Effect of Maize Prolamins on Peripheral Blood Mononuclear Cells from Celiac Disease Patients

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Introduction

CD: Celiac Disease; PBMC: Peripheral Blood Mononuclear Cells; HLA: Human Leucocyte Antigen; Ttg: Tissue Transglutaminase; PHA: Phytohaemaglutinin A; PT: Pepsin-Trypsin; Gd: Gliadin; G33-mer, Immunogenic Peptide of α-gliadin; Z34-mer, Immunogenic Peptide of α-zein

Materials and Methods

Patients

Patients underwent gluten-free diet for at least one month, and a three days challenge with at least 50 g/day gluten was made and blood samples were taken at day 0 and day 6. The ethical committee of the Centro de Investigación en Alimentación y Desarrollo (CIAD A.C.) approved the study and all samples were taken under informed written consent. Whole blood was taken (14 mL) from each patient by venipuncture into Vacutainer tubes (BD Medical Systems, USA). DNA was extracted from 200 μL whole blood by the QiAamp DNA Blood Mini Kit (QIAGEN, USA) and genotyping of HLA-DQ2/DQ8 was done by real time PCR (Step One Plus, Applied Biosystems) using specific primers [8]. Isolation of peripheral blood mononuclear cells (PBMCs) from 12 mL blood was done using Ficoll-Paque PLUS (Amersham-Biosciences, Sweden) density gradient centrifugation technique. Plasma anti-gliadin (Gd) IgG, anti-Gd IgA, anti-zein IgA
and anti-transglutaminase (TG) IgA antibodies were analyzed by a direct enzyme-linked immunosorbent assay (ELISA), as previously reported [9]. IgA anti-gliadin and/or zeins and IgA anti-TG were expressed as an index value and it was calculated based on the mean of absorbance values of control individuals as reported before [9] and index values of 1.0 and above were considered as positive.

Peptide preparation

The immunogenic peptides α-gliadin 33-mer (LQLQFQPQFELPYPQPPELPYPQPF; MW = 3914.51 Da), later referred to as G33-mer, and α-zein 34-mer (LQQAIAASNIPLSPLLFQQSPALSLVQSLVQTIR; MW = 3646.32 Da), later referred to as Z34-mer, were supplied by United Biosystems (USA) with purities of 97.54% and 95.66%, respectively. Gliadins from wheat and zeins from maize (Sigma Chem Co, St. Louis, MO USA) were subjected to pepsin-trypsin (PT) digestion, as previously described [2]. All immunogenic peptides and digested prolamins were treated with transglutaminase (TG) from guinea pig liver (Sigma-Aldrich, St Louis, MO USA) 5 µg/500 mg of protein in CaCl2 2 mM for 60 min at 37°C and then placed on ice. Separation of TG was performed by ultrafiltration (UF cell, Amicon Inc. Beverly, MA. USA.), with a 30 kDa cut-off membrane and peptides were recovered in sterile water.

Cell culture and cytokine assays

Isolated PBMC were incubated at a final concentration of 2 x 10^5 cells/mL on culture plates and cultured in Dulbecco's Modified Medium (D-MEM) containing 10% fetal calf serum (FCS), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, USA) at 37°C in a 5% CO2 atmosphere. The immunogenic peptides were used in the experiments at final concentration of 50 µg/mL and the digested prolams at 100 µg/mL. Phytohemagglutinin A (PHA) (Sigma-Aldrich, USA) was used as positive control at concentration of 25 µg/mL. After 20 h, supernatants were collected and frozen at -70°C prior to cytokine evaluation. ELISA kits were used for IFN-γ (Mabtech, Sweden) detection according to manufacturer.

Statistical analysis

Experiments were performed in triplicate, results are given as mean values that were compared after ANOVA. Statistical significance among days 0 and 6 was compared by Student's one sample T-test and digested fractions compared to untreated cells and the poor response was averaged to simplify the results graphically (Figure 1). As expected, in both CD patients, the α-gliadin immunogenic peptide (G33-mer) increased release of IFN-γ in PBMC respect to controls (p<0.005) at days 0 and 6. Additionally, on patient 1 IFN-γ release was higher at day 6 compared to day 0 (p<0.05), while for patient 2 the IFN-γ increase was similar for both days.

Interestingly, an increase in IFN-γ release by stimulation with Z34-mer peptide was observed at day 0, mostly on patient 2 (p<0.0005) respect to controls, but stimulation diminishes at day 6 in both cases (patients 1 and 2), remaining higher than controls just for patient 2 (p<0.05), as it is shown in Figure 1. Both zeins and gliadins fractions ZFIHI and GFIHI induced a similar IFN-γ release in PBMC at 0 or 6 days; however, such increase was not significant (p>0.05) respect to controls for patient 1, while it was significant (p<0.005) as compared with IFN-γ releasing for PBMC from patient 1 or controls, for patient 2.

Discussion

Both CD patients described as patient 1 and 2, reported extra-intestinal and intestinal symptoms that were alleviated after a gluten-free diet. They also showed some positive indexes for antibodies anti-gliadins, anti-transglutaminase and patient 2 against zeins (Table 1). The deamidation of gluten peptides in lamina propria by tissue transglutaminase is the first step in CD pathogenesis, and after activation of the immune response, IgA anti-TG autoantibodies are induced; they characterize CD [1]. Additionally, in active CD there are antibodies against gliadins, the exogenous antigen. Interestingly, only patient 2 had a positive index for anti-zeins IgA antibodies, as it was previously found in some CD patients by Cabrera-Chávez et al. [9]. Peripheral blood effector T-cells reactive to gliadins were found in both patients before the in vivo gluten challenge and this result agrees with those found by Liu et al. [7] who detected higher levels of IFN-γ in CD patients that carried both haplotypes HLA-DQ2 and/or HLA-DQ8. Furthermore, they also observed that the stimulation of peripheral blood T-cells proliferation is possible without a previous in vivo challenge. Indexes of anti-transglutaminase and anti-gliadin IgA antibodies remained positive, especially on patient 2 (Table 1), since half-life of IgA antibodies last for about 4 months [10], patients possibly did not follow a strict gluten-free diet. Therefore, the in vivo gluten challenge was not effective.

| Subject | Age (year) | Haplotype or alleles | Index of antibodies | Symptoms |
|---------|------------|----------------------|---------------------|----------|
|         |            |                      | IgG anti-Gd | IgA anti-Gd | IgA anti-TG | IgA anti-Zn |         |
| Control 1 | 30 | DQA1*0501, DQB1*0301 | 0.899 | 0.77 | 0.74 | 0.768 | None |
| Control 2 | 30 | DQA1*0501, DQB1*0302/3 | 0.695 | 0.45 | 0.35 | 0.353 | None |
| Control 3 | 27 | DQA1*0301 | 0.796 | 0.72 | 0.66 | 0.619 | None |
| Patient 1 | 31 | HLA-DQ2 | 1.316 | 0.74 | 1.04 | 0.938 | Migraine, fatigue and bloating |
| Patient 2 | 46 | DQA1*0501 | 1.217 | 1.23 | 1.28 | 1.68 | Anemia, constipation, bloating |

Table 1: Characteristics of the three control subjects and the two celiac patients described in Table 1. All the control individuals showed negative indexes (<1.0) anti-Gd IgG, anti-Gd IgA and anti-TG IgA antibodies. Celiac patient 1 presented positive indexes (>1.0) for anti-Gd IgG and anti-TG IgA antibodies, while patient 2 had for anti-Gd IgG, anti-Gd IgA, anti-TG IgA and anti-Zein IgA antibodies (Table 1).

Production of IFN-γ in PBMC of control individuals was not stimulated with any of gliadin or zein immunogenic peptides or PT-digested fractions compared to untreated cells and the poor response was averaged to simplify the results graphically (Figure 1). As expected, in both CD patients, the α-gliadin immunogenic peptide
The immunogenic peptide of α-zeins (Z34-mer) induced an increased release of IFN-γ in both celiac patients, but the stimulus remained after 6 days only on patient 2. Our work team also observed cell stimulation by this proposed immunogenic peptide when duodenal bulb intestinal biopsies were challenged in vitro under cell culture conditions [2]. Cell response to Z34-mer is independent of the gluten challenge and the higher response at day 0 could be explained by the fact that maize is a common constituent food of the gluten-free diet and patients were highly exposed to larger quantities of its protein. On patient 2, the high serum IgA anti-zeins detected (Table 1), suggest a higher sensibility that is reflected on a greater response with respect to patient 1 and controls. Therefore, the response decreases significantly at day 6 (p<0.05, Figure 1) perhaps due to lower consumption of maize by consumption of the gluten challenge.

Table 1: Characteristics of control individuals and celiac disease patients; CD: celiac disease; ND: not done; HLA: human leucocyte antigen; DQA1: alpha-chain DQ alleles; DQB1: beta-chain DQ alleles; IgG: G isotype immunoglobulin; IgA: A isotype immunoglobulin; Gd: gliadins; Zn: zeins; tTG: tissue transglutaminase.

An increase of IFN-γ release was also observed by the PT-digested fraction of zeins (ZFIII) on patient 2, comparable to response to PT-digested gliadins (GFIII). Contrary to Silano et al. [6] who used smaller amounts of PT-digested wheat to obtain a T-cell response, we saw poor response to our PT-digested gliadins despite having used a larger amount of gliadin-digested fraction. It is possible that the immunogenic epitopes in this peptide fraction were insufficient to achieve cell stimulation as consequence of handling and digestion procedure. However, stimuli with gliadin peptides was clearly observed by using the α-gliadin peptide G33-mer that has been demonstrated to have a single dominant epitope that elicits an optimal IFN-γ release in gluten-sensitive T-cells [5].

Z34-mer induced cellular response in a non HLA-DQ8 patient and this was also observed in our previous work on other patients that do not have this haplotype [2], even though it had been shown in silico to have affinity to the HLA-DQ8 tetramer [3]. The amino acid sequences between Z34-mer and G33-mer peptides are quite different. However, they share prolamin features like poor digestion by mammalian proteases and their glutamine residues able to be deamidated by tTG that can increase affinity to HLA-DQ2 or DQ8 molecules in antigen presenting cells and to induce a cellular response. Isolation of PBMC and its posterior stimulation in vitro with zein peptides could be an efficient tool for finding epitopes in maize protein in some non-responsive subjects to the gluten-free diet.

**Conclusion**

In conclusion, in PBMC of a CD patient a cellular response to maize zeins was induced and this response was even higher to that induced by wheat gliadins although independent of the gluten challenge. In vitro stimulation of PBMC with immunogenic peptide Z34-mer is comparable to that of the G33-mer with a dominant epitope that elicits an optimal IFN-γ release in gluten-sensitive T-cells.

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