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

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Abstract

Celiac disease (CD) is an enteropathy induced by wheat prolaminis (gliadins) and in some rare cases by maize prolamins (zeins) possibly due to a similar immune response. The aim was to study the cellular immune response to zeins in comparison to gliadins of peripheral blood mononuclear cells (PBMC) from CD patients. Isolated PBMC from two treated CD patients and three non-CD controls were challenged in vitro with gliadins or zeins and released gamma-interferon (IFN-γ) in culture medium was measured. PBMC were stimulated with gliadin or zein immunogenic peptides or their digested fractions (3-5 kDa). The gliadin peptide G33-mer induced an expected IFN-γ releasing of PBMC from both patients 1 and 2, with a higher level between days 0 and 6 for patients 1 and no differences para patient 2. The zein peptide Z34-mer induced a higher increase of IFN-γ in both CD patients at 0 day, even higher that it of G33-mer for patient 2, and both of them were highly decreased at day 6. Finally, the zein digested fraction induce an IFN-γ release similar to that of gliadin digested fraction in both cases, although negligible for patient 1 and significant for patient 2. In conclusion, the cellular response to zeins was partially similar to it of gliadins after an in vitro challenge.

Keywords:
Celiac disease, T-cell response, Maize prolamins, Zeins

Abbreviations

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

Introduction

CD is an immunologically mediated systemic disorder developed in genetically predisposed individuals, exacerbated by wheat and related cereals as barley and rye. Disease symptoms are promoted by inflammation of the intestinal mucosa, inducing gastrointestinal and/or extra-intestinal manifestations [1]. CD is a lifelong condition and gluten-free diet is the only treatment. One of the most important and gluten-free diet is the only treatment. One of the most important alternative cereals used for the gluten-free bakery products is maize; additionally, its prolamins have been used as a negative control in different studies on CD. By chance, in some of those studies maize prolamins have demonstrated adverse effects [2,3] inducing doubt about the maize use for dietary treatment of CD patients. The response to maize prolamins could be due to similarities between maize (zein) and wheat (gliadin) prolamins both with a high percentage of glutamine able to be deamidated by transglutaminase and proline residues that hinders a full digestion by gastrointestinal proteases [3].

The proposed pathogenesis of CD highlights the role of T-cells, after peptide presentation by dendritic cells to Th1 cells via the HLA-DQ2/8 context, activating them and consequently releasing cytokines, mainly IFN-γ [4]. Therefore, IFN-γ is a marker of cellular response to different gluten peptides by in vitro assays; its advantage is that promotes tissue inflammation and has no autocrine effect on other PBMC, like monocytes [5-7]. Gluten specific T-cells producing IFN-γ can be found in peripheral blood of CD patients in gluten-free diet after a short gluten challenge [5]. Isolation and subsequent in vitro stimulation of these T-cells with a wide variety of dietary peptides, generates a reliable tool to evaluate the cellular response to gluten-free foods [6]. The aim of this study was to evaluate the T-cell response in vitro to maize prolamins in comparison to wheat prolamins of peripheral blood mononuclear cells (PBMC) from CD patients and PBMC from non-CD individuals as controls, after gluten-free diet followed by a three-day gluten challenge.

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 (PBMC’s) 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 on the basis 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 (LQQLQFPQPSPELPYPQPELPYQPQPQPQPF; MW = 3914.51 Da), later referred to as G33-mer, and α-zein 34-mer (LQAIAASNIPSLPILQQPSPALSLQVSLSYQFTIR; 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 10⁵ 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 prolamins 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 statistical significance among treatments by Tukey-Kramer multiple comparison test using the statistical software NCSS, version 2001. The p-values of 0.05 or less were considered as statistically significant.

**Results**

The characteristics of the three control subjects and the two celiac patients are described in Table 1. All the control individuals showed negative indexes (<1.0) of 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 (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 ZFII and GFII 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.

### Table 1: Index values of antibodies and symptoms

<table>
<thead>
<tr>
<th>Subjec</th>
<th>Age (years)</th>
<th>Haplotype or alleles</th>
<th>Index of antibodies</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>IgG anti-Gd</td>
<td>IgA anti-Gd</td>
</tr>
<tr>
<td>Control 1</td>
<td>30</td>
<td>DQA1<em>0501, DQB1</em>0301</td>
<td>0.899</td>
<td>0.77</td>
</tr>
<tr>
<td>Control 2</td>
<td>30</td>
<td>DQA1<em>0501, DQB1</em>0302/3</td>
<td>0.695</td>
<td>0.45</td>
</tr>
<tr>
<td>Control 3</td>
<td>27</td>
<td>DQA1*0301</td>
<td>0.796</td>
<td>0.72</td>
</tr>
<tr>
<td>Patient 1</td>
<td>31</td>
<td>HLA-DQ2</td>
<td>1.316</td>
<td>0.74</td>
</tr>
<tr>
<td>Patient 2</td>
<td>46</td>
<td>DQA1*0501</td>
<td>1.217</td>
<td>1.23</td>
</tr>
</tbody>
</table>
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 sensitivity 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.

<table>
<thead>
<tr>
<th>Control</th>
<th>Patient 1</th>
<th>Patient 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-DQ8</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DQA1</td>
<td>0301</td>
<td>0301</td>
</tr>
<tr>
<td>DQB1</td>
<td>0201</td>
<td>0201</td>
</tr>
<tr>
<td>IgG</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>IgA</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Gd</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Zn</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>tTG</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

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 fraction of 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 glutenin 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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**References**
