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SHORT COMMUNICATION

Two prolamin peptides from durum wheat preclude celiac disease-specific T cell activation by gluten proteins

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Abstract
Purpose Celiac disease (CD) is a permanent intolerance to wheat prolamins and related proteins displayed by genetically susceptible individuals. Blocking or modulation of CD-specific T cell response by altered prolamin peptides are currently considered as a potential alternative to the only effective therapy of CD based on a life-long gluten-free diet. Two prolamin peptides, the 9-mer ASRVAPGQQ and the 10-mer GTVGVAPGQQ sequences, were identified by mass spectrometry in the peptic/tryptic digest of prolamins (PTP) from durum wheat (*Triticum turgidum* ssp. *durum*) cv. Adamello, and investigated for their ability to preclude the stimulation of CD-specific mucosal T cells by gluten proteins.
Methods Gluten-specific polyclonal intestinal T cell lines from five CD children (mean age 5 years) were exposed to 50 μg/ml of a deamidated PTP from whole flour of common wheat (*T. aestivum*) cv. San Pastore, and tested for proliferation and production of interferon-γ (INF-γ) and interleukin 10 (IL-10). The same experiment was performed in the presence of 20 μg/ml of the 9-mer or the 10-mer peptide.
Results T cells exposed to PTP showed a threefold increase in proliferation and INF-γ production, and a significant ($P \leq 0.05$) reduction in IL-10 secretion as compared with control cells incubated with the culture medium. Addition of either the 9-mer or the 10-mer peptide to PTP downregulated T cell proliferation and INF-γ production, and caused a significant ($P \leq 0.05$) increase in IL-10 secretion.
Conclusions The T cell reactivity elicited by PTP is precluded by both the 9-mer and the 10-mer sequence, suggesting that over-expression of these proteolytically stable peptides may result in a wheat flour with reduced toxicity for CD patients.

Keywords Celiac disease · Durum wheat · Peptides · Prolamins · T cells

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Introduction

The gluten proteome contains at least 18 distinct prolamin epitopes that are able to elicit proliferation of specific T cells and secretion of inflammatory cytokines in the small intestine of subjects with celiac disease (CD) [1, 3, 16]. Most of these epitopes resist gastrointestinal digestion, and stimulate a T cell response after deamidation by transglutaminase 2 (TG2) [8, 11]. Minor variations in the amino acid sequence of epitopes involved in the disease process were shown to affect their binding within the HLA-DQ2.5 (or -DQ8) binding cleft, and/or the contact of the epitope/HLA complex with the T cell receptor, suggesting a new treatment of CD based on blocking or modulation of the T cell response by modified immunodominant epitopes, as an

alternative to the present therapy based on a life-long gluten-free diet [2, 6, 11].

Sequence QQPQDAVQPF, a natural analog with the same P/Q-rich scaffold and proteolytic resistance of some immunodominant epitopes, was found to prevent prolamin-induced proliferation of both intestinal and peripheral blood lymphocytes and enterocyte apoptosis in small-bowel mucosa from CD children [12–14]. This 10-mer peptide occurs in the peptic/tryptic digest of prolamin (PTP) from durum wheat (*Triticum turgidum* ssp. *durum*) cv. Adamello as a rare modification of the QQPQQPF motif present in many copies across the entire sequence of ω-gliadins, as suggested by immunoblotting with anti-10-mer polyclonal antibodies [10]. Mass spectrometry of PTP from cv. Adamello also revealed the 9-mer ASRVAPGQQ and the 10-mer GTVGVAPGQQ sequences [5]. In the present work, these peptides are synthesized by the solid phase method and compared for their ability to affect cell proliferation and secretion of both interferon-γ (IFN-γ) and interleukin-10 (IL-10) by CD-specific T cells exposed to a deamidated PTP from common wheat (*T. aestivum*).

Materials and methods

Peptide synthesis and characterization

Peptides in the PTP of durum wheat cv. Adamello were isolated by affinity chromatography and gel filtration as described previously [5]. The 9-mer ASRVAPGQQ and the 10-mer GTVGVAPGQQ sequences were determined by MALDI-TOF MS (Voyager DE-PRO, Applied Biosystems, USA), and synthesized by Primm srl (Milan, Italy) by the solid phase method with the Applied Biosystem Model 431 A.

Peptic/tryptic digestion and tTG deamidation of prolamins

The alcohol-soluble protein fraction (prolamin) was extracted from whole flour of common wheat (*Triticum aestivum*) cv. San Pastore and subjected to sequential digestion as described previously [4]. The peptic/tryptic digest of prolamin (PTP) was freeze-dried, lyophilized and stored at −20 °C until used. Deamidation of PTP (500 μg/ml) was carried for 4 h at 37 °C with 100 μg/ml of Guinea pig liver tTG and 2 mM $CaCl_2$. All reagents were purchased from Sigma Chemical (St. Louis, MO, USA).

Patients

Five celiac patients (3 F and 2 M, mean age at enrolment 5 years) and three normal individuals (2 F and 1 M, mean age at enrolment 5 years) were included in this study. Celiac disease was diagnosed according to the revised ESPGHAN criteria [17]. The study was approved by the Istituto Superiore di Sanità Committee (CE-ISS-05/111, January 9, 2006) and written informed consent was obtained from the parents of the patients. Biopsy specimens from distal duodenum were obtained during upper gastrointestinal endoscopies performed for diagnostic purposes in subjects under study for suspected or known CD at the diagnosis. All CD patients were consuming gluten containing food, and exhibited a positive titer of anti-transglutaminase antibody.

Organ cultures and establishment of intestinal prolamin-specific T cell lines (TCL)

Short term intestinal T cell lines (iTCLs) were generated from antigen-challenged intestinal biopsy specimens as described previously [9]. Small intestinal biopsies from five celiac patients and three normal donors were gently washed three-times with 4 ml of PBS without Ca^{2+} and Mg^{2+} (GIBCO, Italy), oriented on a metal grid in a tissue culture plate (Falcon, Franklin Lakes, NJ, USA) and incubated with 1 mg/ml of PTP in RPMI 1640 (GIBCO, Invitrogen, Carlsbad, CA, USA) for 24 h. Biopsies were then digested with 1 mg/ml collagenase A (Sigma, St. Louis, MO, USA) in RPMI 1640 for 1 h at 37 °C with gentle shaking to produce a single-cell suspension and passed through 70-μm filter to eliminate epithelial cells. Cells obtained from biopsies were cultured in a 96-well plate in RPMI 1640 medium containing antibiotics, non-essential amino acids, sodium pyruvate, glutamine, 10% inactivated human AB serum and 10 ng/ml of IL-2 (Peprotech, London, UK) to promote cell growth. Cells were then stimulated with 1×10^6/ml irradiated (3,500 rad) autologous peripheral blood mononuclear cells (PBMCs). On day 7, iTCLs were restimulated with 50 μg/ml of deamidated PTP and a feeder layer consisting of autologous irradiated PBMCs. Fresh medium was added every 3 days, together with 10 ng/ml of IL-15 (Peprotech, London, UK) to prevent apoptosis. Plasmocin (2.5 mg/ml; Invitrogen, Sial, Italy) was added to all stages of cell culture to prevent mycoplasma infection. iTCLs obtained from normal mucosa failed to proliferate. All the lines established from each biopsy were tested in the resting phase on day 15.

Proliferation assay and cytokine determination

Irradiated (3,500 rad) autologous PBMCs (1×10^5) were prepulsed overnight with the PTP (50 μg/ml) alone or in combination with the 9-mer or the 10-mer peptide (20 μg/ml) in a 100 μl volume of RPMI 1640. PMBCs were then

centrifuged, washed to remove unbound Ag, and used as antigen-presenting cells (APCs) by adding them to a 200 µl solution of RPMI 1640 containing 10% inactivated human AB serum and 5×10^4 gluten-specific T cells in 96-well flat-bottom plates (Falcon, Corning Inc New York, NY, USA). After 48 h of treatment, cell proliferation and cytokine secretion were determined. The proliferation assay was performed by adding 0.5 µCi/well of [^{3}H]-thymidine (Amersham Biosciences) to cultures for 24 h. [^{3}H]-thymidine incorporation was then measured by liquid scintillation counting (Wallac, Inc., UK). Each cell line was tested separately, assays were performed in triplicate, and the results were expressed as mean cpm ± SE. Culture medium alone was the negative control (CTR). Proliferation index (PI) was calculated as the mean cpm in the presence of antigen divided by the mean cpm of CTR. Proliferation was regarded as positive when PI > 2. For cytokine determination, an aliquot of supernatant from each well was collected and stored at –80 °C until used. The production of IFN-γ and IL-10 was measured using commercial ELISA kits (Biosource, Camarillo, CA, USA, and Quantikine R&D Systems, MN, USA, respectively) in accordance with the manufacturer's instructions. Each cell line was tested separately, determinations were performed in triplicate and the results were expressed as mean pg/ml ± SE. Standards were run on each plate. Sensitivity of assay was 15 pg/ml.

Statistical analysis

Data are presented as arithmetic means of at least three independent experiments on the same iTCL ± SE. Statistical analysis was performed by the Student's *t* test using the SPSS (Chicago, IL, USA) software. *P* values smaller than 0.05 were considered as significant.

Results and discussion

MALDI-TOF MS analysis of the peptic/tryptic digest of the alcohol-soluble fraction of endosperm protein from durum wheat cv. Adamello led to the identification of the 9-mer ASRVAPGQQ and the 10-mer GTVGVAPGQQ peptides [5], which were tested here for their effects on short-term intestinal T cell lines obtained from the mucosal explants of five celiac patients. As expected, iTCLs from PTP-challenged intestinal biopsy specimens obtained from three normal donors failed to proliferate and were discarded. When added to the T cell lines, the 9-mer and the 10-mer peptides did not promote cell proliferation and production of INF-γ and IL-10 cytokines, as compared with control cells (CTR) (Fig. 1a–c). In contrast, iTCLs exposed to PTP from common wheat cv. San Pastore exhibited a threefold increase in [^{3}H]-thymidine incorporation and INF-γ production (Fig. 1a, b), as well as a significant decrease in IL-10 secretion (Fig. 1c). On the other hand, exposure to PTP plus 20 µg/ml of either the 9-mer ASRVAPGQQ or the 10-mer GTVGVAPGQQ peptide caused a significant ($P \leq 0.05$) downregulation of T cell

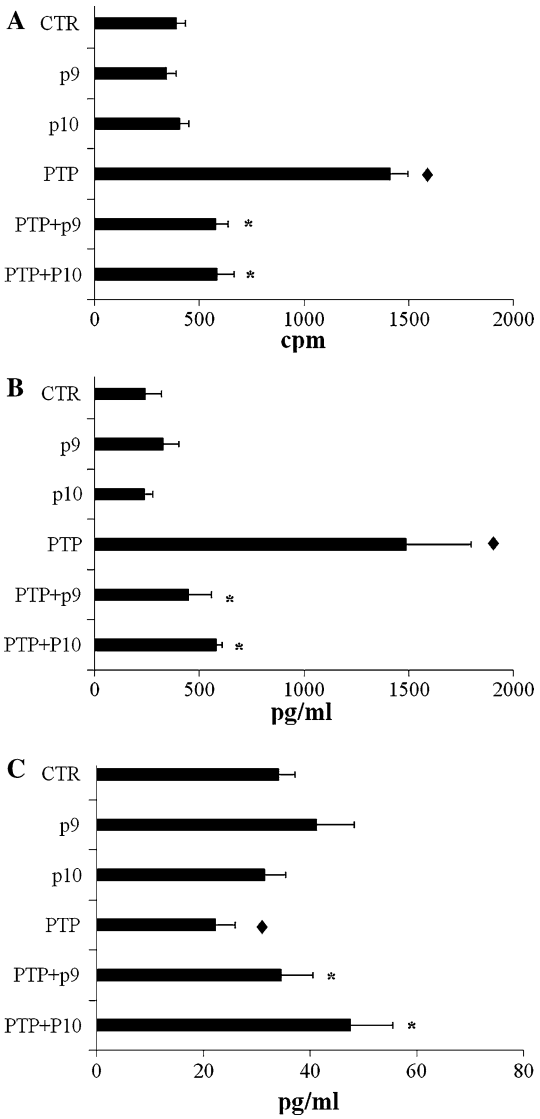

Fig. 1 **a** T cell proliferation, **b** INF-γ and **c** IL-10 production by intestinal T cell lines from mucosa of five CD children after exposure to peptic/tryptic digest of prolamins (PTP) from common wheat cv. S. Pastore 9-mer sequence (p9) ASRVAPGQQ and 10-mer sequence (p10) GTVGVAPGQQ. Cell proliferation was calculated after 72-h stimulation by counting per minute (cpm) tritiated thymidine incorporation. For INF-γ and IL-10 determination, aliquots of supernatant were collected from each T cell line after 48 h of exposure to PTP (50 µg/ml), 9-mer or 10-mer peptide (20 µg/ml each). T cell lines treated with the culture medium alone were the negative control (*CTR*). The results are expressed in terms of mean ± SE (*bar*) of data obtained from three independent experiments on each T cell line. *Filled diamonds* significant at $P < 0.05$ with respect to CTR; *significant at $P < 0.05$ with respect to PTP

proliferation and INF-γ production, as well as an increased secretion of IL-10 cytokine as compared with cells treated with PTP alone (Fig. 1a–c). These findings suggest that the 9-mer and the 10-mer peptides are equally able to preclude prolamin-induced cell proliferation and production of proinflammatory cytokine INF-γ in short-term intestinal T cells from CD children. These peptides are also able to contrast downregulation of anti-inflammatory IL-10 secretion caused by PTP from common wheat flour.

Over 1 million wheat sequences in the database of GenBank were searched to identify sequences related to those of the 9-mer and the 10-mer peptides analyzed here. Both peptides share the PGQQ sequence, which occurs at positions 31–34 of several α-gliadins from both common and durum wheat. This tetramer is similar to the LGQQ sequence at the N-terminal end of the innate immunity eliciting α-gliadin 31–49 peptide [7]. However, the overall sequence identity of the 9-mer and the 10-mer peptides with proteins in the GenBank database was lower than 50%. Therefore, the origin of the 9-mer and the 10-mer peptides remains obscure.

The only effective treatment of CD is based on avoidance of foods containing the prolamin fraction of grain proteins from wheat, rye, barley and triticale. However, the elimination diet is not an easy prescription to follow because prolamins are contained in hundreds of basic foods, and are present as occult traces in many foods and beverages. According to Vader et al. [15], there is a threshold to be overcome to produce a pathological immune response to prolamins in genetically CD-susceptible individuals. This threshold is governed by the number of gluten epitopes that interact with the HLA-DQ2.5 (or -DQ8) molecules in the intestinal mucosa of celiac patients, and by the strength of the T cell response induced by each epitope/HLA complex. Therefore, an ideal, safe wheat should be poor of proteolytic stable, immunodominant epitopes that can activate the intestinal T lymphocytes beyond the pathological threshold. A complementary approach may involve the over-expression of genes coding for prolamins containing proteolytically stable, "protective" peptides such as the ASRVAPGQQ and GTVGVAPGQQ sequences described here, as well as the QQPQDAVQPF peptide present in the peptic/tryptic digest of the alcohol-soluble fraction of endosperm protein from durum wheat cv. Adamello analyzed previously [12–14]. These peptides proved to be able to downregulate T cell proliferation and INF-γ secretion elicited by wheat prolamins. Moreover, they were found to counteract IL-10 downregulation by PTP, and modify the pro/anti-inflammatory cytokine ratio in CD-specific T cell lines exposed to gluten-derived proteins.

Conflict of interest statement The authors declare that they have no conflict of interest.

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