist. Although the choice of these 2 replacements was somewhat arbitrary, our data suggest that these residues are likely to be especially important. The results obtained with the modified peptides pG5 and pG6 showed that alanine and aspartic residues are pivotal for the immunomodulatory effect of p10mer, but more specific experiments are required to define the mechanisms of this effect.

The in vivo studies mentioned (16, 18) concerning the Th1-to-Th2 shift in the treatment of immunomodulated diseases failed because of the difficulties of delivering the effector molecules to the target organs and because of their photolytic instability (36). In contrast, p10mer is resistant to proteolysis by gastrointestinal enzymes and, orally administered, should easily reach the intestinal mucosa to interfere with the action of intestinal T cells that recognize gluten.

In conclusion, we have here described a naturally occurring decapeptide that is able to modulate the pathogenic inflammatory immune response of CD. Improving the expression of such sequences in the genome of wheat cultivars may be a new therapeutical strategy for CD.

The authors’ responsibilities were as follows—MS: designed the study, established the T cell lines, performed enzyme-linked immunosorbent assay measurement of cytokine concentration in the culture medium, interpreted the data, drafted the manuscript, and performed the statistical analysis; MdV: conceived the study, critically revised the manuscript, interpreted the data, and added important points to the discussion; RC and RdB: performed the enzyme-linked immunosorbent assay measurement of cytokine concentrations in the culture medium and the T cell proliferation assay; FM: performed the peptic-tryptic digest of gliadin, the peptide deamidation, and the T-cell proliferations in the culture medium and the T cell proliferation assay; RC: performed the enzyme-linked immunosorbent assay measurement of cytokine concentrations in the culture medium and the T cell proliferation assay; and all authors: approved the final version of the manuscript. None of the authors had a personal or financial conflict of interest.

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